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Research paper

Synthesis and biological evaluation of novel pentacyclic triterpene α-cyclodextrin conjugates as HCV entry inhibitors

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ABSTRACT

Hepatitis C virus (HCV) entry is a key target for the treatment of chronic HCV infection. In our continuing efforts to identify novel potential anti-HCV entry inhibitors, a series of water-soluble triazole-bridged α-cyclodextrin-pentacyclic triterpene conjugates were easily synthesized with moderate to good yields. These novel compounds were fully identified and characterized by 1D and 2D NMR spectroscopy and ESI-HRMS. The anti-HCV entry activities were determined based on HCVpp/VSVGpp entry assays. The best results were found for compounds **15** and **18**, which displayed the most promising anti-HCV entry activities with average IC_{50} values of 1.18 μM and 0.25 μM, respectively. In addition, the *in vitro* cytotoxicity activity of the two compounds against MDCK cells showed no toxicity at 100 μM. Five different binding assays were set up to identify the action mechanism. The results showed that the compounds exert their inhibitory activity at the post-binding step and subsequently prevent virus entry.

1. Introduction

The Hepatitis C virus (HCV) is a small, enveloped, positive-sense single-stranded RNA virus of the *Flaviviridae* family [1]. There are currently ~185 million individuals infected worldwide, many of whom suffer from serious sequelae of persistent HCV infection [2], such as chronic hepatitis, cirrhosis, and liver carcinomas [3]. Historically, the standard of care (SOC) for treating chronic HCV infection has been a combination of pegylated interferon (IFN) and ribavirin (RBV) with sustained viral response (SVR) rates of only 40–50% among genotype 1-infected patients [4]. Recently, HCV therapy has undergone a tremendous revolution with the advent of direct-acting antivirals (DAAs), which directly target proteins involved in replication of the virus. In 2011, the first generation DAAs, NS3/4A protease inhibitors boceprevir and telaprevir, have increased rates of SVR for the most difficult to treat genotype-1 HCV patients [5]. In 2013, approval of the second generation DAAs, NS3/4A protease inhibitors simeprevir and NS5B polymerase inhibitor sofosbuvir, has further improved treatment due to its pangenotypic effect on HCV, better pharmacokinetics, and improved resistance profiles [6]. However, the substantial cost of these DAAs present a substantial barrier to their broad distribution, and viral resistance to these drugs is expected to arise quickly [7–9]. A combination of drugs targeting different stages of the HCV life cycle would be required to eradicate HCV infections globally. Recently, polyphenol dimers [10], diflunisal hydrazide-hydrazones [11], indole [12] and benzofuran [13] derivatives have shown good anti-HCV activity with EC_{50} value at μM or sub-μM level. Viral entry is the first step of the virus-host cell interactions leading to productive infection, and thus it represents an emerging opportunity for the prevention of HCV infection [14,15]. Development of HCV entry inhibitors could be used in tandem with other inhibitors of viral replication, leading to a multifaceted approach to controlling HCV infection more effectively.

We initiated our search for new antivirals by exploring natural products, which have both great structural diversity and existing bioactivity. Pentacyclic triterpenes are the most represented group of phytochemicals, derived biosynthetically from the cyclization of squalene [16]. It has been estimated that more than 20,000 recognized triterpenoids exist in nature [17]. It is generally believed that they might function as defensive agents against herbivores or

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pathogens, or as allelopathic agents [18,19]. Many interesting biological properties have been described for pentacyclic triterpenes, such as hepatoprotective [20], anti-inflammatory [21], anti-tumor [17,22], and immunomodulatory activities. Quite recently, pentacyclic triterpenes have been found to act as effective antiviral agents [23]. Bevirimat (PA-457), a lupane-type triterpene, has been undergoing phase two trials as an anti-HIV entry drug candidate [24]. Moronic acid (MA) and ursolic acid (UA), two oleanane-type triterpenes, also show anti-HIV activity *in vitro* [25,26]. In our previous studies, we found that oleanolic acid (OA) and echinocystic acid (EA), two naturally occurring oleanane-type triterpenes, display substantial activity in inhibiting HCV entry with an IC_{50} of 10 and 1.4 μM, respectively [27,28]. The putative mechanism underlying the inhibition of HCV entry by EA is its tight binding to an HCV envelope protein, E2 ($K_D = 2.4 \times 10^{-5}$ M). Recently Richardson et al. found that naturally occurring terpenoid saikosaponin b2 also efficiently inhibits HCV entry [29].

Despite their promising bioactivity, these pentacyclic triterpenes are bulky, non-polar, and poorly soluble in water. For example, the solubility of BA and OA in water is only about 0.02 μg/mL and 0.05 μg/mL, respectively [30,31]. The poor aqueous solubility is a major drawback for various applications, in particular, the development of drugs. Hence, the synthesis of more water-soluble triterpene derivatives is needed. Some water-soluble derivatives have been synthesized by introducing polar sugar moieties at C-3 and/or C-28 [32]. Other strategies involve cyclodextrin inclusion complexes [33–35]. Alternatively, direct covalent linkage with cyclodextrin has been suggested, which has been widely used in other water insoluble bioactive molecules, such as 5-FU [36] and folic acid [37]. In our recently studies, a series of water-soluble triazole-bridged β-cyclodextrin-pentacyclic triterpene conjugates have been synthesized via click chemistry [38]. Compared with β-cyclodextrin, α-cyclodextrin has greater aqueous solubility at room temperature (14.5 *vs* 1.85 g/100 mL) [39]. The biological activity of such conjugates is expected to increase with increasing solubility.

As a continuation of our ongoing research in the development of novel anti-HCV entry inhibitors [27,28,38,40–42], we describe herein the synthesis, anti-HCV entry activity, and mechanistic studies of new water-soluble α-cyclodextrin-pentacyclic triterpene conjugates. The pentacyclic triterpenes used include oleanolic acid (OA), echinocystic acid (EA), urosolic acid (UA), and betulinic acid (BA) (Fig. 1).

2. Results and discussion

2.1. Chemistry

The α-cyclodextrin-triterpene conjugates were synthesized from α-cyclodextrin **1** in five steps (Scheme 1). Three types of hydroxyl groups are present in α-cyclodextrins, and the ones at *O*-6 are the most basic. Thus, it would undergo substitution using a weak base and a moderate electrophile. Herein, this strategy was used for the preparation of mono-functionalized α-cyclodextrin derivatives. First, α-cyclodextrin **1** was selective monotosylated at *O*-6 according to the procedure described by Quan et al. [43], followed by nucleophilic substitution with NaN_3 in DMF to provide the intermediate 6^A-azide-6^A-deoxy-α-cyclodextrin **3** in quantitative yields and was used without further purification in the next step. Acetylation of the remaining 17 hydroxyl groups of crude **3** with acetic anhydride in the presence of DMAP and pyridine afforded 6^A-azide-6^A-deoxy-per-*O*-acetylated α-cyclodextrin **4**. Compounds **11**–**13**, prepared according to our previously reported method [38], underwent a 'click chemistry' reaction with **4** in THF/H_2O (1:1) in the presence of a catalytic amount of copper sulfate and sodium ascorbate to yield **14**–**16** in 70–82% yields. The acetyl groups were removed upon treatment with CH_3ONa/CH_3OH to afford **17**–**19** in quantitative yields. Similarly, the CuAAC reaction in the synthesis of α-cyclodextrin-BA **26** was carried out from commercially available betulin (Scheme 2).

