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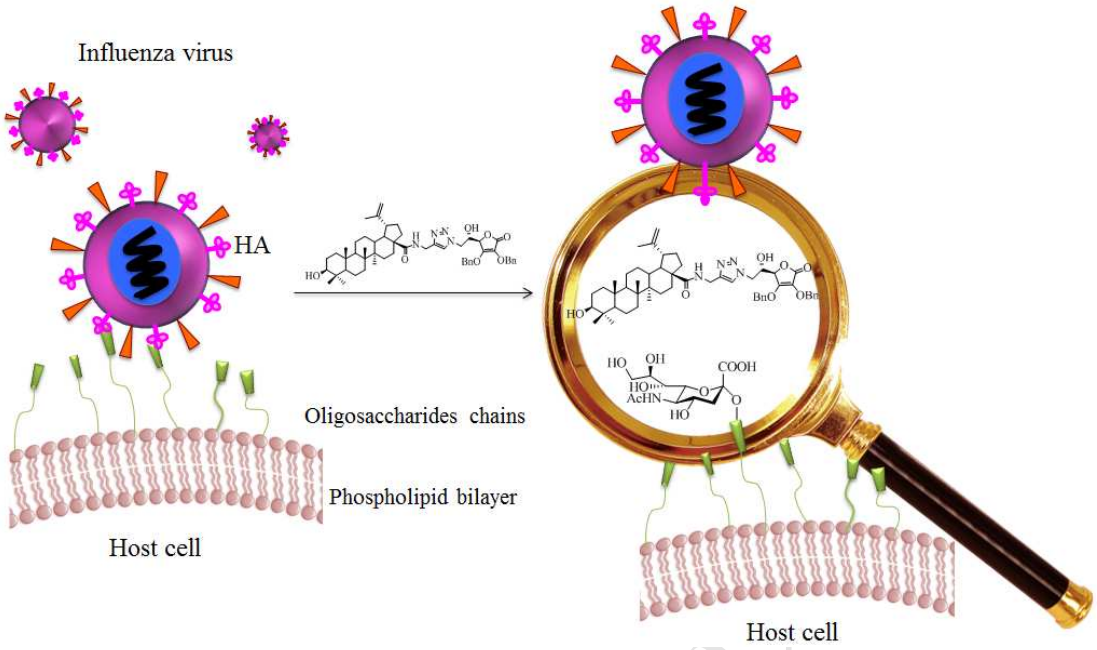
Graphical abstract



Design, synthesis and biological evaluation of novel L-ascorbic acid-conjugated pentacyclic triterpene derivatives as potential influenza virus entry inhibitors

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ABSTRACT:

Since the influenza viruses can rapidly evolve, it is urgently required to develop novel anti-influenza agents possessing a novel mechanism of action. In our previous study, two pentacyclic triterpene derivatives (**Q8** and **Y3**) have been found to have anti-influenza virus entry activities. Keeping the potential synergy of biological activity of pentacyclic triterpenes and L-ascorbic acid in mind, we synthesized a series of novel L-ascorbic acid-conjugated pentacyclic triterpene derivatives (**18**-**26**, **29**-**31**, **35**-**40** and **42**-**43**). Moreover, we evaluated these novel compounds for their anti-influenza activities against A/WSN/33 virus in MDCK cells. Among all evaluated compounds, the 2,3-*O*,*O*-dibenzyl-6-deoxy-L-ascorbic acid-betulinic acid conjugate (**30**) showed the most significant anti-influenza activity with an EC_{50} of 8.7 μM, and no cytotoxic effects on MDCK cells were observed. Time-of-addition assay indicated that compound **30** acted at an early stage of the influenza life cycle. Further analyses revealed that influenza virus-induced hemagglutination of chicken red blood cells was inhibited by treatment of compound **30**, and the interaction between the influenza hemagglutinin (HA) and compound **30** was determined by surface plasmon resonance (SPR) with a dissociation constant of K_D = 3.76 μM. Finally, silico docking studies indicated that compound **30** and its derivative **31** were able to occupy the binding pocket of HA for sialic acid receptor. Collectively, these results suggested that L-ascorbic acid-conjugated pentacyclic triterpenes were promising anti-influenza entry inhibitors, and HA protein associated with viral entry was a promising drug target.

Keywords: Influenza virus, pentacyclic triterpene, L-ascorbic acid, entry inhibitor, hemagglutinin, sialic acid receptor

1. Introduction

Influenza viruses cause substantial morbidity and mortality through routine seasonal spread and seasonal epidemics. Currently, anti-influenza therapies include universal influenza vaccines [1], broadly-neutralizing therapeutic antibodies [2] and small-molecule inhibitors [3]. However, the high mutation rate of the RNA genome of the influenza virus, combined with assortment of its multiple genomic segments, promotes antigenic diversity and new subtypes, allowing the virus to avoid vaccines and become resistant to antiviral drugs [4]. For example, the 2008-2009 H1N1 strain of influenza exhibits ~100% resistance against Tamiflu (oseltamivir) [5]. Therefore, there is a continuous and urgent need for prevention or treatment strategies that can cover a broad range of viral strains and subtypes. As the first step to prevent virus infection, a favorable target for drug discovery is to inhibit influenza virus entry, resulting in an efficient blockage of virus propagation [6-7]. It would be an effective approach of anti-influenza, and it could prevent the infection of influenza as vaccination since many viruses share similar entry routes. Moreover, it also might be a type of broad-spectrum anti-influenza inhibitor, and the resistance is less likely to occur [8]. One possible strategy is to target the sialic acid (also named *N*-acetylneuraminic acid, Neu5Ac) receptor-binding hemagglutinin (HA) glycoprotein to disrupt the interactions between the viral particles and host cell proteins [9]. HA is the major surface envelope protein of influenza viruses A and B, and it carries essential functions in the viral life cycle. The entry of influenza virus is mediated by attachment through HA binding to sialic acid receptors on the host membrane and then internalization of viral particles into the late endosome [10]. A large portion of the sialic acid receptor-binding sites is highly conserved for the recognition of the common ligands. The critical role of HA in influenza entry renders it an attractive target for the development of prophylactic and therapeutic anti-influenza virus drugs that prevent viral infection at the first stage [11-12].

Pentacyclic triterpenes are secondary plant metabolites and biosynthetically derived from the cyclization of squalene [13]. It is generally believed that their physiological functions enhance the immunity of host plants and increase plant resistance to pathogens [14]. Many of these naturally occurring compounds exhibit a variety of antiviral activities, such as anti-HIV [15], anti-HCV [16], anti-influenza [17], anti-HSV [18] and so on. In our previous studies [19-24], we have also found that echinocystic acid (EA, Fig. 1), an oleanane-type triterpene, displays substantial

inhibitory activity on HCV entry with one derivative showing EC_{50} at nanomolar level. In view of significant inhibitory effects of pentacyclic triterpene on broad antiviral spectrum, we sought to learn whether such triterpenes were also active against influenza viruses. Recently, we found that **Q8**, an EA−galactose conjugate, and it analog **Y3** (Fig. 1) are the most potent entry inhibitors against influenza A/WSN/33 virus with EC_{50} values of 5.0 and 14.2 μM, respectively [25]. The molecular basis of their activity is potentially due to their high affinity to HA protein, thus leading to the blockage of HA-SA receptor interaction and the attachment of influenza viruses to host cells.

(Figure 1)

L-ascorbic acid (vitamin C, Fig. 2), which is usually mentioned as an essential nutrient for humans and a reducing agent to scavenge free radicals, has been also shown to have antiviral activity against a broad spectrum of RNA and DNA viruses *in vitro* [26-27]. Some derivatives of L-ascorbic acid, such as 1,2,4-triazole and imidazole [28], 2-*O*-phosphate [29], 2-*O*-glycoside [30] and nucleoside derivatives [31-33], have been found to have antiviral activities. *In vivo*, oral and intravenous administration of L-ascorbic acid has been proved to have clinical improvements in patients with influenza virus [34], hepatitis C virus [35], herpes simplex virus [36] and Epstein-Barr virus infection [37]. In addition, L-ascorbic acid is a weak acid (pK_a = 4.2) with multi-hydroxyl side chain structurally related to sialic acid, which is the primary receptor for influenza A virus during the attachment of viruses to the host cells. In the development of neuraminidase (NA) inhibitors, many analogs of sialic acid (Neu5Ac), such as 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en) and the 4-guanidino analog of Neu5Ac2en (zanamivir), are found to inhibit NA with a potency similar or more stronger than sialic acid (Fig. 2)[38-41]. Therefore, L-ascorbic acid has been considered as a useful synthetic precursor to many molecules of potential biological utility [42-44].

(Figure 2)

To continue our studies towards the development of novel antiviral inhibitors [19-24], we prepared new types of molecules containing pentacyclic triterpene (EA, oleanolic acid (OA), ursolic acid (UA) and betulinic acid (BA)) moieties connected to L-ascorbic acid via triazole and amide linker in the present study. In addition, we report the synthesis, NMR, anti-influenza activity and docking studies in this paper.

2. Results and discussion

2.1 Chemistry

There are four hydroxy groups in the L-ascorbic acid molecule, and two of them are enol hydroxy groups. Many investigations have pointed out that the two hydroxy groups at C-2 and C-3 play particularly important roles to maintain its inherent biological activity *in vivo* [45]. On the contrary, the 6-hydroxy group of L-ascorbic acid has been proved to be unimportant in transport and function of this vitamin [46-48]. Because of these reports, we also focused on the preparation of C-6-*O*-modified L-ascorbic acid derivatives.

(Scheme 1)

Scheme 1 outlines that the 6-deoxy-6-azide-2,3-*O*,*O*-dialkyl-*L*-ascorbic acid (**9** and **10**) and 6-deoxy-6-amino-2,3-*O*,*O*-dibenzyl-*L*-ascorbic acid (**11**), key intermediates for coupling with pentacyclic triterpene (OA, EA, UA and BA), were synthesized from the starting L-ascorbic acid. Briefly, the treatment of L-ascorbic acid with acetyl chloride in acetone was performed to afford the 5,6-ketal of L-ascorbic acid (**2**) according to the procedure described by Spickenreither and Tahir with minor modifications [47-48]. Benzylation or methylation of the C-2 and C-3 hydroxy groups of the lactone ring in **2** was accomplished using K_2CO_3 and methyl iodide or benzyl bromide in DMF to provide **3** and **4**, respectively. Deblocking of the 5,6-*O*,*O*-protected derivative of L-ascorbic acid **3** or **4** with 2N HCl in THF-CH_3OH solution gave 2,3-*O*,*O*-dialkyl-L-ascorbic acid (**5** and **6**). Tosylation of the C-6 hydroxy group in **5** or **6** using TsCl in the presence of triethylamine provided 6-*O*-tosyl-2,3-*O*,*O*-dialkyl-L-ascorbic acid (**7** and **8**). This was followed by nucleophilic substitution with sodium azide in DMF to provide the key intermediates **9** and **10**. Subsequent reduction of the azide group in **10** was carried out through the treatment with Ph_3P in THF/H_2O (1:1) to yield intermediate **11**.

(Scheme 2)

Compounds **15**-**17** and **28** were prepared from OA, EA, UA and BA, respectively, as previously described [19, 21-22]. Coupling of them with key intermediate **9** and **10** via click reaction in THF/H_2O (1:1) in the presence of a catalytic amount of copper sulfate and sodium ascorbate yielded pentacyclic triterpene derivatives of 2,3-*O*,*O*-dialkyl-L-ascorbic acid (**18**-**21**, **23**, **25** and **29**-**30**). Debenzylation of **21**, **23**, **25** and **30** was accomplished by hydrogenolysis, yielding **22**, **24**, **26** and **31**, respectively (Scheme 2). While the hydrogenation of the double bond **20** (29)

of compound **30** did not occur under this condition (300 kPa), whereas a higher pressure, such as 800 kPa, could ensure the reaction to be fully completed [52]. Activation of the carboxyl group of pentacyclic triterpene with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) gave the stable intermediates **32-34** and **41,** and then condensation with intermediate **11** in the presence of EDC thus yielded **35**, **37**, **39** and **42**, followed by hydrogenolysis with 10% Pd/C to afford **36**, **38**, **40** and **43**, respectively (Scheme 3). The structures of all L-ascorbic acid-triterpene conjugates were characterized by ^{1}H NMR, ^{13}C NMR and ESI-HRMS.

