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Caffeic acid derivatives: A new type of influenza neuraminidase inhibitors

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ABSTRACT

Recently, many natural products, especially some plant-derived polyphenols have been found to exert antiviral effects against influenza virus and show inhibitory activities on neuraminidases (NAs). In our research, we took caffeic acid which contained two phenolic hydroxyl groups as the basic fragment to build a small compound library with various structures. The enzyme inhibition result indicated that some compounds exhibited moderate activities against NA and compound **15d** was the best with IC_{50} = 7.2 μM and 8.5 μM against N2 and N1 NAs, respectively. The 3,4-dihydroxyphenyl group from caffeic acid was important for the activity according to the docking analysis. Besides, compound **15d** was found to be a non-competitive inhibitor with K_i = 11.5 ± 0.25 μM by the kinetic study and also presented anti-influenza virus activity in chicken embryo fibroblast cells. It seemed promising to discover more potent NA inhibitors from caffeic acid derivatives to cope with influenza virus.

Influenza virus, especially the highly pathogenic avian influenza A (H5N1) virus is a great threat to human. In recent years, the worldwide spread of H5N1 avian influenza virus in birds and the increasing cases of bird-to-human transmission get heightened concern. The viral surface protein, neuraminidase (NA) plays an important role in the life cycle of influenza virus and has been discovered as an important target for anti-influenza drugs design. Oseltamivir, one kind of NA inhibitors is currently the first line defense drug against influenza. However, more and more influenza virus strains are resistant to oseltamivir, such as the seasonal H1N1 viruses[1] and avian H5N1 strains.[2] In 2009, peramivir was urgently authorized in America to cope with the novel swine-origin H1N1 (A) influenza virus that was also resistant to oseltamivir. At present, this drug has been approved in many countries, but it is mainly administered intravenously, which is very inconvenient for patients.

According to recent studies, it seems hard to discover novel NA inhibitors which would be better than oseltamivir simply based on the NA active site. However, many natural products, especially some small plant-derived polyphenols have been found to exert antiviral effects against influenza virus and show inhibitory activities on NAs (Fig. 1).[3,4] Unlike the classical ones, the structures of these compounds are various and interestingly, most of them act as noncompetitive NA inhibitors, indicating that they likely inhibit the NA activity by binding on some other sites of the enzyme, rather than the catalytic center.

Caffeic acid, which is abundant in nature has a variety of potential pharmacological effects in vitro and in vivo, such as anti-inflammatory, anti-cancer and antiviral activities.[5] Some natural products containing the fragment of caffeic acid, like chlorogenic acid and its analogue also show inhibitory activities against influenza NAs.[6] A simple compound, caffeic acid phenethyl ester (CAPE, Fig. 2) is found to have anti-mitogenic, anti-carcinogenic, anti-inflammatory and especially anti-influenza virus properties in vitro.[7] In 2012, Liu et al. reported some novel dual-targeted bifunctional zanamivir anti-influenza drugs formed by conjugation of zanamivir with caffeic acid for simultaneous inhibition of influenza virus NA and suppression of pro-inflammatory cytokine. These conjugates provided remarkable protection of cells and mice against influenza infections.[8]

Till now, except some natural products, there are no synthetic compounds designed to target influenza NA based on caffeic acid. Therefore, in our study, we took caffeic acid as the basic fragment to design and synthesize a small compound library of caffeic acid derivatives. Through determining the inhibitory effects on the N1 and N2 NAs of influenza virus, we discovered a new kind of NA inhibitors with better activities than the natural products.

Compounds were synthesized using methods presented in Schemes 1–3. Scheme 1 presented the synthesis of compounds **3a**–**3f**. In order to increase the yields of target compounds, caffeic acid was first acetylated to give (*E*)-3-(3,4-diacetoxyphenyl)acrylic acid (compound **1**) that had a relatively better solubility in

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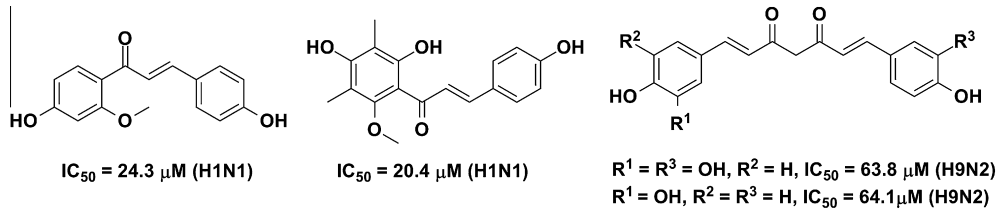


Figure 1. Plant-derived polyphenols with inhibitory activities against influenza NAs.

