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DOI: 10.1039/C7MD00245A

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ARTICLE

Synthesis of novel pentacyclic triterpene-Neu5Ac2en derivatives and investigation of their in vitro anti-influenza entry activity

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Dedicated to Professor Lihe Zhang on the Occasion of His 80th Birthday.

Sialic acid derivatives, analogs and their conjugates are important pharmacophores. Modification of C-4 of sialic acid can lead to derivatives with potent anti-influenza activities, such as Zanamivir. Herein, we described the synthesis of C-4 modified sialic acid derivatives by conjugation with naturally derived pentacyclic triterpenes, an active ingredient of traditional Chinese medicine, and the evaluation of their *in vitro* anti-influenza virus activity in the MDCK cells. Interestingly, a set of configurational isomers was obtained during the de-*O*-acetylation reaction of two pentacyclic triterpene-sialic acid conjugates under Zemplén conditions and a mechanism was proposed. Due to the attachment of Neu5Ac2en moiety, all synthesized conjugates showed lower hydrophobicity than their parent compounds. Compared with ursane- and lupane-type triterpenes, oleanane-type triterpene functionalized Neu5Ac2en conjugates were the most promising. The insertion of a (1,2,3-triazol-4-yl) methyl between the amide bond and Neu5Ac2en caused a substantial decrease in activity. Compound **15a** showed the highest inhibitory activity (IC_{50} = 8.3 μM) and selectivity index (SI = 22.7). Further studies involving hemagglutination inhibition and neuraminidase inhibition suggested that compound **15a** inhibited virus-induced hemagglutination with no effect on the neuraminidase enzymatic activity, indicating that the antiviral activity appears to be mediated by interaction with hemagglutinin at the initial stage of viral infection.

1. Introduction

Influenza A virus, which causes a substantial number of deaths during annual epidemics and occasional pandemics, belongs to the family of single-stranded negative sense RNA viruses or Orthomyxoviridae.[1] The most well-known influenza pandemics in the 20th century, "Spanish Flu (1918/H1N1)", "Asian Flu (1957/H2N2)" and "Hong Kong Flu (1968/H3N2)", resulted in the deaths of more than 50 million people globally.[2] Up to now influenza viruses still infect 3 to 5 million people worldwide every year.[3] Although anti-influenza vaccines are available, their efficacy is limited due to the rapid mutation in the two surface antigens (hemagglutinin (HA) and neuraminidase (NA)), leaving the immune system unable to cope with the essentially new antigens.[4] Currently, only two classes of anti-influenza drugs, including M2 ion-channel inhibitors (amantadine and rimantadine) and NA inhibitors (oseltamivir, zanamivir and peramivir), have been approved by the FDA for interruption of specific processes in influenza infection.[5] However, frequent clinical applications of the marketed anti-influenza drugs combined with the high mutation rate of the RNA genome of the influenza virus lead to the rapid emergence of drug-resistant influenza virus strains.[6] Therefore, there is a continuous and urgent need for discovering new drugs to overcome resistance and looming threats of sporadic outbreaks of pandemic influenza A strains.[7]

The first step in influenza A virus infection involves attachment to cells through binding of viral HA to cell-surface receptors containing sialic acid (also called α-5-*N*-acetylneuraminic acid, Neu5Ac), leading to internalization of viral particles into endosome.[8] One approach to combating cellular infection is the inhibition of the recognition process by using sialic acid analogues that compete with the cell surface sialyl oligosaccharides, for viral HA. Unfortunately, sialic acid and its derivatives, such as α-methyl sialoside, bind only weakly to HA with dissociation constants in the millimolar range.[9] Since the crystal structure of the influenza virus HA complexed with its receptor was first published by Weis et al. in 1988,[10] there have been many efforts to search for sialic acid derivatives with high affinity for HA. Knowles' group reported that the binding affinity of sialic acid can be increased by introducing hydrophobic aglycons at C-2 position, with dissociation constants in the micromolar range.[11] Rudrawar et al also reported that the introduction of hydrophobic group at C-3 of Neu5Ac could lock open the 150-loop of an influenza A virus group-1 sialidase.[12] Therefore, four kinds of hydrophobic pentacyclic triterpene, which are widely distributed in plant kingdom and generally believed to enhance the immunity of host plants and increase plant resistance to pathogens,[13] were selected to conjugate with sialic acid via C-2 in our

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

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DOI: 10.1039/C7MD00245A

previous study.[14] However, those derivatives only showed weak anti-influenza entry activity with IC_{50} ranging from 41.2 to 95.2 μM, possibly due to the fact that when the sialic acid groups bind to the HA polymer backbone, the linking groups are brought too close to the HA surface.

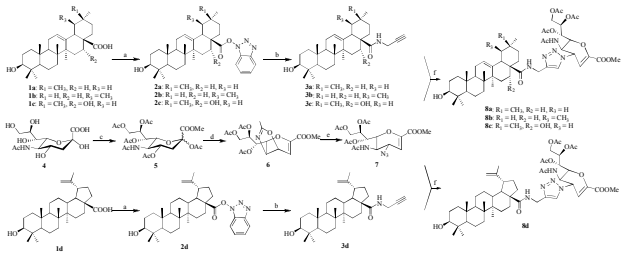
The structures of influenza HA complexed with several different 2-substituted sialic acid derivatives, such as Neu5Ac2N4 and Neu5Ac2N6, have been determined by X-ray crystallography.[15] In all cases the 4-OH of sialic acid points away from the binding site into solution and does not have any interactions with the HA protein, indicating that 4-modified sialosides would not interfere with the binding to HA. For example, Wu et al.[16] reported that a polymeric 4-N-linked sialoside strongly inhibited influenza virus HA activity with Ki of 10^{-6} M. Moreover, the addition of certain functionalities at the C-4 position of unsaturated sialic acid analog, such as 4-amino Neu5Ac2en and 4-guanidino Neu5Ac2en, should enhance its binding with the influenza virus neuraminidase (NA).[17] There have been many efforts to develop anti-influenza inhibitors based on the Neu5Ac2en.[18] However, the report of C-4 triazole modified analogs of Neu5Ac2en is rare. In 2006, Li et al. reported the synthesis of C-4 triazole analogs of Zanamivir and found one derived from 3-hydroxy pentyne showing 61% protective activity (50 μM) against infection by avian influenza virus.[19] One year later, Lu et al. reported the synthesis of C-4 triazole analogs of Neu5Ac2en derivatives as multivalent sialic acid containing scaffolds.[20]

Our recent works on the anti-influenza virus activities of pentacyclic triterpene,[14,21] along with published reports,[15,17,19] lead us to further explore novel sialic acid derivatives in which petacyclic triterpenes are attached at the 4-position of sialic acid. To ensure a stable linkage to the 4-hydroxy group under assay conditions, a triazolyl or amide bond was introduced as linkage between the two moieties. Interestingly, a set of configurational isomers was obtained during the de-*O*-acetylation reaction of **8a** and **8b** under Zemplén conditions. In addition, a five-fold more potent anti-influenza activity was found for compound **15** with better selectivity index (SI = 22.7) when conjugating pentacyclic triterpene to sialic acid via C-4 position. Herein we describe the synthesis and *in vitro* anti-influenza virus activity of those novel pentacyclic triterpene-Neu5Ac2en conjugates

2. Results and discussion

2.1 Chemistry

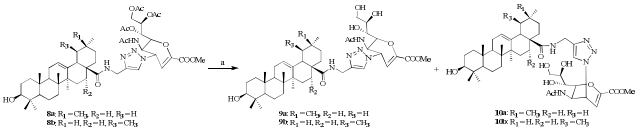
The target pentacyclic triterpene-Neu5Ac2en conjugates were synthesized as described in Scheme 1-5. The pentacyclic triterpenes used to conjugate with Neu5Ac2en included oleanolic acid (OA, **1a**), ursolic acid (UA, **1b**), echinocystic acid (EA, **1c**) and betulinic acid (BA, **1d**). Scheme 1 depicts the synthesis of conjugates **8a-d**. Firstly, alkynyl-functionalized pentacyclic triterpene derivatives **3a-d** were prepared according to published procedures.[22] The key intermediate 4-azido-



Scheme 1. Reagents and conditions: (a) TBTU, DIPEA, THF; (b) propargylamine, K_2CO_3, DMF; (c) H^+-exchange resin, RT, MeOH; then Ac_2O, pyridine, DMAP; (d) TMSOTf, EtOAc; (e) Me_3SiN_3, t-BuOH; (f) $CuSO_4$, sodium *L*-ascorbate, DCM/H_2O (1:1, *v/v*).

Neu5Ac2en **7** was synthesized from commercially available sialic acid **4** in 48% yield through a three-step process described by Chandler and co-workers.[23] The structures of compounds **6** and **7** were confirmed by ^1H and ^{13}C NMR (Supporting Information). Subsequent conjugation of **3a-c** with **7** via copper catalyzed azide-alkyne 1,3-dipolar cycloaddition reaction (CuAAC) provided the 5,7,8,9-tetra-*O*-acetyl protected intermediates **8a-c** with good yield. In a similar way, conjugation of **3d** with **7** afforded intermediate **8d** in 87% yield.

