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Article

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Discovery of N-substituted Oseltamivir Derivatives as Potent and Selective Inhibitors of H5N1 Influenza Neuraminidase

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ABSTRACT

To discover Group-1-specific neuraminidase (NA) inhibitors that are especially involved in combating the H5N1 virus, two series of oseltamivir derivatives were designed and synthesized by targeting the 150-cavity. Among these, compound **20l** was the most potent N1-selective inhibitor, with IC_{50} values of 0.0019 μM, 0.0038 μM and 0.0067 μM against NAs from three H5N1 viruses. These values are better than those of oseltamivir carboxylate. Compound **32** was another potent N1-selective inhibitor that exhibited a 12-fold increase in activity against the H274Y mutant relative to oseltamivir carboxylate. Molecular docking studies revealed that the 150-cavity was an auxiliary binding site that may contribute to the high selectivity of these compounds. The present work is a significant breakthrough in the discovery of potent Group-1-specific neuraminidase inhibitors, which may be further investigated

for the treatment of infection by the H5N1 virus.

INTRODUCTION

Recently, increasing attention has been paid to the possibility of another global flu pandemic. In 2009, a swine-origin H1N1 influenza virus rapidly spread throughout the world, giving rise to a serious public panic.[1] H7N9 avian virus is a lethal avian influenza virus endemic to China that has evolved to infect humans since April 2013.[2] It is known that the highly pathogenic avian influenza A (H5N1) virus, with a 60% mortality, is a great threat to humans. Though there is no evidence of efficient human-to-human transmission, some studies have showed that the H5N1 virus exhibits an increased propensity for acquiring human receptor specificity.[3]

Neuraminidase (NA), which plays an important role in the viral life cycle, is a good target for anti-influenza drug design. To date, three NA inhibitors have been developed as effective treatments of influenza A and B infections: zanamivir (**1**), oseltamivir (**2**) and peramivir (**3,** Figure 1). Of these, orally administered oseltamivir is the first choice and has been widely used since approved in 1999, though recently, resistant viral strains have seriously narrowed the drug's clinical application.[4, 5] Zanamivir and peramivir (which are only approved in Japan, Korea and China at present) are mainly administered by inhalation from a nebulizer or intravenous routes, which is very inconvenient for patients. In addition, viral strains that are resistant to the two drugs are also constantly being reported.[6, 7]

These emerging problems with the current NA inhibitors increase the demand for the development of novel anti-influenza drugs. In 2006, one important discovery

regarding the structure of the influenza virus NA provided a great opportunity for inhibitor design. The X-ray crystallographic structures show that in Group-1 NAs (N1, N4, N5, N8 subtypes), there is a large cavity termed the 150-cavity adjacent to the active site that is not found in Group-2 NAs (N2, N3, N6, N7, N9 subtypes).[8] Previously, NA inhibitors were mainly designed based on the crystal structures of N2 and N9, which are both Group-2 enzymes. This finding is very meaningful for exploring Group-1-specific NA inhibitors, especially against H1N1 and H5N1 influenza virus. The H1N1 virus has caused at least three flu pandemics since the early 20th century, leading to a tremendous loss of life. At present, this virus is variable and still very dangerous to human health. The H5N1 influenza virus, which sees occasional outbreaks in poultry and causes human death, is a significant global pandemic threat. Thus, the development of high potent Group-1-specific NA inhibitors may be of considerable value against H1N1 and H5N1 viral infection.

Enlightened by the new identified cavity of Group-1 NAs, researchers at first tried to screen for potential inhibitors by using various computer-aided drug design tools. A large number of compounds were proposed, but they were only predicted to have high binding affinity for NA (mostly based on N1 from the H5N1 virus) without being biologically evaluated in vivo or in vitro.[9-14] Zanamivir and oseltamivir (OS) are good leads to be optimized in the search for novel inhibitors.[15-18] Based on their structures, a few derivatives with selective NA inhibitory activities have been discovered, such as compounds **4**[19], **5**[20] and **6**.[21] Compounds **4** and **5**, designed with the 150-cavity, are N1-selective inhibitors, but these compounds exhibit low

inhibitory activities. Compound **6** is derived from zanamivir and shows selectivity for the NA of H5N1, but it is not equally potent against the NA of H1N1. In 2013, Lin and co-workers discovered one acylguanidine-modified zanamivir analog (**7**), the hydrophobic substituent (naphthylthiomethyl) of which was thought to interact with the 150-cavity. However, this compound did not exhibit increased activity relative to zanamivir or selectivity for the N1 enzyme.[22] More recently, the group of Martin designed several oseltamivir analogs bearing N-substituted guanidines, two of which presented good activity against NAs from wild-type and oseltamivir-resistant strains. Their findings also suggested that it was hard to access the 150-cavity by appending substituents to the terminal nitrogen atoms of the exocyclic guanidine moiety.[23]

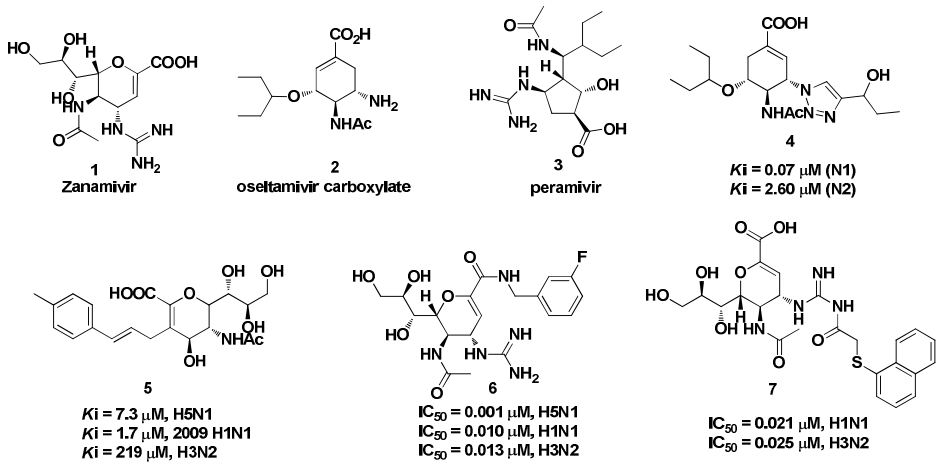
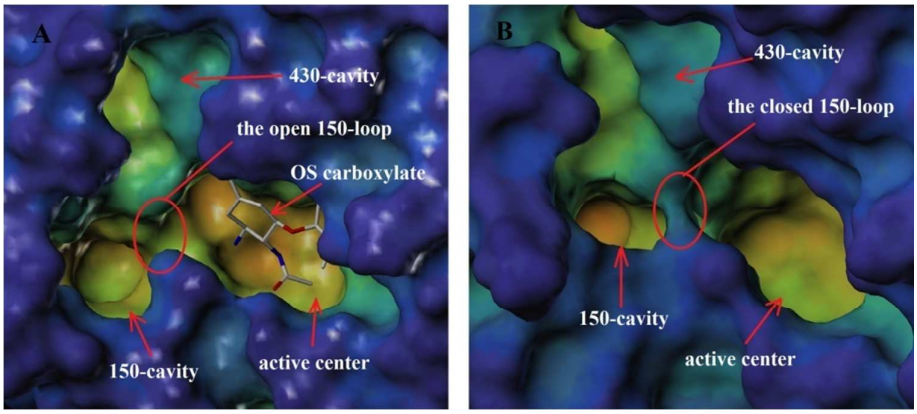


Figure 1. Structures of the approved NA inhibitors (**1**)-(**3**) and compounds **4**-**7**.

DESIGN OF OSELTAMIVIR DERIVATIVES

The main objective of our research was to discover potent Group-1 selective NA inhibitors that act as anti-influenza agents. As shown in Figure 2 (A), the C5-NH_2 of OS carboxylate in the NA active center is very close to the 150-cavity. It is thus feasible to take advantage of the cavity as an auxiliary binding site by modifying the

C5-NH_2 of OS. It should also be considered that the NH_2 group, which forms strong hydrogen bonds with Glu119 and Asp151, is very important for the activity of the molecule. In 2010, Pinto and colleagues synthesized a series of OS analogs bearing substituted triazoles at the C-5 position.[19] The activities of these compounds were dramatically decreased, highlighting the importance of the basic group at the site. Therefore, we planned to develop our compounds with two substituents at the C-5 NH_2: one substituent would occupy the potential binding site and one basic group would bind with Glu119 or Asp151. We thus designed two series of OS derivatives, which were the substituted guanidines and the secondary amines (Figure 2). The acylguanidine-modified zanamivir analogs reported by Lin et al. were also designed to lock the 150-cavity of Group-1 NAs, but the result was unsatisfying.[22] The various substituents at the C-4 guanidine group were introduced by amide bonds, which decreased the alkalinity of the guanidine group. In our study, all the derivatives were designed by modifying the C-5 guanidino group and the amino group with alkyl groups to increase the alkalinity and to reduce the polarity relative to the OS carboxylate. In view of the importance of alkalinity, our design strategy is more reasonable.



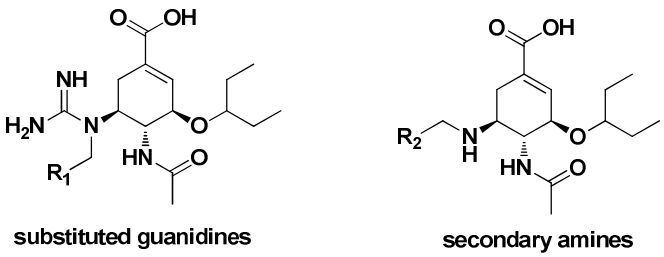


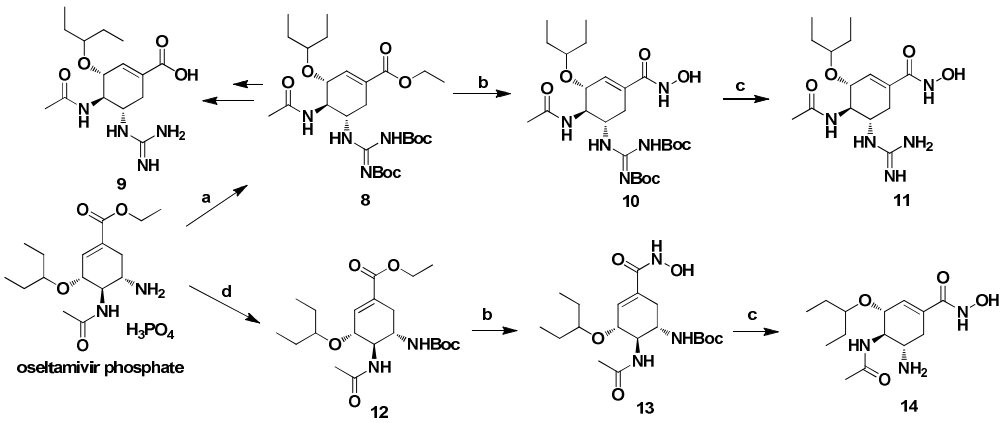
Figure 2. Comparison of the crystal structures of N1 (PDB 2HU0, A) and N2 (PDB 4GZQ, B) and the general structures of the designed compounds.

CHEMISTRY

Compound **9**, with a guanidine group instead of NH_2, was reported to have comparable or even better activities relative to OS carboxylate.[24] We synthesized this compound as one of the positive controls. Another two OS derivatives, (**11**) and (**14**), were prepared by converting the COOH groups of oseltamivir and compound **9** to hydroxamic acid (CONHOH) to explore whether the CONHOH was good for the activity, as shown in Scheme 1.

