Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Monoglycocalix[4]arene-based nanoparticle for tumor selective drug delivery via GLUT1 recognition of hyperglycolytic cancers

Lijuan Zhang,[a]† Shengnan Liu,[a]† Yang Li,[c] Min Liang,[b] Hongxia Zhao,*[a] and Wensheng Yang,*[a]

[a] Institute of Molecular Plus, Frontiers Science Center for Synthetic Biology (Ministry of Education), Tianjin Key Laboratory for Modern Drug Delivery & High-Efficiency, Tianjin University, 92 Weijin Road, Nankai District, Tianjin 300072, P. R. China.

[b] Central Institute of Pharmaceutical Research, CSPC Pharmaceutical Group, 226 Huanhe Road, Shijiazhuang, Hebei 050035, PR China.

[c] Department of Biology, Gudui BioPharma Technology Inc., 5 Lanyuan Road, Huayuan Industrial Park, Tianjin 300384, P. R. China.

Corresponding Authors

* E-mail: zhaohongxia@tju.edu.cn, wsyang@jlu.edu.cn

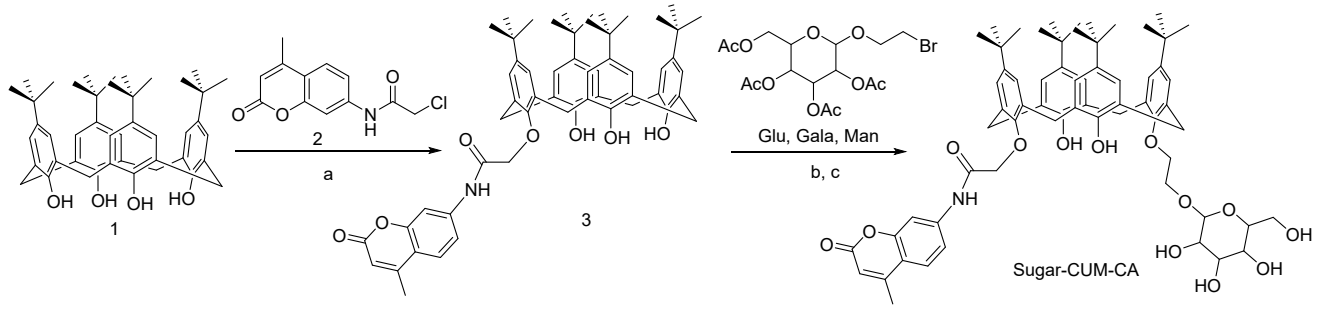
Table of Contents

1. Materials and Instrumentation.

For chemistry, all chemicals were obtained from commercial suppliers and were used as received. If necessary, the reactions were carried out in dry solvents and under an argon atmosphere. ^{1}H and $^{13}CNMR$ spectra were recorded with a Bruker Avance 400 or 600 MHz at the School of Pharmaceutical Science and Technology of Tianjin University, PRC. Data are reported as chemical shifts (δ) in parts per million (ppm) relative to the solvent peak, and scalar coupling constants (*J*) are reported in units of hertz (Hz). Infrared spectra were recorded using a Bruker Tensor II FT-IR spectrometer. Absorptions are reported in wavenumbers (cm^{-1}). Absorptions are reported in wavenumbers (cm^{-1}). Ultraviolet-visible (UV-vis) was respectively recorded with Shimadzu UV-1900 at room temperature with a quartz cuvette having a pathlength of 1 cm as a sample holder. Fluorescence spectra was respectively recorded with Shimadzu RF-6000 systems High-resolution mass spectra (HRMS, m/z) were recorded on a Thermo Scientific™ Q Exactive™ HF spectrometer in positive mode (ESI^{+}).

2. General Methods

2.1 Chemistry



Scheme S1. Synthesis of Sugar-Cum-CA: a) K_2CO_3, CH_3CN, 80 °C; b) KI, K_2CO_3, CH_3CN, 80 °C; c) NaOH, MeOH, H_2O.

5,11,17,23-tetra-tert-butyl-25-((4-methyl-2-oxo-2H-chromen-7-yl)carbamoylmethoxy)-26,27,28-trihydroxycalix[4]arene (3). The suspension of compound 2 (77 mg, 0.3 mmol) in anhydrous CH_3CN (2 ml) was heated to reflux for 30 min, then compound 1 (100 mg, 0.15 mmol) and K_2CO_3 (43 mg, 0.3 mmol) were added. The reaction mixture was further reflux for 6 h. After that, the resulting mixture was allowed to reach room temperature and quenched with H_2O (10 ml), and then it was diluted with EA (20 ml). The organic layer was dried by anhydrous Na_2SO_4 and filtered. The solvent of the filtrate was evaporated in vacuo to give crude product that was purified by silica gel column chromatography using petroleum ether–ethylacetate(EA) to give compound 3 (50 mg, 38%) as white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 11.04 (s, 1H), 10.12 (s, 1H), 9.54 (s, 2H), 8.12 (d, J = 2.0 Hz, 1H), 7.89 (dd, J = 9.0, 2.0 Hz, 1H), 7.61 (d, J = 9.0 Hz, 1H), 7.14 – 7.06 (m, 8H), 6.23 (d, J = 1.2 Hz, 1H), 4.74 (s, 2H), 4.27 (d, J = 14.0 Hz, 2H), 4.22 (d, J = 14.0 Hz, 2H), 3.55 (d, J

= 9.0 Hz, 2H), 3.53 (d, *J* = 9.0 Hz, 2H), 2.44 (d, *J* = 1.2 Hz, 3H), 1.24 (s, 9H), 1.23 (s, 18H), 1.19 (s, 9H).

