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Original article

Structure–activity relationships of 3-O-β-chacotriosyl ursolic acid derivatives as novel H5N1 entry inhibitors

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ABSTRACT

A series of methyl ursolate 3-*O*-β-chacotrioside analogs have been designed, synthesized and evaluated as H5N1 entry inhibitors based on a small molecule inhibitor saponin **3** previously discovered by us. Detailed structure–activity relationships (SARs) studies on the aglycone of compound **3** indicated that both the type of pentacyclic triterpene and the subtle modification of ursolic acid as an aglycon had key influences on the antiviral activity. These results suggested that either the introduction of a disubstituted amide structure at the 17-COOH of ursolic acid or alteration of the C-3 configuration of ursolic acid from 3β-to 3α-forms was helpful to significantly improve the selective index while keeping their antiviral activities.

1. Introduction

H5N1 avian influenza A virus is the cause of significant morbidity among humans, posing a serious threat to public health due to its transmission from animals to human being [1,2]. The two common classes of antiviral drugs used to treat influenza are neuraminidase inhibitors, like zanamivir and oseltamivir, and inhibitors of the viral M2 protein, such as amantadine and rimantadine [3]. It is known that ion channel inhibitors have given rise to the rapid emergence of drug-resistant viral strains which cause CNS side effects [4], and therefore are not recommended for general use. However, resistance of H5N1 to an NA inhibitor oseltamivir has also been recently observed [5]. Therefore, there is an urgent demand for new classes of agents with new mechanism of action to combat avian H5N1 variants.

The most critical and first step of virual infection influenza is viral entry that is mediated by the interaction of viral envelope protein hemagglutinin (HA), the receptor of which is sialic acid sugars on the host cell surface. Therefore, potent entry inhibitors can prevent H5N1 virus from binding to and entering the host cell, which may achieve the inhibition of H5N1 viral infection [6].

In our previous study [7], we have discovered three small molecule H5N1 viral entry inhibitors **1**–**3** (Fig. 1A) using an efficient HIV-based pseudotyping system to screen a saponin library generated from semisynthesis. These compounds show good inhibitory activity against H5N1 entry with the IC_{50} values being 6.00–9.25 μM. Structurally, these compounds bear the same β-chacotriosyl (α-L-rhamnopyranosyl- (1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranosyl) residue that is known as chacotriose. A preliminary structure–activity relationship (SAR) study [7,8] has indicated that both the chacotriosyl residue and the aglycone moiety of compounds **1**–**3** are important to the antiviral activity, though several modifications are tolerated at these positions. The 3-*O*-β-chacotriosyl residue is essential for activity as methylating one or two sugar hydroxyls of the β-chacotriosyl moiety and altering the β-chacotriosyl moiety of compound **1** into α-L-rhamnopyranosyl-(1 → 4)-β- D-glucopyranosyl moiety or α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranosyl moiety resulted in the loss of activity. In addition, replacement of the aglycone moiety of compound **1** with dihydrochlorogenin, dehydroisoandrosterone or stigmasterine led to the loss of activity except in the case of methyl ursolate (i.e., compound **3**), suggesting that the subtle modifications of aglycone may be tolerated without losing antiviral

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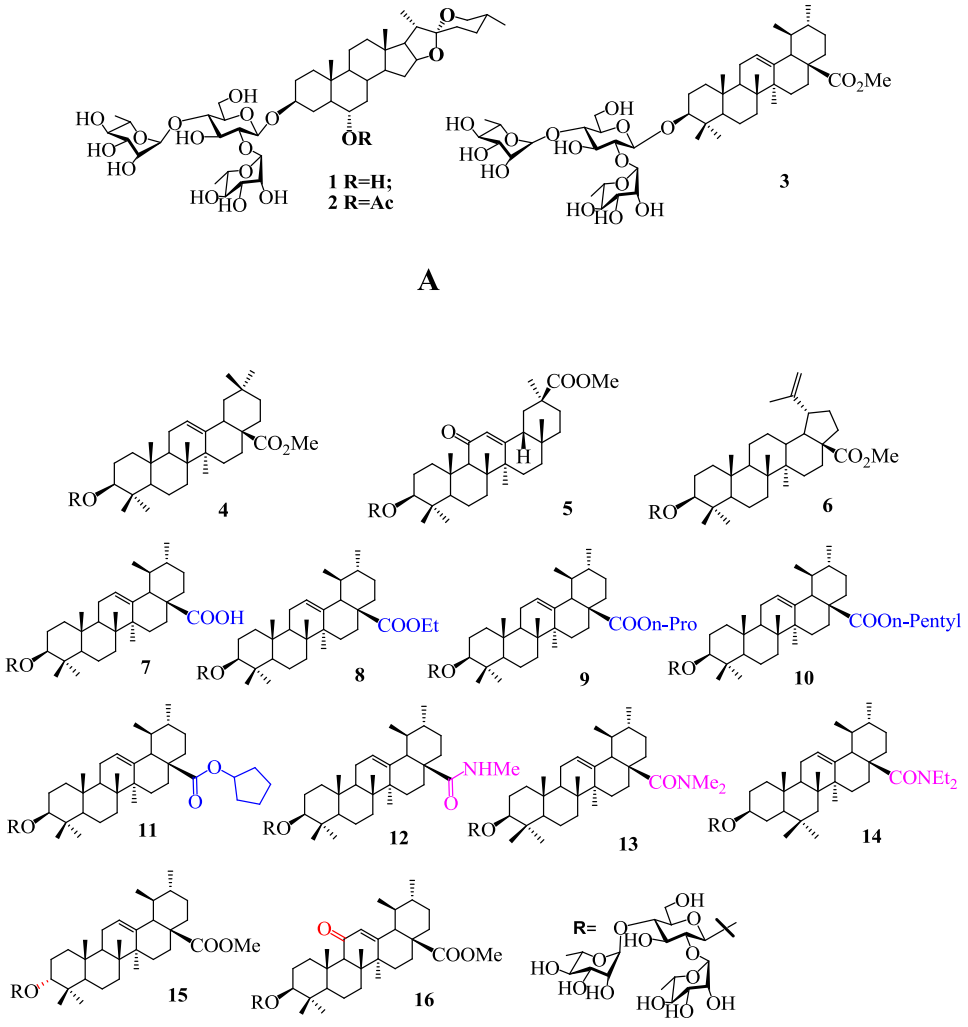


Fig. 1. A. Saponin inhibitors for H5N1 viral entry. B. Saponins designed for SARs studies.

activity. Notably, change of the aglycone moiety of compounds **1** and **2** into pentacyclic triterpene (to afford compound **3**) led to slightly stronger inhibition against HA (QH) [7], suggesting that replacement of the aglycone moiety of compound **1** with pentacyclic triterpene can enhance the activity.

It has been reported that the defense activities of pentacyclic triterpenes stem from their ability to prevent various pathogen and herbivore infections in the host [9]. Certain pentacyclic triterpenoids with conserved structural features displayed *in vitro* anti-influenza virus activity that is comparable to or higher than that of oseltamivir [10,11]. Based on the above results, we chose compound **3** as the lead compound and designed analogs **4**–**16** with the aim to investigate the SARs of pentacyclic triterpenes with different aglycones. Pentacyclic triterpenes can be classified into three major types based on structural features: (a) oleanane type triterpenes, (b) ursane type triterpenes, and (c) lupane type triterpenes. The most well-known member of each category is oleanolic acid (**OA**), ursolic acid (**UA**) and betulinic acid (**BA**), respectively. We first investigated the effect of different types of pentacyclic triterpene as the aglycone residue on the inhibitory activity. In addition, glycyrrhyzinic acid (**GL**), a member of oleanane type triterpenes, is known for multiple pharmacological effects, such as anti-inflammatory [12], antiviral [13,14], and also readily available in large amounts from the extract of liquorice roots. Thus, methyl oleanolate, methyl glycyrrhetinate and methyl betulinate were selected as the aglycone substitutes and the resulting three saponins **4**–**6** were derived (Fig. 1B).

In order to study the influence of esterification of the 17-COOH group of ursolic acid on the inhibitory activity, compounds **7**–**11** (Fig. 1B) were synthesized. Compounds **12**–**14** (Fig. 1B), bioisosteric surrogates of ursolic acid ester, were synthesized to study the effects of acylation at the17-COOH position of ursolic acid with alkyl amines on the activity. To understand the influence of ursane triterpenoids with modified rings A and C on the antiviral activity, we synthesized compounds **15** and **16** (Fig. 1B). Compound **15** bears an α-OH at the C-3 position. Thus, comparison with compound **3** with a β-OH at the same position provides a means to judge the effects of the C-3 configuration on the activity. Compound **16** was derived from the incorporation of a carbonyl group at the C-11 position of compound **3** to determine whether the introduction of an additional hydrogen bond acceptor was beneficial to the activity.

2. Results and discussion

2.1. Chemistry

As depicted in Scheme 1, oxidation of methyl ursolate **17** [7] obtained by treating ursolic acid with iodomethane in the presence of pyridinium chlorochromate (PCC) gave the 3-oxo compound **18**. Meerwein-Ponndorf reduction of compound **18** afforded 3α-ursolic acid methyl ester **19** as the major product (70%), along with compound **17** (18%) as a minor product. The coupling constant of H-3 in the ^{1}H NMR of **19** [δ 3.42 (1H, t, J = 1.7 Hz)] compared with that of **17** [δ 3.23 (1H, dd, J = 11.4, 4.7 Hz)] indicated that H-3 was α orientated in **19** and β orientated in **17**, respectively. Treatment of ursolic acid with allyl bromide in the presence of K_2CO_3 in DMF provided allyl ursolate **20** [15] in good yield. The 3-hydroxy-11-oxo methyl ursolate derivative **21** [16] was prepared following literature procedures.

Compound **4** was prepared following our previous procedure [7]. Compounds **5**–**16** were synthesized via a route similar to that of compound **3** [7,8]. As shown in Scheme 2, glycosylation of the intermediates **19**–**21**, methyl glycyrrhetinate **22** [12] and methyl betulinate **23** [17], with 2,3,4,6-tetra-*O*-benzoyl-D-glucopyranosyl trichloroacetimidate **24** [7], respectively, under the action of TMSOTf gave the 3-*O*-β-glucopyranosides **25**–**29**, respectively. Removal of the benzoyl groups with NaOMe in MeOH afforded **30**–**34** and selective protection of the 3,6-OHs of the β-glucopyranosyl residues with 1-(benzoyloxy)-benzotriazole (1-BBTZ) in the presence of Et_3N afforded intermediates **35**–**39**. Subsequent glycosylation of the 2, 4-OHs in **35**–**39** with 2,3,4-tri-*O*-acetyl-L-rhamnopyranosyl trichloroacetimidate **40** [7] under the "inverse addition conditions" with $BF_3 \cdot Et_2O$ as the promoter, followed by deprotection of the acyl groups with MeONa, afforded target saponins **5**, **6**, **15**, **16** and the important intermediate **41**, respectively.

The target saponins **7**–**11** and **12**–**14** were prepared as depicted in Scheme 3. Intermediate **41** was subjected to $PdCl_2$ to provide the target compound **7**. Treatment of **7** with different halohydrocarbon in the presence of K_2CO_3 in DMF yielded the target compounds **8**–**11**. Protection of all the OHs in **7** by using Ac_2O in the presence of DMAP produced compound **42**. The key intermediate **42** was treated with oxalyl chloride to give 28-acyl chloride, which was then condensed with appropriate amines and finally deprotected with MeONa to yield the corresponding target saponins **12**–**14**.

2.2. Inhibition of the infection of H5N1 pseudovirus

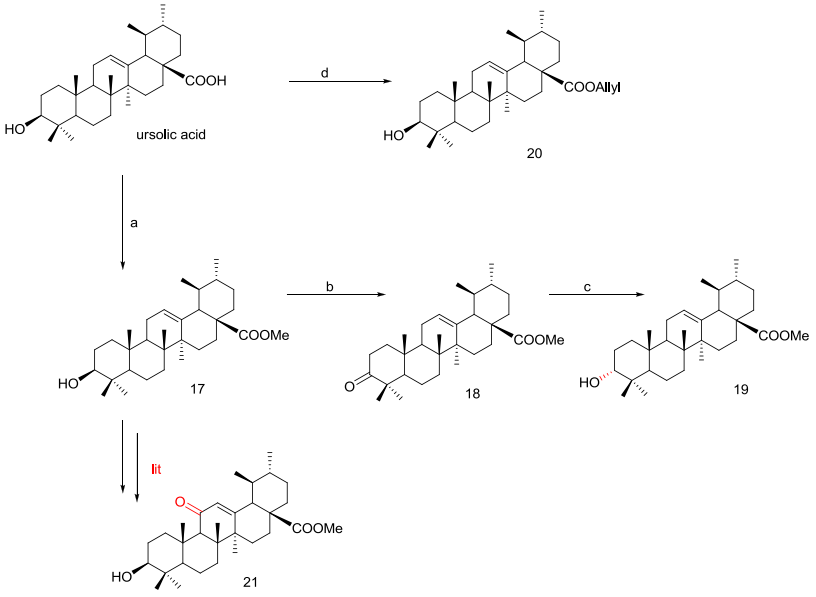
Previously, we have found that methyl ursolate 3-*O*-β-chacotrioside (**3**) could inhibit the highly pathogenic H5N1 avian influenza A virus by blocking viral entry [7]. The inhibitory activity of compound **3** is specific to hemagglutinin, since it has no inhibitory activity toward VSV-G pseudovirus [7].