Commercial oseltamivir phosphate was the primary starting material. In Scheme 1, compound **9** was synthesized using a three-step route that included guanylation, hydrolysis and N-Boc deprotection and was similar to the reported method.[25] The guanylation reaction was performed with *N,N'*-bis(*tert*-butoxycarbonyl)thiourea ($SC(NHBoc)_2$)/$HgCl_2$. Treatment of compound **8** with a solution of NH_2OK in CH_3OH gave intermediate **10**. Compound **13** was synthesized by the same method from Boc-protected oseltamivir **12**. The target compounds **11** and **14** were all prepared as hydrochlorides with 3 M HCl/EtOAc.

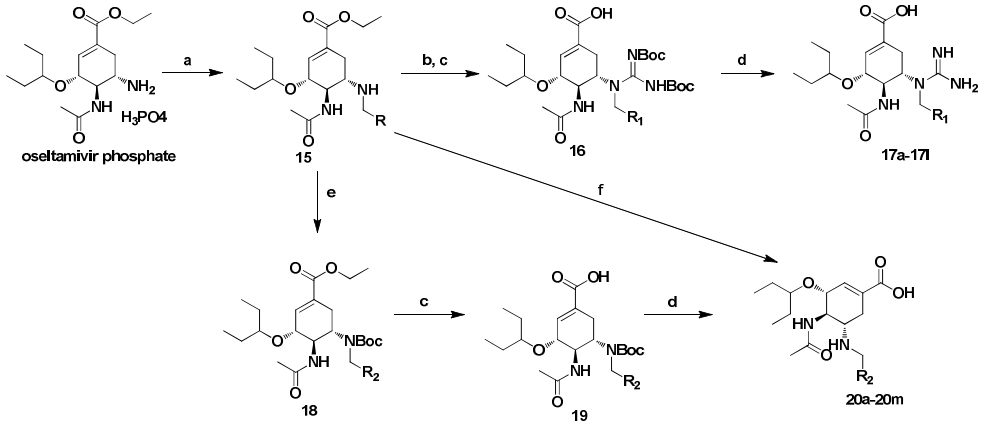
Scheme 1. Synthesis of compounds 9, 11 and 14[a]



[a]Reagents and conditions: (a) $SC(NHBoc)_2$, $HgCl_2$, Et_3N, DMF, rt; (b) NH_2OK, CH_3OH, rt; (c) HCl/EtOAc, CH_3OH, rt; (d) $(Boc)_2O$, Et_3N, CH_2Cl_2, rt.

In Scheme 2, oseltamivir phosphate was reacted with a range of different aldehydes in the presence of $NaBH_3CN$ to afford the key intermediate **15**. The synthesis of compound **17** was achieved in three steps: guanylation, hydrolysis and Boc deprotection. Compound **20** was also synthesized either in three steps (Boc protection, hydrolysis and deprotection) from intermediate **15** as a trifluoroacetate salt or by direct hydrolysis of intermediate **15** with NaOH.

Scheme 2. Synthesis of OS derivatives (17) and (20)[a]

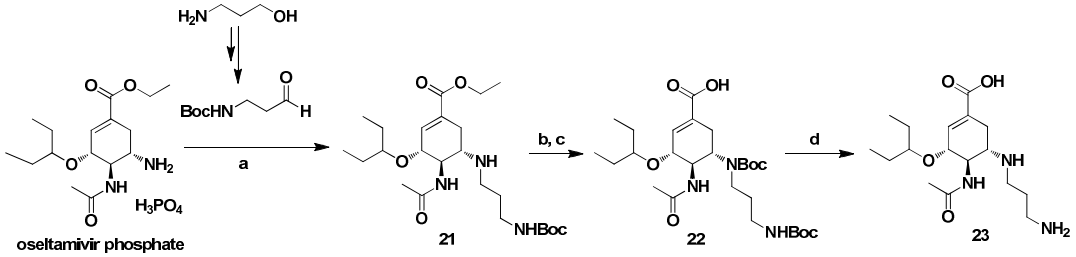


[a]Reagents and conditions: (a) RCHO, $NaBH_3CN$, EtOH, rt; (b) $SC(NHBoc)_2$, $HgCl_2$, Et_3N, DMF, rt; (c) NaOH, CH_3OH, H_2O, 50 °C;(d) CF_3COOH, CH_2Cl_2, rt; (e)

$(Boc)_2O$, Et_3N, CH_2Cl_2, rt; (f) NaOH, CH_3OH, H_2O, 50 °C, 4 h, then CH_3COOH.

Because the R_2 groups of compounds **20a**-**m** were all hydrophobic, we further designed compound **23**, which contained one NH_2 at the end of the substituent. The structure and its synthetic route are shown in Scheme 3. 3-(Boc-amino)propanal was a key reagent that could be prepared from 3-aminopropanol through two steps: Boc protection and PCC (pyridinium chlorochromate) oxidation. The following synthetic procedure was the same as that in Scheme 2.

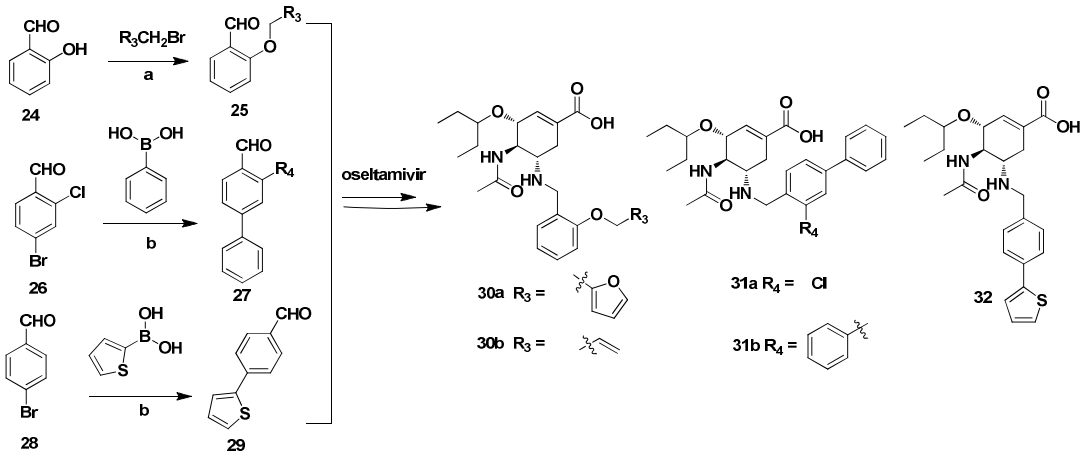
Scheme 3. Synthesis of compounds 23[a]



[a]Reagents and conditions: (a) $NaBH_3CN$, EtOH, rt; (b) $(Boc)_2O$, Et_3N, CH_2Cl_2, rt; (c) NaOH, CH_3OH, H_2O, 50 °C;(d) CF_3COOH, CH_2Cl_2, rt.

Compounds **20h** and **20l** of the second series exhibited good NA inhibitory activities (data shown in Table 2). Therefore, we continued to synthesize another five derivatives, compounds **30**-**32**. In Scheme 4, the two 2-alkoxybenzaldehydes were prepared by reaction of 2-hydroxylbenzaldehyde with 2-(bromomethyl)furan and 3-bromoprop-1-ene and were used for the synthesis of compounds **30a** and **30b**. A Suzuki reaction of the aryl bromides with boronic acid gave another three aldehydes that were used in synthesizing compounds **31** and **32**.

Scheme 4. Synthesis of compounds 30-**32**[a]



[a]Reagents and conditions: (a) acetone, K_2CO_3, reflux; (b) THF, H_2O, K_2CO_3 $Pd(PPh_3)_4$, reflux.

RESULTS AND DISCUSSION

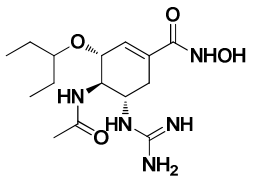
Three N1 enzymes from influenza (H5N1) viral strains and two N2 enzymes from influenza (H9N2) viral strains were the representatives of Group-1 and Group-2 NAs for the NA inhibition assay. In Table 1, OS carboxylate was found to be more potent against N2 (IC_{50} = 0.0028 μM, H9N2-415) than N1 (IC_{50} = 0.017 μM, H5N1-1220), which is consistent with reported data.[26] Compound **9**, by contrast, which has a guanidine group, showed better inhibitory activity against N1 than against N2. The two controls did not equally inhibit the two NA subtypes and showed a certain degree of selectivity.

Compounds **11** and **14,** which had a CONHOH group instead of a COOH group, exhibited significantly decreased activities (> 40-fold) than the two controls. It could be confirmed that CONHOH was not a good group for binding with the S1 pocket. The S1 pocket in the NA active site contains three basic residues, Arg 118, Arg 292,

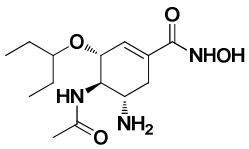
and Arg 371, which can strongly interact with acidic groups, such as a COOH[27] or phosphoryl group.[25] The CONHOH group, with an intramolecular hydrogen bond, is weaker than COOH in acidity, which may have largely contributed to the poor activities of the two compounds.

The first series of OS derivatives (**17a**-**l**) were less potent than the two controls but displayed a certain degree of selectivity for N1. The substituents (R_1CH_2-) at the guanidines of these compounds were unfavorable for the activity and probably affected the binding of the other structural parts to the corresponding pockets of the active site. Compounds **17a** and **17e** were the best two compounds of this series with IC_{50} values of 0.088-0.35 μM (**17a**) and 0.15-0.47 μM (**17e**) against H5N1 and H9N2, respectively. Generally, Compounds **17a**-**e**, which both have aromatic groups at R_1, were better than the alkyl-containing compounds **17f**-**k**.

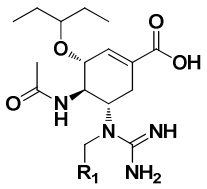
Table 1, NA inhibitory activities of compounds 11, 14 and 17a-l



11

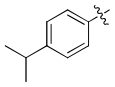
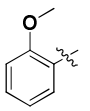
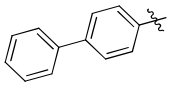
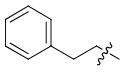


14



17a-17l

Compound	R_1	NA inhibition assay, IC_{50} (μM)[a]				
		H5N1-1220[b]	H5N1-1206[c]	H5N1-QJ[d]	H9N2-415[e]	H9N2-S2[f]
11		> 0.1	> 0.2	> 0.2	0.20 ± 0.015	0.16 ± 0.01
14		> 0.1	> 0.2	0.18	0.11 ± 0.01	0.12 ± 0.01
17a		0.20 ± 0.015	0.088 ± 0.007	0.19 ± 0.013	0.21 ± 0.015	0.35 ± 0.028
17b		1.17 ± 0.11	3.02 ± 0.21	> 3.0	2.60 ± 0.18	2.64 ± 0.25

17c		1.44 ± 0.15	1.56 ± 0.20	3.20 ± 0.24	2.37 ± 0.21	2.16 ± 0.18
17d		0.55 ± 0.032	0.25 ± 0.014	0.55 ± 0.03	0.49 ± 0.044	0.36 ± 0.038
17e		0.15 ± 0.018	0.27 ± 0.029	0.43 ± 0.050	0.47 ± 0.055	0.37 ± 0.045
17f		0.67 ± 0.075	1.14 ± 0.18	3.90 ± 0.42	> 5.0	> 5.0
17g		0.23 ± 0.033	0.39 ± 0.041	0.45 ± 0.053	> 5.0	> 5.0
17h		0.78 ± 0.075	3.62 ± 0.39	> 3.0	> 5.0	> 5.0
17i		1.14 ± 0.20	> 3.0	> 3.0	1.48 ± 0.22	1.23 ± 0.18
17j		0.37 ± 0.045	2.54 ± 0.32	> 3.0	> 5.0	4.88 ± 0.55
17k		0.95 ± 0.12	> 3.0	> 3.0	> 5.0	> 5.0
17l		0.79 ± 0.087	1.78 ± 0.24	> 3.0	> 5.0	> 5.0
OS-C		0.017 ± 0.001	0.013 ± 0.002	0.0090	0.0028	0.0031
9		0.0069 ± 0.001	0.0023	0.0026	0.0064	0.011 ± 0.001

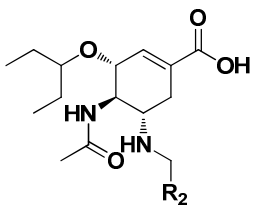
[a]Concentration required to reduce NA activity to 50% of control NA activity (IC_{50}). Values were the mean of three experiments. The standard derivations omitted for conciseness were < 20% of the mean. [b]A/chicken/china/1220/2012(H5N1). [c]A/duck/china/1206/2012(H5N1). [d]A/duck/china/QJ/01(H5N1). [e]A/chicken/china/415/2013(H9N2). [f]A/chicken/shandong/S2/02(H9N2).

