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Design, Synthesis and Biological Evaluation of Pentacyclic Triterpene Dimers as HCV Entry Inhibitors

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A series of triterpene dimers bearing different scaffold were designed and synthesized via CuAAC reaction. Their anti-HCV entry activities were evaluated by HCVpp and VSVpp entry assays. It was found that echinocystic acid (EA) and its dimer were still necessary for maintaining anti-HCV entry activity, and replacement of EA by other triterpenes might significantly decrease its anti-viral activities. Using a linker bearing a piperazine group, compound **14** dramatically increased its potency with IC_{50} at 2.87 nmol/L. In addition, the undesired hemolytic effect of all these compounds was removed.

Keywords pentacyclic triterpene, echinocystic acid, dimer, HCV entry inhibitor

Introduction

Hepatitis C virus (HCV) infection is the leading cause of liver fibrosis and cirrhosis that eventually lead to liver carcinomas.[1] There are more than 170 million people infected with HCV worldwide.[2,3] Several direct anti-viral agents targeting the NS3-4A protease (boceprevir, telaprevir and simeprevir) and the NS5B polymerase (sofosbuvir) were approved by FDA for treating patients infected with HCV.[4-6] However, as a kind of RNA viruses, the genome of HCV bares high mutation rate, which can cause antiviral drugs targeting viral replication to lose activities rapidly.[7,8]

The upstream of virus replication, involving the interaction between virus and host cells, provides a new opportunity for the research and development of antiviral drugs.[9-12] Our previous studies suggested that echinocystic acid (EA), a natural product of oleanane-type triterpenes first isolated from *Echinocystis fabacea* in 1934,[13,14] displays moderate anti-HCV activity (IC_{50}= 1.4 μmol/L) by interruption of the interaction between one HCV envelope protein, E2, and its host receptor CD81, thus blocking entry of the virus into the host cell. The formation of EA dimer via a linker bearing triazole (Figure 1), dramatically increased its potency with IC_{50} at 3.04 nmol/L.[15-19]

However, it is still unknown whether EA moieties of compound **1** for maintaining its high activity was necessary or not, and further structure-activity relationship (SAR) of these tritepene dimers as HCV entry inhibitors was needed to explore. In this paper, as a continuation of our ongoing research in the development of novel anti-HCV entry compounds, we report the design and synthesis of a series of dimers bearing different triterpenes as pharmacophore, and their anti-HCV entry activities were evaluated. This study improved the importance of triterpene dimers as new leads for developing novel HCV entry inhibitors.

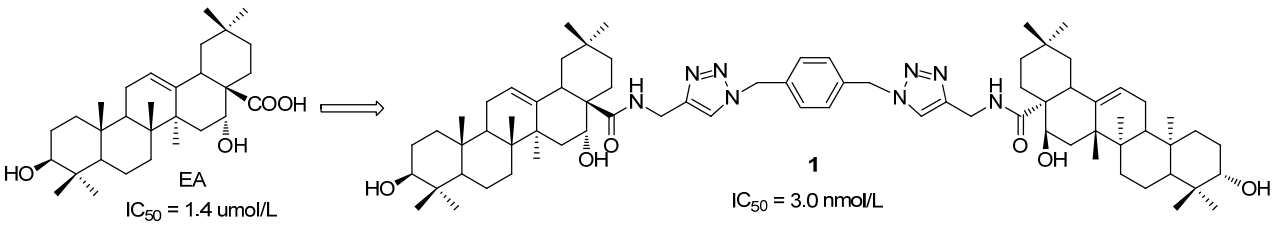

Figure 1 Structures of EA, compound **1** and their anti-HCV activities.

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Experimental

Routine ^{1}H NMR and ^{13}C NMR spectra were recorded. Samples were dissolved in $CDCl_3$ or CD_3OD and tetramethylsilane (TMS) was used as reference. High Resolution Mass Spectra (HRMS) were obtained with an APEX IV FT_MS (7.0 T) spectrometer (Bruker) in positive ESI mode. NMR spectra were recorded at 400 and 100 MHz for ^{1}H and ^{13}C nuclei recorded on a Bruker DRX 400 spectrometer (Rheinstetten, Germany) at ambient temperature. Chemical shifts are reported in parts per million relative to the tetramethylsilane peak recorded as δ 0.00 in $CDCl_3$/TMS solvent, or the residual chloroform (δ 7.26) or methanol (δ 3.31) peaks. The ^{13}C NMR values were referenced to the residual chloroform (δ 77.0), or methanol (δ 49.0) peaks. Melting points were determined with an X-4 apparatus and uncorrected. Optical rotations were measured with a Rudolph Autopol VI polarimeter. Analytical TLC was performed on Merck silica gel 60 F254. Compounds were visualized by staining with a yellow solution containing $Ce(NH_4)_2(NO_3)_6$ (0.5 g) and $(NH_4)_6Mo_7O_{24}$-$4H_2O$ (24.0 g) in 6% H_2SO_4 (500 mL) followed by heating. Chemicals for the synthesis were analytical grade. Echinocystic acid was isolated from *Gleditsia sinensis* Lam, which was characterized by comparing their spectral data with those reported in the literature.

General procedure A for the CuAAC "click" reaction $CuSO_4$ (1 equiv.) and Na-*L*-ascorbate (1.5 equiv.) were added to a solution of compound **3** and the corresponding azide in CH_2Cl_2 (5 mL) and H_2O (5 mL). The resulting solution was stirred vigorously for 12 h at r.t. The reaction mixture was diluted with H_2O (10 mL), then extracted with CH_2Cl_2 (10 mL×3). The combined organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography.