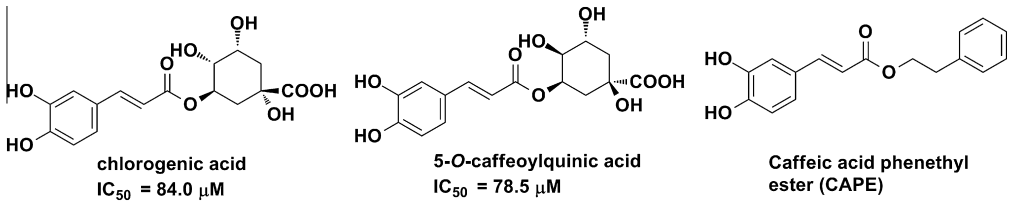
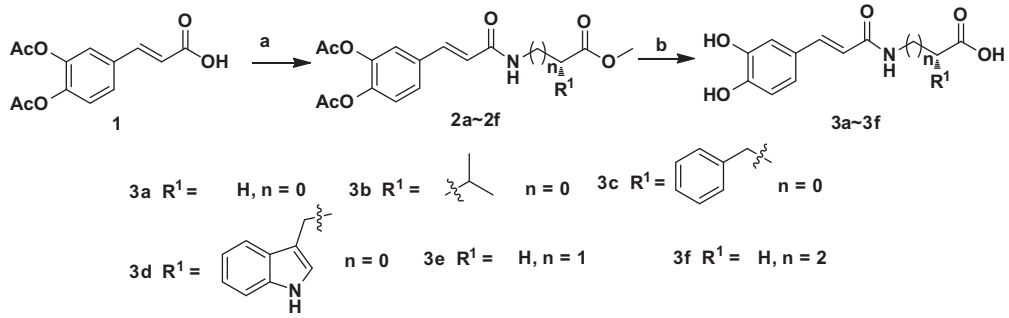


Figure 2. Caffeic acid derivatives with activities against influenza NA or influenza virus.

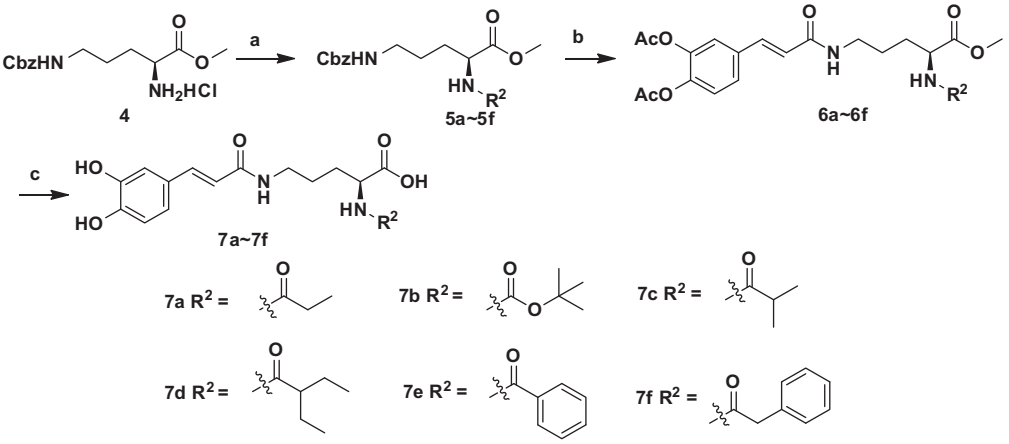
common organic solvents. This compound was then converted to acyl chloride and reacted with different amino acid methyl esters in THF. After hydrolysis with NaOH, compounds **3a**–**3f** were obtained with acceptable yields.

L-Ornithine that contained two amino groups, a carboxyl group and a relative long carbon chain can be modified with caffeic acid and some other structures. In Scheme 2, the α-NH_2 of N′-Cbz-L-ornithine methyl ester hydrochloride (**4**) was first acylated to introduce different fragments on the skeleton of L-ornithine. Under hydrogen atmosphere, the Cbz group was removed with Pd/C, therefore exposing the other NH_2 that was acylated with compound **1**. After hydrolysis with NaOH, compounds **7a**–**7f** were obtained.