As shown in Table 2, the second series of OS derivatives (**20a**-**m**) exhibited selective inhibition against NAs of H5N1 influenza virus and were much more potent than the first series. Compound **20l** was the best compound among the second series, with IC_{50} values of 0.0019 μM, 0.0038 μM and 0.0067 μM against the three types of H5N1 NAs and IC_{50} values of 1.20 μM and 0.58 μM against the two types of H9N2

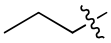
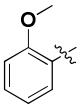
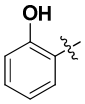
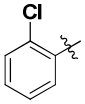
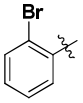
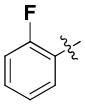
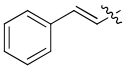
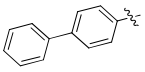
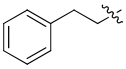
NAs. Obviously, compound **20l** displayed a high degree of selectivity for N1 over N2. For the inhibitory activity against the NA of the H5N1-1220 strains, Compound **20l** was approximately 8-fold more potent than the OS carboxylate and 3-fold more potent than compound **9**.

Several other compounds in the series, such as compounds **20d**, **20e**, **20h** and **20i**, showed comparable or even better inhibitory activities relative to the OS carboxylate against N1. The presence of Cl at the ortho-position of the phenyl group (compound **20h**) enhanced the activity 2- to 10-fold, while the CH_3O, OH, or F at the position (compounds **20f, 20g** and **20j**) decreased the activity. Compounds **20a**-**c**, with alkyl groups at the R_2 position, were also found to be good N1 selective inhibitors but were not as potent as the control. Compound **20a** bears a 3-pentyl group and showed the best activity against N2 (IC_{50} = 0.30 μM and 0.070 μM for H9N2-515 and H9N2-S2, respectively) within the series. Compound **20k** has a double bond in the R_2 group and was approximately 2-fold more active than compound **20m**, which had a single bond at the same position. This suggested that rigid R_2 groups were favorable for NA inhibitory activity. To some extent, this is consistent with the structure-activity relationships (SARs) of most compounds in series 1 and series 2.

Table 2, NA inhibitory activities of compounds 20a-m[a]



NA inhibition assay, IC_{50} (μM)

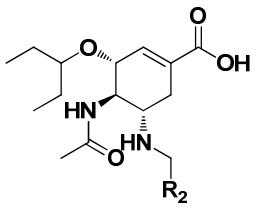
Compound	R_2	H5N1-1220	H5N1-1206	H5N1-QJ	H9N2-415	H9N2-S2
20a		0.027 ± 0.003	0.043 ± 0.004	0.054 ± 0.004	0.30 ± 0.035	0.070 ± 0.009
20b		0.019 ± 0.002	0.056 ± 0.007	0.076 ± 0.008	2.28 ± 0.35	0.63 ± 0.078
20c		0.078 ± 0.009	0.083 ± 0.01	0.099 ± 0.012	> 5.0	1.75± 0.23
20d		0.015 ± 0.003	0.053 ± 0.010	0.054 ± 0.008	0.74 ± 0.11	0.099 ± 0.016
20e		0.0071	0.019 ± 0.004	0.080 ± 0.013	2.44 ± 0.38	0.42 ± 0.08
20f		0.031 ± 0.006	0.117 ± 0.020	> 0.20	1.64 ± 0.31	0.25 ± 0.041
20g		0.021 ± 0.004	0.063 ± 0.009	0.194 ± 0.034	> 5.0	> 5.0
20h		0.0036	0.014 ± 0.002	0.029 ± 0.004	0.98 ± 0.08	0.043 ± 0.007
20i		0.013 ± 0.003	0.038 ± 0.005	0.056 ± 0.007	1.15 ± 0.15	0.042 ± 0.007
20j		0.010 ± 0.002	0.090 ± 0.010	0.108 ± 0.018	2.72 ± 0.31	0.65 ± 0.01
20k		0.014 ± 0.003	0.048 ± 0.009	0.106 ± 0.016	4.02 ± 0.54	3.23 ± 0.52
20l		0.0019	0.0038	0.0067	1.20 ± 0.23	0.58 ± 0.11
20m		0.032 ± 0.006	0.081 ± 0.011	0.190 ± 0.021	> 5.0	> 5.0

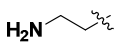
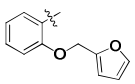
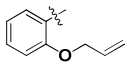
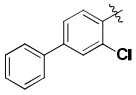
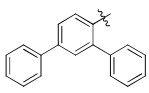
[a]Values were the mean of three independent experiments. The standard derivations omitted for conciseness were < 20% of the mean.

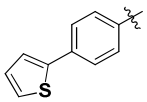
The NA inhibition results of the other six OS derivatives are listed in Table 3. Compound **23** bears one NH_2 and exhibited decreased activity against N1 in comparison to compound **20b**, suggesting that basic groups in the R_2 substituent are not favorable for the activity. Combining the SAR results described above, it can be

concluded that the potential binding region of the R_2 group of the second series was probably hydrophobic. Compounds **30a** and **30b** have large substituents at the ortho-position of the phenyl group and were less potent against N1 than compounds **20f** or **20g**. Among the other three compounds, compound **32** was highly selective and was the most potent against the three types of H5N1 NAs, with IC_{50} values of 0.0021 μM, 0.0043 μM and 0.014 μM. Compounds **31a** and **31b** with substituents (Cl and Ph) at the biphenyl group were less potent than compound **20l** with respect to the N1 inhibitory activity. However, for the N2 inhibitory activity, compound **31b** was much more potent (10 to 100-fold) than compounds **31a** and **20l**, indicating that the Ph at the biphenyl group probably formed some special interactions with the N2 enzyme.

Table 3, NA inhibitory activities of compounds 23 and 30-32[a]



Compound	R_2	NA inhibition assay, IC_{50} (μM)				
		H5N1-1220	H5N1-1206	H5N1-QJ	H9N2-415	H9N2-S2
23		> 0.10	> 0.20	> 0.20	0.95 ± 0.08	1.05 ± 0.17
30a		0.059 ± 0.01	> 0.20	> 0.20	1.53 ± 0.13	0.72 ± 0.08
30b		0.090 ± 0.012	> 0.20	> 0.20	4.70 ± 0.58	1.91 ± 0.17
31a		0.011 ± 0.002	0.027 ± 0.005	0.044 ± 0.005	2.02 ± 0.35	0.34 ± 0.042
31b		0.043 ± 0.006	0.039 ± 0.005	0.058 ± 0.006	0.032 ± 0.004	0.038 ± 0.004

32		0.0021	0.0043	0.014 ± 0.002	3.52 ± 0.53	0.21 ± 0.037

[a]Values were the mean of three independent experiments. The standard derivations omitted for conciseness were < 20% of the mean.

The H274Y mutant NA exhibits high-level resistance to oseltamivir, which is presently a great concern. In Table 4, oseltamivir carboxylate was found to be quite insensitive to this mutant enzyme, with an IC_{50} value of 2.1 μM, which is approximately 1000-fold lower than the IC_{50} against the wild type enzyme. Among these oseltamivir derivatives, compound **32** displayed obvious increased activity (IC_{50} = 0.16 μM) relative to the OS carboxylate towards the H274Y mutant and was deemed a suitable leading compound for further structural optimizations.

Table 4, NA (H274Y, H5N1) inhibitory activities of selected compounds[a]

Compound	**17a**	**17e**	**20l**	**31a**	**32**	**OS-C[b]**
IC_{50} (μM), H5N1(H274Y)	1.0 ± 0.1	1.78 ± 0.1	1.16 ± 0.1	1.91±0.2	0.16 ± 0.02	2.1± 0.2

[a]Values were the mean of three independent experiments. [b]The positive control, oseltamivir carboxylate.

The binding models of compounds **20l** and **32** with the N1 enzyme were analyzed using the computer software programs SYBYL and MOE. As shown in Figure 3 A, the two compounds perfectly bound with the enzyme. For example, the biphenylmethyl group of compound **20l** occupied the 150-cavity while the other structural part of the compound interacted with the active site in a manner similar to the binding pattern of oseltamivir carboxylate. This result was in good agreement with the purpose of our design. In the previous section, we hypothesized that the binding

region of the biphenyl group was hydrophobic. In Figure 3 B, it was clear that most of the residues around the group were hydrophobic, such as Trp 438, Val 116 and Gly 147. The side chain of Gln 136, Arg 128 and Arg 152 also made certain contributions to the hydrophobicity of the cavity. Among these residues, Val 116 seemed to be the most important because it was directly facing the biphenyl groups. Compound **20l** exhibited poor activities against N2, which can be easily explained by the enzyme's structure. Because of the closed 150-loop, the active site of N2 is relatively smaller than that of N1 (Figure 2 B), and would not well accommodate compound **20l** or other compounds bearing a large group at C-5 position. Their structures could be distorted in the active site, resulting in unfavorable interactions with the corresponding pockets.

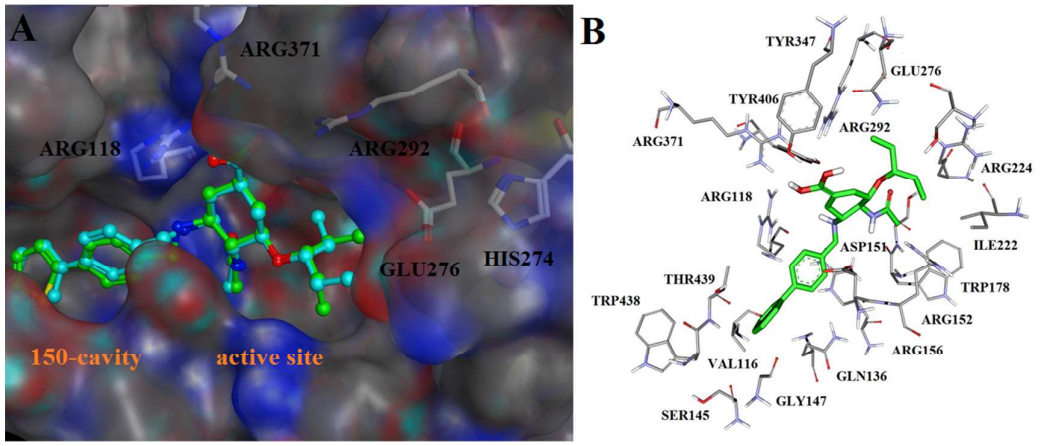


Figure 3. The docking results of compound 20l (light blue) and 32 (green) with N1(PDB 2HU0, A) compared with the binding mode of OS carboxylate and the key residues that may form potential interactions with compound 20l (B).