Due to the safety concerns in studying viral H5N1 pathogens, the single-cycle pseudovirus was used instead of live H5N1 avian influenza virus to evaluate the inhibitory activities of compounds **4**–**16** against H5N1 entry. Compounds **3**–**16** and **41** were evaluated for the inhibitory activity against the entry of H5N1 influenza virus based on an efficient HIV-based pseudotyping system with luciferase report element established by us [18]. This pseudovirus was enveloped with influenza glycoprotein HA and NA, and packed with HIV-backbone [18].

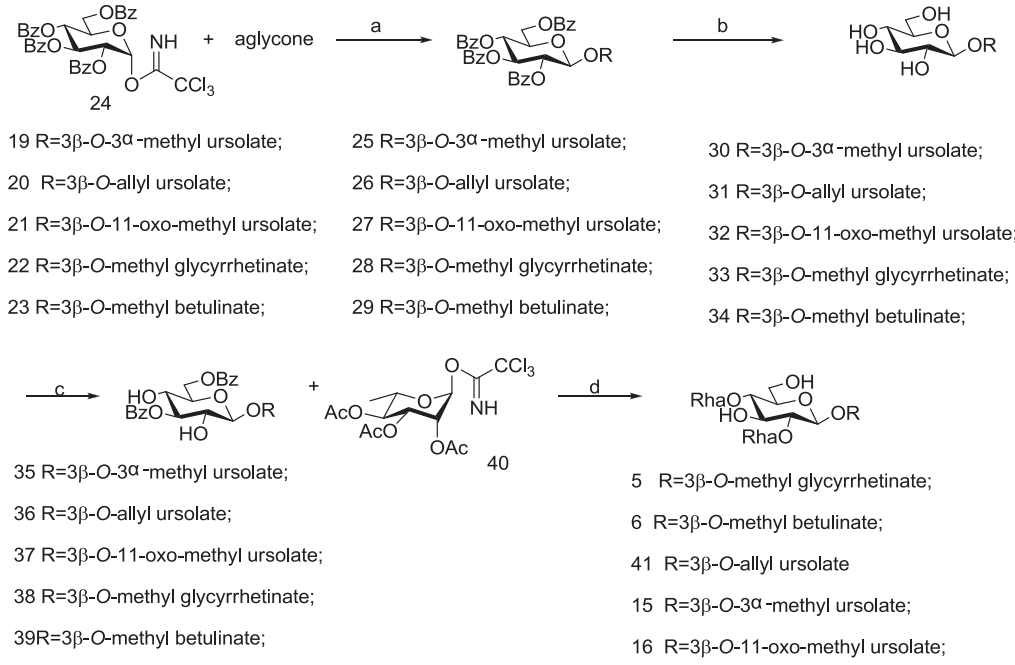
The obtained IC_{50} values were summarized in Table 1 and indicate that all the compounds showed inhibitory activity against H5N1 pseudovirus with a potency ranging from moderate (IC_{50} > 10 μM) to potent (IC_{50} < 4 μM, which is comparable to the IC_{50} of a positive compound **CL-385319** [18–20]). Notably, the active compounds in series **8**–**10** and **13**–**15** display a comparable IC_{50} value with compound **3**, whereas lower cytotoxicity against Madin Darby Canine Kidney (MDCK) cells than compound **3**. Representative compounds **3**, **13** and **15** had no effect on VSV-G enveloped pseudovirus (Fig. 2A), which was similar with both compound **3** and HA targeted compound **CL-385319** [18–20]. These results demonstrate that these compounds did not inhibit HIV-backbone and luciferase reporting activity. In addition, compounds **3**, **13** and **15** at 20 μg/mL did not inhibit neuraminidase (NA) (Fig. 2B), a glycoprotein enveloped in the pseudovirus system. These results suggested that compounds **4**–**15** might interfere with the entry of influenza virus by targeting hemagglutinin, the only other glycoprotein enveloped in the pseudovirus system.

2.3. SAR analysis of compounds *3*–*16*

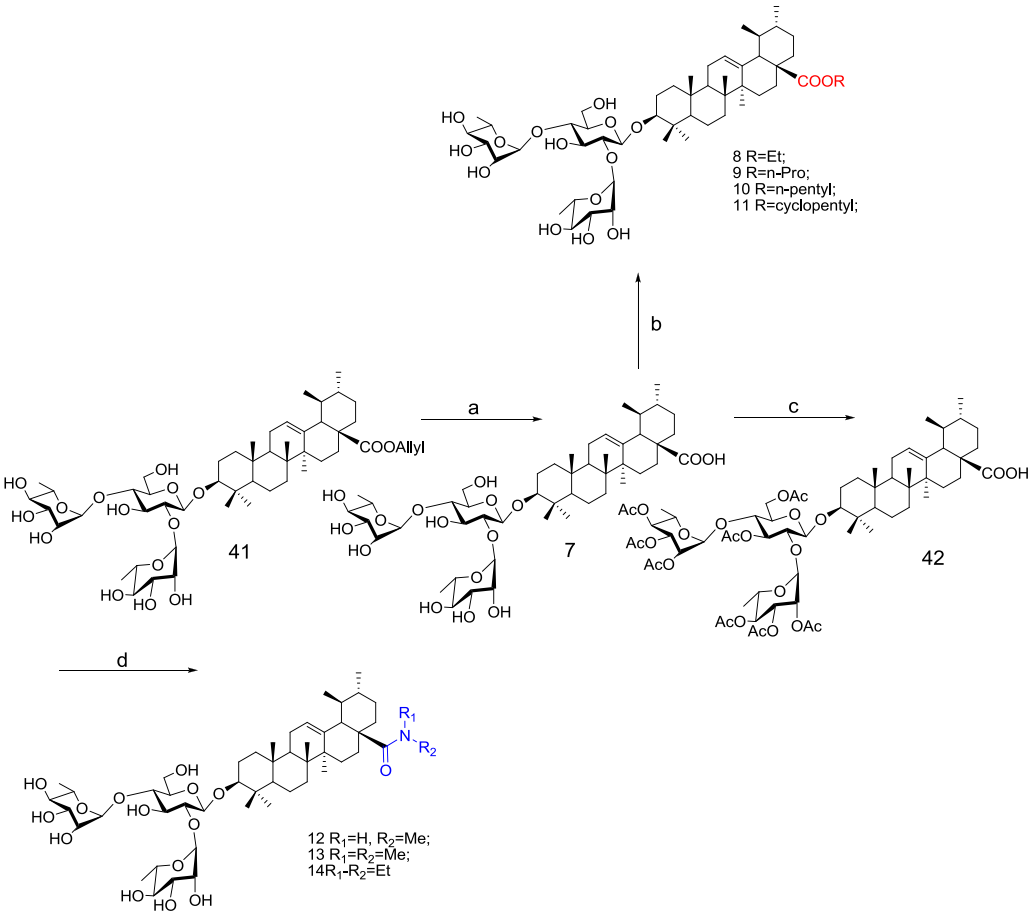
It can be seen from the IC_{50} values that compound **3** and its derivatives **4**–**16** could inhibit infection by H5N1 influenza A virus in MDCK cells in varying extents (IC_{50} values 0.98–22.50 μM). Among them, compound **13** showed the most potent inhibition



Scheme 1. Reagents and conditions: (a) CH_3I, K_2CO_3, DMF; (b) PCC, CH_2Cl_2; (c) Al(O-*i*-Pr)$_3$, *i*-PrOH, reflux; (d) AllylBr, K_2CO_3, DMF.



Scheme 2. Reagents and conditions: (a) TMSOTf, CH_2Cl_2; (b) MeONa, CH_3OH; (c) 1-BBTZ, Et_3N, CH_2Cl_2; (d) (i) $BF_3{\cdot}Et_2O$, CH_2Cl_2, (ii) MeONa, CH_3OH.



Scheme 3. Reagents and conditions: (a) $PdCl_2$, CH_3OH–CH_2Cl_2; (b) R–Br, K_2CO_3, DMF; (c)Ac_2O, DMAP, pyridine; (d) (i) $(COCl)_2$, CH_2Cl_2 (ii) Et_3N, CH_2Cl_2; (iii) CH_3ONa, CH_3OH.

Table 1
IC^a_{50} values (μM) of lead compounds **3**–**16, 41** and **CL-385319** screened in MDCK cells infected with H5N1 pseudovirus and CC^b_{50} cytotoxicity values (μM) of lead compounds **3**–**17** and **41** against MDCK cells (data derived from the mean of three independent assays).

Compound	IC^a_{50}	CC^b_{50}	SI^c
3	1.02 ± 0.13	13.02 ± 0.88	12.8
4	3.74 ± 0.14	16.85 ± 0.51	4.5
5	4.93 ± 0.52	17.12 ± 0.36	3.5
6	2.08 ± 0.29	33.90 ± 0.80	16.3
7	10.09 ± 0.23	26.15 ± 0.80	2.58
8	1.16 ± 0.05	19.05 ± 0.18	16.4
9	2.30 ± 0.16	19.22 ± 0.18	8.4
10	3.72 ± 0.68	20.10 ± 0.85	5.4
11	4.91 ± 0.26	24.25 ± 0.37	4.9
12	22.50 ± 0.12	>500	>15
13	0.98 ± 0.07	48.64 ± 0.35	49.6
14	1.41 ± 0.07	47.04 ± 0.81	50.0
15	1.33 ± 0.15	>200	>162.6
16	5.68 ± 0.11	38.37 ± 0.75	6.76
41	33.95 ± 0.75	21.83 ± 0.68	0.64
CL-385319	4.45 ± 1.25	1480 ± 10	332.3

IC^a_{50} values (μM) of lead compounds **3**–**16** and **41** screened in MDCK cells infected with H5N1 pseudovirus (data derived from the mean of three independent assays).
CC^b_{50} cytotoxicity values (μM) of lead compounds **3**–**16** and **41** against MDCK cells (data derived from the mean of three independent assays).
The selection index (SI^c) was CC^b_{50}/IC^a_{50}.

with an IC_{50} of 0.98 μM and a selection index (SI, CC_{50}/IC_{50}) of 49.6, respectively. Thus, compound **13** is exploitable as a lead compound.

Pentacyclic triterpene as aglycone residue had significant influence on the inhibitory activity. The activity in the order of compounds **3** > **6** > **4** > **5** suggests that ursane type triterpenes are superior to the other kinds of triterpenes as aglycone residue. It was found that esterification of the 17-COOH group of ursolic acid with saturated alkane groups enhanced the inhibitory activity (**8**–**11** > **7**). We supposed that the hydroxyl group of 17-COOH did not contribute to the interaction of the aglycone residue with the receptor. When 17-COOH was substituted by a fatty alkyl group, the length of the carbon chain from one to five led to slightly reduced activity. However, introduction of the allyl group to the 17-COOH position (to give compound **41**), resulted in a loss in the inhibitory activity. In addition, compound **3** showed slightly higher activity than compound **11**, suggesting that the introduction of a bulky group could increase the steric hindrance and decrease binding. Taken together these results suggested that when the 17-COOH was esterified, introduction of short straight saturated alkyl groups was helpful to enhance the inhibitory activity, whereas the length of carbon side chain from one to five had little effect on the antiviral activity but introduction of bulky groups or unsaturated hydrocarbon should be avoided.

The introduction of an amine structure at the 17-COOH position was done to determine whether a disubstituted amide structure was beneficial to inhibition. As a result, compounds **13** and **14** had similar activity as compounds **3** and **8**, while compound **12** has lower activity than compound **13**, possibly due to the polar NH group at the 17-COOH position. This was further supported by the fact that keeping a polar group at the 17-COOH position was not essential for inhibition activity, which should be esterified or amidated. It was interesting to note that there was an excellent change in the IC_{50} of cytotoxicity against MDCK cells *in vitro* between compound **3** and **14**. Compound **14**, amidated from compound **3**, had an improved selective index (12.8–50), indicating that amidation at the 17-COOH position could significantly improve the safety of these compounds while keeping their antiviral activity.

Of the two 3-hydroxyl group isomers, the 3α-form **15** had a similar inhibition as the 3β-form **3**, but 3α-form **15** exhibited significantly decreased cytotoxic activity against MDCK cells *in vitro* compared to compound **3**. These results confirmed that the configuration at C-3 is an important factor for the selection index, rather than for the antiviral activity. As far as selective index is concerned, compound **15** is a more promising lead than compound **3**. Lastly, introduction of an additional oxo group to the 11-position did not improve the inhibition (**16** vs. **3**).

3. Conclusion

Based on our previously discovered small molecule inhibitor **3**, intensive SARs studies on the aglycone of compound **3** were conducted. The results showed that both the type of pentacyclic triterpene and the modification of ursolic acid as aglycon had influence on the antiviral activity. The above results indicated that when the 17-COOH of ursolic acid was esterified or amidated, introduction of short straight saturated alkyl groups or a disubstituted amide structure was helpful in enhancing inhibitory activity, but introduction of bulky groups or unsaturated hydrocarbons should be avoided. The configuration at C-3 was found to be important for the selection index. Compounds with the 3α-form kept antiviral activity and greatly decreased cytotoxicity against MDCK cells compared to compounds with 3β-form. The SARs described herein may serve as the basis for the rational design of more potent ursolic acid derivatives as novel H5N1 entry inhibitors.

