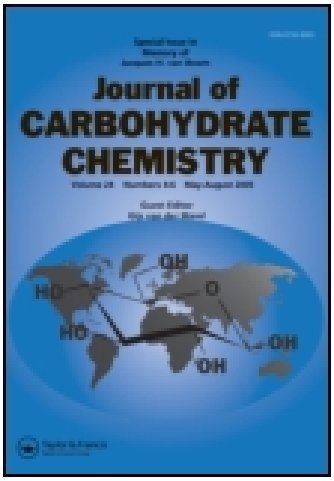

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lcar20

Synthesis and Biological Evaluation of Analogs of Methyl Ursolate 3-O-β-Chacotrioside as H5N1 Viral Entry Inhibitors

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Published online: 31 Oct 2012.

To cite this article: Sumei Ren, Qing Chen, Ning Ding, Wei Zhang, Yingxia Li & Ying Guo (2012) Synthesis and Biological Evaluation of Analogs of Methyl Ursolate 3-O-β-Chacotrioside as H5N1 Viral Entry Inhibitors, Journal of Carbohydrate Chemistry, 31:8, 647-658, DOI: 10.1080/07328303.2012.687060

To link to this article: http://dx.doi.org/10.1080/07328303.2012.687060

Journal of Carbohydrate Chemistry, 31:647–658, 2012

ISSN: 0732-8303 print / 1532-2327 online
DOI: 10.1080/07328303.2012.687060

Synthesis and Biological Evaluation of Analogs of Methyl Ursolate 3-*O*-β-Chacotrioside as H5N1 Viral Entry Inhibitors

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The earliest stage of influenza virus infection is the viral entry to the host cell. In our previous work, we discovered the first three small molecule H5N1 viral entry inhibitors **1–3**. Here, based on saponin **3**, methyl ursolate 3-*O*-β-chacotrioside, several analogs were synthesized and evaluated to understand the structure-activity relationships of this type of compound on the H5N1 viral entry inhibitory activity. The preliminary studies demonstrated that unlike saponins **1** and **2**, it is possible to reduce the 3-*O*-chacotriosyl residue of compound **3** to a disaccharide without affecting the viral entry activities significantly. The results obtained will render new clues to the understanding of the antiviral profile for these types of compounds.

Keywords: Influenza; Entry inhibitors; H5N1; Structure-activity relationship

INTRODUCTION

Avian H5N1 influenza viruses pose a pandemic threat with continuously occurring widespread infections of avian species, as well as sporadic human cases with a mortality rate of approximately 60%.[1] The World Health Organization

Received March 19, 2012; accepted April 17, 2012.
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(WHO) and many individual nations have made tremendous efforts to fully understand the pandemic of the virus and develop effective therapies to control the spread of the virus. However, only two kinds of agents, the M2 ion channel blockers and the neuraminidase inhibitors, have been used to treat influenza. But concern has been raised by the isolation of viable mutant viruses that are resistant to them.[2–5] Therefore, attempts should be made to develop new strategies directed at unexplored targets such as the viral proteins hemagglutinin (HA), the viral polymerase (and endonuclease), and the nonstructural protein NS1.[6] To address the need for new antivirals against influenza, a number of studies have been done to inhibit the interaction of HA with the receptor.[7–10]

By screening a saponin library generated from semisynthesis with an efficient HIV-based pseudotyping system, followed by preliminary structure-activity relationship study, our group had discovered the first two classes of saponins (compounds **1**, **2**, and **3**, Fig. 1) as small molecule H5N1 viral entry inhibitors.[11] Interestingly, these two classes of saponins differ not only in the aglycone structure but also in the mechanism of action. The class of compounds **1** and **2** containing β-chacotrioside linked to the aglycone of chlorogenin is possible by the inhibitors of receptor binding disrupting the interaction of HA with sialic acid sugars on the host cells. In contrast, compound **3**, with a methyl ursolate aglycone, might be possible through interrupting the membrane fusion of virus with the host cells (data not shown).

To further understand the mechanism of actions of these two classes of saponins on the H5N1 viral entry and in order to search for potentially new antiviral agents with high development potentials, extensive structure-activity relationship (SAR) studies around compounds **1**, **2**, and **3** are highly demanded. As part of this continuous work we would like to report here the design, synthesis, and biological evaluations of several analogs of compound **3**. Here, we kept the aglycone of compound **3** intact and focused on the sugar chain. Cutting down one or two sugar units from the chacotrioside resulted in saponins **4**, **5**, and **6**, respectively. The aglycone, compound **7**, was also synthesized (Fig. 2).