Compound 3 Synthesized using methods published previously.[18] m.p. 201.3－23.7 ℃. $[\alpha]_D^{25}$ 60.80 (c＝0.50, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.78, 0.79, 0.91, 0.99, 1.17 (7×CH_3), 0.72－2.04 (m, other aliphatic ring protons), 2.22 (t, J＝2.5 Hz, 1H), 2.54 (dd, J＝3.2, 13.0 Hz, 1H), 3.21 (dd, J＝4.4, 10.7 Hz, 1H), 3.87－3.93 (m, 1H), 4.02－4.08 (m, 1H), 5.42 (brs, 1H), 6.13 (t, J＝4.7 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.3, 15.5, 16.9, 18.2, 23.4, 23.5, 23.8, 25.7, 27.1, 27.2, 28.0, 29.2, 30.6, 32.1, 32.2, 32.9, 34.0, 36.8, 38.4, 38.7, 39.3, 41.9, 42.0, 46.2, 46.6, 47.4, 55.0, 71.5, 78.8, 79.6, 123.1, 144.6, 178.0.

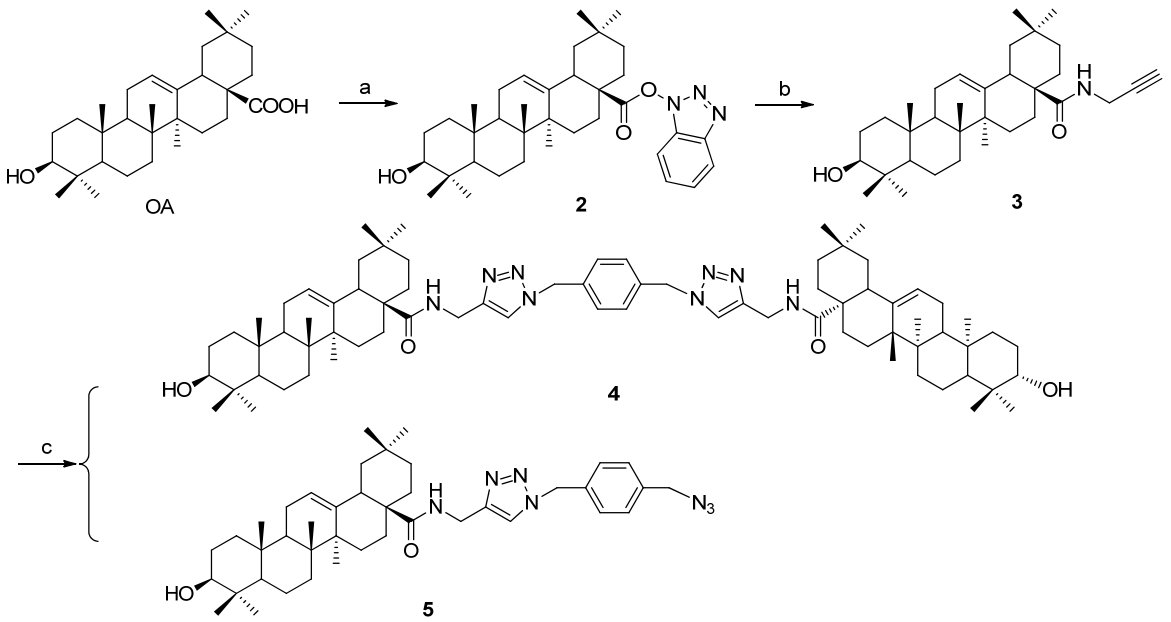
Compound 4 Prepared from **3** (108 mg, 0.22 mmol) and *p*-xylylene diazide (19 mg, 0.1 mmol) according to general procedure A. The product was purified by column chromatography [V(CH_2Cl_2)/V(MeOH)＝25/1] to give **4** as a white solid (99 mg, 84%). m.p. 206.3－208.7 ℃. $[\alpha]_D^{25}$ 43.20 (c＝0.50, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ: 0.46, 0.79, 0.88, 0.91, 0.92, 0.98, 1.14 (14×CH_3), 0.70－2.06 (m, other aliphatic ring protons), 2.76 (dd, J＝3.2, 13.3 Hz, 2H), 3.14 (dd, J＝4.7, 10.8 Hz, 2H), 4.35 (brs, 4H), 5.33 (brs, 2H), 5.55 (dd, J＝15.3, 17.0 Hz, 4H) 7.33 (s, 4H), 7.78 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ: 16.09 (2C), 16.52 (2C), 17.60 (2C), 19.53 (2C), 24.12 (4C), 24.53 (2C), 26.57 (2C), 27.88 (2C), 28.43 (2C), 28.89 (2C), 31.62 (2C), 33.66 (2C), 33.78 (2C), 34.10 (2C), 35.13 (2C), 35.85 (2C), 38.10 (2C), 39.85 (2C), 40.57 (2C), 42.54 (2C), 42.86 (2C), 47.44 (2C), 47.65 (2C), 48.93 (2C), 49.00 (2C), 54.37 (2C), 56.66 (2C), 79.58 (2C), 124.15 (2C), 124.57 (2C), 129.81 (2C), 137.07 (2C), 145.00 (2C), 146.55 (2C), 180.14 (2C). ESI-HRMS calcd for $C_{74}H_{111}N_8O_4$ $[M+H]^+$ 1175.8707, found 1175.8723.