Glu-Cum-CA (4). 2-bromoethyl-2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (128 mg, 0.28 mmol) was synthesized according to the literature [1] and subsequently dissolved in anhydrous CH_3CN. Then the catalytic amount of KI was added. The resulting solution was heated to 80 °C for 30 min and compound 3 (200 mg, 0.23 mmol) and K_2CO_3 (40 mg, 0.23 mmol) were added. The reaction mixture was stirred 3 days and allowed to reach room temperature. The mixture was quenched with H_2O (10 ml) and then it was diluted with EA (20 ml). The organic layers were washed with H_2O (2×10 ml), brine (10 ml), dried over Na_2SO_4 and filtered. After that, the solvent of the filtrate was evaporated in vacuo to give a residue that was purified by silica gel column chromatography to get product Ac-Glu-Cum-CA (120 mg, 42%) as the white solid. A solution of Ac-Glu-Cum-CA (120 mg, 0.09 mmol) in MeOH was cooled to 0 °C and then NaOH aqueous (23 mg, 0.45 mmol NaOH dissolved in 1 ml H_2O) was added. The resulting suspension was stirred 1 h under reflux. After that time, the organic solvent was removed under reduced pressure. The residue was dissolved in EA (20 ml). The organic layers were washed with water (10 ml), dried over Na_2SO_4 and filtered. A pure compound 4 (60 mg, 58.8%) was get after column chromatography with CH_2Cl_2–MeOH as the eluent (20:1, v/v) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.74 (s, 1H), 8.26 (dd, *J* = 9.0, 2.0 Hz, 1H), 7.85 (s, 1H), 7.69 – 7.67 (m, 2H), 7.62 (s, 1H), 7.16 – 7.15 (m, 1H), 7.11 – 7.10 (m, 2H), 7.04 – 7.02 (m, 3H), 6.85 (dd, *J* = 11.0, 2.0 Hz, 2H), 6.24 (d, *J* = 1.0 Hz, 1H), 4.96 (d, *J* = 15.0 Hz, 1H), 4.36 – 4.10 (m, 8H), 3.89 (d, *J* = 8.0 Hz, 1H), 3.83 – 3.78 (m, 2H), 3.70 (dd, *J* = 12.0, 5.0 Hz, 1H), 3.61 (d, *J* = 14.0 Hz, 1H), 3.49 – 3.32 (m, 6H), 3.03 – 2.99 (m, 1H), 2.87 (d, *J* = 14 Hz, 1H), 2.46 (s, 3H), 1.29 (s, 9H), 1.26 (s, 9H), 1.10 (s, 9H), 0.99 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.26, 161.08, 154.27, 152.45, 149.99, 149.12, 148.88, 147.43, 147.16, 143.65, 143.17, 141.91, 132.87, 132.48, 132.03, 129.26, 128.72, 127.09, 126.55, 126.45, 126.10, 125.88, 125.77, 125.30, 124.95, 116.42, 116.13, 113.60, 107.22, 103.34, 77.22, 77.01, 76.79, 76.09, 75.56, 75.52, 74.14, 73.44, 70.64, 67.76, 62.68, 34.23, 33.97, 33.95, 33.93, 32.28, 32.15, 31.66, 31.61, 31.59, 31.56, 30.99, 30.93, 18.61; ESI-MS (m/z): calcd for $C_{64}H_{79}NO_{13}Na$ $(M+Na)^+$: 1092.5551, found: 1092.5439.

Gal-Cum-CA (5). Gal-Cum-CA was synthesized according to Glu-Cum-CA with the two steps yield of 40%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.73 (s, 1H), 8.05 (dd, *J* = 9.0, 2.0 Hz, 1H), 7.88 – 7.87 (m, 2H), 7.63 – 7.62 (m, 2H), 7.16 (d, *J* = 2.0 Hz, 1H) 7.13 (d, *J* = 2.0 Hz, 1H), 7.11 (d, *J* = 2.0 Hz, 1H), 7.05 – 7.03 (m, 3H, ArH), 6.85 (dd, *J* = 14.0, 2.0 Hz, 1H), 6.24 (d, *J* = 1.0 Hz, 1H), 4.91 (d, *J* = 15.0 Hz, 1H), 4.36 – 4.30 (m, 3H), 4.26 – 4.20 (m, 3H), 4.16 – 4.11 (m, 2H), 3.97 (d, *J* = 3.0 Hz, 1H), 3.89 – 3.80 (m, 3H), 3.73 (dd, *J* = 12.0, 4.0 Hz, 1H), 3.65 – 3.60 (m, 2H), 3.56 (dd, *J* = 9.0, 3.0 Hz, 1H), 3.46 (d, *J* = 14.0 Hz, 1H), 3.37 (dd, *J* = 13.0, 5.0 Hz, 2H), 3.21 (t, *J* = 11.0, 5.0 Hz, 1H), 2.45 (s, 3H), 1.29 (s, 9H), 1.27 (s, 9H), 1.11 (s, 9H), 0.99 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.07, 161.03, 154.37, 152.26, 150.14, 149.25, 149.07, 148.78, 147.32, 147.09, 143.72, 143.23,

142.02, 132.93, 132.52, 132.05, 129.40, 128.70, 126.94, 126.66, 126.27, 126.15, 126.13, 125.90, 125.80, 125.57, 125.28, 124.99, 116.27, 115.85, 113.62, 107.28, 103.35, 75.67, 74.39, 74.15, 73.10, 71.07, 68.98, 67.32, 62.65, 60.38, 34.29, 34.00, 33.98, 33.96, 32.36, 32.23, 31.69, 31.63, 31.60, 31.03, 30.96, 21.03, 18.63; ESI-MS (m/z): calcd for $C_{64}H_{79}NO_{13}Na$ $(M+Na)^+$: 1092.5551, found: 1092.5439.

Man-Cum-CA (6). Man-Cum-CA was synthesized according to Glu-Cum-CA with the two steps yield of 43%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.72 (s, 1H), 8.02 (dd, J = 9.0, 2.0 Hz, 1H), 7.76 (d, J = 2.0 Hz, 1H), 7.58 (d, J = 9.0 Hz, 1H), 7.21 (d, J = 4.0 Hz, 2H), 7.10 – 7.06 (m, 4H), 6.87 – 6.85 (m, 4H), 6.14 (s, 1H), 4.77 (s, 1H), 4.70 (d, J = 15.0 Hz, 1H), 4.58 (d, J = 15.0 Hz, 1H), 4.35 (d, J = 13.0 Hz, 1H), 4.30 – 4.27 (m, 2H), 4.22 – 4.18 (m, 3H), 3.92 – 3.71 (m, 12H), 3.46 – 3.36 (m, 6H), 2.37 (s, 3H), 1.28 (s, 9H), 1.27 (s, 9H), 0.99 (s, 9H), 0.98 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 168.04, 161.33, 154.10, 152.66, 149.77, 149.61, 149.36, 149.16, 147.91, 147.82, 142.95, 142.72, 141.37, 132.42, 132.28, 132.18, 128.04, 128.00, 127.90, 127.73, 126.13, 126.05, 126.02, 125.99, 125.56, 125.49, 125.44, 125.37, 116.56, 116.27, 113.54, 107.53, 100.47, 75.30, 74.13, 72.51, 71.62, 70.72, 66.67, 65.92, 61.19, 34.01, 33.92, 33.90, 32.19, 32.11, 31.91, 31.70, 31.65, 31.64, 30.96, 30.95, 29.67, 18.52; ESI-MS (m/z): calcd for $C_{64}H_{79}NO_{13}Na$ $(M+Na)^+$: 1092.5551, found: 1092.5439.