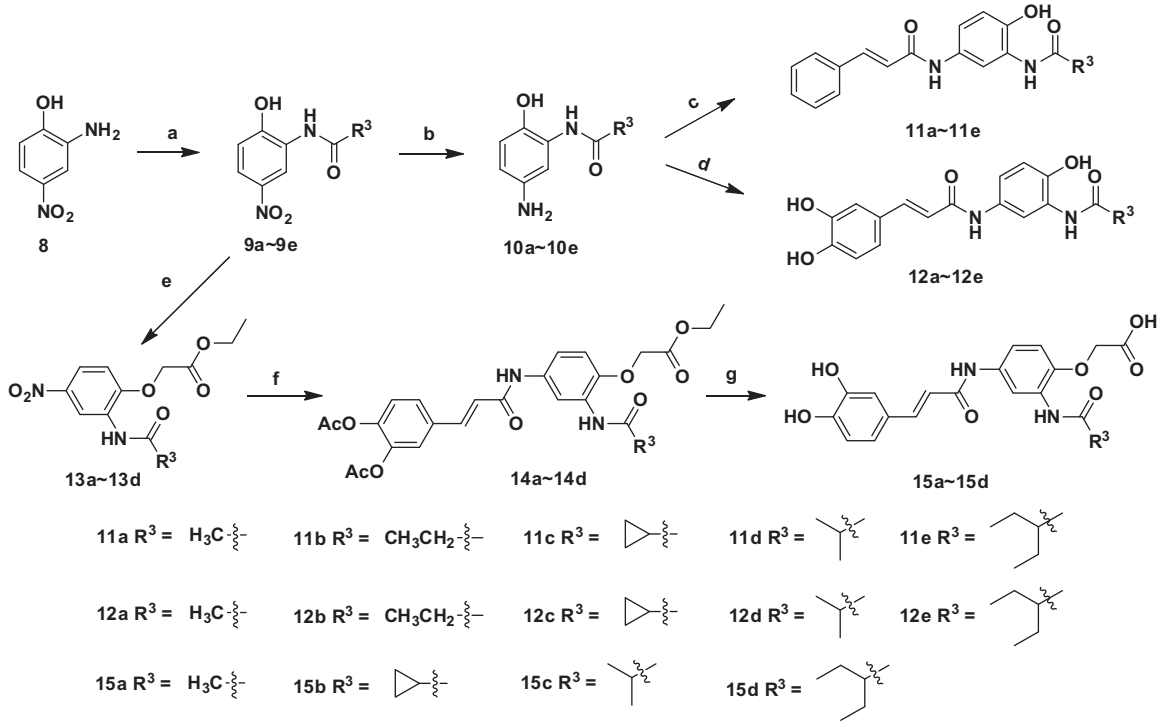
In Scheme 3, compounds **11a**–**11e** bearing cinnamic acid group were prepared for making a comparison with compounds **12a**–**12e** on their NA inhibitory activities. The starting material, 2-amino-4-nitrophenol (compound **8**) was acylated with different acyl chloride followed by reduction with Pd/C in the presence of H_2.



Scheme 1. Reagents and conditions: (a) (1) $(COCl)_2$, DMF, anhydrous THF, rt; (2) amino acid methyl esters, $NaHCO_3$, THF, rt; and (b) NaOH, CH_3OH, H_2O, rt.



Scheme 2. Reagents and conditions: (a) RCOCl, $NaHCO_3$, THF, H_2O, rt; (b) (1) H_2, Pd/C, CH_3COOH, CH_3OH, rt; (2) compound **1**, $(COCl)_2$, DMF, anhydrous THF; (3) $NaHCO_3$, THF, H_2O, rt; and (c) NaOH, CH_3OH, H_2O, rt.