(Scheme 3)

2.2 Biological assays

2.2.1 Anti-influenza A/WSN/33 virus activity

As part of our biological profiling, we evaluated the newly synthesized pentacyclic triterpene-L-ascorbic acid conjugates (**18-26**, **29-31**, **35-40** and **42-43**) against the influenza A/WSN/33 (H1N1) virus that was propagated in MDCK cells by the CellTiter-Glo assay and the cytopathic effect (CPE) reduction assay [50]. Moreover, we employed the CellTiter-Glo screening, an assay used to monitor cell proliferation, to screen and exclude compounds with significant toxicity toward MDCK cells. The CPE screening, an assay to determine the damage to host cells during virus invasion, was used to screen and identify compounds with a reduction of the CPE on influenza A/WSN/33 virus. Except compounds **21**, **23**, **25**, **30**, **31** and **42-43** significantly reduced the viral CPE in the CellTiter-Glo assay, all the other compounds had cytotoxicity at the same concentration (Table 1 and 2). Compound **30** exhibited the most potent anti-influenza virus activity with an EC_{50} of 8.7 μM (Figure 3A), while no cytotoxicity against uninfected MDCK cells was observed at a concentration of 200 μM. The reduction of CPE was confirmed by direct microscopic observation, which detected far less CPE than that in the DMSO control (Figure 3B).

(Table 1)

(Table 2)

(Figure 3)

2.2.2 Structure-activity relationship

Further analysis of the structure-activity relationship of the L-ascorbic acid-triterpene conjugates revealed a clear co-relationship as potential anti-influenza virus agents. Analogues exhibited a variety of anti-influenza virus activities dependent on the substituent groups of L-ascorbic acid, the linker and triterpene skeleton.

Table 1 shows that substituents at C-2 and C-3 hydroxy groups of L-ascorbic acid exerted important effects on the antiviral potency. The synthetic analogs showed high activities when they had a common structural feature, the 2,3-*O*,*O*-dibenzyl substitution of L-ascorbic acid. These results also confirmed that all conjugates (compounds **21, 23, 25, 35, 37** and **39**) with such structural property showed high inhibitory rate of anti-influenza virus at a dose of 50 μM no matter the triterpene skeleton was OA, EA, UA or BA, or the linker moiety was 1,2,3-triazole or amide bond. However, the removal of benzyl groups of C-2 and C-3 hydroxyl groups (compounds **22, 24, 26, 36, 38** and **40**) resulted in the loss of anti-influenza activity. This result might be caused by the reduction of hydrophobicity, which decreased the affinity of L-ascorbic acid-triterpene conjugates to the protein targets. Moreover, most L-ascorbic acid-BA conjugates (compounds **30**-**31** and **42**-**43**) exhibited substantial effects against influenza A/WSN/33 virus activity, except for compound **29** which possessed cytoxicity against uninfected DMCK cells. CPE reduction assay revealed that compound **30** exerted the most potent anti-influenza virus activity with an EC_{50} of 8.7 μM (Fig. 3A), which was in the same order as that found for the reference anti-influenza drug oseltamivir (EC_{50} = 12.5 μM), and no cytotoxicity effects were observed on MDCK cells. In addition, pentacyclic triterpene derivatives of 2,3-*O*,*O*-dimethyl-L-ascorbic acid derivatives (compounds **18**-**20** and **29**) showed obviously cytotoxicity, which was also consistent with our previously studies that per-*O*-methylated cyclodextrin-triterpene conjugates also exhibit some degree of cytotoxicity at 5 μM and strong cytotoxicity at 50 μM in MDCK and 293T cell lines [22]. Clearly, the CH_3O groups at C-2 and C-3 of L-ascorbic acid are an unfavorable group in the scaffold of triterpene-L-ascorbic acid conjugates for antiviral effects. Furthermore, the CPE assay indicated that compound **35**, an analogue of **21** in which the 1,2,3-trizole linker was replaced by an amide bond, had potency almost identical to that of **21**, and similar results were also found for other analogues (**23** *vs.* **37**, **25** *vs.* **39**, **30** *vs.* **42** and **31** *vs.* **43**), indicating the compatibility of 1,2,3-trizole and amide linkers for anti-influenza A/WSN/33 virus.

*2.2.3 Compound **30** inactivates virions and prohibits influenza virus entry*

In order to characterize at which stage(s) of virus life cycle was affected by L-ascorbic acid-triterpene conjugates, we first sought to determine if they acted on viruses or on host cells. A time-of-addition experiment using **30** as a representative was carried out [49]. We administrated compound **30** at various time points (0, 2, 4, 6, 8 h) post infection. The reduction of NP protein level at the interval 0-10 h (covering the whole life cycle) and 0-2 h (covering the entire viral entry process) indicated that **30** was only effective at the attachment or the fusion of virus with host cell (Figure 4A). There was no inhibitory effect for the remaining steps, such as viral genome replication/translation or virion assembly/release. These results suggested that **30** acted in the early stage of the influenza virus replication cycle, including entry and/or an early replication phase.

Then three experiments were conducted in parallel, including the co-treatment, the pre-treatment of cells and the pre-treatment of virus, in order to clarify whether the host cell or the influenza virus was targeted. In the co-treatment assay, compound **30** at a concentration of 50 μM exerted an inhibition rate of 75%, while the pre-treatment of virus led to an inhibition rate of 87% (Figure 4B). We concluded that compound **30** targeted the influenza virus particles but not host cells, which was consistent with our previous study [25].

*2.2.4 Inhibition of HA by compound **30***

Since compound **30** could inhibit the early step of the influenza A virus life cycle by acting on the virus and but not on the cells, we investigated the effect of it on the activity of the influenza A virus surface proteins HA [51]. It is well known that influenza virus HA plays an essential role in virus infection. The attachment mediated by HA initiates the fusion of the viral envelope with the host membrane to release the viral genome into the target cells. Influenza A virus HA can bind to sialic acid on the surface of RBCs, causing agglutination. Therefore, we performed a hemagglutination inhibition (HI) assay to examine whether compound **30** blocked the ability of virus particles to bind to cell receptors. Influenza A virus A/WSN/33 was pretreated with compound **30** for 1 h before the addition of chicken erythrocytes, and a further incubation was conducted at RT for 1 h. Compound **30** inhibited the binding of the influenza A virus A/WSN/33 to chicken erythrocytes, showing similar capability as anti-HA antibody. The

inhibitory effect was in a concentration-dependent manner, indicating that compound **30,** as anti-HA antibody, shared the same target-HA (Figure 4C).

2.2.5 Binding kinetics by surface plasmon resonance (SPR)

We then used SPR experiment to study the kinetics and quantify the interaction between the active compounds **30** and **31** and HA protein.

The results implied that both **30** and **31** could bind to the HA protein immobilized on the CM5 chip, and Figure 4D shows the dose-dependent responses. The K_D values for **30** and **31** were calculated as 3.76 μM and 8.04 μM, respectively. It was found that the weaker inhibitor **31** had less effect K_D than **30**, suggesting that the potency of the compound against the influenza virus was directly correlated to the inhibition of the HA-sialic acid receptor interaction. The affinity of these compounds to HA protein was correlated well to their antiviral effects, supporting that HA protein was a potential target for **30**.

(Figure 4)

2.2.5 Blind docking calculation

Molecular docking is a frequently used tool in computer-aided structure-based rational drug design. In this study, the newly synthesized L-ascorbic acid-triterpene conjugates were docked into the influenza HA protein, which was obtained from the RCSB Protein Data Bank, http://www.rcsb.org/pdb/home/home.do, using AutoDock 4.2 program.

To predict the binding mode of the newly synthesized compounds, we first performed a docking study of compounds **30** and **31**. In view of HA (HA1) as the potential interruption event, we performed blind docking calculations to investigate such possibilities. We employed blind docking calculations in order to figure out the potential interruption event of **30** and **31** within HA (HA1). The docked conformations of HA-**30** were determined based on the minimum free energy analyses. The computer-aided docking data suggested that compounds **30** and **31** exhibited a similar binding mode as **Y3**, occupying the binding pocket for sialic acid receptor (Figure 5) with an estimated binding energy of -9.94 and -8.56 kcal/mol, and an inhibition constant (K_i) = 51.64 and 122.51 nM, respectively. This result suggested that the two compounds, especially **30**, could bind tightly to HA protein. One of the influenza virus receptors is the terminal sialic acid of cellular glycoproteins, which binds the shallow depression located at the top of HA1 domain. For all subtypes of influenza viruses, the HA protein is composed of highly conserved HA1 and HA2

domains, which are essential for sialic acid receptor binding and viral envelope fusion, respectively [53].

The computer-aided docking data indicated that the 3-OH of the BA moiety formed a hydrogen bond with K222, and the carbonyl oxygen atom of BA moiety made a hydrogen bond with H183. For the L-ascorbic acid moiety, besides the hydrophobic interaction, the C-3 *O*-benzyl substitution of L-ascorbic acid occupied the narrow cavity and engaged in π-π stacking interaction with W153 residue. Among these, H183 and L194 were critical for sialic acid binding via hydrogen bonds. Most of the remaining interactions were within the 190 helix (residues 190-198), the 130 loop (residues 135-138) and the 220 loop (residues 221-228), which were all important for sialic acid binding and consistent with the former studies of **Y3**. These docking data were consistent with results from SAR studies, in particular the requirement for *O*-benzyl groups in the L-ascorbic acid residue and the 3-hydroxyl group in the BA moiety.

Docking studies indicated that the 3-hydroxyl group of the betulinic acid, *O*-benzyl groups of the L-ascorbic acid and several other residues were critical in sialic acid binding. To make sure the key residues could be kept in proper distance and position, the 1,2,3-triazole played an important role. The five-membered E-ring of BA perhaps had influence on the hydrogen bond of carbonyl oxygen atom with H183, which also explained why the L-ascorbic acid-BA conjugates had better activity.

(Figure 5)

3. Conclusions

In conclusion, we synthesized a series of L-ascorbic acid-conjugated pentacyclic triterpene (OA, UA, EA and BA) derivatives in the present study, and their anti-influenza activity *in vitro* was tested. A systemic exploration of pharmacological activities of the compounds showed that conjugation of triterpene with *O*-benzyl substitution L-ascorbic acid was the structural requirements for maintenance of high activity. In addition to this, it was clear based on our SAR results that the incorporation of *O*-benzyl substitution groups into L-ascorbic acid considerably enhanced the anti-influenza virus potential with no cytotoxicity. Taken together, compound **30**, a BA-L-ascorbic acid conjugate, showed better anti-influenza virus activity than other types of triterpenes. Similar to **Y3**, the molecular basis of its activity was potentially due to the high affinity to HA protein, thus blocking the attachment of influenza viruses to cells. Docking studies

suggested that these compounds occupied the conserved pocket for sialic acid receptor, which was consistent with the SAR data. There results confirmed that the suitable incorporation of L-ascorbic acid into the triterpene entity enabled an achievement of higher inhibitory potency of anti-viruses. This study suggested that L-ascorbic acid-conjugated pentacyclic triterpenes were promising anti-influenza entry inhibitors, and HA protein associated with viral entry was a promising drug target

4. Experimental section

4.1 General information

Materials. Madin-Darby canine kidney (MDCK) cells and human embryonic kidney 293T (HEK293T) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL. Inc., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Linz Austria) at 37 °C under 5% CO_2. The following virus was used in this study: influenza A/WSN/33 (H1N1) virus.

Mouse anti-NP monoclonal antibody and rabbit anti-GAPDH polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA. USA). Rabbit monoclonal antibodies against influenza virus HA (H1N1) were supplied from Sino Biological Inc. (Beijing, China). The anti-rabbit IgG and anti-mouse IgG conjugated with horseradish peroxidase (HRP) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

OSV of 98% purity (Hoffmann-La Roche Ltd., Basel, Switzerland) was used as reference compounds in CPE reduction assay. OA and UA were kindly supplied by Nanjing Zelang Medical Technology Co., Ltd (Nanjing, China) and Anboruila Biotechnology Co. Ltd (Nanjing, China), respectively. BA was supplied by Aladdin-Reagent Co. Ltd (Shanghai, China). EA was separated from the ethanol/H_2O crude extract of *Gleditsia sinensis Lam*, a traditional Chinese herbal medicine. All the compounds were dissolved in DMSO to 10 mM as a stock solution.

Chemistry. High-resolution mass spectra (HRMS) were obtained with an APEX IV FT_MS (7.0 T) spectrometer (Bruker) in positive ESI mode. NMR spectra were recorded on a Bruker DRX 400 spectrometer at ambient temperature. Chemical shifts of ^{1}H NMR were referenced to the internal standard TMS (δ_H = 0.00) or the solvent signal (δ_H = 3.31 for the central line of MeOD). Chemical shifts of ^{13}C NMR were referenced to the solvent signal (δ_C = 77.00 for the central line of $CDCl_3$, δ_C = 49.00 for the central line of MeOD). Reactions were monitored by

thin-layer chromatography (TLC) on a 60 F_{254} plate (pre-coated with silica gel, layer thickness of 0.2 mm; E. Merck, Darmstadt, Germany) and detected by staining with a yellow solution containing $Ce(NH_4)_2(NO_3)_6$ (0.5 g) and $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (24.0 g) in 6% H_2SO_4 (500 mL), followed by heating.

