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Conjugation of cyclodextrin with fullerene as a new class of HCV entry inhibitors

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ABSTRACT

An α-cyclodextrin–[60]fullerene conjugate with a flexible linker at the secondary face of α-cyclodextrin has been prepared, which displays significant water solubility and, more importantly, acts as a new class of HCV entry inhibitor with IC_{50} at 0.17 μM level.

1. Introduction

Fullerene has been attracting considerable attention in medicinal and material chemistry due to its unique physical and chemical properties. It was found that [60] fullerene was able to occupy the hydrophobic cavity of HIV proteases,[1] and thus inhibit the access of substrates to the catalytic site of the enzyme. Some derivatives of C_{60} also exhibit antibacterial[2] activities by binding specific antibiotics to resistant bacteria and even target certain cancer cells such as melanoma.[3] However, the lack of solubility[4] and formation of aggregates[5] in biologically environments may be a potential drawback for further broad applications, in particular, for development as a therapeutic medicine. There were some attempts to make fullerene water-soluble by chemically linking with hydrophilic appendages or physically forming complex with host molecules.

Cyclodextrins (CDs), cyclic oligosaccharides composed of α (1→4) glucosyl residues (Fig. 1), have been involved in a wide spectrum of applications due to their significant solubility in water.[6] Conjugations of CDs with fullerene have significantly increased fullerene solubility in water via forming water-soluble inclusion complexes.[7] So far, most of the cyclodextrin–fullerene conjugates are linked via the primary external rim of the β- or γ-CD (Fig. 2A) because of the much easier availability of the primary carbon derivatives.[7,8] Alternative conjugation of fullerene to CDs is via the larger secondary rim (Fig. 2B) that may bring unexpected properties. In addition, per-O-methylation of CDs may also alter the chemophysical properties, such as solubility in water and in organic solvents as well as the stability of their inclusion complexes.[9]

Here we reported an ongoing program that conjugated C_{60} with per-O-methylated *a*-CDs via their secondary rims from $2^A,3^B$-diol[10] in a simple and efficient way. To our surprise, such a water-soluble

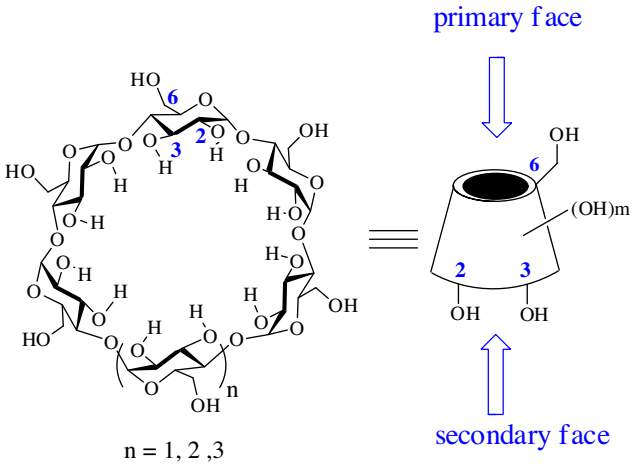

Figure 1. The 2:1 CD–C_{60} conjugates linked via the primary rim of the β- or γ-CD.

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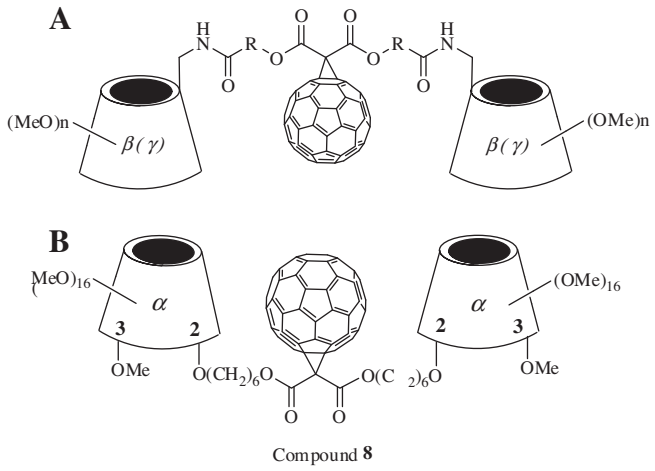


Figure 2. The 2:1 α-CD–C_{60} conjugate.

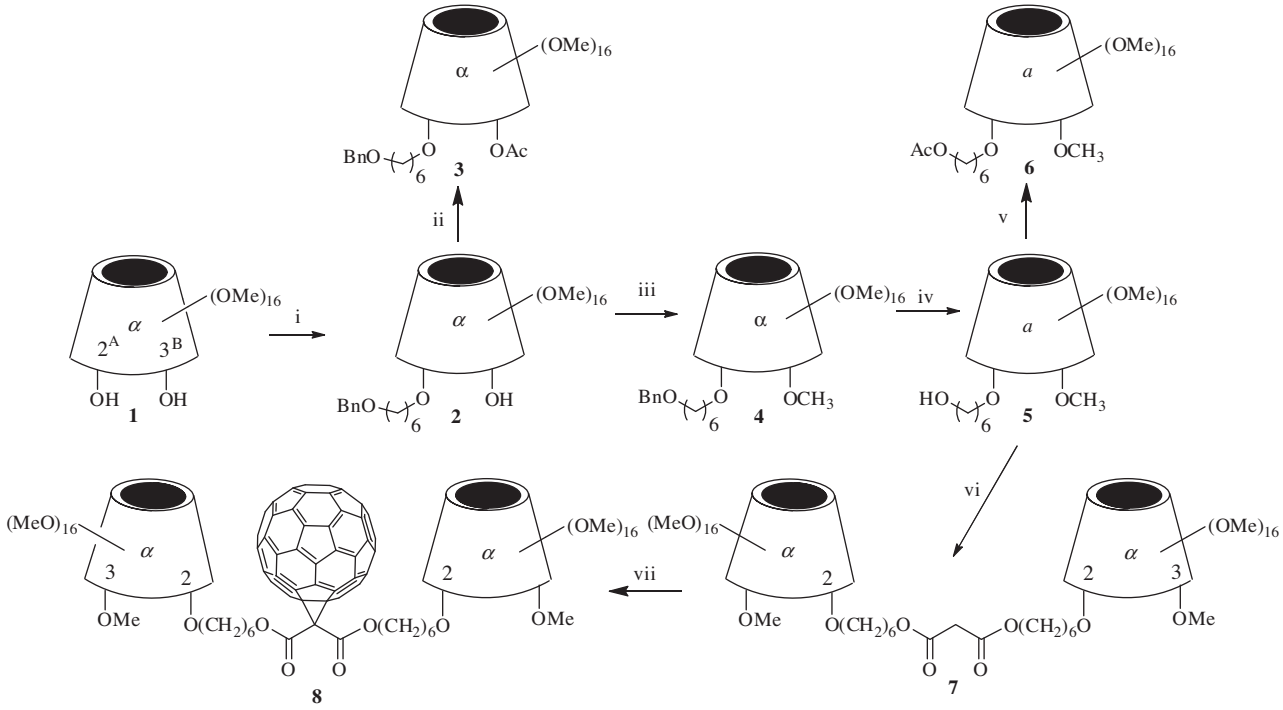
CD–C_{60} conjugate displays significant anti-HCV entry activity with IC_{50} at 0.17 μM level.

