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Design, synthesis and biological evaluation of small molecular polyphenols as entry inhibitors against H_5N_1

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Abstract: To find novel compounds against H_5N_1, three series of known or novel small molecular polyphenols were synthesized and tested *in vitro* for anti-H_5N_1 activity. In addition, the preliminary structure-antiviral activity relationships were elaborated. The results showed that some small molecular polyphenols had better anti-H_5N_1 activity, and could serve as novel virus entry inhibitors against H_5N_1, likely targeting to HA2 protein. Noticeably, compound **4a** showed the strongest activity against H_5N_1 among these compounds, and the molecular modeling analysis also suggested that this compound might target to HA2 protein. Therefore, compound **4a** is well qualified to serve as a lead compound or scaffold for the further development of H_5N_1 entry inhibitor.

Keywords: synthesis; anti-H_5N_1 activity; small molecular polyphenols; entry inhibitor; HA2; molecular modeling

Influenza is considered to be one of the life threatening infectious diseases. In some countries seasonal influenza affects annually up to 40% of the population and 500 million people die from it worldwide every year. New highly-virulent influenza strains can arise unexpectedly to cause world-wide pandemics with markedly increased morbidity and mortality, such as the “avian flu” in 1997 and “swine flu” in 2009. [1] At present, only two classes of antiviral drugs, M2 ion channel blockers and Neuraminidase (NA) inhibitors, are licensed for use against influenza A viruses, but their utility has been limited by side effects and emergence of resistant viral strains. [2] Therefore, the development of new anti-influenza agents represents a crucial defense

strategy to combat seasonal and pandemic influenza strains.

Influenza virus entry inhibitors represent a new class of antiviral agents, which can block virus entry into the target cells. Hemagglutinin (HA) is the important target for developing influenza virus entry inhibitors, and HA is a trimeric glycoprotein in nature and can be cleaved into HA1 and HA2 subunits by host proteases. [2] Although HA1 is the target for developing influenza virus entry inhibitors, the high variability and mutation rate limit its application. Nevertheless, HA2 is for virus–endosome membrane fusion in the acid environment after the virus has been endocytosed into the cell and more conserved so that it may be a better target for developing influenza virus entry inhibitors. [3, 4] Up to now, some influenza virus entry inhibitors have been identified and discovered, which have become more and more exciting research areas.[1, 2, 5]

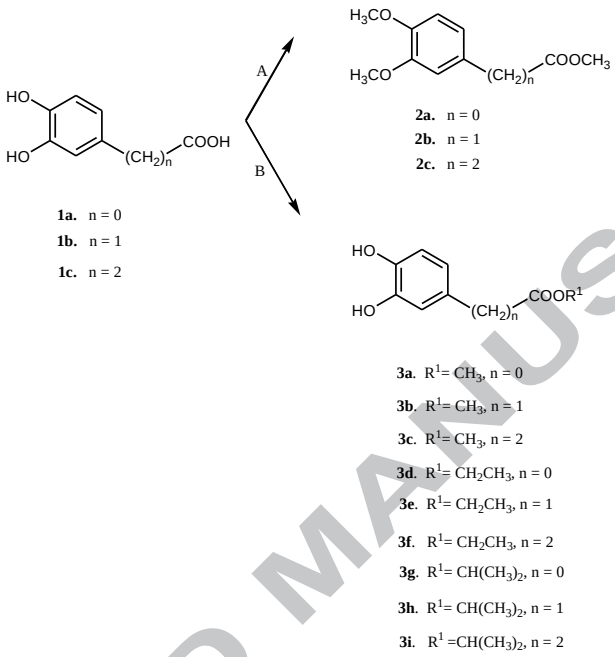
Hydroxytyrosol (HT), 3, 4-dihydroxyphenylethanol, is one of the major polyphenolic components of olive products. [6] In several special studies, it has been demonstrated that HT is antioxidant and antimicrobial and that it has beneficial effects on the cardio-vascular system and in several human diseases. [7-11] Moreover, HT was effective against the enveloped viruses, but not against the non-enveloped viruses. HT could inhibit influenza A viruses including H_1N_1, H_3N_2, H_5N_1, and H_9N_2 subtypes. More recently, it was reported that mechanism of the anti-H_9N_2 effect of HT was involved in morphological change of the virus. [12] Recent studies also had identified HT as the HIV-1 inhibitor, in which HT inhibited not only the fusion between viral and cellular membranes but also the integrase activity of the virus. [13, 14] Therefore, HT

is an interesting scaffold for design of novel antiviral agents.