The structural determination of all α-cyclodextrin-triterpene conjugates was achieved using one and two dimensional NMR spectroscopy and ESI-HRMS. Fig. 2 represents the HSQC spectrum of compound **15**. The characteristic signals have been assigned unambiguously with the aid of $^1H-^1H$ COSY and $^1H-^{13}C$ HSQC spectroscopy. In the aromatic region, the signal at 7.96 ppm integrating to 1H, was assigned to triazole-CH according to the $^1H-^1H$ and $^1H-^{13}C$ correlation spectra. An additional signal was observed for the amide proton at 7.28 ppm (overlapping with $CDCl_3$), indicated that it was indeed connected by a triazole moiety. The ESI-HRMS of compound **15** plus one hydrogen ion clearly shows a *m/z* of 2221.8965.

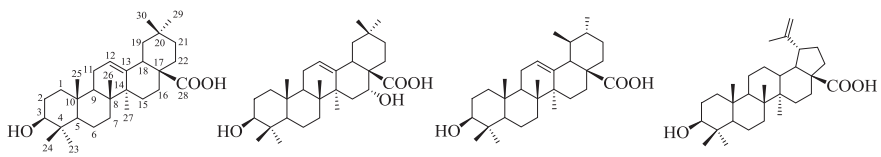
Fig. 3 presents the characteristic portions (80–180 ppm) of the ^{13}C NMR spectra (in $CDCl_3$) of conjugates **14**, **15**, **16**, and **25**. The signals have been fully assigned with the aid of $^1H-^1H$ COSY and $^1H-^{13}C$ HSQC spectra.

2.2. Calculated ALogP

Lipophilicity (Alog*P*) has a major impact on a number of drug-like features, and previous analyses have found that the decreased hit rate observed at high Alog*P* might be due to the limited solubility of the compounds [44]. Lipophilicity governs the interaction between the molecule and the intestinal membrane; the drugs must possess sufficient hydrophobicity for passive transport across the intestinal epithelium. In our study, the calculated Alog*P* values based on Ghose and Crippen's method [45] were determined using Pipeline Pilot software, Vers. 7.5 (Accelrys Corporation, San Diego, USA [46,47]). All conjugates **14**–**19** and **25**–**26** showed increased hydrophilicity to their parent compound (Table 1). In addition, as compared with β-cyclodextrin-triterpene conjugates [38], increased ALog*P* values were observed for α-cyclodextrin-triterpene conjugates. Due to the 16-hydroxyl group, **18** showed the lowest ALog*P* values among the α-cyclodextrin-triterpene conjugates.

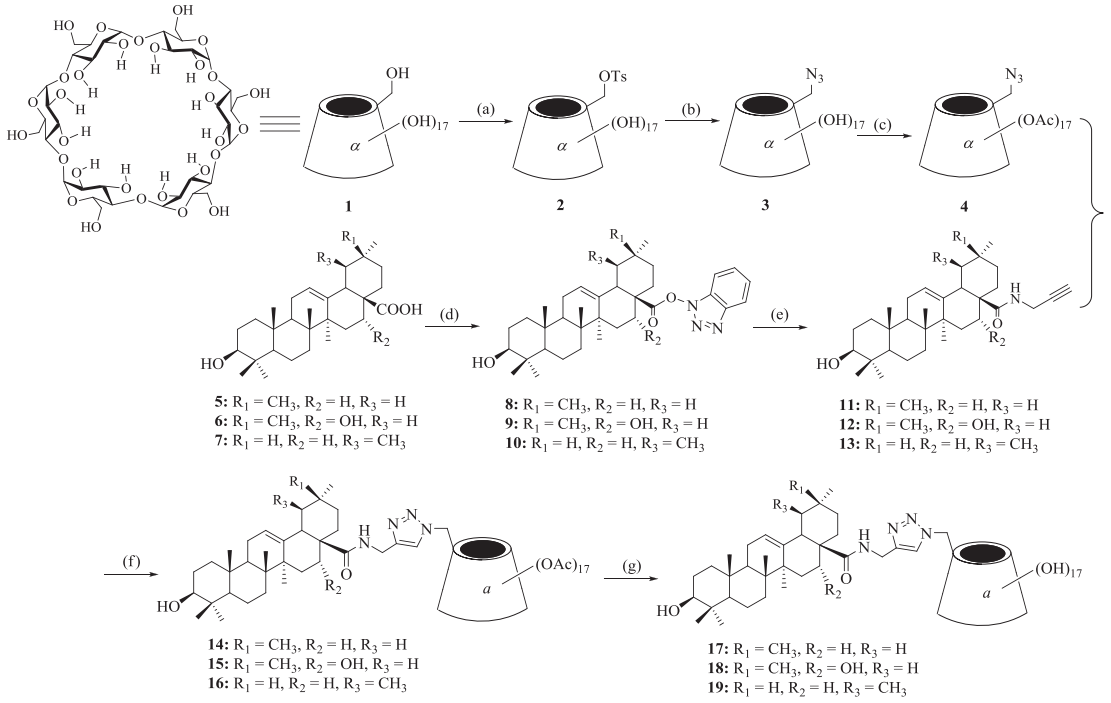
2.3. In vitro anti-HCV entry activity

Cyclodextrins and their derivatives have been widely utilized for

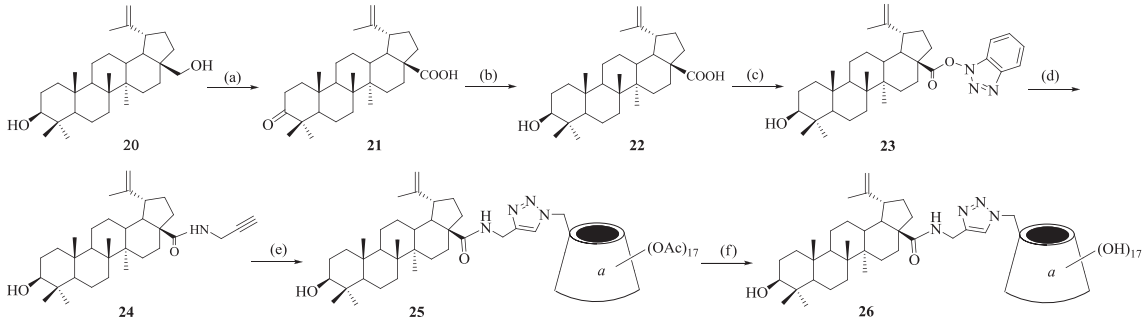


Oleanolic acid (OA) 10 μM: 65% | Echinocystic acid (EA) 10 μM: 90% | Ursolic acid (UA) 10 μM: 30% | Betulinic acid (BA) 10 μM: 75%

Fig. 1. The chemical structures and anti-HCV entry activities of pentacyclic triterpenes [27].



Scheme 1. Reagents and conditions: (a)TsCl, dry pyridine; (b) DMF, NaN_3, 80 °C, 18 h; (c) pyridine, DMAP, Ac_2O; (d) TBTU, DIPEA, THF; (e) propargylamine, K_2CO_3, DMF, 1 h; (f) THF-H_2O (1:1, V/V), $CuSO_4$, Na-*L*-ascorbate; (g) CH_3ONa/CH_3OH, rt, 8–10 h.