Compound 5 Obtained from Scheme 1 as a by-product. m.p. 128.9－131.4 ℃. $[\alpha]_D^{25}$ 37.60 (c＝0.50, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.49, 0.78, 0.86, 0.87, 0.89, 0.98, 1.13 (7×CH_3), 0.69－2.16 (m, other aliphatic ring protons), 2.54 (dd, J＝3.1, 13.3 Hz, 1H), 3.20 (dd, J＝4.4, 10.1 Hz, 1H), 4.32－4.37 (m, 3H), 4.45－4.50 (m, 1H), 5.38 (brs, 1H), 5.44－5.54 (m, 2H), 6.66 (t, J＝5.2 Hz, 1H), 7.27－7.33 (m, 4H), 7.52 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.3, 15.5, 16.4, 18.2, 23.3, 23.5, 23.7, 25.6, 27.0, 27.1, 28.0, 30.6, 32.2, 32.3, 32.9, 34.0, 34.9, 36.8, 38.3, 38.6, 39.2, 41.8, 41.9, 46.1, 46.5, 47.4, 53.6, 54.1, 55.0, 78.7, 122.4, 123.1, 128.4 (2C), 128.7 (2C), 134.6, 136.0, 144.1, 145.1, 178.3.

Compound 6c Obtained according to general procedure A. The product was purified by column chromatography [V(CH_2Cl_2)/V(MeOH)＝25/1] to give **6c** as a white solid (93 mg, 79%). m.p. 193.1－195.7 ℃. $[\alpha]_D^{25}$ 33.60 (c＝0.50, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ: 0.53, 0.78, 0.85, 0.87, 0.93, 0.99, 1.07 (14×CH_3), 0.69－2.02 (m, other aliphatic ring protons), 3.21 (dd, J＝3.6, 10.2 Hz, 2H), 4.32 (dd, J＝5.2, 15.0 Hz, 2H), 4.45 (dd, J＝5.3, 15.0 Hz, 2H), 5.33 (brs, 2H), 5.47 (brs, 4H), 6.67 (t, J＝5.1 Hz, 2H), 7.25 (s, 4H), 7.48 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ: 15.4 (2C), 15.6 (2C), 16.4 (2C), 17.1 (2C), 18.1 (2C), 21.1 (2C), 23.1 (2C), 23.2 (2C), 24.7 (2C), 27.1 (2C), 27.6 (2C), 28.0 (2C), 30.7 (2C), 32.6 (2C), 34.8 (2C), 36.8 (2C), 36.9 (2C), 38.5 (2C), 38.6 (2C), 38.8 (2C), 39.3 (2C), 39.6 (2C), 42.2 (2C), 47.4 (2C), 47.5 (2C), 53.4 (2C), 55.0 (2C), 78.7 (2C), 122.3 (2C), 126.0 (2C), 128.6 (4C), 135.1 (2C), 139.0 (2C), 145.1 (2C), 178.1 (2C). ESI-HRMS calcd for $C_{74}H_{111}N_8O_4$ $[M+H]^+$ 1175.8691, found 1175.8723.

Compound 7c Obtained according to general procedure A. The product was purified by column chromatography [V(CH_2Cl_2)/V(MeOH)＝25/1] to give **7c** as a white solid (83 mg, 71%). m.p. 177.6－179.3 ℃. $[\alpha]_D^{25}$ –2.40 (c＝0.50, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ: 0.75, 0.76, 0.80, 0.93, 0.96, 1.66 (12×CH_3), 0.65－1.99 (m, other aliphatic ring protons), 2.33－2.38 (m, 2H), 3.04－3.10 (m, 2H), 3.18 (dd, J＝5.2, 10.8 Hz, 2H), 4.35－4.47 (m, 4H), 4.58 (s, 2H), 4.71 (s, 2H), 5.47 (dd, J＝15.0, 47.3 Hz, 4H), 6.71 (t, J＝5.6 Hz, 2H), 7.25 (s, 4H), 7.56 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ: 14.5 (2C), 15.4 (2C), 15.7 (2C), 16.1 (2C),

Scheme 1



Reagents and conditions: (a) TBTU, DIEA, THF, r.t., 4 h; (b) 2-propynylamine, Na_2CO_3, DMF, r.t., 20 min; (c) $CuSO_4$, Na-*L*-ascorbate, 1,4-bis(azidomethyl)benzene, CH_2Cl_2/H_2O, r.t., overnight.

18.2 (2C), 19.3 (2C), 20.8 (2C), 25.5 (2C), 27.3 (2C), 27.9 (2C), 29.2 (2C), 30.7 (2C), 33.3 (2C), 34.2 (2C), 34.5 (2C), 37.1 (2C), 37.6 (2C), 38.1 (2C), 38.6 (2C), 38.7 (2C), 40.6 (2C), 42.3 (2C), 46.6 (2C), 50.0 (2C), 50.5 (2C), 53.5 (2C), 55.2 (2C), 55.5 (2C), 78.7 (2C), 109.3 (2C), 122.5 (2C), 128.6 (4C), 135.1 (2C), 145.8 (2C), 150.8 (2C), 176.5 (2C). ESI-HRMS calcd for $C_{74}H_{111}N_8O_4$ $[M+H]^+$ 1175.8691, found 1175.8703.

Compound 8b Starting from compound **8a** (251 mg, 0.5 mmol), compound **8b** was synthesized using methods published previously.[18] The product was purified by column chromatography [*V*(PE)/*V*(EtOAc)=3/1] to give **8b** as a white solid (239 mg, 88%). m.p. 195.1－196.8 ℃. $[\alpha]_D^{25}$ 68.40 (*c*=0.50, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.92, 1.03, 1.08, 1.27, 1.44, 1.49 (s, 7×CH_3), 1.19－2.33 (m, other aliphatic ring protons), 2.54－2.67 (m, 2H), 2.74－2.77 (m, 1H), 2.87 (d, *J*=4.2 Hz, 1H), 3.93－4.05 (m, 2H), 4.56 (br, 1H), 6.30 (t, *J*=3.0, 5.3 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.6, 16.5, 20.4, 22.9, 24.1, 25.9, 29.3, 30.3, 30.9, 31.8, 31.9, 32.0 (2C), 32.9, 35.2, 36.0 (2C), 37.7, 38.7, 39.5, 41.2, 41.5, 48.7 (2C), 51.5, 53.9, 71.0, 74.3, 79.9, 85.9, 174.7, 176.7, 211.1.