As an initial experiment, de-*O*-acetylation of **8a** was carried out under Zemplén conditions.[24] However, two compounds, **9a** and its isomer **10a**, were separated by standard silica gel chromatography in 52% and 31% yield, respectively (Scheme 2). We observed that **9a** was more polar than its isomer **10a** (based on silica gel migratory ability). The structures of the two isomers have been fully ascertained by ESI-HRMS and NMR spectroscopy. Figure 1 (A) represents the characteristic parts (δ = 3.5-6.5 ppm) of the ^1H NMR spectra (in CD_3OD) of conjugates **9a** and **10a**. The chemical shifts of the H_3 and H_5 protons of **10a** were significantly shifted to the lower field compared to its isomer **9a**. In addition, the coupling constant between H_4 and H_5 of **9a** was 9.8 Hz when these two protons are *trans*, while this coupling constant was close to 5.4 Hz when they are *cis*. To further demonstrate the configurations of the two isomers **9a** and **10a**, 2D NOE spectra were also acquired (Figure 1 (B)). The NOE experiment of compound 9a showed a NOE effect between the H_4 proton and the H_6 protons of the carbohydrate ring, while the absence of this effect in its isomer **10a**. On the contrary, an NOE effect of **10a** between H_4 proton and H_5 protons of the carbohydrate ring was observed obviously, while the absence of this effect in its isomers **9a**. Therefore, we concluded that the H_4 of **9a** and **10a** are β-orientation and α-orientation, respectively. Similar results were observed for the de-*O*-acetylation of **8b** under Zemplén conditions and two isomers **9b** and **10b** were also obtained. To the best of our knowledge, there are no other reports about this reaction. We wonder whether the triazolyl group facilitates the formation of these isomers. Therefore, de-*O*-acetylation of **7** was carried out under the same condition (Scheme 3). We found that the reaction finished quickly as shown by TLC analysis, yielding azido compound **11** exclusively in 88% yield. Subsequent direct conjugation with alkynyl-functionalized pentacyclic triterpene derivatives **3c-d** in a similar manner as described in Scheme 1 afforded conjugates **9c-d** with high yields.



Scheme 2. Reagents and conditions: (a) MeONa/MeOH.

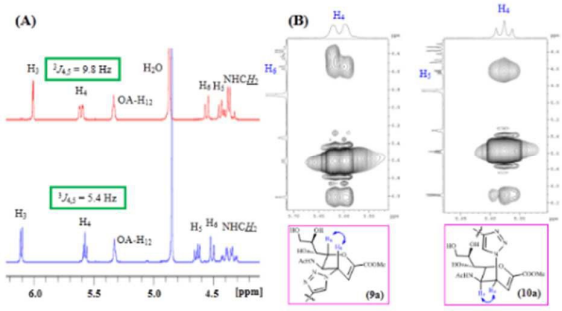


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Figure 1. (A) Characteristic portions of the 400-MHz proton spectra of conjugates **9a** (red) and **10a** (blue). (B) Section of NOESY spectra of compounds **9a** and **10a** in CD_3OD at 25°C.

Scheme 3. Reagents and conditions: (a) MeONa/MeOH; (b) $CuSO_4$, sodium *L*-ascorbate, 3c, THF/H_2O (1:1, v/v); (c) $CuSO_4$, sodium *L*-ascorbate, 3d, THF/H_2O (1:1, v/v).

Based on these results, we propose a plausible mechanism for the formation of the two isomers (Scheme 4). The first step is the removal of the acetate protecting groups by sodium methoxide in methanol to afford triol **9**. Removal of H_4 hydrogen by methoxide forms an allylic carbanion, which is further stabilized by resonance with an extended enolate to form the intermediate **13**. Racemization occurs when a proton attacks from upper or down side of the planar carbanion to generate **9** or **10**, respectively.

Alternatively, reduction of the azide group in **7** was carried out through treatment with Ph_3P in THF/H_2O (1:1) to yield intermediate **14**, which was condensed with intermediate **2a-d** in the presence of EDC to yield **15a-d** in 62-75% yields. Followed by de-*O*-acetylation under Zemplén conditions, **16a-d** were afforded quantitatively (Scheme 5).

2.2 Calculated Alog*P*

Lipophilicity governs the interaction of a given molecule with the intestinal membrane. A compound must possess hydrophobic properties for passive transport across intestinal epithelia. It is known that pentacyclic triterpenes are hydrophobic molecules with relative high lipophilicity. The different substituents such as the sugar part at C_3-OH and/or C_{28}-COOH contribute to different degrees of lipophilicity. In this study, the Alog*P* of pentacyclic triterpene-

Scheme 4. Proposed mechanism for the formation of the two isomers during de-*O*-acetylation reaction under Zemplén conditions.

Scheme 5. Reagents and conditions: (a) PPh_3, THF, H_2O; (b) EDC, Na_2CO_3, DMF, 2a, 2b or 2c, 60°C; (c) EDC, Na_2CO_3, DMF, 2d, 60°C; (d) MeONa/MeOH.

Table 1. Calculated Alog*P* values of pentacyclic triterpene-Neu5Ac2en conjugates.[a]

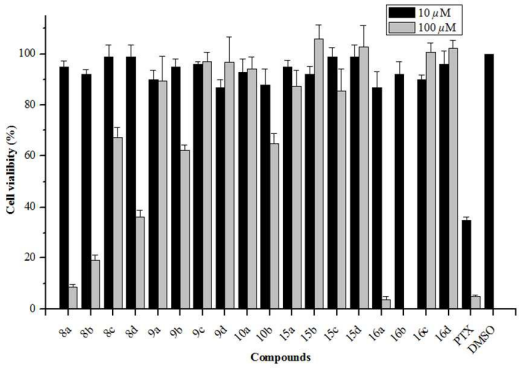
Compound	Alog*P*	Compound	Alog*P*	Compound	Alog*P*
OA	6.447	**9a**	3.619	**15c**	3.673
UA	6.492	**9b**	3.664	**15d**	4.874
EA	5.345	**9c**	2.517	**16a**	3.638
BA	6.546	**9d**	3.718	**16b**	3.683
8a	4.756	**10a**	3.619	**16c**	2.536
8b	4.802	**10b**	3.664	**16d**	3.737
8c	3.654	**15a**	4.775		
8d	4.856	**15b**	4.821		

[a] Alog*P* values (Ghose and Crippen octanol-water partition coefficient at 25°C) were calculated using Pipeline Pilot software, version 7.5 (Accelrys Corp., San Diego, CA, USA).

Neu5Ac2en derivatives were calculated using Pipeline Pilot software version 7.5 (Accelrys Corp., San Diego, CA, USA) and the results are presented in Table 1. All the studied conjugates showed decreased hydrophobicity compared with their parent compounds, with Alog*P* values within the range of 2.52-4.86. Benefiting from the hydrophilic Neu5Ac2en moiety, the Alog*P* value decreased about 2.8 for each series (for example, OA vs **9a** and **16a**), which means that their solubility in water are about 630–fold higher than their parent compounds.

2.3 Cytotoxic activity

After completion of the synthesis, all synthesized conjugates were evaluated for their in vitro anti-influenza activity against A/WSN/33 (H1N1) virus in the MDCK cell line. To exclude the possibility that the observed anti-influenza virus activity is due to non-specific cytotoxic activity, preliminary screening of the synthesized conjugates **8a-d**, **9a-d**, **10a-b**, **15a-d** and **16a-d** was performed to evaluate their cytotoxic activity in MDCK cells based on CellTiter-Glo assay.[21] The commercial antitumor agent paclitaxel (PTX) was used as a positive control. The results of the cytotoxicity test at two different concentrations (10 μM and 100 μM) are shown in Figure 2. No significant cytotoxicity was observed at concentration below 10 μM. However, compounds **8a-b**, **8d** and **16a-b** showed strong cytotoxicity while compounds **8c**, **9b** and **10b** showed moderate cytotoxicity at high concentration (100 μM). Except compound **15b**, most of the UA-Neu5Ac2en conjugates showed strong (**8b** and **16b**) to middle (**9b**



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and **10b**) cytotoxicity at the same concentration. Two

Figure 2. The cytotoxicity screening of pentacyclic triterpene-Neu5Ac2en conjugates **8a-d**, **9a-d**, **10a-b**, **15a-d** and **16a-d** using CellTiter-Glo® Assay. DMSO and paclitaxel were used as negative and positive control, respectively. Error bars indicate standard deviations of triplicate experiments.

BA-Neu5Ac2en conjugates (**8a** and **16a**) also showed strong cytotoxicity to MDCK cells at high concentration (100 μM).