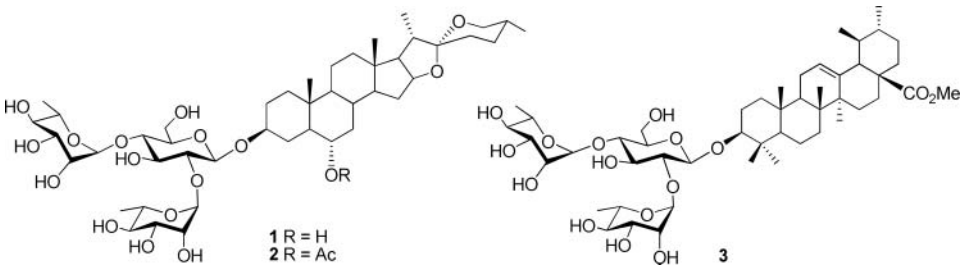


Figure 1: Three saponin inhibitors for H5N1 viral entry.

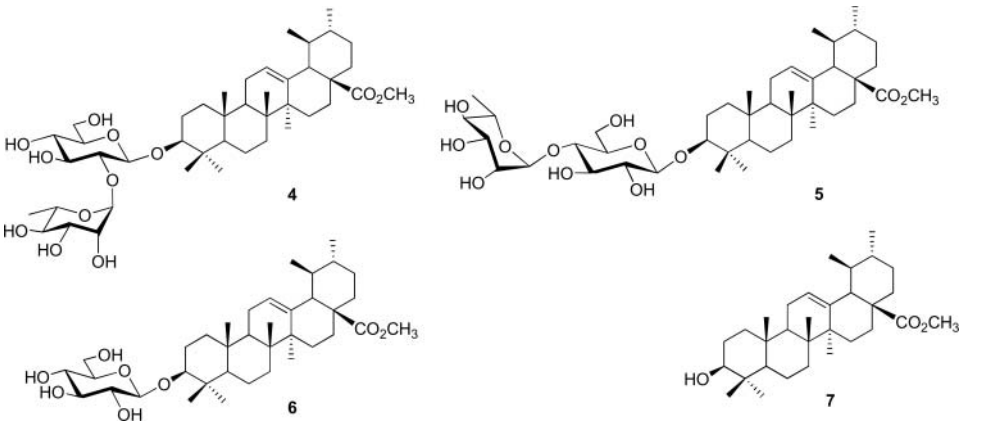
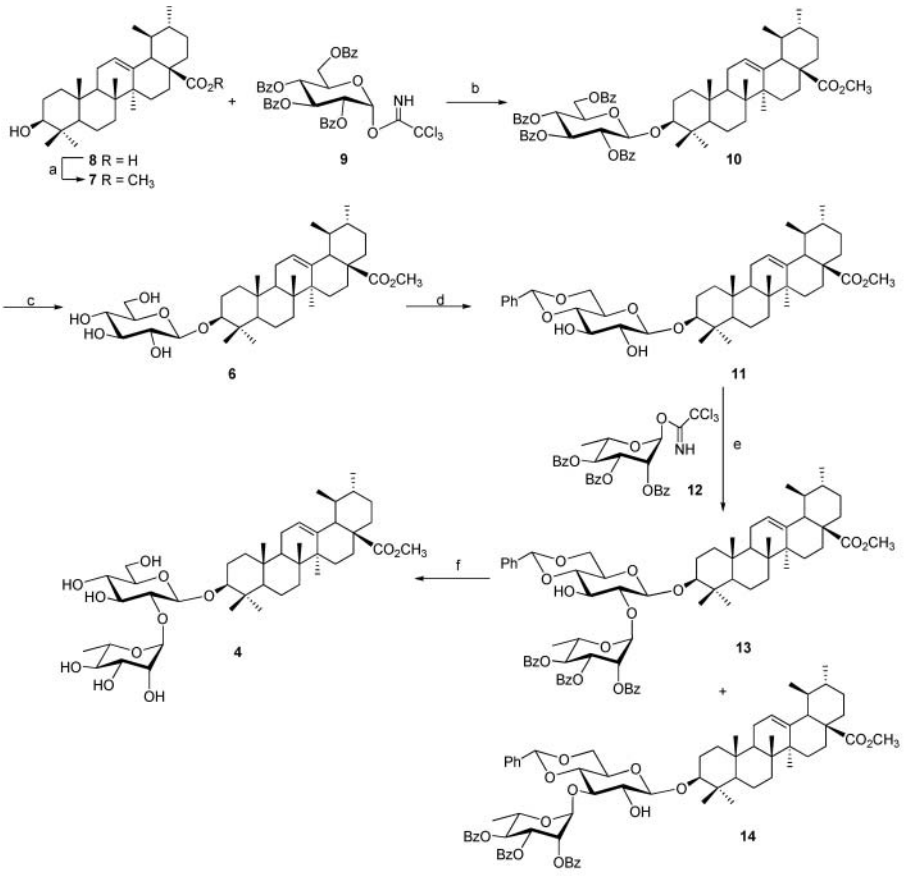


Figure 2: Saponins designed for SAR studies on the sugar chain.

