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RESEARCH ARTICLE

Design, synthesis and biological activity evaluation of novel conjugated sialic acid and pentacyclic triterpene derivatives as anti-influenza entry inhibitors

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Influenza virus is a major human pathogen that causes annual epidemics and occasional pandemics. Recently, plant-derived pentacyclic triterpenes have been shown to act as highly potent anti-viral agents by efficiently preventing the attachment of the virion to the host cells. In this report, we conjugated sialic acid with oleanolic acid (OA), a natural product with broad antiviral entry activity, as well as three other analogs echinocystic acid (EA), ursolic acid (UA) and betulinic acid (BA). A total of 24 conjugated sialic acid and pentacyclic triterpene derivatives with different linkers were synthesized and evaluated for antiviral activity against influenza A/WSN/33 (H1N1) virus in MDCK cell culture. The most potent compound had an IC_{50} of 41.2 μM. Time-of-addition, hemagglutination inhibition (HI), surface plasmon resonance (SPR) and molecular docking assays demonstrated that compound 20a acted as an influenza virus entry inhibitor by preventing the binding of influenza virus hemagglutinin (HA) protein to host cells.

1. Introduction

Influenza A virus, a member of the Orthomyxoviridae family, possesses a segmented, single-stranded RNA-genome with negative orientation. It is a major human pathogen that causes annual epidemics and occasional pandemics, such as the Spanish influenza in 1918, the Asian influenza in 1957, the Hong Kong influenza in 1968, and the Mexico influenza (swine influenza) in 2009.[1] Besides seasonal outbreaks, recently sporadic human infections with highly pathogenic H5N1[2] and H7N9[3] avian influenza viruses have raised concerns about the pandemic potential of these viruses. It was estimated that influenza epidemics cause 250,000 to 500,000 deaths every year worldwide.[4] Although anti-influenza vaccines exist, there are several factors that prevent the eradication through vaccination. One of the major factors is the rapid mutation in the two surface antigens (hemagglutinin and neuraminidase),[5] which results in the failure of vaccines prepared from existing viruses. Currently, two classes of anti-influenza drugs, M2 ion channel inhibitors (amantadine and rimantadine)[6] and neuraminidase inhibitors (oseltamivir, zanamivir[7], and peramivir[8]), have been approved by the FDA for the treatment of influenza virus infection. However, a major problem with both classes of drugs that target viral proteins is the rapid emergence of drug-resistant viral strains which have limited their use.[9] Indeed, most seasonal human H3N2 and H1N1 viruses are now resistant to amantadine/rimantadine.[10,11] In addition, a significant proportion of the seasonal H1N1 viruses, including the pandemic 2008–2009 H1N1 virus,[12] is resistant to the neuraminidase inhibitor oseltamivir[13]. Recently, zanamivir-resistant clinical isolates of influenza viruses with D151, R371 and R152 neuraminidases residue mutations have been also arised.[14-16] A comparison of the IC_{50} of A/Montana/8/2007 that had the change D151V/D with that of its sensitive counterpart, A/Brazil/80/2007, revealed a 150-fold increase in IC_{50}. Four other extreme outliers, A/Argentina/135/2005, A/Oman/6943/2005, A/Canada/270/2007 and B/Hong Kong/36/2005, had the D151V, D151A, D151A/D or R371K change in the NA, respectively. Therefore, there is an urgent need for development of novel antiviral drugs with a different mode of action by targeting a critical step in the viral life cycle.

The cell entry of influenza virus is mediated by attachment through viral hemgglutinin (HA), which binds with sialic acid (SA) on the receptors in the host membrane and thus leads to internalization of viral particles into endosome.[17] Sialic acid (also called N-acetylneuraminic acid, Neu5Ac), a negatively charged nine-carbon carboxylated monosaccharide, is present at the non-reducing terminal positions of carbohydrate chains of glycoproteins and glycolipids on the cell surface. It is well known that sialic acid is involved in many molecular recognition processes, such as viral infection, tumor metastasis, and immune response.[18] Oseltamivir, the most widely used anti-influenza drug to date, is a sialic acid analog. In addition, conjugation of sialic acid with other bioactive

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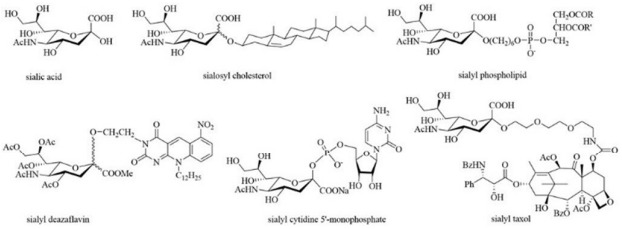


Figure 1. The structures of sialic acid and its conjugates

compounds, such as cholesterol[19], phospholipid[20], taxol[21], deazaflavin[22], and cytidine 5'-monophosphate[23] has been used as a strategy to prepare new biological molecules (figure 1).

Dipsacus asperoides is a perennial herb widely distributed in China, Korea, and Japan. A large number of studies on plants of genus *Dipsacus* have revealed cytoprotective properties, inhibition of HIV-1 reverse transcriptase, anti-hepatitis B, antinociceptive, and antimicrobial effects.[24] Recently, we found that two pentacyclic triterpenes **Y3** and **Q8** (Figure 2),[25] two oleanolic acid-galactose conjugates which were synthesized from the major aglycone separated from *Dipsacus asperoides*, showed broad anti-influenza entry activity with EC_{50} at μM level. The molecular basis of their activity is potentially due to their high affinity to the viral envelope hemgglutinin protein, thus leading to the blockage of hemgglutinin-sialic acid receptor interaction and the attachment of influenza viruses to host cells. As the sugar substituent at C-28 of triterpenes showed an important effect to their anti-influenza activity and sialic acid, a nine-carbon sugar, is involved in many molecular recognition processes,[18-23] we would like to know whether the introduction of sialic acid will promote the anti-influenza entry activity of pentacyclic triterpenes? To continue our studies towards the development of novel antiviral inhibitors,[25-32] we report here a novel class of conjugated sialic acid and pentacyclic triterpene derivatives as potent anti-influenza entry inhibitors via an efficient synthetic route with high yields. The pentacyclic triterpenes used to conjugate with sialic acid included betulinic acid (BA, **1a**), oleanolic acid (OA, **1b**), echinocystic acid (EA, **1c**), and ursolic acid (UA, **1d**).

2. Results and discussion

2.1. Chemistry

The target pentacyclic triterpene-sialic acid conjugates were synthesized as described in Scheme 1-3. Scheme 1 depicts the synthesis of conjugates **9a–d**. Firstly, alkynyl-functionalized pentacyclic triterpene derivatives **3a–d** were prepared according to

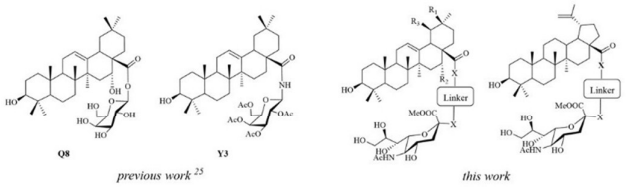
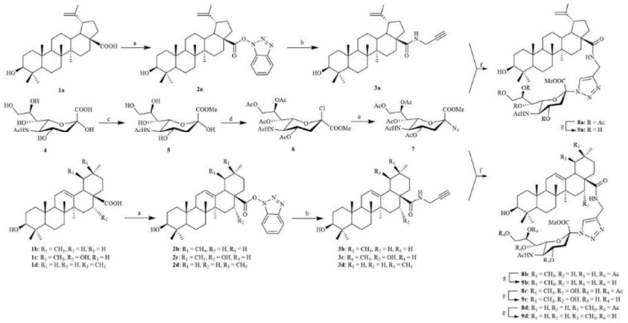


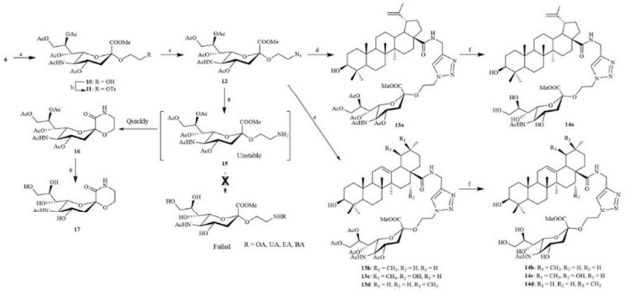
Figure 2. The structure of galactose and sialic acid functionalized pentacyclic triterpene conjugates.



Scheme 1. Synthesis of conjugates **9a–d**: (a) TBTU, DIPEA, THF; (b) propargylamine, K_2CO_3, DMF, 1 h; (c) MeOH, H^+-exchange resin, RT, 40 h; (d) MeOH, AcCl, AcOH, 24 h; (e) NaN_3, TAB-HS, $NaHCO_3$, DCM, H_2O; (f) DCM/H_2O (1:1, v/v), $CuSO_4$, sodium L-ascorbate; (g) MeONa/MeOH, RT, 3 h.

published procedures.[27,30] Azido-functionalized derivative of sialic acid **7** was synthesized from commercially available sialic acid **4** in 55% yield through a three-step process, involving esterification of C-1 carboxylic acid with methanol in the presence of an acidic ion exchange resin,[33] full acetylation of the hydroxy groups with acetyl chloride, and nucleophilic substitution of glycosyl chloride with sodium azide. It is well understood that the doublet-doublet peaks of H_{eq}(3) of an α-glycoside is located in lower magnetic field than those of the β-glycoside in the ^1H NMR spectra of sialic acid derivatives.[19,34,35] Generally the chemical shift of H_{eq}(3) of an α-glycoside is larger than 2.5 ppm while that of a β-glycoside is smaller than 2.5 ppm, which could be used to identify the configuration of sialic acid derivatives.[35] The chemical shifts and coupling constants of H_{eq}(3) of compound **7** is 2.58 (*J* = 13.0, 4.8 Hz), which indicated that it is an α-glycoside. Subsequent conjugation of **3a** with **7**[33,36] via click reaction, followed by deacetylation of the O-acetates under Zemplén conditions,[37] provided the desired conjugate **9a** in 79% yield in two steps. In a similar way, conjugation of **3b–d** with **7**, followed by deacetylation afforded conjugates **9b–d** (Scheme 1).

To increase the length of the linker between pentacyclic triterpene and sialic acid, ethanediol was introduced at C-2 of sialic acid to afford 2'-hydroxyethyl derivative of **6**, which was transformed into



Scheme 2. Synthesis of conjugates **14a–d**: (a) I_2, Ag_2CO_3, Mg_2SO_4, ethanediol, RT, 24 h; (b) p-TsCl, TEA, DMAP, DCM; (c) NaN_3, DMF; (d) DCM/H_2O (1:1, v/v), $CuSO_4$, sodium L-ascorbate, **3a**; (e) DCM/H_2O (1:1, v/v), $CuSO_4$, sodium L-ascorbate, **3b**, **3c** or **3d**; (f) MeONa/MeOH, 1 h; (g) H_2, Pd/C, MeOH.

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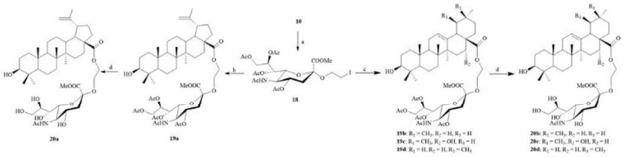

Scheme 3. Synthesis of conjugates 20a–d: (a) I_2, PPh_3, DMF, 60 °C, 24 h; (b) K_2CO_3, DMF, 1a, 60°C, 24 h; (c) K_2CO_3, DMF, 1b, 1c or 1d, 60°C, 24 h; (d) MeONa/MeOH, 1 h.

the corresponding azide 12. Subsequent conjugation with alkynyl-functionalized pentacyclic triterpene derivatives 3a–d in a similar manner as described in Scheme 2 afforded compounds 14a–d in yields ranging from 33% to 40%, as calculated from 6.

In order to replace the rigid triazole linker with amide, reduction of compound 12 by hydrogenolysis with 10% Pd/C or Ph_3P furnished aminoester intermediate 15, which was transformed into the more stable lactam-type six-member ring by-product 16 in high yield (Scheme 2). Alternatively, the hydroxyl group of 10 was easily converted into iodide 18, which reacted with 1a–d in the presence of K_2CO_3 in DMF to afford esters 19a–d in good yields (79-86%). Deacetylation gave the final conjugates 20a–d in quantitative yield (Scheme 3).

2.2. Biological assays

2.2.1 Cell viability test

To explore the effects of sialic acid-conjugated pentacyclic triterpene derivatives as anti-influenza agents, we first determined their cytotoxicity in MDCK cells by the CellTiter-Glo assay. As shown in Figure 3, most compounds showed no apparent cytotoxicity against uninfected MDCK cells at the concentration of 100 μM, except UA and its sialic acid conjugates 8d, 9d, 13d, 14d, 19d and 20d, which possessed certain degree of cytotoxicity at 100 μM. BA showed moderate cytotoxicity to MDCK cells. However, the cytotoxicity of BA dramatically decreased upon conjugation with sialic acid.

2.2.2 Inhibition of influenza virus infectivity

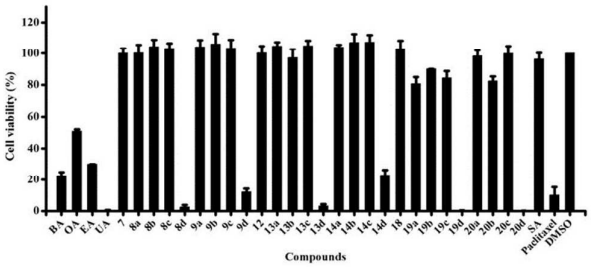

Figure 3. The cytotoxicity screening of sialic acid and pentacyclic triterpene conjugates (100 μM) using CellTiter-Glo® Assay. DMSO and paclitaxel were used as negative and positive control, respectively. Error bars indicate standard deviations of triplicate experiments.

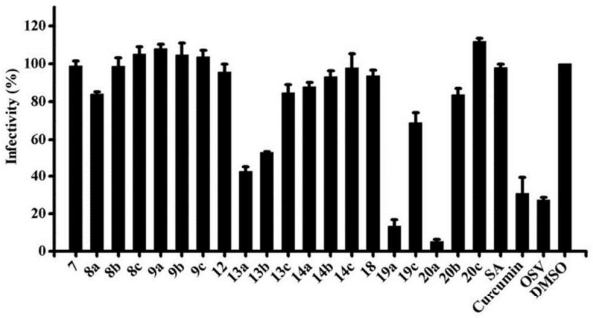

Figure 4. The cytopathic effect-based screening of conjugated sialic acid and pentacyclic triterpene derivatives. MDCK was utilized as the host cell to test A/WSN/33 virus infection; 0.5% DMSO (final concentration) was used as the negative; curcumin, a small-molecule entry inhibitor targeting the HA1 domain,[38-40] as well as OSV (oseltamivir) were utilized as positive controls. Error bars indicate standard deviations of triplicate experiments.

Table 1. The inhibitory effects of sialic acid-pentacyclic triterpene conjugates against influenza A/WSN/33 virus based on cell viability experiment [a]

Compound	Inhibition rate (%)	Cell vialibity (%)
OA	ND [b]	60.9 ± 1.2
EA	ND	40.1 ± 0.1
UA	ND	0.4 ± 0.1
BA	ND	22.8 ± 2.3
SA	2.0 ± 1.9	96.4 ±3.9
BA + SA	ND	20.3 ± 3.1
7	1.0 ± 2.5	100.0 ± 3.4
8a	16.0 ± 1.2	100.2 ± 5.4
8b	1.3 ± 4.5	104.4 ± 4.5
8c	-5.2 ± 3.9	103.3 ± 3.2
8d	ND	2.5 ± 1.2
9a	-8.1 ± 2.3	104.3 ± 4.3
9b	-4.6 ± 6.4	106.2 ± 6.4
9c	-3.6 ± 3.5	103.5 ± 5.3
9d	ND	12.2 ± 2.1
12	4.3 ± 4.1	100.3 ± 4.5
13a	67.6 ± 2.4	104.9 ± 2.4
13b	57.8 ± 1.2	97.1 ± 5.6
13c	15.3 ± 4.3	105.0 ± 3.2
13d	ND	3.3 ± 1.1
14a	12.2 ± 2.3	104.1 ± 1.4
14b	6.7 ± 3.1	107.1 ± 5.4
14c	2.2 ± 3.5	107.4 ± 4.6
14d	ND	20.1 ± 3.2
18	6.3 ± 3.1	103.1 ± 5.2
19a	86.6 ± 3.4	80.7 ± 4.3
19b	18.7 ± 3.8	90.1 ± 0.1
19c	31.2 ± 5.4	84.5 ± 4.3
19d	ND	0.2 ± 0.1
20a	94.8 ± 1.2	98.3 ± 3.7
20b	16.4 ± 3.5	82.4 ± 2.9
20c	-11.8 ± 1.8	99.8 ± 5.1
20d	ND	0.1 ± 0.1
Curcumin [c]	69.3 ± 8.6	98.7 ± 6.6
OSV [d]	72.6 ± 1.3	98.5 ±5.8

[a] Measured at the concentration of 100 μM.
[b] Not detected.
[c] Measured at the concentration of 10 μM.
[d] Measured at the concentration of 50 μM.

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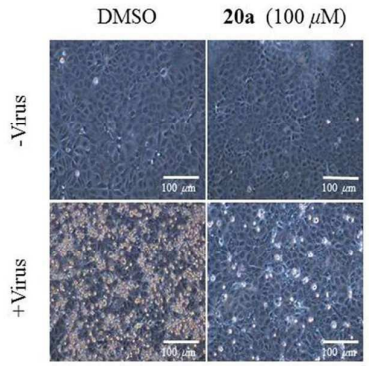

Figure 5. Inhibition of influenza A/WSN/33 virus plaque formation by compound 20a.

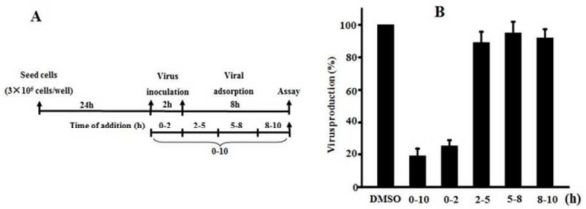

Figure 6. Stage of the viral life cycle targeted by 20a. (A) Time-of-addition experiment was designed to identify at which step life cycle 20a targeted. (B) 20a was added at the indicated time points into MDCK cells preliminarily inoculated with A/WSN/33 viruses at a multiplicity of infection (MOI) of 1 and cell supernatants were collected 11 h post-infection. The viral titer of collected supernatants was estimated by the plaque formation assay.