2.4 *In vitro* anti-influenza virus activity in MDCK cells

Except for compounds **8a-b**, **8d** and **16a-b** with strong cytotoxic activity as described above, the other 13 pentacyclic triterpene-Neu5Ac2en conjugates were evaluated against the influenza virus strain A/WSN/33 (H1N1) that was propagated in MDCK cells by the cytopathic effect (CPE) reduction assay.[25] Curcumin, a small-molecule entry inhibitor targeting the HA1 domain,[25-26] was utilized as positive controls. The concentrations required to inhibit viral replication by 50% (IC_{50}) are summarized in Table 2. We also determined the cytotoxic effects by CellTiter-Glo assay and the cytotoxicity of each compound was expressed as the concentration required to induce 50% cell death (CC_{50}) of the MDCK cells. As shown in Table 2, we found that: 1) Compounds **15a** and **15c** exhibited appreciated effect against influenza virus A/WSN/33
with IC_{50} at 8.3 and 15.5 μM (SI > 22.7), respectively, which were more potent or comparable to that of the diarylheptanoid anti-influenza entry inhibitor curcumin. 2) Conjugation of Neu5Ac2en with oleanane-type pentacyclic triterpene was more effective than with ursane- or lupine-type pentacyclic triterpene. Most of the conjugates with ursolic acid and betulinic acid scaffold showed no anti-influenza activity but cytotoxicity except compound **15b**, which showed potent activity against A/WSN/33 virus with an IC_{50} of 19.2 μM. 3) The insertion of a (1,2,3-triazol-4-yl) methyl between the amide bond and Neu5Ac2en caused a substantial decrease in activity (eg. **15c** vs **8c**, **16c** *vs* **9c**), indicating the linker between pentacyclic triterpene and Neu5Ac2en played an important role in their antiviral activity. 4) Compared with our previous study, the anti-influenza activities of the conjugates of sialic acid to pentacyclic triterpene via C-4 position were almost 5-fold more potent than those linked via C-2 position (IC_{50}: 8.3 μM *vs* 41.2 μM[14]).

Table 2. *In vitro* anti-influenza virus activity and cytotoxicity of the active compounds

Compd	IC_{50} (μM)[a]	CC_{50}(μM)[b]	SI[c]	Compd	IC_{50} (μM)	CC_{50} (μM)	SI
8c	>100	ND	ND	**15a**	8.3±1.3	188.1±10.1	22.7
9a	28.3±2.6	>200	>7.1	**15b**	19.2±3.5	135.9±6.3	7.1
9b	>100	ND	ND	**15c**	15.5±0.5	>200	>32.3
9c	55.0±4.1	>200	>3.6	**15d**	>100	ND	ND
9d	>100	ND	ND	**16c**	40.5±3.9	>200	>4.9
10a	>100	ND	ND	**16d**	>100	ND	ND
10b	>100	ND	ND	curcumin	6.7±1.2	48.3±5.7	7.2

[a] Concentration inhibiting viral replication by 50%. The values are means of at least three independent determinations; the corresponding standard deviations are noted. [b] 50% cytotoxicity concentration. [c] Selectivity index, defined by CC_{50}/IC_{50}.

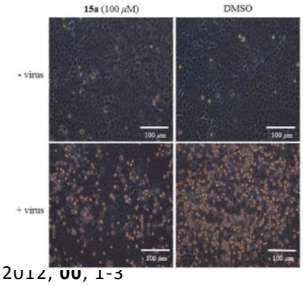


Figure 3. Compound **15a** inhibited virus-induced CPE in MDCK cells. The antiviral efficacy of **15a** was observed in terms of cellular morphology at 40 h post-infection.

In addition, CPE in the virus-infected cells were microscopically observed. Morphologically, the influenza virus-infected cells showed a rounded-up appearance and detached from the dish in the absence of **15a** at 40 h post-infection. Compound **15a** inhibited the virus-induced CPE significantly and no cytotoxicity was morphologically observed at a concentration up to 100 μM (Figure 3).

2.5 Identifying HA as the potential target of compound 15a

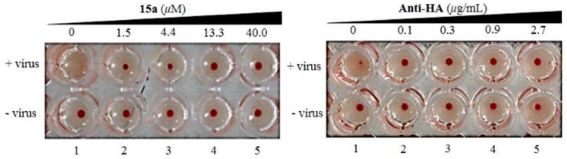
As described in our previous study,[14,21] we found that the molecular basis of the anti-influenza activity of pentacyclic triterpenes is likely due to their high affinity to HA protein, which is a surface glycoprotein of influenza virus and essential for viral attachment to host cells. In this study, a hemagglutination inhibition (HI) assay was performed to evaluate the potential interaction of the envelope HA protein with **15a**. A three-fold serial dilution of compound **15a** from 40 to 1.5 μM was added. As expected, no hemagglutination was elicited by compound **15a** alone (lower row, Figure 4) and the virus caused hemagglutination (lane 1, upper row, Figure 4). However, like anti-HA antibody, compound **15a** inhibited the hemagglutination of red blood cells (RBCs) caused by the influenza virus at the concentration from 40 to 1.5 μM (lane 2-5, upper row, Figure 4), which implied that **15a** and anti-HA antibody had the same target, HA, and the inhibition of viral infection might be resulted from the inhibition of the hemagglutinin-sialic acid receptor interaction.

On the other hand, Neu5Ac2en was found to be an influenza NA inhibitor with a K_i of 4 μM in earlier studies.[27] To examine whether the designed pentacyclic triterpene-Neu5Ac2en derivatives inhibit NA enzymatic activity, untreated or compound **15a**-treated influenza A/WSN/33 virus was tested for enzymatic activity by 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate solution (MUNANA). Zanamivir, a derivative of Neu5Ac2en by introduction of a guanidine group linked to C-4, was used as a positive control. Compared with Zanamivir, compound **15a** showed no inhibition of the enzymatic activity of influenza A/WSN/33 virus (SI Figure 1), implying that NA is not a target of compound 15a in its inhibition of viral infection

3. Experimental

3.1 Chemistry

All chemicals were used as supplied without further purification. The synthesis of compounds **2a-d**, **3a-d**, **4-7** and **14** have been reported previously.[22b,28] High-resolution mass spectra (HRMS) were obtained with an APEX IV FT_MS (7.0T) spectrometer (Bruker) in positive ESI mode. NMR spectra were recorded on a Bruker DRX 400 spectrometer at ambient temperature. ^{1}H NMR chemical shifts are referenced to the internal standard TMS (δ_H = 0.00) or the solvent signal (δ_H = 3.31 for the central line of CD_3OD). ^{13}C NMR chemical shifts are referenced to the



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DOI: 10.1039/C7MD00245A

Figure 4. Comparison of the behaviors of **15a** vs. anti-HA antibody in inhibition of influenza virus-induced aggregation of chicken erythrocytes. **15a** exerted identical capability as anti-HA antibody in hemagglutination inhibition in a dose-dependent manner.

solvent signal (δ_C= 77.00 for the central line of $CDCl_3$, δ_C= 49.00 for the central line of CD_3OD). Reactions were monitored by thin-layer chromatography (TLC) on a precoated silica gel 60 F254 plate (layer thickness 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 $(NH_4)_6Mo_7O_{24}.4H_2O$ (24.0 g) in 6% H_2SO_4 (500 mL), followed by heating. Flash column chromatography was performed on silica gel 60 (200-300 mesh, Qingdao Haiyang Chemical Co. Ltd.). The calculated Alog*P* and water solubility values were determined using Pipeline Pilot software, Vers. 7.5 (Accelrys Corporation, San Diego, CA, USA)

3.1.1 General procedure A for the CuAAC reaction. To a solution of alkyne (0.45 mmol) and azide (0.30 mmol) in DCM/H_2O (1:1 v/v, 12 mL) was added $CuSO_4$ (48 mg, 0.30 mmol) and sodium ascorbate (119 mg, 0.60 mmol). The resulting solution was stirred vigorously for 12 hours at room temperature. The reaction mixture was extracted with DCM (3 × 10 mL). The combined organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography over silica gel.

3.1.2 General procedure B for the de-*O*-acetylation reaction. The per-*O*-acetylated Neu5Ac2en pentacyclic triterpene conjugate was dissolved in MeOH (~5 mL per 100 mg compound) and a solution of MeONa (30% in MeOH, 0.1 equiv [mol acetate]$^{-1}$) was added. The solution was stirred at room temperature for 3 hours. After completion (TLC), the reaction mixture was neutralized with Amberlite IR-120 (H^+) ion-exchange resin, filtered and concentrated. The crude product was purified by column chromatography over silica gel.

3.1.3 Synthesis of 5(S)-acetylamino-4(S)-[4-[(3*β*-hydroxy-olean-12-en-28-oyl)amino]methyl-[1,2,3]triazol-1-yl)]-6(S)-((1R,2R)-(1,2,3-triacetoxy-propyl))-5,6-dihydro-4*H*-pyran-2-carboxylic acid methyl ester (8a). Prepared from **3a** and **7** according to general procedure A, and the residue was purified by chromatography (eluent: PE/Act = 3:2) over silica gel to afford compound **8a** as a white solid in 93% yield. R_f = 0.33 (PE/Act = 3:2); ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 7.79 (s, 1H), 7.31-7.33 (m, 1H, overlap with $CDCl_3$), 6.79 (br s, 1H), 6.03 (s, 1H), 5.69 (d, 1H, *J* = 9.6 Hz), 5.54 (d, 1H, *J* = 4.4 Hz), 5.37-5.36 (m, 2H), 4.71 (td, 2H, *J* = 12.4, 1.9 Hz), 4.46 (dd, 1H, *J* = 15.1, 5.2 Hz), 4.38-4.31 (m, 2H), 4.21 (dd, 1H, *J* = 12.4, 7.3 Hz), 3.82 (s, 3H), 3.23-3.18 (m, 1H), 2.56 (d, 1H, *J* = 10.5 Hz), 2.09 (s, 3H, CH_3CO), 2.06 (2 × s, 6H, 2 × CH_3CO), 2.02-0.92 (m, other aliphatic ring protons), 1.77 (s, 3H, CH_3CO), 1.15, 0.98, 0.90, 0.88, 0.87, 0.78 (s, 3H each, 6 × CH_3), 0.72 (d, 1H, *J* = 11.2 Hz), 0.57 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 178.61, 170.65, 170.41, 170.31, 170.23, 161.24, 146.11, 145.39, 144.35, 123.15, 122.21, 106.82, 78.91, 77.08, 71.38, 67.85, 62.24, 58.44, 55.12, 52.72, 48.21, 47.53, 46.67, 46.22, 41.95, 41.92, 39.36, 38.79, 38.48, 36.96, 35.13, 34.14, 33.03, 32.64, 32.37, 30.74, 28.16, 27.27, 27.18, 25.85, 23.94, 23.63, 23.48, 22.83, 21.00, 20.83, 20.75, 18.29, 16.68, 15.70, 15.41; ESI-HRMS calcd for $C_{51}H_{75}N_5NaO_{12}$ $[M+Na]^+$: 972.5304, found 972.5309.