Scheme 3. Reagents and conditions: (a) R^3COCl, $NaHCO_3$, THF, H_2O, rt; (b) H_2, Pd/C, CH_3OH, rt; (c) (1) cinnamic acid, oxalyl chloride, DMF, anhydrous THF, rt; (2) $NaHCO_3$, THF, H_2O, rt; (d) (1) caffeic acid, oxalyl chloride, DMF, anhydrous THF, rt; (2) $NaHCO_3$, THF, H_2O, rt; (e) ethyl chloroacetate, NaI, K_2CO_3, acetone, reflux; (f) (1) H_2, Pd/C, CH_3OH, rt; (2) compound **1**, oxalyl chloride, DMF, anhydrous THF, rt; (3) $NaHCO_3$, THF, H_2O, rt; and (g) NaOH, CH_3OH, H_2O, rt.

Table 1
In vitro inhibitory effects of compounds **3a**–**3f** against influenza A NAs

Compound	H9N2 IC_{50} (μM)	H1N1 IC_{50} (μM)	Compound	H9N2 IC_{50} (μM)	H1N1 IC_{50} (μM)
3a	43.3	49.5	**3d**	27.2	30.1
3b	37.3	38.3	**3e**	13.5	27.3
3c	39.6	37.4	**3f**	16.8	27.7

Table 2
In vitro inhibitory effects of compounds **7a**–**7f** against influenza A NAs

Compound	H9N2 IC_{50} (μM)	H1N1 IC_{50} (μM)	Compound	H9N2 IC_{50} (μM)	H1N1 IC_{50} (μM)
7a	37.9	31.3	**7d**	44.2	39.2
7b	33.6	34.7	**7e**	37.2	34.3
7c	30.3	31.9	**7f**	28.1	30.2

Reaction of the intermediates **10a**–**10e** with cinnamic acid or caffeic acid gave compounds **11a**–**11e** and **12a**–**12e**. There were four steps involved in the conversion of compounds **9a**, **9c**–**9e** to the final acids **15a**–**15d** and intermediates did not need to be purified. The first step was alkylation of the phenolic hydroxyl group with ethyl chloroacetate to form the corresponding ether. In this step, a catalytic amount of NaI was very beneficial to the reaction. The second step was reduction of the nitro group of the esters to form the anilines. The third step was condensation of the anilines with compound **1** to provide intermediates **14a**–**14d**. The forth step was hydrolysis of intermediates **14a**–**14d** by aqueous NaOH to afford the final acids.

Though the residues within the active site of NA are high conserved, there are still some structural differences between group-1 (N1, N4, N5, N8) and group-2 (N2, N3, N6, N7, N9) NAs in the region adjacent to the enzyme's catalytic center. Therefore, we chose NAs from two kinds of influenza viruses (H9N2 and H1N1) to determine the inhibitory activities of our designed compounds. From the data of Tables 1–3, it could be found that these compounds were superior to the reported caffeic acid derivatives or

Table 3
In vitro inhibitory effects of compounds **11a**–**11e**, compounds **12a**–**12e** and compounds **15a**–**15d** against influenza A NAs

Compound	H9N2 IC_{50} (μM)	H1N1 IC_{50} (μM)	Compound	H9N2 IC_{50} (μM)	H1N1 IC_{50} (μM)
11a	NT	40.2% at 100 μM	**12c**	24.7	18.2
11b	NT	42.9% at 100 μM	**12d**	20.4	19.0
11c	NT	42.6% at 100 μM	**12e**	20.7	22.8
11d	NT	47.2% at 100 μM	**15a**	15.4	12.2
11e	NT	45.2% at 100 μM	**15b**	16.5	13.8
12a	27.4	17.7	**15c**	13.0	11.6
12b	23.6	17.6	**15d**	7.2	8.5

All compounds were tested in triplicate wells per dilution. Oseltamivir carboxylate was used as the positive control with IC_{50} values = 0.0017 μM (H9N2) and 0.010 μM (H1N1). NT: not tested.