Except sialic acid-UA conjugates, the other conjugated sialic acid and pentacyclic triterpene derivatives (8a-8c, 9a-9c, 13a-13c, 14a-14c, 19a-19c and 20a-20c) were evaluated against the influenza A/WSN/33 (H1N1) virus that was propagated in MDCK cells by the cytopathic effect (CPE) reduction assay. Curcumin, a small-molecule entry inhibitor targeting the HA1 domain,[38-40] as well as OSV (oseltamivir) were utilized as positive controls. The screening results are shown in Figure 4 and the results are mentioned in Table 1. Compounds 13a, 13b, 19a and 20a significantly reduced the viral CPE, but the other compounds had little or no activity, indicating that BA is the best scaffold among the three pentacyclic triterpenes for the discovery of potent anti-influenza inhibitors, which also agreed well with our recently published result.[32] The anti-influenza activities of the BA derivatives increased in the order of 20a ≈ 19a > 13a >> 8a ≈ 9a, suggesting that the flexibility of the linker between sialic acid and pentacyclic triterpene played an important role in the activity.

Antiviral activity of compound 20a, the most potent compound found in the initial screening, against influenza A/WSN/33 virus was further confirmed by direct microscopic observation. Far less CPE was observed when treated with 20a than DMSO the negative control (Figure 5).

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

Compound	IC_{50} (μM)[a]	CC_{50} (μM)[b]	SI[c]
13a	78.3 ± 5.1	> 500	> 6.3
13b	95.2 ± 4.6	> 500	> 5.2
19a	61.3 ± 7.3	178.0 ± 16.6	2.9
20a	41.2 ± 2.9	> 500	> 12.1
Curcumin	6.7 ± 1.2	48.3 ± 5.7	7.2
OSV	46.5 ± 3.3	> 500	> 10.8

[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] Selecivity index, defined by CC_{50}/IC_{50}.

Four compounds 13a, 13b, 19a and 20a identified with high inhibition rates in the initial screening were selected for the dose response assays. 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. Among them, compound 20a exhibited the most potent anti-influenza virus activity with an EC_{50} of 41.2 μM (Table 2), which was similar to that of oseltamivir (OSV).

2.2.3 Inhibitor 20a acts at an early stage of the viral life cycle

To determine which step of the virus replication cycle 20a most effectively inhibits, a time-of-addition experiment was performed following the scheme illustrated in Fig 6A. We administrated compound 20a at various time points (0, 2, 4, 6, 8 h) post infection. As shown in Fig 6B, 20a inhibited viral production by up to approximately >75% when added to infected cells at an early time point (up to 2 h post inflection). By contrast, 20a had no inhibitory effects on viral production when added 2 h post-infection or later. The results indicate that the inhibitor 20a only prevents viral attachment or fusion with cellular membranes and inhibits a step during virus entry into the host cell.

To examine whether compound 20a inhibited NA enzymatic activity, untreated or compound-treated influenza A/WSN/33 virus was tested for enzymatic activity by 4-methylumbelliferyl-α-D-N-acetylneuraminic acid sodium salt hydrate solution (MUNANA). Untreated and compound-treated influenza A/WSN/33 virus had similar enzymatic activity, suggesting that the test compound had no effect on NA activity (SI Figure 1).

2.2.4 Inhibition of HA protein by compound 20a

The entry of influenza virus into host cells is mediated by attachment through HA binding with sialic acid on the membrane receptors and subsequent internalization of viral particles into the late endosome.[14] It is conceivable that those conjugated sialic acid and pentacyclic triterpene derivatives could also bind with HA protein, thus inhibiting the interaction of HA with sialic acid on the membrane

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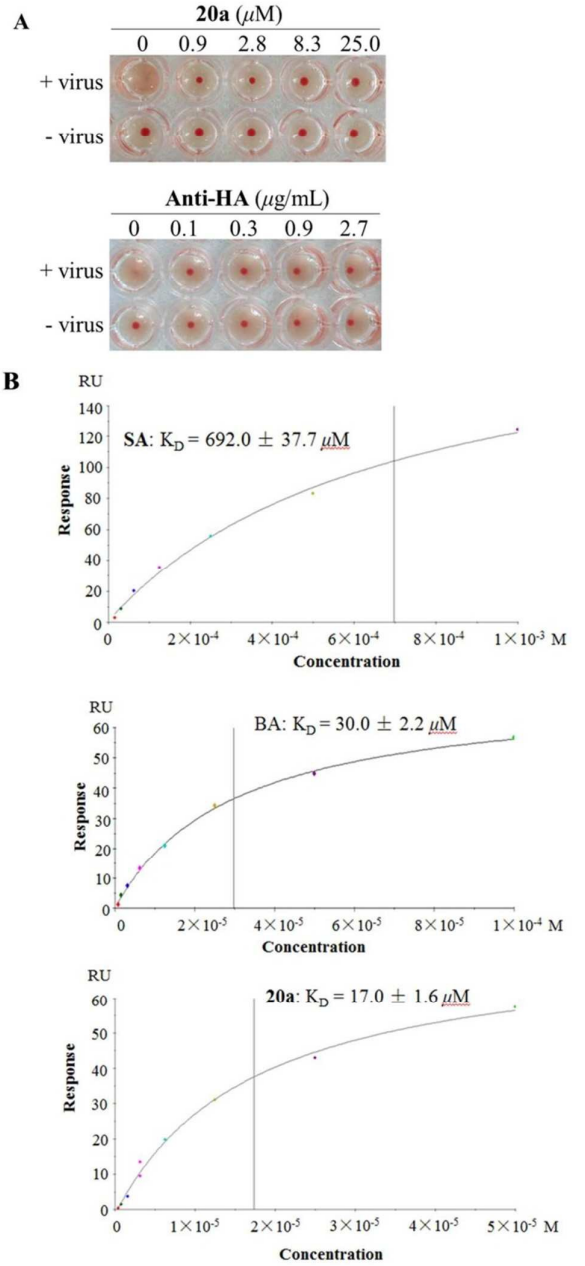

Figure 7. (A) Comparisons of the behaviors of 20a vs anti-HA antibody in inhibition of influenza virus-induced aggregation of chicken erythrocytes. 20a exerted identical capability as anti-HA antibody in inhibition of hemagglutination in a dose dependent manner. (B) Determination of the affinity of sialic acid, BA and their conjugate 20a with HA protein, which was immobilized on a CM5 sensor chip, based on the SPR assay. Their K_D values were labeled on the corresponding curves.

receptors. Therefore, a hemagglutination inhibition (HI) assay,[25] a common assay used to measure influenza-specific antibody levels in blood serum, was conducted to investigate whether the HA protein

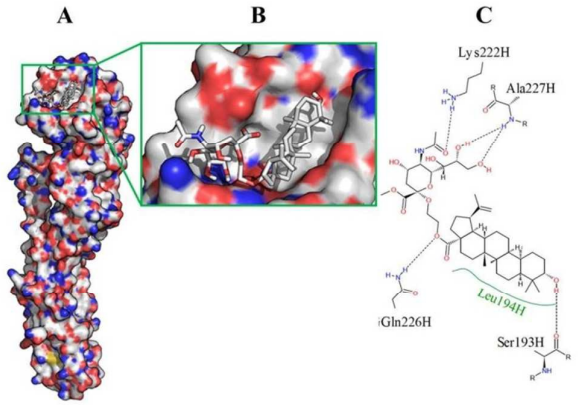

Figure 8. Docking structure of 20a binding with a single chain of HA protein (Protein Data Bank: 1RVT) according to docking calculation. (A) Overview of the protein surface. The inhibitor pocket is highlighted in the green square and compound 20a is shown as sticks. (B) Closer view of the inhibitor pocket. Compound 20a is shown as sticks. (C) 2D interaction plot with black dashes and green curves indicating hydrogen bonds and hydrophobic interactions, respectively.

is the potential target of compound 20a. Results from HAI assays indicated that compound 20a shows the same capability as anti-HA antibody in effectively inhibiting influenza virus-induced aggregation of chicken erythrocytes, and the inhibitory effect is dose-dependent (Figure. 7A). This fact suggested that 20a shares the same target, HA, with anti-HA antibody.

2.2.5 Affinity measurements by Surface Plasmon Resonance (SPR)

After identified that the HA protein is the target of compound 20a, we then characterized the binding affinity of the conjugated sialic acid and pentacyclic triterpene derivatives with HA protein bya SPR-based assay (Biacore T200) as reported earlier.[25,41,42] As shown in Figure7B, the binding curves are fitted well with the Langmuir equation for monovalent binding, which allows the determination of the apparent dissociation constant, K_D. The calculated K_D values for sialic acid and BA binding with HA protein were 692 and 30 μM, respectively, which indicated that BA bound more tightly to HA than SA. In contrast, a higher binding affinity was observed between compound 20a and HA with a calculated K_D value of 17 μM, almost twice as potent as that of BA, suggesting that sialic acid enhanced the binding of BA with HA.

2.2.6 Molecular interaction of compound 20a with HA protein

To understand the molecular basis of the inhibitory properties of sialic acid-pentacyclic triterpene conjugates further, computational analyses of the binding of compound 20a with a single chain of HA protein, which was obtained from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do), was performed using AutoDock 4.2 program. To validate our approach of docking assay, computational analyses of the binding of natural ligand Gal-2-Sia with HA protein was performed firstly. The docked conformation of Gal-2-Sia was determined based on the minimum free energy

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analyses. The computer-aided docking data indicated that the sialic acid moiety of the natural ligand could superimpose with the sialic acid moiety of the ligand bound in the X-ray crystal structure PDBid 1RVT with an estimated binding energy of -4.68 kcal/mol and an inhibition constant (Ki) = 782.56 μM (SI Figure 2). By the same method, the computer-aided docking data indicated that **20a** occupies the binding pocket for sialic acid (Figure 8) with an estimated binding energy of -8.78 kcal/mol and an inhibition constant (Ki) = 228.49 nM, which indicated **20a** could interrupt the recognition of sialic acid with HA. The 2D interaction plot (Figure 8) suggested that the 3-OH of the BA moiety forms a hydrogen bond with Ser193 and the A-ring of BA forms hydrophobic interaction with Leu194. For the sialic acid moiety, the carbonyl oxygen atom of N-acetyl and the 8, 9-OH forms hydrogen bonds with Lys222 and Ala227, respectively. Besides, the oxygen atom of the linker between sialic acid and BA also forms a hydrogen bond with Gln226. Leu194 and Gln226 are critical for sialic acid binding via hydrogen bonds. Most of the remaining interactions are within the 190 helix (residues 190-198) and the 220 loop (residues 221-228), which are important for the recognition of sialic acid. These docking data are consistent with the results from SAR studies, in particular supporting the requirement for the flexibility of the linker between sialic acid and BA.

3. Materials and methods

3.1 Chemistry

All melting points are uncorrected and measured using Electrothermal 4-X apparatus (FuKai, Beijing, China). IR spectra were determined on a Thermo Nicolet Nexus 470 FT-IR spectrometer. The measurements were performed in the range of 4000-500 cm^{-1} at room temperature. 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. ^1H 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 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 F_{254} 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 $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (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 synthesis of compounds **2a-d**, **3a-d**, **4-7** and **10** have been reported previously.[27,30,33,43] The intermediates **11**, **12**, **16-17** and **18** and the sialic acid-pentacyclic triterpene conjugates **8a-d**, **9a-d**, **13a-d**, **14a-d**, **19a-d** and **20a-d** were obtained as follows.

3.1.1 General procedure A for the click 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 deacetylation reaction:

The per-O-acetylated SA-triterpene conjugate was dissolved in dry 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 N-[1-(1-methyl-4,7,8,9-tetra-O-acetyl-N-acetylneuraminyl)-1H-1,2,3-triazol -4-yl] methyl 3β-hydroxy-lup-20(29)-en-28-amide (8a)

Prepared from **3a** and **7** according to general procedure A, and the residue was purified by chromatography (eluent: PE/Act = 1:1) over silica gel to afford compound **8a** as a white solid in 80% yield. R_f = 0.35 (PE/Act = 1:1); Mp: 165-167 °C; IR (KBr, ν, cm^{-1}): 3429, 2945, 1754, 1669, 1226, 1040; ^1H NMR (400 MHz, $CDCl_3$): δ 7.89 (s, 1H), 6.34 (t, 1H, J = 5.4 Hz), 5.53 (d, 1H, J = 9.8 Hz), 5.36-5.44 (m, 2H), 5.19 (td, 1H, J = 4.4, 11.8 Hz), 4.73 (d, 1H, J = 1.7 Hz), 4.58-4.64 (m, 2H), 4.45 (dd, 1H, J = 4.9, 15.5 Hz), 4.26-4.33 (m, 2H), 4.05-4.15 (m, 2H), 3.79 (s, 3H), 3.41 (dd, 1H, J = 4.5, 13.2 Hz), 3.10-3.20 (m, 2H), 2.62 (t, 1H, J = 12.5 Hz), 2.44 (td, 1H, J = 3.5, 12.7 Hz), 2.16, 2.12, 2.07, 2.06, 1.91 (s, 3H each, CH_3CO), 1.67 (s, 3H, CH_3), 0.96 (s, 6H, 2 × CH_3), 0.86, 0.82, 0.75 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.22, 170.81, 170.59, 170.28, 170.10, 170.02, 166.29, 150.88, 145.60, 120.52, 109.40, 88.48, 78.95, 73.82, 68.43, 68.21, 66.92, 62.22, 55.67, 55.32, 54.00, 50.58, 50.08, 49.22, 46.67, 42.42, 40.69, 38.81, 38.68, 38.15, 37.66, 37.15, 36.32, 34.81, 34.31, 33.50, 30.78, 29.42, 27.96, 27.37, 25.55, 23.13, 21.15, 20.88, 20.78, 20.73, 19.34, 18.27, 16.13, 16.01, 15.36, 14.59; ESI-HRMS calcd for $C_{53}H_{79}N_5NaO_{14}$ $[M+Na]^+$: 1032.5516, found 1032.5523.

3.1.4 Synthesis of N-[1-(1-methyl-4,7,8,9-tetra-O-acetyl-N-acetylneuraminyl)-1H-1,2,3-triazol -4-yl] methyl 3β-hydroxy-olean-12-en-28-amide (8b)

Prepared from **3b** and **7** according to general procedure A, and the residue was purified by chromatography (eluent: PE/Act = 1:1) over silica gel to afford compound **8b** as a white solid in 84% yield. R_f = 0.33 (PE/Act = 1:1); Mp: 170-172 °C; IR (KBr, ν, cm^{-1}): 3423, 2954, 1753, 1665, 1226, 1039; ^1H NMR (400 MHz, $CDCl_3$): δ 7.80 (s, 1H), 7.05 (s, 1H), 5.79 (d, 1H, J = 9.5 Hz), 5.59 (s, 1H), 5.37-5.45 (m, 1H), 5.31 (s, 1H), 5.14-5.20 (m, 1H), 4.71 (dd, 1H, J = 6.6, 16.0 Hz), 4.23-4.35 (m, 4H), 4.05-4.16 (m, 2H), 3.78 (s, 3H), 3.42 (dd, 1H, J = 4.4, 13.1 Hz), 3.21-3.25 (m, 1H), 2.92 (dd, 1H, J = 3.6, 13.8 Hz), 2.69 (t, 1H, J = 12.4 Hz), 2.18, 2.12, 2.08, 2.07, 1.91 (s, 3H each, CH_3CO), 1.34, 0.99, 0.95, 0.91, 0.89, 0.78, 0.69 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.86, 170.81, 170.58, 170.35, 169.98, 166.35, 145.5, 143.05, 123.48, 120.30, 88.35, 78.80, 74.83, 73.65, 68.46, 67.98, 66.77, 62.24, 55.14, 53.99, 53.40, 49.31, 49.09, 46.80, 46.52, 41.83, 41.41, 39.67, 38.70, 38.52, 36.86, 36.02, 35.44, 35.24, 34.83, 32.54, 30.05, 28.22, 28.00, 27.12, 26.94, 25.75, 23.32, 23.11, 21.23, 20.78, 20.73, 18.16, 16.84, 15.63, 15.59; ESI-HRMS calcd for $C_{53}H_{80}N_5O_{14}$ $[M+H]^+$: 1010.5696, found: 1010.5704.

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3.1.5 Synthesis of N-[1-(1-methyl-4,7,8,9-tetra-O-acetyl-N-acetylneuraminyl)-1H-1,2,3-triazol -4-yl] methyl 3β,16α-dihydroxy-olean-12-en-28-amide (8c)

Prepared from 3c and 7 according to general procedure A, and the residue was purified by chromatography (eluent: PE/Act = 1:1) over silica gel to afford compound 8c as a white solid in 81% yield. R_f = 0.30 (PE/Act = 1:1); Mp: 173-175 °C; IR (KBr, ν, cm^{-1}): 3424, 2954, 1751, 1663, 1227, 1040; ^1H NMR (400 MHz, $CDCl_3$): δ 7.84 (s, 1H), 7.08 (s, 1H), 6.15 (d, 1H, J = 9.6 Hz), 5.59 (s, 1H), 5.41 (s, 2H), 5.15-5.19 (m, 1H), 4.68-4.70 (m, 1H), 4.24-4.35 (m, 4H), 4.05-4.19 (m, 2H), 3.78 (s, 3H), 3.41-3.44 (m, 1H), 3.22-3.24 (m, 1H), 2.88-2.91 (m, 2H), 2.69 (t, 1H, J = 12.4 Hz), 2.17, 2.12, 2.08, 2.06, 1.90 (s, 3H each, CH_3CO), 1.35, 1.00, 0.95, 0.91, 0.90, 0.79, 0.69 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.87, 170.71, 170.49, 170.34, 170.24, 169.86, 166.24, 143.09, 123.38, 120.27, 88.33, 78.67, 74.71, 73.67, 68.49, 68.03, 66.73, 62.18, 55.04, 53.88, 53.35, 49.14, 48.82, 46.67, 46.48, 41.71, 41.33, 39.54, 38.59, 38.44, 36.76, 35.94, 35.24, 35.15, 34.82, 32.46, 29.99, 28.48, 27.94, 27.01, 26.78, 25.49, 23.24, 22.95, 21.11, 20.69, 20.62, 18.08, 16.71, 15.56, 15.52; ESI-HRMS calcd for $C_{53}H_{80}N_5O_{15}$ $[M+H]^+$: 1026.5645, found 1026.5643.