2. Results and discussion

As shown in Scheme 1, conjugation of *a*-CDs with C_{60} started from 2^A,3^B-dihydroxyl per-*O*-methylated α-CD (2^A,3^B-diol, **1**), a regioselective demethylated intermediate from the commercial per-O-methylated α-CD as previously reported.[10] A flexible hexane-linker was first tethered at 2^A of 2^A,3^B-diol by coupling with sequentially protected hexane-1,6-diol carrying benzyl and tosyl groups. The NMR data, δ 3.25 ppm (dd, $J_{1,2}$ = 3.2 Hz, $J_{2,3}$ = 10.0 Hz, H_3)/71.74 ppm (C_3^B), indicated that the linker was introduced at position 2. Such regioselectivity was further confirmed by its acetylating derivative **3** as its ^{1}H NMR for H_3 of the glucose unit B displayed deshielded signals, δ 5.42 (dd, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 9.6 Hz). Compound **2** was then methylated again at position 3 to convert into 2-alkyl per-O-methylated α-CD **4**, which, after catalytic hydrogenation, removed the benzyl group at the linker in quantitative yields. The afforded 2-hydroxyhexyl α-CD derivative (**6**) was coupled with malonyl dichloride to give α-CD dimer (**7**). At last, C_{60} was attached to the malonate **7** via Bingel–Hirsch cyclopropanation,[12] yielding α-cyclodextrin–[60]fullerene conjugate with a flexible hexane linker (**8**).

Characterization of α-cyclodextrin–[60]fullerene conjugate **8** indicated that 1.5 mg was able to quickly dissolve upon in dichloromethane (DCM) solvent. The UV–vis absorption spectrum in DCM (Fig. 3A) disclosed the typical absorption features[11c] of fullerene [60] mono-adduct: a strong absorption peak at 320 nm, a weak absorption peak at 425 nm and a marginal peak at 470 nm, suggesting it exist as a non-associated species in DCM without aggregate formation. We also found that the same amount of conjugate **8** was able to dissolve in 3 mL of water (0.5 mg/mL) at room temperature with a strong absorption peak at 320 nm (Fig. 3B). It has been estimated that the solubility of C_{60} is just 1.3×10^{-11} mg/mL.[4b] Therefore, conjugation of C_{60} with per-O-methylated α-CDs via their secondary rims increased its solubility over 10 orders of magnitude.

Hepatitis C virus is the leading cause of liver fibrosis and cirrhosis which eventually worsen to liver cancer. Treatment of HCV infection has languished under ribavirin and interferon for almost 30 years with recently approved telaprevir and boceprevir representing the beginning of a new era.[13] However, resistance to individual antivirals is likely to develop that require combination of drugs targeting different stages of HCV viral life cycle. Inhibition of viral entry into HCV-permissive cells and development of HCV entry inhibitors represents an emerging field that could satisfy the tandem mechanism for use with other inhibitors to eradication HCV infection. Here we found that conjugate **8** exhibited significant dose-dependent inhibition against HCVpp entry, a well-established assay for identifying HCV entry inhibitors,[14] with over 95%



Scheme 1. Reagents and conditions: (i) NaH, DMF, $BnO(CH_2)_6OTs$; (ii) Py, Ac_2O; (iii) NaH, CH_3I, DMF, 50 °C; (iv) Pd/C, H_2, CH_3OH; (v) Py, Ac_2O; (vi) malonyl dichloride, Py, CH_2Cl_2, 0 °C → rt; (vii) C_{60}, CBr_4, DBU, toluene, rt.

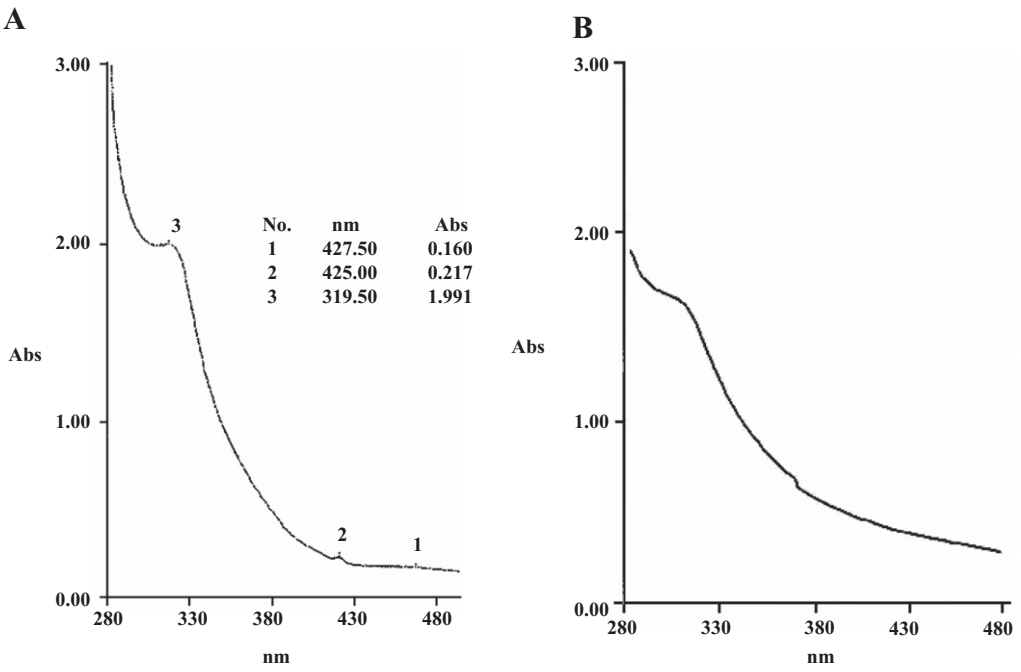


Figure 3. The UV–vis spectrum of conjugate **8** in DCM (A) and water (B).