4.2 General procedures

Compound **2** was synthesized from L-ascorbic acid, according to the procedure described by Spickenreither and Tahir with minor modifications [47-48]. Benzylation or methylation of the C-2 and C-3 hydroxy groups of the lactone ring in **2** was accomplished using K_2CO_3 and benzyl bromide or methyl iodide in DMF to provide **3** or **4**, respectively. Deblocking of 5,6-*O*,*O*-protected-L-ascorbic acid intermediates with 2 N HCl in THF-CH_3OH solution gave 2,3-*O*,*O*-dialkyl-L-ascorbic acid (**5** or **6**). Then tosylation of C-6 hydroxy group in **5** or **6** using TsCl in the presence of triethylamine provided 6-*O*-tosyl-2,3-*O*,*O*-dialkyl-L-ascorbic acid (**7** or **8**). This was followed by nucleophilic substitution with sodium azide in DMF to provide the key intermediates **9** or **10**. Subsequent reduction of the azide group in **10** was carried out through the treatment with Ph_3P in THF/H_2O (1:1) to yield intermediate **11** [47, 54-56]. The detailed synthesis, ^1H and ^{13}C NMR data of compounds **2-8**, **10** and **11** are available in the supporting information.

Compounds **15-17**, **28**, **32-34** and **41** were synthesized using previously published methods [19-23].

4.2.1 General procedure for click chemistry reaction (method A)

The starting material compounds **9** or **10** (1 equiv.), $CuSO_4\cdot 5H_2O$ (1.2 equiv.) and sodium ascorbate (2.4 equiv.) were added to a solution of compound triterpenoid substituted by alkynyl (1.2 equiv.), respectively, in mixture solvant of THF/H_2O (1:1). The mixture was stirred at room temperature over night and monitored by TLC. The organic phase was extracted with CH_2Cl_2, dried over sodium sulfate and concentrated under reduced pressure. The obtained residue was purified by column chromatography on a silica gel using a gradient mixture of petroleum ether/ethyl acetate with increasing polarity as an eluent.

4.2.2 General procedure for reduction reaction (method B)

The starting material (1 equiv.) was dissolved in MeOH (6 mL), and palladium–carbon (0.1 equiv.) was added. The suspension was degassed under vacuum and urged with H_2 for three times, and then it was stirred under H_2 balloon at room temperature for 24 h. The suspension was filtered

through a pad of Celite, and the pad cake was washed with CH_3OH. The combined filtrate was concentrated to dryness. The residue was subjected to flash chromatography to afford the product.

4.2.3 General procedure for amide bond formation reaction (method C)

EDC (1.1 equiv.) was added to a stirred solution of compound **11** (1 equiv.) and triterpenoid substituted by OBt (1 equiv.) and 4-DMAP (1.1 equiv.) in THF, and the mixture was then stirred at room temperature for 12 h. The precipitate was filtered and washed with CH_2Cl_2. Purification by TLC was performed on a silica gel (petroleum ether/ethyl acetate) to yield the product.

*4.2.4 6-azido-6-deoxy-2,3-di-O-methoxy-L ascorbic acid (**9**)*

To solution of compound **7** (500 mg, 1.4 mmol) in DMF, NaN_3 (181 mg, 2.79 mmol) was added at room temperature, and mixture was stirred for 30 min. Temperature was then raised to 60 °C, and reaction solution was stirred overnight. Reaction was terminated and concentrated by evaporation *in vacuo*. Purification of crude product mixture was performed on silica gel column chromatography (petroleum ether/ethyl acetate= 5:1) to yield compound **9** as white oil (240 mg, 75 %). ^{1}H NMR (400 MHz, MeOD): δ 4.77 (s, 1H), 4.73 (d, 1H, J = 1.9 Hz), 4.18 (s, 3H), 3.96 (m, 1H), 3.79 (s, 3H), 3.50 (m, 1H), 3.38 (m, 1H); ^{13}C NMR (100 MHz, MeOD): δ 171.68, 159.64, 124.19, 77.32, 69.33, 61.16, 60.11, 54.10; ESI-HRMS Calcd for $C_8H_{12}N_3O_5$ $[M+H]^+$: 230.0771; found 230.0772.

*4.2.5 1-[2-(3,4-bis-methoxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-4-[N-(3β-hydroxy-olean-12-en-28-oyl)-amino]methyl-1H-1,2,3-triazole (**18**)*

Compound **18** was prepared from compounds **15** (587 mg, 1.19 mmol) and **9** (227 mg, 0.99 mmol) according to general procedure A. The residue was purified by gradient mixture of petroleum ether/ethyl acetate with increasing polarity as an eluent to yield the **18** as a white solid (620 mg, 87 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (s, 1H), 6.82 (s, 1H), 5.30 (s, 1H), 5.19 (d, 1H, J = 6.0 Hz), 4.54 (m, 3H), 4.29 (m, 3H), 4.06 (s, 3H), 3.75 (s, 3H), 3.11 (m, 1H), 2.52 (d, 1H, J = 10.8 Hz), 1.06, 0.89, 0.82, 0.82, 0.78, 0.69, 0.42 (s, each 3H), 0.62 (d, 1H, J = 10.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.31, 169.11, 156.92, 144.20, 143.76, 124.17, 122.99, 122.93, 78.52, 75.45, 68.21, 60.25, 59.25, 54.85, 52.39, 50.02, 47.25, 46.29, 45.93, 41.59, 39.04, 38.47, 38.24, 36.63, 34.63, 33.82, 32.76, 32.26, 32.13, 30.42, 27.87, 26.97, 26.78, 25.53, 23.37, 23.16, 18.01, 16.23, 15.44, 15.07; ESI-HRMS Calcd for $C_{41}H_{63}N_4O_7$ $[M+H]^+$: 723.4691, found 723.4685.

*4.2.6 1-[2-(3,4-bis-methoxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-4-[N-(3β,16α-dihydroxy- -olean-12-en-28-oyl)-amino]methyl-1H-1,2,3-triazole (**19**)*

Compound **19** was prepared from compounds **17** (293 mg, 0.58 mmol) and **9** (110 mg, 0.48 mmol) according to general procedure A. The residue was purified by gradient mixture of petroleum ether/ethyl acetate with increasing polarity as an eluent to yield **19** as a white solid (359 mg, 84 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (s, 1H), 6.97 (s, 1H), 5.45 (s, 1H), 4.87 (s, 1H), 4.53 (m, 3H), 4.29 (s, 4H), 4.07 (s, 3H), 3.76 (s, 3H), 3.14 (d, 1H, J = 6.5 Hz), 2.67 (d, 1H, J = 12.3 Hz), 2.18 (t, 1H, J = 13.0 Hz), 1.28, 0.90, 0.84, 0.82, 0.79, 0.69, 0.45 (s, each 3H), 0.64 (d, 1H, J = 11.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.33, 169.33, 157.06, 144.18, 143.37, 124.08, 123.40, 123.16, 78.73, 75.70, 74.79, 68.53, 60.43, 59.48, 55.06, 53.36, 52.59, 48.89, 46.73, 46.58, 41.66, 41.43, 39.43, 38.64, 38.47, 36.80, 35.14, 35.03, 34.20, 32.51, 30.14, 29.57, 27.97, 27.00, 26.65, 24.99, 23.25, 18.14, 16.57, 15.56, 15.49; ESI-HRMS Calcd for $C_{41}H_{63}N_4O_8$ $[M+H]^+$: 739.4640, found 739.4637.

*4.2.7 1-[2-(3,4-bis-methoxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-4-[N-(3β-hydroxy-urs-12-en-28-oyl)-amino]methyl-1H-1,2,3-triazole (**20**)*

Compound **20** was prepared from compounds **17** (291 mg, 0.59 mmol) and **10** (112 mg, 0.49 mmol) according to general procedure A. The residue was purified by gradient mixture of petroleum ether/ethyl acetate with increasing polarity as an eluent to yield **20** as a white solid (290 mg, 68 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 (s, 1H), 6.79 (s, 1H), 5.25 (s, 1H), 5.19 (m, 1H), 4.55 (s, 2H), 4.47 (m, 1H), 4.25 (m, 3H), 4.06 (s, 3H), 3.75 (s, 3H), 3.11 (br s, 1H), 2.38 (s, 1H), 0.99, 0.89, 0.86, 0.79, 0.77, 0.69, 0.44 (s, each 3H), 0.61 (d, 1H, J = 11.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.15, 169.11, 156.91, 144.17, 138.56, 125.92, 124.05, 123.02, 78.54, 75.49, 68.23, 60.25, 59.26, 54.88, 53.15, 52.43, 50.04, 47.35, 47.25, 42.00, 39.39, 39.23, 38.67, 38.47, 38.41, 36.78, 36.61, 34.54, 32.50, 30.59, 27.92, 27.51, 26.83, 24.45, 23.07, 20.96, 18.00, 16.91, 16.25, 15.49, 15.20; ESI-HRMS Calcd for $C_{41}H_{63}N_4O_7$ $[M+H]^+$: 723.4691, found 723.4692.

*4.2.8 1-[2-(3,4-bis-benzyloxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-4-[N-(3β-hydroxy-olean-12-en-28-oyl)-amino]methyl-1H-1,2,3-triazole (**21**)*

Compound **21** was prepared from compounds **15** (211 mg, 0.43 mmol) and **10** (136 mg, 0.36 mmol) according to general procedure A. The residue was purified by gradient mixture of

petroleum ether/ethyl acetate with increasing polarity as an eluent to yield **21** as a white solid (266 mg, 85 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36-7.32 (m, 8H), 7.22 (m, 2H), 6.74 (br s, 1H), 5.51 (s, 1H), 5.22 (d, 1H, J = 11.8 Hz), 5.06 (s, 2H), 4.54 (s, 1H), 4.35 (br s, 1H), 3.95 (br s, 1H), 3.52 (m, 1H), 3.35 (m, 1H), 3.21 (m, 1H), 2.78 (d, 1H, J = 12.0 Hz), 2.20 (t, 1H, J = 13.3 Hz), 1.34, 0.99, 0.94, 0.90, 0.88, 0.78, 0.74 (s, each 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.55, 169.54, 156.99, 144.30, 143.48, 135.90, 135.31, 129.00, 128.64, 128.58, 127.75, 125.54, 123.34, 121.26, 78.85, 76.60, 74.86, 73.88, 73.44, 68.94, 55.15, 49.25, 46.71, 43.08, 41.84, 41.63, 39.61, 38.72, 38.53, 36.89, 35.23, 35.04, 32.50, 30.12, 29.15, 28.02, 27.09, 26.82, 25.32, 23.37, 18.19, 17.98, 17.00, 15.60; ESI-HRMS Calcd for $C_{53}H_{71}N_4O_7$ $[M+H]^+$: 875.5317, found 875.5318.

*4.2.9 1-[2-(3,4-dihydroxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-4-[N-(3β-hydroxy-olean-12-en-28-oyl)-amino]methyl-1H-1,2,3-triazole (**22**)*

Compound **22** was prepared from **21** (80 mg, 0.09 mmol) according to general procedure B. The residue was purified by column chromatography (CH_2Cl_2/MeOH) to yield **22** as a white solid (50 mg, 80 %). ^{1}H NMR (400 MHz, MeOD): δ 7.85 (s, 1H), 7.73 (t, 1H, J = 5.3 Hz), 5.34 (s, 1H), 4.68 (m, 2H), 4.51 (m, 1H), 4.39 (s, 2H), 4.30 (br s, 1H), 3.14 (dd, 1H, J = 4.3, 10.8 Hz), 2.78 (d, 1H, J = 10.2 Hz), 2.05 (m, 1H), 1.88 (m, 2H), 1.77 (t, 1H, J = 13.6 Hz), 1.15, 0.96, 0.94, 0.91, 0.91, 0.77, 0.54 (s, each 3H), 0.72 (d, 1H, J = 11.3 Hz); ^{13}C NMR (100 MHz, MeOD): δ 180.28, 172.84, 153.61, 146.06, 144.97, 125.61, 124.16, 120.20, 79.63, 77.41, 69.22, 56.66, 53.84, 49.85, 48.94, 47.60, 47.45, 42.81, 42.50, 40.56, 39.80, 38.06, 35.79, 35.04, 34.10, 33.75, 33.60, 31.60, 28.76, 28.43, 27.81, 26.52, 24.49, 24.04, 19.43, 17.52, 16.35, 15.94; ESI-HRMS Calcd for $C_{39}H_{59}N_4O_7$ $[M+H]^+$: 695.4378; found 695.4368.