3.1.6 Synthesis of N-[1-(1-methyl-4,7,8,9-tetra-O-acetyl-N-acetylneuraminyl)-1H-1,2,3-triazol -4-yl] methyl 3β-hydroxy-urs-12-en-28-amide (8d)

Prepared from 3d and 7 according to general procedure A, and the residue was purified by chromatography (eluent: PE/Act = 1:1) over silica gel to afford compound 8d as a white solid in 83% yield. R_f = 0.35 (PE/Act = 1:1); Mp: 162-164 °C; IR (KBr, ν, cm^{-1}): 3423, 2929, 1752, 1662, 1227, 1040; ^1H NMR (400 MHz, $CDCl_3$): δ 7.85 (s, 1H), 6.70 (s, 1H), 5.73 (d, 1H, J = 9.4 Hz), 5.37-5.41 (m, 3H), 5.14-5.21 (m, 1H), 4.63-4.69 (m, 1H), 4.24-4.33 (m, 1H), 4.05-4.17 (m, 1H), 3.79 (s, 3H), 3.40-3.44 (m, 1H), 3.20-3.24 (m, 1H), 2.67 (t, 1H, J = 12.5 Hz), 2.16, 2.12, 2.07, 2.05, 1.91 (s, 3H each, CH_3CO), 1.10, 0.99, 0.95, 0.90, 0.78, 0.66 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.14, 170.71, 170.49, 170.27, 169.97, 169.92, 166.28, 145.11, 139.22, 126.20, 120.55, 88.41, 78.87, 73.77, 68.53, 68.05, 66.89, 62.22, 55.03, 53.95, 53.44, 49.08, 47.67, 47.49, 42.29, 39.67, 39.46, 38.87, 38.66, 38.57, 36.83, 36.11, 34.98, 32.62, 30.84, 28.07, 27.79, 27.13, 24.88, 23.31, 23.26, 23.08, 21.16, 21.11, 20.75, 20.70, 20.67, 18.20, 17.12, 16.47, 15.59, 15.43; ESI-HRMS calcd for $C_{53}H_{80}N_5O_{14}$ $[M+H]^+$: 1010.5696, found 1010.5697.

3.1.7 Synthesis of N-[1-(1-methyl-N-acetylneuraminyl)-1H-1,2,3-triazol-4-yl] methyl 3β-hydroxy- lup-20(29)-en-28-amide (9a)

Prepared from 8a according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 8:1) over silica gel to afford compound 9a as a white solid in 65% yield. R_f = 0.32 (DCM/MeOH = 5:1); Mp: 202-204 °C; IR (KBr, ν, cm^{-1}): 3429, 2942, 2870, 1751, 1642, 1042; ^1H NMR(400 MHz, CD_3OD): δ 8.10 (s, 1H), 4.67 (s, 1H), 4.54 (s, 1H), 4.31-4.40 (m, 2H), 3.96-4.02 (m, 1H), 3.91 (t, 1H, J = 10.39 Hz), 3.75-3.81 (m, 6H), 3.64 (dd, 1H, J = 5.36, 11.73 Hz), 3.53-3.55 (m, 1H), 3.27 (brs, 1H), 3.22 (dd, 1H, J = 4.69, 13.07 Hz), 3.01-3.10 (m, 2H), 2.42-2.49 (m, 1H), 2.07-2.13 (m, 1H), 1.98 (s, 3H, CH_3CO), 1.64, 0.94, 0.90, 0.83, 0.79, 0.71 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 179.02, 175.09, 168.28, 152.24, 146.69, 122.34, 110.03, 90.96, 79.67, 76.48, 71.55, 69.79, 68.06, 64.81, 56.91, 54.24, 53.49, 52.07, 51.38, 48.08, 43.44, 41.97, 41.58, 40.12, 39.93, 39.13, 38.91, 38.31, 35.56, 35.28, 33.94, 31.92, 30.5, 28.64, 28.04, 26.96, 22.71, 22.12, 19.67, 19.46, 16.88, 16.86, 16.15, 15.11; ESI-HRMS calcd for $C_{45}H_{72}N_5O_{10}$ $[M+H]^+$: 842.5274, found 842.5258.

3.1.8 Synthesis of N-[1-(1-methyl-N-acetylneuraminyl)-1H-1,2,3-triazol-4-yl] methyl 3β-hydroxy-olean-12-en-28-amide (9b)

Prepared from 8b according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 8:1) over silica gel to afford compound 9b as a white solid in 89% yield. R_f = 0.30 (DCM/MeOH = 5:1); Mp: 207-209 °C; IR (KBr, ν, cm^{-1}): 3459, 2923, 2864, 1746, 1645, 1037; ^1H NMR (400 MHz, CD_3OD): δ 8.14 (s, 1H), 7.79 (t, 1H, J = 5.5 Hz), 5.34 (brs, 1H), 4.40 (d, 2H, J = 5.0 Hz), 4.02 (td, 1H, J = 4.5, 10.3 Hz), 3.94 (t, 1H, J = 10.3 Hz), 3.79-3.84 (m, 6H), 3.68 (dd, 1H, J = 5.7, 12.0 Hz), 3.57 (d, 1H, J = 9.0 Hz), 3.27-3.31 (m, 1H, overlap with MeOH and CD_3OD), 3.13 (dd, 1H, J = 4.4, 11.5 Hz), 2.79 (d, 1H, J = 9.9 Hz), 2.15 (dd, 1H, J = 11.4, 12.6 Hz), 2.02 (s, 3H, CH_3CO), 1.14, 0.96, 0.95, 0.92, 0.91, 0.77, 0.44 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 180.34, 175.09, 168.25, 146.08, 144.94, 124.23, 122.70, 90.95, 79.68, 76.47, 71.54, 69.78, 68.09, 64.81, 56.69, 54.24, 53.46, 49.85, 47.60, 47.51, 42.80, 42.52, 41.45, 40.55, 39.81, 38.09, 35.71, 35.05, 34.15, 33.77, 33.57, 31.61, 28.76, 28.42, 27.85, 26.47, 24.52, 24.05, 22.71, 19.47, 17.45, 16.35, 16.08; ESI-HRMS calcd for $C_{45}H_{71}N_5NaO_{10}$ $[M+Na]^+$: 864.5093, found: 864.5099.

3.1.9 Synthesis of N-[1-(1-methyl-N-acetylneuraminyl)-1H-1,2,3-triazol-4-yl] methyl 3β,16α- dihydroxy-olean-12-en-28-amide (9c)

Prepared from 8c according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 7:1) over silica gel to afford compound 9c as a white solid in 85% yield. R_f = 0.27 (DCM/MeOH = 5:1); Mp: 208-210 °C; IR (KBr, ν, cm^{-1}): 3454, 2961, 2861, 1728, 1639, 1038; ^1H NMR (400 MHz, CD_3OD): δ 8.13(s, 1H), 7.58-7.61 (m, 1H), 5.47 (s, 1H), 4.36-4.38 (m, 3H), 3.98-4.02 (m, 1H), 3.93 (t, 1H, J = 10.0 Hz), 3.84 (s, 3H), 3.79-3.81 (m, 2H), 3.66-3.70 (m, 1H), 3.56-3.59 (m, 1H), 3.12-3.16 (m, 1H), 2.84-2.87 (m, 1H), 2.36 (t, 1H, J = 13.3 Hz), 2.12-2.18 (m, 1H), 2.02 (s, 3H, CH_3CO), 1.36 (s, 3H, CH_3), 0.96 (s, 6H, 2 × CH_3), 0.93, 0.88, 0.77, 0.47 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 180.07, 175.13, 168.29, 145.94, 124.35, 122.54, 121.07, 90.99, 79.71, 76.52,75.67, 71.58, 69.82, 68.13, 64.82, 56.81, 54.25, 53.49, 49.98, 48.14, 42.77, 42.31, 41.47, 40.72, 39.94, 39.84, 38.09, 36.40, 35.93, 35.86, 35.75, 33.96, 33.27, 31.99, 31.26, 28.71, 27.91, 27.24, 25.29, 24.48, 22.69, 19.46, 17.53, 16.33, 16.25; ESI-HRMS calcd for $C_{45}H_{72}N_5O_{11}$ $[M+H]^+$: 858.5223, found 858.5120.

3.1.10 Synthesis of N-[1-(1-methyl-N-acetylneuraminyl)-1H-1,2,3-triazol-4-yl] methyl 3β-hydroxy-urs-12-en-28-amide (9d)

Prepared from 8d according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 8:1) over silica gel to afford compound 9d as a white solid in 89% yield. R_f = 0.32 (DCM/MeOH = 5:1); Mp: 205-207 °C; IR (KBr, ν, cm^{-1}): 3427, 2928, 2872, 1752, 1642, 1038; ^1H NMR (400 MHz, CD_3OD): δ 8.08 (s, 1H), 7.65 (s, 1H), 5.29 (d, 1H, J = 9.6 Hz), 4.33-4.34 (m, 1H), 3.95-4.02 (m, 1H), 3.88-3.93 (m,1H), 3.80 (s, 3H), 3.75-3.77 (m, 2H), 3.62-3.66 (m, 1H), 3.53-3.55 (m, 1H), 3.23-3.27 (m, 2H), 3.10 (dd, 1H, J = 4.8, 11.3 Hz), 2.09-2.16 (m, 1H), 1.99 (s, 3H, CH_3CO), 1.06, 0.93, 0.89, 0.87, 0.85, 0.74, 0.42 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 180.22, 175.10, 168.23, 146.06, 139.61, 127.42, 122.66, 90.93, 79.66, 76.45, 71.55, 69.82, 68.09, 64.79, 56.68, 54.26, 54.11, 53.44, 48.92, 48.88, 43.19, 41.39, 40.79, 40.76, 40.26, 39.96, 39.80, 38.54, 38.04,

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35.70, 34.11, 31.87, 28.89, 28.79, 27.87, 25.26, 24.35, 24.08, 22.73, 21.59, 19.43, 17.73, 17.42, 16.41, 16.20; ESI-HRMS calcd for $C_{45}H_{72}N_5O_{10}$ $[M+H]^+$: 842.5274, found 842.5256.

3.1.11 1-methyl-2-O-(2-hydroxyethyl)-4,7,8,9-tetra-O-acetyl-N-acetylneuraminic acid (10)[43]

Ethylene glycol (25 mL, 449 mmol), Ag_2O_3 (662 mg, 2.4mmol), $MgSO_4$ (1.2 g), and an iodine crystal were stirred with the exclusion of moisture and light for 0.5 hour at room temperature before the sialyl chloride 6 (1.6 mmol) was added. The reaction mixture was stirred at room temperature for 24 hours, diluted with DCM (25 mL), filtered through Celite, and washed with water. The solvent was removed by evaporation, and the mixture was purified by chromatography (eluent: PE/Act = 1:1) over silica gel to afford the glycoside 10 as a white solid in 65% yield. R_f = 0.44 (PE/Act = 1:2); Mp: 150-152 °C; IR (KBr, ν, cm^{-1}): 3539, 3440, 1754, 1239, 1211, 1037; ^1H NMR (400 MHz, $CDCl_3$): δ 5.31 - 5.42 (m, 3H), 4.84 - 4.91 (m, 1H), 4.34 (dd, 1H, J = 2.4, 12.4 Hz), 4.17 (dd, 1H, J = 1.9, 10.7 Hz), 4.04-4.11 (m, 2H), 3.84-3.89 (m, 1H), 3.81 (s, 3H), 3.69-3.75 (m, 2H), 3.44-3.49 (m, 1H), 2.61 (dd, 1H, J = 4.6, 12.8 Hz), 2.26 (t, 1H, J = 6.1 Hz), 2.14, 2.15, 2.04, 2.05 (s, 3H each, CH_3CO), 1.99 (t, 1H, J = 12.4 Hz), 1.89 (s, 3H, CH_3CO); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.09, 170.84, 170.38, 170.26, 170.21, 168.47, 98.86, 72.73, 69.07, 68.74, 67.39, 66.75, 62.52, 61.68, 53.00, 49.46, 38.00, 23.26, 21.21, 20.95, 20.91, 20.88.

3.1.12 1-methyl-2-O-[2-(4-methylbenzenesulfonate) ethyl)]-4,7,8,9-tetra-O-acetyl-N-acetylneuraminic acid (11)

The glycoside 10 (1.0 g, 1.9 mmol), Et_3N (0.4 μL, 2.8 mmol), DMAP (68 mg, 0.56 mmol) were dissolved in DCM 30 mL. The resulting mixture was cooled to 0 °C, and a solution of TsCl (540 mg, 2.8 mmol) in 10 mL of DCM was added dropwise. The mixture was allowed to cool at room temperature and stirred overnight. The solution was washed with sat. aq. $NaHCO_3$ (2 × 30 mL) and H_2O (2 × 30 mL). The combined aqueous layers were extracted with DCM (2 × 30 mL) and the combined organic layers were dried over Na_2SO_4, then filtered and concentrated. The residue was purified by chromatography (eluent: PE/Act = 3:2) over silica gel to afford compound 11 as a white solid in 90% yield. R_f = 0.40 (PE/Act = 1:1); Mp: 81-83 °C; IR (KBr, ν, cm^{-1}): 3392, 2960, 1746, 1371, 1225, 1040; ^1H NMR (400 MHz, $CDCl_3$): δ 7.80 (d, 2H, J = 8.2 Hz), 7.36 (d, 2H, J = 8.2 Hz), 5.29-5.37 (m, 2H), 5.20 (brs, 1H), 4.81-4.87 (m, 1H), 4.27 (dd, 1H, J = 2.4, 12.5 Hz), 4.01-4.17 (m, 5H), 3.90-3.95 (m, 1H), 3.79 (s, 3H), 3.52-3.58 (m, 1H), 2.50 (dd, 1H, J = 4.6, 12.8 Hz), 2.46 (s, 3H), 2.15, 2.13 (s, 3H each, CH_3CO), 2.04 (s, 6H, 2 × CH_3CO), 1.88 (s, 3H, CH_3CO), 1.74 (t, 1H, J = 12.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.04, 170.76, 170.38, 170.21, 170.18, 168.05, 144.91, 133.12, 129.93, 128.21, 98.71, 72.59, 69.31, 69.03, 68.31, 67.27, 62.74, 62.47, 53.05, 49.46, 37.86, 23.30, 21.74, 21.25, 20.97, 20.95, 20.88; ESI-HRMS calcd for $C_{29}H_{39}NNaO_{16}S$ $[M+Na]^+$: 712.1882, found: 712.1879.

3.1.13 1-methyl-2-O-(2-azidoethyl)-4,7,8,9-tetra-O-acetyl-N-acetylneuraminic acid (12)

NaN_3 (60 mg, 0.87 mmol), and compound 11 (200 mg, 0.29 mmol) were dissolved in DMF (10 mL). The resulting mixture was heated to 80 °C and stirred overnight. The solution was then concentrated and purified by chromatography (eluent: PE/Act = 2:1) over silica gel to afford compound 12 as a white solid in 82% yield. R_f = 0.42 (PE/Act = 1:1); Mp: 163-165 °C; IR (KBr, ν, cm^{-1}): 3266, 2960, 2104, 1751, 1209, 1037; ^1H NMR (400 MHz, $CDCl_3$): δ 5.39-5.43 (m, 1H), 5.31-5.35 (m, 2H), 4.86-4.92 (m, 1H), 4.31 (dd, 1H, J = 2.6, 12.4 Hz), 4.04-4.15 (m, 3H), 3.96-4.01 (m, 1H), 3.82 (s, 3H), 3.47-3.52 (m, 1H), 3.36-3.42 (m, 1H), 3.28-3.33 (m, 1H), 2.63 (dd, 1H, J = 4.6, 12.8 Hz), 2.15, 2.15, 2.04, 2.03 (s, 3H each, CH_3CO), 1.98 (t, 1H, J = 12.6 Hz), 1.89 (s, 3H, CH_3CO); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.05, 170.74, 170.31, 170.24, 170.18, 168.24, 98.78, 72.64, 69.02, 68.44, 67.30, 64.21, 62.52, 52.94, 50.60, 49.44, 38.03, 23.26, 21.21, 20.92, 20.89, 20.84; ESI-HRMS calcd for $C_{22}H_{33}N_4O_{13}$ $[M+H]^+$: 561.2039, found: 561.2040.

3.1.14 Synthesis of N-{1-[2-O-(1-methyl-4,7,8,9-tetra-O-acetyl-N-acetylneuraminyl) ethyl]-1H- 1,2,3-triazol-4-yl} methyl 3β-hydroxy-lup-20(29)-en-28-amide (13a)

Prepared from 3a and 12 according to general procedure A, and the residue was purified by chromatography (eluent: PE/Act = 1:1) over silica gel to afford compound 13a as a white solid in 94% yield. R_f = 0.38 (PE/Act = 2:3); Mp: 150-152 °C; IR (KBr, ν, cm^{-1}): 3428, 2946, 2870, 1749, 1226, 1044; ^1H NMR (400 MHz, $CDCl_3$): δ 7.64 (s, 1H), 6.41 (t, 1H, J = 5.5 Hz), 5.46 (d, 1H, J = 9.6 Hz), 5.32-5.36 (m, 1H), 5.27-5.29 (m, 1H, overlap with DCM), 4.79-4.86 (m, 1H), 4.70 (d, 1H, J = 1.6 Hz), 4.40-4.55 (m, 5H), 4.26 (dd, 1H, J = 2.5, 12.4 Hz), 3.99-4.13 (m, 4H), 3.63-3.68 (m, 4H), 3.06-3.16 (m, 2H), 2.52 (dd, 1H, J = 4.7, 12.8 Hz), 2.36 (td, 1H, J = 3.3, 12.7 Hz), 2.12, 2.10, 2.01, 2.00, 1.85 (s, 3H each, CH_3CO), 1.64, 0.93, 0.92 (s, 3H each, CH_3), 0.77 (s, 6H, 2 × CH_3), 0.72 (s, 3H, CH_3), 0.63 (d, 1H, J = 9.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.40, 171.00, 170.76, 170.37, 170.21, 170.17, 167.98, 150.98, 144.99, 123.25, 109.50, 98.85, 79.01, 72.72, 68.86, 68.20, 67.24, 63.47, 62.59, 55.72, 55.43, 53.02, 50.66, 50.30, 50.18, 49.30, 46.88, 42.53, 40.81, 38.93, 38.79, 38.29, 37.93, 37.84, 37.27, 34.81, 34.40, 33.62, 30.97, 29.49, 28.09, 27.48, 25.68, 23.24, 21.23, 20.96, 20.92, 20.87, 19.51, 18.38, 16.24, 15.93, 15.52, 14.72; ESI-HRMS calcd for $C_{55}H_{84}N_5O_{15}$ $[M+H]^+$: 1054.5958, found 1054.5967.