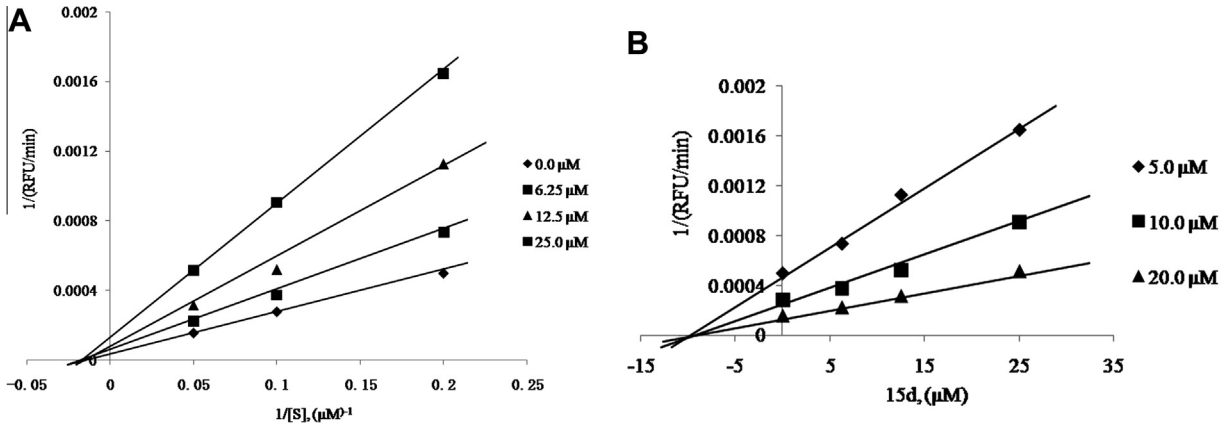


Figure 3. Graphical determination of the type of inhibition for compound **15d**. (A) Lineweaver–Burk plot for the inhibition of compound **15** on NA from H9N2 influenza virus for the hydrolysis of substrate in the presence of different concentrations for lines from bottom to top: 25, 12.5, 6.25 and 0.0 μM. (B) Dixon plot for inhibition of compound **15d** on NA from H9N2 influenza virus for the hydrolysis of substrate in the presence of different concentrations of substrate for lines from bottom to top: 20, 10.0, and 5.0 μM.

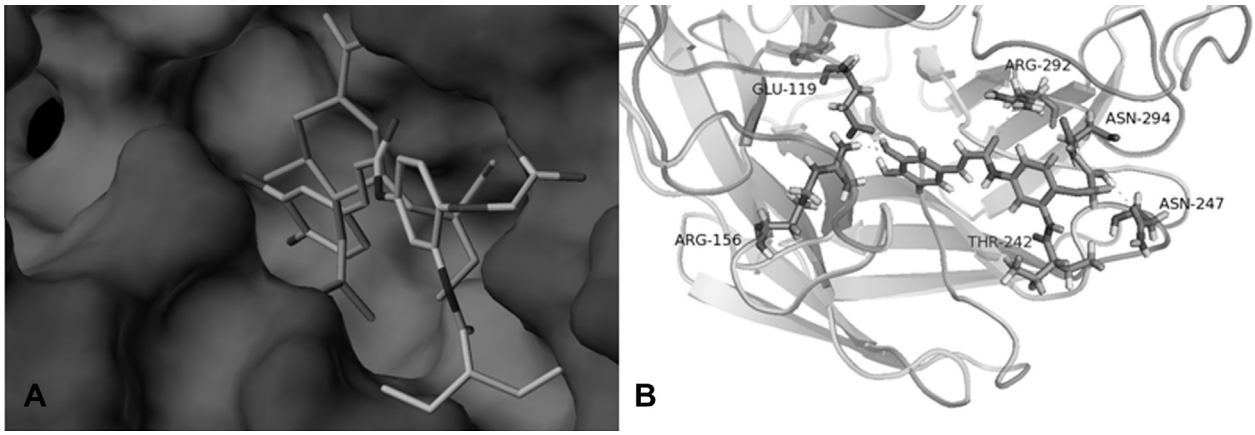


Figure 4. Docking analysis of compound **15d**. (A) Oseltamivir (the smaller compound) and **15d** binding to the active site of NA (PDB ID: 2HU0), and (B) residues that formed hydrogen bonds with compound **15d**.

caffeic acid (IC_{50} >100 μM against N2 and N1, data not shown) on the NA inhibitory activities. Besides, most of these compounds presented similar activities against N1 and N2 NAs, except **3e** and **3f** in Table 1. The two compounds contained chain-like amino acids, 3-aminopropanoic acid and 4-aminopropanoic acid, which may favor selective inhibition of the N2 NA.