3.1.4 Synthesis of 5(S)-acetylamino-4(S)-[4-[(3*β*-hydroxy-urs-12-en-28-oyl)amino]methyl-[1,2,3]triazol-1-yl)]-6(S)-((1R,2R)-(1,2,3-triacetoxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (8b). Prepared from **3b** and **7** according to general procedure A, and the residue was purified by chromatography (eluent: PE/Act = 3:2) over silica gel to afford compound **8b** as a white solid in 93% yield. R_f = 0.35 (PE/Act = 3:2); ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 7.74 (s, 1H), 7.18 (d, 1H, *J* = 8.9 Hz), 6.76 (brs, 1H), 6.03 (d, 1H, *J* = 1.6 Hz), 5.70 (d, 1H, *J* = 9.5 Hz), 5.53 (dd, 1H, *J* = 4.7, 1.3 Hz), 5.33-5.38 (m, 2H), 4.70-4.74 (m, 2H), 4.45 (dd, 1H, *J* = 14.6, 5.2 Hz), 4.27-4.34 (m, 2H), 4. 20 (dd, 1H, *J* = 12.5, 7.2 Hz), 3.82 (s, 3H), 3.20-3.22 (m, 1H), 2.09, 2.07, 2.06 (s, 3H each, 3 × CH_3CO), 1.92-0.97 (m, other aliphatic ring protons), 1.78 (s, 3H, CH_3CO), 1.09, 0.99, 0.94, 0.89 (s, 3H each, 4 × CH_3), 0.86 (d, 3H, CH_3, *J* = 6.3 Hz), 0.78, 0.61 (s, 3H each, 2 × CH_3), 0.71 (d, 1H, *J* = 11.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 178.50, 170.70, 170.44, 170.28, 161.28, 146.14, 145.40, 139.27, 126.10, 121.96, 106.85, 78.99, 77.02, 71.38, 67.92, 62.26, 58.35, 55.17, 53.50, 52.77, 48.39, 47.69, 47.56, 42.40, 39.76, 39.58, 39.02, 38.82, 38.66, 37.21, 36.97, 35.12, 32.78, 30.92, 28.22, 27.86, 27.25, 25.02, 23.39, 22.94, 21.28, 21.04, 20.86, 20.79, 18.31, 17.26, 16.75, 15.75, 15.57; ESI-HRMS calcd for $C_{51}H_{76}N_5O_{12}$ $[M+H]^+$: 950.5485; found 950.5493.

3.1.5 Synthesis of 5(S)-acetylamino-4(S)-[4-[(3*β*,16*α*-dihydroxy-olean-12-en-28-oyl)amino] methyl-[1,2,3]triazol-1-yl]]-6(S)-((1R,2R)-(1,2,3-triacetoxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (8c). Prepared from **3c** and **7** according to general procedure A, and the residue was purified by chromatography (eluent: PE/Act = 3:2) over silica gel to afford compound **8c** as a white solid in 91% yield. R_f = 0.30 (PE/Act = 3:2); ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 7.74 (s, 1H), 7.49 (d, 1H, J = 8.2 Hz), 7.09 (br s, 1H), 6.04 (s, 1H), 5.68 (br s, 1H), 5.57 (d, 1H, *J* = 2.3 Hz), 5.54 (br t, 1H), 5.36-5.35 (m, 1H), 4.81 (d, 1H, *J* = 10.8 Hz), 4.66 (d, 1H, *J* = 10.5 Hz), 4.57 (dd, 1H, *J* = 9.5, 4.3 Hz), 4.46-4.39 (m, 1H), 4.29 (d, 1H, *J* = 12.6 Hz), 4.25-4.20 (m, 2H), 3.82 (s, 3H), 3.24 (br s, 2H), 2.88 (d, 1H, *J* = 12.4 Hz), 2.14 (t, 1H, *J* = 13.1 Hz), 2.09 (s, 3H, CH_3CO), 2.07 (s, 6H, 2 × CH_3CO), 1.91-1.90 (m, 3H), 1.68-0.98 (m, other aliphatic ring protons), 1.72 (s, 3H, CH_3CO), 1.33, 0.99, 0.92, 0.90, 0.89, 0.79, 0.70 (s, 3H each, 7 × CH_3), 0.74 (d, 1H, *J* = 11.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 178.31, 170.76, 170.64, 170.60, 170.05, 161.06, 146.00, 145.57, 142.99, 123.25, 121.51, 106.75, 78.77, 77.32, 74.34, 71.75, 67.85, 62.09, 58.69, 55.18, 52.66, 49.27, 47.70, 46.77, 46.37, 41.84, 41.34, 39.69, 38.69, 38.50, 36.85, 35.55, 34.98, 34.65, 32.43, 29.97, 27.99, 27.11, 26.90, 25.86, 23.27, 22.60, 20.91, 20.73, 20.68, 18.11, 17.00, 15.61, 15.56; ESI-HRMS calcd for $C_{51}H_{76}N_5O_{13}$ $[M+H]^+$: 966.5434; found 966.5440.

3.1.6 Synthesis of 5(S)-acetylamino-4(S)-[4-[(3*β*-hydroxy-lup-20(29)-en-28-oyl)amino]methyl-[1,2,3]triazol-1-yl]]-6(S)-((1R,2R)-(1,2,3-triacetoxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (8d). Prepared from **3d** and **7** according to general procedure A, and the residue was purified by chromatography (eluent: PE/Act = 3:2) over silica gel to afford compound **8d** as a white solid in 87% yield. R_f = 0.35 (PE/Act = 3:2); ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 7.73 (s, 1H), 6.90 (s, 1H), 6.69 (s, 1H), 6.00 (s, 1H), 5.79 (s, 1H), 5.54 (d, 1H, *J* = 4.2 Hz), 5.38 (br t, 1H), 4.78 (d, 1H, *J* = 9.4 Hz), 4.71-4.74 (m, 2H), 4.59 (s, 1H), 4.45 (br s, 2H), 4.24-4.19 (m, 2H), 3.81 (s, 3H), 3.19-3.17 (m, 1H), 3.08 (t, 1H, *J* = 8.3 Hz), 2.45 (t, 1H, *J* = 10.5 Hz), 2.09 (s, 3H, CH_3CO), 2.06 (s, 6H, 2 × CH_3CO), 2.00 (d, 1H, *J* = 12.8 Hz), 1.80 (s, 3H, CH_3CO), 1.79-0.88 (m, other aliphatic ring protons), 1.67, 0.96, 0.95, 0.87, 0.80, 0.76 (s, 3H each, 6 × CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 176.89, 170.75, 170.66, 170.50, 170.27, 161.18, 150.78, 146.26, 121.95, 109.60, 106.77, 79.03, 76.84, 71.25, 67.88, 62.15, 58.30, 55.72, 55.44, 52.83, 50.66, 50.11, 48.73, 46.77, 42.52, 40.85, 38.93, 38.80, 38.34, 37.80, 37.25, 34.88, 34.49, 33.49, 30.88, 29.52, 28.10, 27.48, 25.68, 22.99, 21.05 (2C), 20.87, 20.80, 19.49, 18.36, 16.28, 16.22, 15.49, 14.72; ESI-HRMS calcd for $C_{51}H_{75}N_5NaO_{12}$ $[M+Na]^+$: 972.5304, found 972.5298.