3.1.15 Synthesis of N-{1-[2-O-(1-methyl-4,7,8,9-tetra-O-acetyl-N-acetylneuraminyl) ethyl]-1H- 1,2,3-triazol-4-yl} methyl 3β-hydroxy-olean-12-en-28-amide (13b)

Prepared from 3b and 12 according to general procedure A, and the residue was purified by chromatography (eluent: PE/Act = 1:1) over silica gel to afford compound 13b as a white solid in 98% yield. R_f = 0.37 (PE/Act = 2:3); Mp: 153-155 °C; IR (KBr, ν, cm^{-1}): 3424, 2951, 2867, 1749, 1225, 1045; ^1H NMR (400 MHz, $CDCl_3$): δ 7.61 (s, 1H), 6.62 (t, 1H, J = 5.2 Hz), 5.55 (d, 1H, J = 9.8 Hz), 5.31-5.36 (m, 2H), 5.26-5.29 (m, 1H, overlap with DCM), 4.77-4.83 (m, 1H), 4.39-4.54 (m, 3H), 4.30 (dd, 1H, J = 5.1, 15.0 Hz), 4.25 (dd, 1H, J = 2.5, 12.4 Hz), 3.97-4.11 (m, 4H), 3.59-3.63 (m, 4H), 3.17 (t, 1H, J = 5.1 Hz), 2.46-2.52 (m, 2H), 2.11, 2.09, 2.00, 1.98, 1.83 (s, 3H each, CH_3CO), 1.11, 0.94, 0.85, 0.84, 0.83, 0.74 (s, 3H each, CH_3), 0.67 (d, 1H, J = 10.8 Hz), 0.53 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.30, 170.91, 170.71, 170.36, 170.18, 170.12, 167.94, 144.42, 144.31, 123.51, 123.33, 98.87, 78.95, 72.71, 68.82, 68.16, 67.23, 63.53, 62.59, 55.16, 52.95, 50.22, 49.28, 47.60, 46.67, 46.28, 42.14, 41.99, 39.39, 38.80, 38.53, 37.92, 36.99, 35.02, 34.13, 33.04, 32.51, 32.41, 30.76, 28.17, 27.34, 27.22, 25.83, 23.87, 23.63, 23.53, 23.20, 21.19, 20.93, 20.90, 20.83, 18.33, 16.57, 15.69, 15.43; ESI-HRMS calcd for $C_{55}H_{84}N_5O_{15}$ $[M+H]^+$: 1054.5958, found 1054.5959.

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3.1.16 Synthesis of N-{1-[2-O-(1-methyl-4,7,8,9-tetra-O-acetyl-N-acetylneuraminyl) ethyl]-1H- 1,2,3-triazol-4-yl} methyl 3β,16α-dihydroxy-olean-12-en-28-amide (13c)

Prepared from 3c and 12 according to general procedure A, and the residue was purified by chromatography (eluent: PE/Act = 1:1) over silica gel to afford compound 13c as a white solid in 95% yield. R_f = 0.38 (PE/Act = 1:2); Mp: 156-158 °C; IR (KBr, ν, cm^{-1}): 3410, 2950, 2863, 1749, 1226, 1040; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.62 (s, 1H), 6.92 (t, 1H, J = 5.0 Hz), 5.54-5.56 (m, 2H), 5.31-5.37 (m, 2H), 4.84 (td, 1H, J = 4.6, 12.2), 4.27-4.58 (m, 6H), 4.02-4.15 (m, 4H), 3.64-3.68 (m, 4H), 3.20-3.22 (m, 1H), 2.74 (d, 1H, J = 10.6 Hz), 2.53 (dd, 1H, J = 4.6, 12.8 Hz), 2.15, 2.13, 2.04, 2.03, 1.87 (s, 3H each, CH_3CO), 1.36, 0.99, 0.91 (s, 3H each, CH_3), 0.90 (s, 6H, 2 × CH_3), 0.78, 0.61 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.91, 171.00, 170.76, 170.43, 170.22, 167.99, 144.30, 143.57, 123.74, 123.38, 98.90, 78.97, 75.50, 72.77, 68.92, 68.25, 67.27, 63.55, 62.61, 55.29, 53.02, 50.25, 49.33, 49.04, 46.98, 46.81, 41.87, 41.71, 39.67, 38.85, 38.66, 37.98, 37.03, 35.43, 35.30, 32.69, 30.37, 29.87, 28.18, 27.30, 26.92, 25.09, 23.48, 23.25, 21.23, 20.95, 20.87, 18.33, 16.82, 15.72; ESI-HRMS calcd for $C_{55}H_{83}N_5NaO_{16}$ $[M+Na]^+$: 1092.5727, found 1092.5736.

3.1.17 Synthesis of N-{1-[2-O-(1-methyl-4,7,8,9-tetra-O-acetyl-N-acetylneuraminyl) ethyl]-1H- 1,2,3-triazol-4-yl} methyl 3β-hydroxy-urs-12-en-28-amide (13d)

Prepared from 3d and 12 according to general procedure A, and the residue was purified by chromatography (eluent: PE/Act = 1:1) over silica gel to afford compound 13d as a white solid in 92% yield. R_f = 0.38 (PE/Act = 2:3); Mp: 149-151 °C; IR (KBr, ν, cm^{-1}): 3431, 2951, 2872, 1750, 1226, 1038; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.59 (s, 1H), 6.61 (t, 1H, J = 5.1 Hz), 5.56 (d, 1H, J = 9.7 Hz), 5.26-5.35 (m, 3H), 4.77-4.84 (m, 1H), 4.38-4.54 (m, 3H), 4.32 (dd, 1H, J = 5.0, 15.0 Hz), 4.25 (dd, 1H, J = 2.5, 12.4 Hz), 3.97-4.11 (m, 4H), 3.59-3.67 (m, 4H), 3.17 (t, 1H, J = 5.3 Hz), 2.49 (dd, 1H, J = 4.6, 12.8 Hz), 2.11, 2.09, 2.00, 1.98, 1.83 (s, 3H each, CH_3CO), 1.04, 0.94, 0.89, 0.84 (s, 3H each, CH_3), 0.82 (d, 3H, CH_3, J = 6.4 Hz), 0.74 (s, 3H, CH_3), 0.67 (d, 1H, J = 11.5 Hz), 0.55 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.16, 170.91, 170.70, 170.36, 170.18, 170.13, 167.94, 144.36, 139.15, 126.29, 123.31, 98.87, 78.98, 72.72, 68.85, 68.18, 67.26, 63.51, 62.60, 55.20, 53.72, 52.94, 50.22, 49.29, 47.71, 47.61, 42.41, 39.78, 39.59, 39.10, 38.80, 38.69, 37.94, 37.12, 36.98, 34.99, 32.80, 30.91, 28.21, 27.90, 27.27, 24.97, 23.36, 23.19, 21.28, 21.18, 20.93, 20.89, 20.83, 18.32, 17.26, 16.61, 15.73, 15.56; ESI-HRMS calcd for $C_{55}H_{83}N_5NaO_{15}$ $[M+Na]^+$: 1076.5778, found 1076.5784.

3.1.18 Synthesis of N-{1-[2-O-(1-methyl-N-acetylneuraminyl) ethyl]-1H-1,2,3-triazol-4-yl} methyl 3β-hydroxy-lup-20(29)-en-28-amide (14a)

Prepared from 13a according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound 14a as a white solid in 75% yield. R_f = 0.52 (DCM/MeOH = 4:1); Mp: 179-181 °C; IR (KBr, ν, cm^{-1}): 3433, 2942, 1742, 1640, 1545, 1043; ^{1}H NMR (400 MHz, CD_3OD): δ 7.83 (s, 1H), 4.71 (brs, 1H), 4.52-4.58 (m, 3H), 4.41 (s, 2H), 4.16-4.19 (m, 1H), 3.75-3.91 (m, 7H), 3.57-3.68 (m, 3H), 3.50 (d, 1H, J = 9.0 Hz), 3.08-3.14 (m, 2H), 2.64 (dd, 1H, J = 4.6, 12.8 Hz), 2.50 (t, 1H, J = 10.1 Hz), 2.15 (d, 1H, J = 13.5 Hz), 2.00 (s, 3H, CH_3CO), 1.81-1.87 (m, 2H), 1.69, 0.99, 0.95, 0.86, 0.84, 0.76 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ178.98, 175.15, 170.44, 152.24, 146.74, 125.04, 110.02, 100.22, 79.62, 75.04, 72.17, 70.09, 68.35, 64.78, 63.90, 56.91, 56.88, 53.69, 53.50, 52.07, 51.51, 51.41, 48.06, 43.47, 41.99, 41.47, 40.10, 39.94, 39.21, 38.95, 38.32, 35.59, 35.46, 34.00, 31.97, 30.55, 28.65, 28.03, 26.98, 22.68, 22.14, 19.72, 19.45, 16.83, 16.68, 16.15, 15.13; ESI-HRMS calcd for $C_{47}H_{75}N_5NaO_{11}$ $[M+Na]^+$: 908.5355, found 908.5354.

3.1.19 Synthesis of N-{1-[2-O-(1-methyl-N-acetylneuraminyl) ethyl]-1H-1,2,3-triazol-4-yl} methyl 3β-hydroxy-olean-12-en-28-amide (14b)

Prepared from 13b according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound 14b as a white solid in 80% yield. R_f = 0.50 (DCM/MeOH = 4:1); Mp: 175-177 °C; IR (KBr, ν, cm^{-1}): 3445, 2945, 1743, 1646, 1550, 1036; ^{1}H NMR (400 MHz, CD_3OD): δ 7.84 (s, 1H), 7.77 (brs, 1H), 5.35 (brs, 1H), 4.54 (brs, 2H), 4.40 (d, 2H, J = 4.9 Hz), 4.16-4.19 (m, 1H), 3.76-3.90 (m, 7H), 3.57-3.67 (m, 3H), 3.49 (d, 1H, J = 9.0 Hz), 3.14 (dd, 1H, J = 3.8, 11.1 Hz), 2.80 (d, 1H, J = 10.4 Hz), 2.62 (dd, 1H, J = 4.3, 12.7 Hz), 2.00 (s, 3H, CH_3CO), 1.15, 0.97, 0.95 (s, 3H each, CH_3), 0.91 (s, 6H, 2 × CH_3), 0.77, 0.53 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 180.28, 175.15, 170.43, 146.08, 145.03, 125.46, 124.17, 100.25, 79.62, 75.03, 72.17, 70.04, 68.36, 64.78, 63.88, 56.66, 53.70, 53.50, 51.51, 47.60, 47.47, 42.82, 42.56, 41.42, 40.57, 39.82, 38.09, 35.87, 34.99, 34.19, 33.76, 33.58, 31.64, 28.75, 28.46, 27.83, 26.49, 24.51, 24.06, 22.68, 19.45, 17.47, 16.35, 15.95; ESI-HRMS calcd for $C_{47}H_{76}N_5O_{11}$ $[M+H]^+$: 886.5536; found 886.5534.

3.1.20 Synthesis of N-{1-[2-O-(1-methyl-N-acetylneuraminyl) ethyl]-1H-1,2,3-triazol-4-yl} methyl 3β,16α-dihydroxy-olean-12-en-28-amide (14c)

Prepared from 13c according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound 14c as a white solid in 82% yield. R_f = 0.49 (DCM/MeOH = 4:1); Mp: 181-183 °C; IR (KBr, ν, cm^{-1}): 3444, 2947, 1741, 1649, 1527, 1035; ^{1}H NMR (400 MHz, CD_3OD): δ 7.83 (s, 1H), 5.47 (brs, 1H), 4.53-4.54 (m, 2H), 4.37 (s, 3H), 4.15-4.19 (m, 1H), 3.75-3.91 (m, 7H), 3.57-3.68 (m, 3H), 3.50 (d, 1H, J = 8.9 Hz), 3.15 (dd, 1H, J = 4.9, 10.9 Hz), 2.87 (d, 1H, J = 10.4 Hz), 2.62 (d, 1H, J = 4.7, 12.8 Hz), 2.37 (t, 1H, J = 13.1 Hz), 2.00 (s, 3H, CH_3CO), 1.37 (s, 3H, CH_3), 0.97 (s, 6H, 2 × CH_3), 0.92, 0.89, 0.77, 0.56 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 179.97, 175.14, 170.43, 145.77, 144.94, 125.31, 124.23, 100.26, 79.64, 75.61, 75.05, 72.18, 70.09, 68.38, 64.81, 63.86, 56.77, 53.72, 53.48, 51.53, 49.91, 48.12, 48.05, 42.77, 42.31, 41.41, 40.72, 39.92, 39.83, 38.08, 36.34, 35.96, 35.88, 33.94, 33.29, 32.01, 31.25, 28.73, 27.88, 27.29, 25.34, 24.47, 22.69, 19.43, 17.56, 16.34, 16.15; ESI-HRMS calcd for $C_{47}H_{75}N_5NaO_{12}$ $[M+Na]^+$: 924.5304, found 924.5301.

3.1.21 Synthesis of N-{1-[2-O-(1-methyl-N-acetylneuraminyl) ethyl]-1H-1,2,3-triazol-4-yl} methyl 3β-hydroxy-urs-12-en-28-amide (14d)

Prepared from 13d according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound 14d as a white solid in 85% yield. R_f = 0.52 (DCM/MeOH = 4:1); Mp: 176-178 °C; IR (KBr, ν, cm^{-1}): 3443, 2929, 1742, 1641, 1551, 1042; ^{1}H NMR (400 MHz, CD_3OD): δ 7.81 (s, 1H), 7.62 (t, 1H, J = 5.6 Hz), 5.33 (brs, 1H), 4.50-4.57 (m, 2H), 4.33-

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4.42 (m, 2H), 4.14-4.19 (m, 1H), 3.74-3.91 (m, 7H), 3.61-3.68 (m, 2H), 3.58 (dd, 1H, J = 1.4, 10.4 Hz), 3.49 (dd, 1H, J = 1.3, 9.0 Hz), 3.14 (dd, 1H, J = 4.8, 11.1 Hz), 2.62 (dd, 1H, J = 4.7, 12.8 Hz), 1.99 (s, 3H, CH_3CO), 1.10 (s, 3H, CH_3), 0.96 (s, 6H, 2 × CH_3), 0.92 (s, 3H, CH_3), 0.90 (d, 3H, CH_3, J = 6.4 Hz), 0.77, 0.55 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 180.16, 175.15, 170.45, 146.03, 139.74, 127.35, 125.36, 79.65, 75.07, 72.21, 70.11, 68.37, 64.82, 63.85, 56.68, 54.22, 53.73, 53.48, 51.52, 48.94, 48.86, 43.24, 41.40, 40.82, 40.27, 39.96, 39.82, 38.59, 38.07, 35.81, 34.12, 31.85, 28.93, 28.77, 27.88, 25.30, 24.35, 24.07, 22.68, 21.58, 19.42, 17.72, 17.52, 16.40, 16.08; ESI-HRMS calcd for $C_{47}H_{75}N_5NaO_{11}$ $[M+Na]^+$: 908.5355, found 908.5364.

3.1.22 5-N-acetyl-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosy- loyl-1''→2'-lactam (16)

Compound 12 (250 mg, 0.45 mmol) was dissolved in dry MeOH (20 mL) and 10% palladium-carbon (40 mg) was added. The reaction mixture was shaken under hydrogen gas (0.35 MPa) overnight. After removal of catalyst by filtration with aid of celite, the filtrate was concentrated and purified by chromatography (eluent: PE/Act = 1:1) over silica gel to afford compound 16 as a white solid in 79% yield. R_f = 0.30 (PE/Act = 1:2); Mp: 249-251 °C; IR (KBr, ν, cm^{-1}): 3372, 3247, 1738, 1669, 1370, 1224; ^{1}H NMR (400 MHz, CD_3OD): δ 5.57-5.64 (m, 1H), 5.29-5.36 (m, 2H), 4.36 (dd, 1H, J = 1.6, 10.6 Hz), 4.21-4.29 (m, 2H), 3.98-4.04 (m, 2H), 3.78 (dd, 1H, J = 4.5, 11.8 Hz), 3.49 (td, 1H, J = 4.7, 12.2 Hz), 3.14 (dd, 1H, J = 3.0, 12.4 Hz), 2.33, (dd, 1H, J = 5.6, 13.2 Hz), 2.10, 2.05, 2.00, 1.97, 1.85 (s, 3H each, CH_3CO), 1.66 (t, 1H, J = 11.5 Hz); ^{13}C NMR (100 MHz, CD_3OD): δ 173.56, 172.36, 172.03, 171.72, 171.14, 168.56, 97.09, 73.11, 72.16, 69.20, 68.55, 63.61, 59.17, 50.21, 41.91, 39.21, 22.67, 20.92, 20.86, 20.80, 20.62; ESI-HRMS calcd for $C_{21}H_{31}N_2O_{12}$ [M+H]+: 503.1872; found 503.1867.