RESULTS AND DISCUSSION

The synthetic route toward target compounds **4**, **6**, and **7** is depicted in Scheme 1. Starting from ursolic acid **8**, compound **7** was generated by protecting the 28-CO_2H with a methyl group. The free hydroxyl group of **7** was then glycosylated with 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl trichloroacetimidate **9**[12] under the promotion of trimethylsilyl trifluoromethanesulfonate (TMSOTf) to afford **10**. Removal of benzoyl groups in **10** furnished target saponin **6**.[11] Treatment of 3β-*O*-(D-glucopyranosyl)-ursolic-28-methyl ester **6** with benzyaldehyde dimethyl acetal catalyzed by camphor sulfonic acid (CSA) at 60°C in acetonitrile gave the 4,6-*O*-benzylidene-protected glycoside **11**. The next step was to introduce an L-rhamnopyranosyl residue to the 2-OH of **11**. As we know, fully differentiating 2,3-diols of D-glucopyranoside is still a challenge and usually needs extensive protecting group manipulations. To rapidly access our designed target compound **4**, we employed 1.0 equiv. of 2,3,4-tri-*O*-acetyl-L-rhamnopyranosyl trichloroacetimidate **12**[13] as donor for glycosylation with equal equivalents of acceptor **11**, affording the disaccharide glycosides **13** (37%) and **14** (63%) simultaneously. These two compounds can be easily separated by silica gel column chromatography. Removal of the benzylidene group in **13** by hydrogenolysis in the presence of Pd/C, followed by removal of the benzoyl groups with MeONa in CH_3OH/CH_2Cl_2, afforded target saponin **4**.

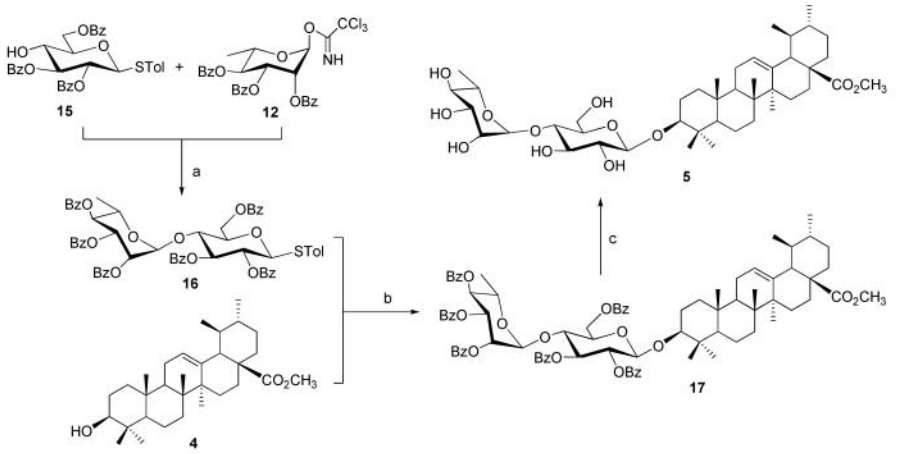
The synthesis of compound **5** is shown in Scheme 2. The known trichloroacetamidate **12** and thioglycoside **15**[11] were coupled under the promotion of TMSOTf to afford disaccharide **16**,[10] which was coupled with acceptor **7** promoted by *N*-iodosuccinimide (NIS) and trifluoromethanesulfonic acid (TfOH) in CH_2Cl_2 to give the intermediate **17**. Final removal of the benzoyl groups with NaOMe in CH_3OH/CH_2Cl_2 obtained the target saponin **5**.



Reagents and conditions: (a) CH_3I, NaH, DMF, 91%; (b) TMSOTf, CH_2Cl_2, 0 C, 70%; (c) NaOMe, CH_3OH/CH_2Cl_2, 90%; (d) $PhCH(OCH_3)_2$, CAS, CH_3CN, 65%; (e) TMSOTf, CH_2Cl_2, 0 C, 37% for **13** and 63% for **14**; (f) (i) Pd/C-H_2, CH_3OH/CH_2Cl_2; (ii) NaOMe, CH_3OH/CH_2Cl_2, 62% for 2 steps.

Scheme 1: Synthesis of compounds **4**, **6**, and **7**.