It is interesting to observe that compound **20l** was more potent than **32** against the wild type H5N1 NAs, but about six-fold less potent against the H274Y mutant. The molecular basis of oseltamivir resistance caused by the mutation H274Y has been

elucidated by Collins et al.[28] In the NA active site, the replacement of histidine with a bulkier tyrosine residue could disrupt the hydrophobic pocket formed by the methylene of Glu 276, thus preventing oseltamivir from making hydrophobic contact with the pocket. With respect to the two compounds, the only difference between them was the C-4 substituent on the phenyl ring linked to the C-5 NH_2 of oseltamivir carboxylate. Compound **20l** had a higher activity against the wild type N1 enzymes than compound **32** and almost completely occupied the 150-cavity due to the biphenylmethyl group. The 4-thiophen-2-ylphenyl group of compound **32** at the same position was relatively small. In the case of binding with wild type H5N1 NAs, compound **32** could not bind tightly like **20l** and thus presented lower activities. However, in the case of binding with the H274Y mutant enzyme, the 4-thiophen-2-ylphenyl group of compound **32** was more prone to moving, thereby adjusting the molecule to the changes that were caused by the mutation. Further explanation would be better assisted by the crystal structures of the enzyme-inhibitor complexes.

Finally, we compared the amino acid sequences of two NAs (H5N1-1220 and H5N1-1206) with the protein (2HU0) that was used for the docking analysis. We found that the NA of H5N1-1220 showed a high degree (> 97%) of similarity to 2HU0. Only 8 of the 368 residues were different between these two proteins, and not included in the active site or the 150-loop region. The NA of H5N1-1206, with 12 different residues, showed a slightly lower degree of similarity to 2HU0 (96.7%, data shown in the supporting information). The amino acid sequence of the two proteins

made a great contribution to the credibility of the docking analysis.

CONCLUSION

Because the 150-cavity was first reported, many studies have been conducted to explore N1 selective NA inhibitors but no outstanding progress has been made. In this manuscript, we designed and synthesized two series of oseltamivir derivatives with modifications at the C-5 NH_2 position. The secondary amine derivatives were found to be much better than the N-substituted guanidines. To the best of our knowledge, this work contributes the most selective N1 inhibitors, such as compound **20l**, which possesses IC_{50} values of 0.0019 μM, 0.0038 μM and 0.0067 μM against the three types of H5N1. Moreover, compound **32** exhibited increased activity against the H274Y mutant. Collectively, we provide one rational design strategy for the discovery of Group-1-specific NAs inhibitors. Considering the interesting anti-mutant potential of compound **32**, we hope that detailed SARs studies and further derivatization of compounds **32** and **20l** based on this strategy might lead to more potent compounds against drug-resistant NAs.

EXPERIMENTAL SECTION

Materials Used for Biological Experiments. Influenza virus (A/chicken/china/1220/2012, A/duck/china/1206/2012, A/duck/china/QJ/01. A/chicken/china/415/2013 and A/chicken/shandong/S2/02) were kindly provided by the College of Veterinary Medicine, South China Agricultural University, P.R. China and handled under biosafety level 3 (BSL-3) conditions. The H5N1 NA (H274Y) was obtained from Sino Biological Inc. The substrate used in the enzyme inhibition assay,

2'-(4-methylumbelliferyl)-α-D-acetylneuraminic acid sodium salt hydrate (4-MU-NANA) (Sigma, M8639), was purchased from Sigma.

NA Enzyme Inhibitory Assay. The NA inhibition assay was performed according to the standard method.[29] Influenza virus suspensions (H5N1-1220, H5N1-1206, H5N1-QJ, H9N2-415, H9N2-S2) obtained from the allantoic fluid of embryonated chicken eggs were inactivated and used for biological evaluation. The substrate, 2'-(4-methylumbelliferyl)-α-D-acetylneuraminic acid sodium salt hydrate (4-MU-NANA) (Sigma, M8639), was cleaved by NA to yield a quantifiable fluorescent product. The tested compounds were dissolved in DMSO and diluted to the corresponding concentrations in MES buffer (32.5 mM 2-(N-morpholino)-ethanesulfonic acid, 4 mM $CaCl_2$, pH 6.5). In a 96-well plate, 10 μL of the diluted virus supernatant, 70 μL of MES buffer and 10 μL of compounds at different concentrations were added successively and incubated for 5 min at 37 °C. The reaction was started by the addition of the substrate. After incubation for 30 to 60 min, the reaction was terminated by adding 150 μL 0.2 M glycine-NaOH (pH 10.2) in water. Fluorescence was recorded (excitation at 360 nm and emission at 450 nm) and substrate blanks were subtracted from the sample readings. The 50% inhibitory concentration (IC_{50}) was calculated by plotting the percent of inhibition of NA activity versus the inhibitor concentration.

General Information on Synthesis. Oseltamivir phosphate was provided by Shandong Qidu Pharmaceutical Co., Ltd. The other commercially available chemicals and reagents were purchased from Aladdin, TCI, J&K, ENERGY CHEMICAL and

Sinopharm Chemical Reagent Co., Ltd with purities of at least 97%. All the tested compounds were found to be > 95% pure by HPLC analysis, which was performed on a Shimadzu HPLC instrument using a C18 column (5 μm，4.6 mm × 250 mm) with two solvent systems (either methanol/water (0.1% CF_3COOH in methanol) with a gradient elution of 55% to 90% methanol over 30 minutes at 1.0 mL/min, or acetonitrile/buffer (0.2 M KH_2PO_4, pH = 6) with a gradient elution of 10% to 80% acetonitrile over 25 minutes at 1.0 mL/min). The ^{1}H-NMR spectra were determined using either a Brucker Avance 300 spectrometer or the 600 model with TMS as an internal standard. The solvents for NMR were DMSO-d_6 (δ 2.5 for 1H) and CD_3OD (δ 3.3 for ^{1}H). ESI-MS was determined using an API 4000 LC/MS spectrometer (Applied Biosystems, USA). HRMS analysis was performed using an Agilent 6520 Q-TOF LC/MS spectrometer (Agilent, Germany). All reactions were monitored by thin-layer chromatography (TLC) on 25.4 × 76.2 mm silica gel plates (GF-254). The silica gel used for column chromatography was 200-300 mesh. Melting points were determined using an electrothermal melting point apparatus and were uncorrected.

Synthetic Methods. Synthesis of compound **9**. Compound **9** was synthesized according to the reported method.[25]

(3*R*,4*R*,5*S*)-4-acetamido-5-guanidino-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid trifluoroacetate salt (9). White solid (yield 52%), mp 140 °C. ^{1}H NMR (D_2O, 300 MHz): δ 6.79 (s, 1H), 4.29 (d, 1H, J = 8.7 Hz), 3.87 (dd, 1H, J = 10.8, 8.7 Hz), 3.72-3.82 (m, 1H), 3.44-3.52 (m, 1H), 2.80 (dd, 1H, J = 17.4, 5.1 Hz), 2.29-2.41 (m, 1H), 1.98 (s, 3H), 1.32-1.58 (m, 4H), 0.83 (t, 3H, J = 7.5 Hz), 0.78 (t, 3H, J = 7.5

Hz). ^{13}C NMR (D_2O, 75 MHz): δ 174.82, 169.41, 156.91, 138.33, 128.76, 84.34, 75.47, 54.91, 50.65, 29.93, 25.67, 25.30, 22.07, 8.61, 8.57. HRMS calcd for $C_{15}H_{27}N_4O_4$ $[M + H]^+$: 327.2032, found: *m*/*z* 327.2025.

Synthesis of compounds **11** and **14**. To a solution of compound **8** (0.55 g, 1.0 mmol) in 5.0 mL of anhydrous methanol, a solution of NH_2OK (0.14 g, 6.0 mmol) in 3.5 mL of anhydrous methanol was added. The mixture was stirred for 0.5 h and then concentrated under vacuum. The solution was acidified with 1 M HCl to pH 5-6 and extracted with EtOAc (3 × 10 mL). The organic layers were dried over $MgSO_4$ and evaporated under vacuum. The crude product was purified by column chromatography to give compound **10**, which was dissolved in 20.0 mL HCl/EtOAc. The mixture was stirred at room temperature overnight. The precipitate was filtered and then dried to give compound **11** as a white solid. Compound **14** was prepared using the same method from intermediate **12**.

(3*R*,4*R*,5*S*)-4-acetamido-5-guanidino-N-hydroxy-3-(pentan-3-yloxy)cyclohex-1-enecarboxamide hydrochloride (11). White solid (yield 59%), mp 142-143°C. ^{1}H NMR (D_2O, 300 MHz): δ 6.27 (s, 1H), 4.33 (d, 1H, *J* = 8.4 Hz), 3.69-3.87 (m, 2H), 3.40-3.47 (m, 1H), 2.66 (dd, 1H, *J* = 17.1, 5.1 Hz), 2.30–2.39 (m, 1H), 1.93 (s, 3H), 1.28-1.51 (m, 4H), 0.73-0.82 (m, 6H). ^{13}C NMR (D_2O, 75 MHz): δ 174.74, 156.81, 132.69, 84.46, 75.38, 54.93, 50.51, 29.89, 25.59, 25.20, 21.98, 8.52, 8.45. HRMS calcd for $C_{15}H_{28}N_5O_4$ $[M + H]^+$: 342.2141, found: *m*/*z* 342.2134.

(3*R*,4*R*,5*S*)-4-acetamido-5-amino-N-hydroxy-3-(pentan-3-yloxy)cyclohex-1-enecarboxamide hydrochloride (14). White solid (yield 49%), mp 136-138°C. ^{1}H

NMR (D_2O, 300 MHz): δ 6.34 (s, 1H), 4.26 (d, 1H, J = 9.0 Hz), 3.98-4.05 (m, 1H), 3.54-3.62 (m, 1H), 3.44-.53 (m, 1H), 2.81 (dd, 1H, J = 17.1, 5.1 Hz), 2.46-2.56 (m, 1H), 2.03 (s, 3H), 1.35-1.58 (m, 4H), 0.76-0.85 (m, 6H). ^{13}C NMR (D_2O, 75 MHz): δ 175.27, 166.63, 132.83, 128.22, 84.47, 75.05, 52.65, 49.07, 28.23, 25.46, 25.06, 22.42, 8.52, 8.47. HRMS calcd for $C_{14}H_{26}N_3O_4$ $[M+H]^+$: 300.1923, found: m/z 300.1915.

The general procedure for the synthesis of compounds **17a-l**. To a solution of oseltamivir phosphate (0.82 g, 2.0 mmol) and aldehyde (2.4 mmol, 1.2 eq) in 25 mL ethanol, $NaBH_3CN$ (0.25 g, 4.0 mmol, 2 eq) was slowly added. The reaction was stirred at room temperature for 6 h and then concentrated. To the residue, 20 mL saturated $NaHCO_3$ solution was added and the mixture was extracted with EtOAc. The combined extracts were dried over anhydrous $MgSO_4$ and concentrated to give the crude product, intermediate **15**. Compound **15l** (containing a biphenyl group at R_2), white solid (84%), ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.53-7.60 (m, 4H), 7.30-7.46 (m, 5H), 6.80 (s, 1H), 5.50 (d, 1H, J = 7.2 Hz), 4.18-4.26 (m, 3H), 3.93-3.98 (m, 1H), 3.72-3.82 (m, 2H), 3.33-3.41 (m, 1H), 3.15-3.24 (m, 1H), 2.80 (dd, 1H, J = 17.7, 5.1 Hz), 2.22-2.35 (m, 1H), 2.01 (s, 3H), 1.46-1.56 (m, 4H), 1.30 (t, 3H, J = 7.2 Hz), 0.90 (t, 6H, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.65, 166.52, 140.92, 139.95, 139.29, 137.19, 129.38, 128.75, 128.57, 127.35, 127.17, 127.03, 81.77, 74.53, 60.88, 55.81, 53.60, 50.25, 30.41, 26.15, 25.77, 23.76, 14.24, 9.52, 9.42. HRMS calcd for $C_{29}H_{39}N_2O_4$ $[M+H]^+$: 479.2910, found: m/z 479.2904.