3.1.23 5-N-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyloyl-1''→2'-lactam (17)

Prepared from 16 according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 4:1) over silica gel to afford compound 17 as a white solid in 83% yield. R_f = 0.35 (DCM/MeOH = 3:2); Mp: 256-258 °C; IR (KBr, ν, cm^{-1}): 3358, 3282, 1650, 1559, 1104, 1032; ^{1}H NMR (400 MHz, D_2O + CD_3OD): δ 4.40-4.47 (m, 1H), 4.27 (td, 1H, J = 3.8, 11.4 Hz), 4.04, (dd, 1H, J = 1.0, 10.6 Hz), 3.73-3.90 (m, 4H), 3.61-3.66 (m, 1H), 3.47-3.56 (m, 2H), 3.29-3.34 (m, 1H, overlap with CD_3OD), 2.39 (dd, 1H, J = 5.6, 13.4 Hz), 2.06 (s, 3H, CH_3CO), 1.63 (dd, 1H, J = 10.7, 13.5 Hz); ^{13}C NMR (100 MHz, D_2O + CD_3OD): δ 175.87, 169.54, 97.24, 74.15, 71.24, 69.73, 68.62, 64.72, 59.71, 53.48, 41.48, 41.37, 22.88; ESI-HRMS calcd for $C_{13}H_{22}N_2O_8$ $[M+H]^+$: 335.1449; found 335.1453.

3.1.24 1-methyl-2-O-(2-iodoethyl)-4,7,8,9-tetra-O-acetyl-N-acetylneuraminic acid (18)

Compound 10 (1.2 g, 2.2 mmol) was dissolved in DMF (20 mL), then I_2 (2.3 g, 9.0 mmol) and Ph_3P (2.4 g, 9.0 mmol) were added. The reaction mixture was stirred at 65 °C overnight. After removal of DMF under vacuum, the residue was purified by chromatography (eluent: PE/Act = 2:1) over silica gel to afford compound 18 as a white solid in 60% yield. R_f = 0.36 (PE/Act = 1:1); Mp: 138-140 °C; IR (KBr, ν, cm^{-1}): 3284, 1750, 1653, 1210, 1040, 603; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.25-5.40 (m, 3H), 4.85-4.91 (m, 1H), 4.30 (dd, 1H, J = 2.5, 12.5 Hz), 4.01-4.11 (m, 4H), 3.82 (s, 3H), 3.53-3.59 (m, 1H), 3.20-3.29 (m, 2H), 2.63 (dd, 1H, J = 4.6, 12.8 Hz), 2.15 (s, 6H, 2 × CH_3CO), 2.05, 2.04 (s, 3H each, CH_3CO), 1.98 (t, 1H, J = 12.6 Hz), 1.89 (s, 3H, CH_3CO); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.94, 170.61, 170.19, 170.07, 170.00, 168.02, 98.60, 72.57, 68.93, 68.44, 67.19, 65.57, 62.35, 52.84, 49.30, 37.82, 23.15, 21.09, 20.80, 20.76, 2.55; ESI-HRMS calcd for $C_{22}H_{33}INO_{13}$ $[M+H]^+$: 646.0991; found 646.0995.

3.1.25 O-[2-O-(1-methyl-4,7,8,9-tetra-O-acetyl-N-acetylneuraminyl)] ethyl 3β-hydroxy-lup-20 (29)-en-28-oate (19a)

To a solution of 1a (192 mg, 0.42 mmol) and 18 (226 mg, 0.35 mmol) in DMF (15 mL), K_2CO_3 (97 mg, 0.70 mmol) was added. The resulting solution 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 19a as a white solid in 81% yield. R_f = 0.50 (PE/Act = 1:1); Mp: 135-137 °C; IR (KBr, ν, cm^{-1}): 3376, 2949, 1750, 1371, 1226, 1040; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.32-5.42 (m, 3H), 4.81-4.88 (m, 1H), 4.74 (dd, 1H, J = 1.4 Hz), 4.60 (s, 1H), 4.27-4.31 (m, 2H), 3.97-4.17 (m, 5H), 3.80 (s, 3H), 3.44-3.49 (m, 1H), 3.18 (t, 1H, J = 5.6 Hz), 2.98-3.04 (m, 1H), 2.59 (dd, 1H, J = 4.6, 12.7 Hz), 2.15, 2.14, 2.05, 2.03, 1.88 (s, 3H each, CH_3CO), 1.69, 0.96, 0.95, 0.91, 0.82, 0.75 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.78, 170.91, 170.59, 170.21, 170.07, 169.99, 168.07, 150.56, 109.55, 98.59, 78.91, 72.40, 69.04, 68.27, 67.15, 62.89, 62.36, 62.28, 56.47, 55.28, 52.74, 50.46, 49.38, 49.28, 46.92, 42.34, 40.65, 38.79, 38.65, 38.13, 38.09, 37.14, 36.89, 34.26, 31.98, 30.52, 29.60, 27.96, 27.37, 25.43, 23.15, 21.10, 20.80, 20.73, 19.31, 18.26, 16.05, 15.97, 15.36, 14.66; ESI-HRMS calcd for $C_{52}H_{79}NNaO_{16}$ $[M+Na]^+$: 996.5291, found 996.5288.

3.1.26 O-[2-O-(1-methyl-4,7,8,9-tetra-O-acetyl-N-acetylneuraminyl)] ethyl 3β-hydroxy-olean- 12-en-28-oate (19b)

To a solution of 1b (178 mg, 0.39 mmol) and 18 (210 mg, 0.33 mmol) in DMF (15 mL), K_2CO_3 (90 mg, 0.65 mmol) was added. The resulting solution 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 19b as a white solid in 85% yield. R_f = 0.48 (PE/Act = 1:1); Mp: 139-141 °C; IR (KBr, ν, cm^{-1}): 3375, 2948, 1750, 1370, 1228, 1040; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.30-5.45 (m, 4H), 4.82-4.88 (m, 1H), 4.21-4.31 (m, 2H), 3.96-4.11 (m, 5H), 3.80 (s, 3H), 3.43 (m, 1H), 3.19-3.22 (m, 1H), 2.87 (dd, 1H, J = 3.8, 13.7 Hz), 2.59 (dd, 1H, J = 4.6, 12.8 Hz), 2.15, 2.14, 2.04, 2.03, 1.88 (s, 3H each, CH_3CO), 1.13, 0.98, 0.93, 0.91, 0.90, 0.78, 0.73 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.46, 170.88, 170.57, 170.24, 170.05, 169.99, 168.10, 143.65, 122.32, 98.55, 78.92, 72.39, 69.06, 68.36, 67.16, 62.91, 62.72, 62.23, 55.15, 52.72, 49.24, 47.58, 46.55, 45.79, 41.54, 41.18, 39.27, 38.66, 38.37, 38.03, 36.97, 33.82, 33.06, 32.60, 32.27, 30.64, 28.06, 27.60, 27.12, 25.86, 23.55, 23.36, 23.11, 22.82, 21.08, 20.80, 20.72, 18.23, 16.78, 15.58, 15.26; ESI-HRMS calcd for $C_{52}H_{79}NNaO_{16}$ $[M+Na]^+$: 996.5291, found 996.5293.

3.1.27 O-[2-O-(1-methyl-4,7,8,9-tetra-O-acetyl-N-acetylneuraminyl)] ethyl 3β,16α-dihydroxy- olean-12-en-28-oate (19c)

To a solution of 1c (190 mg, 0.40 mmol) and 18 (216 mg, 0.34 mmol) in DMF (15 mL), K_2CO_3 (93 mg, 0.67 mmol) was added. The resulting solution 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 19c as a

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white solid in 84% yield. R_f = 0.46 (PE/Act = 1:1); Mp: 143-145 °C; IR (KBr, ν, cm^{-1}): 3382, 2950, 1750, 1371, 1229, 1039; ^1H NMR (400 MHz, $CDCl_3$): δ 5.37-5.42 (m, 3H), 5.32 (d, 1H, J = 9.6 Hz), 4.82-4.89 (m, 1H), 4.46 (brs, 1H), 4.31 (dd, 1H, J = 2.5, 12.4 Hz), 4.04-4.20 (m, 2H), 3.95-3.99 (m, 1H), 3.81 (s, 3H), 3.42-3.47 (m, 1H), 3.22 (dd, 1H, J = 4.2, 11.2 Hz), 3.07 (dd, 1H, J = 3.9, 14.4 Hz), 2.57 (dd, 1H, J = 4.6, 12.8 Hz), 2.15 (s, 6H, 2 × CH_3CO), 2.04, 2.03, 1.88 (s, 3H each, CH_3CO), 1.34, 0.99, 0.97, 0.92, 0.91, 0.78, 0.75 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.63, 170.92, 170.70, 170.25, 170.12, 170.07, 168.06, 142.53, 122.74, 98.50, 78.89, 74.36, 72.40, 68.98, 68.29, 67.15, 63.18, 62.53, 62.40, 55.24, 52.80, 49.25, 49.04, 46.78, 46.21, 41.32, 40.68, 39.57, 38.69, 38.47, 37.87, 36.98, 35.37, 35.22, 32.79, 32.72, 30.26, 29.62, 28.05, 27.15, 27.00, 25.03, 23.29, 23.13, 21.10, 20.81, 20.73, 18.23, 17.04, 15.60, 15.49; ESI-HRMS calcd for $C_{52}H_{79}NNaO_{17}$ $[M+Na]^+$: 1012.5240, found 1012.5241.

3.1.28 O-[2-O-(1-methyl-4,7,8,9-tetra-O-acetyl-N-acetylneuraminyl)] ethyl 3β-hydroxy-urs-12- en-28-oate (19d)

To a solution of 1d (68 mg, 0.15 mmol) and 18 (80 mg, 0.12 mmol) in DMF (10 mL), K_2CO_3 (34 mg, 0.25 mmol) was added. The resulting solution 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 19d as a white solid in 87% yield. R_f = 0.49 (PE/Act = 1:1); Mp: 136-138 °C; IR (KBr, ν, cm^{-1}): 3390, 2948, 1750, 1371, 1227, 1041; ^1H NMR (400 MHz, $CDCl_3$): δ 5.25-5.41 (m, 4H), 4.82-4.87 (m, 1H), 4.29 (dd, 1H, J = 2.5, 12.4 Hz), 3.94-4.20 (m, 6H), 3.80 (s, 3H), 3.43-3.48 (m, 1H), 3.21 (dd, 1H, J = 4.6, 10.8 Hz), 2.60 (dd, 1H, J = 4.6, 12.8 Hz), 2.23 (d, 1H, J = 11.2 Hz), 2.15, 2.14, 2.04, 2.03, 1.89 (s, 3H each, CH_3CO), 1.08, 0.98, 0.95, 0.92 (s, 3H each, CH_3), 0.86 (d, 3H, CH_3, J = 6.4 Hz), 0.78, 0.75 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.29, 170.91, 170.58, 170.20, 170.06, 169.96, 168.12, 138.00, 125.57, 98.60, 78.97, 72.41, 69.07, 68.31, 67.16, 62.95, 62.79, 62.25, 55.17, 52.75, 49.30, 47.93, 47.53, 41.91, 39.52, 38.97, 38.82, 38.69, 38.57, 38.06, 36.93, 36.54, 32.92, 30.65, 28.11, 27.96, 27.20, 24.06, 23.58, 23.28, 23.17, 21.15, 21.10, 20.83, 20.80, 20.74, 18.23, 17.02, 16.86, 15.63, 15.43; ESI-HRMS calcd for $C_{52}H_{79}NNaO_{16}$ $[M+Na]^+$: 996.5291, found 996.5296.

3.1.29 O-[2-O-(1-methyl-N-acetylneuraminyl)] ethyl 3β-hydroxy-lup-20(29)-en-28-oate (20a)

Prepared from 19a according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound 20a as a white solid in 88% yield. R_f = 0.21 (DCM/MeOH = 8:1); Mp: 176-178 °C; IR (KBr, ν, cm^{-1}): 3434, 2942, 1738, 1698, 1642, 1033; ^1H NMR (400 MHz, CD_3OD + $CDCl_3$): δ 4.72-4.73 (m, 2H, overlap with water), 4.60 (brs, 1H), 4.16-4.27 (m, 2H), 3.97-4.02 (m, 1H), 3.61-3.90 (m, 9H), 3.52-3.54 (m, 1H), 3.47 (dd, 1H, J = 1.4, 10.4 Hz), 3.15 (dd, 1H, J = 5.8, 10.2 Hz), 2.97-3.02 (m, 1H), 2.74 (dd, 1H, J = 4.7, 12.8 Hz), 2.16-2.26 (m, 2H), 2.03 (s, 3H, CH_3CO), 1.69, 0.99, 0.95, 0.93, 0.84, 0.75 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, CD_3OD + $CDCl_3$): δ 177.10, 174.90, 170.25, 151.07, 110.14, 99.45, 79.19, 74.50, 71.63, 69.47, 67.85, 64.14, 63.29, 62.71, 57.33, 56.16, 53.41, 53.26, 51.27, 50.04, 47.77, 43.02, 41.37, 40.93, 39.53, 39.45, 39.03, 37.79, 37.54, 34.98, 32.62, 31.14, 30.26, 28.35, 27.48, 26.20, 22.61, 21.53, 19.57, 18.92, 16.53, 16.42, 15.85, 15.12; ESI-HRMS calcd for $C_{44}H_{71}NNaO_{12}$ $[M+Na]^+$: 828.4868, found 828.4866.

3.1.30 O-[2-O-(1-methyl-N-acetylneuraminyl)] ethyl 3β-hydroxy-olean-12-en-28-oate (20b)

Prepared from 19b according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound 20b as a white solid in 86% yield. R_f = 0.20 (DCM/MeOH = 8:1); Mp: 175-177 °C; IR (KBr, ν, cm^{-1}): 3498, 2951, 1742, 1709, 1632, 1036; ^1H NMR (400 MHz, CD_3OD + $CDCl_3$): δ 5.27 (brs, 1H), 4.17-4.22 (m, 1H), 4.06-4.10 (m, 1H), 3.95-4.00 (m, 1H), 3.46-3.89 (m, 13H), 3.17 (dd, 1H, J = 5.3, 10.6 Hz), 2.85 (dd, 1H, J = 3.9, 13.6 Hz), 2.74 (dd, 1H, J = 4.6, 12.9 Hz), 2.03 (s, 3H, CH_3CO), 1.15, 0.98, 0.94, 0.93, 0.92, 0.78, 0.74 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, CD_3OD + $CDCl_3$): δ 178.98, 174.93, 170.29, 144.26, 123.32, 99.50, 79.25, 74.55, 71.66, 69.50, 67.88, 64.17, 63.78, 62.60, 56.09, 53.41, 53.30, 47.54, 46.46, 42.32, 42.09, 40.98, 40.07, 39.37, 39.30, 37.65, 34.41, 33.41, 33.11, 31.23, 28.49, 28.29, 27.33, 26.35, 24.08, 23.92, 23.51, 22.61, 18.98, 17.41, 16.08, 15.75; ESI-HRMS calcd for $C_{44}H_{71}NNaO_{12}$ $[M+Na]^+$: 828.4868, found 828.4876.

3.1.31 O-[2-O-(1-methyl-N-acetylneuraminyl)] ethyl 3β,16α-dihydroxy-olean-12-en-28-oate (20c)

Prepared from 19c according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound 20c as a white solid in 84% yield. R_f = 0.19 (DCM/MeOH = 8:1); Mp: 177-179 °C; IR (KBr, ν, cm^{-1}): 3455, 2948, 1729, 1645, 1449, 1037; ^1H NMR (400 MHz, CD_3OD): δ 5.30 (brs, 1H), 4.48 (brs, 1H), 4.13-4.18 (m, 1H), 3.94-4.03 (m, 2H), 3.50-3.85 (m, 12H), 3.15 (dd, 1H, J = 5.0, 11.0 Hz), 3.01 (dd, 1H, J = 3.9, 14.2 Hz), 2.69 (dd, 1H, J = 4.7, 12.7 Hz), 2.29 (t, 1H, J = 13.3 Hz), 2.00 (s, 3H, CH_3CO), 1.37 (s, 3H, CH_3), 0.97 (s, 6H, 2 × CH_3), 0.95, 0.88, 0.77, 0.74 (s, 3H each, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 178.58, 175.19, 170.70, 144.76, 123.59, 100.02, 79.66, 74.99, 74.95, 72.27, 70.07, 68.42, 64.62, 64.47, 63.05, 56.80, 53.74, 53.51, 49.88, 49.85, 48.11, 47.55, 42.51, 42.03, 41.58, 40.67, 39.88, 39.80, 38.12, 36.42, 36.19, 34.17, 33.45, 32.54, 31.37, 28.78, 27.86, 27.31, 24.98, 24.48, 22.68, 17.76, 16.37, 16.10; ESI-HRMS calcd for $C_{44}H_{72}NO_{13}$ $[M+H]^+$: 822.4998, found 822.5001.

3.1.32 O-[2-O-(1-methyl-N-acetylneuraminyl)] ethyl 3β-hydroxy-urs-12-en-28-oate (20d)

Prepared from 19d according to general procedure B, and the residue was purified by chromatography (eluent: DCM/MeOH = 10:1) over silica gel to afford compound 20d as a white solid in 85% yield. R_f = 0.21 (DCM/MeOH = 8:1); Mp: 175-177 °C; IR (KBr, ν, cm^{-1}): 3495, 2949, 1732, 1655, 1453, 1035; ^1H NMR (400 MHz, CD_3OD): δ 5.24 (t, 1H, J = 3.3 Hz), 4.10-4.16 (m, 1H), 3.95-4.06 (m, 2H), 3.74-3.86 (m, 6H), 3.59-3.69 (m, 3H), 3.50-3.56 (m, 2H), 3.15 (dd, 1H, J = 4.8, 11.1 Hz), 2.70 (dd, 1H, J = 4.6, 12.8 Hz), 2.22 (d, 1H, J = 11.3 Hz), 2.00 (s, 3H, CH_3CO), 1.11 (s, 3H, CH_3), 0.97 (s, 6H, 2 × CH_3), 0.96 (s, 3H, CH_3), 0.88 (d, 3H, CH_3, J = 6.4 Hz), 0.78 (s, 6H, 2 × CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 179.12, 175.20, 170.76, 139.40, 127.13, 100.11, 79.68, 75.01, 72.31, 70.11, 68.48, 64.68, 64.47, 63.12, 56.73, 54.29, 53.78, 53.46, 43.16, 41.69, 40.89, 40.31, 40.01, 39.83, 38.09, 37.89, 34.23, 31.67, 29.14, 28.80, 27.89, 25.20, 24.42, 24.20, 22.67, 21.55, 19.46, 17.82, 17.64, 16.40, 16.10; ESI-HRMS calcd for $C_{44}H_{71}NNaO_{12}$ $[M+Na]^+$: 828.4868, found 828.4873.