*4.2.10 1-[2-(3,4-bis-benzyloxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-4-[N-(3β,16α-dihydroxy-olean-12-en-28-oyl)-amino]methyl-1H-1,2,3-triazole (**23**)*

Compound 23 was prepared from **17** (211 mg, 0.43 mmol) and **10** (136 mg, 0.36 mmol) according to general procedure A. The residue was purified by gradient mixture of petroleum ether/ethyl acetate with increasing polarity as an eluent to yield the **23** as a white solid (266 mg, 85 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (br s, 1H), 7.37-7.32 (m, 8H), 7.20 (br s, 2H), 6.98 (s, 1H), 5.52 (s, 1H), 5.12 (d, 2H, J = 11.7 Hz), 5.08 (d, 2H, J = 11.4 Hz), 4.57 (m, 3H), 4.35 (m, 4H), 3.20 (m, 1H), 2.73 (d, 1H, J = 12.6 Hz), 2.26 (t, 1H, J = 13.3 Hz), 1.34, 0.97, 0.91, 0.89, 0.85, 0.75, 0.53 (s, each 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.32, 169.35, 156.53, 144.27, 143.42,

135.76, 135.08, 129.02, 128.77, 128.73, 128.65, 127.76, 125.92, 123.57, 121.40, 78.86, 76.01, 75.08, 73.97, 73.66, 68.57, 55.13, 52.73, 48.99, 46.82, 46.65, 41.75, 41.56, 39.52, 38.71, 38.52, 36.87, 35.24, 35.02, 32.56, 32.51, 30.22, 29.62, 28.01, 27.11, 26.73, 25.04, 23.33, 18.20, 16.67, 15.56; ESI-HRMS Calcd for $C_{53}H_{71}N_4O_8$ $[M+H]^+$: 891.5266, found 891.5266.

*4.2.11 1-[2-(3,4-dihydroxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-4-[N-(3β,16α-dihydroxy-olean-12-en-28-oyl)-amino]methyl-1H-1,2,3-triazole (**24**)*

Compound 24 was prepared from **23** (104 mg, 0.12 mmol) according to general procedure B. The residue was purified by column chromatography (CH_2Cl_2/MeOH) to yield **24** as a white solid (65 mg, 78 %). ^{1}H NMR (400 MHz, MeOD): δ 7.84 (s, 1H), 7.56 (t, 1H, J = 5.2 Hz), 5.46 (s, 1H), 4.69 (dd, 1H, J = 4.4, 13.9 Hz), 4.63 (d, 1H, J = 1.5 Hz), 4.51 (m, 1H), 4.37 (s, 3H), 4.29 (m, 1H), 3.14 (dd, 1H, J = 4.8, 11.3 Hz), 2.87 (m, 1H), 2.37 (t, 1H, J = 13.1 Hz), 1.36, 0.96, 0.95, 0.92, 0.88, 0.77, 0.56 (s, each 3H), 0.73 (d, 1H, J = 11.1 Hz); ^{13}C NMR (100 MHz, MeOD): δ 180.10, 172.98, 154.12, 145.82, 144.82, 125.54, 124.24, 120.07, 79.62, 77.44, 75.55, 69.21, 56.74, 53.84, 49.92, 49.85, 48.08, 48.02, 42.73, 42.24, 40.69, 39.89, 39.80, 38.03, 36.31, 35.90, 33.88, 33.29, 31.83, 31.20, 28.72, 27.84, 27.31, 25.35, 24.43, 19.39, 17.60, 16.34, 16.13; ESI-HRMS Calcd for $C_{39}H_{59}N_4O_8$ $[M+H]^+$: 711.4327, found 711.4311.

*4.2.12 1-[2-(3,4-bis-benzyloxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-4-[N-(3β-hydroxy-urs-12-en-28-oyl)-amino]methyl-1H-1,2,3-triazole (**25**)*

Compound 25 was prepared from **17** (193 mg, 0.38 mmol) and **10** (123 mg, 0.32 mmol) according to general procedure A. The residue was purified by gradient mixture of petroleum ether/ethyl acetate with increasing polarity as an eluent to yield **25** as a white solid (236 mg, 84 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (s, 1H), 7.35-7.18 (m, 10 H), 6.81 (br s, 1H), 5.32 (s, 1H), 5.16 (d, 1H, J = 11.7 Hz), 5.05 (s, 2H), 4.52 (m, 6H), 3.16 (m, 1H), 1.06, 0.95, 0.92, 0.85, 0.83, 0.74, 0.52 (s, each 3H) , 0.67 (d, 1H, J = 11.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.20, 169.26, 156.59, 144.19, 138.67, 135.67, 135.02, 128.77, 128.40, 127.49, 125.97, 124.15, 121.21, 78.64, 75.84, 73.78, 73.36, 68.31, 54.92, 53.25, 52.54, 47.41, 47.29, 42.06, 39.45, 39.27, 38.71, 38.50, 38.41, 36.83, 36.64, 34.62, 32.53, 30.63, 27.95, 27.57, 26.89, 24.53, 23.11, 21.01, 18.04, 16.96, 16.33, 15.51, 15.25; ESI-HRMS Calcd for $C_{53}H_{71}N_4O_7$ $[M+H]^+$: 875.5317, found 875.5317.

*4.2.13 1-[2-(3,4-dihydroxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-4-[N-(3β-hydroxy-urs-12-en-28-oyl)-amino]methyl-1H-1,2,3-triazole (**26**)*

Compound 26 was prepared from **25** (200 mg, 0.23 mmol) according to general procedure B. The residue was purified by column chromatography (CH_2Cl_2/MeOH) to yield **26** as a white solid (141 mg, 89 %). ^{1}H NMR (400 MHz, MeOD): δ 7.85 (s, 1H), 5.32 (s, 1H), 4.68 (d, 1H, J = 1.9 Hz), 4.50 (m, 1H), 4.37 (d, 2H, J = 2.0 Hz), 4.31 (m, 1H), 3.15 (dd, 1H, J = 5.0, 11.3 Hz), 2.13 (d, 1H, J = 10.6 Hz), 2.07 (t, 1H, J = 9.7 Hz), 1.10, 0.96, 0.92, 0.90, 0.89, 0.77, 0.56 (s, each 3H), 0.71 (d, 1H, J = 11.3 Hz); ^{13}C NMR (100 MHz, MeOD): δ 180.05, 172.93, 154.03, 145.90, 139.59, 127.26, 125.54, 120.05, 79.58, 77.46, 69.24, 56.61, 54.06, 53.83, 49.85, 48.86, 48.75, 43.15, 40.72, 40.68, 40.16, 39.91, 39.75, 38.42, 37.97, 35.69, 34.06, 31.83, 28.80, 27.80, 25.23, 24.28, 24.14, 21.65, 19.36, 17.77, 17.50, 16.41, 16.06; ESI-HRMS Calcd for $C_{39}H_{59}N_4O_7$ $[M+H]^+$: 695.4378; found 695.4363.

*4.2.14 1-[2-(3,4-bis-methoxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-4-[N-(3β-hydroxy-lup-20(29)-en-28-oyl)-amino]methyl-1H-1,2,3-triazole (**29**)*

Compound 29 was prepared from **28** (222 mg, 0.45 mmol) and **9** (86 mg, 0.38 mmol) according to general procedure A. The residue was purified by gradient mixture of petroleum ether/ethyl acetate with increasing polarity as an eluent to yield the **29** as a white solid (157 mg, 58 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.88 (br s, 1H), 7.05 (br s, 1H), 4.70 (m, 2H), 4.57 (m, 2H), 4.43 (s, 2H), 4.33 (m, 1H), 4.14 (s, 3H), 3.84 (s, 3H), 3.18 (m, 2H), 3.05 (m, 1H), 2.31 (t, 1H, J = 10.8 Hz), 1.65, 0.95, 0.93, 0.78, 0.75, 0.73 (s, each 3H), 0.65 (d, 1H, J = 9.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.83, 169.20, 156.89, 150.78, 145.78, 123.24, 123.09, 109.36, 78.87, 75.61, 68.61, 60.46, 59.55, 55.63, 55.29, 53.39, 52.94, 50.50, 49.99, 46.71, 42.36, 40.66, 38.80, 38.67, 38.14, 37.73, 37.11, 34.23, 33.29, 30.76, 29.35, 27.94, 27.27, 25.55, 20.83, 19.37, 18.25, 16.09, 15.69, 15.37, 14.58; ESI-HRMS Calcd for $C_{41}H_{63}N_4O_7$ $[M+H]^+$: 723.4691, found 723.4688.

*4.2.15 1-[2-(3,4-bis-benzyloxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-4-[N-(3β-hydroxy-lup-20(29)-en-28-oyl)-amino]methyl-1H-1,2,3-triazole (**30**)*

Compound **30** was prepared from **28** (233 mg, 0.47 mmol) and **5** (150 mg, 0.39 mmol) according to general procedure A. The residue was purified by gradient mixture of petroleum ether/ethyl acetate with increasing polarity as an eluent to yield **30** as a white solid (201 mg, 59 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (s, 1H), 7.37-7.27 (m, 8H), 7.20 (m, 2H), 6.67 (br s,

1H), 5.15 (d, 1H, *J* = 11.7 Hz), 5.08 (d, 1H, *J* = 11.2 Hz), 4.72 (s, 1H), 4.59 (s, 2H), 4.53 (m, 2H), 4.41 (m, 2H), 4.35 (s, 1H), 3.16 (dd, 1H, *J* = 4.7, 11.0 Hz), 3.08 (m, 1H), 2.35 (t, 1H, *J* = 10.2 Hz), 1.66, 0.94, 0.94, 0.79, 0.78, 0.73 (s, each 3H), 0.64 (d, 1H, *J* = 8.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.71, 169.29, 156.51, 150.74, 145.18, 135.74, 135.08, 129.03, 128.73, 128.69, 128.61, 127.79, 123.94, 121.31, 109.41, 78.92, 75.82, 73.92, 73.60, 68.37, 55.63, 55.30, 52.69, 50.52, 50.00, 46.71, 42.39, 40.68, 38.78, 38.65, 38.18, 37.74, 37.11, 34.58, 34.26, 33.39, 30.79, 29.36, 27.92, 27.29, 25.56, 20.85, 19.40, 18.23, 16.09, 15.81, 15.34, 14.58; ESI-HRMS Calcd for $C_{53}H_{71}N_4O_7$ $[M+H]^+$: 875.5317, found 875.5314.

*4.2.16 1-[2-(3,4-dihydroxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-4-[N-(3β-hydroxy-lup-20(29)-en-28-oyl)-amino]methyl-1H-1,2,3-triazole (**31**)*

Compound **31** was prepared from **30** (143 mg, 0.16 mmol) according to general procedure B. The residue was purified by column chromatography (CH_2Cl_2/MeOH) to yield **31** as a white solid (100 mg, 89 %). ^{1}H NMR (400 MHz, MeOD): δ 7.78 (br s, 1H), 4.68 (m, 2H), 4.66 (m, 1H), 4.56 (m, 2H), 4.40 (br s, 2H), 4.29 (br s, 1H), 3.12 (dd, 1H, *J* = 4.8, 11.1 Hz), 2.48 (t, 1H, *J* = 10.4 Hz), 2.27 (br s, 1H), 2.14 (d, 1H, *J* = 13.2 Hz), 1.67, 0.97, 0.94, 0.84, 0.83, 0.75 (s, each 3H), 0.68 (d, 1H, *J* = 8.1 Hz); ^{13}C NMR (100 MHz, MeOD): δ 180.86, 172.89, 153.57, 152.22, 146.08, 125.34, 120.23, 109.96, 79.65, 77.40, 69.24, 56.89, 53.96, 52.05, 51.39, 48.04, 43.45, 41.96, 40.09, 39.92, 39.17, 38.91, 38.28, 35.53, 34.01, 31.89, 30.53, 28.61, 28.01, 26.96, 22.10, 19.67, 19.41, 16.78, 16.64, 16.11, 15.09; ESI-HRMS Calcd for $C_{39}H_{59}N_4O_7$ $[M+H]^+$: 695.4384; found 695.4382.

*4.2.17 N-(3β-hydroxy-olean-12-en-28-oyl)-6-amino-6-deoxy-2,3-bis-benzyloxy-L-ascorbic acid (**35**)*

Compound **35** was prepared from **32** (245 mg, 0.54 mmol) and **11** (173 mg, 0.49 mmol) according to general procedure C. The residue was purified by gradient mixture of petroleum ether/ethyl acetate with increasing polarity as an eluent to yield the **35** as a white solid (187 mg, 48 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36-7.32 (m, 8H), 7.21 (m, 2H), 6.75 (br s, 1H), 5.51 (br s, 1H), 5.17 (d, 2H, *J* = 11.8 Hz), 5.06 (s, 2H), 4.54 (s, 1H), 4.35 (s, 1H), 3.95 (br s, 1H), 3.51 (m, 1H), 3.35 (m, 1H), 3.22 (m, 1H), 2.78 (d, 1H, *J* = 11.3 Hz), 2.22 (t, 1H, *J* = 13.3 Hz), 1.35, 0.99, 0.94, 0.90, 0.88, 0.78, 0.74 (s, each 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.48, 169.54, 156.99, 143.45, 135.88, 135.29, 128.96, 128.55, 127.69, 123.28, 121.23, 78.81, 76.58, 74.83, 73.86, 73.74, 73.39, 68.88, 55.12, 49.20, 46.68, 43.06, 41.80, 41.57, 39.57, 38.69, 38.51, 36.85, 35.20, 35.01,

32.50, 30.10, 29.18, 28.00, 27.05, 26.80, 25.29, 23.34, 18.17, 16.98, 15.58; ESI-HRMS Calcd for $C_{50}H_{68}NO_7$ $[M+H]^+$: 794.4996; found 794.4969.