Compound 8c Obtained according to general procedure A. The product was purified by column chromatography [*V*(CH_2Cl_2)/*V*(MeOH)=25/1] to give **8c** as a white solid (83 mg, 41.4%). m.p. 213.6－216.1 ℃. $[\alpha]_D^{25}$ 36.80 (*c*=0.50, MeOH). ^{1}H NMR (400 MHz, Pyr-d_5) δ: 0.98, 0.99, 1.06, 1.20, 1.33, 1.40, 1.64 (s, 14×CH_3), 1.23－2.71 (m, other aliphatic ring protons), 3.49 (d, *J*=4.0 Hz, 2H), 3.56－3.65 (m, 2H), 4.75－4.92 (m, 4H), 5.25 (br, 2H), 5.68 (d, *J*=15.2 Hz, 2H), 5.73 (d, *J*=15.2 Hz, 2H), 7.32 (s, 4H), 8.21 (s, 2H), 9.29 (t, *J*=5.1 Hz, 2H); ^{13}C NMR (100 MHz, Pyr-d_5) δ: 15.2 (2C), 16.4 (2C), 20.3 (2C), 22.9 (2C), 24.4 (2C), 25.8 (2C), 30.8 (2C), 30.9 (2C), 31.7 (2C), 32.1 (2C), 32.1 (2C), 33.5 (2C), 35.6 (2C), 36.5 (2C), 36.6 (2C), 37.8 (2C), 39.0 (2C), 39.4 (2C), 41.3 (2C), 41.9 (2C), 48.8 (2C), 49.1 (2C), 51.9 (2C), 53.1 (2C), 53.5 (2C), 74.0 (2C), 85.1 (2C), 123.5 (2C), 128.5 (4C), 136.2 (2C), 146.7 (2C), 173.9 (2C), 177.6 (2C), 210.8 (2C). ESI-HRMS calcd for $C_{74}H_{107}N_8O_{10}$ $[M+H]^+$ 1267.8095, found 1267.8105.

Compound 9b Starting from compound **9a** (200 mg, 0.4 mmol), compound **9b** was synthesized using methods published previously.[18] The product was purified by column chromatography [*V*(PE)/*V*(EtOAc)=3/1] to give **9b** as a white solid (179 mg, 83%). m.p. 143.2－145.3 ℃. $[\alpha]_D^{25}$ 55.10 (*c*=0.50, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.72, 0.76, 0.83, 0.94, 0.94, 0.98, 1.11, 1.14 (s, 7×CH_3), 0.69－1.91 (m, other aliphatic ring protons), 2.24 (t, *J*=2.4 Hz, 1H), 2.29 (d, *J*=13.8 Hz, 1H), 2.56－2.72 (m, 2H), 2.97－3.11 (m, 1H), 3.21 (dd, *J*=4.5, 11.5 Hz, 1H), 3.96－4.09 (m, 2H), 4.12－4.20 (m, 1H), 4.82 (d, *J*=5.5 Hz, 1H), 6.77 (t, *J*=5.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.3, 16.4, 17.3, 17.9, 19.8, 25.3, 26.9, 27.5, 27.8, 29.3, 29.5, 30.8, 32.2, 33.3, 33.8, 36.2, 36.8, 37.7, 38.7, 38.8 (2C), 43.6, 44.5, 49.1, 49.5, 54.9, 71.3, 71.5, 75.6, 78.3, 79.8, 176.0, 177.2.

Compound 9c Obtained according to general procedure A. The product was purified by column chromatography [*V*(CH_2Cl_2)/*V*(MeOH)=25/1] to give **9c** as a white solid (69 mg, 54%). m.p.>250 ℃. $[\alpha]_D^{25}$ 41.50 (*c*=0.50, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ: 0.76, 0.85, 0.87, 0.89, 0.93, 0.98, 1.14 (s, 14×CH_3), 0.71－1.90 (m, other aliphatic ring protons) 2.24 (d, *J*=14.0 Hz, 2H), 2.67 (dd, *J*=13.6, 9.8 Hz, 2H), 2.83－2.88 (m, 2H), 3.15 (dd, *J*=9.4, 6.5 Hz, 2H), 4.18 (dd, *J*=7.4, 3.3 Hz, 2H), 4.37 (d, *J*=15.2 Hz, 2H), 4.43 (d, *J*=15.1 Hz,

2H), 4.98 (d, J=6.2 Hz, 2H), 5.57 (s, 4H), 7.33 (s, 4H), 7.79 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ: 16.2 (2C), 16.8 (2C), 17.7 (2C), 19.1 (2C), 19.5 (2C), 26.6 (2C), 27.8 (2C), 28.5 (2C), 29.3 (2C), 30.7 (2C), 31.9 (2C), 33.1 (2C), 35.2 (2C), 35.6 (2C), 36.0 (2C), 37.2 (2C), 38.0 (2C), 38.2 (2C), 40.0 (2C), 40.0 (2C), 40.1 (2C), 44.3 (2C), 45.8 (2C), 50.2 (2C), 50.8 (2C), 54.4 (2C), 56.2 (2C), 72.9 (2C), 78.0 (2C), 79.1 (2C), 129.8 (4C), 137.2 (2C), 178.9 (2C). ESI-HRMS calcd for $C_{74}H_{111}N_8O_{10}$ $[M+H]^+$ 1271.8398, found 1271.8418.