3.2 Bioassays

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3.2.1 Cytotoxicity assay

The assay was performed as previously described[44] with some modifications. Cells were seeded in 96-well plates containing 1% FBS overnight and cultured with increasing amounts of the test compounds for 40 hours. Cell viability was assessed using the CellTiter-Glo assay mixture as recommended by the supplier, and the plates were read using a plate reader (Tecan Infinite M2000 PRO; Tecan Group Ltd., Mannedorf, Switzerland) 2.3. CellTiter-Glo® luminescent cell viability assay. 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 were seeded into 96-well plates, incubated overnight and infected with influenza virus (MOI = 0.1). Cells were suspended in DMEM supplemented with 1% FBS, containing test compound and 2 μg/mL TPCK-treated trypsin, and a final DMSO concentration of 1% was added in each well. After 40 hours of incubation, CellTiter-Glo reagent (Promega Corp., Madison, WI, USA) was added, and assessed with the CellTiter-Glo assay as described above.

3.2.3 Time-of-addition assay

MDCK cells were seeded into 6-well plates at a density of 5×10^5 cells per well 24 h prior to infection and incubated at 37 °C under 5% CO_2. Then MDCK cells were infected with WSN virus at an MOI of 1. Compound was added at 0-10, 0-2, 2-5, 5-8 or 8-10 h post infection. Cell supernatants were collected 11 h post-infection. The viral titer of collected supernatants was estimated by the plaque formation assay.

3.2.4 Plaque assay

Confluent cultures of cells from MDCK cells in 12-well plates were infected with the collected viral supernatants in addition-of-time assay for 1 h at 37 °C. Cell monolayers were washed with PBS and overlaid with DMEM containing 0.6% low-melting-point agarose and 2 μg/mL TPCK-treated trypsin, and incubated at 37 °C. After 4 days, the cells were fixed with 4% paraformaldehyde, strained with crystal violet (Sigma-Aldrich, St Louis, MI, USA). Visible plaques were counted, and the virus titers were determined.

3.2.5 The hemagglutination inhibition (HI) assay

HI assay was performed as described previously.[25] 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 mL of freshly prepared chicken red blood cells (cRBC) (1% v/v in saline) was added to each well. The mixture was incubated for 30 min at room temperature before observing cRBC aggregation on the plate.

3.2.6 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.

3.2.7 Surface plasmon resonance (SPR) assay

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

3.2.8 Docking simulation

The docking simulation was performed via AutoDock 4.2 (Scripps Research Institute),[45] and assessed by OpenSource PyMOL 1.5.x (Schrödinger, LLC) and PoseView web service (Universität Hamburg)[46]. Afterwards, structure of the protein (Protein Data Bank: 1RVT) was obtained from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). Protein preparation was performed as follows. After the removal of HETATM atoms, hydrogen atoms and the crystal cells, the protein was further processed via AutoDock Tools 1.5.6 rc3 (ADT, Scripps Research Institute) to add Polar hydrogen and Kollman charge. Ligand preparation was performed as follows. Polar hydrogen and Gasteiger charge were given, and there was no further adjustment on the ligand torsion tree. Grid box covering the HA head was prepared with ADT with parameters as follows: grid box center coordinate: 87.320, -9.696, -34.751; box size: 74 × 78 × 80; grid point spacing: 0.375 Å. Lamarckian genetic algorithm was applied to the docking simulation with modifications of the parameters described below due to the compounds' highly flexible nature: number of individuals in population: 300; maximum number of energy evaluations: 25,000,000; number of GA runs: 100. A hundred docking output conformations for one compound were clustered with a maximum RMS tolerance of 2.0 Å. To avoid isolated conformations, the best binding conformation from the largest cluster was used for further analysis.

Conclusions

In conclusion, a total of 24 conjugated sialic acid and pentacyclic triterpene derivatives were synthesized and assayed for their anti-influenza A/WSN/33 virus activity by CPE assay. Four sialic acid derivatives **13a**, **13b**, **19a** and **20a** were found to significantly reduce the viral CPE. The most promising result was observed for compound **20a**, in which sialic acid was linked to BA at C_{28} carboxylic acid by a flexible glycol linker. The IC_{50} of

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20a was 41.2 μM, which was comparable to that of oseltamivir (OSV). Time-of-addition experiment indicates that 20a acts at an early stage of the viral life cycle. Noticeably, compound 20a binds tightly to influenza virus HA protein with a dissociation constant of K_D = 17 μM by SPR assay, thus disrupting the interaction of HA with sialic acid.

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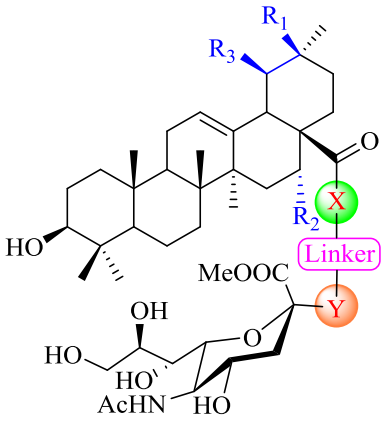
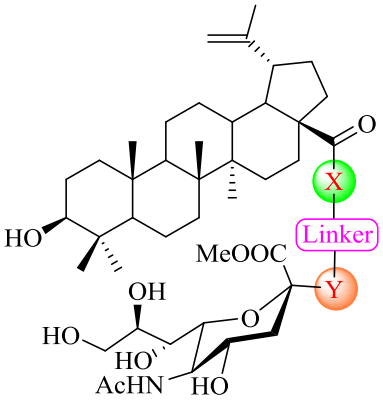
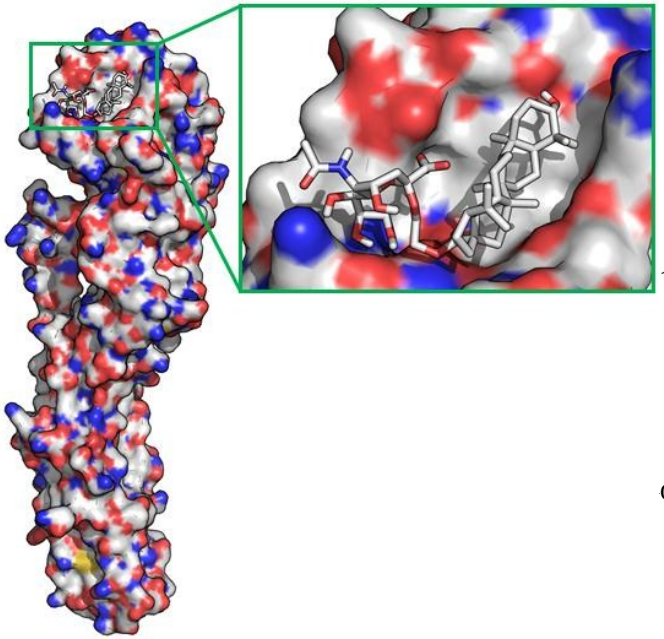
Notes and references

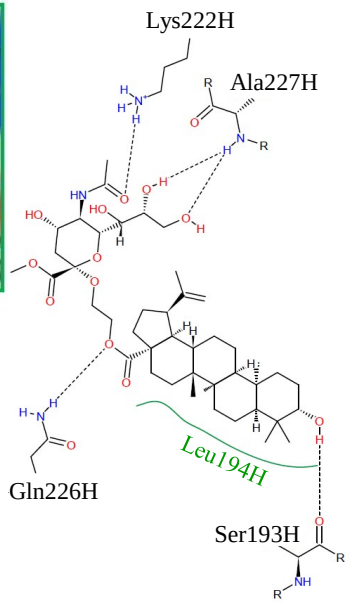
1. A. Loregian, B. Mercorelli, G. Nannetti, C. Compagnin, G. Palù, Cell Mol. Life Sci., 2014, 71, 3659.
2. J. S. Peiris, M. D. Jong, Y. Guan, Clin. Microbiol. Rev., 2007, 20, 243.
3. R. Gao, B. Cao, Y. Hu, N. Engl. J. Med., 2013, 368, 1888.
4. G. A. Poland, R. M. Jacobson, P. V. Targonski, Vaccine, 2007, 25, 3057.
5. T. Bedford, S. Riley, I. G. Barr, S. Broor, M. Chadha, N. J. Cox, R. S. Daniels, C. P. Gunasekaran, A. C. Hurt, A. Kelso, A. Klimov, N. S. Lewis, X. Li, J. W. McCauley, T. Odagiri, V. Potdar, A. Rambaut, Y. Shu, E. Skepner, D. J. Smith, M. A. Suchard, M. Tashiro, D. Wang, X. Xu, P. Lemey, C. A. Russell, Nature, 2015, 523, 217.
6. N. S. Moorthy, V. Poongavanam, V. Pratheepa, Mini-Rev. Med. Chem., 2014, 14, 819.
7. W. B. Dreitlein, J. Maratos, J. Brocavich, Clin. Ther., 2001, 23, 327.
8. M. M. McLaughlin, E. W. Skoglund, M. G. Ison, Expert Opin. Pharmacother., 2015, 16, 1889.
9. A. C. Hurt, H. T. Ho, I. Barr, Expert Rev. Anti-Infect. Ther., 2006, 4, 795.
10. R. A. Bright, M. J. Medina, X. Xu, G. Perez-Oronoz, T. R. Wallis, X. M. Davis, L. Povinelli, N. J. Cox, A. I. Klimov, Lancet, 2005, 366, 1175.
11. G. Dong, C. Peng, J. Luo, C. Wang, L. Han, B. Wu, G. Ji, H. He, PLoS ONE, 2015, 10, e0119115.
12. V. E. Vander, F. F. Stelma, C. A. Boucher, N. Engl. J. Med., 2010, 363, 1381.
13. A. Nitsch-Osuch, L. B. Brydak, Acta Biochim. Pol., 2014, 61, 505.
14. L. V. Gubareva, M. N. Matrosovich, M. K. Brenner, R. C. Bethell, R. G. Webster, J. Infect. Dis., 1998, 178, 1257.
15. L.V. Gubareva, R. G. Webster, F. G. Hayden, Antimicrob Agents Chemother., 2001, 45, 3403.
16. T. G. Sheu, V. M. Deyde, M. Okomo-Adhiambo, R. J. Garten, X. Xu, R. A. Bright, E. N. Butler, T. R. Wallis, A. I. Klimov, L. V. Gubareva, Antimicrob Agents Chemother., 2008, 52, 3284.
17. J. J. Skehel, D. C. Wiley, Annu. Rev. Biochem., 2000, 69, 531.
18. R. Schauer, Zoology, 2004, 107, 49.
19. S. Sato, S. Fujita, K. Furuhata, H. Ogura, S. Yoshimura, M. Itoh, Y. Shitori, Chem. Pharm. Bull., 1987, 35, 4043.
20. M. Koketsu, T. Nitoda, H. Sugino, L. R. Juneja, M. Kim, T. Yamamoto, N. Abe, T. Kajimoto, C. H. Wong, J. Med. Chem., 1997, 40, 3332.
21. T. Takahashi, H. Tsukamoto, H. Yamada, Bioorg. Med. Chem. Lett., 1998, 8, 113.
22. Y. Ikeuchi, M. Sumiya, T. Kawamoto, N. Akimoto, Y. Mikata, M. Kishigami, S. Yano, T. Sasaki, F. Yoneda, Bioorg. Med. Chem., 2000, 8, 2027.
23. H. Yu, H. Yu, R. Karpel, X. Chen, Bioorg. Med. Chem., 2004, 12, 6427.
24. Y. M. Zhao, Y. P. Shi, Chem. Biodivers., 2011, 8, 414.
25. M. R. Yu, L.L. Si, Y. F. Wang, Y. M. Wu, F. Yu, P. X. Jiao, Y. Y. Shi, H. Wang, S. L. Xiao, G. Fu, K. Tian, Y. T. Wang, Z. H. Guo, X. S. Ye, L. H. Zhang, D. M. Zhou, J. Med. Chem., 2014, 57, 10058.
26. S. L. Xiao, L. L. Si, Z. Y. Tian, P. X. Jiao, Z. B. Fan, K. Meng, X. S. Zhou, H. Wang, R. Y. Xu, X. Han, G. Fu, Y. M. Zhang, L. H. Zhang, D. M. Zhou, Biomaterials, 2016, 78, 74.
27. F. Yu, Q. Wang, Z. Zhang, Y. Y. Peng, Y. Y. Qiu, Y. Y. Shi, Y. X. Zheng, S. L. Xiao, H. Wang, X. X. Huang, L. Y. Zhu, K. B. Chen, C. K. Zhao, C. L. Zhang, M. R. Yu, D. A. Sun, L. H. Zhang, D. M. Zhou, J. Med. Chem., 2013, 56, 4300.
28. H. Wang, Q. Wang, S. L. Xiao, F. Yu, M. Ye, Y. X. Zheng, C. K. Zhao, D. A. Sun, L. H. Zhang, D. M. Zhou, Eur. J. Med. Chem., 2013, 64, 160.
29. F. Yu, Y. Y. Peng, Q. Wang, Y. Y. Shi, L. L. Si, H. Wang, Y. X. Zheng, E. Lee, S. L. Xiao, M. R. Yu, Y. B. Li, C. L. Zhang, H. L. Tang, C. G. Wang, L. H. Zhang, D. M. Zhou, Eur. J. Med. Chem., 2014, 77, 258.
30. S. L. Xiao, Q. Wang, L. L. Si, Y. Y. Shi, H. Wang, F. Yu, Y. M. Zhang, Y. B. Li Y. X. Zheng, C. L. Zhang, C. G. Wang, L. H. Zhang, D. M. Zhou, ChemMedChem, 2014, 9, 1060.
31. H. Wang, F. Yu, Y. Y. Peng, Q. Wang, X. Han, R. Y. Xu, X. S. Zhou, C. X. Wan, Z. B. Fan, P. X. Jiao, Y. M. Zhang, L. H. Zhang, D. M. Zhou, S. L. Xiao, Eur. J. Med. Chem., 2015, 102, 594.
32. H. Wang, R. Y. Xu, Y. Y. Shi, L. L. Si, P. X. Jiao, Z. B. Fan, X. Han, X. Y. Wu, X. S. Zhou, F. Yu, Y. M. Zhang, L. R. Zhang, L. H. Zhang, D. M. Zhou, S. L. Xiao, Eur. J. Med. Chem., 2016, 110, 376.
33. R. Kuhn, P. Lutz, D. L. MacDonald, Chem. Ber., 1966, 99, 611.
34. K. Suzuki, R. Kobayashi, K. Furuhata, H. Ogura, Chem. Pharm. Bull., 1990, 38, 2083.
35. U. Dabrowski, H. Friebolin, R. Brossmer, M. Supp, Tetrahedron Lett., 1979, 48, 4637.
36. H. Ogura, K. Furuhata, M. Itoh, Y. Shitori, Carbohydr. Res., 1986, 158, 37.
37. G. Zemplén, Ber. Dtsch. Chem. Ges., 1926, 59, 1254.
38. D. Y. Chen, J. H. Shien L. Tiley, S. S. Chiou, S. Y. Wang, T. J. Chang, Y. J. Lee, K. W. Chan, W. L. Hsu, Food Chem., 2010, 119, 1346.
39. J. Yang, M. M. Li, X. T. Shen, S. W. Liu, Viruses, 2013, 5, 352.
40. J. L. Ou, Y. Mizushina, S. Y. Wang, D. Y. Chuang, M. Nadar, W. L. Hsu, FEBS J., 2013, 280, 5829.
41. X. Q. Chen, L. L. Si, D. Liu, P. Proksch, L. H. Zhang, D. M. Zhou, W. H. Lin, Eur. J. Med. Chem., 2015, 93, 182.
42. S. W. Niu, L. L. Si, D. Liu, A. Zhou, Z. W. Zhang, Z. Z. Shao, S. M. Wang, L. H. Zhang, D. M. Zhou, W. H. Lin, Eur. J. Med. Chem., 2016, 108, 229.
43. Kiyoshi, N. Yoshihiro, A. Kazuo, Carbohydr. Res., 1992, 224, 123.
44. B. S. Hamilton, G. R. Whittaker, S. Daniel, Viruses, 2012, 4, 1144.
45. K. Wittine, M. Stipković Babić, D. Makuc, J. Plavec, S. Kraljević Pavelić, M. Sedić, K. Pavelić, P. Leyssen, J. Neyts, J. Balzarini, M. Mintas, Bioorg. Med. Chem., 2012, 20, 3675.
46. K. Stierand, M. Rarey, ACS Med. Chem. Lett., 2010, 1, 540.