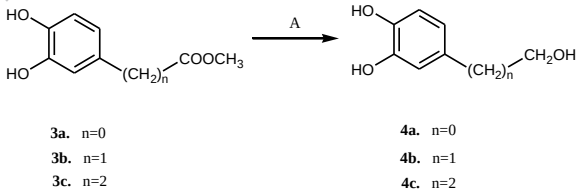
In order to further know about the antiviral activity of HT, HT was synthesized according to previous report (Schemes.1-2)[13] and was tested *in vitro* antiviral activity.[15, 16] In the antiviral screening experiments, we found that HT (compound **4b**) has a strong inhibitory effect on H_5N_1, and could inhibit H_5N_1 virus production most effectively at the virus entry step of cell infection (Fig.1). Interestingly, the starting material (compound **1b)** showed no activity against H_5N_1, but the intermediate (compound **3b)** exhibited potent activity against H_5N_1. The results implied that esterification of carboxylic acid, reduction of carboxylic acid to alcohol or other modification of carboxylic acid in small molecular polyphenols could significantly influence the anti-H_5N_1 activity. Therefore, the preliminary results stimulated interest in exploration of HT and its analogs in which phenolic hydroxyls, the length and functional groups of the side chain were altered to study their influence on the anti-H_5N_1 activity. In the meantime, our previous results showed that some amino acids were important moities in polyphenols as antiviral entry inhibitors, such as gossypol derivatives.[17-19]

Therefore, three series of known or novel small molecular polyphenols, such as aryl ester derivatives (compounds **2a**-**c** and **3a**-**i**), aromatic alcohol derivatives (compounds **4a**-**c**) and amino acids derivatives (compounds **5a**-**b**, **7a**-**b** and **8a**-**b**), were designed and synthesized respectively (Schemes.1-4), and their preliminary structure-activity relationships of antiviral activity were also investigated. Small molecular polyphenols were evaluated for their capability to inhibit the H_5N_1

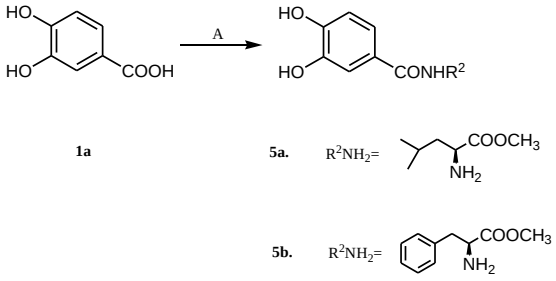
(Influenza strain A/Vietnam/1194/2004) replication in MDCK cells.[15, 16] Amantidine and Zanamivir were used as the reference drugs. The results, expressed as IC_{50}, CC_{50} and SI (selectivity index), are illustrated in Table 1.



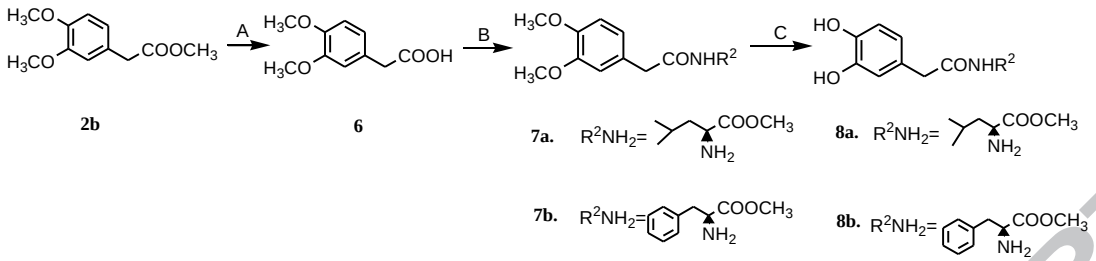
Scheme 1. Reagents and Conditions: (A) $(CH_3)_2SO_4$, K_2CO_3, acetone, 70℃, 6h, 85-95% yield; (B) For compounds **3a-c**: $SOCl_2$, methanol, rt, 8h, 95-97% yield; For compounds **3d-f**: $SOCl_2$, ethanol, rt, 8h, 90-95% yield; For compounds **3g-i**: $SOCl_2$, isopropanol, rt, 8h, 90-95% yield.



Scheme 2. Reagents and Conditions: (A) $LiAlH_4$, THF, 0℃, 8h, 72-75% yield.