Compound 10b Starting from compound **10a** (195 mg, 0.4 mmol), compound **10b** was synthesized using methods published previously.[21] The product was purified by column chromatography [V(PE)/V(EtOAc)=3/1] to give **10b** as a white solid (189 mg, 90%). m.p. 158.9－160.3 ℃. $[\alpha]_D^{25}$ 51.70 (c=0.50, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ: 0.74, 0.85, 0.89, 0.96, 0.97, 1.04, 1.08 (s, 7×CH_3), 0.71－2.06 (m, other aliphatic ring protons), 2.27 (d, J=13.8 Hz, 1H), 2.56 (s, 1H), 2.65 (dd, J=13.1, 11.3 Hz, 1H), 2.69－2.80 (m, 1H), 3.15 (dd, J=8.4, 7.6 Hz, 1H), 3.82－3.95 (m, 1H), 4.02－4.15 (m, 1H), 5.03 (d, J=6.7 Hz, 1H), 8.18 (t, J=5.1 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ: 16.1, 16.3, 17.2, 18.2, 19.1, 22.5, 23.7, 27.7, 28.5, 29.6, 29.6, 31.3, 32.3, 33.8, 34.4, 35.2, 35.6, 36.6, 38.1, 39.8, 39.9, 39.9, 44.4, 45.7, 47.8, 50.4, 55.8, 71.8, 78.0, 79.1, 81.2, 178.8, 179.2.

Compound 10c Obtained according to general procedure A. The product was purified by column chromatography [V(CH_2Cl_2)/V(MeOH)=25/1] to give **10c** as a white solid (68 mg, 59%). m.p. 199.1－201.5 ℃. $[\alpha]_D^{25}$ 35.90 (c=0.50, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ: 0.71, 0.76, 0.80, 0.87, 0.95, 0.97, 1.01 (s, 14×CH_3), 0.68－2.03 (m, other aliphatic ring protons), 2.22 (d, J=13.8 Hz, 2H), 2.55 (dd, J=13.3, 11.0 Hz, 2H), 2.66－2.75 (m, 2H), 3.14 (dd, J=9.3, 6.9 Hz, 2H), 4.38 (d, J=15.0 Hz, 2H), 4.44 (d, J=15.0 Hz, 2H), 4.90 (d, J=6.8 Hz, 2H), 5.58 (s, 4H), 7.35 (s, 4H), 7.85 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ: 16.3 (2C), 16.4 (2C), 17.2 (2C), 17.6 (2C), 19.1 (2C), 22.5 (2C), 23.8 (2C), 27.7 (2C), 28.6 (2C), 29.6 (2C), 31.3 (2C), 32.3 (2C), 33.8 (2C), 34.6 (2C), 35.2 (2C), 35.5 (2C), 35.7 (2C), 36.7 (2C), 38.2 (2C), 39.8 (2C), 39.9 (2C), 44.4 (2C), 45.6 (2C), 47.8 (2C), 50.4 (2C), 54.3 (2C), 55.8 (2C), 77.9 (2C), 79.1 (2C), 124.7 (2C), 129.8 (4C) (2C), 137.2 (2C), 146.8 (2C), 178.7 (2C), 179.3 (2C). ESI-HRMS calcd for $C_{74}H_{111}N_8O_8$ $[M+H]^+$ 1239.8501, found 1239.8519.

Compound 11b Starting from compound **11a** (207 mg, 0.4 mmol), compuond **11b** was synthesized using methods published previously.[18] The product was purified by column chromatography [V(PE)/V(EtOAc)=3/1] to give **11b** as a white solid (168 mg, 76%). m.p. 171.4－173.5 ℃. $[\alpha]_D^{25}$ 63.90 (c=0.50, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.94, 0.95, 1.11, 1.15, 1.40, 1.50, (s, 7×CH_3), 1.01－1.96 (m, other aliphatic ring protons), 2.21－2.31 (m, 2H), 2.45－2.55 (m, 1H), 2.61－2.79 (m, 3H), 3.00－3.10 (m, 1H), 3.96－4.10 (m, 2H), 4.12－4.20 (m, 1H), 4.89 (d, J=5.6 Hz, 1H), 6.76 (t, J=5.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 16.2, 19.4, 19.5, 23.0, 25.7, 26.9, 27.2, 29.4, 29.5, 30.5, 31.9, 32.1, 32.2, 33.4, 36.1, 36.7, 37.4, 40.5, 40.8, 43.7, 44.2, 49.5 (2C), 52.0, 71.2, 71.6, 75.6, 79.7, 85.3, 174.7, 175.4, 177.0.

Compound 11c Obtained according to general procedure A. The product was purified by column chromatography [V(CH_2Cl_2)/V(MeOH)=25/1] to give **11c** as a white solid (38 mg, 29%). m.p.＞250 ℃. $[\alpha]_D^{25}$ 41.70 (c=50, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ: 0.86, 0.92, 0.93, 1.09, 1.15, 1.39, 1.50, (s, 7×CH_3), 1.00－1.99 (m, other aliphatic ring protons), 2.24 (d, J=14.9 Hz, 2H), 2.49－2.35 (m, 2H), 2.71－2.93 (m, 6H), 4.18 (dd, J=7.3, 3.3 Hz, 2H), 4.38 (d, J=15.2 Hz, 2H), 4.43 (d, J=15.1 Hz, 2H), 5.03 (d, J=6.3 Hz, 2H), 5.57 (s, 4H), 7.32 (s, 4H), 7.81 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ: 16.8 (2C), 19.3 (2C), 20.1 (2C), 24.2 (2C), 26.6 (2C), 27.5 (2C), 29.2 (2C), 30.7 (2C), 30.7 (2C), 32.9 (2C), 33.0 (2C), 33.1 (2C), 33.4 (2C), 35.6 (2C), 35.9 (2C), 37.2 (2C), 37.9 (2C), 38.2 (2C), 41.7 (2C), 42.1 (2C), 44.6 (2C), 45.5 (2C), 50.5 (2C), 50.8 (2C), 52.6 (2C), 54.3 (2C), 72.9 (2C), 77.9 (2C), 87.4 (2C), 124.3 (2C), 129.7 (4C), 137.2 (2C), 146.9 (2C), 177.6 (2C), 178.5 (2C), 179.5 (2C). ESI-HRMS calcd for $C_{74}H_{107}N_8O_{12}$ $[M+H]^+$ 1299.7970, found 1299.8003.