Graphical abstract





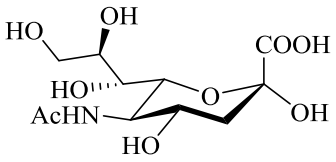




Compound 20a:

Anti-influenza: IC_{50} = 41.2 μM

Figure 1



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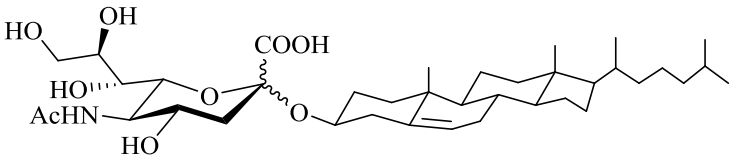

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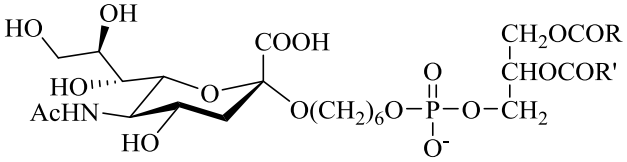

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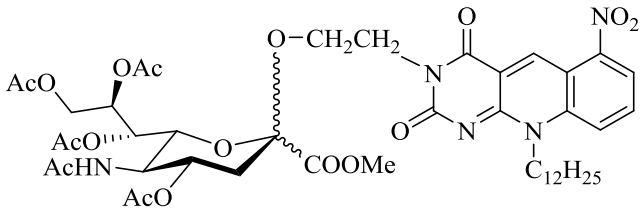

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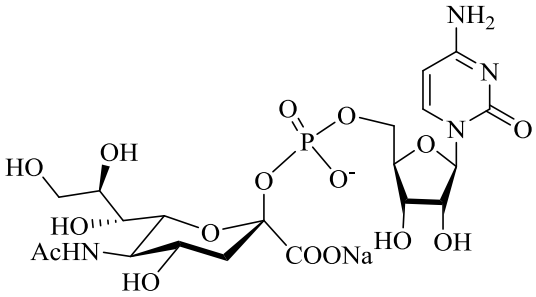

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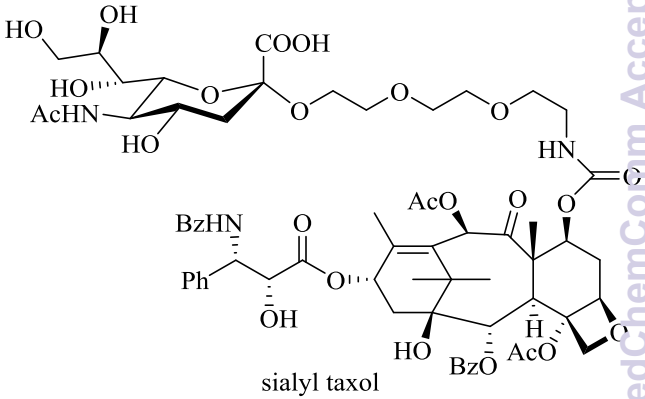

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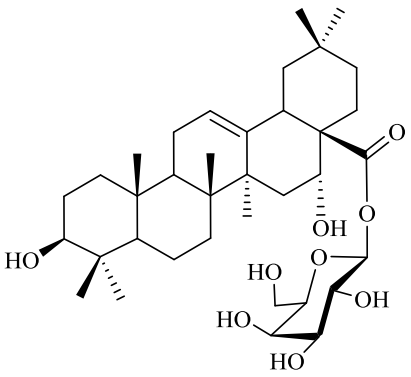
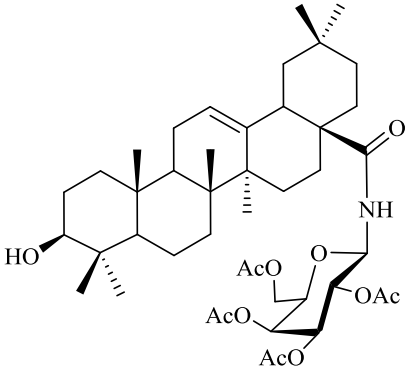
Figure 2

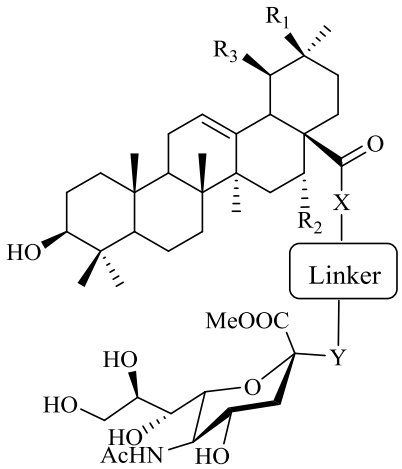


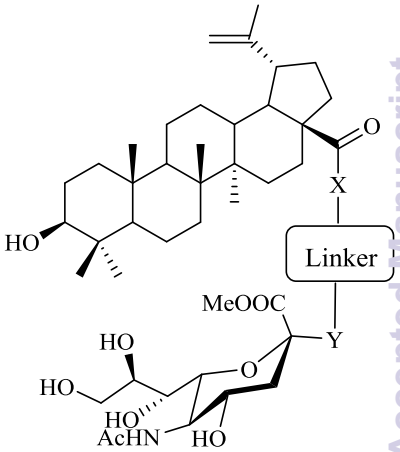
Q8



Y3

previous work [25]





this work

Figure 3

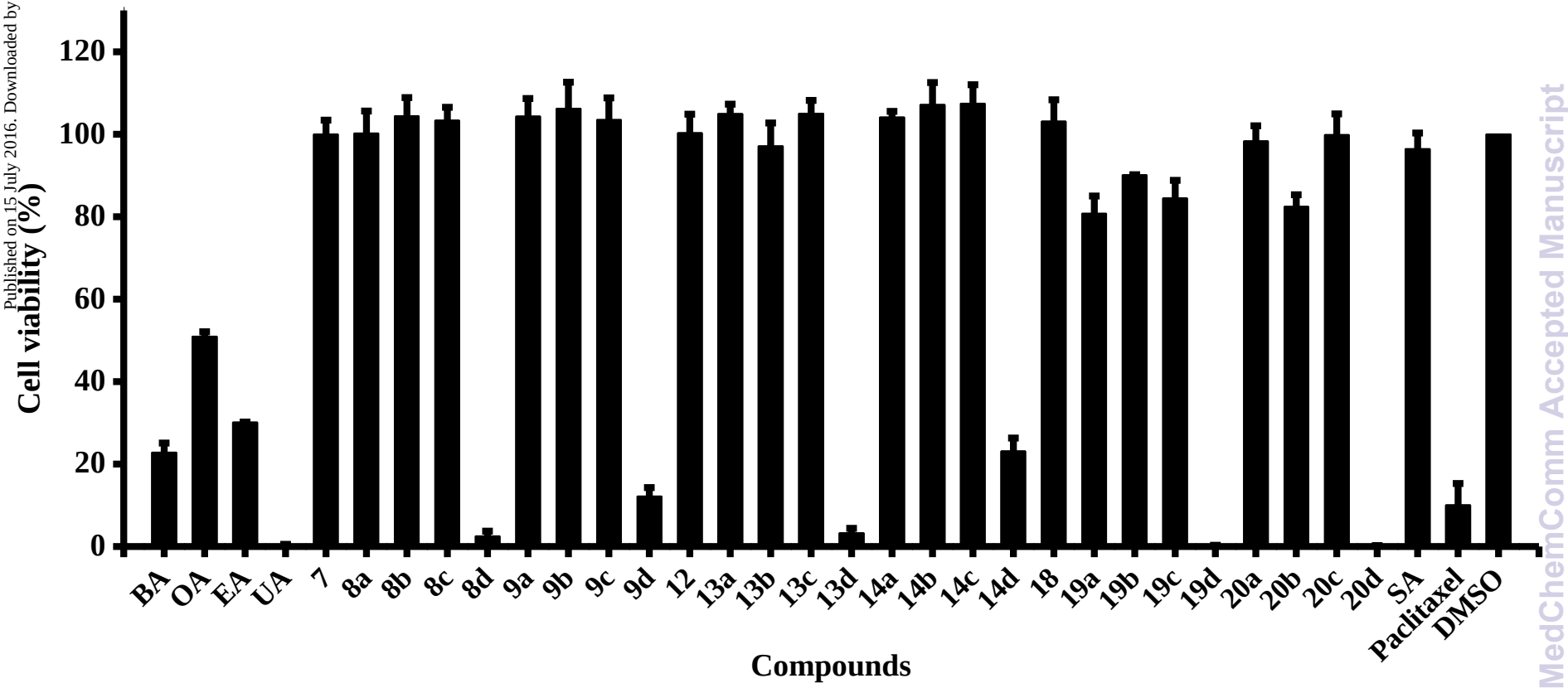


Figure 4

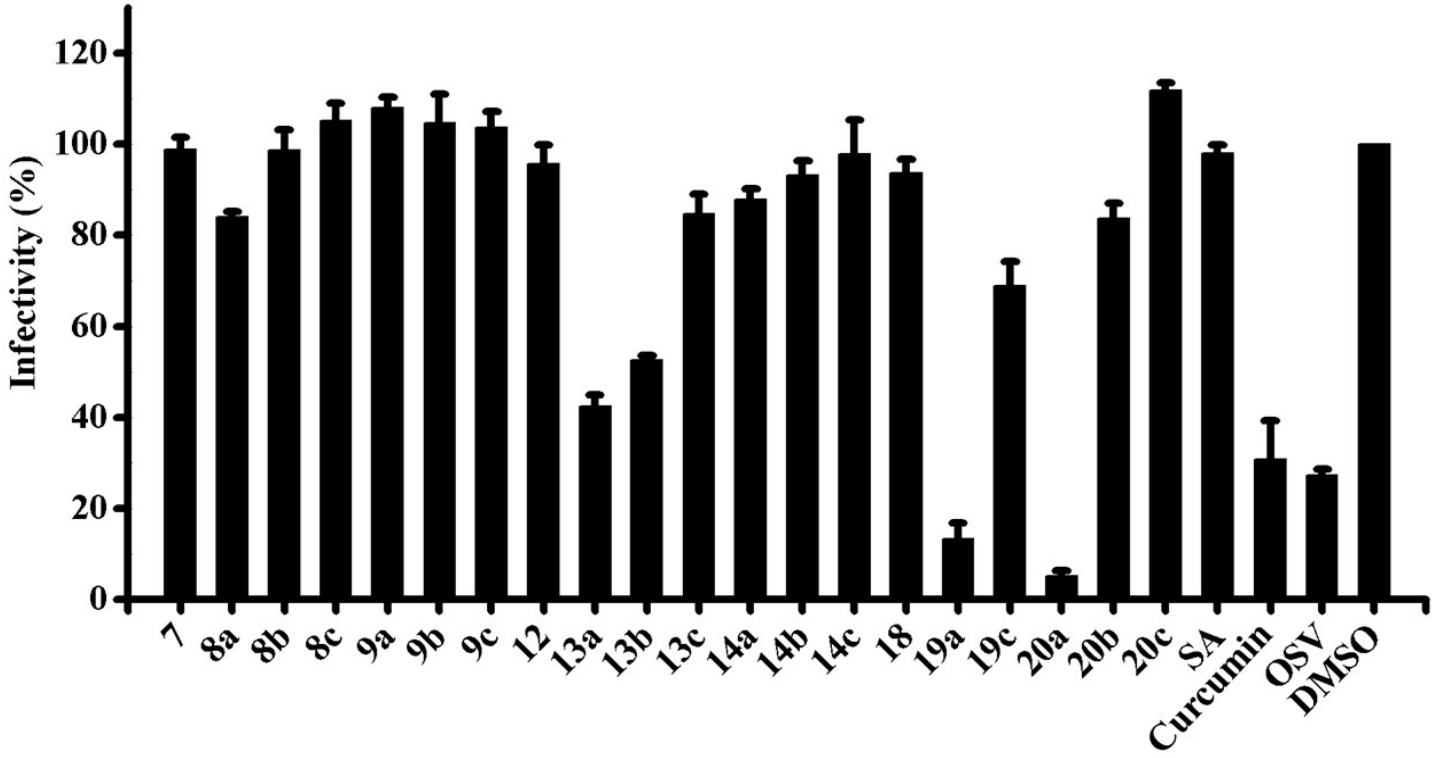


Figure 5

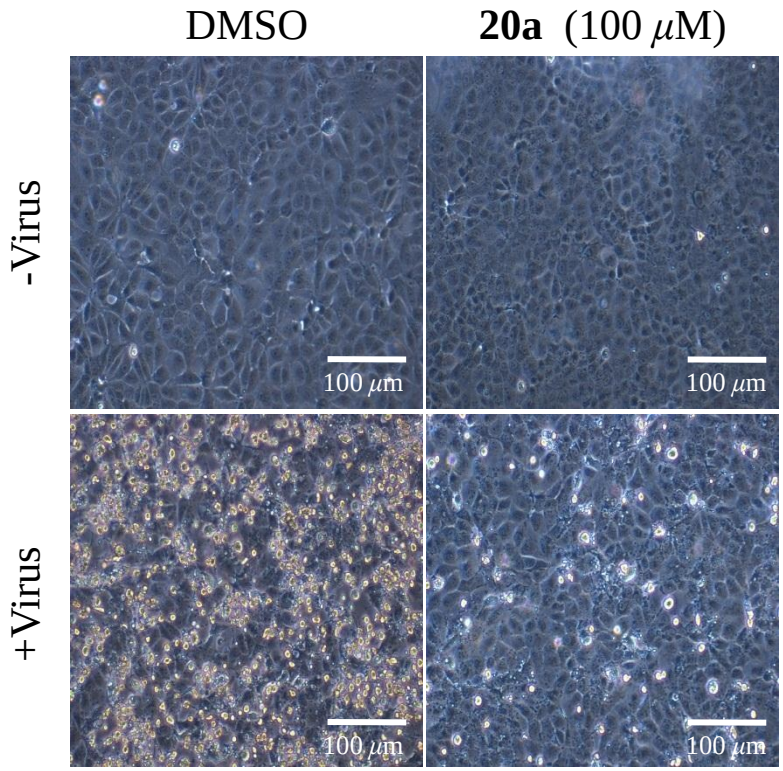


Figure 6

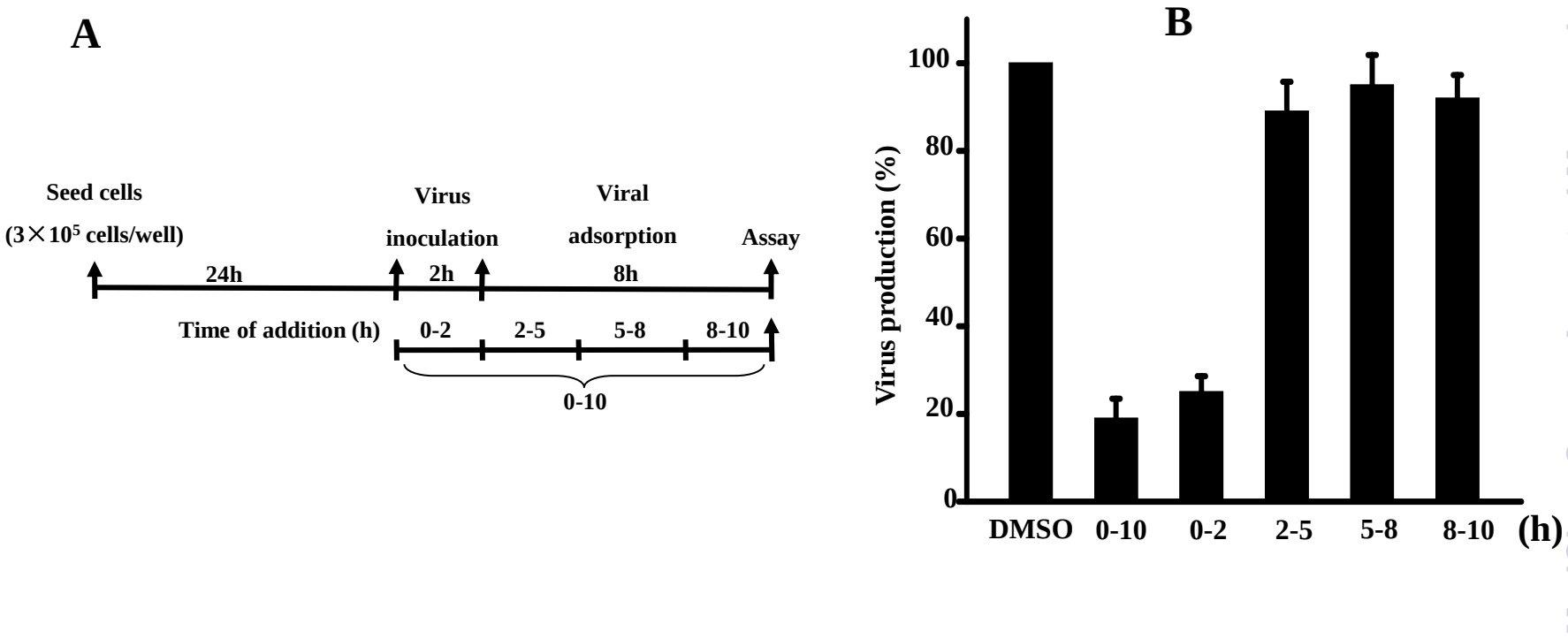


Figure 7(A)