Without further purification, intermediate **15** (1.0 mmol), Et_3N (0.29 ml, 2.0 mmol, 2 eq) and $SC(NHBoc)_2$ (0.33 g, 1.2 mmol, 1.2 eq) were dissolved in 15 ml

DMF, followed by the addition of $HgCl_2$ (0.41 g, 1.5 mmol, 1.5 eq). The reaction mixture was stirred at room temperature for 3 h and was subsequently diluted with EtOAc and filtered through a pad of Celite. The filtrate was evaporated under reduced pressure to give a colorless oil, which was dissolved in 15 ml CH_3OH and 5 ml H_2O. NaOH (0.24 g, 6.0 mmol, 6 eq) was added and the solution was stirred at room temperature for 1 h. After adjusting the pH to 5-6 with 1 M HCl, the mixture was extracted with EtOAc. The combined extracts were dried and concentrated. Chromatographic purification afforded compound **16**.

A solution of compound **16** (0.20 mmol) and TFA (1.0 mL, 13.0 mmol) in CH_2Cl_2 (1.0 mL) was stirred at room temperature for 24 h. The solvent was evaporated and dried under vacuum. The residue was triturated in ether to give a white solid, which was collected by filtration. The solid was dried to give compounds **17a**-**l**.

(3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-(1-(thiophen-2-ylmethyl)guanidino)cylohex-1-enecarboxylic acid trifluoroacetate salt (17a). White solid (63%), mp 128-132 °C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.38 (dd, 1H, J = 5.1, 1.2 Hz), 7.07 (dd, 1H, J = 3.6, 1.2 Hz), 7.00 (dd, 1H, J = 5.1, 3.6 Hz), 6.84 (s, 1H), 4.8 (s, 2H), 4.25-4.32 (m, 2H), 4.13-4.20 (m, 1H), 3.39-3.47 (m, 1H), 2.85 (dd, 1H, J = 17.4, 5.1 Hz), 2.60-2.71 (m, 1H), 2.01 (s, 3H), 1.49-1.58 (m, 4H), 0.95 (t, 3H, J = 7.2 Hz), 0.91 (t, 3H, J = 7.2 Hz). ^{13}C NMR (CD_3OD, 75 MHz): δ 171.80, 166.92, 157.73, 138.36, 136.92, 128.25, 126.02, 125.36, 124.49, 81.90, 74.99, 55.93, 52.14, 42.22, 27.89, 25.37, 24.85, 21.07, 7.93, 7.67. HRMS calcd for $C_{20}H_{31}N_4O_4S$ $[M+H]^+$: 423.2066,

found: *m*/*z* 423.2065.

(3*R*,4*R*,5*S*)-4-acetamido-5-(1-benzylguanidino)-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid trifluoroacetate salt (17b). White solid (36%), mp 122-124°C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.56 (br, 1H), 8.01 (d, 1H, J = 8.7 Hz), 7.51 (br, 4H), 7.37 (t, 2H, J = 7.5 Hz), 7.26 (t, 1H, J = 7.5 Hz),7.14 (d, 1H, J = 7.5 Hz), 6.62 (s, 1H), 4.49-4.64 (m, 2H), 4.12-4.26 (m, 2H), 3.98-4.11 (m, 1H), 3.35-3.43 (m, 1H), 2.51-2.63 (m, 1H), 2.30-2.40 (m, 1H), 1.84 (s, 3H), 1.40-1.50 (m, 4H), 0.85 (t, 3H, J = 7.2 Hz), 0.80 (t, 3H, J = 7.2 Hz). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 169.37, 166.94, 157.88, 137.33, 136.94, 128.88, 128.40, 126.87, 125.76, 81.16, 75.16, 56.00, 51.64, 45.86, 27.97, 25.72, 25.10, 22.76, 9.40, 8.86. HRMS calcd for $C_{22}H_{33}N_4O_4$ $[M+H]^+$: 417.2502, found: *m*/*z* 417.2548.

(3*R*,4*R*,5*S*)-4-acetamido-5-(1-(4-isopropylbenzyl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid trifluoroacetate salt (17c). White solid (62%), mp 127-130°C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.62 (br, 1H), 7.99 (d, 1H, J = 8.1 Hz), 7.46 (br, 4H), 7.24 (d, 2H, J = 8.1 Hz), 7.05 (d, 2H, J = 8.1 Hz), 6.62 (s, 1H), 4.44-4.59 (m, 2H), 4.11-4.25 (m, 2H), 3.98-4.10 (m, 1H), 3.35-3.43 (m, 1H), 2.80-2.94 (m, 1H), 2.56-2.67 (m, 1H), 2.30-2.42 (m, 1H), 1.84 (s, 3H), 1.39-1.50 (m, 4H), 1.19 (d, 6H, J = 6.9 Hz), 0.85 (t, 3H, J = 7.2 Hz), 0.80 (t, 3H, J = 7.2 Hz). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 169.35, 166.97, 157.80, 146.98, 137.35, 134.19, 128.90, 126.33, 125.71, 81.17, 75.15, 55.98, 51.71, 45.66, 33.01, 27.96, 25.71, 25.09, 23.87, 23.81, 22.76, 9.40, 8.85. HRMS calcd for $C_{25}H_{39}N_4O_4$ $[M+H]^+$: 459.2971, found: *m*/*z* 459.2966.

(3*R*,4*R*,5*S*)-4-acetamido-5-(1-(2-methoxybenzyl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid trifluoroacetate salt (17d). White solid (54%), mp 126-130°C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.62 (br, 1H), 8.00 (d, 1H, J = 8.1 Hz), 7.58 and 7.37 (br, 4H), 7.27 (dt, 1H, J = 8.4, 1.5 Hz), 6.89-7.03 (m, 3H), 6.60 (s, 1H), 4.36-4.51 (m, 2H), 4.14-4.26 (m, 2H), 3.95-4.08 (m, 1H), 3.82 (s, 3H), 3.35-3.43 (m, 1H), 2.49-2.58 (m, 1H, partially merged in DMSO), 2.03-2.16 (m, 1H), 1.82 (s, 3H), 1.33-1.51 (m, 4H), 0.84 (t, 3H, J = 7.2 Hz), 0.80 (t, 3H, J = 7.2 Hz). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 169.40, 166.86, 157.94, 156.22, 137.30, 128.75, 128.12, 124.95, 124.16, 120.05, 110.58, 81.22, 75.02, 55.54, 55.22, 51.57, 42.24, 27.66, 25.73, 25.15, 22.69, 9.37, 8.90. HRMS calcd for $C_{23}H_{35}N_4O_5$ $[M + H]^+$: 447.2607, found: *m*/*z* 447.2602.

(3*R*,4*R*,5*S*)-5-(1-([1,1'-biphenyl]-4-ylmethyl)guanidino)-4-acetamido-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid trifluoroacetate salt (17e). White solid (58%), mp 124-128°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.60-7.68 (m, 4H), 7.41-7.47 (m, 2H), 7.30-7.37 (m, 3H), 6.85 (s, 1H), 4.61-4.86 (m, 2H), 4.31-4.51 (m, 2H), 4.16-4.23 (m, 1H), 3.41-3.49 (m, 1H), 2.84 (dd, 1H, J = 17.4, 5.1 Hz), 2.47-2.57 (m, 1H), 2.02 (s, 3H), 1.46-1.61 (m, 4H), 0.95 (t, 3H, J = 7.5 Hz), 0.92 (t, 3H, J = 7.5 Hz). ^{13}C NMR (CD_3OD, 75 MHz): δ 171.82, 166.97, 157.86, 139.95, 139.85, 137.02, 134.16, 128.13, 128.01, 126.61, 126.50, 126.00, 125.58, 81.94, 74.88, 55.68, 52.06, 27.54, 25.37, 24.88, 21.09, 7.93, 7.71. HRMS calcd for $C_{28}H_{37}N_4O_4$ $[M + H]^+$: 493.2815, found: *m*/*z* 493.2807.

(3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-(1-propylguanidino)cyclohex-

1-enecarboxylic acid trifluoroacetate salt (17f). White solid (49%), mp 128-132°C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.69 (br, 1H), 7.89 (d, 1H, J = 7.8 Hz), 7.32 (br, 4H), 6.62 (s, 1H), 4.14-4.20 (m, 1H), 3.91-4.00 (m, 2H), 3.32-3.40 (m, 1H), 3.06-3.20 (m, 2H), 2.51-2.56 (m, 2H, partially emerged in DMSO), 1.79 (s, 3H), 1.52-1.68 (m, 1H), 1.23-1.50 (m, 5H), 0.75-0.87 (m, 9H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 169.12, 166.96, 156.99, 137.09, 129.03, 80.96, 75.21, 55.50, 51.52, 44.33, 28.01, 25.64, 25.02, 22.66, 20.91, 10.62, 9.36, 8.75. HRMS calcd for $C_{18}H_{33}N_4O_4$ $[M + H]^+$: 369.2502, found: m/z 369.2515.

(3*R*,4*R*,5*S*)-4-acetamido-5-(1-isobutylguanidino)-3-(pentan-3-yloxy)cyclohex -1-enecarboxylic acid trifluoroacetate salt (17g). White solid (43%), mp 140-143°C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.69 (br, 1H), 7.93 (d, 1H, J = 8.4 Hz), 7.40 (br, 4H), 6.61 (s, 1H), 4.12-4.21 (m, 1H), 3.92-4.18 (m, 1H), 3.82-3.90 (m, 1H), 3.31-3.40 (m, 1H), 3.22 (dd, 1H, J = 15.3, 6.0 Hz), 2.81-2.90 (m, 1H), 2.51-2.60 (m, 2H), 1.90-2.00 (m, 1H), 1.79 (s, 3H), 1.32-1.49 (m, 4H), 0.75-0.87 (m, 12H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 169.20, 167.09, 158.16, 136.99, 129.17, 80.91, 75.12, 55.50, 52.22, 48.87, 28.62, 25.66, 25.02, 22.78, 19.66, 19.55, 9.44, 8.77. HRMS calcd for $C_{19}H_{35}N_4O_4$ $[M + H]^+$: 383.2658, found: m/z 383.2655.

(3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-(1-pentylguanidino)cyclohex-1 -enecarboxylic acid trifluoroacetate salt (17h). White solid (38%), mp 124-127°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 6.83 (s, 1H), 4.27 (d, 1H, J = 6.9 Hz), 4.11-4.20 (m, 1H), 4.02-4.09 (m, 1H), 3.37-3.45 (m, 1H), 3.21-3.28 (m, 2H), 2.75 (dd, 1H, J = 17.1, 5.1 Hz), 2.56-2.65 (m, 1H), 1.96 (s, 3H), 1.70-1.85 (m, 1H), 1.49-1.59 (m, 5H),

1.28-1.47 (m, 4H), 0.87-0.97 (m, 9H). ^{13}C NMR (CD_3OD, 75 MHz): δ 171.59, 167.06, 157.20, 137.06, 128.21, 81.88, 75.00, 55.43, 52.08, 43.31, 27.86, 27.62, 27.03, 25.35, 24.83, 21.55. 20.99, 12.38, 7.91, 7.65. HRMS calcd for $C_{20}H_{37}N_4O_4$ $[M + H]^+$: 397.2815, found: *m*/*z* 397.2808.

(3*R*,4*R*,5*S*)-4-acetamido-5-(1-butylguanidino)-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid trifluoroacetate salt (17i). White solid (41%), mp 116-118°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 6.83 (s, 1H), 4.27 (d, 1H, J = 6.9 Hz), 4.11-4.18 (m, 1H), 4.02-4.10 (m, 1H), 3.33-3.45 (m, 1H), 3.22-3.30 (m, 2H), 2.75 (dd, 1H, J = 17.1, 5.1 Hz), 2.57-2.67 (m, 1H), 1.97 (s, 3H), 1.70-1.80 (m, 1H), 1.45-1.60 (m, 5H), 1.31-1.43 (m, 2H), 0.89-1.01 (m, 9H). ^{13}C NMR (CD_3OD, 75 MHz): δ 171.60, 167.06, 157.19, 137.05, 128.22, 81.87, 75.00, 55.43, 52.10, 29.40, 27.61, 25.35, 24.83, 20.99, 18.95, 12.21, 7.92, 7.65. HRMS calcd for $C_{19}H_{35}N_4O_4$ $[M + H]^+$: 383.2658, found: *m*/*z* 383.2652.