Compound 13 Starting from **5** (50 mg, 0.07 mmol) and EA-propargylamine conjugate (45 mg, 0.09 mmol) according to general procedure A. The product was purified by column chromatography [V(CH_2Cl_2)/V(MeOH)=25/1] to give **13** as a white solid (60 mg, 69%). m.p. 182.1－183.7 ℃. $[\alpha]_D^{25}$ 30.40 (c=0.50, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.50, 0.78, 0.86, 0.87, 0.87, 0.88, 0.89, 0.90, 0.99, 0.99, 1.14 (14×CH_3), 0.69－2.05 (m, other aliphatic ring protons), 2.24 (t, J=13.3 Hz, 1H), 2.54 (dd, J=3.7, 13.6 Hz, 1H), 2.69 (dd, J=2.5, 13.2 Hz, 1H), 3.20－3.23 (m, 2H), 4.32－4.50 (m, 5H), 5.38－5.55 (m, 6H), 6.68 (t, J=5.2 Hz, 1H), 6.90 (t, J=5.1 Hz, 1H), 7.25 (s, 4H), 7.43 (s, 1H), 7.49 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.3, 15.6 (3C), 16.4, 16.6, 18.2 (2C), 23.3, 23.4, 23.5, 23.7, 24.9, 25.7, 26.7, 27.1 (3C), 28.0, 29.7, 30.2, 30.6, 32.2, 32.4, 32.5 (2C), 32.9, 34.0, 34.9 (2C), 35.1, 35.2, 36.8, 38.4, 38.5, 38.7, 39.2, 39.4, 41.5, 41.7, 41.8, 41.9, 46.1, 46.5, 46.6, 46.9, 47.4, 48.8, 53.5 (2C), 55.0, 55.1, 75.0, 78.7, 78.8, 122.3, 122.6, 123.1, 123.4, 128.7 (2C), 128.7 (2C), 135.2 (2C), 143.6, 144.1, 145.2, 145.3, 178.0, 178.5. ESI-HRMS calcd for $C_{74}H_{111}N_8O_5$ $[M+H]^+$ 1191.8645, found 1191.8672.

Compound 14 Obtained according to general procedure A. The product was purified by column chromatography [V(CH_2Cl_2)/V(MeOH)=25/1] to give **14** as a white solid (66 mg, 53%). m.p. 185.4－187.7 ℃. $[\alpha]_D^{25}$ 20.80 (c=0.50, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.58, 0.78, 0.89, 0.90, 0.92, 0.99, 1.36 (14×CH_3), 0.71－2.11 (m, other aliphatic ring protons), 2.25 (t, J=13.3 Hz, 2H), 2.50 (brs, 8H), 2.74 (dd, J=

3.4, 13.6 Hz, 2H), 2.81 (t, J=6.2 Hz, 4H), 3.21 (dd, J=2.8, 10.1 Hz, 2H), 2.32−4.49 (m, 10H), 5.53 (t, J=3.0 Hz, 2H), 6.92 (t, J=5.3 Hz, 2H), 7.61 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.6 (2C), 15.6 (2C), 16.7 (2C), 18.2 (2C), 23.3 (2C), 25.0 (2C), 26.8 (2C), 27.1 (2C), 28.0 (2C), 29.8 (2C), 30.2 (2C), 32.5 (2C), 32.6 (2C), 35.1 (2C), 35.3 (2C), 36.9 (2C), 38.5 (2C), 38.7 (2C), 39.5 (2C), 41.5 (2C), 41.7 (2C), 46.6 (2C), 46.8 (2C), 47.6 (2C), 48.9 (2C), 52.9 (4C), 55.1 (2C), 57.3 (2C), 75.2 (2C), 78.8 (2C), 122.7 (2C), 123.5 (2C), 143.5 (2C), 144.4 (2C), 177.9 (2C). ESI-HRMS calcd for $C_{74}H_{119}N_{10}O_6$ $[M+H]^+$ 1243.9278, found 1243.9285.

HCV and VSV pseudovirus entry assay

All compounds were tested by using the HCVpp and VSVpp entry assay as described. Briefly, pseudotyped viruses were produced by cotransfecting 293T cells with plasmid encoding HCV E1, E2 or vesicular stomatitis G protein (VSVG) and the envelope and Vpr deficient HIV vector carrying a luciferase reporter gene inserted into the Nef position 72 h after transfection, HCVpp or VSVpp was harvested from the supernatant of the transfected cells. For compound library screening, infections were performed in 96-well plates by adding diluted HCVpp or VSVpp into 5×10^3 Huh-7 cells/well in the presence or absence of test compounds, followed by incubation for 72 h at 37 ℃. Luciferase activity, reflecting the degree of the pseudoparticles into host cells, was measured 3 days after infection using the Bright-Glo Reagent (Promega). Test compounds were serially diluted to give a final concentration of 1 μmol/L and 5 μmol/L in 1% dimethyl sulfoxide (DMSO). Maximum activity (100% of control) and background were derived from control wells containing DMSO alone or from uninfected wells, respectively. The individual signals in each of the compound test wells were then divided by the averaged control values (wells lacking inhibitor) after background subtraction, and multiplied by 100% to determine percent activity. The corresponding inhibition values were then calculated by subtracting this value from 100%. The specificity of the compounds for inhibiting HCV was determined by evaluating inhibition of VSVpp infection in parallel. Each sample was done in duplicate, and experiments were repeated at least three times.