2.2. FT-IR characterization of the Sugar-Cum-CAs

FT-IR spectroscopy measurements were performed with a Bruker spectrometer (INVENIO-S) operating in total reflection mode. The broadband infrared source is modulated by an interferometer and all the wavelengths are simultaneously analyzed. Take a fully dried powder sample of about 3 mg, cover it with germanium crystals, rotate the fixing button of the sampler, press the sample, and scan with a resolution of 4 cm^{-1} in the wavenumber range of 4000 cm^{-1} ~ 400 cm^{-1}, and the number of sample scans is 32 scans, collect the attenuated total reflection infrared spectrum of the sample. The final infrared spectrum was obtained after atmospheric compensation, baseline correction, and AB-TR conversion of the raw data. All spectral operations were performed using OPUS software 8.5.29.

2.3. UV-vis and fluorescence characterization of the Sugar-Cum-CAs

The UV-vis and fluorescence spectroscopic analyses were performed by using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific). A 100 μM DMSO stock solution of each compound was prepared by ultrasonication for 15 min. The stock solution was further diluted with

PBS to 10 μM working solutions. A 96-well black polystyrene microplate (Thermo Fisher) was used for UV-vis absorption recording (200-600 nm) and fluorescence measurements in the ranges of λ_{ex} = 200–450 and λ_{em} = 300–600 nm.

2.4. Preparation of Sugar-Cum-CA-PEG nanoparticles and loading with DTX

Mother solutions of 50 mM PEG and 50 mM of Sugar-Cum-CA in ethanol were prepared respectively. 40 μL of injection solution containing 4 μL of each of the above solutions diluted with ethanol was prepared accordingly. Then, the above solution was rapidly injected into 2 mL deionized water under ultrasonic treatment at 25 °C for 2 minutes. The supernatant was then collected after centrifugation at 2000 rcf for 2 minutes. The final product was obtained after lyophilization.

For drug-loaded NPs, mother solutions of 50 mM PEG, 50 mM of Sugar-Cum-CA and 50 mM DTX in ethanol were prepared respectively. 40 μL of injection solution containing 4 μL of each of the above solutions diluted with ethanol was prepared accordingly. Then, the above solution was rapidly injected into 2 mL deionized water under ultrasonic treatment at 25 °C for 2 minutes. The supernatant was then collected after centrifugation at 2000 rcf for 2 minutes. The final product was obtained after lyophilization.

2.5. Characterization of DTX loaded nanoparticles

The particle sizes and morphologies of the DTX and PEGylated DTX NPs were examined via transmission electron microscope (TEM) (FEI Talos F200X) at the Institute of Molecular Plus of Tianjin University, PRC. The accelerating voltage used for the analysis was 200kV. Hydrodynamic sizes of the NPs were measured in aqueous solutions using a dynamic light scattering (DLS) instrument (Malvern Zetasizer Nano-ZS90). For preparation of DLS test samples: the sample solution is filtered by a 0.2 μm filter membrane to remove insoluble material. Ultrasonic treatment for 5 minutes to make it disperse evenly, put the samples in the cuvette for test, and the optical path is 1 cm. For TEM analysis: TEM sample was prepared according to the following procedure: 6 μL nanoparticles suspension was placed in front side of the copper mesh, allowed to stand for 1 minute, and the excess suspension was blotted dry with filter paper and repeated three times. The measurements were carried out after naturally drying.

2.6. Drug loading and entrapment rates analysis

Determination of drug loading (DL, %) and entrapment efficiency (EE, %) rates were analyzed by HPLC and calculated based on the standard curve of DTX. To 100 μL of NP solution, add 400 μL of methanol, sonicate for 30 minutes to destroy the drug loaded nanoparticles and dissolve DTX completely into methanol. Filter with 0.2 μm filter membrane, take the filtrate and inject into HPLC to determine the content of DTX. Total mass of the DTX was determined based on the standard curve. The drug loading (DL, %) and entrapment efficiency (EE, %) of nanoparticles are calculated as follows:

$$DL\,(\%) = \frac{\text{Mass of DTX in drug loaded nanoparticles}}{\text{Total mass of DTX feeding}} \times 100\%$$

$$EE\,(\%) = \frac{\text{Mass of DTX in drug loaded nanoparticles}}{\text{Total mass of drug loaded nanoparticles}} \times 100\%$$

2.7. Cell lines and cell culture

Human non-small-cell lung cancer cell A549, human esophageal carcinoma cell Eca-109, human gastric cancer cell NUGC-4 and human bronchial epithelium cell BEAS-2B were purchased from ATCC. A549, Eca-109 and NUGC-4 cells were cultured at 37 °C in RPMI 1640 medium (High Glucose; Gibco, Invitrogen). Cell culture was supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen) and 1% penicillin/streptomycin solution (Gibco, Invitrogen) in humidified atmosphere with 5% CO_2. BEAS-2B cells were cultured in Dulbecco's modified Eagle's medium (DMEM 1x, High Glucose; Gibco, Invitrogen) at 37°C with 10% fetal bovine serum and 100 $U.mL^{-1}$ penicillin-streptomycin under a 5% CO_2 environment.