Using the HIV-based particles pseudotyped with a highly pathogenic H5N1 HA model (A/Viet Nam/1203/2004), the influences of compounds **4–7** on HA-mediated viral entry were evaluated, while VSVG/HIV was applied to evaluate the specificity of H5N1 inhibitors. The results are summarized in Table 1. Compounds **4** and **5**, each bearing a disaccharide sugar chain, showed good inhibitory activity. The position of the terminal L-rhamnosyl residue on the interglucose has no important effect on the activity (IC_{50}, 22.6 vs. 22.1 μM)). This result is very interesting because during the SAR studies around compounds **1** and **2** we found that removing any of the two L-rhamnosyl residues from



Reagents and conditions: (a) TMSOTf, CH_2Cl_2, 0 C, 90%; (b) NIS, TfOH, CH_2Cl_2, 0 C, 45%; (c) NaOMe, CH_3OH/CH_2Cl_2, 91%.

Scheme 2: Synthesis of compound **5**.

the trisaccharide chain led to total inactivation.[11] The finding is also attractive since analogs with reduced sugar chains are easier to access and modify. However, we failed to further reduce the sugar chain from compounds **4** and **5**, since compound **6** with a monosaccharide residue displayed strong cytotoxicity under the testing concentrations. Not surprisingly, the aglycon itself (i.e., methyl ursolate **7**) did not show viral entry inhibition at the concentration of 50 μM.

Table 1: Effect of compounds on HA mediated viral entry.

Compounds	HA(Viet)[a]/HIV IC_{50} (μM)	VSVG[b]/HIV Conc. (30 μM)
3	16.6	99.6%[c]
4	22.6	106.1%
5	22.1	91.1%
6	[d]	[d]
7	>50	103%

[a]HA(Viet), HA, H5N1 (A/Viet Nam/1203/2004).
[b]VSVG, vesicular stomatitis virus glycoprotein.
[c]The percentages in the table presented the infectivity compared to the same amount solvent as 100%.
[d]Cytotoxicity was observed at the final concentration of 10 μM.

CONCLUSION

In this work, based on our previously discovered small molecule inhibitor saponin **3**, several analogs were synthesized and evaluated as inhibitors of H5N1 viral entry. The results demonstrated that unlike the class of saponins **1** and **2**, it's possible to reduce the 3-*O*-chacotriosyl residue of compound **3** to a disaccharide without affecting the viral entry activities significantly. This work offered more lead compounds for the following studies on the action mechanisms and will render new clues to the understanding of the antiviral profile for these types of compounds.

EXPERIMENTAL PROTOCOLS

General Methods

All chemical reagents were used as supplied unless indicated. Solvents used in organic reactions were distilled under an inert atmosphere. Unless otherwise noted, all reactions were carried out at rt and were performed under a positive pressure of argon. Crushed 4 Å molecular sieves were activated by thorough flame-drying and cooled in vacuo prior to use. Flash column chromatography was performed on silica gel (200–300 mesh, Qingdao, China). Amberlite 15 (H^+ form) was used where acidic ion-exchange resin is indicated. Analytical thin layer chromatography (TLC) was performed on glass plates precoated with a 0.25-mm thickness of silica gel. ^{1}H NMR and ^{13}C NMR spectra were taken on a Jeol JNM-ECP 600 or a Bruker Avance III 400 spectrometer at rt. Chemical shifts of the ^{1}H NMR spectra are expressed in ppm relative to the solvent residual signal 7.26 in $CDCl_3$ or to tetramethylsilane ($\delta = 0.00$). Chemical shifts of the ^{13}C NMR spectra are expressed in ppm relative to the solvent signal 77.00 in $CDCl_3$ or to tetramethylsilane ($\delta = 0.00$) unless otherwise noted. COSY, HMQC, and HMBC were routinely used to definitively assign the signals of ^{1}H NMR and ^{13}C NMR spectra. Electrospray ionization (ESI) mass spectra were recorded on a Global Q-TOF mass spectrometer.

Synthesis of 3β-O-(4,6-O-benzylidene-β-D-Glucopyranosyl)-Ursolic-28-Methyl Ester (11)

Compound **6** (438.9 mg, 0.69 mmol) was dissolved in dried CH_3CN (50 mL) containing $PhCH(OCH_3)_2$ (0.208 mL, 1.39 mmol) and CSA (29.0 mg, 0.13 mmol). The reaction mixture was stirred for 2 h at rt, then neutralized with Et_3N and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH gradient elution) to give crude **11** as a white solid (323.0 mg, 65%): $R_f = 0.27$ (1:6, EtOAc/petroleum