Scheme 2. Reagents and conditions: (a) H_2CrO_4, acetone, 0 °C to rt, 18 h; (b) $NaBH_4$, THF, rt, 2.5 h; (c) TBTU, DIPEA, THF; (d) Propargylamine, K_2CO_3, DMF, 1 h; (e) THF-H_2O (1:1, V/V), $CuSO_4$, Na-*L*-ascorbate, **4;** (f) CH_3ONa/CH_3OH, rt, 8–10 h.

improving drug solubility, stability, and absorption, due to their remarkable ability to form inclusion complexes. Our previous studies suggested that EA displayed substantial activity in inhibiting HCV entry [27], and the introduction of β-cyclodextrin at the C-17 carboxylic acid may further enhance its potency [38]. To further investigate these molecules, a series of α-cyclodextrin-triterpene conjugates and their acetylated derivatives were synthesized and their anti-HCV entry activities were evaluated.

As a preliminary screen for anti-HCV entry activity, all conjugates were tested for their capacities to inhibit entry of HCVpp (Huh-7 cells) at two concentrations (1 and 5 μM) (Fig. 4A). DMSO was used as the negative control. CD81 was the positive control to indicate maximum blocking of HCVpp entry. In this assay, we found that compounds **15** and **18** exhibited significant HCVpp entry inhibition with more than 80% and 95% of virus entry blocked at 5 μM, respectively. Compounds **25** and **26** showed moderate inhibition (~60%) of HCVpp entry, whereas compounds **16** and **19** showed no inhibition of HCVpp entry at concentrations of 5 μM. To determine the specificity of α-cyclodextrin-triterpene conjugates toward HCV, their ability to block entry of VSVG/HIV (vesicular stomtatis virus G protein) pseudovirions into Huh7 cells was tested. The VSVG virus has a broad host range and can infect multiple cell types. We found that, with the exception of **25** and **26**, the other α-cyclodextrin-triterpene derivatives **14**–**19** did not block VSVG viral entry (Fig. 4B), indicating BA derivatives **25** and **26** were nonselective antiviral agents against HCV.

After the preliminary screen at two concentrations (1 and 5 μM), the anti-HCV entry activities of **15** and **18** were further characterized in a dose-dependent assay. As shown in Fig. 5, both compounds exhibited significant inhibition against HCVpp entry with IC_{50} values of 1.18 μM and 0.25 μM, respectively. Compared with their parent compound EA (IC_{50} = 1.4 μM [27]), the introduction of α-cyclodextrin or per-*O*-acetylated α-cyclodextrin increased the potency by 1.2–5.6-fold.

Furthermore, we investigated the toxicity of compounds **15** and **18** against the MDCK cell (a Madin-Daby canine kidney cell) line using the alamarBlue assay. As shown in Table 2, no significant toxicity was observed at concentrations of 100 μM for both compounds. Compared with their parent compounds, both compounds showed high selectivity indexes (SI > 85 and 400, respectively),

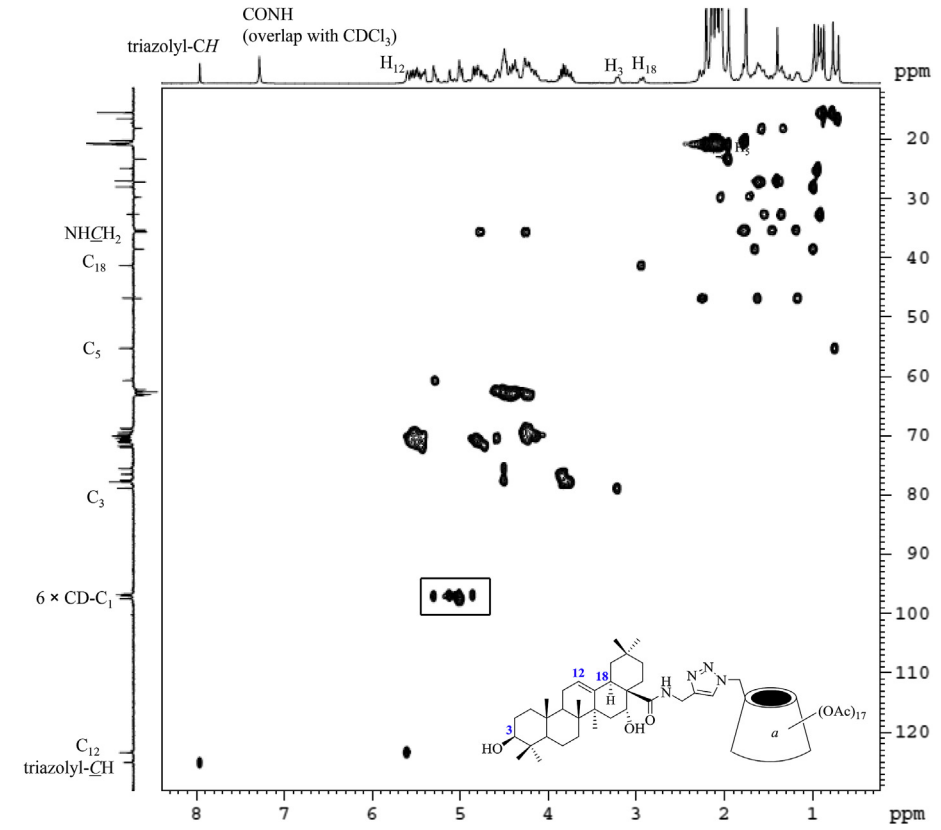


Fig. 2. A portion of the 400 MHz HSQC ($CDCl_3$, 25 °C) spectrum of **15**, with the 1D ^1H and ^{13}C NMR spectra along the side and the top, respectively. The frame corresponds to correlation $^1J_{CH}$ CD-C_1 and CD-H_1.

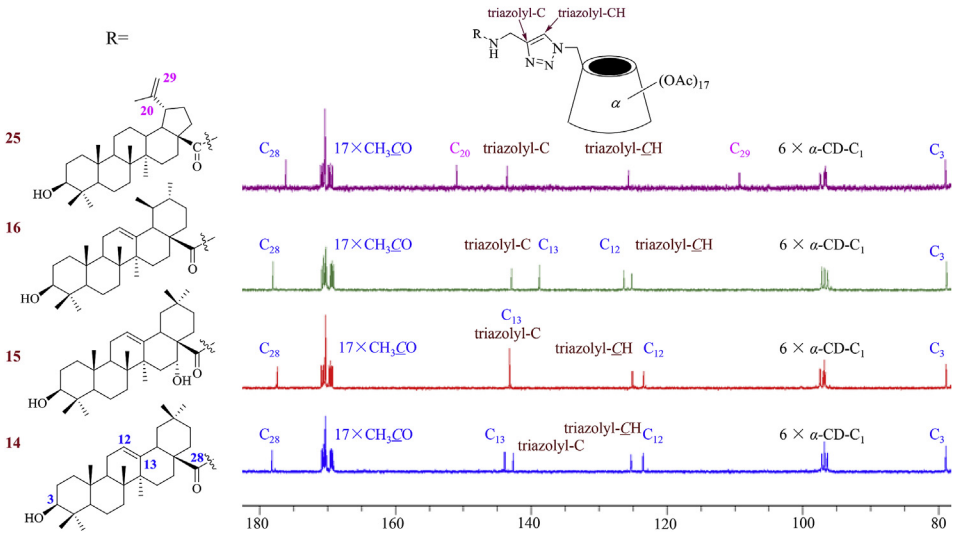


Fig. 3. Characteristic portions of the 400-MHz ^{13}C NMR spectra of **14**, **15**, **16**, and **25** recorded in $CDCl_3$ at 25 °C.

which might have been due to the increased solubility of the triterpene derivatives upon the introduction of α-cyclodextrin.