*4.2.18 N-(3β-hydroxy-olean-12-en-28-oyl)-6-amino-6-deoxy-L-ascorbic acid (**36**)*

Compound **36** was prepared from **35** (40 mg, 0.05 mmol) according to general procedure B. The residue was purified by column chromatography (CH_2Cl_2/MeOH) to yield **36** as a white solid (23 mg, 74 %). ^{1}H NMR (400 MHz, MeOD): δ 7.31 (br s, 1H), 5.50 (br s, 1H), 4.53 (br s, 1H), 4.32 (br s, 1H), 3.92 (br s, 1H), 3.46 (m, 1H), 3.15 (d, 1H, J = 10.4 Hz), 2.91 (d, 1H, J = 13.0 Hz), 2.35 (t, 1H, J = 12.2 Hz), 1.37, 0.97, 0.97, 0.97, 0.89, 0.83, 0.78 (s, each 3H); ^{13}C NMR (100 MHz, MeOD): δ 180.10, 173.47, 157.75, 144.98, 124.05, 121.26, 80.12, 79.65, 68.46, 56.70, 47.57, 47.46, 42.80, 42.36, 40.66, 38.11, 35.17, 34.26, 33.86, 33.67, 31.63, 28.77, 27.85, 26.54, 24.53, 24.20, 23.87, 19.47, 17.78, 16.35, 15.94; ESI-HRMS Calcd for $C_{36}H_{56}NO_7$ $[M+H]^+$: 614.4057; found 614.4061.

*4.2.19 N-(3β,16α-dihydroxy-olean-12-en-28-oyl)-6-amino-6-deoxy-2,3-bis-benzyloxy-L-ascorbic acid (**37**)*

Compound **37** was prepared from **33** (257 mg, 0.55 mmol) and **11** (176 mg, 0.50 mmol) according to general procedure C. The residue was purified by gradient mixture of petroleum ether/ethyl acetate with increasing polarity as an eluent to yield **37** as a white solid (209 mg, 52 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36-7.31 (m, 8H), 7.22 (m, 2H), 6.77 (t, 1H, J = 5.3 Hz), 5.51 (m, 1H), 5.17 (d, 1H, J = 11.8 Hz), 5.06 (s, 2H), 4.54 (d, 1H, J = 1.4 Hz), 4.35 (br s, 1H), 3.95 (br s, 1H), 3.51 (m, 1H), 3.37 (m, 1H), 3.21 (m, 1H), 2.79 (d, 1H, J = 10.6 Hz), 2.20 (t, 1H, J = 13.4 Hz), 1.34, 0.98, 0.94, 0.90, 0.87, 0.77, 0.74 (s, each 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.44, 169.59, 157.04, 143.42, 135.86, 135.28, 128.93, 128.53, 127.66, 123.24, 121.21, 78.79, 76.58, 74.75, 73.86, 73.36, 68.78, 55.11, 49.18, 46.66, 43.01, 41.78, 41.52, 39.55, 38.67, 38.49, 36.83, 35.18, 34.98, 32.49, 30.08, 29.15, 27.99, 27.02, 26.78, 25.30, 23.32, 18.16, 16.96, 15.57; ESI-HRMS Calcd for $C_{50}H_{68}NO_8$ $[M+H]^+$: 810.4939, found 810.4930.

*4.2.20 N-(3β,16α-dihydroxy-olean-12-en-28-oyl)-6-amino-6-deoxy-L-ascorbic acid (**38**)*

Compound **38** was prepared from **37** (100 mg, 0.12 mmol) according to general procedure B. The residue was purified by column chromatography (CH_2Cl_2/MeOH) to yield **38** as a white solid (62 mg, 80 %). ^{1}H NMR (400 MHz, MeOD): δ 7.31 (t, 1H, J = 5.1 Hz), 5.50 (s, 1H), 4.58 (d, 1H, J = 1.6 Hz), 4.32 (br s, 1H), 3.93 (br s, 1H), 3.44 (m, 1H), 3.16 (dd, 1H, J = 4.8, 11.1 Hz), 2.91

(dd, 1H, *J* = 3.1, 13.6 Hz), 2.33 (t, 1H, *J* = 13.2 Hz), 1.38, 0.98, 0.98, 0.96, 0.89, 0.83, 0.78 (s, each 3H); ^{13}C NMR (100 MHz, MeOD): δ 180.81, 173.16, 154.88, 144.77, 124.28, 119.84, 79.62, 78.20, 75.44, 68.93, 56.76, 50.21, 49.85, 48.20, 47.98, 42.85, 42.38, 40.81, 39.94, 39.81, 38.07, 36.23, 36.10, 33.94, 33.27, 31.39, 31.15, 28.73, 27.84, 27.38, 25.62, 24.49, 19.43, 17.86, 16.33, 16.17; ESI-HRMS Calcd for $C_{36}H_{56}NO_8$ $[M+H]^+$: 630.4000, found 630.3991.

*4.2.21 N-(3β-hydroxy-urs-12-en-28-oyl)-6-amino-6-deoxy-2,3-bis-benzyloxy-L-ascorbic acid (**39**)*

Compound **39** was prepared from **34** (165 mg, 0.36 mmol) and **11** (117 mg, 0.34 mmol) according to general procedure C. The residue was purified by gradient mixture of petroleum ether/ethyl acetate with increasing polarity as an eluent to yield **39** as a white solid (109 mg, 40 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38-7.32 (m, 8H), 7.22 (m, 2H), 6.34 (t, 1H, *J* = 5.4 Hz), 5.31 (br s, 1H), 5.15 (d, 2H, *J* = 11.7 Hz), 5.09 (s, 2H), 4.55 (d, 1H, *J* = 2.0 Hz), 3.95 (br s, 1H), 3.52 (m, 1H), 3.31 (m, 1H), 3.21 (m, 2H), 1.09, 0.99, 0.95, 0.87, 0.86, 0.78, 0.72 (s, each 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 180.03, 169.25, 156.86, 139.42, 135.93, 135.35, 129.07, 128.63, 128.59, 127.74, 125.92, 121.26, 78.92, 76.55, 73.84, 73.41, 69.37, 55.09, 53.76, 47.80, 47.47, 43.06, 42.39, 39.68, 39.49, 38.97, 38.71, 38.58, 37.30, 36.88, 32.67, 30.79, 28.10, 27.78, 27.14, 24.74, 23.35, 23.27, 21.17, 18.21, 17.17, 16.85, 15.60, 15.44; ESI-HRMS Calcd for $C_{50}H_{68}NO_7$ $[M+H]^+$: 794.4990, found 794.4979.

*4.2.22 N-(3β-hydroxy-urs-12-en-28-oyl)-6-amino-6-deoxy-L-ascorbic acid (**40**)*

Compound **40** was prepared from **39** (85 mg, 0.11 mmol) according to general procedure B. The residue was purified by column chromatography (CH_2Cl_2/MeOH) to yield **40** as a white solid (57 mg, 86 %). ^{1}H NMR (400 MHz, MeOD): δ 7.30 (t, 1H, *J* = 5.2 Hz), 5.36 (br s, 1H), 4.60 (br s, 1H), 3.95 (m, 1H), 3.38 (m, 2H), 3.15 (dd, 1H, *J* = 4.3, 10.9 Hz), 2.14 (d, 1H, *J* = 10.7 Hz), 2.07 (dd, 1H, *J* = 4.0, 13.5 Hz), 1.13, 0.98, 0.96, 0.92, 0.91, 0.82, 0.78, 0.74 (s, each 3H); ^{13}C NMR (100 MHz, MeOD): δ 180.98, 173.07, 154.33, 139.86, 127.46, 120.05, 79.69, 78.02, 69.03, 56.70, 54.33, 43.56, 43.35, 40.89, 40.22, 39.99, 39.83, 38.55, 38.09, 34.18, 31.91, 29.00, 28.77, 27.89, 25.34, 24.40, 24.04, 21.56, 19.44, 17.93, 17.69,16.36, 16.03; ESI-HRMS Calcd for $C_{36}H_{56}NO_7$ $[M+H]^+$: 614.4051, found 614.4057.

*4.2.23 N-(3β-hydroxy-lup-20(29)-en-28-oyl)-6-amino-6-deoxy-2,3-bis-benzyloxy-L-ascorbic acid (**42**)*

Compound **42** was prepared from **41** (230 mg, 0.72 mmol) and **11** (179 mg, 0.50 mmol) according to general procedure C. The residue was purified by gradient mixture of petroleum ether/ethyl acetate with increasing polarity as an eluent to yield **42** as a white solid (210 mg, 53 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38-7.34 (m, 8H), 7.23 (m, 2H), 6.11 (br s, 1H), 5.20 (m, 4H), 4.73 (s, 1H), 4.59 (s, 1H), 4.56 (m, 1H), 4.12 (dd, 1H, *J* = 7.1, 14.3 Hz), 3.97 (m, 1H), 3.57 (m, 1H), 3.42 (m, 1H), 3.17 (dd, 2H, *J* = 4.9, 11.0 Hz), 3.07 (td, 1H, *J* = 4.3, 11.9 Hz), 2.42 (td, 1H, *J* = 3.2, 12.6 Hz), 1.68, 1.26, 0.96, 0.95, 0.87, 0.79, 0.75 (s, each 3H), 0.67 (d, 1H, *J* = 6.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.31, 169.28, 156.89, 150.75, 135.93, 135.35, 129.07, 128.71, 128.63, 128.60, 127.85, 121.30, 109.42, 78.96, 73.82, 73.52, 69.54, 60.39, 55.73, 55.35, 50.59, 50.04, 46.62, 42.69, 42.45, 40.70, 38.83, 38.69, 38.22, 37.69, 37.17, 34.35, 33.67, 30.82, 29.68, 29.45, 29.32, 27.96, 27.37, 26.89, 25.58, 21.03, 20.90, 19.47, 18.27, 16.12, 16.00, 15.36, 14.62, 14.17; ESI-HRMS Calcd for $C_{50}H_{68}NO_7$ $[M+H]^+$: 794.4990; found 794.4980.

*4.2.24 N-(3β-hydroxy-lup-20(29)-en-28-oyl)- 6-amino-6-deoxy-L-ascorbic acid (**43**)*

Compound **43** was prepared from **42** (100 mg, 0.13 mmol) according to general procedure B. The residue was purified by column chromatography (CH_2Cl_2/MeOH) to yield **43** as a white solid (57 mg, 72 %). ^{1}H NMR (400 MHz, MeOD): δ 7.75 (t, 1H, *J* = 5.5 Hz), 4.60 (s, 1H), 3.95 (m, 1H), 3.44 (m, 1H), 3.13 (dd, 1H, *J* = 5.0, 11.2 Hz), 2.59 (td, 1H, *J* = 3.5, 12.8 Hz), 2.28 (m, 1H), 2.14 (m, 1H), 0.99, 0.97, 0.95, 0.88, 0.86, 0.77, 0.75 (s, each 3H), 0.71 (d, 1H, *J* = 9.4 Hz); ^{13}C NMR (100 MHz, MeOD): δ 180.18, 173.33, 157.97, 119.94, 79.69, 77.90, 69.26, 57.47, 56.89, 51.95, 50.93, 49.85, 45.31, 43.69, 43.07, 42.08, 40.12, 39.96, 39.69, 38.77, 38.34, 35.71, 34.00, 31.22, 30.69, 28.61, 28.42, 28.05, 23.98, 23.40, 22.27, 19.47, 16.88, 16.74, 16.11, 14.94; ESI-HRMS Calcd for $C_{36}H_{58}NO_7$ $[M+H]^+$: 616.4208; found 616.4204.

4.3 Biological Assays

4.3.1 CPE reduction assay

The assay was performed as previously described [50] with some modifications. MDCK cells were seeded into 96-well plates, incubated overnight and infected with influenza virus (MOI = 0.1). Cells were suspended in DMEM supplemented with 1% FBS, containing test compound and 2 μg/mL TPCK-treated trypsin, and a final DMSO concentration of 1% was added in each well. After 40 h of incubation, CellTiter-Glo reagent (Promega Corp., Madison, WI, USA) was

added, and the plates were read using a plate reader (Tecan Infinite M2000 PRO; Tecan Group Ltd., Mannedorf, Switzerland).

4.3.2 Cytotoxicity test

Cells were grown in 96-well plates containing 1% FBS overnight and then cultured with increasing amounts of the test compounds for 40 h. Cytotoxicity was assessed with the CellTiter-Glo assay as above described.