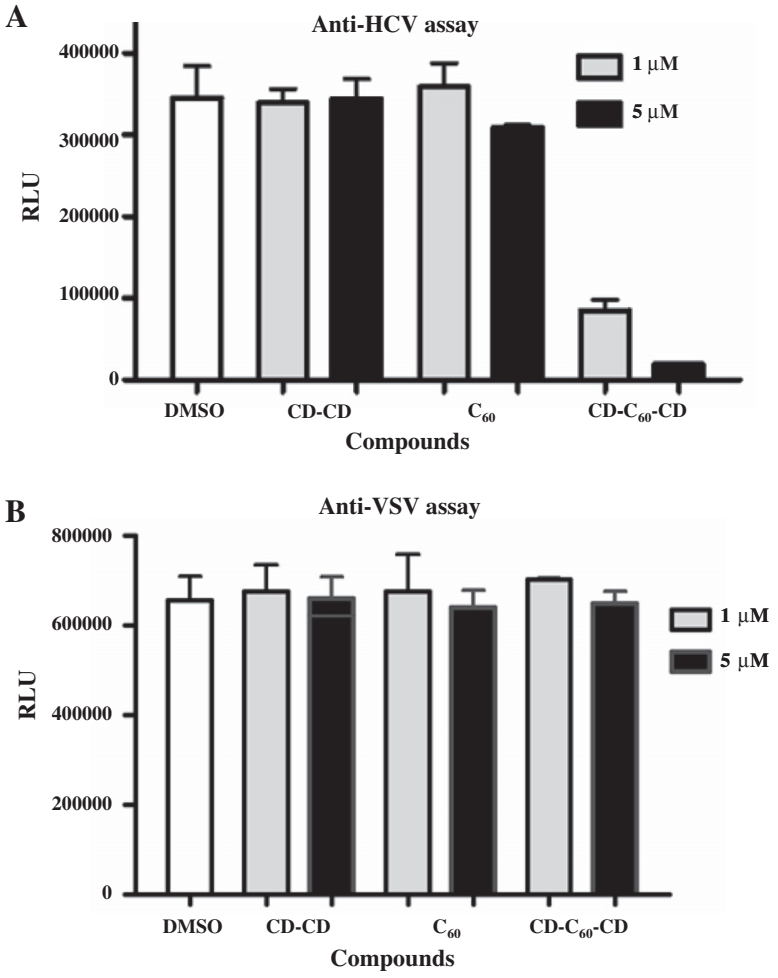


Figure 4. Anti-HCV activity of conjugate **8** based on HCVpp/VSV-Gpp entry assay.

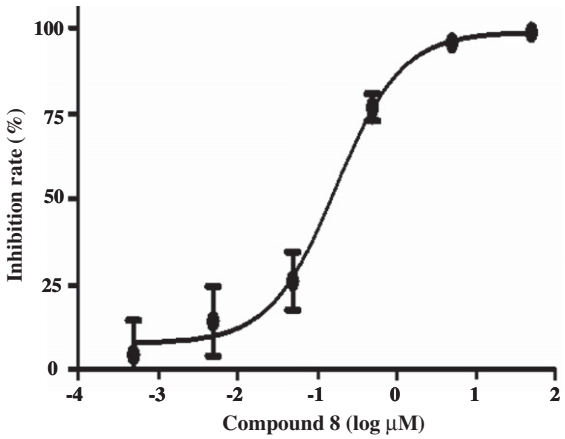


Figure 5. The inhibition curve plateaus for obtaining the IC_{50}, which covered concentration 0.5 nM, 5 nM, 50 nM, 0.5 μM, 5 μM and 50 μM. Each concentration was tested triplicate and results were expressed as mean and standard deviation from triplicate assays.

of virus entry being blocked at 5 μM (Fig. 4A). To determine the specificity of α-cyclodextrin–[60]fullerene conjugate toward HCV, its ability to block entry of the 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 the α-cyclodextrin–[60]fullerene conjugate did not block VSVG viral entry (Fig. 4B), indicating it is a selective antiviral agent against the HCV. Further biological evaluation of the α-cyclodextrin–[60]fullerene conjugate showed that it did exhibit significant inhibition against HCVpp entry with an IC_{50} value of 0.17 μM (Fig. 5), comparable with or even better than other reported HCV entry inhibitors such as IM2281.[14] α-Cyclodextrin–[60]fullerene conjugate may represent a new class of HCV entry inhibitor and continuous conjugations of fullerene with a series of cyclodextrins including α-, β-, γ- and other non-natural isoforms are ongoing.

Aimed at understanding at which stage of infection the cyclodextrin–fullerene conjugate exerts its inhibitory activity, five different assay conditions were set up as previously reported.[15] In the first condition—that is the standard antiviral assay or co-treatment—the cyclodextrin–fullerene conjugate was present in the culture medium during the entire viral entry process. Briefly, cells were infected with HCVpp or VSVpp in the presence of 10 μM cyclodextrin–fullerene conjugate and incubated for 72 h at 37 °C to allow entry of the virus. In the second condition—the pretreatment assay—cells were first pretreated with 10 μM cyclodextrin–fullerene conjugate at 37 °C for 3 h, washed to remove unbound compound and then exposed to viruses at 37 °C for 72 h. In the third condition—the prebinding assay—cells were exposed to viruses in the presence of cyclodextrin–fullerene conjugate at 4 °C for 3 h (this step was for virus binding not entry since entry is high temperature-dependent), washed to remove unbound viruses and compound and then cultured at 37 °C for 72 h. In the forth condition—the post-binding or pre-entry assay—cells were first incubated with viruses at 4 °C for 3 h, washed to remove unbound virus and then treated with cyclodextrin–fullerene conjugate at 37 °C for 72 h. In the last condition—post-entry assay—cells were first treated with viruses at 37 °C for 6 h to make virus entry into cells. After washing unbound viruses, infected cells were treated with the compound at 37 °C for 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 and IM2841 were two non-relevant compounds as negative control[14] and 0.5% DMSO (final concentration) was used for normalization in each condition. In comparison to the standard antiviral assay, a short pretreatment of the cells with compound prior to virus infection (pretreatment) or co-treatment of cells with mixture of virus and compound at 4 °C (prebinding) resulted in very weak if any activity (Fig. 6), suggesting that cyclodextrin–fullerene conjugate exerts its inhibitory activity post virus binding, significantly different from CD81 antibody which interferes with virus attachment to the target cells via occupation of the surface receptors. In the post-binding or pre-entry condition, a high activity of cyclodextrin–fullerene conjugate was noted, suggesting that cyclodextrin–fullerene conjugate interfere with the subsequent step following virus attachment to target cells, presumably the viral envelop-cell membrane fusion. While in the post-entry condition, no antiviral activity was observed, indicating that cyclodextrin–fullerene conjugate does not interferes with the multiple processes post viral entry and further confirming cyclodextrin–fullerene conjugate exerts its inhibitory function. In conclusion, these data suggest that cyclodextrin–fullerene conjugate exerts its inhibitory activity post-virus binding and pre-virus entry, potentially at virus-target cell fusion.