2.4. Mechanism investigation of HCV entry assay

In order to understand at which stage of infection the α-cyclodextrin-triterpene conjugates exert their inhibitory activity, five different assays, including the standard antiviral assay, pre-treatment assay, pre-binding assay, post-binding (pre-entry) assay, and post-entry assay were performed as previously reported [48,49].

In all five conditions (Fig. 6), 0.5% DMSO (final concentration) was used for normalization. The CD81 antibody was utilized as a positive control due to its blocking of the HCV virus entry by binding to CD81 receptor. With the exception of the post-entry assay, high activity of the CD81 antibody was observed in the

Table 1
Calculated ALog*P* of pentacyclic triterpene and their α-cyclodextrin conjugates[a].

Compound	ALog *P*	Compound	ALog *P*	Compound	ALog *P*
OA	6.447	**14**	2.327	**17**	−3.553
EA	5.345	**15**	1.226	**18**	−5.673
UA	6.492	**16**	2.373	**19**	−3.508
BA	6.546	**25**	2.427	**26**	−4.472

[a] ALog*P* (The Ghose and Crippen octanol-water partition coefficient at 25 °C) were calculated using Pipeline Pilot software, Vers. 7.5 (Accelrys Corporation, San Diego, USA).

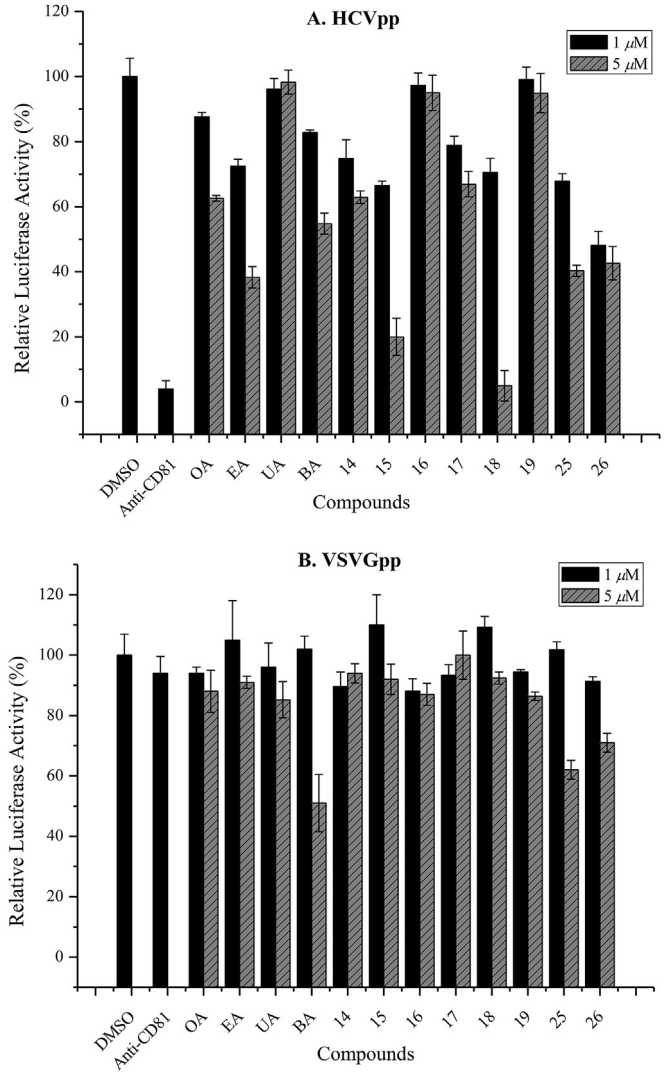


Fig. 4. Anti-HCV entry activity of α-cyclodextrin-pentacyclic triterpene conjugates based on HCVpp/VSVGpp entry assays.

assays. IM2865 was a non-relevant compound used as the negative control [14], and no significant inhibitory activity was observed in all conditions. Compared with the standard antiviral assay, only very weak activity (~10–30%) of **15** and **18** was observed in the pre-treatment, pre-binding, and post-entry assays. A strong decrease in HCV infection (>90%) was noted in the post-binding assay, indicating that **15** and **18** exert their inhibitory activity at the post-binding (pre-entry) step and interfere with subsequent steps following virus attachment to target cells, presumably viral envelop-cell membrane fusion.

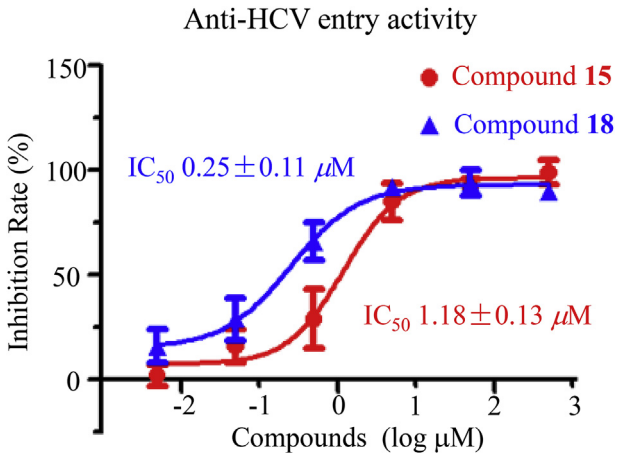


Fig. 5. Inhibition curves of **15** and **18**. Concentration of **15** and **18** were 5 nM, 50 nM, 0.5 μM, 5 μM, 50 μM, and 500 μM. Each concentration was tested in triplicate, and the results are expressed as the mean and standard deviation.

Table 2
In vitro anti-HCV entry activity and cytotoxicity of compounds **15** and **18**.

Compound	IC_{50} (μM)[a]	CC_{50} (μM)[b]	SI[c]
15	1.18 ± 0.13	>100	>85
18	0.25 ± 0.11	>100	>400
EA	1.40 ± 0.17 [27]	15.41 ± 0.54	11.0

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

3. Conclusion

In this study, we reported the synthesis, anti-HCV entry activity, cytotoxicity, and mechanistic studies of novel α-cyclodextrin-pentacyclic triterpene conjugates. All new conjugates were unambiguous characterized by 1D and 2D NMR and ESI-HRMS. By conjugating with water soluble α-cyclodextrin, these derivatives showed decreased hydrophobicity (ALog *P*) and reasonable anti-HCV entry activities. Compounds **15** and **18** displayed the most promising anti-HCV entry activities with average IC_{50} values of 1.18 μM and 0.25 μM, respectively. No significant cytotoxicity of compounds **15** and **18** were observed at concentrations of 100 μM. Moreover, entry assays indicated that they exert their inhibitory activity at the post-binding step and subsequently prevent virus entry. Our results provide a rationale for further exploring the anti-HCV entry activity of pentacyclic triterpenes, which is widely found in plants, as potent HCV inhibitors.