3.1.7 Synthesis of 5(S)-acetylamino-4(S)-[4-[(3*β*-hydroxy-olean-12-en-28-oyl)amino]methyl-[1,2,3]triazol-1-yl]]-6(S)-((1R,2R)-(1,2,3-trihydroxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (9a) and 5-acetylamino-4(R)-[4-[(3*β*-hydroxy-olean-12-en-28-oyl)amino]methyl-[1,2,3]triazol-1-yl]]6(S)-((1R,2R)6-(1,2,3-trihydroxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (10a). Prepared from **8a** according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound **9a** and **10a** as white solids in 52% and 31% yield, respectively. Compound **9a**: R_f = 0.18 (DCM/MeOH = 10:1); ^{1}H NMR (400 MHz, CD_3OD): *δ* 7.82 (s, 1H), 6.01 (d, 1H, *J* = 2.4 Hz), 5.61 (dd, 1H, *J* = 9.8, 2.2 Hz), 5.33 (t, 1H, *J* = 3.4 Hz), 4.56 (d, 1H, *J* = 10.9 Hz), 4.46-4.33 (m, 3H), 3.95-3.90 (m, 1H), 3.85-3.81 (m, 4H), 3.68 (dd, 1H, *J* = 11.5, 6.2 Hz), 3.65 (d, 1H, *J* = 8.7 Hz), 3.13 (dd, 1H, *J* = 11.4, 4.5 Hz), 2.80 (dd, 1H, *J* = 13.0, 3.6 Hz), 2.06 (dt, 1H, *J* = 13.6, 3.9 Hz), 1.89-1.85 (m,3H), 1.87 (s, 3H, CH_3CO), 1.77 (t, 1H, *J* = 13.7 Hz), 1.62-0.95 (m, other aliphatic ring protons), 1.15, 0.96, 0.94 (s, 3H each, 3 × CH_3), 0.91 (s, 6H, 2 × CH_3), 0.77 (s, 3H, CH_3), 0.73 (d, 1H, *J* = 11.1 Hz), 0.53 (s, 3H, CH_3); ^{13}C NMR

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DOI: 10.1039/C7MD00245A

(100 MHz, CD_3OD): δ 180.36, 173.76, 163.51, 147.58, 146.68, 145.07, 124.13, 123.29, 107.29, 79.67, 78.44, 71.16, 69.71, 64.78, 59.95, 56.70, 53.07, 50.09, 48.96, 47.63, 47.51, 42.86, 42.52, 40.63, 39.84, 38.10, 35.86, 35.08, 34.21, 33.79, 33.54, 31.61, 28.74, 28.46, 27.85, 26.39, 24.51, 24.03, 22.55, 19.45, 17.75, 16.34, 15.97; ESI-HRMS calcd for $C_{45}H_{70}N_5O_9$ $[M+H]^+$: 824.5168; found 824.5174. Compound **10a:** R_f = 0.21 (DCM/MeOH = 10:1); ^{1}H NMR (400 MHz, CD_3OD): δ 7.80 (t, 1H, *J* = 5.6 Hz), 7.77 (s, 1H), 6.11 (d, 1H, *J* = 5.4 Hz), 5.58 (t, 1H, *J* = 5.4 Hz), 5.33 (br t, 1H), 4.64 (dd, 1H, *J* = 11.1, 5.4 Hz), 4.51 (dd, 1H, *J* = 11.1, 1.0 Hz), 4.37 (dq, 2H, *J* = 15.0, 5.4 Hz), 3.94 (ddd, 1H, *J* = 8.8, 5.3, 2.8 Hz), 3.85-3.81 (m, 4H), 3.66 (dd, 1H, *J* = 11.4, 5.4 Hz), 3.61 (dd, 1H, *J* = 9.4, 0.8 Hz), 3.13 (dd, 1H, *J* = 11.4, 4.5 Hz), 2.79 (dd, 1H, *J* = 13.3, 3.4 Hz), 2.07 (dt, 1H, *J* = 13.6, 3.4 Hz), 1.88 (dd, 1H, *J* = 8.8, 3.2 Hz), 1.85 (s, 3H, CH_3CO), 1.77 (t, 1H, *J* = 13.6 Hz), 1.64-0.98 (m, other aliphatic ring protons), 1.15, 0.96, 0.93, 0.92, 0.90, 0.78, (s, 3H each, 6 × CH_3), 0.73 (d, 1H, *J* = 11.2 Hz), 0.49 (s, 3H, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 180.46, 173.54, 163.79, 148.09, 145.86, 145.03, 125.51, 124.16, 104.61, 79.66, 73.83, 71.45, 69.77, 64.79, 56.69, 54.48, 53.10, 48.97, 48.36, 47.56, 47.50, 42.85, 42.60, 40.60, 39.83, 38.10, 35.93, 35.05, 34.26, 33.80, 33.53, 31.61, 28.74, 28.45, 27.85, 26.43, 24.51, 24.02, 22.42, 19.45, 17.48, 16.36, 16.00; ESI-HRMS calcd for $C_{45}H_{69}N_5NaO_9$ $[M+Na]^+$: 846.4987, found 846.4993.

3.1.8 Synthesis of 5(S)-acetylamino-4(S)-[4-[(3*β*-hydroxy-urs-12-en-28-oyl)amino]methyl-[1,2,3]triazol-1-yl]]-6(S)-((1R,2R)-(1,2,3-trihydroxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (9b) and 5-acetylamino-4(R)-[4-[(3*β*-hydroxy-urs-12-en-28-oyl)amino]methyl-[1,2,3]triazol-1-yl]]-6(S)-((1R,2R)-(1,2,3-trihydroxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (10b). Prepared from **8b** according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound **9b** and **10b** as white solids in 49% and 28% yield, respectively. Compound **9b:** R_f = 0.19 (DCM/MeOH = 10:1); ^{1}H NMR (400 MHz, CD_3OD): δ 7.83 (s, 1H), 6.02 (d, 1H, *J* = 2.3 Hz), 5.62 (d, 1H, *J* = 9.2 Hz), 5.32 (br t, 1H), 4.56 (d, 1H, *J* = 10.9 Hz), 4.46-4.36 (m, 3H), 3.92 (ddd, 1H, *J* = 8.6, 5.1, 2.8 Hz), 3.83 (dd, 1H, *J* = 11.5, 2.8 Hz), 3.81 (s, 3H), 3.64-3.70 (m, 2H), 3.14 (dd, 1H, *J* = 11.0, 5.0 Hz), 2.14 (d, 1H, *J* = 5.6 Hz), 2.03-2.10 (m, 1H), 1.87 (s, 3H, CH_3CO), 1.68-0.95 (m, other aliphatic ring protons), 1.10 (s, 3H, CH_3), 0.96 (s, 6H, 2 × CH_3), 0.92 (s, 3H, CH_3), 0.89 (d, 3H, *J* = 6.4 Hz, CH_3), 0.77 (s, 3H, CH_3), 0.72 (d, 1H, *J* = 11.0 Hz), 0.56 (s, 3H, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 180.17, 173.72, 163.49, 147.54, 146.63, 139.71, 127.28, 123.19, 107.30, 79.62, 78.40, 71.15, 69.67, 64.76, 59.96, 56.67, 54.11, 53.09, 50.04, 49.85, 48.91, 43.22, 40.82, 40.26, 39.94, 39.82, 38.55, 38.04, 35.80, 34.11, 31.88, 28.92, 28.77, 27.87, 25.25, 24.33, 24.04, 22.58, 21.59, 19.41, 17.74, 17.73, 16.41, 16.13; ESI-HRMS calcd for $C_{45}H_{70}N_5O_9$ $[M+H]^+$: 824.5168; found 824.5163. Compound **10b:** R_f = 0.22 (DCM/MeOH = 10:1); ^{1}H NMR (400 MHz, CD_3OD): δ 7.75 (s, 1H), 6.12 (d, 1H, *J* = 5.4 Hz), 5.57 (t, 1H, *J* = 5.4 Hz), 5.31 (br t, 1H), 4.63 (dd, 1H, *J* = 11.0, 5.4 Hz), 4.51 (d, 1H, *J* = 11.1 Hz), 4.30-4.39 (m, 2H), 3.95-3.92 (m, 1H), 3.83-3.81 (m, 4H), 3.66 (dd, 1H, *J* = 11.3, 5.4 Hz), 3.60 (d, 1H, *J* = 9.3 Hz), 3.14 (dd, 1H, *J* = 10.8, 4.70 Hz), 2.14-2.04 (m, 2H), 1.85 (s, 3H, CH_3CO), 1.72-0.97 (m, other aliphatic ring protons), 1.10 (s, 3H, CH_3), 0.96 (s, 6H, 2 × CH_3), 0.93 (s, 3H, CH_3), 0.89 (d, 3H, *J* = 6.3 Hz, CH_3), 0.78 (s, 3H, CH_3), 0.72 (d, 1H, *J* = 11.1 Hz), 0.51 (s, 3H, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 180.29, 173.56, 163.81, 148.10, 145.77, 139.71, 127.35, 125.51, 104.61, 79.67, 73.85, 71.46, 69.78, 64.80, 56.68, 54.49, 54.22, 53.10, 48.95, 48.88, 48.39, 43.27, 40.83, 40.80, 40.35, 39.98, 39.84, 38.66, 38.06, 35.78, 34.13, 31.87, 28.91, 28.75, 27.89, 25.27, 24.34, 24.00, 22.40, 21.54, 19.42, 17.69, 17.49, 16.42, 16.15; ESI-HRMS calcd for $C_{45}H_{69}N_5NaO_9$ $[M+Na]^+$: 846.4987, found 846.4988.