ether); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.51–7.48 (m, 2H), 7.39–7.27 (m, 3H), 5.53 (s, 1H), 5.25 (s, 1H), 4.47 (d, 1H, J = 7.6 Hz), 4.31 (dd, 1H, J = 10.4, 4.9 Hz), 3.85–3.77 (m, 2H), 3.61 (s, 3H), 3.57–3.54 (m, 2H), 3.48–3.44 (m, 1H), 3.21 (dd, 1H, J = 11.6, 4.6 Hz), 2.81 (s, 1H), 2.51 (s, 1H), 2.23 (d, 1H, J = 11.3 Hz), 1.07, 1.03, 0.70 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 178.1, 140.0, 138.1, 129.3, 128.3, 126.3, 125.5, 105.2, 101.9, 90.0, 80.5, 75.2, 73.1, 68.8, 66.3, 55.5, 52.8, 51.5, 48.1, 47.6, 42.0, 39.5, 39.2, 38.8, 38.6, 36.7, 36.6, 32.9, 30.6, 29.7, 28.3, 28.0, 25.9, 24.2, 23.6, 23.3, 21.2, 18.1, 17.0, 16.9, 16.7, 15.4; MALDI-HRMS calcd for $C_{44}H_{64}NaO_8$ $[M+Na]^+$ 743.4493, found 743.4622.

Synthesis of 3β-O-[2-O-(2,3,4-Tri-O-benzoyl-α-L-rhamnopyranosyl)-4,6-O- benzylidene-β-D-glucopyranosyl]-ursolic-28-methyl ester (13) and 3β-O-[4-O- (2,3,4-tri-O-benzoyl-α-L-rhamnopyranosyl)-4,6-O-benzylidene-β-D-Glucopyranosyl] -Ursolic-28-Methyl Ester (14)

A mixture of **11** (100.0 mg, 0.14 mmol), **12** (103.0 mg, 0.17 mmol), and powdered 4 Å molecular sieves in dried CH_2Cl_2 (15 mL) was stirred at rt for 30 min and then cooled to 0°C. TMSOTf (4.8 μL, 27.6 μmol) was added slowly. The reaction was stirred for 1 h at 0°C. After completion of the reaction (monitored by TLC), the reaction was quenched with Et_3N, and then filtered. The filtrate was concentrated and purified by silica gel column chromatography (EtOAc/petroleum ether, 1:10–1:7) to give **13** (61.0 mg, 37%) and **14** (104.0 mg, 63%). Compound **13**: white solid; R_f = 0.24 (CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) for 13: δ 8.09 (d, 2H, J = 7.3 Hz), 7.97 (d, 2H, J = 7.3, Hz), 7.82 (d, 2H, J = 7.3 Hz), 7.60–7.21 (m, 14H), 5.90 (dd, 1H, J = 10.1, 3.1 Hz), 5.83 (s, 1H),, 5.77 (s, 1H), 5.69 (t, 1H, J = 10.1 Hz), 5.54 (s, 1H), 4.67 (d, 1H, J = 7.3 Hz), 4.58–4.55 (m, 1H), 4.34 (dd, 1H, J = 4.6 Hz), 4.08 (t, 1H, J = 8.0 Hz), 3.85 (t, 1H, J = 8.2, 7.9 Hz), 3.78 (t, 1H, J = 10.1 Hz), 3.61 (s, 3H), 3.56 (t, 1H, J = 9.5, 9.2 Hz), 3.49–3.42 (m, 1H), 3.26 (dd, 1H), 3.12 (brs, 1H), 2.24 (d, 1H, J = 11.0 Hz), 1.09, 0.75 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 178.1, 165.7, 165.6, 165.6, 138.2, 137.0, 133.4, 133.3, 133.0, 129.9, 129.7, 129.5, 129.4, 129.3, 129.2, 128.5, 128.4, 128.2, 126.2, 125.6, 114.1, 104.6, 101.8, 97.5, 89.9, 80.7, 77.7, 77.2, 75.0, 71.8, 70.6, 69.8, 68.8, 67.1, 65.7, 55.9, 52.9, 51.5, 48.1, 47.6, 42.0, 39.5, 39.2, 39.0, 39.0, 38.9, 36.7, 36.6, 33.0, 31.9, 30.6, 29.7, 29.4, 28.0, 26.3, 24.2, 23.6, 23.3, 22.7, 21.2, 18.1, 17.5, 17.0, 16.9, 16.6, 15.5, 14.1; MALDI-HRMS calcd for $C_{71}H_{86}NaO_{15}$ $[M+Na]^+$ 1201.5859, found 1201.6154. Compound **14**: white solid; R_f = 0.17 (CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) for 14: δ 8.08 (d, 2H, J = 8.3 Hz), 7.82 (d, 4H, J = 6.6 Hz), 7.60 (t, 1H, J = 7.0 Hz), 7.54–7.41 (m, 6H), 7.35 (t, 2H, J = 7.9, 7.6 Hz), 7.28–7.21 (m, 5H), 5.83 (dd, 1H, J = 10.1, 3.7 Hz), 5.73 (s, 1H), 5.64 (s, 1H), 5.58 (t, 1H, J = 10.1 Hz), 5.53 (s, 1H), 5.25 (s, 1H), 4.53 (dd, 1H, J = 9.9, 6.1 Hz), 4.47 (d, 1H, J = 7.6 Hz),