4. Experimental

4.1. Chemistry

4.1.1. General information

The syntheses of **2**–**4**, **11**–**13**, and **21**–**24** were performed as previously reported (Supporting information) [27,38,41]. High resolution mass spectra (ESI-HRMS) were obtained with an APEX IV FT_MS (7.0 T) spectrometer (Bruker) in positive ESI mode. NMR spectra were recorded on a Bruker DRX 400 spectrometer at ambient temperature. ^1H NMR chemical shifts were referenced to the internal standard TMS (δ_H = 0.00) or the solvent signal (δ_H = 3.31 for the central line of MeOD). ^{13}C NMR chemical shifts were

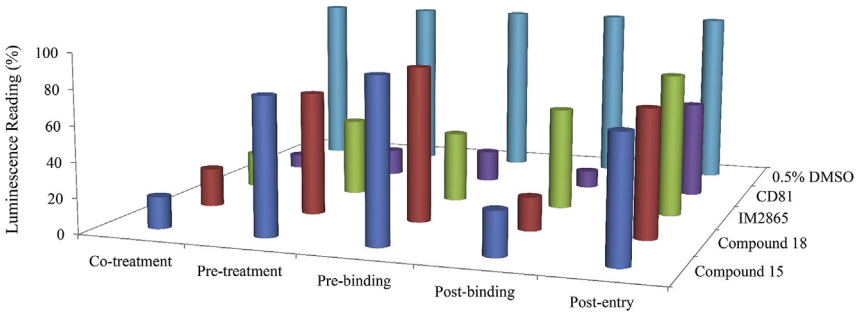


Fig. 6. Mechanistic studies of compounds **15** and **18**-mediated blocking of HCVpp entry by five different assays. CD81 antibody, an entry inhibitor targeting host cell membrane, was utilized as the positive control and IM2865, a non-relevant compound [14], were used as the negative control. 0.5% DMSO (final concentration) was used for normalization in each condition.

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 MeOD). Reactions were monitored by thin-layer chromatography (TLC) on a pre-coated silica gel 60 F_{254} plate (layer thickness 0.2 mm; E. Merck, Darmstadt, Germany) and detected by detected by staining with a yellow solution containing $Ce(NH_4)_2(NO_3)_6$ (0.5 g) and $(NH_4)_6Mo_7O_{24}4H_2O$ (24.0 g) in 6% H_2SO_4 (500 mL) followed by heating. Flash column chromatography was performed on silica gel 60 (200–300 mesh, Qingdao Haiyang Chemical Co. Ltd). The calculated Alog *P* and water solubility values were determined using Pipeline Pilot software, Vers. 7.5 (Accelrys Corporation, San Diego, CA, USA).

4.1.2. General procedure A for the click reaction

To a solution of alkyne (0.10 mmol) and azide (0.067 mmol) in 1:1 THF:H_2O (6 mL) was added $CuSO_4$ (10.5 mg, 0.067 mmol) and sodium ascorbate (26.6 mg, 0.13 mmol). The resulting solution was stirred vigorously for 12 h at room temperature. The reaction mixture was extracted with CH_2Cl_2 (10 mL × 3). The combined organics was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography.

4.1.3. General procedure B for the deacetylation reaction

The per-*O*-acetylated α-cyclodextrin-triterpene conjugate was dissolved in dry methanol (~5 mL per 100 mg of compound) and a solution of sodium methoxide (30% in methanol, 0.1 eq per mol of acetate) was added. The solution was stirred at room temperature for 4–6 h. 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 RP column chromatography (eluted by CH_3OH).

*4.1.4. Synthesis of [1-(6^A-deoxy-per-O-acetylated α-cyclodextrin-6-yl)-1H-1,2,3-triazol-4-yl]methyl 3β-hydroxy-olean-12-en-28-amide (**14**)*

Prepared from **4** and **11** according to general procedure A, the residue was purified by flash chromatography (eluent: DCM/CH_3OH = 30:1) to afford **14** as a white solid in 68% yield. R_f = 0.37 (DCM:CH_3OH = 20:1); ^1H NMR (400 MHz, $CDCl_3$): δ 7.96 (s, 1H), 7.09 (br s, 1H), 5.38–5.61 (m, 7H), 5.29 (br s, 1H), 5.19–5.28 (m, 1H), 5.14 (br s, 1H), 5.03 (d, 2H, *J* = 3.0 Hz), 5.00–5.03 (m, 3H), 4.14–4.86 (m, 29H), 3.72–3.87 (m, 5H), 2.22 (2 × s), 2.14, 2.13, 2.09, 2.08, 2.07, 2.06, 2.04, 2.01, 1.99, 1.96, 1.78, 1.74 (17 × $COCH_3$), 1.18, 0.98, 0.94, 0.90, 0.89, 0.87, 0.77, 0.70 (s, each 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.18, 170.88, 170.78, 170.65, 170.63, 170.55, 170.43, 170.32, 170.30, 170.18, 169.62, 169.52, 169.50, 169.45, 169.36, 169.21, 143.89, 142.64, 125.28, 123.52, 97.20, 97.12, 96.80, 96.41, 96.33, 78.89, 77.74, 77.52, 77.40, 77.34, 76.53, 76.01, 72.02, 71.62, 71.24, 70.89, 70.76, 70.55, 70.54, 70.26, 70.23, 69.91, 69.85, 69.75, 69.59, 68.91, 68.82, 63.21, 63.08, 62.98, 62.74, 62.68, 62.39, 60.63, 55.10, 47.59, 46.55, 46.29, 42.09, 41.89, 39.40, 38.71, 38.44, 36.89, 35.55, 34.11, 32.95, 32.52, 32.42, 30.65, 28.05, 27.38, 27.17, 25.80, 23.83, 23.47, 21.00, 20.93, 20.84, 20.76, 20.73, 20.62, 20.60, 20.23, 20.18, 19.06, 18.22, 16.31, 15.49, 15.29; ESI-HRMS (*m*/*z*) Calcd for $C_{103}H_{145}N_4O_{48}$ $[M+H]^+$: 2205.9023. Found 2205.9021.