Scheme 3. Reagents and Conditions: (A) EDCI, HOBT, DCM, R^2NH_2, 0℃-rt, 7h, 68-77% yield.



Scheme 4. Reagents and Conditions: (A) 20% Na_2CO_3, THF, 80℃, 10h, then 4M aq. HCl, 93-95% yield; (B): EDCI, HOBT, TEA, DCM, R^2NH_2, 0℃-rt, 7h, 78-81% yield; (C) BBr_3, DCM, -78℃, 8h, 72-78% yield.

Table 1

Inhibition of small molecular polyphenols on replication of H_5N_1 (Influenza strain A/Vietnam/1194/2004) in MDCK cells

Compound	IC^a_{50} (μM)	CC^b_{50} (μM)	SI^c
Amantidine	10.78	218.92	20.27
Zanamivir	0.88	>100.03	>113.64
1a	—	—	NA
1b	—	—	NA
1c	—	—	NA
2a	—	>169.99	NA
2b	—	>158.65	NA
2c	—	>148.73	NA
3a	18.27	>183.07	>10.02
3b	19.53	>169.99	>8.70
3c	23.78	>237.98	>10.01
3d	16.11	>169.99	>10.55
3e	16.86	>158.64	>9.41
3f	21.47	>216.34	>10.08
3g	14.89	>158.65	>10.65

3h	15.22	>148.72	>9.77
3i	19.52	>198.30	>10.16
4a	1.78	>142.66	>80.15
4b	17.53	>172.11	>9.82
4c	22.91	>198.31	>8.66
5a	22.23	>118.57	>5.33
5b	—	>105.77	NA
7a	—	—	NA
7b	—	—	NA
8a	41.78	>183.07	>4.38
8b	—	>169.99	NA

[a]IC_{50}: compound concentration required to achieve 50% inhibition of replication of H_5N_1, as determined by plaque reduction assays.

[b]CC_{50}: compound concentration required to cause 50% death of uninfected MDCK cells, as determined by the MTT method.

[c]SI: selectivity index as CC_{50}/ IC_{50} . NA: no activity. —: no calculation.

All data represent average values for three separate experiments. The variation of these results under the standard procedures is below ±10%.

As shown in Table 1, aromatic acid derivatives (compounds **1a-c**) had no activity against H_5N_1. However, the derivatives of aryl ester (compounds **3a-i**) and aromatic alcohol (compounds **4a-c**) exhibited potent inhibitory activity, while some amino acids derivatives (compounds **5a** and **8a**) showed moderate activity against H_5N_1. The results verified the hypothesis that esterification of carboxylic acid or reduction of carboxylic acid to alcohol in small molecular polyphenols could significantly enhance the anti-H_5N_1 activity besides amino acid-activated amidation of

carboxylic acid in small molecular polyphenols. Among amino acid derivatives, it was also observed that instead of the L-Phenylalanine methyl ester derivatives, the L-Leucine methyl ester derivatives could enhance the anti-H_5N_1 activity.

Interestingly, methylation of diphenol hydroxyls at the 3 and 4 positions of benzyl ring of aryl ester derivatives abolished their anti-H_5N_1 activity, such as compounds **2a-c**. Likewise, methylation of diphenol hydroxyls at the 3 and 4 positions of benzyl ring of amino acid derivatives also led inactive, such as compound **7a**. The results suggested that phenol hydroxyls might play a very important role in the anti-H_5N_1 activity of small molecular polyphenols, whereas introduction of alkyl groups in the hydroxyls should be avoided.

Among aryl ester derivatives, it was observed that the increase of the chain length at the 1 position of phenyl ring from one carbon to three carbons resulted in slightly decrease in activity against H_5N_1, while increasing the length or bulk of the O-substituted (R^2) of the aryl ester derivatives from methyl group to isopropyl group resulted in a slightly increased activity against H_5N_1. Noticeably, there were not significant difference in anti-H_5N_1 activity and selectivity index among these compounds, but compound **3g** (IC_{50}=14.89μM) profiled relatively better anti-H_5N_1 activity.

However, increasing the chain length at the 1 position of phenyl ring from one carbon to three carbons resulted in significantly decreasing the activity against H_5N_1 among aromatic alcohol derivatives. Amino acids derivatives also had the similar antiviral effect. For example, compound **4a** (IC_{50}=1.78μM) was almost 10-fold or

12-fold more active against H_5N_1 than compound **4b** (IC_{50}=17.53μM) or compound **4c** (IC_{50}=22.91μM) respectively, and compound **5a** (IC_{50}=22.23μM) was also almost 2-fold more active against H_5N_1 than compound **8a** (IC_{50}=41.78μM).