2.8. Anticancer activity assay

The antiproliferative activity of the non-drug loaded Sugar-Cum-CA and DTX-loaded NPs against A549, Eca-109 and NUGC-4 cancer cells was evaluated by 3-(4,5-dimethylthiazol-2-cyl)-2,5-di-phenyl tetrazolium bromide (MTT) assay. Approximately 1×10^4 cells per well were transferred to 96-well plates. After 12 h, the test drug samples were added to triplicate wells at different

concentrations (0.001 up to 20 μM), with 0.1% DMSO as control. DTX concentration was based on the encapsulated DTX amount. After 72 h incubation, 20 □L of the MTT solution (5 mg/mL) was added to each well. The plate was shaken for 1 min and incubated for another 4 h at 37 °C. Cells were lysed by the MTT lysis buffer (15% SDS, 0.015 M HCl). The uptake of MTT was measured as the absorbance at 570 nm by a multi-well-reading UV–Vis spectrometer. Experimental conditions were performed in five replicates (5 wells of the 96-well plate for each experimental condition). All experiments were performed three times.

2.9. Confocal fluorescence imaging study

A549 cancer cells and corresponding normal human bronchial epithelium BEAS-2B cells were aliquoted into 6-well plates. Confocal experiments were performed by addition of 500 nM of Glu-Cum-CA-PEG-DTX into the cell culture medium. After 30 min of incubation at 37 °C, the cells were washed 3 times with cold PBS and seeded in glass bottom dish (NEST, China). The chamber slides were then mounted and sealed for confocal microscopic analysis using an Olympus FV1000-IX81 confocal-laser scanning microscope with 358 nm excitation through a 100 × 1.4 NA oil immersion objective lens.

2.10. Statistical analysis

Statistical analysis for *in vitro* assay data was performed with GraphPad Prism 7 software and data were expressed as mean ± standard deviation. Statistically significant differences between experimental groups were determined using GraphPad Prism 7 and as previously described in our studies. [1b, 2] The symbols *, ** and *** indicate *P* values less than 0.05, 0.01 and 0.001 respectively.

3. Analytical Data of the Compounds

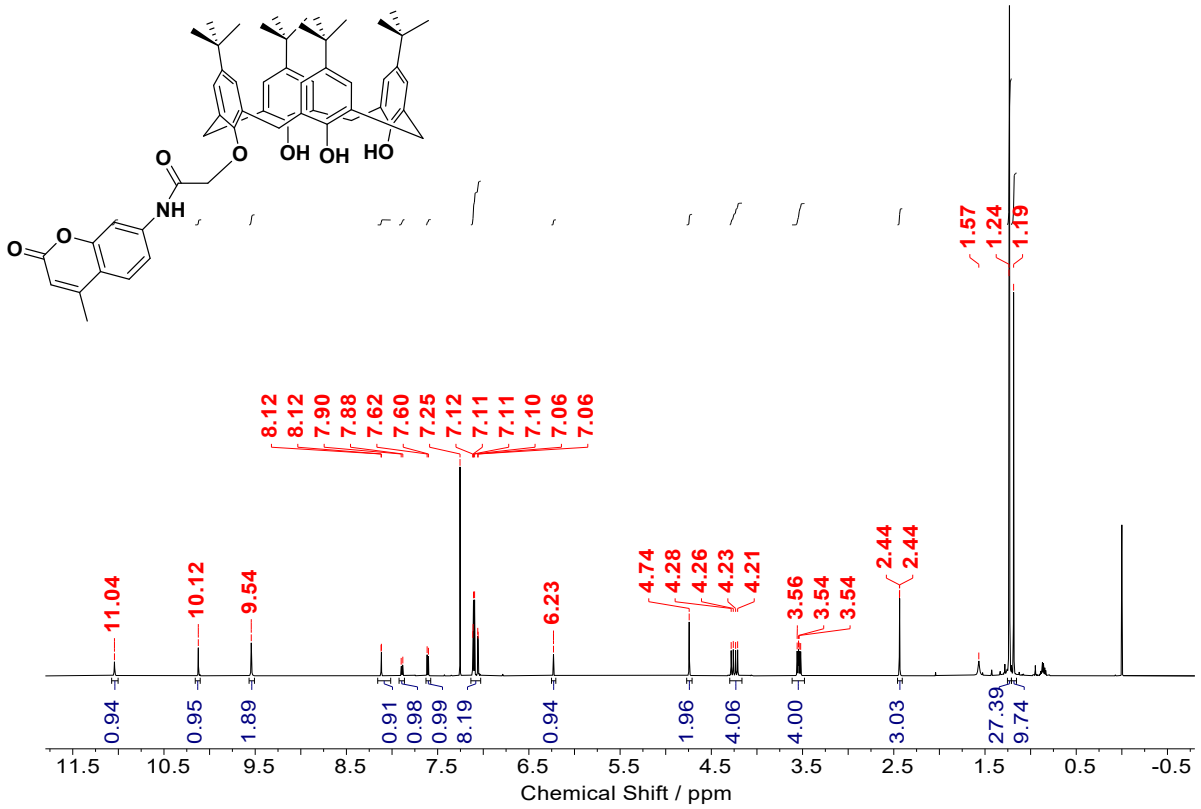


Figure S1. ^{1}H NMR (600 MHz, 298 K) spectrum of compound **3** in $CDCl_3$.