*4.1.5. Synthesis of [1-(6^A-deoxy-per-O-acetylated α-cyclodextrin-6-yl)-1H-1,2,3-triazol-4-yl]methyl 3β,16α-dihydroxy-olean-12-en-28-amide (**15**)*

Prepared from **4** and **12** according to general procedure A, the residue was purified by flash chromatography (eluent: DCM/CH_3OH = 35:1) to afford **15** as a white solid in 60% yield. R_f = 0.29 (eluent: DCM/CH_3OH = 15:1); ^1H NMR (400 MHz, $CDCl_3$): δ 7.96 (s, 1H), 7.28 (br s, 1H, overlap with $CDCl_3$), 5.60 (br s, 1H), 5.40–5.92 (m, 6H), 5.30 (br s, 1H), 5.28 (t, 1H, *J* = 10.7 Hz), 5.12 (d, 1H, *J* = 2.9 Hz), 5.01 (br s, 2H), 4.98 (d, 1H, *J* = 2.7 Hz), 4.85 (br s, 1H), 4.74–4.82 (m, 4H), 4.71 (dd, 1H, *J* = 2.8, 10.3 Hz), 4.11–4.61 (m, 24H), 3.72–3.88 (m, 5H), 2.21, 2.20, 2.15, 2.13, 2.12, 2.09, 2.07, 2.05, 2.04, 2.03, 1.95, 1.75 (17 × $COCH_3$), 1.40, 0.98, 0.91, 0.90, 0.87, 0.76, 0.70 (s, each 3H), 0.75 (m, 1H), 0.99–2.25 (m, other aliphatic ring protons); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.39, 170.92, 170.86, 170.66, 170.57, 170.38, 170.31, 170.28, 169.82, 169.77, 169.59, 169.48, 169.35, 169.27, 143.18, 125.09, 123.43, 97.44, 97.41, 96.96, 96.79, 96.66, 78.87, 77.79, 77.68, 77.53, 76.53, 76.43, 75.55, 71.99, 71.71, 71.18, 71.01, 70.95, 70.75, 70.57, 70.42, 70.37, 70.12, 70.06, 69.89, 69.51, 68.92, 68.78, 63.21, 63.03, 62.62, 62.25, 60.67, 55.24, 48.89, 46.85, 46.80, 41.65, 41.29, 39.60, 38.72, 38.52, 36.91, 35.63, 35.34, 32.64, 30.17, 29.75, 28.02, 27.21, 27.01, 24.92, 23.37, 20.97, 20.88, 20.83, 20.81, 20.77, 20.73, 20.59, 20.20, 18.15, 16.54, 15.50; ESI-HRMS Calcd for $C_{103}H_{145}N_4O_{49}$ $[M+H]^+$: 2221.8972. Found 2221.8965.

*4.1.6. Synthesis of [1-(6^A-deoxy-per-O-acetylated α-cyclodextrin-6-yl)-1H-1,2,3-triazol-4-yl]methyl 3β-dihydroxy-urs-12-en-28-amide (**16**)*

Prepared from **4** and **13** according to general procedure A, the residue was purified by flash chromatography (eluent: DCM/CH_3OH = 40:1) to afford **16** as a white solid in 66% yield. R_f = 0.30 (DCM:CH_3OH = 20:1); ^1H NMR (400 MHz, $CDCl_3$): δ 7.94 (s, 1H), 7.30 (br s, 1H), 5.36–5.60 (m, 6H), 5.29 (d, 1H, *J* = 3.5 Hz), 5.25 (t, 1H, *J* = 9.8 Hz), 5.12 (d, 1H, *J* = 3.5 Hz), 5.03 (d, 2H, *J* = 3.0 Hz), 5.00 (d, 1H, *J* = 3.4 Hz), 4.86 (d, 1H, *J* = 3.1 Hz), 4.77–4.84 (m, 3H), 4.72 (dd, 1H, *J* = 3.3, 10.3 Hz), 4.11–4.68 (m, 23H), 3.72–3.87 (m, 5H), 2.22, 2.19, 2.17, 2.14 (2 × s), 2.13, 2.10, 2.09, 2.08, 2.07, 2.06, 2.05 (2 × s), 2.00, 1.96, 1.78, 1.73 (each s, 3H, 17 × $COCH_3$), 1.12 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 0.97 (d, 3H, *J* = 6.4 Hz, CH_3), 0.88 (s, 3H, CH_3), 0.87 (d, 3H, *J* = 6.5 Hz, CH_3), 0.77 (s, 3H, CH_3), 0.74 (s, 3H, CH_3), 0.73 (d, 1H,

J = 10.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.01, 170.83, 170.65, 170.58, 170.40, 170.28, 170.27, 170.25, 170.13, 169.57, 169.46, 169.39, 169.30, 169.28, 169.10, 142.89, 138.77, 126.36, 125.17, 97.21, 97.19, 96.79, 96.77, 96.34, 96.29, 78.81, 77.65, 77.40, 77.20, 76.14, 71.91, 71.57, 71.03, 70.86, 70.81, 70.74, 70.54, 70.45, 70.26, 70.20, 69.82, 69.61, 69.59, 68.99, 68.72, 63.18, 63.01, 62.86, 62.69, 62.60, 62.35, 60.64, 55.05, 53.61, 47.63, 47.51, 42.23, 39.57, 39.49, 39.03, 38.65, 38.54, 37.06, 36.81, 35.46, 32.67, 30.75, 28.03, 27.82, 27.13, 24.84, 23.31, 23.28, 21.10, 20.96, 20.79, 20.77, 20.70, 20.67, 20.57, 20.54, 20.30, 20.20, 18.14, 17.12, 16.36, 15.50, 15.35; HRMS Calcd for $C_{103}H_{145}N_4O_{48}$ $[M+H]^+$: 2205.9023. Found 2205.9077.

4.1.7. Synthesis of [1-(6^A-deoxy-α-cyclodextrin-6-yl)-1H-1,2,3-triazol-4-yl]methyl 3β-hydroxy-olean-12-en-28-amide (**17**)

Prepared from **14** according to general procedure B, the residue was purified by RP flash chromatography (eluent: methanol) to afford **17** as a white solid in 90% yield. ^{1}H NMR (400 MHz, CD_3OD): δ 8.02 (s, 1H), 5.37 (s, 1H), 4.93–4.95 (m, 4H), 4.85 (m, 1H), 4.61 (s, 1H), 4.40 (m, 2H), 4.35 (t, J = 9.1 Hz), 3.75–4.09 (m, 24H), 3.64 (d, J = 9.2 Hz), 3.50–3.60 (m, 7H), 3.43 (t, 1H, J = 9.4 Hz) 3.12–3.18 (m, 2H), 2.80 (d, J = 11.0 Hz), 2.08 (t, 1H, J = 13.7 Hz), 1.92 (m, 2H), 1.79 (t, 1H, J = 13.4 Hz), 1.17 (s, 3H), 0.97 (2 × s, 6H), 0.94 (s, 3H), 0.91 (s, 3H), 0.78 (s, 3H), 0.75 (m, 1H), 0.73 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ 180.71, 145.21, 127.37, 127.35, 124.27, 103.78, 103.66, 103.62, 103.60, 103.55, 102.53, 83.37, 83.21, 83.18, 82.98, 79.74, 75.31, 75.26, 74.98, 74.57, 74.10, 74.01, 73.90, 73.77, 73.70, 73.66, 73.45, 72.53, 67.96, 62.05, 61.95, 61.91, 61.84, 56.73, 49.05, 47.66, 42.89, 42.64, 40.67, 39.84, 38.15, 35.79, 35.07, 33.95, 33.74, 33.56, 31.61, 30.73, 28.74, 28.53, 27.88, 26.50, 24.56, 24.15, 19.47, 17.70, 16.33, 15.93; ESI-HRMS (m/z) Calcd for $C_{69}H_{111}N_4O_{31}$ $[M+H]^+$: 1491.7227. Found 1491.7223.