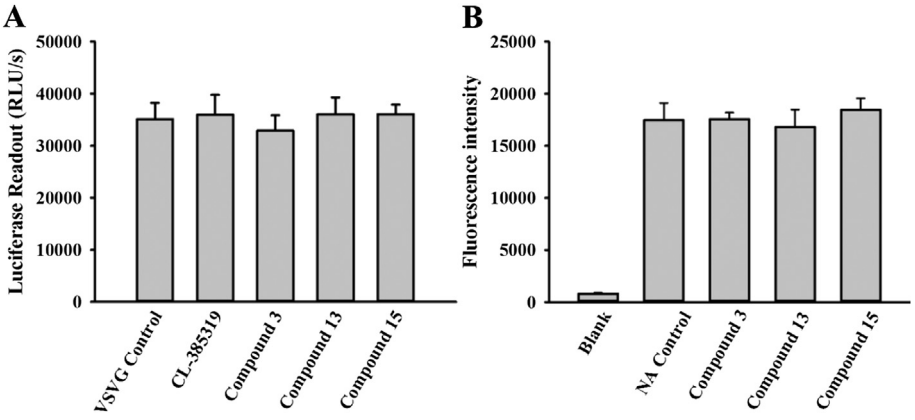


Fig. 2. A. Representative compounds **3**, **13** and **15** did not inhibit VSVG pseudovirus. B. Representative compounds **3**, **13** and **15** did not inhibit neuraminidase activity.

4. Experimental protocols

4.1. General methods

Solvents were purified in a conventional manner. Thin layer chromatography (TLC) was performed on precoated E. Merck silica gel 60 F254 plates. Flash column chromatography was performed on silica gel (200–300 mesh, Qingdao, China). ^{1}H NMR and ^{13}C NMR spectra were taken on a JEOL JNM-ECP 600 spectrometer with tetramethylsilane as an internal standard, and chemical shifts are recorded in ppm values. Mass spectra were recorded on a Q-TOF Global mass spectrometer.

4.2. 3- Hydroxy-urs-12-en-28-oic acid methyl ester (**17**)

To a solution of UA (1.00 g, 2.19 mmol) in dry DMF (20 mL) was added potassium carbonate (0.60 g, 4.38 mmol). After 20 min of stirring at room temperature, iodomethane (0.41 mL, 6.57 mmol) was added and the mixture was stirred for an additional 5 h. The solvents were evaporated and the crude residue was dissolved in a mixture of CH_2Cl_2 (100 mL) and hydrochloric acid (30 mL, 1.0 M). The aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layer was washed with brine (100 mL), dried (Na_2SO_4) and concentrated under reduced pressure. Recrystallization from EtOH provided the known compound **17** (0.98 g, 95%) as a colorless solid; ^{1}H NMR ($CDCl_3$): δ 5.26 (t, 1H, J = 3.6 Hz, H-12), 3.62 (s, 3H, OCH_3), 3.23 (dd, 1H, J = 11.4, 4.7 Hz, H-3), 2.24 (d, 1H, J = 11.5 Hz, H-18), 1.09, 1.00, 0.93, 0.80, 0.76 (each s, each 3H, CH_3), 0.95 (d, 3H, J = 6.2 Hz, CH_3), 0.88 (d, 3H, J = 6.6 Hz, CH_3).

4.3. 3-Oxo-urs-12-en-28-oic acid methyl ester (**18**)

To a solution of compound **17** (2.50 g, 5.31 mmol) in CH_2Cl_2 (80 mL) was added PCC (1.72 g, 7.97 mmol). After being stirred at room temperature for 8 h, the mixture was concentrated and partitioned with H_2O and CH_2Cl_2. The CH_2Cl_2 layer was concentrated and purified by chromatography on a silica gel column eluted with *n*-hexane-acetone (15:1) to give **18** (2.15 g, 86%) as a colorless solid; R_f 0.53 (1:8, EtOAc-petroleum ether); ^{1}H NMR ($CDCl_3$): δ 5.29 (*t*-like, 1H, H-12), 3.63 (s, 3H, OCH_3), 2.53–2.59 (m, 1H, H-2a), 2.37–2.41 (m, 1H, H-2b), 2.26 (d, 1H, J = 11.5 Hz, H-18), 1.10 (s, 6H, 2 × CH_3), 1.05 (s, 6H, 2 × CH_3), 0.96 (d, 3H, J = 6.2 Hz, CH_3), 0.88 (d, 3H, J = 6.3 Hz), 0.81 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ 217.8 (C-3), 178.0 (C-28), 138.3 (C-13), 125.3 (C-12), 55.3, 53.0, 51.5, 48.1, 47.4, 46.8, 42.1, 39.5, 39.3, 39.1, 38.9, 36.7, 36.6, 34.2, 32.5, 30.7, 28.0, 26.6, 24.2, 23.5, 23.4, 21.5, 21.2, 19.6, 17.0, 16.9, 15.2; HRESIMS calcd for $C_{31}H_{48}O_3Na$ 491.3496; found 491.3498.

4.4. 3α-Hydroxyurs-12-en-28-oic acid methyl ester (**19**)

A mixture of compound **18** (2.10 g, 4.63 mmol), freshly prepared Al (O-*i*-Pr)$_3$ (18.9 g, 92.57 mmol), catalytic amount of $AlCl_3$ (61.7 mg, 0.46 mmol) and dry *i*-PrOH (50 mL) was refluxed for 4 h. After the mixture was cooled to room temperature, 1 M HCl (50 mL) was added. The mixture was extracted with EtOAc (3 × 100 mL). The combined extract was washed with saturated $NaHCO_3$ (3 × 100 mL) and brine (3 × 100 mL), dried over Na_2SO_4, filtered, and concentrated to afford a yellow solid, which was purified by flash chromatography (petroleumether-EtOAc, 6:1) to give **19** as a white solid (1.49 g, 70%), together with **17** as a minor product. R_f 0.48 (1:4, EtOAc-petroleum ether); ^{1}H NMR ($CDCl_3$): δ 5.26 (t, 1H, J = 3.6 Hz, H-12), 3.62 (s, 3H, OCH_3), 3.42 (t, 1H, J = 1.7 Hz, H-3), 2.24 (d, 1H, J = 11.3 Hz, H-18), 1.10, 0.96, 0.94, 0.85, 0.76 (each s, each 3H, CH_3), 0.95 (d, 3H, J = 6.4 Hz, CH_3), 0.87 (d, 3H, J = 6.5 Hz, CH_3); ^{13}C NMR ($CDCl_3$): δ 178.1 (C-28), 138.1 (C-13), 125.6 (C-12), 76.1 (C-3), 52.9, 51.4, 49.0, 48.1, 47.4, 42.1, 39.7, 38.9, 37.3, 36.7, 33.1, 32.8, 30.7, 28.3, 25.2, 24.2, 23.7, 23.2, 22.3, 21.2, 18.3, 17.0, 16.9, 15.2; HRESIMS calcd for $C_{31}H_{50}O_3Na$ 493.3652; found 493.3659.

4.5. 3-Hydroxy-urs-12-en-28-oic acid allyl ester (**20**)

To a solution of UA (1.00 g, 2.19 mmol) in dry DMF (20 mL) was added potassium carbonate (0.60 g, 4.38 mmol). After 4 h of stirring at room temperature, allyl bromide (0.48 mL, 6.57 mmol) was added and the mixture was stirred for an additional 8 h. The solvents were evaporated and the crude residue was dissolved in a mixture of CH_2Cl_2 (100 mL) and hydrochloric acid (30 mL, 1.0 M). The aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL), the combined organic layers were washed with brine (100 mL), dried (Na_2SO_4), filtered and the solvent was evaporated. Recrystallization from EtOH provided compound **20** (0.99 g, 91%) as a colorless solid; ^{1}H NMR ($CDCl_3$): δ 5.86–5.91 (m, 1H, CH=), 5.32 (dd, 1H, J = 17.0, 1.7 Hz, CH_2=CH-1), 5.23 (t, 1H, J = 3.3 Hz, H-12), 5.21 (dd, 1H, J = 10.5, 1.3 Hz, CH_2=CH-2), 4.51 (d, 2H, J = 5.6 Hz, OCH_2–CH=), 3.22 (dd, 1H, J = 11.2, 4.7 Hz, H-3), 2.26 (d, 1H, J = 11.3 Hz, H-18), 1.99 (td, 1H, J = 17.8, 4.3 Hz), 1.08, 1.00, 0.91, 0.79, 0.64 (each s, each 3H, CH_3), 0.94 (d, 3H, J = 6.3 Hz, CH_3), 0.86 (d, 3H, J = 6.2 Hz, CH_3).

4.6. 3-Hydroxy-11-oxo-urs-12-en-28-oic acid methyl ester (**21**)

Compound **21** was prepared following the same procedure as described by C.M Ma [16]. ^{1}H NMR ($CDCl_3$): δ 5.58 (brs, 1H, H-12), 3.56 (s, 3H, OCH_3), 3.18 (dd, 1H, J = 10.5, 5.7 Hz, H-3), 2.75 (dt, 1H, J = 14.0, 3.5 Hz, H-1a), 2.36 (d, 1H, J = 11.0 Hz, H-18), 2.25 (s, 1H, H-9), 1.22, 1.08, 0.95, 0.86, 0.75 (each s, each 3H, CH_3), 0.93 (d, 3H, J = 6.3 Hz, CH_3), 0.82 (d, 3H, J = 6.2 Hz, CH_3).

4.7. General procedure for the preparation of **25**–**29**

To a solution of compounds **19**–**23** (1 eq), 2,3,4,6-tetra-*O*-benzoyl-D-glucopyranosyl trichloroacetimidate **24** (1.3 eq) and 4 Å molecular sieves in dry CH_2Cl_2 was added TMSOTf (0.1 eq) at 0 °C under argon. The reaction mixture was stirred for 1 h and warmed to room temperature for 1 h. The reaction was quenched by Et_3N and concentrated. The residue was purified by silica gel column chromatography (petroleum ether-EtOAc, 6:1) to afford compounds **25**–**29**, respectively.

4.7.1. 3α-O-(2,3,4,6-tetra-O-benzoyl-β-D-glucopyranosyl)-urs-12-en-28-oic acid methyl ester (**25**)

Compound **25** was synthesized as a white solid in 93% yield; R_f 0.42 (1:4, EtOAc-petroleum ether); ^{1}H NMR ($CDCl_3$): δ 7.29–8.03 (m, 20H, Ar–H), 5.94 (t, 1H, J = 9.7 Hz, H-3′), 5.66 (t, 1H, J = 9.6 Hz, H-4′), 5.12 (t, 1H, J = 3.7 Hz, H-12), 4.79 (d, 1H, J = 7.9 Hz, H-1′), 4.66 (dd, 1H, J = 11.9, 3.5 Hz, H-6′-1), 4.51 (dd, 1H, J = 11.9, 5.5 Hz, H-6′-2), 4.08–4.12 (m, 1H, H-5′), 3.59 (s, 3H, OCH_3), 3.35 (t-like, 1H, J = 1.6 Hz, H-3), 2.21 (d, 1H, J = 11.2 Hz, H-18), 1.07, 0.94, 0.78, 0.76, 0.65 (each s, each 3H, CH_3), 0.96 (d, 3H, J = 6.2 Hz, CH_3), 0.95 (d, 3H, J = 6.6 Hz, CH_3); ^{13}C NMR ($CDCl_3$):δ 178.1 (C-28), 166.1, 165.8, 165.3, 165.0, 138.2 (C-13), 133.4, 133.2, 133.1, 133.0, 129.8, 129.7 (three), 129.6, 128.9 (two), 128.4, 128.3, 128.2 (two), 125.5 (C-12), 99.0 (C-1′), 82.9, 73.0, 71.9, 71.8, 70.4, 63.1, 52.9, 51.4, 49.0, 48.1, 47.0, 41.9, 39.5, 39.0, 38.9, 36.7 (two), 36.5, 33.0, 32.7, 30.6, 28.6, 28.0, 24.2, 23.6, 22.9, 22.1, 21.4, 21.2, 17.9, 17.3, 16.8, 15.3; HRESIMS calcd for $C_{65}H_{76}O_{12}Na$ 1071.5229; found 1071.5233.

4.7.2. 3β-O-(2,3,4,6-Tetra-O-benzoyl-β-D-glucopyranosyl)-urs-12-en-28-oic acid allyl ester (**26**)

Similarly, **26** was prepared as a white solid in 92% yield; R_f 0.57 (1:3, EtOAc-petroleum ether); ^{1}H NMR ($CDCl_3$): δ 7.29–8.06 (m,

20H, Ar–H), 5.93 (t, 1H, J = 9.7 Hz, H-3′), 5.83–5.92 (m, 1H, CH =), 5.60 (t, 1H, J = 9.7 Hz, H-2′), 5.58 (dd, 1H, J = 9.7, 7.9 Hz, H-2′), 5.29–5.32 (m, 1H, CH_2=CH-1), 5.27 (t, 1H, J = 3.5 Hz, H-12), 5.19–5.23 (m, 1H, CH_2=CH-2), 4.86 (d, 1H, J = 7.9 Hz, H-1′), 4.50–4.59 (m, 4H, H-6′, OCH_2), 4.15–4.18 (m, 1H, H-5′), 3.08 (dd, 1H, J = 11.9, 4.8 Hz, H-3), 2.27 (d, 1H, J = 12.3 Hz, H-18), 1.06, 0.85, 0.71, 0.70, 0.63 (each s, each 3H, CH_3), 0.97 (d, 3H, J = 6.2 Hz, CH_3), 0.92 (d, 3H, J = 6.6 Hz, CH_3); ^{13}C NMR ($CDCl_3$): δ 177.7 (C-28), 166.6, 166.5, 165.9, 165.6, 138.6 (C-13), 134.1, 133.8, 133.7, 133.2, 130.7, 130.5, 130.4 (two), 130.3 (two), 130.0, 129.5, 129.4, 129.3, 129.0, 128.9 (two), 126.2 (C-12), 118.4, 103.9 (C-1′), 91.2, 79.7, 73.6, 72.7, 72.6, 71.0, 65.4, 64.1, 56.1, 55.8, 53.5, 48.7, 48.2, 42.6, 40.1, 39.7, 39.3, 39.1, 37.3, 37.1, 33.6, 31.3, 28.7, 28.5, 28.2, 26.4, 24.8, 24.1, 23.8, 21.8, 18.6, 17.6, 16.8, 15.9; HRESIMS calcd for $C_{67}H_{78}O_{16}Na$ 1097.5385; found 1097.5383.