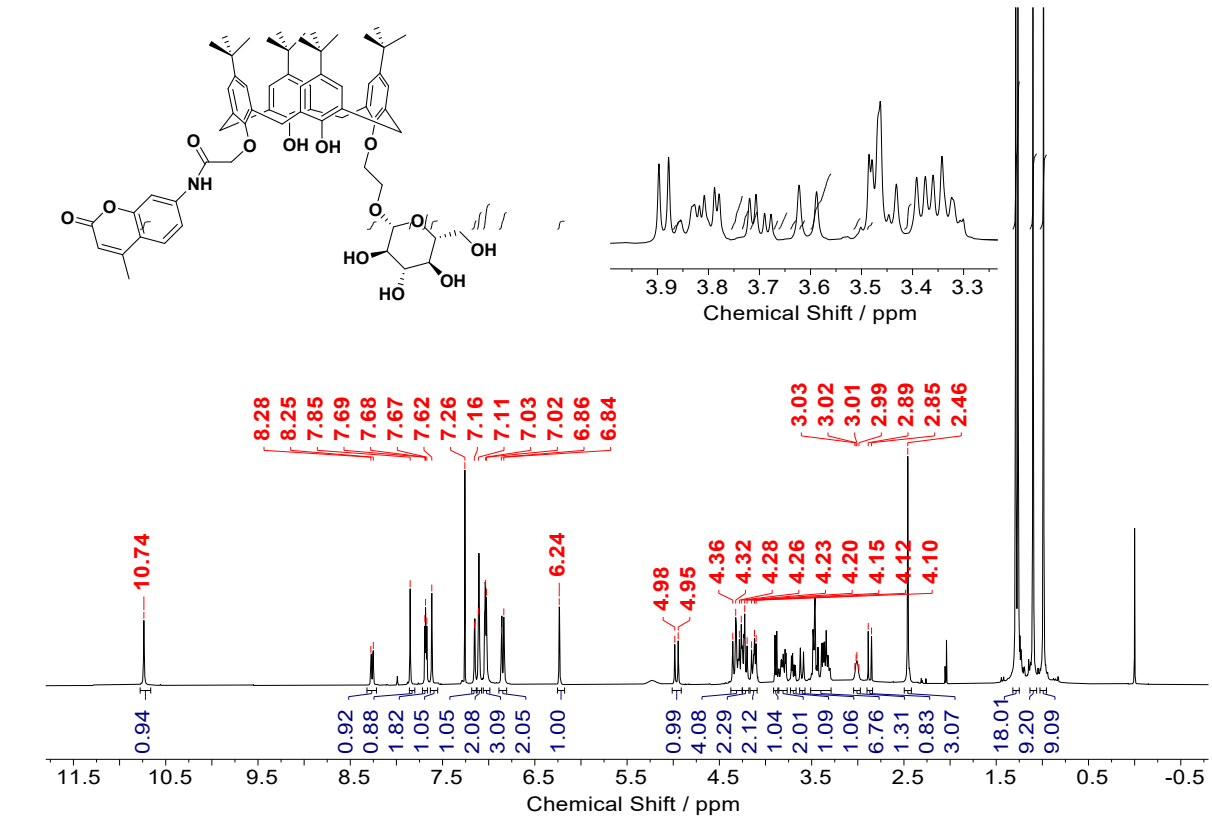


Figure S2. ^{1}H NMR (600 MHz, 298 K) spectrum of **Glu-Cum-CA** in $CDCl_3$.

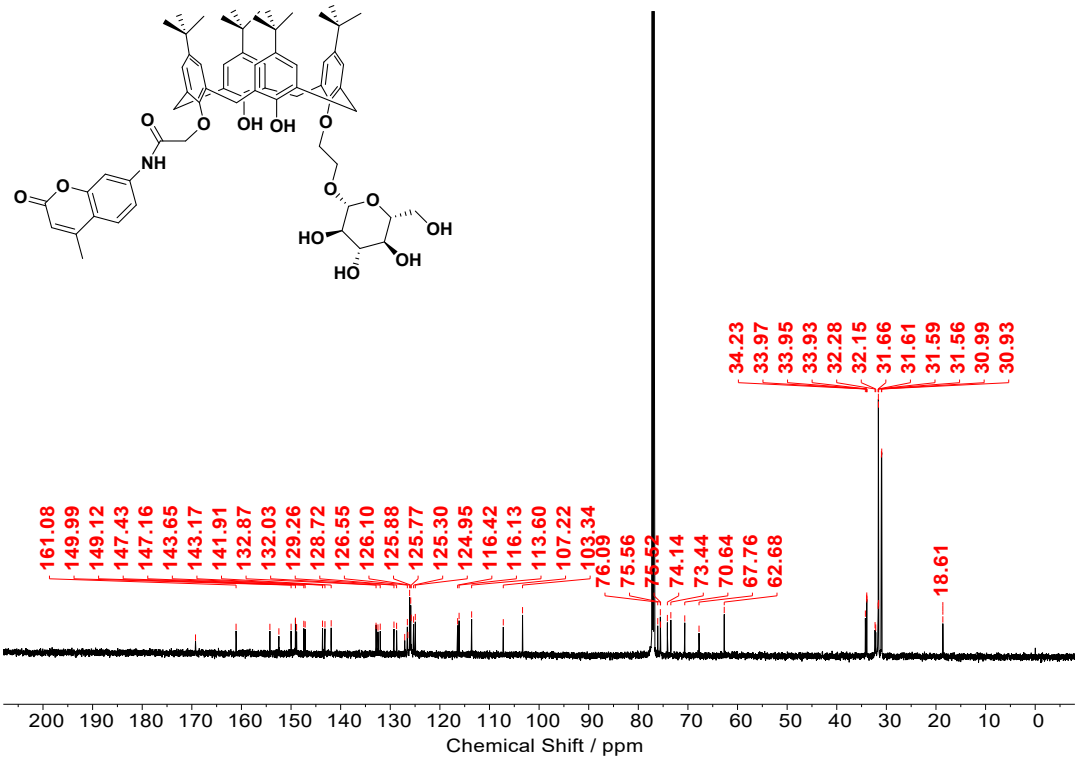


Figure S3. ^{13}C NMR (150 MHz, 298 K) spectrum of **Glu-Cum-CA** in $CDCl_3$.