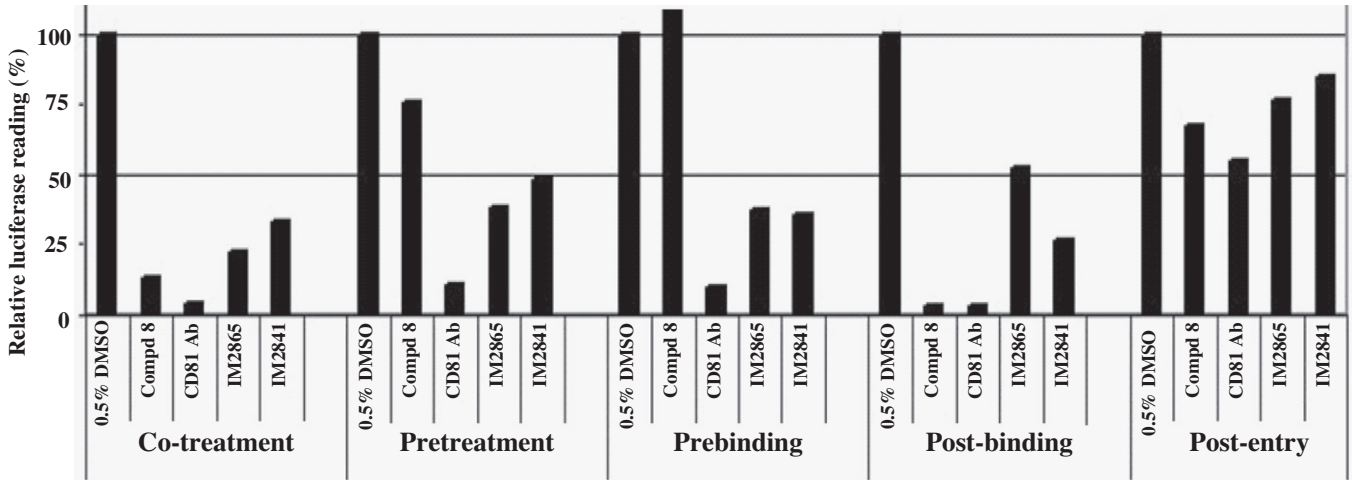


Figure 6. Mechanistic studies of conjugate **8**-mediated blocking of HCVpp entry by five different assay conditions. CD81 antibody, an entry inhibitor targeting host cell membrane, was utilized as a positive control and IM2865 and IM2841, two non-relevant compounds[14], were used as negative control. 0.5% DMSO (final concentration) was used for normalization in each condition.

3. Conclusion

We reported here the synthesis of α-CD/C_{60} conjugate via the secondary rim of α-CD that fullerene was directly tethered. Such conjugate exists significant solubility in both DCM and water. More importantly, it displays high activity for blocking hepatitis C virus entry with IC_{50} at 0.17 μM.

4. Experimental

4.1. General

Optical rotations were measured at 20 ± 2 °C with a Perkin Elmer Model 343 digital polarimeter, using a 10 cm, 1 mL cell. 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 on a Bruker DRX 400 spectrometer at ambient temperature. ^{1}H NMR chemical shifts are referenced to the internal standard TMS (δ_H = 0.00). ^{13}C NMR chemical shifts are referenced to the solvent signal (δ_C = 77.00 for the central line of $CDCl_3$). 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 charring with a 10% solution of sulfuric acid in ethanol. Flash column chromatography was performed on Silica Gel 60 (230–400 mesh, E. Merck or 200–300 mesh, Qingdao Haiyang Chemical Co. Ltd). The UV–vis absorption spectra were obtained by a TU-1901 UV–Vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd, Beijing, China).