4.7.3. 3β-O-(2,3,4,6-Tetra-O-benzoyl-β-D-glucopyranosyl)-oxo-urs-12-en-28-oic acid methyl ester (**27**)

Similarly, **27** was prepared as a white solid in 90% yield; R_f 0.52 (1:3, EtOAc-petroleum ether); ^{1}H NMR ($CDCl_3$): δ 7.29–8.03 (m, 20H, Ar–H), 5.92 (t, 1H, J = 9.6 Hz, H-3′), 5.62 (brs, 1H, H-12), 5.59 (t, 1H, J = 9.7 Hz, H-4′), 5.57 (dd, 1H, J = 9.7, 7.9 Hz, H-2′), 4.87 (d, 1H, J = 8.0 Hz, H-1′), 4.65 (dd 1H, J = 11.8, 4.2 Hz, H-6-1′), 4.50 (dd, 1H, J = 11.8, 7.0 Hz, H-6-2′), 4.15–4.18 (m, 1H, H-5′), 3.61 (s, 3H, OCH_3), 3.13 (dd, 1H, J = 11.3, 5.0 Hz, H-3), 2.73 (dt, 1H, J = 13.7, 3.3 Hz, H-1a), 2.43 (d, 1H, J = 11.2 Hz, H-18), 2.31 (s, 1H, H-9), 1.27, 1.08, 0.87, 0.71, 0.68 (each s, each 3H, CH_3), 0.99 (d, 3H, J = 6.4 Hz, CH_3), 0.91 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$): δ 199.6 (C-11), 177.2 (C-28), 166.1, 165.9, 165.3, 164.9, 162.6 (C-13), 133.4, 133.3, 133.2, 133.1, 133.0, 130.6 (C-12), 129.8, 129.7, 129.6, 128.5, 128.4, 128.3 (two), 103.1 (C-1′), 90.5, 73.0, 72.1, 72.0, 70.3, 63.4, 61.4, 55.2, 52.7, 51.9, 47.7, 44.6, 43.7, 39.0, 38.6, 36.8, 36.0, 32.9, 30.3, 28.3, 27.6, 25.8, 23.9, 21.0, 18.9, 17.2, 17.1, 16.2, 16.1; HRESIMS calcd for $C_{65}H_{74}O_{13}Na$ 1085.5022; found 1085.5030.

4.7.4. Methyl 3β-O-(2,3,4,6-Tetra-O-benzoyl-β-D-glucopyranosyl)-11-oxo-olean-12 -en-30-oate (**28**)

Similarly, **28** was prepared as a white solid in 88% yield; R_f 0.50 (1:3, EtOAc-petroleum ether); ^{1}H NMR ($CDCl_3$): δ 7.34–8.01 (m, 20H, Ar–H), 5.70 (brs, 1H, H-12), 5.63 (t, 1H, J = 9.7 Hz, H-3′), 5.51 (t, 1H, J = 9.7 Hz, H-4′), 4.62 (d, 1H, J = 8.2 Hz, H-1′), 4.61 (dd, 1H, J = 11.9, 3.2 Hz, H-6′-1), 4.46 (dd, 1H, J = 11.9, 7.1 Hz, H-6′-2), 4.01–4.08 (m, 1H, H-2′), 3.87–3.91 (m, 1H, H-5′), 3.72 (s, 3H, OCH_3), 3.24 (dd, 1H, J = 11.7, 4.7 Hz, H-3), 2.77 (dt, 1H, J = 13.7, 3.5 Hz, H-1), 2.54 (d, 1H, J = 3.0 Hz, H-9), 2.29 (s, 1H), 2.11 (dd, 1H, J = 13.6, 3.6 Hz, H-18), 2.08 (dd, 1H, J = 13.4, 4.6 Hz, H-15), 2.01–2.03 (m, 1H, H-21), 1.95–1.97 (m, 1H, H-19), 1.90–1.93 (m, 1H, H-2), 1.37, 1.18, 1.15, 1.14, 1.05, 0.87, 0.83 (each s, each 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ 199.9 (C-11), 177.0 (C-30), 169.1 (C-13), 166.5, 166.0, 165.5, 133.4, 133.3, 133.2, 129.9, 129.8, 129.7, 129.6, 129.2, 128.9 (C-12), 128.5 (two), 128.4 (two), 104.9 (C-1′), 90.4, 75.0, 73.3, 72.0, 69.9, 63.5, 61.8, 55.3, 51.8, 48.4, 45.4, 44.4, 43.2, 41.2, 39.3, 39.0, 37.8, 36.8, 32.7, 31.8, 31.2, 28.5, 28.4, 28.2, 26.5, 26.4, 25.9, 23.4, 18.7, 17.4, 16.7, 16.3; HRESIMS calcd for $C_{65}H_{74}O_{13}Na$ 1085.5022; found 1085.5023.

4.7.5. Methyl betulinate 3β-O-2,3,4,6-Tetra-O-benzoyl-β-D-glucopyranoside (**29**)

Similarly, **29** was prepared as a white solid in 93% yield; R_f 0.40 (1:4, EtOAc-petroleum ether); ^{1}H NMR ($CDCl_3$): δ 7.29–8.04 (m, 20H, Ar–H), 5.92 (t, 1H, J = 9.6 Hz, H-3′), 5.60 (t, 1H, J = 9.7 Hz, H-4′), 5.57 (dd, 1H, J = 9.6, 8.0 Hz, H-2′), 4.87 (d, 1H, J = 7.9 Hz, H-1′), 4.77 (d, 1H, J = 2.2 Hz, H-29-1), 4.65–4.66 (m, 1H, H-29-2), 4.61 (dd, 1H, J = 11.9, 3.4 Hz, H-6′-1), 4.55 (dd, 1H, J = 11.9, 6.8 Hz, H-6′-2), 4.13–4.18 (m, 1H, H-5′), 3.67 (s, 3H, OCH_3), 3.08 (dd, 1H, J = 11.8, 4.5 Hz, H-3), 2.99–3.03 (m, 1H, H-19), 2.17–2.24 (m, 2H), 1.87–1.94 (m, 2H), 1.80–1.85 (m, 1H), 0.93, 0.87, 0.76, 0.67, 0.62 (each s, each 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ 176.7 (C-28), 166.1, 165.9, 165.3, 165.0, 150.7 (C-20), 133.4, 133.2, 133.1, 129.9, 129.8, 129.7, 128.4 (two), 128.3 (two), 109.5 (C-29), 103.2 (C-1′), 90.7, 73.0, 72.1, 72.0, 70.3, 63.5, 56.6, 55.6, 51.3, 50.5, 49.4, 47.0, 42.3, 40.7, 38.8, 38.6, 38.3, 37.0, 36.8, 34.3, 34.2, 32.1, 30.6, 29.6, 27.5, 26.0, 25.6, 20.9, 19.5, 18.1, 16.0 (two), 15.9, 14.6; HRESIMS calcd for $C_{65}H_{76}O_{12}Na$ 1071.5229; found 1071.5238.

4.8. General procedure for the preparation of **30**–**34**

Compounds **25**–**29** was dissolved in CH_2Cl_2 and CH_3OH (V:V = 1:1) and then NaOMe was added until pH = 10, respectively. After stirred at r.t. for 8 h, the solution was neutralized with Dowex 50 × 8 (H^+) resin until pH = 7, filtered and concentrated. Then the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH, 10:1) to give compounds **30**–**34**, respectively.

4.8.1. 3α-O-(β-g-Glucopyranosyl)-urs-12-en-28-oic acid methyl ester (**30**)

Compound **30** was synthesized as a white solid in 94% yield; R_f 0.35 (8:1, $CHCl_3$–MeOH); ^{1}H NMR ($CDCl_3$): δ 5.26 (t, 1H, J = 3.5 Hz, H-12), 4.29 (d, 1H, J = 7.8 Hz, H-1′), 3.88 (dd, 1H, J = 12.0, 3.1 Hz, H-6′-1), 3.83 (dd, 1H, J = 11.8, 3.5 Hz, H-6′-2), 3.64 (t, 1H, J = 9.2 Hz, H-4′), 3.62 (s, 3H, OCH_3), 3.58 (t-like, 1H, J = 9.2, 8.9 Hz, H-3′), 3.41(t-like, 1H, J = 8.8, 8.2 Hz, H-2′), 3.36 (t, 1H, J = 1.7 Hz, H-3), 3.29–3.32 (m, 1H, H-5′), 2.24 (d, 1H, J = 11.2 Hz, H-18), 1.11, 0.95, 0.94, 0.86, 0.75 (each s, each 3H, CH_3), 0.96 (d, 3H, J = 6.3 Hz, CH_3), 0.88 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$): δ 178.1 (C-28), 138.1 (C-13), 125.6 (C-12), 100.4 (C-1′), 82.4, 76.4, 75.2, 73.6, 70.0, 62.0, 52.9, 51.5, 49.8, 48.1, 47.2, 42.0, 39.7, 39.1, 38.9, 36.9 (two), 36.7, 33.5, 32.7, 30.7, 28.9, 28.0, 24.2, 23.9, 23.2, 22.7, 21.2, 18.1, 17.1, 16.9, 15.4; HRESIMS calcd for $C_{37}H_{60}O_8Na$ 655.4180; found 655.4184.

4.8.2. 3β-O-(β-D-Glucopyranosyl)-urs-12-en-28-oic acid allyl ester (**31**)

Similarly, **31** was prepared as a white solid in 93% yield; R_f 0.33 (8:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.90–5.96 (m, 1H, CH=CH_2), 5.33 (dd, 1H, J = 17.2, 1.6 Hz, CH=CH_2-1), 5.15 (t, 1H, J = 3.4 Hz, H-12), 5.22–5.23 (m, 1H, CH=CH_2-2), 4.50 (d, 2H, J = 5.6 Hz, OCH_2–CH=), 4.34 (d, 1H, J = 7.8 Hz, H-1′), 3.85 (dd, 1H, J = 11.9, 2.2 Hz, H-6′-1), 3.67 (dd, 1H, J = 11.9, 5.5 Hz, H-6′-2), 3.28–3.36 (m, 2H, H-3′, H-4′), 3.24–3.27 (m, 1H, H-5′), 3.28–3.36 (m, 2H, H-3, H-2′), 2.25 (dd, 1H, J = 11.2, 4.1 Hz, H-18), 1.13, 1.07, 0.97, 0.86, 0.79 (each s, each 3H, CH_3), 0.98 (d, 3H, J = 6.3 Hz, CH_3), 0.90 (d, 3H, J = 6.3 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 177.5 (C-28), 138.1 (C-13), 132.3, 125.7 (C-12), 117.0, 105.3 (C-1′), 89.4, 76.9, 76.2, 74.3, 70.2, 64.7, 61.4, 55.6, 53.0, 41.8, 39.5, 39.0, 38.8, 36.6, 36.3, 32.9, 30.2, 27.7, 27.2, 25.5, 22.9, 22.6, 20.1, 17.9, 16.2, 15.7; HRESIMS calcd for $C_{39}H_{62}O_8Na$ 681.4337; found 681.4345.

4.8.3. 3β-O-(β-D-Glucopyranosyl)-oxo-urs-12-en-28-oic acid methyl ester (**32**)

Similarly, **32** was prepared as a white solid in 90% yield; R_f 0.30 (8:1, $CHCl_3$–MeOH); ^{1}H NMR ($CDCl_3$): δ 5.59 (s, 1H, H-12), 4.35 (d, 1H, J = 7.8 Hz, H-1′), 3.78–3.83 (m 2H, H-6′), 3.56–3.60 (m, 1H, H-5′), 3.62 (s, 3H, OCH_3), 3.51 (t, 1H, J = 9.0 Hz, H-4′), 3.41 (t-like, 1H, J = 8.2 Hz, H-3′), 3.27 (d, 1H, J = 9.3 Hz, H-3), 3.16 (t, 1H, J = 8.2 Hz, H-2′), 2.77 (d, 1H, J = 13.1 Hz, H-1a), 2.42 (d, 1H, J = 11.2 Hz, H-18), 2.30 (s, 1H, H-9), 1.30, 1.12, 1.02, 0.90, 0.84 (each s, each 3H, CH_3), 0.98 (d, 3H, J = 6.4 Hz, CH_3), 0.88 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$): δ 199.7 (C-11), 177.1 (C-30), 162.7 (C-13), 130.7 (C-12), 105.2 (C-1′), 89.7, 76.4, 75.3, 73.9, 69.5, 61.7, 61.4, 55.3, 52.7, 51.8, 47.1, 44.6, 43.7, 39.5, 39.2, 38.7, 38.5, 36.9, 36.0, 33.0, 30.3, 28.4, 28.0, 26.1, 24.0, 21.0 (two), 18.9, 17.3, 17.2, 16.7, 16.3; HRESIMS calcd for

$C_{37}H_{58}O_9Na$ 669.3973; found 669.3976.