(3*R*,4*R*,5*S*)-4-acetamido-5-(1-isopentylguanidino)-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid trifluoroacetate salt (17j). White solid (33%), mp 117-119°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 6.83 (s, 1H), 4.26 (d, 1H, J = 6.9 Hz), 4.03-4.19 (m, 2H), 3.38-3.45 (m, 1H), 3.21-3.33 (m, 2H), 2.75 (dd, 1H, J = 17.1, 5.1 Hz), 2.53-2.63 (m, 1H), 1.97 (s, 3H), 1.61-1.81 (m, 2H), 1.45-1.59 (m, 4H), 1.20-1.33 (m, 1H), 0.87-1.00 (m, 12H). ^{13}C NMR (CD_3OD, 75 MHz): δ 171.64, 167.04, 157.19, 137.10, 128.17, 81.94, 75.03, 55.40, 52.05, 42.35, 35.99, 27.59, 25.61, 25.35, 24.83, 21.20, 20.98, 20.51, 7.92, 7.66. HRMS calcd for $C_{20}H_{37}N_4O_4$ $[M + H]^+$: 397.2815, found: *m*/*z* 397.2808.

(3*R*,4*R*,5*S*)-4-acetamido-5-(1-(2-ethylbutyl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid trifluoroacetate salt (17k). White solid (68%), mp 147-148°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 6.82 (s, 1H), 4.22-4.38 (m, 1H), 4.11-4.18 (m, 1H), 3.98-4.08 (m, 1H), 3.36-3.45 (m, 1H), 3.26-3.30 (m, 1H), 3.10 (dd, 1H, J = 15.9, 9.3 Hz), 2.81 (dd, 1H, J = 17.1, 4.8 Hz), 2.55-2.65 (m, 1H), 1.98 (s, 3H), 1.73-1.83 (m, 1H), 1.47-1.59 (m, 4H), 1.30-1.44 (m, 4H), 0.87-0.98 (m, 12H). ^{13}C NMR (CD_3OD, 75 MHz): δ 171.66, 167.07, 158.56, 136.92, 128.24, 81.83, 75.27, 56.95, 52.51, 38.09, 28.22, 25.31, 24.78, 22.45, 22.15, 21.10, 9.65, 8.76, 7.93, 7.61. HRMS calcd for $C_{21}H_{39}N_4O_4$ $[M+H]^+$: 411.2971, found: *m*/*z* 411.2966.

(3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-(1-(3-phenylpropyl)guanidino)cyclohex-1-enecarboxylic acid trifluoroacetate salt (17l). White solid (44%), mp 75-78°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.15-7.32 (m, 5H), 6.79 (s, 1H), 4.24 (d, 1H, J = 8.1 Hz), 4.08-4.18 (m, 1H), 3.93-4.00 (m, 1H), 3.34-3.44 (m, 1H), 3.21-3.33 (m, 2H, partially merged in H_2O), 2.53-2.77 (m, 3H), 2.38-2.47 (m, 1H), 2.05-2.12 (m, 1H), 1.96 (s, 3H), 1.67-1.79 (m, 1H), 1.46-1.58 (m, 4H), 0.93 (t, 3H, J = 7.5 Hz), 0.89 (t, 3H, J = 7.5 Hz). ^{13}C NMR (CD_3OD, 75 MHz): δ 171.58, 166.98, 157.22, 140.34, 136.97, 128.13, 127.69, 127.54, 125.30, 81.91, 74.93, 55.45, 52.00, 42.80, 31.65, 29.14, 27.39, 25.34, 24.83, 20.99, 7.90, 7.66. HRMS calcd for $C_{24}H_{37}N_4O_4$ $[M+H]^+$: 445.2815, found: *m*/*z* 445.2810.

The general procedure for the synthesis of compounds **20a-m**. Method A: To a solution of intermediate **15** (1.0 mmol) and Et_3N (0.29 ml, 2 eq) in 20 ml THF was added $(Boc)_2O$ (0.22 g, 1.0 mmol) in one portion. The reaction mixture was stirred

for 10 h at room temperature and then concentrated. The residue was then hydrolyzed with NaOH, followed by chromatographic purification to give compound **19**, which was treated with TFA to give the target compounds according to the previous method. Method B: To a solution of compound **15** (1.0 mmol) in 15 mL CH_3OH and 5 mL H_2O, NaOH (0.24 g, 6 mmol, 6 eq) was added. The reaction mixture was stirred at 50 °C for 4 h. After the reaction was complete, the pH was adjusted to 5-6 with acetic acid. The solvent was evaporated under vacuum and the obtained residue was purified by column chromatography with CH_2Cl_2 and CH_3OH to give the target compounds.

(3*R*,4*R*,5*S*)-4-acetamido-5-((2-ethylbutyl)amino)-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid (20a). White solid (32%), mp 138 °C. ^{1}H NMR (CD_3OD, 300 MHz): δ 6.89 (s, 1H), 4.29 (d, 1H, J = 7.2 Hz), 4.17 (dd, 1H, J = 10.8 Hz, 8.1 Hz), 3.61-3.72 (m, 1H), 3.44-3.52 (m, 1H), 3.02-3.10 (m, 2H), 2.96 (dd, 1H, J = 17.4 Hz, 5.4 Hz), 2.57-2.66 (m, 1H), 2.07 (s, 3H), 1.34-1.70 (m, 9H), 0.89-0.99 (m, 12H). ^{13}C NMR (CD_3OD, 75 MHz): δ 172.83, 166.65, 136.49, 126.90, 81.87, 74.06, 54.99, 50.62, 37.41, 25.24, 25.14, 24.75, 22.17, 22.04, 21.43, 8.66, 8.62, 7.92, 7.64. HRMS calcd for $C_{20}H_{37}N_2O_4$ $[M+H]^+$: 369.2753, found: m/z 369.2749.

(3*R*,4*R*,5*S*)-4-acetamido-5-(butylamino)-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid acetate salt (20b). White solid (21%), mp 85-86°C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.91 (d, 1H, J = 8.7 Hz), 6.57 (s, 1H), 4.02 (d, 1H, J = 8.1 Hz), 3.61-3.72 (m, 1H), 3.30-3.38 (m, 1H), 2.80-2.89 (m, 1H), 2.60-2.69 (m, 2H), 2.49-2.58 (m, 1H, partially emerged in DMSO), 2.00-2.12 (m, 1H), 1.90 (s, 3H), 1.84 (s, 3H), 1.23-1.45 (m, 8H), 0.76-0.89 (m, 9H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ

177.25, 174.93, 173.00, 141.75, 134.98, 86.01, 80.53, 60.00, 58.72, 50.22, 36.21, 34.97, 30.83, 30.33, 28.23, 26.33, 24.95, 19.00, 14.64, 14.12. HRMS calcd for $C_{18}H_{33}N_2O_4$ $[M + H]^+$: 341.2440, found: *m*/*z* 341.2433.

(3*R*,4*R*,5*S*)-4-acetamido-5-(isobutylamino)-3-(pentan-3-yloxy)cyclohex-1-ene carboxylic acid acetate salt (20c). White solid (31%), mp 48-50°C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.92 (d, 1H, J = 9.0 Hz), 6.47 (s, 1H), 3.97 (d, 1H, J = 8.1 Hz), 3.58-3.68 (m, 1H), 3.28-3.36 (m, 1H), 2.56-2.74 (m, 2H), 2.28-2.40 (m, 2H), 1.92-2.03 (m, 1H), 1.87 (s, 3H), 1.82 (s, 3H), 1.53-1.66 (m, 1H), 1.33-1.49 (m, 4H), 0.81-0.86 (m, 12H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 177.67, 174.73, 174.19, 139.60, 137.18, 85.86, 81.00, 60.54, 59.33, 58.94, 36.13, 33.17, 30.90, 30.38, 28.14, 26.81, 25.73, 25.65, 14.67, 14.14. HRMS calcd for $C_{18}H_{33}N_2O_4$ $[M + H]^+$: 341.2440, found: *m*/*z* 341.2434.

(3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-((thiophen-2-ylmethyl)amino) cyclohex-1-enecarboxylic acid acetate salt (20d). White solid (27%), mp 86-88°C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.80 (d, 1H, J = 9.0 Hz), 7.37 (dd, 1H, J = 4.5, 1.8 Hz), 6.93-6.97 (m, 2H), 6.59 (s, 1H), 3.94-4.01 (m, 2H), 3.85-3.91 (m, 1H), 3.66-3.76 (m, 1H), 3.31-3.38 (m, 1H), 2.71-2.80 (m, 1H), 2.64 (dd, 1H, J = 17.4, 4.8 Hz), 2.01-2.11 (m, 1H), 1.91 (s, 3H), 1.85 (s, 3H), 1.33-1.49 (m, 4H), 0.83 (t, 3H, J = 7.5 Hz), 0.79 (t, 3H, J = 7.5 Hz). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 177.22, 174.83, 172.84, 150.35, 142.52, 134.61, 131.77, 129.74, 129.56, 86.06, 80.58, 59.59, 59.17, 49.78, 35.63, 30.85, 30.39, 28.23, 26.27, 14.65, 14.17. HRMS calcd for $C_{19}H_{29}N_2O_4S$ $[M + H]^+$: 381.1848, found: *m*/*z* 381.1843.

(3*R*,4*R*,5*S*)-4-acetamido-5-(benzylamino)-3-(*pentan*-3-yloxy)cyclohex-1-enec arboxylic acid trifluoroacetate salt (20e). White solid (52%), mp 80-82°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.45-7.51 (m, 5H), 6.90 (s, 1H), 4.40-4.45 (m, 2H), 4.18-4.30 (m, 3H), 3.57-3.67 (m, 1H), 3.42-3.50 (m, 1H), 3.05 (dd, 1H, J = 17.4 Hz, 5.7 Hz), 2.62-2.73 (m, 1H), 2.06 (s, 3H), 1.49-1.60 (m, 4H), 0.93 (t, 3H, J = 7.2 Hz), 0.91 (t, 3H, J = 7.2 Hz). ^{13}C NMR (CD_3OD, 75 MHz): δ 173.02, 166.58, 136.78, 130.25, 129.01, 128.85, 128.48, 126.69, 81.86, 74.00, 54.32, 50.94, 25.25, 25.21, 24.71, 21.41, 7.88, 7.63. HRMS calcd for $C_{21}H_{31}N_2O_4$ $[M + H]^+$: 375.2284, found: *m*/*z* 375.2280.

(3*R*,4*R*,5*S*)-4-acetamido-5-((2-methoxybenzyl)amino)-3-(pentan-3-yloxy)cycl ohex-1-enecarboxylic acid trifluoroacetate salt (20f). White solid (39%), mp 75-78°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.48 (t, 1H, J = 7.8 Hz), 7.40 (d, 1H, J = 7.8 Hz), 7.13 (d, 1H, J = 7.8 Hz), 7.04 (t, 1H, J = 7.8 Hz), 6.90 (s, 1H), 4.31-4.40 (m, 2H), 4.21-4.30 (m, 1H), 4.12-4.19 (m, 1H), 3.96 (s, 3H), 3.56-3.66 (m, 1H), 3.42-3.50 (m, 1H), 3.07 (dd, 1H, J = 17.4 Hz, 5.4 Hz), 2.65-2.76 (m, 1H), 2.07 (s, 3H), 1.49-1.60 (m, 4H), 0.93 (t, 3H, J = 7.5 Hz), 0.91 (t, 3H, J = 7.5 Hz). ^{13}C NMR (CD_3OD, 75 MHz): δ 172.88, 166.57, 157.43, 136.63, 130.97, 130.73, 126.75, 120.38, 118.06, 110.28, 81.76, 73.79, 54.45, 54.39, 51.09, 43.85, 25.17, 25.05, 24.69, 21.36, 7.86, 7.61. HRMS calcd for $C_{22}H_{33}N_2O_5$ $[M + H]^+$: 405.2389, found: *m*/*z* 405.2384.