4.3.3 Time-of-addition experiment

MDCK cells were seeded into 6-well plates at a density of 5×10^5 cells per well 24 h prior to infection and incubated at 37 °C under 5% CO_2. Then MDCK cells were infected with WSN virus at an MOI of 1. Compound was added at 0-10, 0-2, 2-5, 5-8 or 8-10 h post infection. The cell lysates were harvested and applied to Western blotting for NP analysis.

4.3.4 HI assay

Compound from a 3-fold serial dilution in saline was mixed with an equal volume of influenza virus (2 HA units) in the V-bottomed 96-well microplates. Subsequently, 50 μL of freshly prepared chicken red blood cells (cRBC) (1% v/v in saline) was added to each well. The mixture was incubated for 30 min at room temperature before observing cRBC aggregation on the plate.

4.3.5 SPR

Interactions between the influenza HA and the compounds were analyzed using the Biacore T200 system (GE Healthcare, Uppsala, Sweden) at 25 °C. Recombinant influenza HA (Sino Biological Inc., Beijing, China) was immobilized on a sensor chip (CM5) using an amine coupling kit (GE Healthcare, Buckinghamshire, UK). Final HA immobilized levels were typically ~16,000 RU. Subsequently, compounds were injected as analytes at various concentrations, and PBS-P (10 mM phosphate buffer with 2.7 mM KCl and 137 mM NaCl, 0.05% surfactant P20, pH 4.5) was used as running buffer. For binding studies, analytes were applied at corresponding concentrations in running buffer at a flow rate of 30 μL/min with a contact time of 60 s and a dissociation time of 60 s. Chip platforms were washed with running buffer and 50% DMSO. Data were analyzed with the Biacore evaluation software (T200 version 1.0) by curve fitting using a binding model of 1:1.

4.3.6 Docking simulation

The docking simulation was performed via AutoDock 4.2 (Scripps Research Institute) [28], and assessed by OpenSource PyMOL 1.5.x (Schrödinger, LLC) and PoseView web service (Universität Hamburg) [39]. Afterwards, structure of the protein (Protein Data Bank: 1RVT) was obtained from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). Protein preparation was performed as follows. After the removal of HETATM atoms, hydrogen atoms and the crystal cells, the protein was further processed via AutoDock Tools 1.5.6 rc3 (ADT, Scripps Research Institute) to add Polar hydrogen and Kollman charge. Ligand preparation was performed as follows. Polar hydrogen and Gasteiger charge were given, and there was no further adjustment on the ligand torsion tree. Grid box covering the HA head was prepared with ADT with parameters as follows: grid box center coordinate: 87.320, -9.696, -34.751; box size: 74 × 78 × 80; grid point spacing: 0.375 Å. Lamarckian genetic algorithm was applied to the docking simulation with modifications of the parameters described below due to the compounds' highly flexible nature: number of individuals in population: 300; maximum number of energy evaluations: 25,000,000; number of GA runs: 100. A hundred docking output conformations for one compound were clustered with a maximum RMS tolerance of 2.0 Å. To avoid isolated conformations, the best binding conformation from the largest cluster was used for further analysis.

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Figure Legends

Figure 1. Structures and anti-influenza virus activity of pentacyclic triterpene derivatives.

Figure 2. Structures of L-ascorbic acid, sialic acid and its analogs.

Figure 3. Inhibition of viral plaque formation induced by compound **30**

(A) The inhibition curve for compound **30** was around 8.7 μM. (B) Validation of the protection of MDCK cells from influenza A/WSN/33 virus by compound **30**.

Figure 4. Mechanism of compound **30** as an inhibitor of influenza viruses and identification of HA as potential target. (A) Time-of-addition experiments was designed to identify at which step life cycle **30** targeted, such as virus entry (0-2 h), viral genome replication and translation (2-8 h) or progeny virion release (8-10 h). Detection of the expression levels of influenza NP protein in infected MDCK cells at five time intervals, 0-10, 0-2, 2-5, 5-8, and 8-10 h. Only at two intervals, 0-10 h (covering the whole life cycle) and 0-2 h (covering the entry step), the NP expression was significantly reduced (around 90% and 80%, respectively) as compared with DMSO control. (B) Three experiments, namely co-treatment, pretreatment of cell, and pretreatment of virus, were designed to clarify whether the host cell or the influenza virus was targeted. (C) Comparisons of the behaviors of **30** vs. anti-HA antibody in inhibition of influenza virus-induced aggregation of chicken erythrocytes. **30** exerted identical capability as anti-HA antibody in hemagglutination inhibition in a dose-dependent manner. (D) Characterization of the affinity between lead compounds (**30** and **31**) and HA protein, which was immobilized on a CM5 sensor chip. Their K_D values are labeled in the corresponding curves.

Figure 5. Structural representative of **30** binding within HA protein (Protein Data Bank: 1RVT) according to blind docking calculation. (A) Overview of HA protein. The inhibitor pocket is highlighted in yellow square. Protein and compound **30** are shown as orange surface and green spheres, respectively. (B) Closer view of the inhibitor pocket. Compound **30** is shown as green sticks. (C) 2D interaction plot black dashes, green dashes and green curves indicate hydrogen bonds, π–π stacking and hydrophobic interactions correspondingly.

Scheme 1. Reagents and conditions: (a) Me_2CO, AcCl, rt. 2-3 h; 7^oC, 4-8 h; (b) CH_3I or $PhCH_2Br$, K_2CO_3, DMF; (c) 2 N HCl, THF-MeOH (1:1, V/V); (d) TsCl, Et_3N, CH_2Cl_2; (e) NaN_3, DMF, 60^oC, 24 h; (f) PPh_3, THF-H_2O (1:1, V/V).

Scheme 2. Reagents and conditions: (a) i) TBTU, DIEA, THF, rt, overnight; ii) 2-propynylamine, Na_2CO_3, DMF, rt, 20 min; (b) $CuSO_4$, Na-L-ascorbate, THF-H_2O (1:1, V/V); (c) H_2, Pd/C, 300 kPa, MeOH.

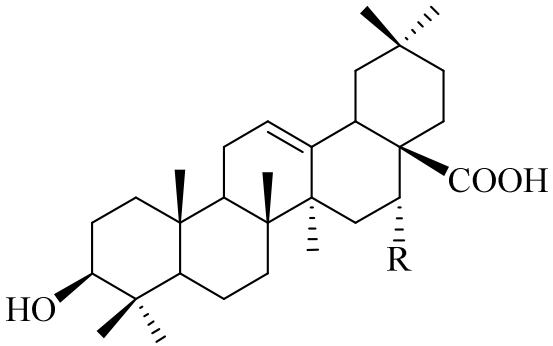
Scheme 3. Reagents and conditions: (a)TBTU, DIEA, THF, rt, overnight; (b) EDC·HCl, THF r.t. 1 h; 60^oC, 20 h; (c) H_2, Pd/C, 300 kPa, MeOH.

Table 1. Inhibitory effects of compounds against influenza A/WSN/33 virus

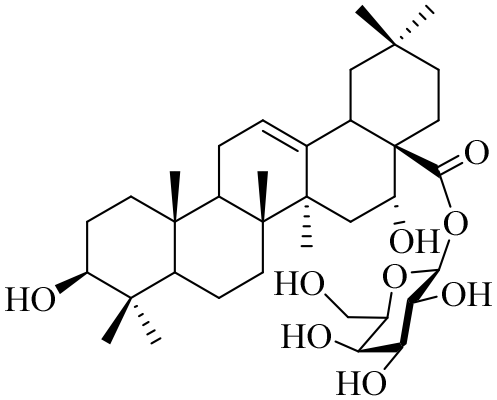
Compounds	Inhibition rate (%)	EC_{50} (μM)	CC_{50} (μM)
21	91.3	19.2	>200
23	87.0	56.6	>200
25	90.8	25.7	>200
30	89.6	8.7	>200
31	93.1	41.3	>200
35	81.9	25.3	>200
37	71.1	30.3	>200
39	88.1	26.4	>200
42	89.6	23.7	>200
43	84.4	35.6	>200
OSV-P	86.5	12.5	>200

Table 2. CC_{50} (μM) values of analogs against MDCK cell line using CellTiter-Glo assay

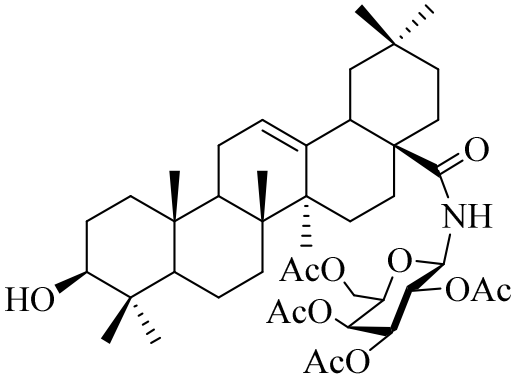
Compounds	CC_{50} (μM)	Compounds	CC_{50} (μM)
18	19.6	**26**	41.1
19	5.6	**29**	43.9
20	52.5	**36**	41.9
22	46.8	**38**	29.7
24	59.3	**40**	13.4



R = OH, echinocystic acid (**EA**)
R = H, oleanolic acid (**OA**)