4.2. Synthesis of 2^A-*O*-(6-benzyloxyhexyl)-3^B-hydroxyl-per-*O*-methylated α-CD (2)

A mixture of 2^A,3^B-diol **1** (100 mg, 0.08 mmol), NaH (60%, 4.0 mg, 0.1 mmol) in dry DMF (3 mL) under nitrogen was stirred at 0 °C for 1 h. Diluted 6-(Benzyloxy)hexan-1-ol *p*-methylbenzenesulfonate (33 mg, 0.09 mmol) by dry DMF (2 mL) was dropped into the above mixture and stirred for 18 h. CH_3OH was added dropwise to quench the reaction and the solvent was removed by evaporation. The residue was dissolved in CH_2Cl_2, washed with brine and water, dried over $MgSO_4$ and concentrated. The crude product was purified by column chromatography (PE/Acetone = 3:2) to afford **2** (86 mg, 76%) as a white amorphous solid: R_f = 0.41 (PE/Acetone = 1:1); $[\alpha]_D$ +139 (c = 1.0 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.30–1.40 (m, 4H), 1.57–1.69 (m, 4H), 3.14–3.18 (m, 4H, 4 × H_2), 3.25 (dd, 1H, $J_{1,2}$ = 3.2 Hz, $J_{2,3}$ = 10.0 Hz, H_2^B), 3.29–3.21 (m, 1H, H_2^A), 3.41 (3 × s, 18H, 6 × $OCH_3(C_6)$), 3.43(m, 2H, CH_2OBn), 3.48 (2 × s, 6H, 2 × OCH_3 (C_2)), 3.49 (s, 6H, 2 × OCH_3 (C_2)), 3.53 (s, 3H, OCH_3 (C_2)), 3.57 (m, 1H, H_4^B), 3.60 (m, 1H, H_3^A), 3.62 (s, 3H, OCH_3 (C_3)), 3.63 (s, 6H, 2 × OCH_3 (C_3)), 3.64 (2 × s, 6H, 2 × OCH_3 (C_3)), 3.68 (m, 2H, CD-OCH_2–), 3.43–3.90 (m, 27H, 4 × H_3, 5 × H_4, 6 × H_5, 6 × H_{6a}, 6 × H_{6b}), 4.07 (t, 1H, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 8.8 Hz, H_3^B), 4.49 (s, 2H, $PhCH_2$–), 4.95 (d, 1H, $J_{1,2}$ = 3.2 Hz, H_1^A), 4.97 (br s, 1H, D_2O exchangeable, OH), 5.02 (d, 1H, $J_{1,2}$ = 3.2 Hz, H_1), 5.04–5.06 (m, 3H, 3 × H_1), 5.10 (d, 1H, $J_{1,2}$ = 3.2 Hz, H_1^B), 7.25–7.29 (m, 1H, arom-H), 7.32–7.34 (m, 4H, 4 × arom-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.52, 25.88 (2C, $C_{3'}$-CH_2, $C_{4'}$-CH_2), 29.65, 29.69 (2C, $C_{2'}$-CH_2, $C_{5'}$-CH_2), 57.63, 57.68, 57.83, 57.85 (5C, 5 × OCH_3 (C_2)), 58.95, 58.99, 59.02, 59.05, 59.10 (6C, 6 × OCH_3 (C_6)), 61.79, 61.85, 61.94 (5C, 5 × OCH_3 (C_3)), 70.01, 71.14, 71.19, 71.26, 71.35, 71.56 (6C, 6 × C_5), 70.29 (–CH_2OBn), 71.42, 71.65 (6C, 6 × C_6), 71.74 (C_3^B), 72.83 ($PhCH_2O$–), 72.91 (CD-OCH_2–), 81.14, 81.24, 82.10, 82.20, 82.28, 82.32, 82.38, 82.47, 82.55, 82.79, 83.68 (17C, 6 × C_2, 5 × C_3, 6 × C_4), 100.02, 100.07, 100.27, 100.30 (5C, 5 × C_1), 101.39(C_1^A), 127.42, 127.58, 128.30, 138.63 (6C, 6 × arom-C); ESI-HRMS (m/z) Calcd for $C_{65}H_{114}NO_{31}$ $[M+NH_4]^+$: 1404.7369. Found 1404.7356. Calcd for $C_{65}H_{110}O_{31}Na$ $[M+Na]^+$: 1409.6923. Found 1409.6910.

4.3. Synthesis of 2^A-*O*-(6-benzyloxyhexyl)-3^B-*O*-acetyl-per-*O*-methylated α-CD (3)

A mixture of **2** (27 mg, 0.02 mmol), Ac_2O (0.5 mL) in anhydrous pyridine (1 mL) was stirred at room temperature for 18 h. After removal of the solvent by evaporation, the residue was dissolved in CH_2Cl_2, washed with brine and water, dried over $MgSO_4$ and concentrated, the crude product was purified by flash chromatography (CH_2Cl_2/CH_3OH = 40:1) to afford **3** (26 mg, 94%) as a white amorphous solid: R_f = 0.52 (PE/Acetone = 1:1); $[\alpha]_D$ +141 (c = 1.0 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.38–1.41 (m, 4H), 1.61–1.66 (m, 4H), 2.12 (s, 3H, CH_3CO), 3.10–3.19 (m, 5H, 5 × H_2), 3.24 (dd, 1H, $J_{1,2}$ = 3.2 Hz, $J_{2,3}$ = 10.0 Hz, H_2^B), 3.40 (br s, 18H, 6 × $OCH_3(C_6)$), 3.43 (s, 3H, OCH_3 (C_2)), 3.46 (s, 3H, OCH_3 (C_2)), 3.47 (m, 2H, –CH_2OBn), 3.48 (s, 3H, OCH_3 (C_2)), 3.49 (br s, 6H, 2 × OCH_3 (C_2)), 3.57 (s, 3H, OCH_3 (C_3)), 3.59 (s, 3H, OCH_3 (C_3)), 3.61 (s, 3H, OCH_3 (C_3)), 3.63 (s, 3H, OCH_3 (C_3)), 3.65 (m, 2H, CD-OCH_2–), 3.69 (s, 3H, OCH_3 (C_3)), 3.74 (m, 1H, H_4^B), 3.41–3.94 (m, 27H, 5 × H_3, 5 × H_4, 6 × H_5, 6 × H_{6a}, 5 × H_{6b}), 4.04 (dd, 1H, $J_{5,6b}$ = 2.4 Hz, $J_{6a,6b}$ = 10.4 Hz, H_{6b}), 4.50 (s, 2H, $PhCH_2O$–), 4.84 (d, 1H, $J_{1,2}$ = 2.8 Hz, H_1), 5.00 (d, 1H, $J_{1,2}$ = 3.2 Hz, H_1), 5.04–5.05 (m, 2H, 2 × H_1), 5.08 (d, 1H, $J_{1,2}$ = 3.2 Hz, H_1), 5.10 (d, 1H, $J_{1,2}$ = 3.2 Hz, H_1^B), 5.42 (t, 1H, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 9.6 Hz, H_3^B), 7.28–7.29 (m, 1H, arom-H), 7.34–7.35 (m, 4H, 4 × arom-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.57 (CH_3CO), 25.66, 26.07 (2C, $C_{3'}$-CH_2, $C_{4'}$-CH_2), 29.77, 29.89 (2C, $C_{2'}$-CH_2, $C_{5'}$-CH_2), 57.50, 57.71, 57.79, 57.99, 58.24 (5C, 5 × OCH_3 (C_2)), 58.81, 58.94, 58.97, 59.05, 59.10 (6C, 6 × OCH_3 (C_6)), 61.52, 61.62, 61.69, 61.89, 62.10 (5C, 5 × OCH_3 (C_3)), 70.32 (–CH_2OBn), 70.78, 71.35, 71.48, 71.65, 71.90 (7C, CD-OCH_2-, 6 × C_6), 70.95 (C_3^B), 71.08, 71.14, 71.43, 71.51 (6C, 6 × C_5), 72.89 ($PhCH_2O$–), 79.33 (C_2^B), 79.79 (C_4^B), 80.68, 80.89, 80.93, 81.29, 82.21, 82.26, 82.35, 82.50, 82.57, 82.63, 82.76, 82.90 (15C, 5 × C_2, 5 × C_3, 5 × C_4), 99.85, 100.12, 100.20, 100.31[2C], 100.53 (6C, 6 × C_1), 127.49, 127.59, 128.33, 138.62 (6C, 6 × arom-C), 170.86 (CH_3CO); ESI-HRMS (m/z) Calcd for $C_{67}H_{116}NO_{32}$ $[M+NH_4]^+$: 1446.7475. Found 1446.7455. Calcd for $C_{67}H_{112}O_{32}Na$ $[M+Na]^+$: 1451.7029. Found 1451.7014.