(3*R*,4*R*,5*S*)-4-acetamido-5-((2-hydroxybenzyl)amino)-3-(pentan-3-yloxy)cycl ohex-1-enecarboxylic acid (20g). White solid (36%), mp 148-150°C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.90 (d, 1H, J = 9.3 Hz), 7.04-7.10 (m, 2H), 6.68-6.73 (m, 2H), 6.60 (s, 1H), 4.03 (d, 1H, J = 8.1 Hz), 3.88-3.93 (m, 1H), 3.64-3.77 (m, 2H),

3.31-3.39 (m, 1H), 2.70-2.79 (m, 2H), 2.04-2.14 (m, 1H), 1.86 (s, 3H), 1.23-1.53 (m, 4H), 0.83 (t, 3H, J = 7.2 Hz), 0.76 (t, 3H, J = 7.2 Hz). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 174.85, 172.75, 162.71, 142.67, 134.37, 133.82, 133.16, 129.40, 123.61, 120.69, 86.10, 80.57, 60.01, 58.98, 52.92, 34.86, 30.88, 30.33, 28.23, 14.68, 14.11. HRMS calculated for calcd for $C_{21}H_{31}N_2O_5$ $[M + H]^+$: 391.2233, found: m/z 391.2228.

(3*R*,4*R*,5*S*)-4-acetamido-5-((2-chlorobenzyl)amino)-3-(pentan-3-yloxy)cycloh ex-1-enecarboxylic acid acetate salt (20h). White solid (28%), mp 75-77°C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.83 (d, 1H, J = 9.0 Hz), 7.49 (dd, 1H, J = 7.2, 1.8 Hz), 7.41 (dd, 1H, J = 7.2, 1.8 Hz), 7.31 (dt, 1H, J = 7.2, 1.2 Hz), 7.27 (dt, 1H, J = 7.2, 1.2 Hz), 6.61 (s, 1H), 4.01 (d, 1H, J = 8.4 Hz), 3.77-3.88 (m, 2H), 3.62-3.73 (m, 1H), 3.30-3.38 (m, 1H), 2.66-2.73 (m, 2H), 1.98-2.08 (m, 1H), 1.91 (s, 3H), 1.83 (s, 3H), 1.30-1.49 (m, 4H), 0.83 (t, 3H, J = 7.2 Hz), 0.78 (t, 3H, J = 7.2 Hz). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 177.21, 174.83, 172.77, 143.23, 142.71, 137.81, 135.37, 134.36, 134.32, 133.63, 132.28, 86.03, 80.42, 59.84, 59.54, 52.55, 35.73, 30.86, 30.34, 28.21, 26.27, 14.68, 14.12. HRMS calcd for $C_{21}H_{30}ClN_2O_4$ $[M + H]^+$: 409.1894, found: m/z 409.1889.

(3*R*,4*R*,5*S*)-4-acetamido-5-((2-bromobenzyl)amino)-3-(pentan-3-yloxy)cyclo hex-1-enecarboxylic acid trifluoroacetate salt (20i). White solid (45%), mp 86-88°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.74 (dd, 1H, J = 8.1, 1.2 Hz), 7.60 (dd, 1H, J = 7.5, 1.5 Hz), 7.49 (dt, 1H, J = 7.5, 1.2 Hz), 7.40 (dt, 1H, J = 7.8, 1.5 Hz), 6.91 (s, 1H), 4.46-4.60 (m, 2H), 4.28-4.33 (m, 1H), 4.17-4.25 (m, 1H), 3.75-3.85 (m, 1H), 3.43-3.51 (m, 1H), 3.10 (dd, 1H, J = 17.4, 5.7 Hz), 2.72-2.84 (m, 1H), 2.07 (s, 3H),

1.50-1.61 (m, 4H), 0.94 (t, 3H, J = 7.5 Hz), 0.91 (t, 3H, J = 7.5 Hz). ^{13}C NMR (CD_3OD, 75 MHz): δ 173.14, 166.55, 136.76, 132.74, 131.29, 130.92, 130.15, 127.77, 126.71, 123.94, 81.84, 74.00, 55.27, 51.10, 25.37, 25.22, 24.72, 21.50, 7.91, 7.65. HRMS calcd for $C_{21}H_{30}BrN_2O_4$ $[M + H]^+$: 453.1389, found: m/z 453.1377.

(3*R*,4*R*,5*S*)-4-acetamido-5-((2-fluorobenzyl)amino)-3-(pentan-3-yloxy)cycloh ex-1-enecarboxylic acid trifluoroacetate salt (20j). White solid (42%), mp 192-194°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.49-7.58 (m, 2H), 7.23-7.33 (m, 2H), 6.90 (s, 1H), 4.37-4.48 (m, 2H), 4.19-4.30 (m, 2H), 3.67-3.76 (m, 1H), 3.42-3.50 (m, 1H), 3.07 (dd, 1H, J = 17.4 Hz, 5.4 Hz), 2.66-2.77 (m, 1H), 2.06 (s, 3H), 1.50-1.61 (m, 4H), 0.94 (t, 3H, J = 7.5 Hz), 0.91 (t, 3H, J = 7.5 Hz). ^{13}C NMR (CD_3OD, 75 MHz): δ 173.03, 166.58, 162.57, 159.29, 136.85, 131.53, 131.41, 131.37, 126.73, 124.37, 124.32, 117.81, 117.61, 115.21, 114.93, 81.87, 74.17, 54.79, 50.88, 40.75, 25.33, 25.20, 24.70, 21.41, 7.92, 7.63. HRMS calcd for $C_{21}H_{30}FN_2O_4$ $[M + H]^+$: 393.2190, found: m/z 393.2180.

(3*R*,4*R*,5*S*)-4-acetamido-5-(cinnamylamino)-3-(pentan-3-yloxy)cyclohex-1-e necarboxylic acid (20k). White solid (32%), mp 144-146°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.21-7.52 (m, 5H), 6.92 (d, 1H, J = 15.9 Hz), 6.84 (s, 1H), 6.26-6.37 (m, 1H), 4.26 (d, 1H, J = 7.8 Hz), 4.07-4.14 (m, 1H), 3.95-4.03 (m, 1H), 3.84-3.92 (m, 1H), 3.59-3.71 (m, 1H), 3.43-3.51 (m, 1H), 3.08 (dd, 1H, J = 17.1, 5.4 Hz), 2.49-2.59 (m, 1H), 2.08 (s, 3H), 1.49-1.60 (m, 4H), 0.93 (t, 3H, J = 7.5 Hz), 0.91 (t, 3H, J = 7.5 Hz). ^{13}C NMR (CD_3OD, 75 MHz): δ 174.99, 168.60, 140.15, 136.87, 129.96, 129.85, 129.71, 129.43, 127.97, 119.01, 83.65, 75.87, 55.85, 53.53, 47.79, 27.57, 27.13, 26.62,

23.40, 9.78, 9.57. HRMS calcd for $C_{23}H_{33}N_2O_4$ $[M + H]^+$: 401.2440, found: *m*/*z* 401.2433.

(3*R*,4*R*,5*S*)-5-(([1,1'-biphenyl]-4-ylmethyl)amino)-4-acetamido-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid trifluoroacetate salt (20l). White solid (45%), mp 117-119°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.74 (d, 2H, *J* = 8.4 Hz), 7.65 (dd, 2H, *J* = 7.2, 1.2 Hz), 7.58 (d, 2H, *J* = 8.4 Hz), 7.47 (t, 2H, *J* = 7.2 Hz), 7.38 (t, 1H, *J* = 7.2 Hz), 6.90 (s, 1H), 4.44-4.50 (m, 1H), 4.30-4.35 (m, 1H), 4.19-4.26 (m, 2H), 3.59-3.69 (m, 1H), 3.42-3.50 (m, 1H), 3.08 (dd, 1H, *J* = 17.1, 5.4 Hz), 2.65-2.75 (m, 1H), 2.07 (s, 3H), 1.50-1.61 (m, 4H), 0.94 (t, 3H, *J* = 7.5 Hz), 0.89 (t, 3H, *J* = 7.5 Hz). ^{13}C NMR (CD_3OD, 75 MHz): δ 173.02, 166.67, 142.01, 139.36, 136.66, 129.55, 129.08, 128.11, 127.05, 126.93, 126.80, 126.10, 81.84, 73.95, 54.28, 51.03, 25.32, 25.21, 24.71, 21.41, 7.87, 7.63. HRMS calcd for $C_{27}H_{35}N_2O_4$ $[M + H]^+$: 451.2597, found: *m*/*z* 451.2595.

(3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-((3-phenylpropyl)amino)cyclohex-1-enecarboxylic acid (20m). White solid (51%), mp 79-80°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.18-7.40 (m, 5H), 6.89 (s, 1H), 4.24 (d, 1H, *J* = 9.0 Hz), 4.08 (dd, 1H, *J* = 10.8 Hz, 8.1 Hz), 3.57-3.66 (m, 1H), 3.41-3.49 (m, 1H), 3.13-3.23 (m, 1H), 3.00-3.10 (m, 1H), 2.94 (dd, 1H, *J* = 17.4, 5.4 Hz), 2.65-2.77 (m, 2H), 2.42-2.54 (m, 1H), 1.89-2.14 (m, 5H), 1.46-1.62 (m, 4H), 0.93 (t, 3H, *J* = 7.5 Hz), 0.89 (t, 3H, *J* = 7.5 Hz). ^{13}C NMR (CD_3OD, 75 MHz): δ 172.95, 166.60, 139.60, 136.63, 127.78, 127.49, 126.69, 125.57, 81.85, 73.71, 54.19, 51.17, 43.59, 31.66, 26.81, 25.23, 25.17, 24.68, 21.30, 7.82, 7.63. HRMS calcd for $C_{23}H_{35}N_2O_4$ $[M + H]^+$: 403.2597, found: *m*/*z*

403.2590.

Synthesis of compound **23**. To a mixture of 3-aminopropan-1-ol (0.75 g, 10 mmol) and Et_3N (2.8 mL, 20 mmol, 2 eq) in 50 mL THF was added $(Boc)_2O$ (2.4 g, 11 mmol, 1.1 eq) in one portion. The reaction mixture was stirred at room temperature for 6 h. After the reaction was complete, the solvent was evaporated and the residue was extracted with EtOAc (3 × 20 mL). The combined extracts were dried over $MgSO_4$ and then concentrated. The obtained residue was dissolved in 50 mL CH_2Cl_2, followed by the addition of PCC (3.2 g, 15.0 mmol, 1.5 eq). The mixture was stirred at room temperature for 4 h and then filtered through a pad of Celite to give a dark solution. 3-(Boc-amino)propanal was obtained by chromatographic purification with petroleum ether and ethyl acetate.[30] Compound **23** was synthesized according to the methods above.