4.1.8. Synthesis of [1-(6^A-deoxy-α-cyclodextrin-6-yl)-1H-1,2,3-triazol-4-yl]methyl 3β,16α-dihydroxy-olean-12-en-28-amide (**18**)

Prepared from **15** according to general procedure B, the residue was purified by RP flash chromatography (eluent: methanol) to afford **18** as a white solid in 88% yield. ^{1}H NMR (400 MHz, CD_3OD): δ 7.97 (s, 1H), 5.49 (br t, 1H, H_{12}), 5.09 (d, 1H, J = 3.1 Hz), 4.95–4.97 (m, 3H), 4.93 (d, 1H, J = 3.3 Hz), 4.81 (t, 1H, J = 10.2 Hz, overlap with H_2O), 4.60 (d, 1H, J = 3.4 Hz), 4.40 (m, 1H), 4.36 (m, 1H), 4.34 (m, 1H), 4.32 (m, 1H), 4.05 (m, 1H), 3.77–4.06 (m, 21H), 3.75 (t, 1H, J = 9.6 Hz), 3.63 (t, 1H, J = 9.2 Hz), 3.47–3.57 (m, 7H), 3.44 (t, 1H, J = 9.2 Hz), 3.13–3.18 (m, 2H), 2.92 (dd, 1H, J = 3.1, 13.9 Hz), 2.32 (t, 1H, J = 13.2 Hz), 1.00–2.02 (m, other aliphatic ring protons), 1.36 (s, 3H), 0.97 (s, 6H), 0.95 (s, 3H), 0.89 (s, 3H), 0.78 (s, 3H), 0.75 (m, 1H), 0.74 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ 180.40, 144.87, 144.66, 127.23, 124.29, 103.76, 103.67, 103.66, 103.59, 103.56, 102.52, 83.33, 83.32, 83.19, 83.16, 82.95, 79.71 (2C), 75.52, 75.31, 75.25, 74.97, 74.52, 74.09, 74.01, 73.88, 73.76, 73.69, 73.65, 73.40, 72.54, 72.48, 67.82, 62.06, 61.93, 61.89, 61.82, 56.83, 50.15, 48.25, 47.99, 42.86, 42.32, 40.86, 39.95, 39.85, 38.10, 36.19, 36.12, 36.03, 33.86, 33.29, 31.15, 28.72, 27.91, 27.41, 25.74, 24.51, 19.45, 17.77, 16.35, 16.17; ESI-HRMS (m/z) Calcd for $C_{69}H_{111}N_4O_{32}$ $[M+H]^+$: 1507.7176. Found 1507.7202.

4.1.9. Synthesis of [1-(6^A-deoxy-α-cyclodextrin-6-yl)-1H-1,2,3-triazol-4-yl]methyl 3β-dihydroxy-urs-12-en-28-amide (**19**)

Prepared from **16** according to general procedure B, the residue was purified by RP flash chromatography (eluent: methanol) to afford **19** as a white solid in 92% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 (s, 1H), 7.50 (br s, 1H), 5.35 (br t, 1 Hz), 5.09 (d, 1H, J = 2.9 Hz), 4.95–4.97 (m, 3H), 4.93 (d, 1H, J = 3.2 Hz), 4.78 (t, 1H, J = 10.3 Hz), 4.61 (d, 1H, J = 3.2 Hz), 4.30–4.45 (m, 3H), 4.04 (m, 1H), 4.09 (m, 1H), 3.73–3.96 (m, 16H), 3.63 (t, 1H, J = 9.3 Hz), 3.48–3.57 (m, 7H), 3.45 (t, 1H, J = 9.2 Hz), 3.17–3.18 (m, 2H), 2.15 (m, 1H), 1.57 (m, 1H), 1.13 (s, 3H), 0.97–0.98 (2 × s, 9H), 0.92 (d, 3H, J = 6.2 Hz), 0.79 (s, 6H), 0.75 (d, 1H, J = 11.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 180.38, 145.28, 139.93, 127.36, 126.54, 103.73, 103.66, 103.62, 103.57, 103.54, 102.51, 83.31, 83.27, 83.17, 83.12, 82.93, 79.79, 79.70, 75.27, 75.23, 74.96, 74.51, 74.07, 73.99, 73.86, 73.74, 73.66, 73.61, 73.34, 72.54, 67.39, 62.12, 61.92, 61.83, 61.80, 56.69, 54.10, 48.98, 43.24, 40.85, 40.81, 40.15, 39.96, 39.82, 38.27, 38.08, 36.32, 36.20, 34.06, 31.87, 28.97, 28.78, 27.89, 25.31, 24.40, 24.15, 21.65, 19.44, 17.75, 17.70, 16.41, 16.06; ESI-HRMS (m/z) Calcd for $C_{69}H_{111}N_4O_{31}$ $[M+H]^+$: 1491.7227. Found 1491.7215.

4.1.10. Synthesis of N-[1-(6^A-deoxy-per-O-acetylated α-cyclodextrin-6-yl)-1H-1,2,3-triazol-4-yl]methyl 3β-hydroxy-lup-20(29)-en-28-amide (**25**)

Prepared from **4** and **24** according to general procedure A, the residue was purified by flash chromatography (eluent: DCM/EtOAc = 1:1) to afford **25** as a white solid in 71% yield. R_f = 0.30 (DCM:CH_3OH = 20:1); R_f = 0.30 (DCM:CH_3OH = 20:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.97 (s, 1H), 6.54 (t, 1H, J = 4.8 Hz), 5.37–5.59 (m, 6H), 5.30 (d, 1H, J = 3.6 Hz), 5.22–5.27 (m, 1H), 5.12 (d, 1H, J = 3.5 Hz), 5.02–5.03 (m, 2H), 5.00 (d, 1H, J = 3.5 Hz), 4.87 (d, 1H, J = 2.9 Hz), 4.77–4.84 (m, 3H), 4.74 (m, 1H), 4.72 (dd, 1H, J = 10.4, 3.3 Hz), 4.14–4.59 (m, 23H), 3.81–3.88 (m, 3H), 3.79 (t, 1H, J = 4.0 Hz), 3.74 (t, 1H, J = 6.4 Hz), 3.12–3.19 (m, 2H), 2.58 (td, 1H, J = 12.4, 2.9 Hz), 2.22, 2.19, 2.17, 2.15, 2.14, 2.13, 2.10, 2.09, 2.07, 2.06, 2.05, 2.05, 2.01, 1.96, 1.79, 1.78 (each s, 17 × $COCH_3$), 1.69, 0.98, 0.95, 0.92, 0.81, 0.75 (each s, 6 × CH_3), 0.89–2.14 (m, other aliphatic ring protons), 0.67 (d, 1H, J = 11.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.14, 171.02, 170.84, 170.70, 170.63, 170.47, 170.41, 170.37, 169.84, 169.72, 169.58, 169.55, 169.33, 169.25, 150.97, 143.52, 125.64, 109.32, 97.45, 97.33, 96.83, 96.67, 96.61, 96.47, 78.97, 77.73, 77.51, 77.38, 76.53, 76.30, 71.99, 71.62, 71.07, 70.90, 70.82, 70.52, 70.25, 69.97, 69.91, 69.76, 69.68, 69.01, 68.75, 63.25, 63.09, 62.92, 62.65, 62.28, 60.73, 55.64, 55.41, 50.67, 50.19, 46.50, 42.43, 40.69, 38.84, 38.68, 38.29, 37.51, 37.18, 35.04, 34.40, 33.48, 30.82, 29.42, 27.99, 27.41, 25.62, 21.05, 20.92, 20.89, 20.84, 20.80, 20.78, 20.64, 20.49, 20.34, 19.49, 19.14, 18.23, 16.12, 16.09, 15.32, 14.61; ESI-HRMS (m/z) Calcd for $C_{103}H_{145}N_4O_{48}$ $[M+H]^+$: 2205.9023. Found 2205.9061.