*4.8.4. Methyl 3β-O-(β-D-glucopyranosyl)-11-oxo-olean-12-en-30-oate (**33**)*

Similarly, **33** was prepared as a white solid in 89% yield; R_f 0.27 (10:1, $CHCl_3$–MeOH); ^1H NMR ($CDCl_3$): δ 5.66 (s, 1H, H-12), 4.37 (d, 1H, J = 7.5 Hz, H-1′), 3.82–3.89 (m, 2H, H-6′), 3.69 (s, 3H, OCH_3), 3.62 (t, 1H, J = 9.2 Hz, H-3′), 3.60 (t, 1H, J = 9.1 Hz, H-4′), 3.44 (t-like, 1H, J = 9.2, 8.0 Hz, H-2′), 3.29–3.30 (m, 1H, H-5′), 3.20 (t-like, 1H, J = 9.8, 7.8 Hz, H-3), 2.78 (d, 1H, J = 15.2 Hz, H-1), 2.34 (s, 1H, H-9), 2.06–2.09 (m, 1H, H-18), 2.00–2.03 (m, 1H, H-15), 1.36, 1.15, 1.14, 1.12, 1.05, 0.86, 0.81 (each s, each 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ 200.1 (C-11), 176.7 (C-30), 169.2 (C-13), 128.5 (C-12), 104.9 (C-1′), 89.5, 76.3, 75.2, 74.0, 61.8, 60.4, 55.2, 51.8, 48.4, 45.4, 44.1, 43.2, 41.1, 39.5, 39.2, 37.7, 36.8, 32.8, 31.8, 31.1, 28.5, 28.3, 28.1, 26.5, 26.1, 23.4, 18.7, 17.4, 16.7, 16.4; HRESIMS calcd for $C_{37}H_{58}O_9Na$ 669.3973; found 669.3982.

*4.8.5. Methyl betulinate 3β-O-β-D-glucopyranoside (**34**)*

Similarly, **34** was prepared as a white solid in 95% yield; R_f 0.31 (10:1, $CHCl_3$–MeOH); ^1H NMR ($CDCl_3$): δ 4.76 (brs, 1H, H-29-1), 4.63 (brs, 1H, H-29-2), 4.34 (d, 1H, J = 7.3 Hz, H-1′), 3.78–3.85 (m, 2H, H-6′), 3.62 (t, 1H, J = 8.8 Hz), 3.55–3.58 (m, 1H), 3.43 (t, 1H, J = 6.8 Hz), 3.28–3.29 (m, 1H, H-5′), 3.66 (s, 3H, OCH_3), 3.10 (dd, 1H, J = 10.3, 4.1 Hz, H-3), 2.99–3.03 (m, 1H, H-19), 2.19–2.25 (m, 2H), 1.89–1.90 (m, 2H), 1.81–1.84 (m, 1H), 1.17, 0.97, 0.96, 0.92, 0.82, 0.79 (each s, each 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ 176.6 (C-28), 150.4 (C-20), 109.6 (C-29), 105.1 (C-1′), 90.2, 76.2, 75.2, 73.8, 69.5, 61.5, 56.5, 55.7, 51.2, 50.5, 49.5, 47.0, 42.4, 40.7, 39.2, 38.8, 38.3, 37.0, 36.9, 34.4, 32.2, 30.6, 29.7, 27.9, 26.3, 25.5, 20.9, 19.4, 18.2, 16.5, 16.2, 16.0, 14.7; HRESIMS calcd for $C_{37}H_{60}O_8Na$ 655.4180; found 655.4188.

*4.9. General procedure for the preparation of **35**–**39***

To a mixture of **30**–**34** (1 eq) and 1-BBTZ (3 eq) in dried CH_2Cl_2 (100 mL) was added Et_3N (4 eq). After stirred at r.t. for 24 h, the mixture was concentrated and purified by silica gel column chromatography (EtOAc/petroleum ether/CH_2Cl_2, 1:8:2) to provide **35**–**39** as white solids, respectively.

*4.9.1. 3α-O-(3,6-Di-O-benzoyl-β-D-glucopyranosyl)-urs-12-en-28-oic acid methyl ester (**35**)*

Compound **35** was synthesized as a white solid in 74% yield; R_f 0.32 (1:4, EtOAc-petroleum ether); ^1H NMR ($CDCl_3$): δ 7.46–8.11 (m, 10H, Ar–H), 5.23–5.26 (m, 2H, H-12, H-3′), 4.71 (dd, 1H, J = 11.9, 5.3 Hz, H-6′-1), 4.66 (dd, 1H, J = 11.9, 2.6 Hz, H-6′-2), 4.45 (d, 1H, J = 7.7 Hz, H-1′), 3.74–3.80 (m, 2H, H-4′, H-2′), 3.69–3.72 (m, 1H, H-5′), 3.61 (s, 3H, OCH_3), 3.41 (t, 1H, J = 1.1 Hz, H-3), 2.22 (d, 1H, J = 11.2 Hz, H-18); 1.07, 0.94, 0.93, 0.81, 0.74 (each s, each 3H, CH_3), 0.95 (d, 3H, J = 6.3 Hz, CH_3), 0.85 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$): δ 178.1 (C-28), 167.7, 166.8, 138.1 (C-13), 133.5, 133.2, 130.0, 129.9, 129.8, 129.7, 129.5, 128.5, 128.4 (two), 128.3, 126.3, 125.5 (C-12), 100.4 (C-1′), 82.3, 78.6, 74.3, 72.2, 69.9, 63.7, 52.9, 51.4, 49.9, 48.1, 47.4, 42.0, 39.7, 39.1, 38.9, 36.9, 36.8, 36.7, 33.7, 32.8, 30.7, 28.5, 28.0, 24.2, 23.8, 23.2, 22.4, 21.2, 18.1, 17.0, 16.9, 15.4; HRESIMS calcd for $C_{51}H_{68}O_{10}Na$ 863.4705; found 863.4702.

*4.9.2. 3β-O-(3,6-Di-O-benzoyl-β-D-glucopyranosyl)-urs-12-en-28-oic acid allyl ester (**36**)*

Similarly, **36** was prepared as a white solid in 71% yield; R_f 0.31 (1:4, EtOAc-petroleum ether); ^1H NMR ($CDCl_3$): δ 7.44–8.08 (m, 10H, Ar–H), 5.87–5.94 (m, 1H, C*H*=CH_2), 5.32 (dd, 1H, J = 17.2, 1.6 Hz, CH=C*H*$_2$-1), 5.28 (t, 1H, J = 3.2 Hz, H-12), 5.21–5.24 (m, 2H, CH=C*H*$_2$-2, H-3′), 4.68 (d, 2H, J = 3.6 Hz, OC*H*$_2$–CH=), 4.49–4.52 (m, 3H, H-6′ × 2, H-1′), 3.73–3.79 (m, 3H, H-5′, H-4′, H-2′), 3.15 (dd, 1H, J = 11.8, 4.4 Hz, H-3), 2.28 (d, 1H, J = 11.1 Hz, H-18); 1.08, 1.00, 0.89, 0.81, 0.74 (each s, each 3H, CH_3), 0.98 (d, 3H, J = 6.3 Hz, CH_3), 0.93 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$): δ 177.2 (C-28), 167.8, 166.6, 144.0, 138.1 (C-13), 133.5, 133.1, 132.6, 130.1, 130.0, 129.8, 128.5, 128.4, 128.3, 125.6 (C-12), 117.8, 104.8 (C-1′), 90.1, 78.8, 74.2, 72.7, 70.2, 64.8, 63.9, 55.5, 52.9, 48.1, 47.6, 42.1, 39.6 39.1, 38.9, 38.8, 38.5, 36.7, 36.6, 33.1, 30.6, 28.2, 28.0, 25.9, 24.2, 23.5, 23.3, 21.2, 18.1, 17.0, 16.1, 15.4; HRESIMS calcd for $C_{53}H_{70}O_{10}Na$ 889.4867; found 889.4861.

*4.9.3. 3β-O-(3,6-Di-O-benzoyl-β-D-glucopyranosyl)-oxo-urs-12-en-28-oic acid methyl ester (**37**)*

Similarly, **37** was prepared as a white solid in 72% yield; R_f 0.36 (1:3, EtOAc-petroleum ether); ^1H NMR ($CDCl_3$): δ 7.45–8.09 (m, 10H, Ar–H), 5.63 (s, 1H, H-12), 5.22 (t, 1H, J = 9.2 Hz, H-3′), 4.71 (dd, 1H, J = 11.6, 1.3 Hz, H-6-1′), 4.63 (dd, 1H, J = 11.6, 5.6 Hz, H-6-2′), 4.50 (d, 1H, J = 7.8 Hz, H-1′), 3.74–3.78 (m, 3H, H-3′, H-4′, H-5′), 3.62 (s, 3H, OCH_3), 3.27 (brs, 1H, H-2′), 3.19 (dd, 1H, J = 11.8, 4.6 Hz, H-3), 2.74 (dt, 1H, J = 13.6, 3.4 Hz, H-1a), 2.45 (d, 1H, J = 11.6 Hz, H-18), 2.23 (s, 1H, H-9), 1.30, 1.21, 1.01, 0.91, 0.84 (each s, each 3H, CH_3), 0.99 (d, 3H, J = 6.4 Hz, CH_3), 0.92 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$): δ 199.6 (C-11), 177.2 (C-28), 167.8 (C-13), 166.8, 162.7, 133.5, 133.2, 130.7 (C-12), 130.0, 129.8, 129.7, 129.4, 128.5 (two), 104.8 (C-1′), 90.0, 78.6, 74.2, 72.8, 70.0, 63.9, 61.4, 55.3, 52.7, 51.8, 47.7, 44.6, 43.7, 39.3, 39.1, 38.6 (two), 36.8, 36.0, 33.0, 30.3, 28.4, 28.2, 25.8, 23.9, 21.0 (two), 18.9, 17.3, 17.1, 16.6, 16.1; HRESIMS calcd for $C_{51}H_{66}O_{11}Na$ 877.4497; found 877.4506.

*4.9.4. Methyl 3β- O-(3,6-Di-O-benzoyl-β-D-glucopyranosyl)-11-oxo-olean-12-en-30-oate (**38**)*

Similarly, **38** was prepared as a white solid in 70% yield; R_f 0.35 (1:3, EtOAc-petroleum ether); ^1H NMR ($CDCl_3$): δ 7.45–8.07 (m, 10H, Ar–H), 5.70 (s, 1H, H-12), 5.24 (t, 1H, J = 8.1 Hz, H-3′), 4.72 (dd, 1H, J = 11.6, 1.1 Hz, H-6-1′), 4.62 (dd, 1H, J = 11.8, 5.7 Hz, H-6-2′), 4.52 (d, 1H, J = 7.8 Hz, H-1′), 3.74–3.78 (m, 3H, H-2′, H-4′, H-5′), 3.70 (s, 3H, OCH_3), 3.22 (dd, 1H, J = 11.8, 4.6 Hz, H-3), 2.77 (dt, 1H, J = 13.3, 2.9 Hz, H-1), 2.29 (s, 3H, H-9), 2.09–2.11 (m, 1H, H-18), 2.02–2.04 (m, 1H, H-15), 1.36, 1.18, 1.14, 1.13, 1.03, 0.86, 0.82 (each s, each 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ 199.9 (C-11), 176.9 (C-30), 169.1 (C-13), 167.7, 133.5, 133.3, 130.0, 129.8, 128.5 (C-12), 128.4, 104.8 (C-1′), 90.0, 78.5, 74.2, 72.8, 70.0, 63.9, 61.8, 55.3, 51.8, 48.4, 45.3, 44.1, 43.3, 41.2, 39.2, 39.0, 37.7, 36.9, 32.7, 31.8, 31.1, 28.5, 28.4, 28.2, 26.5, 26.4, 25.9, 23.4, 18.7, 17.3, 16.7, 16.3; HRESIMS calcd for $C_{51}H_{66}O_{11}Na$ 877.4497; found 877.4501.

*4.9.5. Methyl betulinate 3β-O-3,6-Di-O-benzoyl-β-D-glucopyranoside (**39**)*

Similarly, **39** was prepared as a white solid in 71% yield; R_f 0.38 (1:3, EtOAc-petroleum ether); ^1H NMR ($CDCl_3$): δ 8.08 (td, 4H, J = 8.2, 1.2 Hz, Ar–H), 7.59 (t, 2H, J = 7.8 Hz, Ar–H), 7.46 (td, 4H, J = 8.3, 1.4 Hz, Ar–H), 5.23 (t, 1H, J = 8.8 Hz, H-3′), 4.78 (d, 1H, J = 1.7 Hz, H-29-1), 4.70 (dd, 1H, J = 11.7, 2.2 Hz, H-6′-1), 4.61–4.65 (m, 1H, H-6′-2), 4.65 (s, 1H, H-29-2), 4.50 (d, 1H, J = 7.8 Hz, H-1′), 3.73–3.77 (m, 3H, H-2′, H-4′, H-5′), 3.68 (s, 3H, OCH_3), 3.14 (dd, 1H, J = 11.9, 4.5 Hz, H-3), 3.00–3.04 (m, 1H, H-19), 2.24–2.26 (m, 1H), 2.18–2.23 (m, 1H), 1.74, 0.98, 0.96, 0.91, 0.79, 0.79 (each s, each 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ 176.7 (C-28), 167.8, 166.6, 150.6 (C-20), 133.5, 133.1, 130.0 (two), 129.8 (two), 129.4, 128.4 (two), 128.3 (two), 109.5 (C-29), 104.8 (C-1′), 90.2, 78.7, 74.2, 72.8, 70.1, 64.0, 56.6, 55.7, 51.3, 49.5, 46.9, 42.4, 40.7, 39.0, 38.6, 38.3, 37.0, 36.8, 34.3, 32.2, 30.7, 29.7, 28.0, 26.0, 25.5, 20.9, 19.5, 18.1, 16.4, 16.1, 15.9, 14.7; HRESIMS calcd for $C_{51}H_{68}O_{10}Na$ 863.4705; found 863.4713.