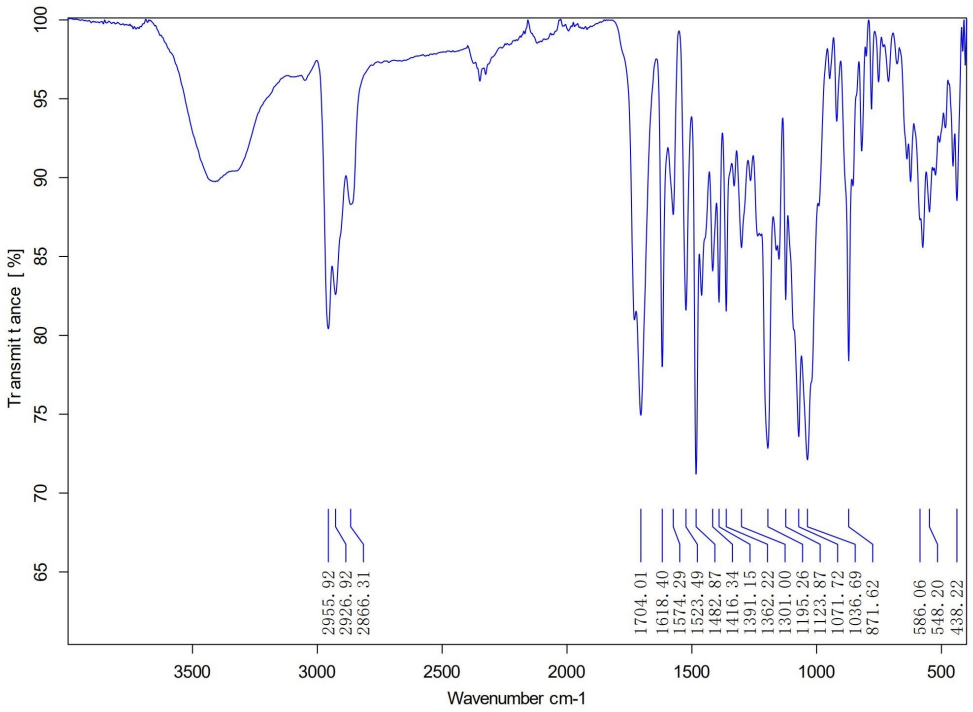


Figure S4. IR spectrum of **Glu-Cum-CA**.

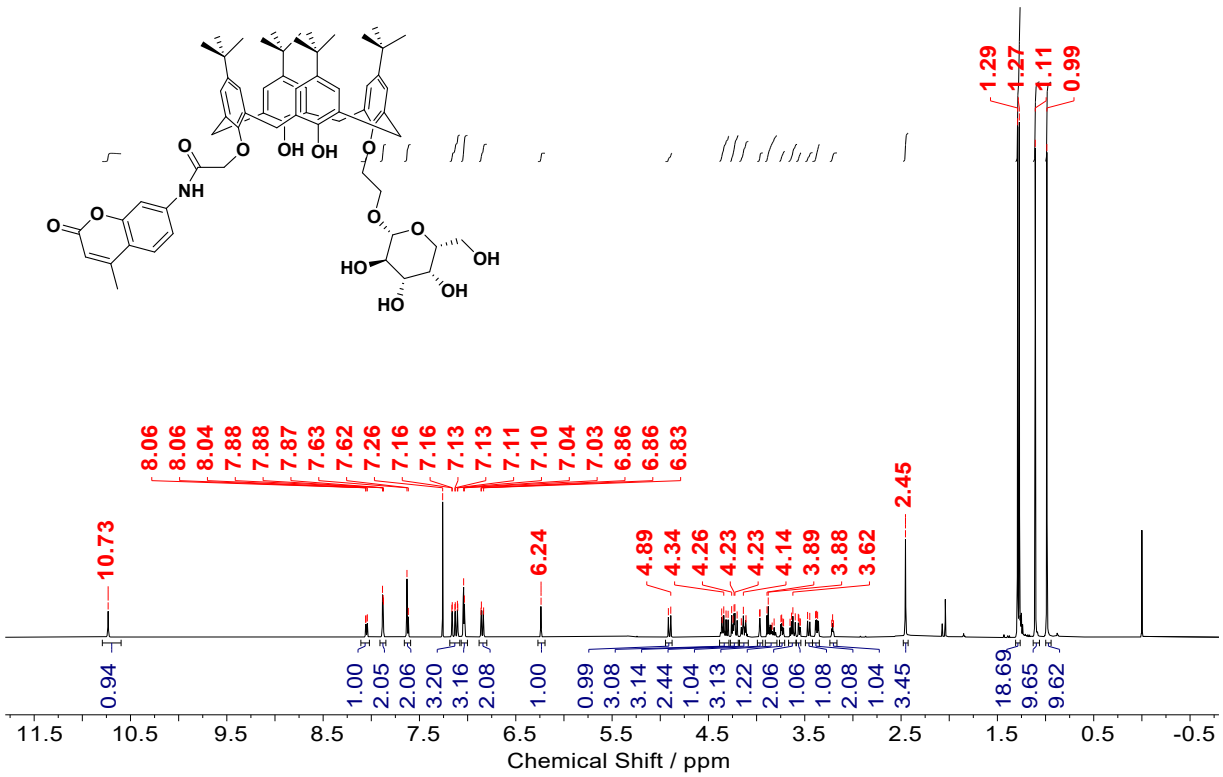


Figure S5. ^{1}H NMR (600 MHz, 298 K) spectrum of **Gal-Cum-CA** in $CDCl_3$.

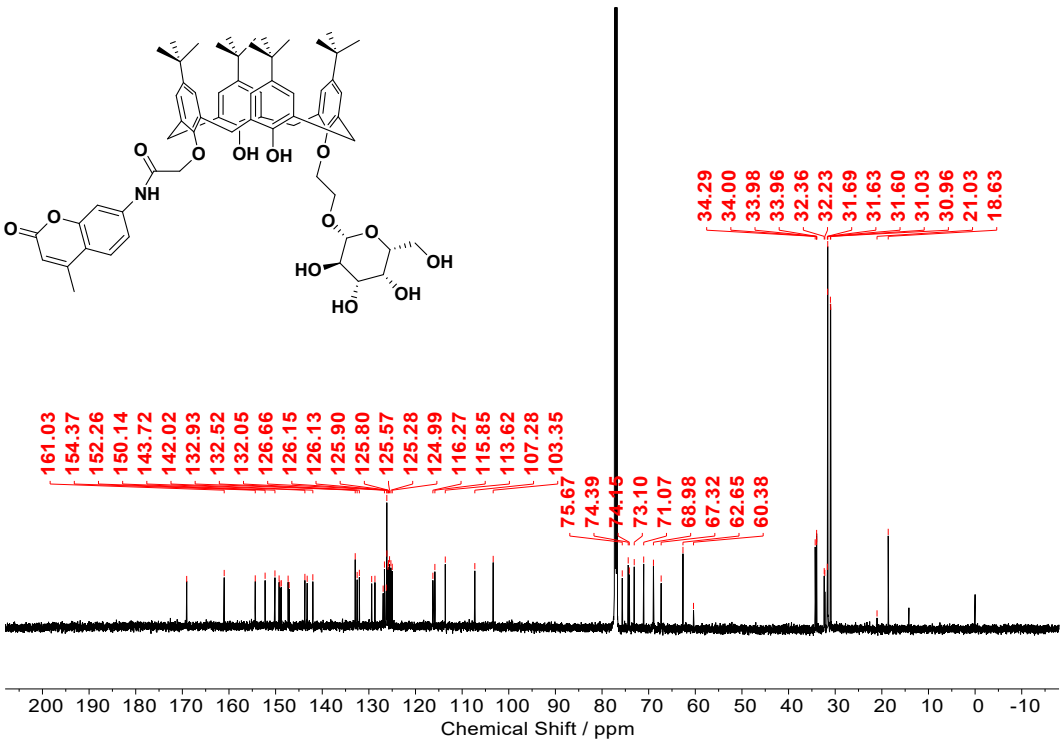


Figure S6. ^{13}C NMR (150 MHz, 298 K) spectrum of **Gal-Cum-CA** in $CDCl_3$.