4.4. Synthesis of 2^A-*O*-(6-benzyloxyhexyl)-per-*O*-methylated α-CD (4)

To a mixture of **2** (103 mg, 0.07 mmol) and NaH (60%, 8.9 mg, 0.22 mmol), Ac_2O (0.5 mL) in anhydrous DMF (4 mL) was added CH_3I (15 μL, 0.22 mmol). The reaction mixture was stirred at room temperature for 2 h, then at 50 °C for 8 h. CH_3OH (0.5 mL) was added to quench the reaction. After removal of the solvent by evaporation, the residue was purified by flash chromatography (PE/Acetone = 5:2) to afford **7** (96 mg, 83%) as a white amorphous solid: R_f = 0.39 (PE/Acetone = 1:1); $[\alpha]_D$ +136 (c = 1.0 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.34–1.41 (m, 4H), 1.57–1.62 (m, 4H), 3.15–3.18 (m, 6H, 6 × H_2), 3.38 (s, 3H, $OCH_3(C_6)$), 3.40 (br s, 15H, 5 × $OCH_3(C_6)$), 3.50 (br s, 15H, 5 × OCH_3 (C_2)), 3.64 (br s, 18H, 6 × OCH_3 (C_3)), 3.45 (m, 2H, –CH_2OBn), 3.77 (m, 2H, CD-OCH_2–), 3.44–3.88 (m, 30H, 6 × H_3, 6 × H_4, 6 × H_5, 6 × H_{6a}, 6 × H_{6b}), 4.49 (s, 2H, $PhCH_2$–), 5.03–5.07 (m, 6H, 6 × H_1), 7.27–7.29 (m, 1H, arom-H), 7.33–7.40 (m, 4H, 4 × arom-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 26.02, 26.12 (2C, $C_{3'}$-CH_2, $C_{4'}$-CH_2), 29.61, 29.74 (2C, $C_{2'}$-CH_2, $C_{5'}$-CH_2), 57.80, 57.82 (5C, 5 × OCH_3 (C_2)), 58.92, 58.95, 59.00 (6C, 6 × OCH_3 (C_6)), 61.76 (6C, 6 × OCH_3 (C_3)), 70.33 (–CH_2OBn), 71.15, 71.18 (6C, 6 × C_5), 71.39, 71.46 (7C, CD-OCH_2–, 6 × C_6), 72.83 ($PhCH_2$–), 81.20, 81.24, 82.11, 82.16, 82.20, 82.38, 82.43 (18C, 6 × C_2, 6 × C_3, 6 × C_4), 99.89, 100.07, 100.12, 100.16 (6C, 6 × C_1), 127.44, 127.54, 128.29, 138.59 (6C, 6 × arom-C);

ESI-HRMS (m/z) Calcd for $C_{67}H_{116}NO_{32}$ ($M+NH_4^+$): 1418.7526. Found 1418.7566. Calcd for $C_{67}H_{112}O_{32}Na$ $[M+Na]^+$: 1423.7080. Found 1423.7119. Calcd for $C_{67}H_{112}O_{32}K$ $[M+K]^+$: 1439.6814. Found 1439.6833.

4.5. Synthesis of 2^A-*O*-(6-hydroxyhexyl)-per-*O*-methylated α-CD (5)

To a solution of **4** (784 mg, 0.56 mmol) in methanol (20 mL) was added 116 mg (10%, 0.11 mmol, 0.2 equiv) of palladium–carbon. The suspension was degassed under vacuum and urged with H_2 three times, then stirred under H_2 balloon at room temperature for 16 h. The suspension was filtered through a pad of Celite and the pad cake was washed with CH_3OH (4 mL × 3). The combined filtrate was concentrated to dryness. The residue was subjected to flash chromatography (PE/Acetone = 5:2) to afford **5** (698 mg, 95%) as a white foam. R_f = 0.29 (PE/Acetone = 1:1); $[\alpha]_D$ +136 (c = 1.0 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.36–1.46 (m, 4H), 1.54–1.68 (m, 4H), 3.15–3.19 (m, 5H, 5 × H_2), 3.22 (dd, 1H, $J_{1,2}$ = 3.0 Hz, $J_{2,3}$ = 9.5 Hz, H_2^A), 3.40 (br s, 18H, 6 × $OCH_3(C_6)$), 3.44 (m, 2H, –CH_2OH), 3.49 (2 × s, 15H, 5 × OCH_3 (C_2)), 3.62 (s, 3H, OCH_3 (C_3)), 3.64 (2 × s, 12H, 4 × OCH_3 (C_3)), 3.65 (s, 3H, OCH_3 (C_3)), 3.66 (m, 2H, CD-OCH_2–), 3.46–3.85 (m, 29H, 6 × H_3, 6 × H_4, 6 × H_5, 6 × H_{6a}, 5 × H_{6b}), 3.91 (dd, 1H, $J_{5,6b}$= 3.8 Hz, $J_{6a,6b}$ = 10.8 Hz, H_{6b}), 4.97 (d, 1H, H_1^A), 5.04–5.06 (m, 5H, 5 × H_1); ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.41, 25.60 (2C, $C_{3'}$-CH_2, $C_{4'}$-CH_2), 29.69, 30.00 (2C, $C_{2'}$-CH_2, $C_{5'}$-CH_2), 57.82, 57.89, 57.93, 58.11 (5C, 5 × OCH_3 (C_2)), 58.92, 58.97, 58.99 (6C, 6 × OCH_3 (C_6)), 61.61, 61.72, 61.79, 61.81, 62.18 (6C, 6 × OCH_3 (C_3)), 62.67 (–CH_2OH), 70.64, 71.45, 71.47, 71.49, 71.57 (7C, CD-OCH_2-, 6 × C_6), 71.16, 71.19, 71.25 (6C, 6 × C_5), 81.21, 81.27, 81.31, 81.39, 81.86, 82.19, 82.21, 82.27, 82.34, 82.35, 82.40, 82.46, 82.48, 82.53, 82.55 (18C, 6 × C_2, 6 × C_3, 6 × C_4), 100.05[2C], 100.12, 100.25, 100.37, 100.51 (6C, 6 × C_1); ESI-HRMS (m/z) Calcd for $C_{59}H_{107}O_{31}$ $[M+H]^+$: 1311.6791. Found 1311.6803. Calcd for $C_{59}H_{110}NO_{31}$ $[M+NH_4]^+$: 1328.7062. Found 1328.7096. Calcd for $C_{59}H_{106}O_{31}Na$ $[M+Na]^+$: 1333.6616. Found 1333.6668.