4.10. General procedure for the preparation of **5, 6, 15, 16, 41**

To a mixture of **35**–**39** (1 eq) and 4 Å molecular sieves in dried CH_2Cl_2 (40 mL) at −40 °C under argon was added $BF_3 \cdot Et_2O$ (1.5 eq), followed by a solution of imidate **40** (5 eq) in CH_2Cl_2 (10 mL). After stirring at - 40 °C for 2 h and then at r.t. for 1 h, the reaction was quenched with Et_3N. The solid was filtered, and the filtrate was concentrated under vacuum to give yellow oil. The oil was subjected to column chromatography on silica gel (EtOAc-petroleum ether, 1:4) to give the desired crude trisaccharide. The above trisaccharide mixture was dissolved in CH_2Cl_2 and CH_3OH (V:V = 1:1) and then NaOMe was added until pH = 10. After stirred at r.t. for 24 h, the solution was neutralized with Dowex 50 × 8 (H^+) resin until pH = 7, filtered and concentrated. Then the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH, 6:1) to afford compounds **5**, **6**, **15**, **16** and **41**, respectively.

4.10.1. Methyl 3β- O-[2,4-Di-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-11- oxo-olean-12-en-30-oate (**5**)

Compound **5** was synthesized as a white solid in 51% yield for two steps; R_f 0.35 (4:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.58 (s, 1H, H-12), 5.38 (d, 1H, J = 1.3 Hz, Rha-H-1), 4.60 (brs, 1H, Rha-H-1), 4.44 (d, 1H, J = 7.7 Hz, H-1′), 3.97–4.00 (m, 2H), 3.91–3.93 (m, 1H), 3.85 (dd, 1H, J = 3.2, 1.7 Hz, Rha-H-2), 3.81 (dd, 1H, J = 12.0, 1.5 Hz, H-6′-1), 3.76 (dd, 1H, J = 9.5, 3.3 Hz, Rha-H-3), 3.67 (dd, 1H, J = 12.1, 4.1 Hz, H-6′-2), 3.63 (dd, 1H, J = 9.4, 3.2 Hz, Rha-H-3), 3.61 (s, 3H, OCH_3), 3.60 (t, 1H, J = 8.9 Hz), 3.55 (t, 1H, J = 8.9 Hz), 3.38–3.48 (m, 2H), 3.21 (dd, 1H, J = 11.8, 4.3 Hz, H-3), 3.09–3.13 (m, 1H, H-5′), 2.72 (dt, 1H, J = 13.5, 2.8 Hz, H-1), 2.46 (s, 1H, H-19), 1.28 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.25 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.43, 1.16, 1.15, 1.14, 1.08, 0.90, 0.83 (each s, each 3H, CH_3), 0.99 (d, 3H, J = 6.4 Hz, CH_3), 0.91 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 201.2 (C-11), 171.2 (C-30), 171.1 (C-13), 121.5 (C-12), 104.1 (C-1′), 101.7 (Rha-C-1), 100.6 (Rha-C-1), 88.1, 79.1, 78.1, 77.9, 76.8, 75.1, 72.6, 72.3, 71.0, 70.7, 70.6, 69.4, 68.7, 61.8, 60.6, 55.3, 50.9, 48.5, 45.3, 43.9, 43.2, 41.0, 39.2, 39.1, 37.6, 36.7, 32.4, 31.5, 30.6, 27.7, 27.1, 27.0, 26.1, 25.9, 25.8, 22.4, 17.9, 17.0, 16.6, 16.5, 15.7, 15.6; HRESIMS calcd for $C_{49}H_{78}O_{17}Na$ 961.5131; found 961.5138.

4.10.2. Methyl betulinate 3β-O-2,4-Di-O-(α-L-rhamnopyranosyl)-β-D- glucopyranoside (**6**)

Similarly, **6** was prepared as a white solid in 55% yield for two steps; R_f 0.38 (4:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.37 (d, 1H, J = 1.2 Hz, Rha-H-1), 4.86 (s, 1H, Rha-H-1), 4.73 (s, 1H, H-29-1), 4.64 (s, 1H, H-29-2), 4.43 (d, 1H, J = 7.7 Hz, H-1′), 3.96–4.00 (m, 2H), 3.90–3.94 (m, 1H), 3.84 (dd, 1H, J = 3.2, 1.8 Hz, Rha-H-2), 3.80 (d, 1H, J = 11.2 Hz, H-6′-1), 3.76 (dd, 1H, J = 9.5, 3.0 Hz, Rha-H-3), 3.66–3.68 (m, 2H), 3.67 (s, 3H, OCH_3), 3.63 (dd, 1H, J = 9.4, 2.9 Hz, Rha-H-3), 3.59 (t, 1H, J = 8.3 Hz), 3.55 (t, 1H, J = 9.2 Hz), 3.39–3.47 (m, 3H), 3.16 (dd, 1H, J = 11.2, 3.5 Hz, H-3), 2.98–3.03 (m, 1H, H-19), 2.21–2.24 (m, 2H), 1.95–1.97 (m, 1H), 1.85–1.90 (m, 2H), 1.28 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.22 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.71, 1.04, 1.01, 0.95, 0.88, 0.85 (each s, each 3H, CH_3), 0.98 (d, 3H, J = 6.4 Hz, CH_3), 0.90 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 176.8 (C-28), 150.4 (C-20), 108.9 (C-29), 104.1 (C-1′), 101.7 (Rha-C-1), 100.6 (Rha-C-1), 89.0, 79.1, 78.1, 77.8, 76.8, 75.0, 72.5, 72.3, 71.0, 70.7, 70.5, 69.4, 68.6, 60.6,56.5, 56.0, 50.6, 50.4, 49.3, 42.1, 40.5, 38.9 (two), 38.3, 36.7, 36.5, 34.2, 31.7, 30.2, 29.4, 27.0, 26.0, 25.5, 20.7, 18.1, 17.9, 16.6, 16.5, 15.6, 15.5, 15.2, 13.7; HRESIMS calcd for $C_{49}H_{80}O_{16}Na$ 947.5339; found 947.5338.

4.10.3. 3β-O-[2,4-Di-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-12-en-28-oic acid allyl ester (**41**)

Similarly, **41** was prepared as a white solid in 54% yield for two steps; R_f 0.48 (3:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.90–5.97 (m, 1H, C*H*=CH_2), 5.37 (d, 1H, J = 1.3 Hz, Rha-H-1), 5.32–5.35 (m, 1H, CH=C*H*$_2$-1), 5.25 (t, 1H, J = 3.3 Hz, H-12), 5.21–5.23 (m, 1H, CH=C*H*$_2$-2), 4.86 (s, 1H, Rha-H-1), 4.50 (d, 2H, J = 5.2 Hz, O*CH*$_2$–CH=), 4.44 (d, 1H, J = 7.7 Hz, H-1′), 3.98–4.00 (m, 2H), 3.91–3.93 (m, 1H), 3.85 (dd, 1H, J = 3.1, 1.8 Hz, Rha-H-2), 3.81 (dd, 1H, J = 12.1, 1.7 Hz, H-6′-1), 3.76 (dd, 1H, J = 9.5, 3.4 Hz, Rha-H-3), 3.67 (dd, 1H, J = 12.1, 4.1 Hz, H-6′-2), 3.64 (dd, 1H, J = 9.4, 3.3 Hz, Rha-H-3), 3.60 (t, 1H, J = 8.7 Hz), 3.55 (t, 1H, J = 9.3 Hz), 3.46 (t, 1H, J = 8.2 Hz), 3.38–3.44 (m, 2H), 3.33–3.34 (m, 1H), 3.19 (dd, 1H, J = 11.6, 4.1 Hz, H-3), 2.25 (d, 1H, J = 11.2 Hz, H-18), 1.27 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.22 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.13, 1.07, 0.97, 0.87, 0.79 (each s, each 3H, CH_3), 0.98 (d, 3H, J = 6.4 Hz, CH_3), 0.90 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 175.9 (C-28), 136.6 (C-13), 130.9, 124.2 (C-12), 115.4, 102.5 (C-1′), 100.2 (Rha-C-1), 99.2 (Rha-C-1), 87.4, 75.2, 71.1, 70.7, 69.5, 67.9, 67.2, 63.2, 59.1, 54.4, 51.5, 40.3, 38.0, 37.5, 35.1, 34.9, 31.4, 28.8, 26.2, 25.6, 22.4, 21.2, 16.4, 15.0, 14.7, 13.3; HRESIMS calcd for $C_{51}H_{82}O_{16}Na$ 973.5495; found 973.5493.

4.10.4. 3α-O-[2,4-Di-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-12-en-28-oic acid methyl ester (**15**)

Similarly, **15** was prepared as a white solid in 53% yield for two steps; R_f 0.49 (3:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.28 (d, 1H, J = 1.3 Hz, Rha-H-1), 5.22 (t, 1H, J = 3.2 Hz, H-12), 4.86 (d, 1H, J = 1.3 Hz, Rha-H-1), 4.39 (d, 1H, J = 7.7 Hz, H-1′), 4.02 (dd, 1H, J = 3.3, 1.7 Hz, Rha-H-2), 3.88–3.95 (m, 2H), 3.85 (dd, 1H, J = 3.2, 1.8 Hz, Rha-H-2), 3.82 (dd, 1H, J = 11.9, 1.9 Hz, H-6′-1), 3.76 (dd, 1H, J = 9.5, 3.4 Hz, Rha-H-3), 3.67 (dd, 1H, J = 12.0, 4.5 Hz, H-6′-2), 3.64 (dd, 1H, J = 9.4, 3.3 Hz, Rha-H-3), 3.61 (s, 3H, OCH_3), 3.58 (t, 1H, J = 8.7 Hz), 3.54 (t, 1H, J = 9.3 Hz), 3.49 (t, 1H, J = 0.9 Hz, H-3), 3.42 (t, 1H, J = 9 0.5 Hz), 3.34–3.39 (m, 2H), 3.28–3.31 (m, 1H, H-5′), 2.22 (d, 1H, J = 11.2 Hz, H-18), 1.28 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.25 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.18, 0.98, 0.97, 0.88, 0.76 (each s, each 3H, CH_3), 0.99 (d, 3H, J = 6.4 Hz, CH_3), 0.91 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 178.6 (C-28), 138.1 (C-13), 125.7 (C-12), 101.7 (C-1′), 100.6 (Rha-C-1), 99.6 (Rha-C-1), 81.2, 79.3, 78.2, 76.2, 75.1, 72.5, 72.3, 71.0, 70.8, 70.7, 69.4, 68.8, 60.8, 53.0, 50.7, 50.0, 46.5, 41.9, 39.5, 39.0, 36.7, 36.5, 33.1, 32.6, 30.3, 27.8, 27.7, 23.9, 22.9, 20.6, 20.1, 17.9, 17.3, 16.5, 16.4, 16.3, 14.8; HRESIMS calcd for $C_{49}H_{80}O_{16}Na$ 947.5339; found 947.5346.

4.10.5. 3β-O-[2,4-Di-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-oxo-urs-12-en-28-oic acid methyl ester (**16**)

Similarly, **16** was prepared as a white solid in 52% yield for two steps; R_f 0.46 (3:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.54 (s, 1H, H-12), 5.37 (d, 1H, J = 1.3 Hz, Rha-H-1), 4.86 (d, 1H, J = 1.3 Hz, Rha-H-1), 4.44 (d, 1H, J = 7.8 Hz, H-1′), 3.97–3.99 (m, 2H), 3.89–3.93 (m, 1H), 3.84 (dd, 1H, J = 3.2, 1.9 Hz, Rha-H-2), 3.80 (dd, 1H, J = 11.8, 1.6 Hz, H-6-1′), 3.75 (dd, 1H, J = 9.6, 3.4 Hz, Rha-H-3), 3.67 (dd, 1H, J = 11.9, 4.1 Hz, H-6-2′), 3.63 (dd, 1H, J = 9.6, 3.3 Hz, Rha-H-3), 3.62 (s, 3H, OCH_3), 3.59 (t, 3H, J = 8.7 Hz), 3.55 (t, 3H, J = 8.8 Hz), 3.46 (t, 3H, J = 8.2 Hz), 3.38–3.44 (m, 2H), 3.20 (dd, 1H, J = 11.8, 4.3 Hz, H-3), 2.70 (dt, 1H, J = 13.5, 2.9 Hz, H-1a), 2.43 (d, 1H, J = 11.7 Hz, H-18), 2.41 (s, 1H, H-9), 1.36, 1.14, 1.07, 0.93, 0.88 (each s, each 3H, CH_3), 1.28 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.23 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.00 (d, 3H, J = 6.3 Hz, CH_3), 0.90 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 199.3 (C-11), 175.9 (C-28), 163.0 (C-13), 128.6 (C-12), 102.3 (C-1′), 99.9 (Rha-C-1), 98.9 (Rha-C-1), 87.0, 77.5, 76.3, 75.2, 73.5, 71.0, 70.7, 69.5, 69.2, 69.1, 67.8, 67.1, 59.9, 59.0, 53.8, 51.5, 49.5, 43.1, 42.2, 37.6, 37.5, 37.1, 36.7, 35.2, 34.2, 31.2, 28.3, 26.5, 25.5, 24.2, 22.1, 18.4, 18.3, 16.6, 15.4, 15.1, 15.0, 14.6, 14.3, 14.0; HRESIMS calcd for $C_{49}H_{78}O_{17}Na$ 961.5131; found 961.5138.