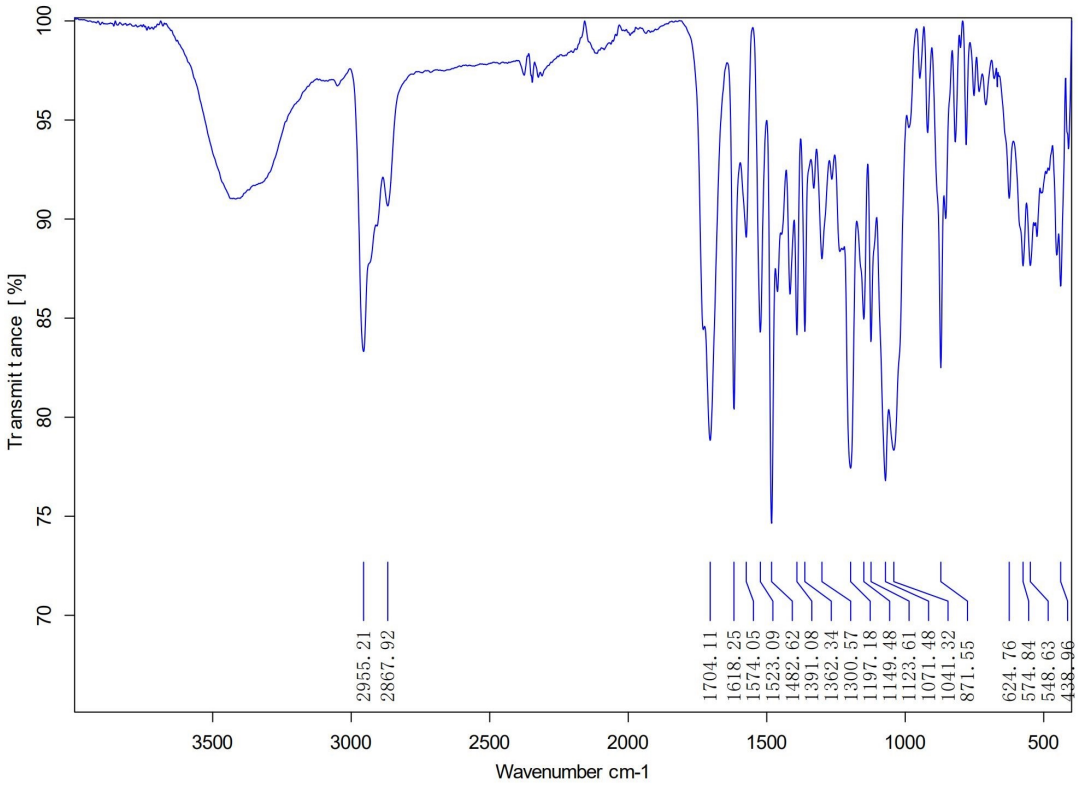


Figure S7. IR spectrum of compound **Gal-Cum-CA**.

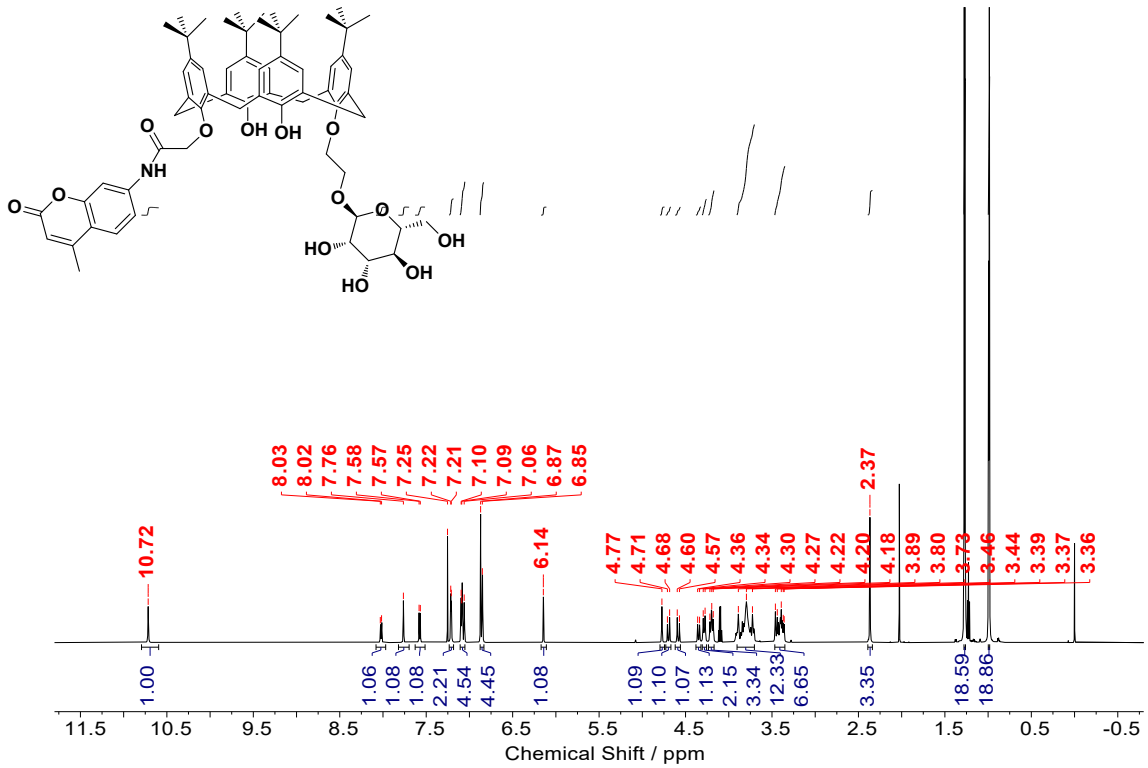


Figure S8. ^{1}H NMR (600 MHz, 298 K) spectrum of **Man-Cum-CA** in $CDCl_3$.