4.6. Synthesis of 2^A-*O*-(6-acetyloxyhexyl)-per-*O*-methylated α-CD (6)

A mixture of **5** (37 mg, 0.03 mmol), Ac_2O (0.5 mL) in anhydrous pyridine (1 mL) was stirred at room temperature for 18 h. After removal of the solvent by evaporation, the residue was dissolved in CH_2Cl_2, washed with brine and water, dried over $MgSO_4$ and concentrated, the crude product was purified by flash chromatography (CH_2Cl_2/CH_3OH = 50:1) to afford **6** (35 mg, 90%) as a white amorphous solid. R_f = 0.32 (PE/Acetone = 1:1); $[\alpha]_D$ +140 (c = 1.0 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.35–1.44 (m, 4H), 1.61–1.66 (m, 4H), 2.04 (s, 3H, CH_3CO), 3.14–3.19 (m, 5H, 5 × H_2), 3.22 (dd, 1H, $J_{1,2}$ = 2.8 Hz, $J_{2,3}$ = 9.6 Hz, H_2^A), 3.40 (br s, 18H, 6 × $OCH_3(C_6)$), 3.49 (2 × s, 12H, 4 × OCH_3 (C_2)), 3.50 (s, 3H, OCH_3 (C_2)), 3.62 (s, 3H, OCH_3 (C_3)), 3.64 (2 × s, 9H, 3 × OCH_3 (C_3)), 3.64 (s, 3H, OCH_3 (C_3)), 3.65 (s, 3H, OCH_3 (C_3)), 3.74 (m, 2H, CD-OCH_2–), 3.47–3.85 (m, 29H, 6 × H_3, 6 × H_4, 6 × H_5, 6 × H_{6a}, 5 × H_{6b}), 3.90 (dd, 1H, $J_{5,6b}$ = 4.0 Hz, $J_{6a,6b}$ = 10.4 Hz, H_{6b}), 4.05 (t, 2H, $J_{5',6'}$ = 4.0 Hz, $J_{6a',6b'}$ = 10.4 Hz, –CH_2OAc), 4.97 (d, 1H, $J_{1,2}$ = 2.8 Hz, H_1^A), 5.04–5.06 (m, 5H, 5 × H_1); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.99 (CH_3CO), 25.61, 25.80 (2C, $C_{3'}$-CH_2, $C_{4'}$-CH_2), 28.62, 29.94 (2C, $C_{2'}$-CH_2, $C_{5'}$-CH_2), 57.81, 57.86, 57.90, 58.09 (5C, 5 × OCH_3 (C_2)), 58.93, 58.97 (6C, 6 × OCH_3 (C_6)), 61.61, 61.75, 61.80, 61.83, 62.16 (6C, 6 × OCH_3 (C_3)), 64.51 (–CH_2OAc), 70.52, 71.42, 71.47, 71.52 (7C, CD-OCH_2-, 6 × C_6), 71.11, 71.15, 71.17, 71.23 (6C, 6 × C_5), 81.14, 81.19, 81.24, 81.26, 81.91, 82.16, 82.18, 82.24, 82.29, 82.38, 82.46, 82.47, 82.51, 82.54 (18C, 6 × C_2, 6 × C_3, 6 × C_4), 100.05 [2C], 100.12, 100.26, 100.39, 100.46 (6C, 6 × C_1); ESI-HRMS (m/z) Calcd for $C_{61}H_{112}NO_{32}$ $[M+NH_4]^+$: 1370.7162. Found 1370.7151. Calcd for $C_{61}H_{108}O_{32}Na$ $[M+Na]^+$:1375.6716. Found 1375.6721. Calcd for $C_{61}H_{108}O_{32}K$ $[M+K]^+$: 1391.6450. Found 1391.6446.

4.7. Synthesis of per-*O*-methylated α-CD dimer (7)

A solution of **6** (121 mg, 0.09 mmol), Ac_2O (0.5 mL) in anhydrous CH_2Cl_2 (10 mL) in ice-bath was added pyridine (9.0 μL, 0.11 mmol) and malonyl dichloride (5.0 μL, 0.046 mmol) under nitrogen. The mixture was stirred at room temperature for 12 h. After removal of the solvent by evaporation, the residue was purified by flash chromatography (PE/Acetone = 1:1) to provide **7** (78 mg, 63%) as a white foam. R_f = 0.12 (PE/Acetone = 1:1); $[\alpha]_D$ +136 (c = 1.0 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.37–1.43 (m, 8H), 1.56–1.67 (m, 8H), 3.15–3.18 (m, 10H, 10 × H_2), 3.22 (dd, 2H, $J_{1,2}$ = 2.4 Hz, $J_{2,3}$ = 9.0 Hz, 2 × H_2^A), 3.37 (s, 2H, –$COCH_2$-CO–), 3.40 (br s, 36H, 12 × $OCH_3(C_6)$), 3.49 (br s, 30H, 10 × OCH_3 (C_2)), 3.62 (s, 6H, 2 × OCH_3 (C_3)), 3.64 (s, 24H, 8 × OCH_3 (C_3)), 3.65 (s, 6H, 2 × OCH_3 (C_3)), 3.49–3.92 (m, 64H, 12 × H_3, 12 × H_4, 12 × H_5, 12 × H_{6a}, 12 × H_{6b}, 2 × CD-OCH_2–), 4.13 (t, 4H, $J_{5',6'}$ = 6.7 Hz, –$CH_2OCOCH_2COOCH_2$–), 4.97 (d, 2H, $J_{1,2}$ = 2.4 Hz, 2 × H_1^A), 5.05 (br s, 10H, 10 × H_1); ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.56, 25.65 (4C, 2 × $C_{3'}$-CH_2, 2 × $C_{4'}$-CH_2), 28.46, 29.91 (4C, 2 × $C_{2'}$-CH_2, 2 × $C_{5'}$-CH_2), 41.53 (–OCOCH_2COO–), 57.78, 57.79, 57.85, 57.89, 58.08 (10C, 10 × OCH_3 (C_2)), 58.92, 58.96 (12C, 12 × OCH_3 (C_6)), 61.60, 61.74, 61.79, 61.82, 62.15 (12C, 12 × OCH_3 (C_3)), 65.53 (2C, –CH_2OCOCH_2COOCH_2–), 70.47, 71.41, 71.45, 71.52 (14C, 2 × CD-OCH_2-, 12 × C_6), 71.10, 71.14, 71.15, 71.21 (12C, 12 × C_5), 81.12, 81.19, 81.23, 81.89, 82.15, 82.22, 82.28, 82.36, 82.44, 82.46, 82.50, 82.53 (36C, 12 × C_2, 12 × C_3, 12 × C_4), 100.03, 100.04, 100.10, 100.25, 100.38, 100.43 (12C, 12 × C_1), 166.66 (2C, –OCOCH_2COO–); ESI-HRMS (m/z) Calcd for $C_{121}H_{214}O_{64}$ $[M/2+H]^+$: 1362.7000. Found 1362.7059. Calcd for $C_{121}H_{212}O_{64}Na_2$ $[M/2+Na]^+$: 1367.6554. Found 1367.6599.