3.1.9 Synthesis of 5(S)-acetylamino-4(S)-[4-[(3*β*,16*α*-dihydroxy-olean-12-en-28-oyl)amino]methyl-[1,2,3]triazol-1-yl]]-6(S)-((1R,2R)-(1,2,3-trihydroxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (9c). To a solution of **3c** (229 mg, 0.45 mmol) and **11** (99 mg, 0.30 mmol) in THF/H_2O (1:1 *v/v*, 12 mL) was added $CuSO_4$ (48 mg, 0.30 mmol) and sodium ascorbate (119 mg, 0.60 mmol). The resulting solution was stirred vigorously for 12 hours at room temperature. After removal of THF and water, the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound **9c** as a white solid in 85% yield. R_f = 0.15 (DCM/MeOH = 10:1); ^{1}H NMR (400 MHz, CD_3OD): δ 7.82 (s, 1H), 7.57 (t, 1H, *J* = 5.2 Hz), 5.99 (d, 1H, *J* = 2.4 Hz), 5.63 (dd, 1H, *J* = 9.8, 2.2 Hz), 5.45 (br t, 1H), 4.56 (d, 1H, *J* = 10.9 Hz), 4.46-4.30 (m, 4H), 3.92 (ddd, 1H, *J* = 8.4, 5.1, 2.8 Hz), 3.85-3.81 (m, 4H), 3.68 (dd, 1H, *J* = 11.6, 5.3 Hz), 3.66 (d, 1H, *J* = 9.4 Hz), 3.14 (dd, 1H, *J* = 11.0, 4.9 Hz), 2.88 (dd, 1H, *J* = 13.6, 3.3 Hz), 2.35 (t, 1H, *J* = 13.2 Hz), 1.98-1.89 (m, 4H), 1.87 (s, 3H, CH_3CO), 1.64-1.01 (m, other aliphatic ring protons), 1.36, 0.97, 0.96, 0.92, 0.88, 0.77 (s, 3H each, 6 × CH_3), 0.74 (d, 1H, *J* = 11.2 Hz), 0.56 (s, 3H, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 180.12, 173.73, 163.49, 147.55, 146.56, 144.92, 124.17, 122.96, 107.31, 79.64, 78.45, 75.56, 71.15, 69.69, 64.77, 59.96, 56.79, 53.08, 50.01, 49.96, 48.11, 48.05, 42.77, 42.26, 40.75, 39.92, 39.83, 38.07, 36.38, 36.10, 35.97, 33.93, 33.29, 31.92, 31.23, 28.73, 27.88, 27.29, 25.38, 24.46, 22.57, 19.43, 17.77, 16.35, 16.20; ESI-HRMS calcd for $C_{45}H_{70}N_5O_{10}$ $[M+H]^+$: 840.5117; found 840.5125.

3.1.10 Synthesis of 5(S)-acetylamino-4(S)-[4-[(3*β*-hydroxy-lup-20(29)-en-28-oyl)amino]methyl-[1,2,3]triazol-1-yl]]-6(S)-((1R,2R)-(1,2,3-trihydroxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (9d). To a solution of **3d** (222 mg, 0.45 mmol) and **11** (99 mg, 0.30 mmol) in THF/H_2O (1:1 v/v, 12 mL) was added $CuSO_4$ (48 mg, 0.30 mmol) and sodium ascorbate (119 mg, 0.60 mmol). The resulting solution was stirred vigorously for 12 hours at room temperature. After removal of THF and water, the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound **9d** as a white solid in 83% yield. R_f = 0.19 (DCM/MeOH = 10:1); ^{1}H NMR (400 MHz, CD_3OD): δ 7.82 (s, 1H), 6.02 (d, 1H, *J* = 2.4 Hz), 5.61 (dd, 1H, *J* = 9.6, 2.0 Hz), 4.70 (s, 1H), 4.58-4.53 (m, 2H), 4.49-4.42 (m, 2H), 4.29 (d, 1H, *J* = 15.1 Hz), 3.94 (ddd, 1H, *J* = 8.9, 5.1, 2.9 Hz), 3.78-3.85 (m, 4H), 3.69 (dd, 1H, *J* = 11.4, 5.2 Hz), 3.66 (d, , 1H, *J* = 9.4 Hz), 3.07-3.13 (m, 2H), 2.51 (dt, 1H, *J* = 12.7, 3.0 Hz), 2.12 (td, 1H, *J* = 13.6, 3.0 Hz), 1.88 (s, 3H, CH_3CO), 1.86-1.78 (m, 2H), 1.72-0.90 (m, other aliphatic ring protons), 1.68, 0.98, 0.94, 0.87, 0.84, 0.76 (s, 3H each, 6 × CH_3), 0.68 (dd, 1H, *J* = 8.4, 2.7 Hz); ^{13}C NMR (100 MHz, CD_3OD): δ 179.27, 173.87, 163.51, 152.28, 147.58, 123.35, 110.02, 107.34, 79.66, 78.52, 71.15, 69.71, 64.78, 59.95, 56.93, 53.10, 52.07, 51.40, 50.10, 48.13, 43.46, 42.08, 40.11, 39.96, 39.20, 39.01, 38.32, 35.65, 35.54, 33.92, 31.95, 30.58, 28.66, 28.05, 27.00, 22.58, 22.16, 19.65, 19.46, 16.99, 16.85, 16.18, 15.12; ESI-HRMS calcd for $C_{45}H_{70}N_5O_9$ $[M+H]^+$: 824.5168; found 824.5173.

3.1.11 Synthesis of 5(S)-acetylamino-4(S)-[(3*β*-hydroxy-olean-12-en-28-oyl)amino]-6(S)-((1R,2R)-(1,2,3-triacetoxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (15a). To a solution of **14** (188.5 mg, 0.44 mmol) and **2a** (321 mg, 0.56 mmol) in DMF (20 mL), Na_2CO_3 (201 mg, 1.9 mmol) was added. The resulting mixture was stirred vigorously for 24 hours at 60 ^{o}C. After removal of DMF under vacuum, the residue was purified by chromatography (eluent: PE/Act = 2:1) over silica gel to afford compound **15a** as a white solid in 66% yield. R_f = 0.33 (PE/Act = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.15-6.16 (m, 1H), 6.04 (d, 1H, *J* = 7.3 Hz), 5.91 (d, 1H, *J* = 1.9 Hz), 5.49 (d, 1H, *J* = 4.5 Hz), 5.31-5.30 (m, 2H), 4.70-4.72 (m, 2H), 4.26-4.22 (m, 2H), 4.18 (dd, 1H, *J* = 12.4, 7.4 Hz), 3.79 (s, 3H), 3.21 (dd, 1H, *J* = 9.8, 3.3 Hz), 2.86 (dd, 1H, *J* = 10.4, 3.4 Hz), 2.10, 2.07, 2.06, 1.89 (s, 3H each, 4 ×CH_3CO), 1.87-0.97 (m, other aliphatic ring protons), 1.13, 0.99, 0.90, 0.89, 0.88, 0.78, 0.76 (s, 3H each, 7 × CH_3), 0.73 (d, 1H, *J* = 11.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.54, 171.44, 170.55, 170.40, 170.03, 161.82, 144.30, 143.48, 122.62, 110.78, 78.96, 77.32, 71.51, 67.86, 62.21, 55.20, 52.37, 49.19, 47.58, 46.74, 46.14, 45.98, 41.81, 40.97, 39.36, 38.73, 38.40, 37.04, 34.05, 33.37, 32.97, 32.81, 30.61, 28.11, 27.34, 27.15, 25.80, 23.53, 23.41, 23.15, 20.89, 20.75, 20.65, 18.30, 17.23, 15.61, 15.30; ESI-HRMS calcd for $C_{48}H_{72}N_2NaO_{12}$ $[M+Na]^+$: 891.4977, found 891.4984.

3.1.12 Synthesis of 5(S)-acetylamino-4(S)-[(3β-hydroxy-urs-12-en-28-oyl)amino]-6(S)-((1R,2R)-(1,2,3-triacetoxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (15b). To a solution of **14** (188.5 mg, 0.44 mmol) and **2b** (321 mg, 0.56 mmol) in DMF (20 mL), Na_2CO_3 (201 mg, 1.9 mmol) was added. The resulting mixture was stirred vigorously for 24 hours at 60 ^{o}C. After removal of DMF under vacuum, the residue was purified by chromatography (eluent: PE/Act = 2:1) over

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DOI: 10.1039/C7MD00245A

silica gel to afford compound **15b** as a white solid in 75% yield. R_f = 0.35 (PE/Act = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.60 (d, 1H, J = 8.8 Hz), 6.11 (d, 1H, J = 7.2 Hz), 5.88 (s, 1H), 5.50 (d, 1H, J = 4.2 Hz), 5.29 (br t, 1H), 5.25 (s, 1H), 4.72-4.70 (m, 2H), 4.30-4.32 (m, 2H), 4.18 (dd, 1H, J = 12.5, 7.6 Hz), 3.78 (s, 3H), 3.22-3.20 (m, 1H), 2.31 (s, 1H), 2.19-2.11 (m, 2H), 2.10, 2.07, 2.06, 1.90 (s, 3H each, 4 × CH_3CO), 1.94-0.91 (m, other aliphatic ring protons), 1.08, 0.99, 0.93, 0.91 (s, 3H each, 4 × CH_3), 0.85 (d, 3H, J = 6.1 Hz, CH_3), 0.79, 0.78 (s, 3H each, 2 × CH_3), 0.72 (d, 1H, J = 11.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.49, 171.42, 170.53, 170.43, 170.01, 161.78, 144.07, 137.67, 125.77, 110.91, 78.89, 77.25, 71.49, 67.80, 62.22, 55.10, 52.54, 52.31, 49.15, 47.62, 47.42, 46.11, 42.10, 39.55, 39.15, 38.66, 38.51, 37.60, 36.91, 33.09, 30.73, 28.12, 27.72, 27.10, 24.26, 23.36, 23.27, 23.17, 21.10, 20.85, 20.72, 20.64, 18.23, 17.41, 16.98, 15.68, 15.38; ESI-HRMS calcd for $C_{48}H_{73}N_2O_{12}$ $[M+H]^+$: 869.5158; found 869.5150.