Since **3e** and **3f** showed better activities relative to compounds **3a**–**3d**, we further designed and synthesized compounds **7a**–**7f** that had a relative long chain based on the skeleton of L-ornithine. As shown in Table 2, however, the activities of compounds **7a**–**7f** were lower than those of **3e** and **3f**. The result did not come up to our expectations. It could be found that the size of substituents at the α-NH_2 had little influence on the activity and generally, the skeleton of L-ornithine was unfavorable to NA inhibition.

In Table 3, it could be clearly found that compounds **11a**–**11e** were less potent than compounds **12a**–**12f**, which proved that caffeic acid was appropriate for being modified as NA inhibitors. Compounds **15a**–**15e** with the structural fragment of phenoxy acetic acid instead of phenol exhibited improved activities relative to compounds **12a**–**12f**. The introduction of the carboxyl group obviously increased the potency, which was consistent with the structure–activity relationship of classical NA inhibitors. Among the four compounds, **15d** with 2-ethylbutanamide group was the most potent against N2 and N1 NA with IC_{50} = 7.2 and 8.5 μM, respectively. In

Table 4
In vitro anti-influenza virus (A/Chicken/Shandong/LY/08) activities in Chicken embryo fibroblast cells using the CPE reduction assay

Compound	EC_{50}[a] (μM)	CC_{50}[b] (μM)	Compound	EC_{50}[a] (μM)	CC_{50}[b] (μM)
3d	>100	89.5	**12e**	22.6	>100
3e	>100	>100	**15a**	NT	60.4
7c	NT	65.0	**15b**	NT	86.7
7f	NT	87.7	**15c**	NT	94.3
12a	NT	78.2	**15d**	31.2	>100
12b	58.6	>100	OS carboxylate	0.5	>10.0

NT: not tested.
[a] EC_{50}: concentration required to achieve 50% protection against virus-induced cytopathic effect.
[b] CC_{50}: concentration of 50% cellular toxicity.

the kinetic study, compound **15d** was found to be a non-competitive inhibitor with *K*i = 11.5 ± 0.25 μM against the N2 NA (H9N2) by the means of Lineweaver–Burk and Dixon plots analysis (Fig. 3).

The docking analysis (Fig. 4A) performed by the means of SYBYL-X 1.1 indicated that the 3,4-dihydroxyphenyl group of **15d** could penetrate deeply into the active site of NA, and presented hydrogen bonding interactions with residues Glu119 and Arg156 (Fig. 4B). To some extent, this can explain the reason why compounds **12a–12f** were potent than compounds **11a–11e**. However, the other parts of **15d** were mainly on the surface of the active sites, which was not favorable to the activity. Notably, the acetic acid group had strong interactions with residue Arg292, Asn294 and Asn247, a little like that of oseltamivir, interacting with residue Arg118, Arg292, and Arg371. These interactions likely contributed the higher activities of compounds **15a–15d** compared with compounds **12a–12f**.

Generally, the NA inhibitory activities of compounds **12a–12e** and **15a–15e** were better than those of compounds **3a–3f** and **7a–7f**. It seemed that caffeic acid derivatives bearing rigid structural fragments, especially with carboxyl groups were superior to those containing flexible long chains with branched groups. Compound **15d** could be a new lead for further modifications to discover more potent inhibitors.

In addition, some compounds were further evaluated for their anti-influenza virus activities as well as the cellular toxicity by the CPE reduction assay (Table 4). Compound **15d** with the best enzyme inhibitory activity showed moderate anti-influenza virus activity with EC_{50} value of 31.2 μM against H9N2. Due to the obvious toxicity of compounds **7c**, **7f**, **12a** and **15a–15c**, their anti-influenza virus activities were not determined. It was noteworthy that compound **12e** with low cytotoxicity displayed more potent activity relative to compound **15d**, which was not consistent with the enzyme inhibition result. As mentioned above, many plant-derived polyphenols have been found to exert antiviral effects against influenza virus. It seemed that the phenolic hydroxyl group of compound **12e** was important for the antiviral activity.

In summary, we synthesized some caffeic acid derivatives and discovered a new type of influenza NA inhibitors. Compound **15d** was the best inhibitor among the designed caffeic acid derivatives and could be as a lead for further modifications.

NA is still an important target for anti-influenza drugs design. Considering the current anti-influenza situation, it is still necessary to discover novel NA inhibitors. The caffeic derivatives seem promising to be a new choice for development of anti-influenza virus agents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.04.033.

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