Notably, an aromatic alcohol derivative, compound **4a** (IC_{50}=1.78μM, SI>80.15), exhibited excellent activity against H_5N_1 among these derivatives, which was superior to Amantidine (IC_{50}=10.78μM, SI=20.27) and next to Zanamivir (IC_{50}=0.88μM, SI>113.64).

Compounds **3g**, **4a-b** and **5a** had better selectivity indexes and comparable potency in the corresponding derivatives, thus, compounds **3g**, **4a-b** and **5a** were chosen to investigate their mechanism of action against H_5N_1.

To determine whether Neuraminidase protein was the target of these compounds, compounds **3g**, **4a-b** and **5a** were tested *in vitro* for the inhibition of Neuraminidase activity using Neuraminidase Inhibitors Identification Kit P0309 (Beyotime Institute of Biotechnology). The preliminary results demonstrated that these compounds could not significantly inhibit the Neuraminidase activity at concentrations of up to 20μg/ml, indicating that Neuraminidase protein might not be the primary target of small molecular polyphenols.

Therefore, time-of-addition assay was carried out to investigate the possible target phase of small molecular polyphenols in the H_5N_1 life cycle.[20] Compounds **3g**, **4a-b** and **5a** were added to the confluent monolayers of MDCK cells at three time points: before viral adsorption (pre-treatment), at the same time as virus infection (simultaneous treatment) and after the viral adsorption (post treatment).

As shown in Fig.1, MDCK cells pre-treated with the compounds **4a** and **4b** prior to infection had slightly virus inhibition at -4 h and -2 h point. But compounds **3g** and **5a** had no inhibition effect when they were added to cells before virus infection. The result suggested that compounds **4a** and **4b** could slightly provoke the cell factors to resist the virus.

Interestingly, these compounds all had the significant inhibitory effect on H_5N_1 when they were added to the cells with virus simultaneously. Compound **4a** almost reached the 100% inhibition effect. And the three others also had the inhibition effect over 50% at this concentration.

However, these compounds have no significant inhibitory activity on H_5N_1 if they were added 2 h or longer after virus adsorption. These compounds had only 20% inhibition effect 2 h after infection. And when they were added to the infected cells 4 h or 8 h after infection, all of them had no inhibition effect at all. That means that these compounds had not effect on H_5N_1 virus replication stage.

The data from the timing assays suggested that small molecular polyphenols inhibited H_5N_1 virus production most effectively at the virus entry step of cell infection, and probably affected the attachment protein, such as HA protein.

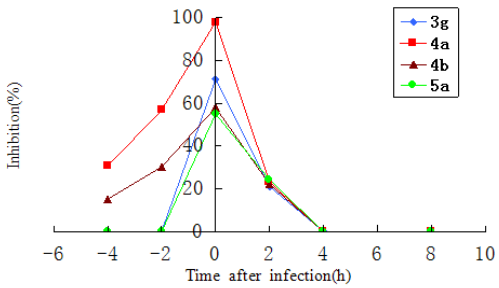


Figure 1. Compounds **3g**, **4a-b** and **5a** inhibited H_5N_1 (Influenza strain A/Vietnam/1194/2004) entry. Inhibition of H_5N_1 entry was determined by a time-of-addition assay.[20]

Furthermore, we investigated whether these small molecular polyphenols could inhibit hemagglutination(HA) by interfering with the H5 HA adsorption to RBCs. The hemagglutination inhibition assay was employed to evaluate the effects of compounds **3g**, **4a-b** and **5a** on viral adsorption to target cells.[18] Both H5 standard antigen and antiserum were provided by Beijing KangNong XingMu technology development center, China. Inhibitory activities of these compounds were tested at a serial 2-fold dilution (ranging from 100 to 0.098μg/ml). The results showed that compounds **3g**, **4a-b** and **5a** could not inhibit the adsorption of H5 HA to chicken RBCs even at the highest concentration of 100μg/ml, while the H5-positive control antisera could effectively inhibit H5 HA adsorption to chicken RBCs with a titer of 1:128. The results suggested that small molecular polyphenols**,** such as compounds **3g**, **4a-b** and **5a**, might not target the receptor binding domain of the HA1 subunit. On the other hand, HT (compound **4b**) is active against enveloped viruses, but not against nonenveloped viruses. HT could inhibit the fusion between HIV-1 and cellular membranes.[13] Taken together, we postulated that small molecular polyphenols could target to the membranes fusion step medicated by the more conserved hemagglutinin transmembrance subunit HA2, and not HA1.