4.36 (dd, 1H, J = 10.7, 5.2 Hz), 4.01 (t, 1H, J = 9.5, 8.9 Hz), 3.86 (t, 1H, J = 10.4, 10.1 Hz), 3.79–3.71 (m, 2H), 3.61 (s, 3H), 3.54–3.46 (m, 1H), 3.21 (dd, 1H, J = 11.3, 4.6 Hz), 2.60 (d, 1H, J = 3.1 Hz), 2.23 (d, 1H, J = 11.6 Hz), 1.07, 0.74 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 178.0, 165.6, 138.1, 137.2, 133.4, 133.2, 133.0, 129.9, 129.7, 129.6, 129.5, 129.4, 129.3, 129.0, 128.5, 128.3, 128.2, 128.1, 126.1, 125.5, 105.6, 101.7, 97.6, 90.2, 79.0, 77.2, 76.4, 76.2, 71.8, 70.8, 70.1, 68.9, 66.8, 66.2, 60.4, 55.5, 52.9, 51.5, 48.1, 47.6, 42.0, 39.5, 39.0, 38.8, 38.6, 36.7, 36.6, 32.9, 30.6, 29.7, 28.3, 28.0, 25.9, 24.2, 23.6, 23.3, 21.2, 18.1, 17.0, 16.9, 16.7, 15.4, 14.2; MALDI-HRMS calcd for $C_{71}H_{86}NaO_{15}$ $[M+Na]^+$ 1201.5859, found 1201.6154.

Synthesis of 3β-O-[2-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-Ursolic- 28-Methyl Ester (4)

A mixture of **13** (81.0 mg, 0.068 mmol) and 10% Pd–C (30.0 mg) in CH_3OH/CH_2Cl_2 (15 mL, v:v = 1:1) was stirred under 1 atm of H_2 for 5 h. The reaction mixture was then filtered, and the filtrate was concentrated to dryness to give a white solid. The solid was dissolved in CH_3OH/CH_2Cl_2 (8 mL, v:v = 1:1), and then NaOMe was added until pH = 9. After stirring at 35°C for 8 h, the solution was neutralized with ion-exchange resin (H^+) and then filtered and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 10:1) to afford **4** as a white solid (33.4 mg, 62%): R_f = 0.13 (CH_2Cl_2/MeOH, 4:1); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 5.23 (s, 1H), 5.12 (brs, 1H), 4.96 (brs, 1H), 4.62 (brs, 1H), 4.55 (s, 1H), 4.45 (brs, 1H), 4.40 (brs, 1H), 4.23 (d, 1H, J = 7.4 Hz), 3.82–3.78 (m, 1H), 3.66 (s, 1H), 3.61 (d, 1H, J = 11.7 Hz), 3.49 (s, 3H), 3.46–3.44 (m, 1H), 3.28–3.16 (m, 4H), 3.04–3.01 (m, 3H), 2.12 (d, 1H, J = 8.6 Hz), 1.03 (d, 3H, J = 9.0 Hz), 1.02, 0.92, 0.89, 0.84, 0.72, 0.64 (each s, each 3H), 0.79 (d, 3H, J = 5.9 Hz); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 177.4, 138.4, 125.3, 104.3, 100.2, 88.3, 78.6, 77.0, 76.7, 72.5, 70.9, 70.8, 70.8, 68.4, 61.5, 55.7, 52.9, 51.8, 47.8, 47.4, 42.0, 39.0, 38.9, 38.7, 36.6, 33.0, 31.7, 30.4, 29.4, 29.1, 27.9, 27.8, 26.2, 24.2, 23.7, 23.3, 22.5, 21.4, 18.3, 18.1, 17.4, 17.0, 16.7, 15.7, 14.4; MALDI-HRMS calcd for $C_{43}H_{70}NaO_{12}$ $[M+Na]^+$ 801.4759, found 801.4999.

Synthesis of 2,3,4-tri-O-benzoyl-α-L-rhamnopyranosyl-(1$\rightarrow$4)-2,3,6-tri-O-benzoyl- 1-(4-tolyl) thio-β-D-glucopyranoside (16)

A mixture of **12** (700.0 mg, 1.17 mmol), **15** (871.0 mg, 1.40 mmol), and powdered 4 Å molecular sieves in dried CH_2Cl_2 (50 mL) was stirred at rt for 30 min and then cooled to 0°C. TMSOTf (20 μL, 117 μmol) was added slowly. The reaction was stirred for 1 h at 0°C. After completion of the reaction (monitored by TLC), the reaction was quenched with Et_3N and then filtered. The filtrate was concentrated and purified by silica gel column chromatography