4.11. 3β-O-[2,4-Di-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-12-en-28-oic acid (7)

Compound **41** (475 mg, 0.50 mmol) was dissolved in 1:1 MeOH/CH_2Cl_2 (20 mL), $PdCl_2$ (22.6 mg, 0.25 mmol) was then added. The mixture was vigorously stirred at r.t. for 36 h and then filtered over a celite pad and concentrated under vacuum. The mixture was subjected to column chromatography on silica gel (1:5, MeOH–CH_2Cl_2) to give compound **7** (415 mg, 91%) as a colorless solid; R_f 0.42 (3:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.38 (d, 1H, J = 1.2 Hz, Rha-H-1), 5.24 (t, 1H, J = 3.3 Hz, H-12), 4.86 (d, 1H, J = 1.2 Hz, Rha-H-1), 4.45 (d, 1H, J = 7.7 Hz, H-1′), 3.97–4.01 (m, 2H), 3.91–3.94 (m, 1H), 3.85 (dd, 1H, J = 3.1, 1.8 Hz, Rha-H-2), 3.81 (dd, 1H, J = 12.1, 1.7 Hz, H-6′-1), 3.76 (dd, 1H, J = 9.5, 3.4 Hz, Rha-H-3), 3.67 (dd, 1H, J = 12.1, 4.1 Hz, H-6′-2), 3.64 (dd, 1H, J = 9.4, 3.3 Hz, Rha-H-3), 3.60 (t, 1H, J = 8.7 Hz), 3.55 (t, 1H, J = 9.3 Hz), 3.46 (t, 1H, J = 8.1 Hz), 3.38–3.44 (m, 2H), 3.33–3.34 (m, 1H), 3.19 (dd, 1H, J = 11.7, 4.1 Hz, H-3), 2.21 (d, 1H, J = 11.3 Hz, H-18), 2.05 (dd, 1H, J = 13.4, 4.2 Hz), 1.28 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.23 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.13, 1.07, 0.97, 0.87, 0.86 (each s, each 3H, CH_3), 0.98 (d, 3H, J = 6.0 Hz, CH_3), 0.90 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 180.2 (C-28), 138.2 (C-13), 125.5 (C-12), 104.1 (C-1′), 101.6 (Rha-C-1), 100.6 (Rha-C-1), 89.0, 79.1, 77.8, 76.8, 75.0, 72.5, 72.3, 71.0, 70.7, 70.6, 69.4, 68.5, 60.6, 55.9, 53.0, 41.8, 39.4, 39.0, 38.9, 36.7, 36.4, 32.9, 30.4, 27.8, 27.1, 25.8, 23.0, 22.7, 20.2, 17.9, 16.6, 16.5, 16.5, 16.3, 15.8, 14.8; HRESIMS calcd for $C_{48}H_{78}NaO_{16}$ 933.5182; found 933.5184.

4.12. General procedure for the preparation of 8–11

A mixture of compound **41** (1 mmol) and K_2CO_3 (2 mmol) in DMF (10 mL) was stirred at room temperature for 4 h. The bromoalkane (4 mmol) was then dripped slowly into the mixture. After being stirred for another 12 h, the reaction mixture was poured into the 40 mL distilled water and partitioned with butyl alcohol (3 × 80 mL). The organic layer was washed with saturated sodium chloride, dried over Na_2SO_4, and purified via silica gel column chromatography (MeOH/CH_2Cl_2, 1:8) to provide **8**–**11** as white solids, respectively.

4.12.1. 3β-O-[2,4-Di-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-12-en-28-oic acid ethyl ester (8)

Compound **8** was synthesized as a white solid in 96% yield; R_f 0.48 (3:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.38 (d, 1H, J = 1.3 Hz, Rha-H-1), 5.25 (t, 1H, J = 3.4 Hz, H-12), 4.86 (d, 1H, J = 1.3 Hz, Rha-H-1), 4.45 (d, 1H, J = 7.7 Hz, H-1′), 4.06 (q, 2H, J = 7.0 Hz, O$CH_2$$CH_3$), 3.97–4.01 (m, 2H), 3.90–3.95 (m, 1H), 3.85 (dd, 1H, J = 3.2, 1.8 Hz, Rha-H-2), 3.79–3.83 (m, 2H), 3.76 (dd, 1H, J = 9.5, 3.4 Hz, Rha-H-3), 3.68 (dd, 1H, J = 12.0, 4.1 Hz, H-6′-1), 3.64 (dd, 1H, J = 9.4, 3.2 Hz, Rha-H-3), 3.59 (t, 1H, J = 8.7 Hz), 3.55 (t, 1H, J = 8.9 Hz), 3.46 (t, 1H, J = 8.1 Hz), 3.39–3.44 (m, 2H), 3.19 (dd, 1H, J = 11.8, 4.2 Hz, H-3), 2.24 (d, 1H, J = 11.2 Hz, H-18), 1.28 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.26 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.13, 0.98, 0.97, 0.88, 0.74 (each s, each 3H, CH_3), 1.23 (d, 3H, J = 6.4 Hz, CH_3), 0.91 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 177.9 (C-28), 138.0 (C-13), 125.7 (C-12), 104.1 (C-1′), 101.6 (Rha-C-1), 100.5 (Rha-C-1), 88.8, 79.1, 77.8, 76.8, 75.0, 72.5, 72.3, 71.0, 70.7, 70.6, 69.4, 68.6, 60.6, 60.5, 55.9, 53.0, 41.8, 39.5, 39.0, 38.8, 36.5, 36.4, 32.9, 30.3, 27.7, 27.1, 25.8, 23.9, 23.0, 22.7, 20.1, 17.9, 16.7, 16.6, 16.5, 16.2, 15.8, 14.8, 13.1; HRESIMS calcd for $C_{50}H_{82}O_{16}Na$ 961.5495; found 961.5496.

4.12.2. 3β-O-[2,4-Di-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-12-en-28-oic acid n-propyl ester (9)

Similarly, **9** was prepared as a white solid in 92% yield; R_f 0.49 (3:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.38 (brs, 1H, Rha-H-1), 5.28 (t, 1H, J = 3.4 Hz, H-12), 4.85 (brs, 1H, Rha-H-1), 4.45 (d, 1H, J = 7.9 Hz, H-1′), 3.98–4.00 (m, 2H), 3.96 (t, 2H, J = 6.4 Hz, O$CH_2$$CH_2$), 3.90–3.93 (m, 1H), 3.85 (dd, 1H, J = 3.2, 1.8 Hz, Rha-H-2), 3.82 (d, 1H, J = 11.0 Hz, H-6-1′), 3.76 (dd, 1H, J = 9.5, 3.3 Hz, Rha-H-3), 3.67 (dd, 1H, J = 12.0, 3.9 Hz, H-6-2′), 3.63 (dd, 1H, J = 9.4, 3.1 Hz, Rha-H-3), 3.60 (t, 1H, J = 8.6 Hz), 3.55 (t, 1H, J = 8.9 Hz), 3.44 (t, 1H, J = 8.5 Hz), 3.38–3.43 (m, 2H), 3.19 (dd, 1H, J = 11.6, 4.0 Hz, H-3), 2.25 (d, 1H, J = 11.2 Hz, H-18), 1.28 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.23 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.13, 1.07, 0.97, 0.88, 0.79 (each s, each 3H, CH_3), 0.98 (d, 3H, J = 6.2 Hz, CH_3), 0.96 (t, 3H, J = 6.2 Hz, $CH_2$$CH_3$), 0.90 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 178.1 (C-28), 138.2 (C-13), 125.7 (C-12), 104.0 (C-1′), 101.7 (Rha-C-1), 100.7 (Rha-C-1), 89.0, 79.1, 77.8, 76.8, 75.0, 72.5, 72.3, 71.0, 70.7, 70.6, 69.4, 68.6, 65.8, 60.6, 55.9, 53.0, 41.8, 39.5, 39.0, 38.8, 36.6, 36.4, 32.9, 30.3, 27.7, 21.1, 25.8, 23.9, 23.0, 22.7, 21.6, 20.1, 17.9, 16.6, 16.5 (two), 16.2, 15.8, 14.8, 9.6; HRESIMS calcd for $C_{51}H_{84}O_{16}Na$ 975.5652; found 975.5656.

4.12.3. 3β-O-[2,4-Di-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-12-en-28-oic acid n-pentyl ester (10)

Similarly, **10** was prepared as a white solid in 94% yield; R_f 0.49 (3:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.37 (d, 1H, J = 1.4 Hz, Rha-H-1), 5.24 (t, 1H, J = 3.4 Hz, H-12), 4.86 (d, 1H, J = 1.5 Hz, Rha-H-1), 4.45 (d, 1H, J = 7.7 Hz, H-1′), 3.96–4.03 (m, 4H), 3.90–3.94 (m, 1H), 3.85 (dd, 1H, J = 3.2, 1.8 Hz, Rha-H-2), 3.81 (dd, 1H, J = 12.1, 1.9 Hz, H-6′-1), 3.76 (dd, 1H, J = 9.5, 3.4 Hz, Rha-H-3), 3.67 (dd, 1H, J = 12.1, 4.1 Hz, H-6′-2), 3.63 (dd, 1H, J = 9.5, 3.3 Hz, Rha-H-3), 3.60 (t, 1H, J = 8.6 Hz), 3.55 (t, 1H, J = 8.9 Hz), 3.46 (t, 1H, J = 8.2 Hz), 3.39–3.44 (m, 2H), 3.32–3.24 (m, 1H), 3.18 (dd, 1H, J = 11.6, 4.2 Hz, H-3), 2.24 (d, 1H, J = 11.2 Hz, H-18), 2.07 (td, 1H, J = 13.6, 4.6 Hz), 1.28 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.22 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.13, 1.07, 0.96, 0.88, 0.79 (each s, each 3H, CH_3), 0.98 (d, 3H, J = 6.4 Hz, CH_3), 0.94 (d, 3H, J = 7.0 Hz, $CH_2$$CH_3$), 0.90 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 177.8 (C-28), 138.1 (C-13), 125.7 (C-12), 104.2 (C-1′), 101.8 (Rha-C-1), 100.6 (Rha-C-1), 88.9, 79.1, 77.8, 76.9, 76.8, 75.0, 72.5, 72.3, 71.0, 70.7, 70.6, 69.4, 68.6, 60.6, 55.9, 53.0, 41.9, 39.6, 39.0, 38.8, 36.6, 36.4, 33.0, 32.1, 31.9, 30.3, 27.6, 27.1, 25.8, 23.9, 23.2, 23.1, 23.0, 22.5, 20.1, 17.9, 16.7, 16.6, 16.5, 16.2, 15.8, 14.8; HRESIMS calcd for $C_{53}H_{88}O_{16}Na$ 1003.5965; found 1003.5967.

4.12.4. 3β-O-[2,4-Di-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-12-en-28-oic acid cyclopentyl ester (11)

Similarly, **11** was prepared as a white solid in 92% yield; R_f 0.49 (3:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.38 (d, 1H, J = 1.5 Hz, Rha-H-1), 5.24 (t, 1H, J = 3.4 Hz, H-12), 4.86 (d, 1H, J = 1.6 Hz, Rha-H-1), 4.45 (d, 1H, J = 7.7 Hz, H-1′), 3.96–3.99 (m, 1H), 3.98 (dd, 1H, J = 3.4, 1.8 Hz, Rha-H-2), 3.90–3.92 (m, 1H), 3.85 (dd, 1H, J = 3.2, 1.8 Hz, Rha-H-2), 3.81 (dd, 1H, J = 12.1, 1.9 Hz, H-6′-1), 3.76 (dd, 1H, J = 9.5, 3.4 Hz, Rha-H-3), 3.67 (dd, 1H, J = 12.1, 4.1 Hz, H-6′-2), 3.64 (dd, 1H, J = 9.5, 3.3 Hz, Rha-H-3), 3.60 (t, 1H, J = 8.6 Hz), 3.55 (t, 1H, J = 8.9 Hz), 3.46 (t, 1H, J = 8.1 Hz), 3.38–3.44 (m, 2H), 3.32–3.24 (m, 1H), 3.19 (dd, 1H, J = 11.9, 4.2 Hz, H-3), 2.20 (d, 1H, J = 11.2 Hz, H-18), 2.07 (td, 1H, J = 13.6, 4.6 Hz), 1.28 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.23 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.13, 1.07, 0.98, 0.88, 0.82 (each s, each 3H, CH_3), 0.97 (d, 3H, J = 6.4 Hz, CH_3), 0.90 (d, 3H, J = 7.0 Hz, $CH_2$$CH_3$), 0.90 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 178.0 (C-28), 138.3 (C-13), 125.7 (C-12), 103.9 (C-1′), 101.6 (Rha-C-1), 100.6 (Rha-C-1), 89.0, 79.1, 77.8, 76.7, 76.8, 75.0, 72.5, 72.3, 71.0, 70.7, 70.6, 69.3, 68.6, 64.2, 60.6, 55.9, 53.0, 41.8, 39.5, 39.0, 38.8 (two), 36.6, 36.4, 32.9, 30.3, 28.1, 28.0, 27.7, 27.1, 25.8, 23.9, 22.9, 22.7, 21.9, 20.1, 17.9, 16.6, 16.5 (two), 16.2, 15.8, 14.7, 13.0; HRESIMS calcd for $C_{53}H_{86}O_{16}Na$ 1001.5808; found 1001.5810.