4.1.11. Synthesis of N-[1-(6^A-deoxy-α-cyclodextrin-6-yl)-1H-1,2,3-triazol-4-yl]methyl 3β-hydroxy-lup-20(29)-en-28-amide (**26**)

Prepared from **25** according to general procedure B, the residue was purified by RP flash chromatography (eluent: methanol) to afford **26** as a white solid in 87% yield. ^{1}H NMR (400 MHz, CD_3OD): δ 8.17 (s, 1H), 5.11 (d, 1H, J = 3.1 Hz), 4.92–4.97 (m, 4H), 4.71 (br s, 1H), 4.62 (d, 1H, J = 3.4 Hz), 4.54–4.58 (m, 2H), 4.32–4.38 (m, 2H), 3.73–4.11 (m, 23H), 3.62 (t, 1H, J = 7.5 Hz), 3.48–3.56 (m, 6H), 3.42 (t, 1H, J = 9.2 Hz), 3.18 (dd, 1H, J = 10.1, 3.3 Hz), 3.05–3.14 (m, 2H), 2.57 (t, 1H, J = 9.4 Hz), 2.15 (d, 1H, J = 12.9 Hz), 1.82–1.88 (m, 2H), 1.69, 0.99, 0.95, 0.93, 0.86, 0.76 (each s, 6 × CH_3), 0.92–1.73 (m, other aliphatic ring protons), 0.70 (d, 1H, J = 8.9 Hz); ^{13}C NMR (100 MHz, CD_3OD): δ 179.44, 152.30, 144.72, 127.91, 110.04, 103.81, 103.67, 103.61, 102.50, 83.52, 83.34, 83.19, 83.17, 83.00, 79.69, 79.64, 75.31, 75.27, 75.21, 74.98, 74.56, 74.07, 74.00, 73.89, 73.81, 73.77, 73.74, 73.70, 73.65, 73.48, 72.51, 72.38, 68.49, 61.92, 61.88, 61.83, 57.01, 56.92, 54.80, 53.15, 52.11, 51.43, 48.08, 43.51, 42.01, 40.10, 39.96, 39.09, 38.96, 38.34, 35.57, 34.91, 33.97, 31.92, 30.61, 28.61, 28.04, 27.00, 22.16, 19.63, 19.43, 16.80, 16.13, 15.04; ESI-HRMS Calcd for $C_{69}H_{111}N_4O_{31}$ $[M+H]^+$: 1653.7755. Found 1653.7821; $C_{75}H_{120}N_4NaO_{36}$ $[M+Na]^+$: 1675.7574. Found 1675.7494.

4.2. Biological assays

4.2.1. HCV and VSV pseudovirus entry assays

All compounds were tested using the HCV and VSV pseudo

particle (HCVpp and VSVGpp) entry assay as described previously [27,28,40–42]. Briefly, pseudotyped viruses were produced by co-transfecting plasmids expressing HCV E1E2 or vesicular stomatis G protein (VSVG) with pNL4-3 HIV proviral DNA (AIDS Reagent Program, NIH, Bethesda, MD). The envelope protein and the Vpr deficient HIV vector carrying a luciferase reporter gene was inserted into the Nef position in 293 T producer cells. For compound library screening, Huh-7 cells (5×10^3 cells/well) were seeded into 96-well plates for 24 h, and were then infected with HCVpp or VSVGpp in the presence or absence of compounds, followed by incubation at 37 °C. Test compounds were diluted to a final concentration of 1 μM and 5 μM in 1% dimethyl sulfoxide (DMSO). Luciferase activity, reflecting the amount of pseudovirus entering into host cells, was measured 3 days after infection using the Bright-Glo Reagent (Promega). Maximum activity (100% of control) and background were determined from the control wells containing DMSO alone or from uninfected wells, respectively. CD81 was used as the positive control as previously reported [14]. The individual signals in each of the compound test wells were then divided by the averaged control values (wells lacking inhibitor), background subtracted, 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 VSVGpp infection in parallel. Each sample was analyzed in duplicate, and the experiments were repeated at least three times.

4.2.2. Cytotoxicity assays

All of the reported conjugates were evaluated for cytotoxicity in MDCK Madin-Daby canine kidney cells. The cells (1×10^4 cells per well) were seeded in 96-well tissue culture plates and incubated for 16 h at 37 °C in an atmosphere of 5% CO_2 to allow the cells to adhere to the surface of the wells. Then, the culture medium was replaced with fresh medium containing the compounds at the concentrations of 0.1 μM, 1 μM, 10 μM, and 100 μM in triplicate, and control wells contained the equivalent volume of the medium 1% DMSO. They were then incubated for 48 h at 37 °C in an atmosphere of 5% CO_2. Following the incubation, alamarBlue (10 μL per well) was added aseptically to the wells, and the cultures were returned to incubator. After 2 h, the fluorescence intensities were measured with an excitation wavelength of 530 nm, and an emission wavelength of 590 nm. The percent of cell viability was calculated using the equation:

$$\text{Cell Viability } (\%) = F/F_0) \times 100\%$$

F: the fluorescence of the compounds, F_0: the fluorescence of DMSO.

4.2.3. Binding assays

Huh-7 cells were infected with HCVpp under five different experimental conditions: a co-treatment assay, a pre-treatment assay, a pre-binding assay, a post-binding assay, and a post-entry assay. The co-treatment assay: Huh-7 cells were incubated with 50 μL compound and 50 μL HCVpp for 72 h at 37 °C. The pre-treatment assay: Huh-7 cells were first incubated with 50 μL compound (final concentration of 10 μM) and 50 μL culture medium (DMEM containing 10% FBS) at 37 °C for 3 h. Subsequently, cells were washed with medium to remove unbound compound and then exposed to 50 μL HCVpp and 50 μL medium at 37 °C for 72 h. The prebinding assay: Huh-7 cells were first incubated with 50 μL compound and 50 μL HCVpp at 4 °C for 3 h (for virus binding only since virus entry is high temperature-dependent). After the incubation period, cells were washed to remove unbound virus and compound, followed by addition of 100 μL medium, and cultured at 37 °C for 72 h to allow viral internalization and replication. The post-binding assay: Huh-7 cells were first incubated with 50 μL HCVpp and 50 μL medium at 4 °C for 3 h. After incubation, cells were washed with culture medium thoroughly to remove unbound virus and then exposed to 50 μL medium and 50 μL compound at 37 °C for 72 h. The post-entry assay: Huh-7 cells were first treated with 50 μL HCVpp and 50 μL medium at 37 °C for 6 h to allow virus entry into cells but not reverse transcription and integration of the viral genome within cells. After the incubation period, cells were washed to remove unbound virus, and incubated at 37 °C with 50 μL compound and 50 μL medium for further 72 h. In all five conditions, CD81 antibody was utilized as a positive control due to its blocking HCV virus entry via binding to CD81 receptor. IM2865 was a non-relevant compound as negative control and 0.5% DMSO (final concentration) was used for normalization in each condition.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.08.020.

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