(EtOAc/petroleum ether, 1:10) to give crude **16** as a white solid (1.1 g, 90%): R_f = 0.54 (1:3, EtOAc/petroleum ether); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.10 (d, 2H, J = 6.8 Hz), 7.94 (d, 6H, J = 7.2 Hz), 7.86–7.81 (m, 4H), 7.61–7.33 (m, 17H), 7.27–7.24 (m, 3H), 6.89 (d, 2H, J = 7.6 Hz), 5.85 (t, 1H, J = 9.6, 9.2 Hz), 5.66 (dd, 1H, J = 10.4, 3.2 Hz), 5.55–5.53 (m, 1H), 5.49 (t, 1H, J = 10.0 Hz), 5.20 (d, 1H, J = 1.2 Hz), 5.14 (dd, 1H, J = 12.4, 1.6 Hz), 4.93 (d, 1H, J = 10.0 Hz), 4.65 (dd, 1H, J = 12.4, 4.0 Hz), 4.21 (t, 1H, J = 9.6, 9.2 Hz), 4.07 (dd, 1H, J = 9.6, 2.4 Hz), 3.97–3.93 (m, 1H), 2.24 (s, 3H), 0.74 (d, 3H, J = 6.0 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 165.9, 165.6, 165.5, 165.1, 139.4, 134.9, 134.7, 134.6, 134.5, 134.3, 133.4, 133.2, 133.1, 133.0, 129.9, 129.8, 129.7, 129.5, 129.4, 128.4, 128.3, 128.2, 98.8, 85.7, 75.1, 71.3, 71.1, 70.9, 69.5, 67.8, 62.5,60.3, 21.1, 21.0, 17.1, 14.2; ESIMS: calcd for $[M+Na]^+$ m/z 1079.3; found: 1079.1.

Synthesis of 3β-O-[4-O-(2,3,4-Tri-O-benzoyl-α-L-rhamnopyranosyl)-2,3,6-tri-O- benzoyl-β-D-glucopyranosyl]-Ursolic-28-Methyl Ester (17)

A mixture of **16** (300.0 mg, 0.28 mmol), **7** (111.0 mg, 0.24 mmol), and powdered 4 Å molecular sieves in dried CH_2Cl_2 (25 mL) was stirred at rt for 30 min and then cooled to 0°C. NIS (80.0 mg, 0.35 mmol) and TfOH (8.4 μL, 94 μmol) were added. The reaction was stirred for 2 h. After completion of the reaction (monitored by TLC), the reaction mixture was filtered and concentrated. Then the residue was diluted with CH_2Cl_2 (100 mL) and washed with aqueous $Na_2S_2O_3$ (50 mL), satd aq $NaHCO_3$ (50 mL), and brine (2 × 50 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (EtOAc/petroleum ether, 1:10) to give crude **17** as a white amorphous solid (150.0 mg, 45%): R_f = 0.27 (1:6, EtOAc/petroleum ether); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.10 (d, 2H, J = 7.2 Hz), 7.97 (t, 4H, J = 7.2, 6.8 Hz), 7.91 (d, 2H, J = 6.8 Hz), 7.89 (d, 2H, J = 6.8 Hz), 7.83 (d, 2H, J = 7.2 Hz), 7.57–7.33 (m, 14H), 7.30–7.24 (m, 4H), 5.85 (t, 1H, J = 9.6, 9.2 Hz), 5.69 (dd, 1H, J = 10.0, 3.2 Hz), 5.57–5.55 (m, 1H), 5.51 (t, 1H, J = 10.0 Hz), 5.44 (t, 1H, J = 8.0, 7.6 Hz), 5.25 (t, 1H, J = 3.6, 3.2 Hz), 5.20 (d, 1H, J = 1.2 Hz), 4.97 (dd, 1H, J = 12.0, 2.0 Hz), 4.80 (d, 1H, J = 7.6 Hz), 4.73 (dd, 1H, J = 12.0, 6.0 Hz), 4.23 (t, 1H, J = 9.6, 9.2 Hz), 4.06–4.02 (m, 1H), 3.98 (dd, 1H, J = 9.6, 6.0 Hz), 3.59 (s, 3H), 3.05 (dd, 1H, J = 11.6, 4.4Hz), 2.23 (d, 1H, J = 11.2 Hz), 1.04, 0.97, 0.80, 0.57 (each s, each 3H), 0.76 (d, 3H, J = 6.0 Hz), 0.68 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 138.0, 133.5, 133.3, 133.2, 133.1, 133.0, 130.0, 129.9, 129.7, 129.4, 129.3, 129.2, 129.1, 128.5, 128.4, 128.3, 128.3, 125.6, 103.1, 98.7, 90.3, 73.9, 73.3, 72.6, 71.3, 71.0, 69.5, 67.7, 62.7, 55.4, 53.4, 52.9, 51.5, 48.1, 47.5, 41.9, 39.4, 39.1, 38.9, 38.7, 38.4, 36.6, 36.5, 32.9, 31.9, 30.7, 29.7, 29.7, 29.4, 28.0,

27.6, 25.7, 24.2, 23.6, 23.2, 22.7, 21.2, 18.0, 17.0, 16.8, 16.1, 15.3, 14.1; ESIMS: calcd for $[M+K]^+$ m/z 1441.6; found: 1442.1.