On the basis of the results, we used the neutral-pH crystal structure of the HA of H_5N_1 (Influenza strain A/Vietnam/1194/2004, PDB: 2IBX) [21] as a reference for our modeling work. Using Molegro Virtual Docker (version 5.0), the predicted binding modes of compounds **4a** and **4b** are shown in Fig.2, respectively. The docking results of compounds **4a** and **4b** are summarized in Table 2, which shows that compounds **4a**

and **4b** can fit inside the same cavity in the stem region of the HA2 near the fusion peptide through Van der Waals interactions, hydrophobic contacts, and hydrogen bonding networks. The MolDockScore of compound **4b** was very close to that of compound **4a**, but compound **4a** formed more favorable hydrogen bonding networks with HA protein residues than compound **4b**, which helped to enhance the binding affinities to HA protein. As shown in Fig.2, at the top of cavity, compound **4a** occupied the binding site with the diphenol hydroxyls forming stable hydrogen bonds with Leu2, Asp109, and Arg322, respectively. Hydroxyl group at compound **4a** also could form stable hydrogen bonds with Arg322 and Leu108, respectively. However, one of phenol hydroxyls at the compound **4b** only formed stable hydrogen bonds with Asn117 and Asp120 at the bottom of cavity respectively. As expected, the analysis of binding models also gave our results support. Our study results suggested that phenol hydroxyls might play a very important role in the anti-H_5N_1 activity of small molecular polyphenols, whereas introduction of alkyl groups in the hydroxyls should be avoided. These interactions, identified by docking simulations, rationalized the anti-H_5N_1 activity of small molecular polyphenols, such as compounds **4a** and **4b**.

The results also show that compound **4a** can be potential anti-H_5N_1 entry inhibitors, likely targeting to the HA2 protein. Noticeably, compound **4a** is a small molecule with molecular weight of 140.14 and is different from the other known entry inhibitors.[1, 2, 5]

Table 2

Results of the molecular modeling study for compounds **4a** and **4b**

Compound	MolDock Score	Total Energy	E-Intra	Steric Energy	Hbond number	Hbond Energy	Hbond Interaction residues
4a	-70.174	-70.1735	9.46595	-70.1618	5	-9.47759	Leu2, Leu108, Asp109, Arg322(2)
4b	-68.0979	-68.6509	8.30568	-74.7874	2	-2.16921	Asn117, Asp120

MolDock Score: The energy score using docking (arbitrary units); E-Intra: energy between a ligand atom and the other atoms in the ligand; Steric Energy: steric energy; Hbond energy: Hydrogen bonding energy.

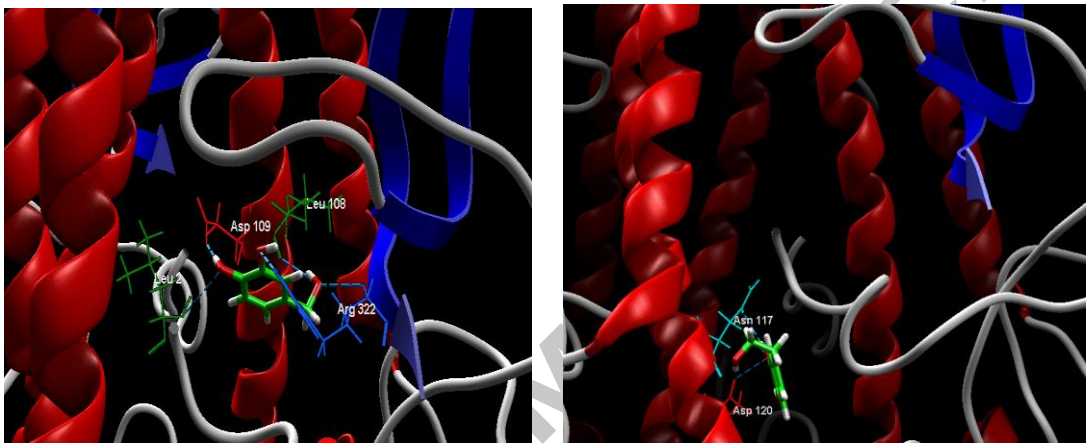


Figure 2. The binding modes of compound **4a** (left) or compound **4b** (right) with H5 HA protein. The modeling is based on the published crystal structure of HA of H_5N_1 (Influenza strain A/Vietnam/1194/2004, PDB: 2IBX). The HA1 subunit (A, C, E chains) is colored in blue. The HA2 subunit (B, D, F chains) is colored in red. Compounds **4a** and **4b** are shown in green stick model and hydrogen bonds with compounds **4a** and **4b** are in blue dashed lines. Residues Leu2, Leu108, Asp109, Asn117, Asp120 and Arg322 are indicated on the protein structure and other residues are not shown.