4.8. Synthesis of 2:1 per-*O*-methylated α-CD/fullerene conjugate (8)

To a solution of **7** (148 mg, 0.06 mmol) and C_{60} (198 mg, 0.28 mmol) in anhydrous toluene, DBU (20 μL, 0.14 mmol) was added and the mixture was stirred at room temperature for 24 h. The reaction mixture was directly chromatographed, eluting first with toluene to recover the excess of C_{60}, then PE/Acetone = 3:2 to provide compound **8** (66 mg, 36%) as brown solid. R_f = 0.19 (PE/Acetone = 1:1); $[\alpha]_D$ + 156 (c = 1.0 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.41–1.50 (m, 8H), 1.62–1.65 (m, 4H), 1.83–1.85 (m, 4H), 3.14–3.19 (m, 10H, 10 × H_2), 3.22 (dd, 2H, $J_{1,2}$ = 3.2 Hz, $J_{2,3}$ = 8.6 Hz, 2 × H_2^A), 3.40 (br s, 36H, 12 × $OCH_3(C_6)$), 3.49 (2 × s, 30H, 10 × OCH_3 (C_2)), 3.62 (s, 6H, 2 × OCH_3 (C_3)), 3.64 (2 × s, 24H, 8 × OCH_3 (C_3)), 3.65 (s, 6H, 2 × OCH_3 (C_3)), 3.49–3.91 (m, 64H, 12 × H_3, 12 × H_4, 12 × H_5, 12 × H_{6a}, 12 × H_{6b}, 2 × CD-OCH_2–), 4.49 (t, 4H, $J_{5',6'}$ = 6.6 Hz, 2 × –CH_2OCO–), 4.97 (d, 2H, $J_{1,2}$ = 3.0 Hz, 2 × H_1^A), 5.05 (br s, 10H, 10 × H_1); ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.60, 25.84 (4C, 2 × $C_{3'}$-CH_2, 2 × $C_{4'}$-CH_2), 28.61, 30.01 (4C, 2 × $C_{2'}$-CH_2, 2 × $C_{5'}$-CH_2), 57.80, 57.87, 57.91, 58.12 (10C, 10 × OCH_3 (C_2)), 58.94, 58.98, 59.00 (12C, 12 × OCH_3 (C_6)), 61.65, 61.76, 61.81, 61.84, 62.20 (12C, 12 × OCH_3 (C_3)), 67.36 (2C, 2 × –CH_2OCO–), 70.44, 71.42, 71.47, 71.51, 71.55 (17C, 2 × CD-OCH_2-, 2 × C_{60}-sp^3C, bridgehead C, 12 × C_6), 71.14, 71.17, 71.23 (12C, 12 × C_5), 81.14, 81.24, 81.92, 82.16, 82.18, 82.23, 82.29, 82.39, 82.45, 82.47, 82.49, 82.55 (36C, 12 × C_2, 12 × C_3, 12 × C_4), 100.05, 100.12, 100.24, 100.41 (12C, 12 × C_1), 138.95, 140.95, 141.88, 142.20, 142.99, 143.00, 143.09, 143.89, 144.60, 144.64, 144.68, 144.88, 145.18, 145.25, 145.40, 145.64 (58C, C_{60}-sp^2C), 163.65 (2C, 2 × COO–); ESI-HRMS (m/z) Calcd for $C_{181}H_{218}N_2O_{64}$ $[M/2+NH_4]^+$: 1721.6914. Found 1721.6924.

4.9. Anti-HCV assay

Pseudotyped viruses were produced by cotransfecting plasmid expressing HCV E1E2 or vesicular stromatis G protein (VSVG) with pNL4-3 HIV proviral DNA (AIDS Reagent Program, NIH, Bethesda, MD), the envelope and Vpr deficient HIV vector carrying a luciferase reporter gene inserted into the Nef position in 293T producer cells. For compound library screening, Huh-7 cells (5×10^3 cells/well) were seeded into 96-well plates for 24 h, and then were 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 amounts of the pseudoviruses entering into host cells, was measured 3 days after infection using the Bright-Glo Reagent (Promega). Maximum activity (100% of control) and background were derived from control wells containing DMSO alone or from uninfected wells, respectively. CD81 and IM2281 were used two 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), 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 VSVGpp infection in parallel. Each sample was done in duplicate, and experiments were repeated at least three times.

IC_{50} was calculated as the inhibitor concentration needed to decrease the luciferase activity by 50% compared to the luciferase activity without any inhibitor.

4.10. Binding assays

Huh-7 cells were infected with HCVpp under five different experimental conditions: a co-treatment assay, a pretreatment assay, a prebinding assay, a post-binding assay and a post-entry assay (Fig. 6). The co-treatment assay: Huh-7 cells were incubated with 50 μL compound and 50 μL HCVpp for 72 h at 37 °C. The pretreatment 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 and IM2841 were two non-relevant compounds as negative control and 0.5% DMSO (final concentration) was used for normalization in each condition.

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Supplementary data

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