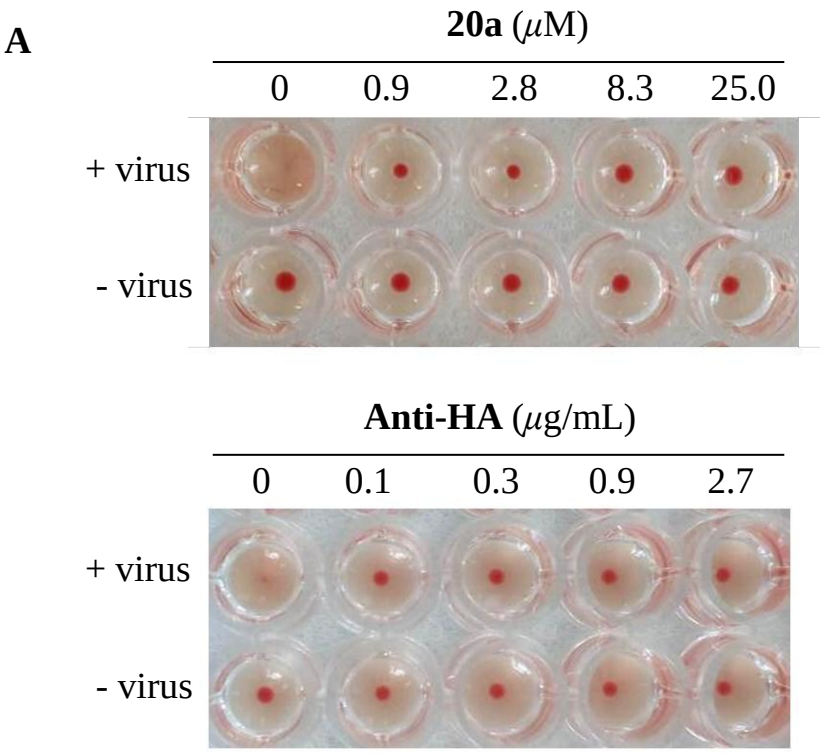
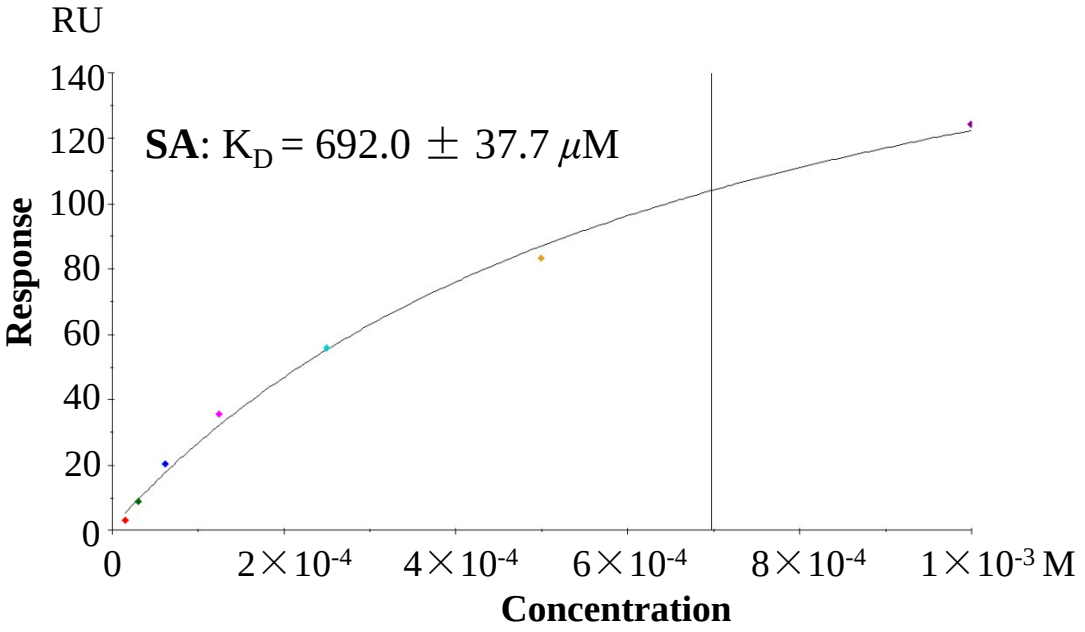
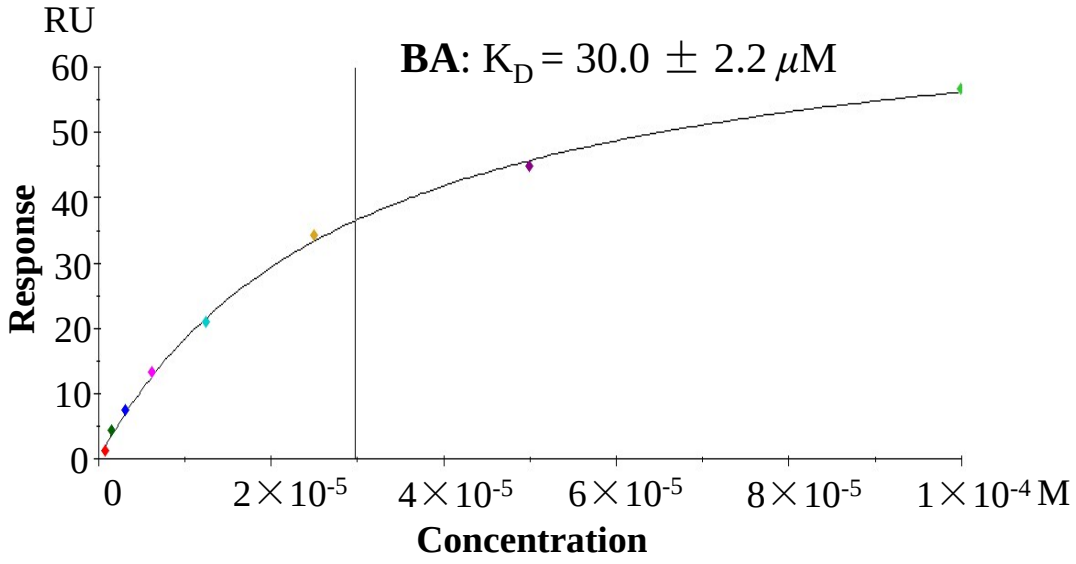


Figure 7(B)





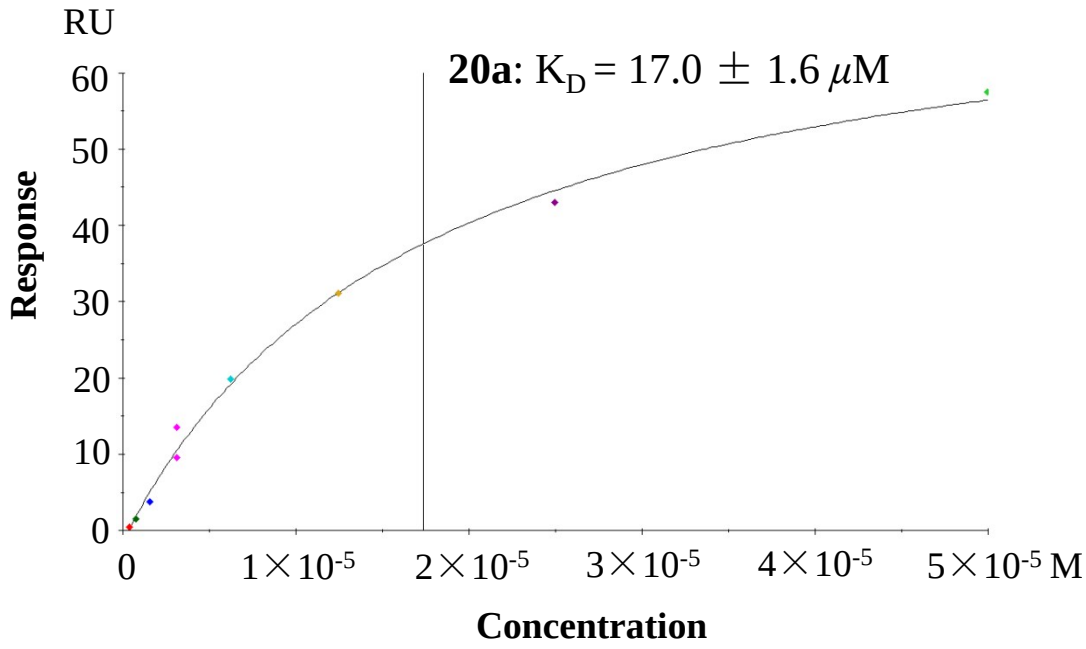
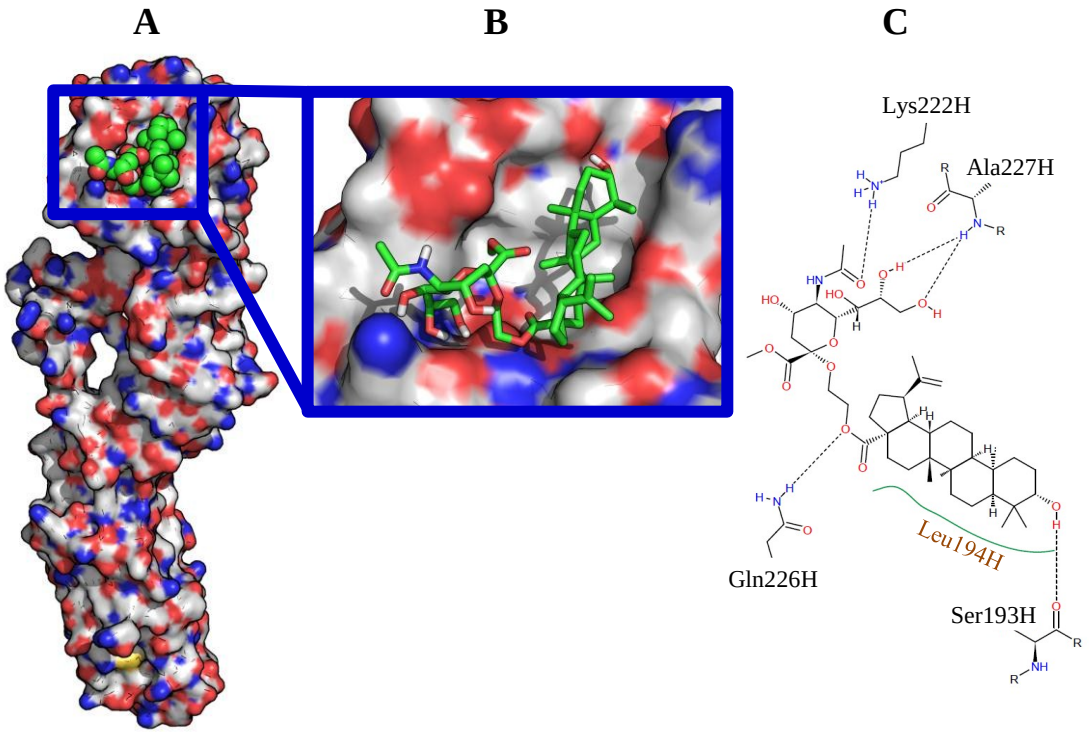
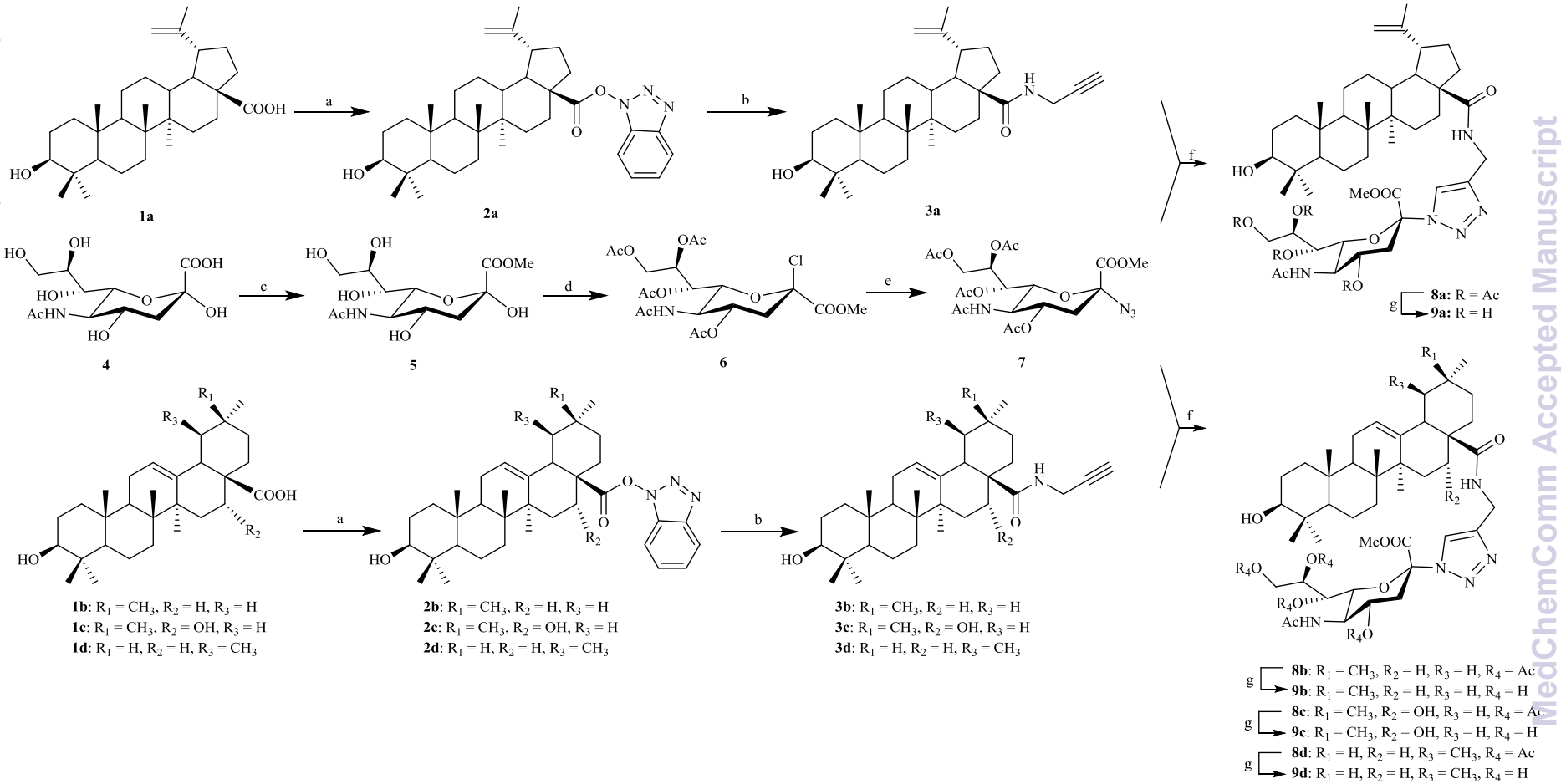


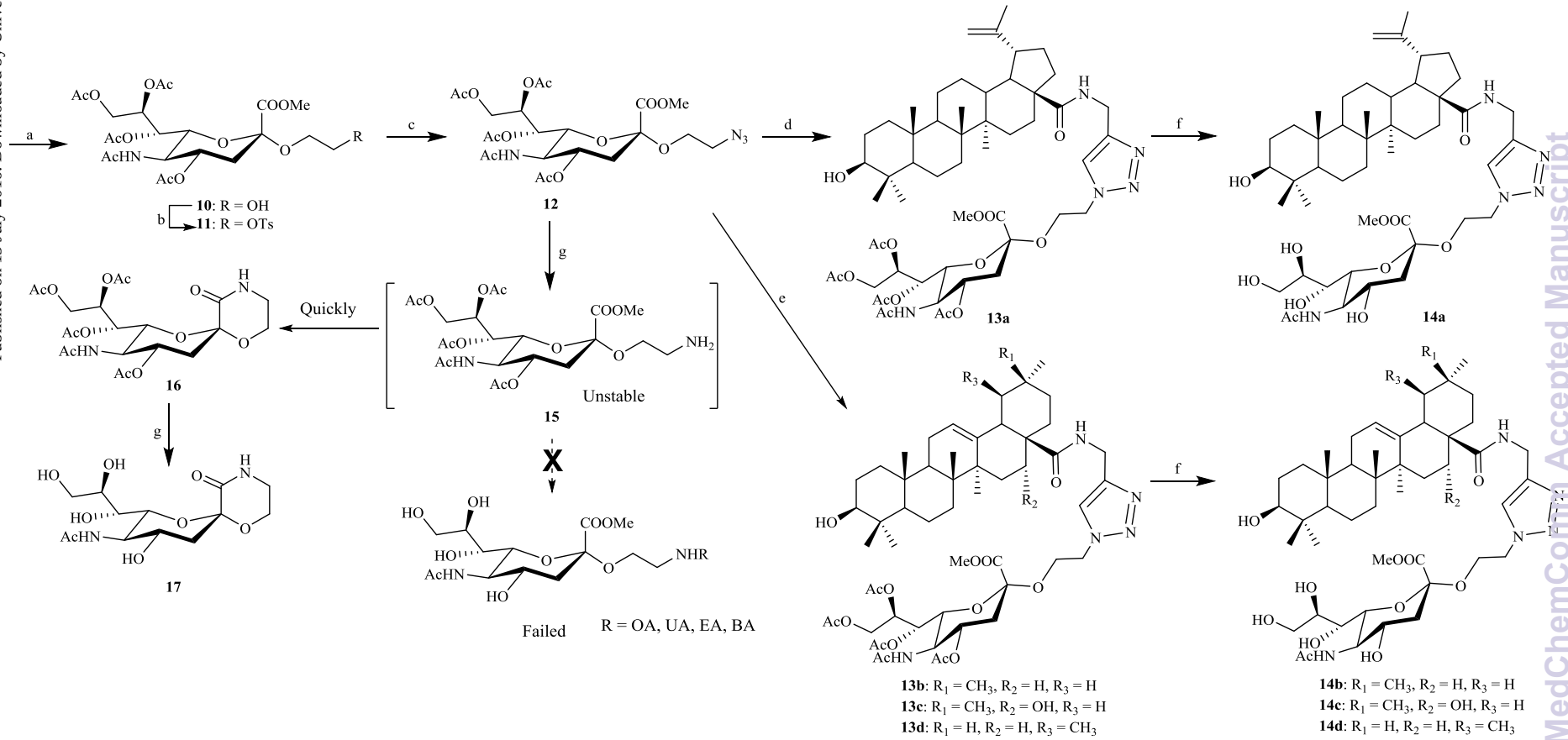
Figure 8



Scheme 1



Scheme 2



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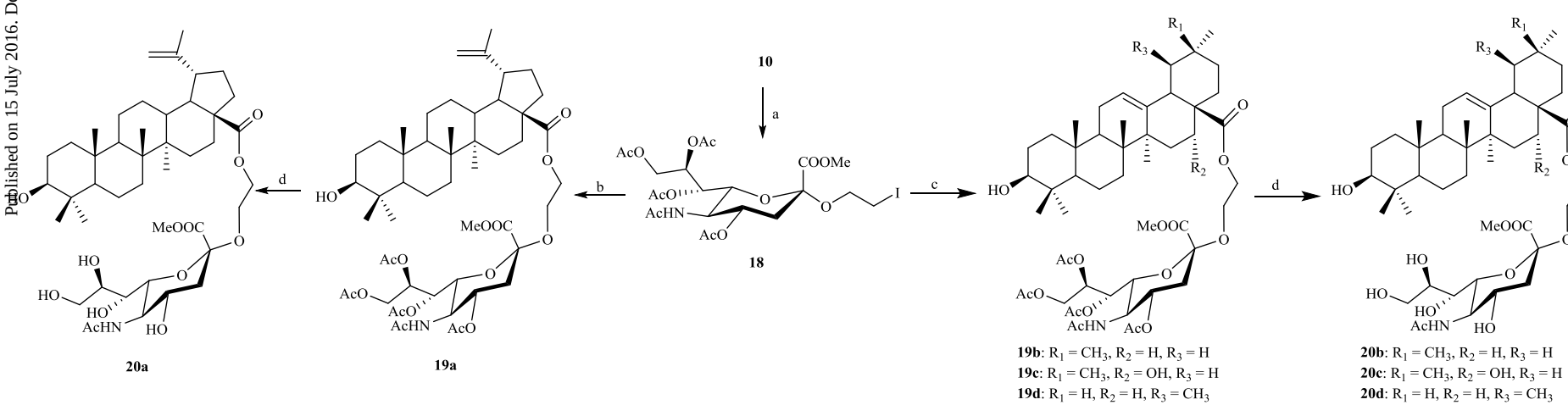

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Table of contents

A total of 24 novel sialic acid-pentacyclic triterpene conjugates were synthesized and evaluated as anti-influenza virus entry inhibitors.