Interestingly, it was reported that the structure of H_9N_2 virus could be disrupted by HT. Electron microscopic analysis revealed morphological abnormalities in the HT-treated H_9N_2 virus. [12] In the time-of-addition assay, however, the addition of HT to the cells simultaneously with the viral inoculation caused only a marginal antiviral effect, and Pretreatment of the cells with HT did not affect the titer of the H_9N_2 virus

subsequently inoculated onto the cells, [12] which were different from our timing assays against H_5N_1. The results implied that the antiviral effects of HT on the H_9N_2 virus might be different from those of small molecular polyphenols on H_5N_1 virus. Therefore, more investigation must be taken to elucidate the entry mechanism of small molecular polyphenols against H_5N_1.

In summary, three series of known or novel small molecular polyphenols were designed and synthesized, and further screened *in vitro* for their anti-H_5N_1activity. The results verified the hypothesis that esterification of carboxylic acid or reduction of carboxylic acid to alcohol in small molecular polyphenols could significantly enhance the anti-H_5N_1 activity besides amino acid-activated amidation of carboxylic acid in small molecular polyphenols**.** On the other hand**,** the preliminary SAR studies showed that the anti-H_5N_1 activity of small molecular polyphenols depended on the availability of the phenolic groups, carbon chain length and the carbon chain functional groups at small molecular polyphenols. Small molecular polyphenols impaired the virus entry step of cell infection, likely targeting to HA2 protein, but the detail mechanism of action against H_5N_1 need further study. Among these derivatives, an aromatic alcohol derivative, compound **4a**, exhibits excellent activity against H_5N_1 and may serve as a lead compound or scaffold for the further development of H_5N_1 entry inhibitor.

Acknowledgment

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20. Time-of-addition assay：The time-of-addition experiment was carried out with MDCK cells. The MDCK cells were plated in 24-wells plates (2×10^4 cells/well) and incubated at 37℃/5% CO_2 for 24 h to allow the cells to form a monolayer on the bottom of the well. Then the compounds (the final concentration of 30μM) were added to the medium at the time point -4 h, -2 h (pre-treatment group), 0 h (simultaneous treatment group), 2 h, 4 h, 8 h (post treatment group). The untreated wells infected with virus at the same dilution were taken as the control group. In pre-treatment group, compounds were incubated with cell for 2 h and were removed 2 h or 4 h prior to virus infection. Then the medium with compounds were removed and the MDCK cells were washed twice with PBS. The H_5N_1 virus (80 PFU/well) were added to the cells and incubated at 37℃/5 % CO_2 for 2 h. In

simultaneous treatment group, the virus (80 PFU/well) and compounds were added to the cells simultaneously, and then incubated at 37℃/5 % CO_2 for 2 h. In post treatment group, the cells were first infected with virus (80 PFU/well) for 2 h. Then 2 h, 4 h and 8 h after the infection, the virus suspension was aspirated and cells were washed twice with PBS. The plaque medium containing test compounds (30μM) was added to cells, and remained throughout the time of addition experiments. After those treatments, the inhibitor rate of virus was determined by plaque assay. The cells were washed twice with PBS, overlaid with plaque medium (medium with 1% penicillin-streptomycin, 0.5% BSA, 1μg/ml TPCK and 1% agarose), and incubated at 37℃/ 5% CO_2 for 48 h. After the agarose was removed, the cells were stained with crystal violet. Plaques were counted to measure the effect of compounds. Every condition was tested in triplicate. The inhibition ratio was obtained using the equation: Inhibition (%) = $(1- C_{test} / C_{control}) \times 100\%$, C_{test} is the count of plaques in compounds added group; $C_{control}$ is the count of plaques in the control group.

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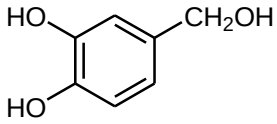

compound **4a**

Compound **4a** exhibits excellent activity against H_5N_1 and may serve as a lead compound or scaffold for the further development of H_5N_1 entry inhibitor.