4.13. 3β-O-[2,4-Di-O-(2,3,4-tri-O-acetyl-α-L-rhamnopyranosyl)-β-(3,6-two-O-acetyl)-D-glucopyranosyl] -12-en-28-oic acid (**42**)

Compound **7** (455 mg, 0.50 mmol) was dissolved in pyridine (10 mL), Ac_2O (0.76 mL, 8.0 mmol) and DMAP (30.5 mg, 0.25 mmol) were then added, and the mixture was vigorously stirred at r.t. for 12 h. The mixture was concentrated and dissolved in CH_2Cl_2 (100 mL), then washed with 1 M HCl (3 × 50 mL), saturated $NaHCO_3$ (3 × 50 mL) and brine (2 × 50 mL), dried over Na_2SO_4, filtered, and concentrated to afford a yellow solid, which was purified by flash chromatography [petroleumether-EtOAc (2:1)] to afford **42** as a white solid (0.58 g, 94%); R_f 0.36 (1:2, EtOAc-petroleum ether); ^{1}H NMR ($CDCl_3$): δ 5.23–5.26 (m, 3H), 5.18 (dd, 1H, J = 10.1, 3.1 Hz, Rha-H-3), 5.11 (dd, 1H, J = 3.2, 17 Hz, Rha-H-2), 5.01–5.06 (m, 4H), 4.81 (d, 1H, J = 1.2 Hz, Rha-H-1), 4.53 (d, 1H, J = 7.3 Hz, H-1′), 4.49 (d, 1H, J = 12.1 Hz, H-6′-1), 4.22–4.28 (m, 2H), 3.85–3.88 (m, 1H), 3.77 (t, 1H, J = 9.3 Hz), 3.68 (t, 1H, J = 8.3 Hz), 3.60–3.62 (m, 1H), 3.16 (dd, 1H, J = 11.5, 4.1 Hz, H-3), 2.55 (d, 1H, J = 12.4 Hz, H-18), 2.14, 2.13, 2.12, 2.11, 2.05, 2.02, 1.99, 1.98 (each s, each 3H, each CH_3CO), 1.18 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.16 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.09, 1.03, 0.92, 0.81, 0.77 (each s, each 3H, CH_3), 0.95 (d, 3H, J = 6.0 Hz, CH_3), 0.86 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$): δ 182.8 (C-28), 170.5, 170.2, 170.1 (two), 170.0 (three), 169.6, 137.8 (C-13), 125.8 (C-12), 103.6 (C-1′), 99.4 (Rha-C-1), 97.0 (Rha-C-1), 90.0, 77.9, 75.7, 75.4, 72.1, 71.1, 70.6, 69.9, 69.7, 68.6, 68.5, 67.9, 66.7, 62.1, 55.9, 52.6, 47.9, 47.6, 42.0, 39.5, 39.0, 38.8, 36.7, 32.9, 30.6, 28.0, 27.8, 26.0, 24.1, 23.6, 23.3, 21.4, 21.2, 20.9 (two), 20.8 (two), 20.7 (two), 18.2, 17.2, 17.1, 17.0, 16.9, 16.2, 15.6; HRESIMS calcd for $C_{64}H_{94}O_{24}Na$ 1269.6027; found 1269.6031.

4.14. General procedure for the preparation of **12**–**14**

To a solution of compound **42** (1.5 mmol) in 20 mL dried CH_2Cl_2 was added oxalyl chloride (1 mL). The mixture was stirred at room temperature for 36 h under argon and then concentrated to dryness under reduced pressure. Hexane (3 × 10 mL) was added to the residue, then concentrated to dryness. To a dried CH_2Cl_2 (10 mL) solution of methylamine hydrochloride, dimethylamine hydrochloride or diaethylamine (2.0 mmol) was added to the above acid chloride in the presence of triethylamine (4.0 mmol). The reaction mixture was stirred at room temperature for 5 h under argon and then concentrated. The obtained residue was dissolved in 2:1 MeOH/CH_2Cl_2 (15 mL) and then NaOMe was added until pH = 10. After stirred at r.t. for 12 h, the solution was neutralized with Dowex 50 × 8 (H^+) resin until pH = 7, filtered and concentrated. Then the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH, 6:1) to give compounds **12**–**14**.

4.14.1. N-{3β-O-[2,4-Di-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-urs-12-en-28-oyl}- methylamine (**12**)

Compound **12** was synthesized as a white solid in 92% yield for three steps; R_f 0.30 (4:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.36 (d, 1H, J = 1.2 Hz, Rha-H-1), 5.32 (t, 1H, J = 3.4 Hz, H-12), 4.84 (d, 1H, J = 1.2 Hz, Rha-H-1), 4.42 (d, 1H, J = 7.7 Hz, H-1′), 3.95–3.98 (m, 2H), 3.88–3.92 (m, 1H), 3.84 (dd, 1H, J = 3.1, 1.8 Hz, Rha-H-2), 3.79 (dd, 1H, J = 12.1, 1.7 Hz, H-6-2′), 3.75 (dd, 1H, J = 9.5, 3.4 Hz, Rha-H-3), 3.62–3.67 (m, 2H), 3.58 (t, 1H, J = 8.7 Hz), 3.53 (t, 1H, J = 9.3 Hz), 3.43 (t, 1H, J = 8.5 Hz), 3.36–3.40 (m, 2H), 3.30–3.32 (m, 1H), 3.16 (dd, 1H, J = 11.5, 4.1 Hz, H-3), 2.64 (s, 3H, $NHCH_3$), 2.09 (d, 1H, J = 11.0 Hz, H-18), 1.27 (d, 3H, J = 6.0 Hz, CH_3), 1.26 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.20 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.10, 1.04, 0.96, 0.95, 0.84, 0.76 (each s, each 3H, each CH_3), 0.89 (d, 3H, J = 6.2 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 179.6 (C-28), 138.6 (C-13), 125.8 (C-12), 104.0 (C-1′), 101.5 (Rha-C-1), 100.6 (Rha-C-1), 89.1, 78.8, 78.0, 76.6, 75.0, 72.5, 72.3, 71.0, 70.7, 70.6, 69.2, 68.7, 63.8, 60.6, 55.8, 52.7, 41.8, 39.4 (two), 38.8, 38.7, 37.1, 36.4, 32.6, 30.5, 29.3, 27.5, 27.2, 25.8, 23.9, 23.0, 22.8, 20.2, 17.9, 16.6, 16.5, 16.4, 16.1, 15.8, 14.7, 14.0; HRESIMS calcd for $C_{49}H_{81}O_{15}NNa$ 946.5498; found 946.5505.

4.14.2. N-{3β-O-[2,4-Di-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-urs-12-en-28-oyl}- dimethylamine (**13**)

Similarly, **13** was prepared as a white solid in 94% yield for three steps; R_f 0.45 (3:1, $CHCl_3$–MeOH); ^{1}H NMR (DMSO-d6): δ 5.18 (s, 1H, Rha-H-1), 5.05 (brs, 1H, H-12), 4.67 (s, 1H, Rha-H-1), 4.26 (d, 1H, J = 7.7 Hz, H-1′), 3.77–3.81 (m, 3H), 3.60 (dd, 1H, J = 3.1, 1.7 Hz, Rha-H-2), 3.56 (d, 1H, J = 11.1 Hz, H-6-2′), 3.47 (dd, 1H, J = 9.5, 3.1 Hz, Rha-H-3), 3.40–3.42 (m, 2H), 3.36–3.37 (m, 2H), 3.27 (t, 1H, J = 7.7 Hz), 3.18 (t, 1H, J = 9.3 Hz), 3.17 (t, 1H, J = 9.4 Hz), 3.13–3.15 (m, 1H), 3.02 (dd, 1H, J = 10.8, 2.9 Hz, H-3), 2.87 (s, 6H, 2 × CH_3), 2.36 (d, 1H, J = 10.5 Hz, H-18), 1.09 (d, 3H, J = 6.1 Hz, Rha-H-6), 1.05 (d, 3H, J = 6.1 Hz, Rha-H-6), 1.00, 0.92, 0.83, 0.72, 0.62 (each s, each 3H, CH_3), 0.88 (d, 3H, J = 6.0 Hz, CH_3), 0.80 (d, 3H, J = 6.0 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 176.1 (C-28), 128.8 (C-13), 128.6 (C-12), 104.1 (C-1′), 101.7 (Rha-C-1), 100.6 (Rha-C-1), 89.0, 79.1, 77.8, 76.8, 75.1, 72.5, 72.3, 71.0, 70.7, 70.6, 69.4, 68.6, 60.6, 56.0, 48.7, 47.5, 39.3, 38.9 (two), 38.0, 36.4, 33.6, 32.8, 30.2, 27.8, 27.1, 25.8, 23.0, 20.2 17.9, 16.6, 16.5, 16.1, 15.8, 14.8; HRESIMS calcd for $C_{50}H_{83}O_{15}NNa$ 960.5655; found 960.5664.

4.14.3. N-{3β-O-[2,4-Di-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-urs-12-en-28-oyl}- diethylamine (**14**)

Similarly, **14** was prepared as a white solid in 93% yield for three steps; R_f 0.46 (3:1, $CHCl_3$–MeOH); ^{1}H NMR (CD_3OD): δ 5.37 (d, 1H, J = 1.3 Hz, Rha-H-1), 5.21 (t, 1H, J = 3.1 Hz, H-12), 4.86 (d, 1H, J = 1.2 Hz, Rha-H-1), 4.44 (d, 1H, J = 7.8 Hz, H-1′), 3.98–4.01 (m, 2H), 3.91–3.93 (m, 1H), 3.85 (dd, 1H, J = 3.1, 1.8 Hz, Rha-H-2), 3.81 (dd, 1H, J = 12.0, 1.7 Hz, H-6-1′), 3.76 (dd, 1H, J = 9.6, 3.4 Hz, Rha-H-3), 3.67 (dd, 1H, J = 12.1, 4.1 Hz, H-6-2′), 3.64 (dd, 1H, J = 9.4, 3.3 Hz, Rha-H-3), 3.60 (t, 1H, J = 8.7 Hz), 3.55 (t, 1H, J = 9.3 Hz), 3.46 (t, 1H, J = 8.5 Hz), 3.36–3.44 (m, 3H), 3.29–3.31 (m, 4H, NCH_2), 3.18 (dd, 1H, J = 11.6, 4.1 Hz, H-3), 2.41 (d, 1H, J = 11.0 Hz, H-18), 1.28 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.22 (d, 3H, J = 6.2 Hz, Rha-H-6), 1.13, 1.06, 0.97, 0.87, 0.81 (each s, each 3H, CH_3), 0.98 (d, 3H, J = 6.4 Hz, CH_3), 0.91 (d, 3H, J = 6.4 Hz, CH_3); ^{13}C NMR (CD_3OD): δ 176.1 (C-28), 128.9 (C-13), 128.6 (C-12), 104.1 (C-1′), 101.7 (Rha-C-1), 100.6 (Rha-C-1), 89.0, 79.1, 77.9, 76.7, 75.0, 72.5, 72.3, 71.0, 70.7, 70.6, 69.4, 68.6, 60.6, 56.0, 52.7, 41.8, 39.4, 38.8 (two), 36.5, 32.6, 30.3, 29.3, 27.5, 27.1, 25.8, 23.0, 22.8, 20.3, 17.9, 16.6 (two), 16.5, 16.4, 16.1, 15.8, 14.8; HRESIMS calcd for $C_{52}H_{87}O_{15}NNa$ 988.5968; found 988.5976.

4.15. Measurement of the inhibitory activity against H5N1pseudovirus

MDCK cells and 293T cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing glutamine, supplemented with 10% fetal calf serum (FCS). The H5N1 pseudoviruses were prepared by transfecting HA plasmid from the H5 subtype strain A/Thailand/Kan353/2004 (H5N1) strain and the NA plasmid from the N1 subtype strain A/Thailand/Kan353/2004. Briefly, 293T cells (70–80% confluent) were co-transfected with 1 μg HA plasmid, 1 μg NA plasmid and 3 μg HIV backbone plasmid (pNL4-3.luc.R_E_) into six-well plate with polyethylenimine (PEI) [18]. Forty-eight hours after transfection, the culture supernatants were harvested and centrifuged at 2000 g for 10 min. Aliquots were stored at −80 °C. For measuring the inhibitory activities of test compounds, MDCK cells (10^4/well) were seeded in 96-well plates and grown overnight. Tested compounds at indicated concentrations were incubated with pseudotyped particles for 30 min at 37 °C. Subsequently, the virus-compound mixture was transferred

to the cells and incubated for an additional 48 h. Cells were washed with phosphate buffer saline (PBS) and lysed with luciferase cell culture lysis reagent (Promega, Madison, WI). Aliquots of cell lysates were transferred to 96-well flat bottom luminometer plates (Costar), followed by the addition of luciferase assay substrate (Promega). The luciferase activity was measured in a microplate luminometer (Genios Pro, Tecan, US). As a negative control, VSV-G pseudotyped particles were incubated with the tested compound instead of H5N1 pseudovirus.

4.16. Neuraminidase activity assay

The neuraminidase activity was measured by a fluorescence based assay using a Neuraminidase Inhibitors Screen Kit by following the manufacturer's instruction (Beyotime Institute of Biotechnology, China). Briefly, 10 μL of purified N1-typed neuraminidase was added to 70 μL of detection buffer, followed by adding 10 μL of a test compound and 10 μL of neuraminidase substrate sequentially. After incubation at 37 °C for 30 min, the fluorescence intensity was measured at an excitation wavelength of 340 nm and an emission wavelength of 535 nm using a microplate reader (Genios Pro, Tecan, US).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.02.029.

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