Hemolytic assay

Hemolytic activity was measured as follows: 2% rabbit red blood cells in erythrocyte buffer (130 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4) were incubated with serially diluted compounds. After incubating for 60 min at 37 ℃, hemolysis was monitored by measuring absorption at 540 nm with a microplate reader Infinite M200 Pro. Percentage of hemolysis was then calculated as the routine method.

Results and Discussion

As shown in Scheme 1, the 28-COOH of oleanolic acid (OA) was firstly activated by TBTU (**2**) then reacted with 2-propynylamine to give compound **3** with high yield.[20] OA dimer (**4**) was prepared from compound **3** according to our reported method, which underwent a 'click reaction' with 1,4-bis(azidomethyl)-benzene in CH_2Cl_2/H_2O (V/V=1/1) in the presence of a catalytic amount of copper sulfate and sodium ascorbate.[15,16] By the same strategy, a series of dimers bearing different triterpene scaffolds, such as ursolic acid (UA), betulinic acid (BA) *etc*. (compounds **6c**−**11c**), were designed and synthesized (Table 1).

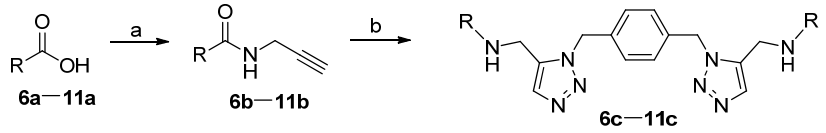
To further investigate whether the EA moiety of compound **1** can be replaced by OA or not, compound **13**, with half OA and half EA, was synthesized and its anti HCV entry activity was also evaluated (Scheme 2).

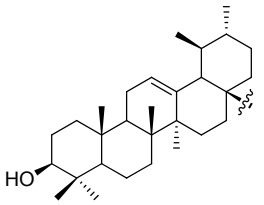
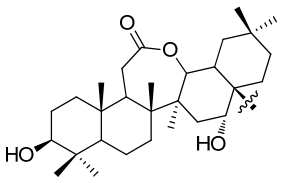
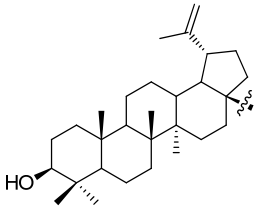
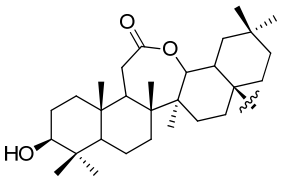
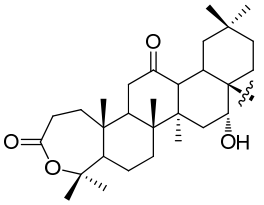
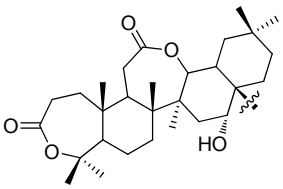
As a preliminary screen for anti-HCV entry activity, all compounds were tested for their capacities to inhibit HCV at two concentrations (1 μmol/L and 5 μmol/L) using an HCV pseudo-particle (HCVpp) entry assays. vesicular stomatitis virus G protein pseudo particle (VSVpp) was also tested in parallel to determine the specificity and toxicity as reported.[10,22,23] 1% DMSO was used as negative control, and CD81 antibody was utilized as a positive control because it blocks HCV virus entry via binding to CD81 receptors (Figure 2). In this assay (Figure 2A), we found both compound **4** and **6c** exhibited certain activities of inhibiting the entry of HCV into host cells, but were less potent than that of EA. Although compound **7c**, with replacement of EA with BA, displayed significant anti-HCV activity at 1 μmol/L, it also decreased the report reading of VSVpp infected cells (Figure 2B), suggesting compound **7c** was a nonselective antiviral agent against HCV or toxicity rather than potency enhancement. Furthermore, the anti-HCV activity of the dimers **8c**−**11c** completely abolished the activity of compound **1**. Interestingly, biological evaluation showed that compound **13**, a heterodimer containing both EA and OA moiety, displayed a further potency enhancement than that of EA but was much less potent than compound **1**. All these results provided the evidence that EA moiety played an irreplaceable role in maintaining high anti-HCV entry activity, and any replacement of EA by other triterpenes might significantly decrease its potency.

In our previous studies, we found the rigidity, length, and hydrophobicity of the spacer all had significant influence on the potency of EA dimers to HCVpp entry, and this dramatically affects their overall anti-HCV activity.[16] However, phenyl group is hydrophobic, which may affect the potential pharmacokinetic properties of compound **1**. To increase its solubility and maintain some rigidity, compound **14**, replacing the phenyl group of the linker with a piperazine, was design and synthesized (Scheme 3).