Synthesis of 3β-O-[4-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-Ursolic-28-Methyl Ester (5)

Compound **17** (105.0 mg, 0.075 mmol) was dissolved in CH_3OH/CH_2Cl_2 (10 mL, v:v = 1:1), and then NaOMe was added until pH = 9. After stirring at 35°C for 8 h, the solution was neutralized with ion-exchange resin (H^+) and then filtered and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 10:1–4:1) to afford **5** as a white solid (53.0 mg, 91%): $R_f = 0.29$ (CH_2Cl_2/MeOH, 4:1); ^{1}H NMR (CD_3OD, 400 MHz): δ 5.23 (t, 1H, $J = 3.5$, 3.1 Hz), 4.85 (d, 1H, $J = 1.6$ Hz), 4.33 (d, 1H, $J = 7.8$ Hz), 3.98–3.94 (m, 1H), 3.83–3.77 (m, 2H), 3.67–3.60 (m, 2H), 3.59 (s, 3H), 3.52 (t, 1H, $J = 9.4$, 9.0 Hz), 3.45–3.37 (m, 2H), 3.22 (t, 1H, $J = 8.6$, 8.2 Hz), 3.17 (dd, 1H, $J = 11.7$, 9.3 Hz), 2.22 (d, 1H, $J = 11.3$ Hz), 1.26 (d, 3H, $J = 5.9$ Hz), 1.11, 1.05, 0.84, 0.79 (each s, each 3H), 0.96 (s, 6H), 0.88 (d, 3H, $J = 6.3$ Hz); ^{13}C NMR (CD_3OD, 100 MHz): δ 177.1, 138.1, 128.5, 105.4, 100.7, 88.2, 75.5, 74.4, 72.1, 70.9, 70.8, 68.8, 60.4, 55.1, 52.6, 51.5, 47.6, 47.1, 41.7, 38.9, 38.6, 38.5, 36.4, 32.7, 30.1, 29.2, 28.9, 27.8, 25.7, 23.9, 23.5, 23.0, 21.2, 17.9, 17.2, 16.8, 16.7, 15.4; MALDI-HRMS calcd for $C_{43}H_{70}NaO_{12}$ $[M+Na]^+$ 801.4759, found 801.5149.

Plasmids and Cell Lines

A codon-optimized HA gene is from A/Viet Nam/1203/2004 (H5N1) and was cloned into pcDNA3. NA A/PR/8/34 influenza virus (H1N1) in the vector pEF6/V5-His-TOPO was kindly provided by John C. Olsen (University of North Carolina, Chapel Hill).

A549 and 293T cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 μg/mL streptomycin and 100 units/mL penicillin (GIBCO) at 37°C, 5% CO_2.

Production of HA/HIV Pseudovirions

Human embryonic kidney 293T cells were transiently cotransfected with 8 μg hemagglutinin envelope expression plasmid with 5 μg NA and 10 μg Env-deficient HIV vector (pNL4–3-Luc-R^-E^-) in 100-mm plates by a standard $Ca_3(PO_4)_2$ protocol. Sixteen hours posttransfection, cells were washed by PBS without Ca^{2+} and Mg^{2+}, and then 10 mL fresh medium was added into each plate. Forty-eight hours posttransfection, the supernatants were collected and

filtered through a 0.45-micron pore size filter (Nalgene) and the pseudovirions were directly used for infection.

Infection Assay of HA/HIV Pseudovirions

To investigate the effect of the test compounds, A549 cells were seeded into 24-well plates at 50,000 cells per well and tested compounds were added to each well incubating for 15 min followed by adding HA/HIV or VSVG/HIV pseudovirions to each well and incubating at 37°C. Cells were lysed in 50 μL of cell culture lysis reagent (Promega) 48 h postinfection. The luciferase activity was measured with a luciferase assay kit (Promega) and an FB15 luminometer (Berthold detection system) according to the supplier's protocols.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (81072525 and 30701035), the central level, scientific research institutes for basic R & D special fund business (2010ZD03 and 2011ZD06) and Shanghai outstanding academic leaders program (11XD1400200). Sumei Ren and Qing Chen contributed equally to this work.

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