(3*R*,4*R*,5*S*)-4-acetamido-5-((3-aminopropyl)amino)-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid trifluoroacetate salt (23). White solid (35%), mp 85-88 °C. ^{1}H NMR (D_2O, 300 MHz): δ 6.81 (s, 1H), 4.26 (d, 1H, J = 8.7 Hz), 4.02-4.10 (m, 1H), 3.55-3.65 (m, 1H), 3.44-3.50 (m, 1H), 3.17-3.27 (m, 1H), 3.07-3.13 (m, 1H), 3.01 (t, 2H, J = 7.8 Hz), 2.91 (dd, 1H, J = 17.1, 5.4 Hz), 2.40-2.51 (m, 1H), 1.89-2.14 (m, 5H), 1.34-1.55 (m, 4H), 0.80 (t, 3H, 7.2 Hz), 0.76 (t, 3H, 7.2 Hz). ^{13}C NMR (D_2O, 75 MHz): δ 175.42, 168.88, 137.98, 127.22, 84.30, 75.14, 54.77, 51.65, 41.22, 36.47, 25.71, 25.30, 24.94, 23.54, 22.45, 8.40, 8.36. HRMS calcd for $C_{17}H_{32}N_3O_4$ $[M+H]^+$: 342.2393, found: *m*/*z* 342.2384.

Synthesis of compounds **30**. The two 2-alkoxybenzaldehydes, (**25a**) and (**25b**),

were prepared according to the reported method.[31] Compounds **30a** and **30b** were synthesized using Method B.

Synthesis of aldehydes (**27**) and (**29**). To a solution of 4-bro-2-chlorobenzaldehyde (1.09 g, 5.0 mmol) in 15 mL THF, phenylboronic acid (0.61 g, 5.0 mmol, 1.0 eq), K_2CO_3 (1.04 g, 7.5 mmol, 1.5 eq) dissolved in 7.5 mL of water and $Pd(PPh_3)_4$ (0.12 g, 0.1 mmol) were added successively. The reaction mixture was heated at 80 °C for 4 h, followed by the addition of 20 mL water. The solution was extracted with EtOAc and the combined extracts were dried over $MgSO_4$. Chromatographic purification with petroleum ether and ethyl acetate gave 3-chloro-[1,1'-biphenyl]-4-carbaldehyde (**27a**) as a white solid, mp 90 °C. [1,1':3',1''-terphenyl]-4'-carbaldehyde (**27b**) was prepared in the same way using phenylboronic acid (1.22 g, 10.0 mmol, 2.0 eq). 4-(thiophen-2-yl)benzaldehyde (**29**) was synthesized from thiophen-2-ylboronic acid and 4-bromobenzaldehyde using the same method for preparing compound **27a**.

Synthesis of compounds **31** and **32**. Compounds **31** and **32** were synthesized using Method A.

(3*R*,4*R*,5*S*)-4-acetamido-5-(((3-chloro-[1,1'-biphenyl]-4-yl)methyl)amino)-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid trifluoroacetate salt (31a). White solid (44%), mp 93-96°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.83 (d, 1H, J = 1.8 Hz), 7.71 (dd, 1H, J = 8.1, 1.8 Hz), 7.64-7.69 (m, 3H), 7.40-7.53 (m, 3H), 6.92 (s, 1H), 4.49-4.60 (m, 2H), 4.19-4.32 (m, 2H), 3.74-3.84 (m, 1H), 3.43-3.52 (m, 1H), 3.12 (dd, 1H, J = 17.4, 5.7 Hz), 2.72-2.84 (m, 1H), 2.08 (s, 3H), 1.52-1.62 (m, 4H), 0.94 (t, 3H,

J = 7.5 Hz), 0.92 (t, 3H, *J* = 7.5 Hz). ^{13}C NMR (CD_3OD, 75 MHz): δ 175.03, 168.49, 146.04, 139.78, 138.61, 136.35, 133.63, 130.23, 129.67, 129.35, 128.92, 128.67, 128.06, 127.43, 83.75, 75.90, 57.16, 53.01, 46.56, 27.30, 27.14, 26.64, 23.38, 9.83, 7.56. HRMS calcd for $C_{27}H_{34}ClN_2O_4$ $[M+H]^+$: 485.2207, found: *m*/*z* 485.2201.

(3*R*,4*R*,5*S*)-5-(([1,1':3',1''-terphenyl]-4'-ylmethyl)amino)-4-acetamido-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid trifluoroacetate salt (31b). White solid (55%), mp 96-98°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.36-7.83 (m, 13H), 6.80 (s, 1H), 4.46-4.52 (m, 1H), 4.30-4.36 (m, 1H), 4.11-4.19 (m, 1H), 3.99 (dd, 1H, *J* = 10.8, 8.1 Hz), 3.36-3.47 (m, 2H), 2.45 (dd, 1H, *J* = 17.4, 5.7 Hz), 2.20-2.30 (m, 1H), 2.02 (s, 3H), 1.45-1.59 (m, 4H), 0.82-0.97 (m, 6H). ^{13}C NMR (CD_3OD, 75 MHz): δ 173.07, 166.27, 143.22, 141.89, 139.05, 138.82, 136.44, 129.33, 128.56, 128.51, 128.27, 128.19, 127.53, 127.22, 126.53, 126.39, 126.17, 126.09, 81.68, 73.52, 53.88, 51.31, 44.23, 25.11, 24.99, 24.63, 21.36, 7.83, 7.60. HRMS calcd for $C_{33}H_{39}N_2O_4$ $[M+H]^+$: 527.2910, found: *m*/*z* 527.2918.

(3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-((4-(thiophen-2-yl)benzyl)amino)cyclohex-1-enecarboxylic acid trifluoroacetate salt (32). White solid (51%), mp 94-96°C. ^{1}H NMR (CD_3OD, 300 MHz): δ 7.72 (d, 2H, *J* = 8.4 Hz), 7.49 (d, 2H, *J* = 8.4 Hz), 7.45 (d, 1H, *J* = 3.6 Hz), 7.42 (d, 1H, *J* = 5.1 Hz), 7.10 (dd, 1H, *J* = 5.1, 3.6 Hz), 6.88 (s, 1H), 4.38-4.44 (m, 1H), 4.17-4.28 (m, 3H), 3.57-3.67 (m, 1H), 3.40-3.48 (m, 1H), 3.05 (dd, 1H, *J* = 17.1, 5.7 Hz), 2.62-2.72 (m, 1H), 2.05 (s, 3H), 1.48-1.59 (m, 4H), 0.91 (t, 3H, *J* = 7.2 Hz), 0.89 (t, 3H, *J* = 7.2 Hz). ^{13}C NMR (CD_3OD, 75 MHz): δ 173.55, 167.11, 142.73, 137.33, 135.75, 130.27, 129.58, 127.96, 127.19,

125.95, 125.37, 123.76, 82.36, 74.52, 54.78, 51.50, 25.80, 25.71, 25.21, 21.94, 8.40, 8.15. HRMS calcd for $C_{25}H_{33}N_2O_4S$ $[M + H]^+$: 457.2161, found: *m/z* 457.2255.

ASSOCIATED CONTENT

Supporting Information

The amino acid sequences of the NAs (H5N1-1220 and H5N1-1206), the ^1H NMR and ^{13}C NMR spectra for the new compounds and the HPLC chromatograms associated with this paper is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

NA, neuraminidase; Boc, butyloxycarbonyl; MUNANA, 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid; OS, oseltamivir; PCC, pyridinium chlorochromate; SAR, structure-activity relationships; TLC, thin-layer chromatography.

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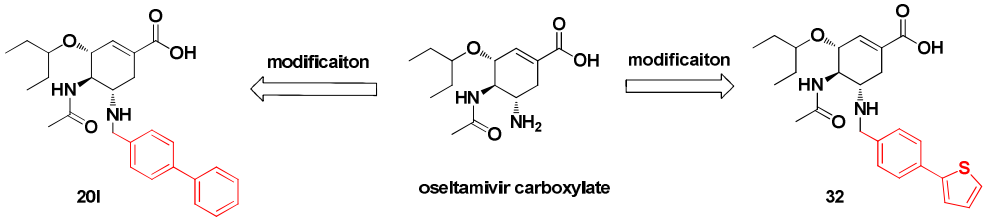
Table of Contents graphic:



20l

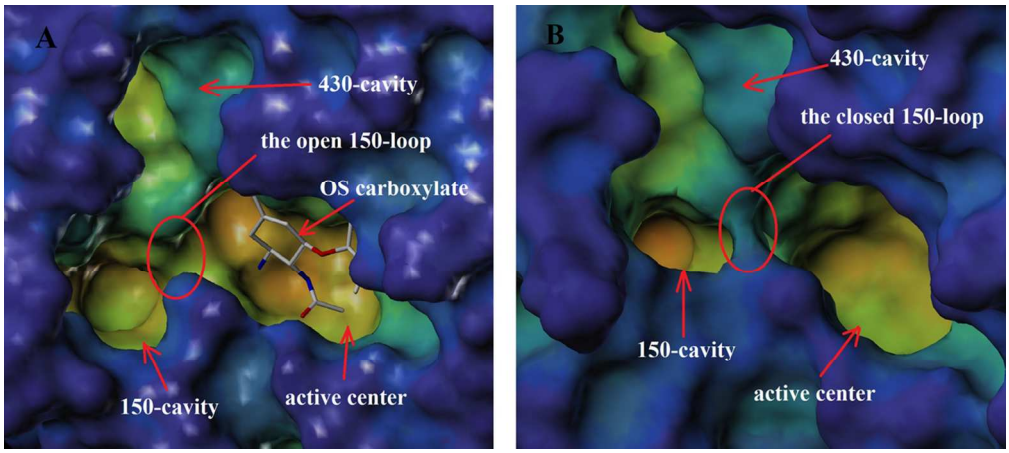
IC_{50} = 1.9 nM (NA, H5N1-1220)
IC_{50} = 580 nM (NA, H9N2-S2)
IC_{50} = 1160 nM (NA, H5N1-H274Y)

oseltamivir carboxylate

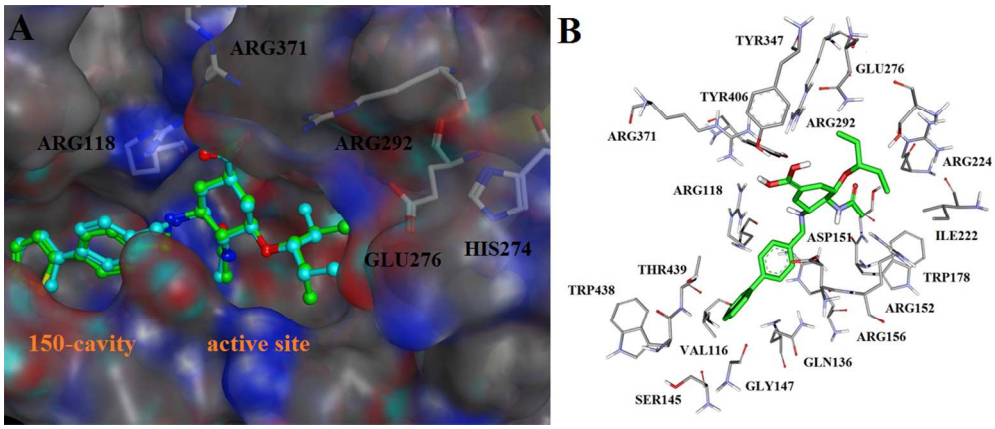
IC_{50} = 17 nM (NA, H5N1-1220)
IC_{50} = 3.1 nM (NA, H9N2-S2)
IC_{50} = 2100 nM (NA, H5N1-H274Y)

32

IC_{50} = 2.1 nM (NA, H5N1-1220)
IC_{50} = 210 nM (NA, H9N2-S2)
IC_{50} = 160 nM (NA, H5N1-H274Y)



Comparison of the crystal structures of N1 (PDB 2HU0, A) and N2 (PDB 4GZQ, B)
109x48mm (300 x 300 DPI)



The doking results of compound 20l (light blue) and 32 (green) with N1(PDB 2HU0, A) compared with the binding mode of OS carboxylate and the key residues that may form potential interactions with compound 20l (B).
110x46mm (299 x 299 DPI)