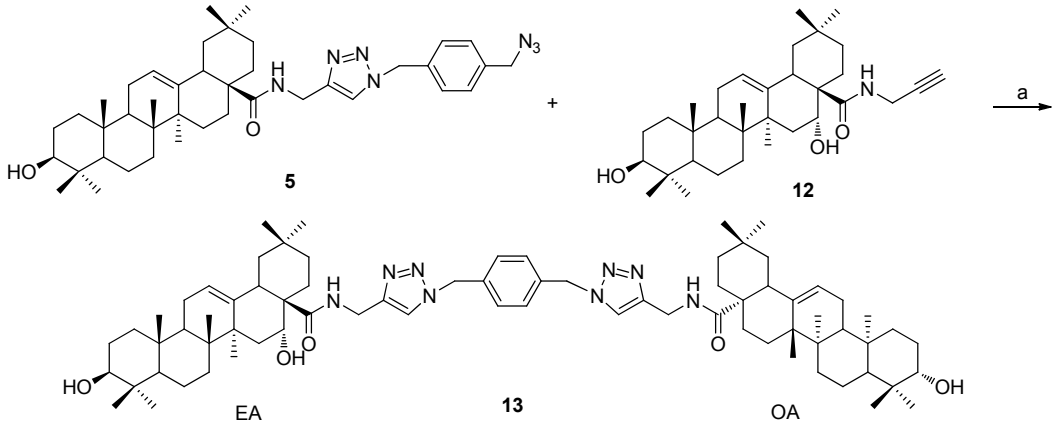
As shown in Figure 2, compound **14** exhibited significantly higher potency with IC_{50} at 2.87 nmol/L, two orders of magnitudes more potent than EA (1.4 μmol/L), and no significant cytotoxicity was observed. To our

Table 1 The structures of compounds **6c**—**11c**



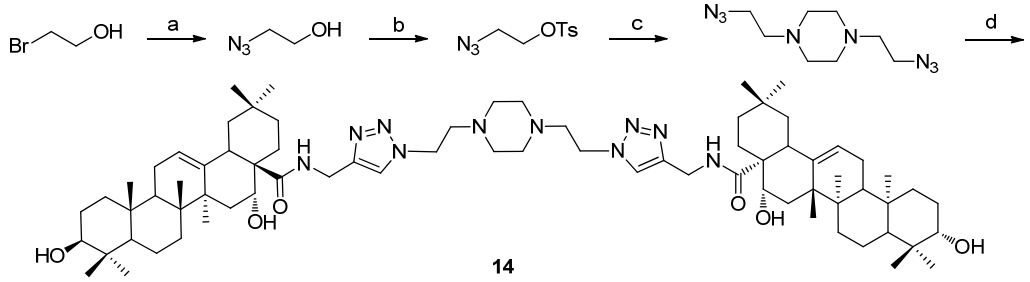
Compd	R	Compd	R
6		**9**	
7		**10**	
8		**11**	

Scheme 2



Reagents and conditions: (a) $CuSO_4$, Na-L-ascorbate, CH_2Cl_2/H_2O, r.t., overnight.

Scheme 3



Reagents and conditions: (a) NaN_3, Acetone/H_2O, 60 °C, overnight; (b) TsCl, TEA, CH_2Cl_2, overnight; (c) piperazine, TEA, DMF, 50 °C, overnight; (d) Na-*L*-ascorbate, CH_2Cl_2/H_2O, r.t., overnight.

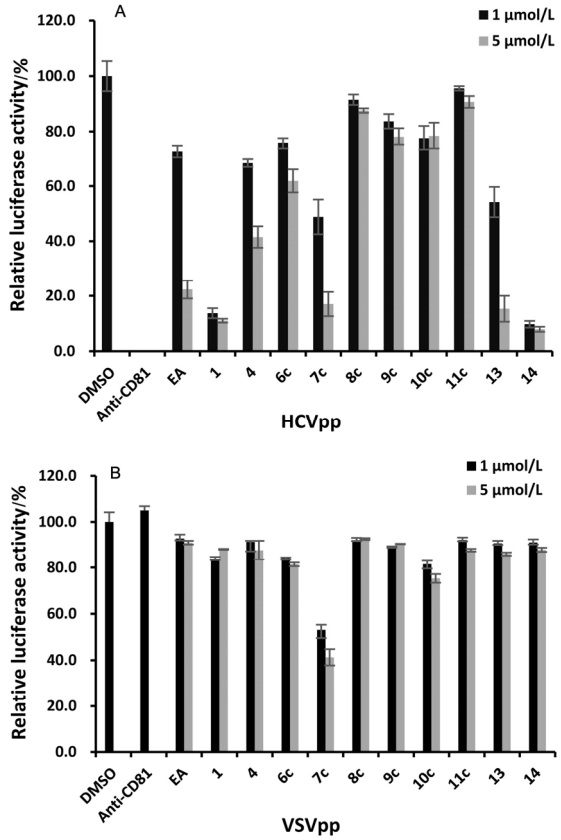


Figure 2 Anti-HCV entry activities of triterpene dimers.

knowledge, this is the most potent compound based on HCVpp entry assay. Further explorations of this new lead are ongoing to obtain promising HCV entry inhibitors.

We know that the triterpenoids may have crucial influence on the hemolytic properties, and we also found that EA gains substantial hemolytic side effect with CC_{50} at 15 mmol/L, which may restrict triterpenoids used as potential anti-HCV entry inhibitor. Thus, the hemolytic properties of all these triterpene dimers were tested with the concentration gradient of 0, 6.25, 12.5, 25 and 50 mmol/L. The results showed that undesired hemolytic effect of all these dimers was removed (S Figure 1).

Conclusions

In this study, we reported the design, synthesis and anti-HCV entry activity studies of novel triterpene dimers. Compounds **4**, **6c**—**11c** and **13** showed no improvement but detrimental effect on potency of compound **1**. The results confirmed that EA moiety of triterpene dimer is highly conserved, and any modification or replacement might significantly decrease or even eliminate its anti-HCV entry activity. Based on all these data, compound **14**, bearing a piperazine group in the linker, was design and synthesized, which is the most potent compound based on HCVpp entry assay. In addition, the undesired hemolytic effect of all these compounds was removed. As a novel HCV entry inhibitor, compound **14** is worth for further exploration.

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