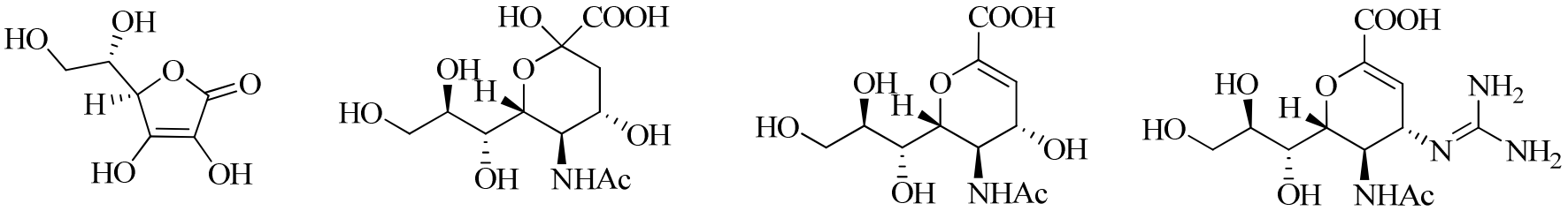


Q_8
anti-IAV(A/WSN/33)
EC_{50} = 5.0 μM



Y3
anti-IAV(A/WSN/33)
EC_{50} = 14.2 μM

Figure 1



L-ascorbic acid | sialic acid (Neu5Ac) | Neu5Ac2en | zanamivir

Figure 2

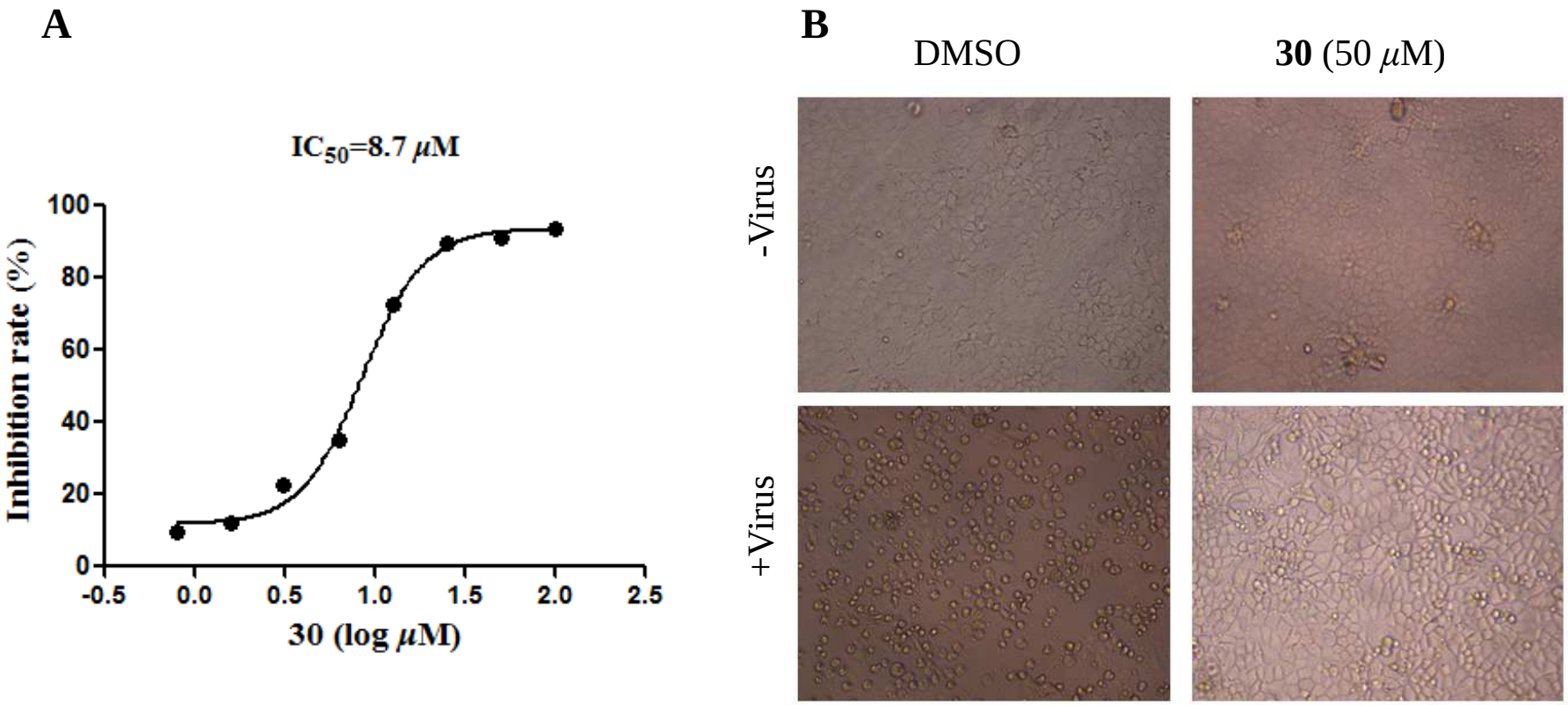
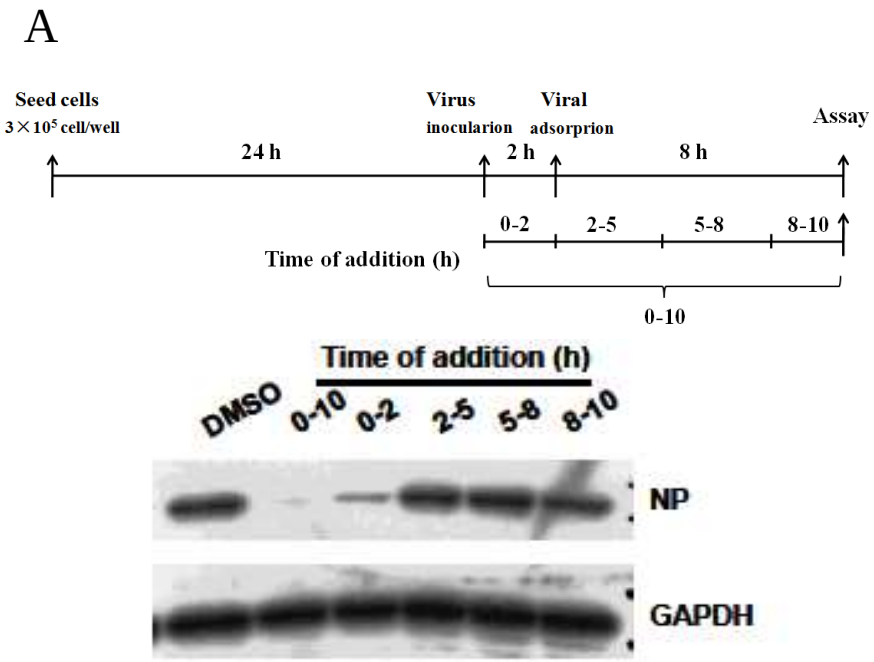
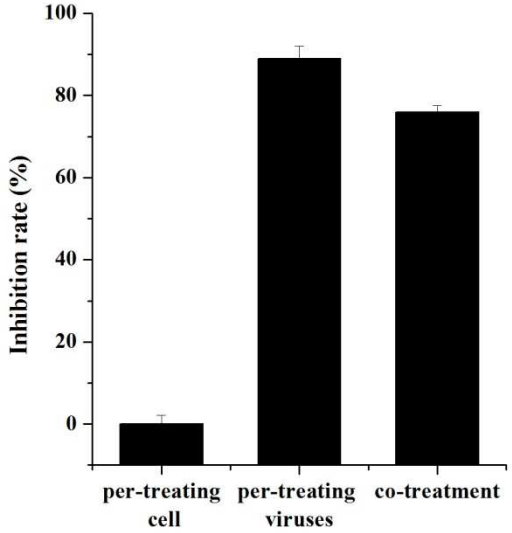
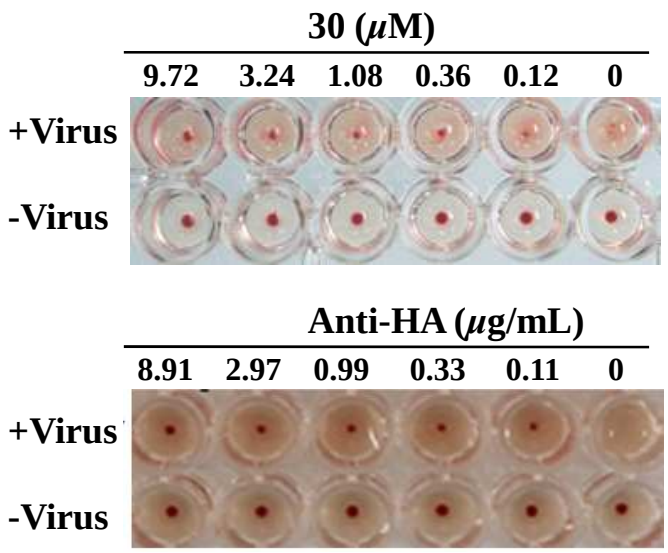

A
IC50=8.7 μM
Inhibition rate (%)
100
80
60
40
20
0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
30 (log μM)
B
DMSO
30 (50 μM)
-Virus
+Virus

Figure 3.


A
Seed cells
3×10^5 cell/well
24 h
Virus
inocularion
Viral
adsorprion
2 h
8 h
Assay
0-2
2-5
5-8
8-10
Time of addition (h)
0-10
Time of addition (h)
DMSO
0-10
0-2
2-5
5-8
8-10
NP
GAPDH


B
Inhibition rate (%)
100
80
60
40
20
0
per-treating cell
per-treating viruses
co-treatment


C
30 (μM)
9.72
3.24
1.08
0.36
0.12
0
+Virus
-Virus
Anti-HA (μg/mL)
8.91
2.97
0.99
0.33
0.11
0
+Virus
-Virus

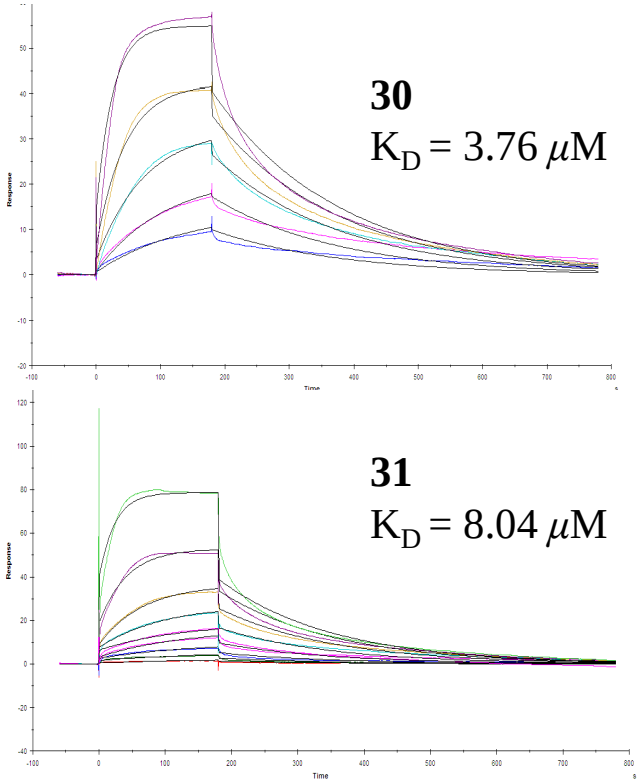
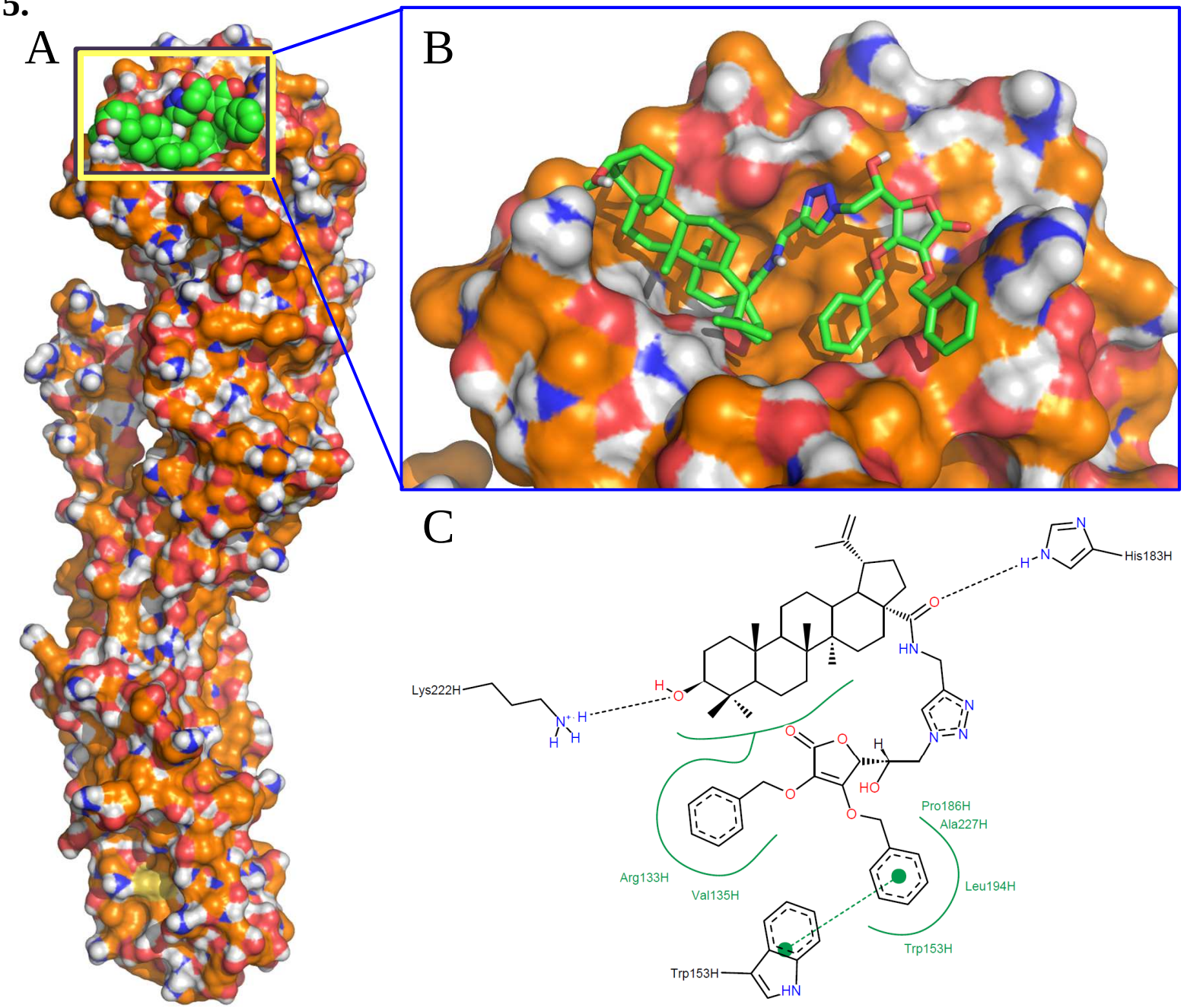
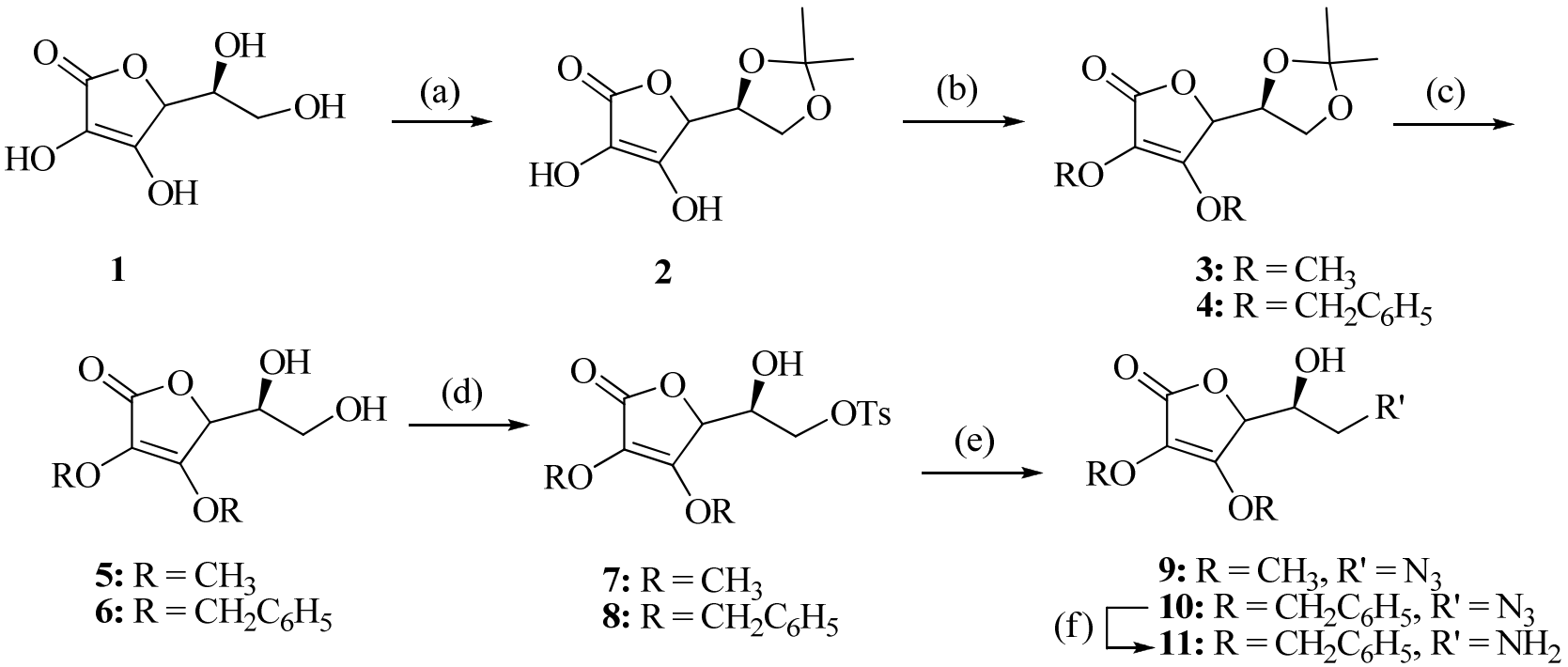