3.1.13 Synthesis of 5(S)-acetylamino-4(S)-[(3*β*,16*α*-dihydroxy-olean-12-en-28-oyl)amino]-6(S)-((1R,2R)-(1,2,3-triacetoxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (15c). To a solution of **14** (188.5 mg, 0.44 mmol) and **2c** (330 mg, 0.56 mmol) in DMF (20 mL), Na_2CO_3 (201 mg, 1.9 mmol) was added. The resulting mixture was stirred vigorously for 24 hours at 60 °C. After removal of DMF under vacuum, the residue was purified by chromatography (eluent: PE/Act = 2:1) over silica gel to afford compound **15c** as a white solid in 68% yield. R_f = 0.30 (PE/Act = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.80 (d, 1H, J = 6.9 Hz), 6.59 (d, 1H, J = 8.6 Hz), 5.86 (s, 1H), 5.47 (s, 2H), 5.30 (br s, 1H), 4.82-4.75 (m, 2H), 4.29-4.08 (m, 4H), 3.79 (s, 3H), 3.23 (br s, 1H), 3.11 (d, 1H, J = 14.3 Hz), 2.45 (s, 1H), 2.10, 2.06, 2.05, 1.89 (s, 3H each, 4 × CH_3CO), 1.95-0.96 (m, other aliphatic ring protons), 1.25, 1.00, 0.95, 0.92, 0.89, 0.84, 0.79 (s, 3H each, 7 × CH_3), 0.74 (d, 1H, J = 10.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.79, 171.19, 170.68, 170.47, 170.05, 161.90, 144.30, 141.77, 123.08, 111.16, 78.86, 77.66, 73.62, 71.77, 68.03, 62.33, 55.32, 52.34, 49.94, 48.79, 47.12, 46.49, 45.67, 42.08, 41.03, 40.00, 38.74, 38.61, 36.94, 36.15, 33.84, 32.65, 32.39, 29.63, 28.04, 27.23, 27.08, 26.61, 23.29, 23.21, 20.84, 20.76, 20.73, 18.21, 17.64, 15.76, 15.66; ESI-HRMS calcd for $C_{48}H_{73}N_2O_{13}$ $[M+H]^+$: 885.5107; found 885.5108.

3.1.14 Synthesis of 5(S)-acetylamino-4(S)-[(3*β*-hydroxy-lup-20(29)-en-28-oyl)amino]-6(S)-((1R,2R)-(1,2,3-triacetoxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (15d). To a solution of **14** (188.5 mg, 0.44 mmol) and **2d** (321 mg, 0.56 mmol) in DMF (20 mL), Na_2CO_3 (201 mg, 1.9 mmol) was added. The resulting mixture was stirred vigorously for 24 hours at 60 °C. After removal of DMF under vacuum, the residue was purified by chromatography (eluent: PE/Act = 2:1) over silica gel to afford compound **15d** as a white solid in 62% yield. R_f = 0.35 (PE/Act = 2:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.35 (d, 1H, J = 7.5 Hz), 6.03 (d, 1H, J = 8.4 Hz), 5.86 (d, 1H, J = 2.0 Hz), 5.50 (d, 1H, J = 3.2 Hz), 5.32-5.28 (m, 1H), 4.83 (t, 1H, J = 8.2 Hz), 4.70-4.74 (m, 2H), 4.58 (s, 1H), 4. 30-4.23 (m, 2H), 4.17 (dd, 1H, J = 12.5, 7.5 Hz), 3.80 (s, 3H), 3.22-3.18 (m, 1H), 3.04 (dt, 1H, J = 11.2, 4.1 Hz), 2.40 (dt, 1H, J = 9.5, 3.1 Hz), 2.15 (s, 1H), 2.09, 2.08, 2.06, 1.90, (s, 3H each, 4 × CH_3CO), 1.93-0.85 (m, other aliphatic ring protons), 1.66, 0.97, 0.96, 0.93, 0.83, 0.76 (s, 3H each, 6 × CH_3), 0.68 (d, 1H, J = 9.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.06, 171.29, 170.58, 170.43, 170.01, 161.82, 150.58, 144.47, 111.21, 109.46, 78.95, 77.53, 71.54, 67.82, 62.18, 55.73, 55.33, 52.43, 50.57, 50.14, 48.12, 46.74, 46.45, 42.38, 40.71, 38.83, 38.69, 37.89, 37.69, 37.17, 34.31, 33.37, 30.79, 29.37, 27.97, 27.33, 25.52, 23.06, 20.88, 20.75, 20.62, 19.31, 18.25, 16.10, 16.00, 15.43, 14.60; ESI-HRMS calcd for $C_{48}H_{73}N_2O_{12}$ $[M+H]^+$: 869.5158; found 869.5160.

3.1.15 Synthesis of 5(S)-acetylamino-4(S)-[(3*β*-hydroxy-olean-12-en-28-oyl)amino]-6(S)-((1R,2R)-(1,2,3-trihydroxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (16a). Prepared from **15a** according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound **16a** as a white solid in 86% yield. R_f = 0.31 (DCM/MeOH = 10:1); ^{1}H NMR (400 MHz, CD_3OD): δ 5.83 (d, 1H, J = 2.5 Hz), 5.28 (t, 1H, J = 3.3 Hz), 4.74 (dd, 1H, J = 9.4, 2.5 Hz), 4.55 (s, 1H), 4.31-4.22 (m, 2H), 3.89 (ddd, 1H, J = 8.6, 5.3, 2.9 Hz), 3.82 (dd, 1H, J = 11.4, 2.9 Hz), 3.78 (s, 3H), 3.66 (dd, 1H, J = 11.4, 5.4 Hz), 3.60 (d, 1H, J = 10.0 Hz), 3.15 (dd, 1H, J = 11.1, 4.9 Hz), 2.91 (dd, 1H, J = 9.9, 3.5 Hz), 2.11 (dt, 1H, J = 14.5, 4.0 Hz), 1.96 (s, 3H, CH_3CO), 1.93-1.89 (m, 2H), 1.73-0.97 (m, other aliphatic ring protons), 1.17, 0.98, 0.96, 0.93, 0.90, 0.84, 0.78 (s, 3H each, 7 × CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 180.69, 174.46, 164.28, 145.66, 145.03, 123.83, 111.77, 79.71, 78.54, 71.17, 70.02, 64.88, 56.77, 52.82, 49.64, 49.00, 48.36 47.65, 47.33, 42.96, 42.35, 40.71, 39.84, 38.21, 35.11, 34.63, 34.17, 33.54, 31.57, 28.76, 28.60, 27.87, 26.40, 24.56, 23.98, 23.48, 22.82, 19.51, 18.29, 16.33, 15.96; ESI-HRMS calcd for $C_{42}H_{66}N_2NaO_9$ $[M+Na]^+$: 765.4661, found 765.4669.

3.1.16 Synthesis of 5(S)-acetylamino-4(S)-[(3*β*-hydroxy-urs-12-en-28-oyl)amino]-6(S)-((1R,2R)-(1,2,3-trihydroxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (16b). Prepared from **15b** according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound **16b** as a white solid in 87% yield. R_f = 0.32 (DCM/MeOH = 10:1); ^{1}H NMR (400 MHz, CD_3OD): δ 7.49 (d, 1H, J = 7.9 Hz), 5.81 (d, 1H, J = 2.5 Hz), 5.26 (t, 1H, J = 3.2 Hz), 4.73 (dt, 1H, J = 9.8, 2.4 Hz), 4.28 (t, 1H, J = 10.5 Hz), 4.23 (t, 1H, J = 10.7 Hz), 3.89 (ddd, 1H, J = 8.5, 5.2, 2.8 Hz), 3.82 (dd, 1H, J = 11.4, 2.9 Hz), 3.77 (s, 3H), 3.66 (dd, 1H, J = 11.4, 5.4 Hz), 3.59 (d, 1H, J = 10.0 Hz), 3.15 (dd, 1H, J = 11.0, 4.8 Hz), 2.26 (d, 1H, J = 11.0 Hz), 2.12 (dt, 1H, J = 14.0, 3.6 Hz), 1.97 (s, 3H, CH_3CO), 1.96-0.98 (m, other aliphatic ring protons), 1.12, 0.98, 0.97, 0.96 (s, 3H each, 4 × CH_3), 0.89 (d, 3H, J = 6.4 Hz, CH_3), 0.87, 0.78 (s, 3H each, 2 × CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 180.64, 174.36, 164.23, 145.58, 139.38, 127.02, 111.83, 79.61, 78.52, 71.09, 69.90, 64.82, 56.70, 53.83, 52.82, 49.05, 48.18, 43.28, 40.92, 40.55, 40.17, 39.98, 39.82, 38.93, 38.11, 34.47, 31.93, 28.97, 28.82, 27.87, 24.65, 24.43, 24.06, 22.95, 21.62, 19.47, 18.47, 17.73, 16.44, 16.15; ESI-HRMS calcd for $C_{42}H_{67}N_2O_9$ $[M+H]^+$: 743.4841; found 743.4846.