D
30
K_D = 3.76 μM
31
K_D = 8.04 μM

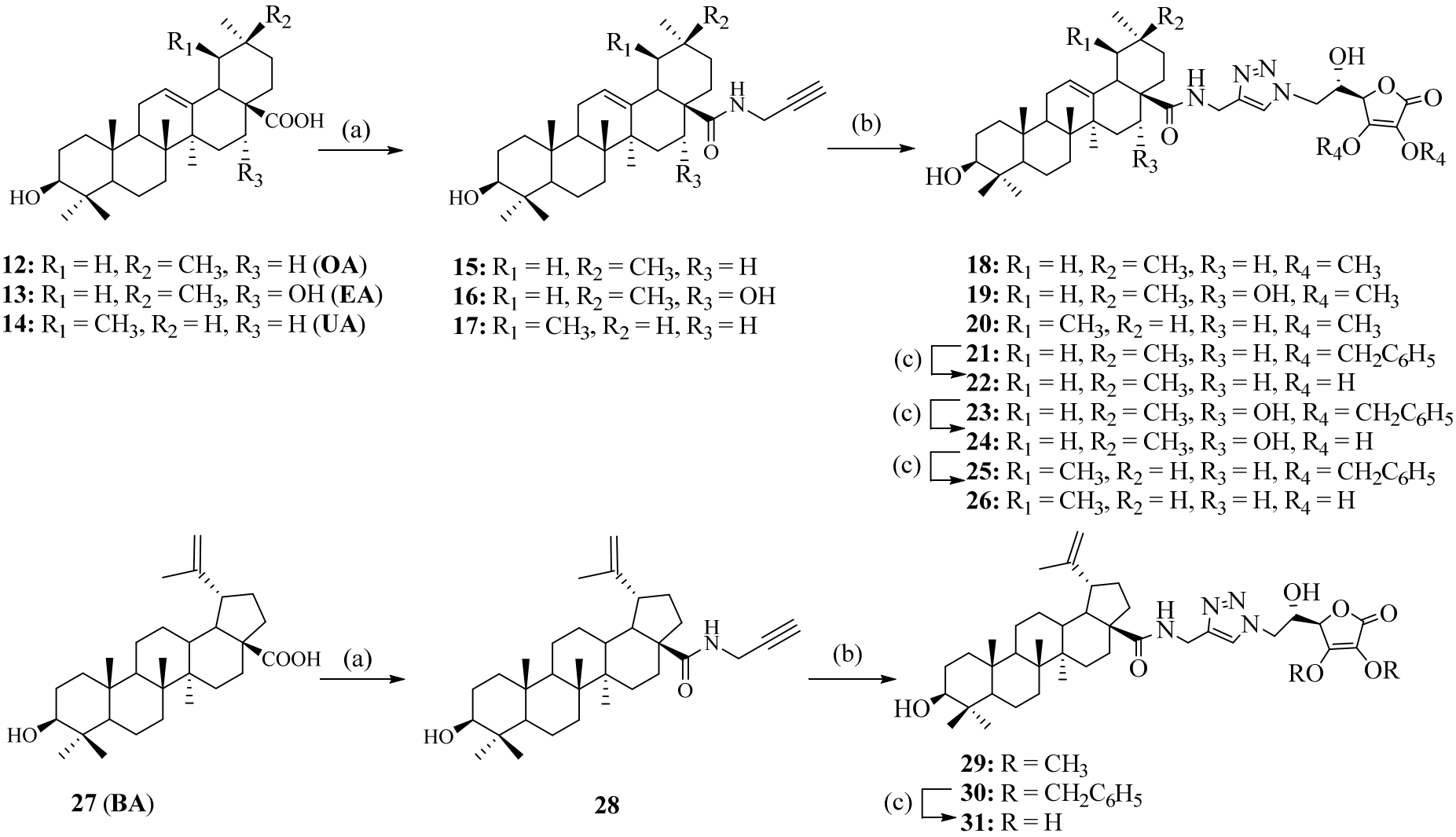
Figure 4

Figure 5.


A
B
C
Lys222H
His183H
Pro186H
Ala227H
Arg133H
Val135H
Leu194H
Trp153H
Trp153H



Scheme 1. Reagents and conditions: (a) Me_2CO, AcCl, rt. 2-3 h; 7°C, 4-8 h; (b) CH_3I or $PhCH_2Br$, K_2CO_3, DMF; (c) 2 N HCl, THF-MeOH (1:1, V/V); (d) TsCl, Et_3N, CH_2Cl_2; (e) NaN_3, DMF, 60°C, 24 h; (f) PPh_3, THF-H_2O (1:1, V/V).



12: R_1 = H, R_2 = CH_3, R_3 = H (**OA**)
13: R_1 = H, R_2 = CH_3, R_3 = OH (**EA**)
14: R_1 = CH_3, R_2 = H, R_3 = H (**UA**)

15: R_1 = H, R_2 = CH_3, R_3 = H
16: R_1 = H, R_2 = CH_3, R_3 = OH
17: R_1 = CH_3, R_2 = H, R_3 = H

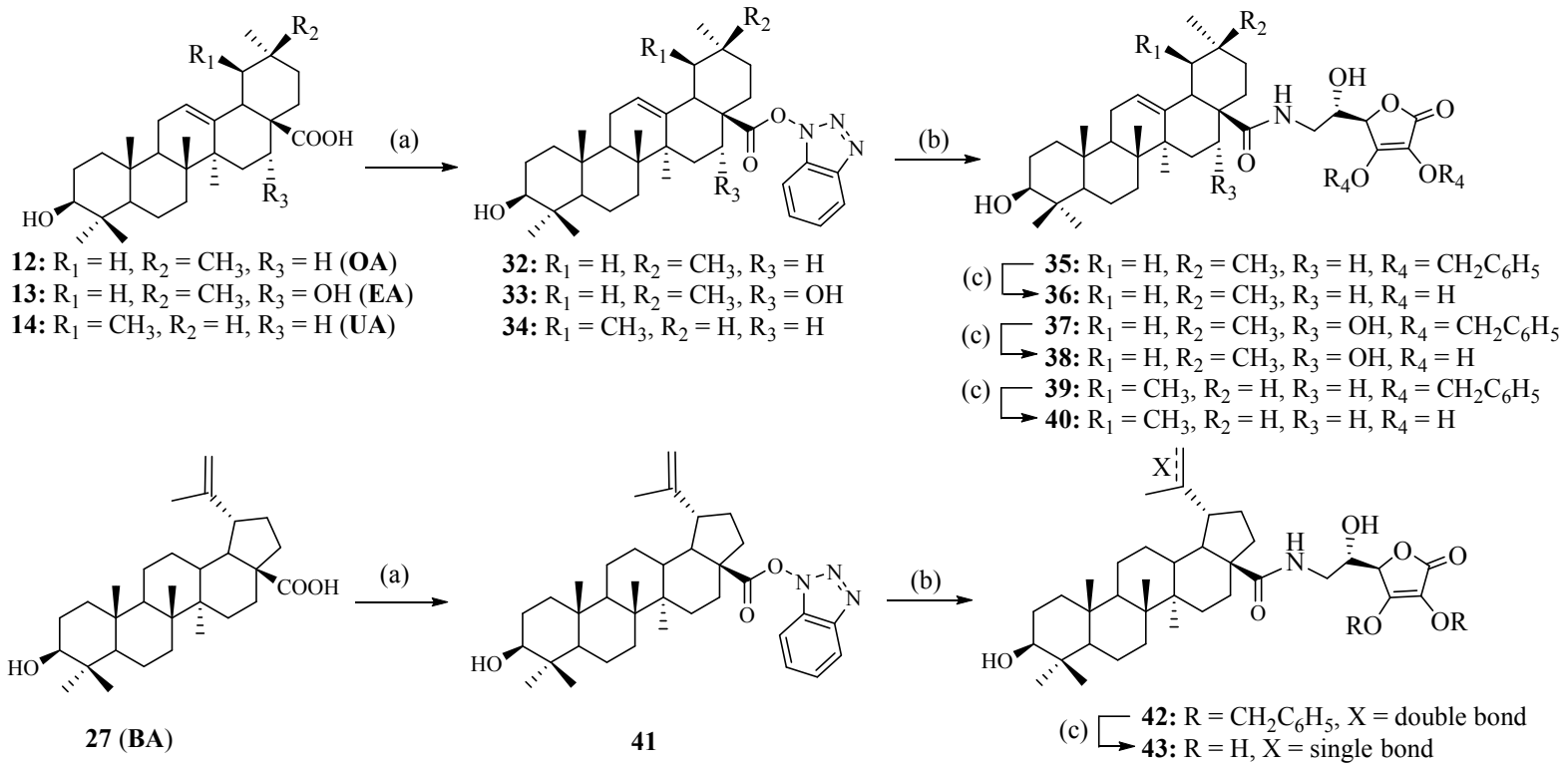
18: R_1 = H, R_2 = CH_3, R_3 = H, R_4 = CH_3
19: R_1 = H, R_2 = CH_3, R_3 = OH, R_4 = CH_3
20: R_1 = CH_3, R_2 = H, R_3 = H, R_4 = CH_3
(c) **21:** R_1 = H, R_2 = CH_3, R_3 = H, R_4 = $CH_2C_6H_5$
22: R_1 = H, R_2 = CH_3, R_3 = H, R_4 = H
(c) **23:** R_1 = H, R_2 = CH_3, R_3 = OH, R_4 = $CH_2C_6H_5$
24: R_1 = H, R_2 = CH_3, R_3 = OH, R_4 = H
(c) **25:** R_1 = CH_3, R_2 = H, R_3 = H, R_4 = $CH_2C_6H_5$
26: R_1 = CH_3, R_2 = H, R_3 = H, R_4 = H

27 (**BA**)

28

29: R = CH_3
(c) **30:** R = $CH_2C_6H_5$
31: R = H

Scheme 2. Reagents and conditions: (a) i) TBTU, DIEA, THF, rt, overnight; ii) 2-propynylamine, Na_2CO_3, DMF, rt, 20 min; (b) $CuSO_4$, Na-L-ascorbate, THF-H_2O (1:1, V/V); (c) H_2, Pd/C, 300 kPa, MeOH.



Scheme 3. Reagents and conditions: (a)TBTU, DIEA, THF, rt, overnight; (b) EDC·HCl, THF r.t. 1 h; 60°C, 20 h; (c) H_2, Pd/C, 300 kPa, MeOH.

Highlights

- A total of 20 novel L-ascorbic acid-conjugated pentacyclic triterpene derivatives were synthesized via triazole or amide linker.
- The anti-influenza activities of those conjugates against A/WSN/33 virus were evaluated.
- Compound **30** displayed the highest anti-influenza A/WSN/33 (H1N1) activity with an IC_{50} at 8.7 μM.
- Mechanistic studies indicated that compound **30** could tightly bind with HA protein (K_D = 3.76 μM), thus disrupting the interaction of HA with sialic acid receptor.