3.1.17 Synthesis of 5(S)-acetylamino-4(S)-[(3*β*,16*α*-dihydroxy-olean-12-en-28-oyl)amino]-6(S)-((1R,2R)-(1,2,3-trihydroxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (16c). Prepared from **15c** according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound **16c** as a white solid in 84% yield. R_f = 0.29 (DCM/MeOH = 10:1); ^{1}H NMR (400 MHz, CD_3OD): δ 7.39 (d, 1H, J = 7.8 Hz), 5.78 (d, 1H, J = 2.4 Hz), 5.40 (br t, 1H), 4.68-4.70 (m, 1H), 4.23-4.24 (m, 3H), 3.89 (ddd, 1H, J = 8.6, 5.2, 2.8 Hz), 3.82 (dd, 1H, J = 11.3, 2.9 Hz), 3.76 (s, 3H), 3.66 (dd, 1H, J = 11.5, 5.4 Hz), 3.59 (d, 1H, J = 9.4 Hz), 3.16 (dd, 1H, J = 11.0, 5.0 Hz), 3.09 (dd, 1H, J = 14.1, 3.4 Hz), 2.20 (t, 1H, J = 13.5 Hz), 1.97 (s, 3H, CH_3CO), 1.92 (dd, 1H, J = 5.9, 2.4 Hz), 1.84-1.01 (m, other aliphatic ring protons), 1.34, 0.98, 0.97, 0.95, 0.89, 0.86, 0.78 (s, 3H each, 7 × CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 180.43, 174.29, 164.18, 145.59, 144.34, 123.68, 111.61, 79.62, 78.47, 74.78, 71.10, 69.95, 64.83, 56.86, 52.82, 50.48, 49.77, 48.43, 48.33, 47.58, 42.91, 41.89, 40.98, 39.97, 39.83, 38.14, 36.42, 36.03, 34.17, 33.26, 31.00, 30.69, 28.76, 27.89, 27.59, 26.26, 24.49, 22.90, 19.47, 18.24, 16.37, 16.27; ESI-HRMS calcd for $C_{42}H_{67}N_2O_{10}$ $[M+H]^+$: 759.4790; found 759.4787.

3.1.18 Synthesis of 5(S)-acetylamino-4(S)-[(3*β*-hydroxy-lup-20(29)-en-28-oyl)amino]-6(S)-((1R,2R)-(1,2,3-trihydroxy-propyl))-5,6-dihydro-4H-pyran-2-carboxylic acid methyl ester (16d). Prepared from **15d** according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound **16d** as a white solid in 81% yield. R_f = 0.32 (DCM/MeOH = 10:1); ^{1}H NMR (400 MHz, CD_3OD): δ 7.69 (d, 1H, J = 8.5 Hz), 5.76 (d, 1H, J = 2.4 Hz), 4.83-4.86 (m, 1H, overlap with water), 4.69 (br s, 1H), 4.59 (br s, 1H), 4.25 (t, 1H, J = 10.6 Hz), 14.22 (t, 1H, J = 10.6 Hz), 3.90 (ddd, 1H, J = 8.6, 5.2, 2.8 Hz), 3.82 (dd, 1H, J = 11.4, 2.9 Hz), 3.78 (s, 3H), 3.66 (dd, 1H, J = 11.4, 5.4 Hz), 3.60 (d, 1H, J = 9.4 Hz), 3.13 (dd, 1H, J = 10.9, 5.1 Hz), 3.07 (dt, 1H, J = 11.1, 4.3 Hz), 2.59 (ddd, 1H, J = 12.8, 3.2 Hz), 2.14 (d, 1H, J = 10.9 Hz), 1.96 (s, 3H, CH_3CO), 1.87-0.92 (m, other aliphatic ring protons), 1.68 (s, 3H, CH_3), 1.00 (2 × s, 6H, 2 × CH_3), 0.96, 0.87, 0.76 (s, 3H each, 3 × CH_3), 0.71 (d, 1H, J = 8.7 Hz); ^{13}C NMR (100 MHz, CD_3OD): δ 179.32, 174.31, 164.19, 152.18, 145.80, 112.11, 110.05, 79.63, 78.60, 71.10, 69.90, 64.84, 57.10, 56.91, 52.85, 52.11, 51.45, 48.69, 48.66, 48.11, 43.49, 42.02, 40.13, 39.95, 39.16, 38.91, 38.35, 35.64, 33.75, 31.98, 30.56, 28.66, 28.05, 26.98, 22.78, 22.20, 19.68,

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19.47, 16.84, 16.79, 16.16, 15.16; ESI-HRMS calcd for $C_{42}H_{66}N_2NaO_9$ $[M+Na]^+$: 765.4661, found 765.4667.

3.2 Bioassays

3.2.1 Cytotoxicity assay. The assay was performed as previously described with some modifications.[28] Cells were seeded in 96-well plates in DMEM supplemented with 10% FBS and cultured overnight at 37 °C in 5% CO_2. Then the test compounds were added and the cells were further incubated at 37 °C in 5% CO_2 for 40 hours. Cell viability was assessed using the CellTiter-Glo assay kit as recommended by the supplier, and the plates were read using a plate reader (Tecan Infinite M2000 PRO; Tecan Group Ltd., Mannedorf, Switzerland) Viability was calculated using the background-corrected absorbance as follows:
Viability (%) = A of experiment well/A of control well × 100%.

3.2.2 CPE reduction assay. MDCK cells (ATCC CCL-34, Manassas, VA, USA) were seeded into 96-well plates in DMEM supplemented with 10% FBS and incubated overnight at 37 °C under 5% CO_2. The culture medium was replaced by test compound and influenza virus (MOI = 0.1)-DMEM supplemented with 1% FBS and 2 μg/mL TPCK-treated trypsin. The final concentration of DMSO was 1%. After 40 hours of incubation, CellTiter-Glo reagent (Promega Corp., Madison, WI, USA) was added, and assessed by the CellTiter-Glo assay as described above.

3.2.3 The hemagglutination inhibition (HI) assay. HI assay was performed as described previously.[30] Briefly, 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 (cRBCs) (1% v/v in saline) (Beijing Yuabio Biotechnology Co., Ltd, China) was added to each well. The mixture was incubated for 30 min at room temperature before observing cRBC aggregation on the plate.

3.2.4 The neuraminidase (NA) inhibition assay. The NA inhibition assay was performed utilizing NAs from the influenza A/WSN/33 virus. Influenza virus NA activity was determined by quantifying the fluorescent product resulting from the cleavage of the substrate 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate solution (MUNANA, Sigma, Saint Louis, MO, USA) by NA. The reaction mixture consisted of the tested compounds, virus (as the source of NAs) and 20 μM 4-MUNANA in 32.5 mM MES buffer (containing 4 mM $CaCl_2$, pH = 6.5) in a 96-well plate. After incubation for 30 min at 37 °C, the reaction was terminated with 150 μL 34 mM NaOH and the fluorescence of the mixture was recorded for the excitation wavelength 360 nm and emission wavelength 460 nm.

Conclusions

A series of pentacyclic triterpene-Neu5Ac2en derivatives were synthesized and evaluated for their anti-influenza virus activity by CPE assay. During the de-*O*-acetylation reaction of **8a** or **8b** under Zemplén conditions, a pair of configurational isomers was separated and their configurations were characterized by detailed NMR and HRMS analysis. Benefiting from the Neu5Ac2en group, all the new conjugates showed decreased hydrophobicity with Alog*P* values within the range of 2.52-4.86. The most potent inhibitory activity against influenza A/WSN/33 (H1N1) virus was displayed by compounds **15a** and **15c** with IC_{50} at 8.3 and 15.5 μM, respectively, which were comparable to the known anti-influenza entry inhibitor curcumin, but almost 2.7~5.0-fold more potent than its analogs in our previous study.[14] Compound **15a** showed inhibition of the hemagglutination of red blood cells while no inhibition was observed on NA activity, implying that compound **15a** targeted hemagglutinin-related functions such as hemagglutinin-sialic acid interactions during viral entry. These findings indicate that compound **15a** could be a potential anti-influenza entry inhibitor, although more studies are necessary to investigate its anti-influenza effect in vivo.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (Grants Nos. 81373271, 81361168002, 81573269 and 21572015). The authors gratefully acknowledge Dr. Hongwei Jin (State Key Laboratory of Natural and Biomimetic Drugs, Peking University) for carrying out the Alog*P* determinations.

Conflict of Interest

The authors declare no competing interests.

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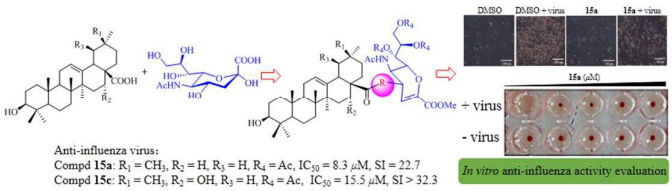
Graphical abstract



The C-4 of sialic acid is not important for its binding with hemagglutinin and could be replaced with hydrophobic moieties.