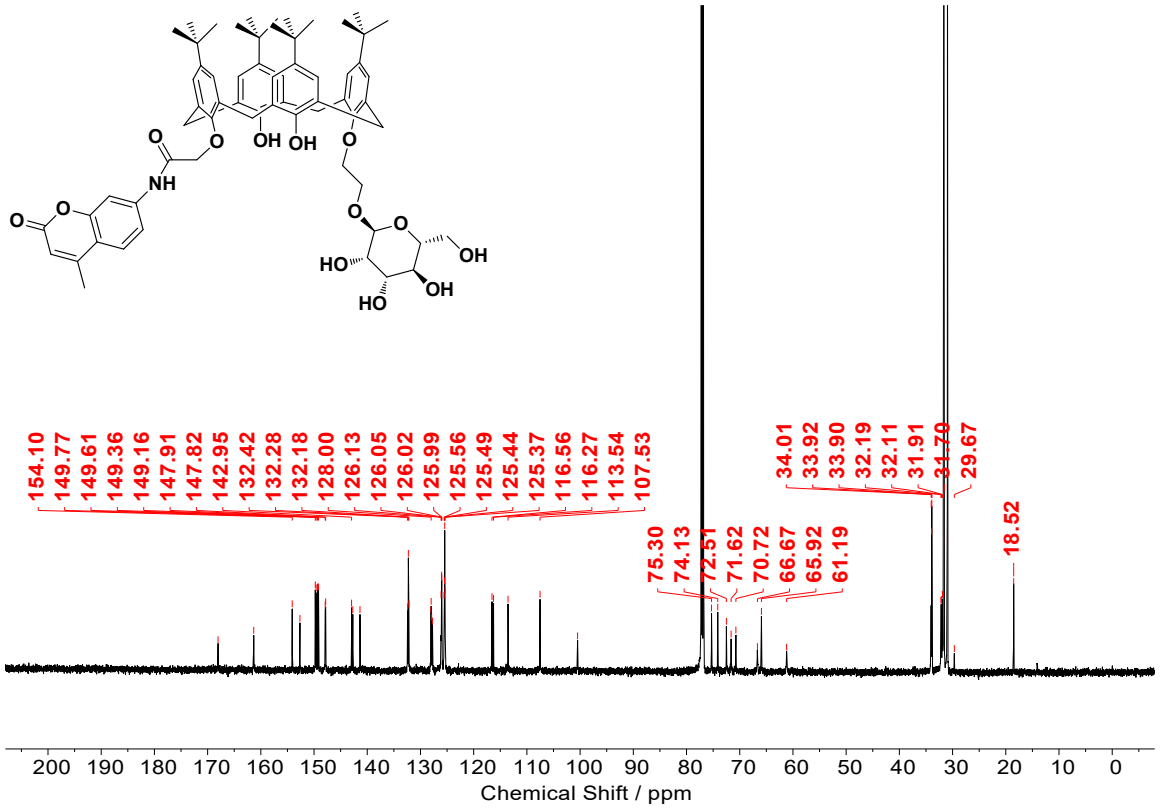


Figure S9. ^{13}C NMR (150 MHz, 298 K) spectrum of **Man-Cum-CA** in $CDCl_3$.

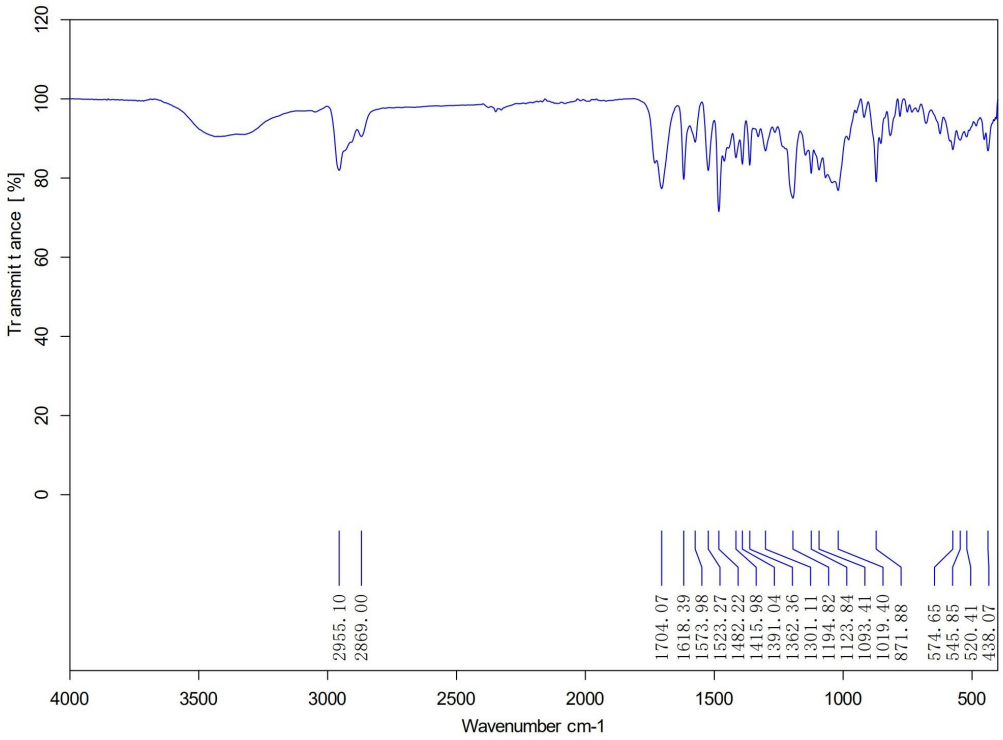


Figure S10. IR spectrum of **Man-Cum-CA**.

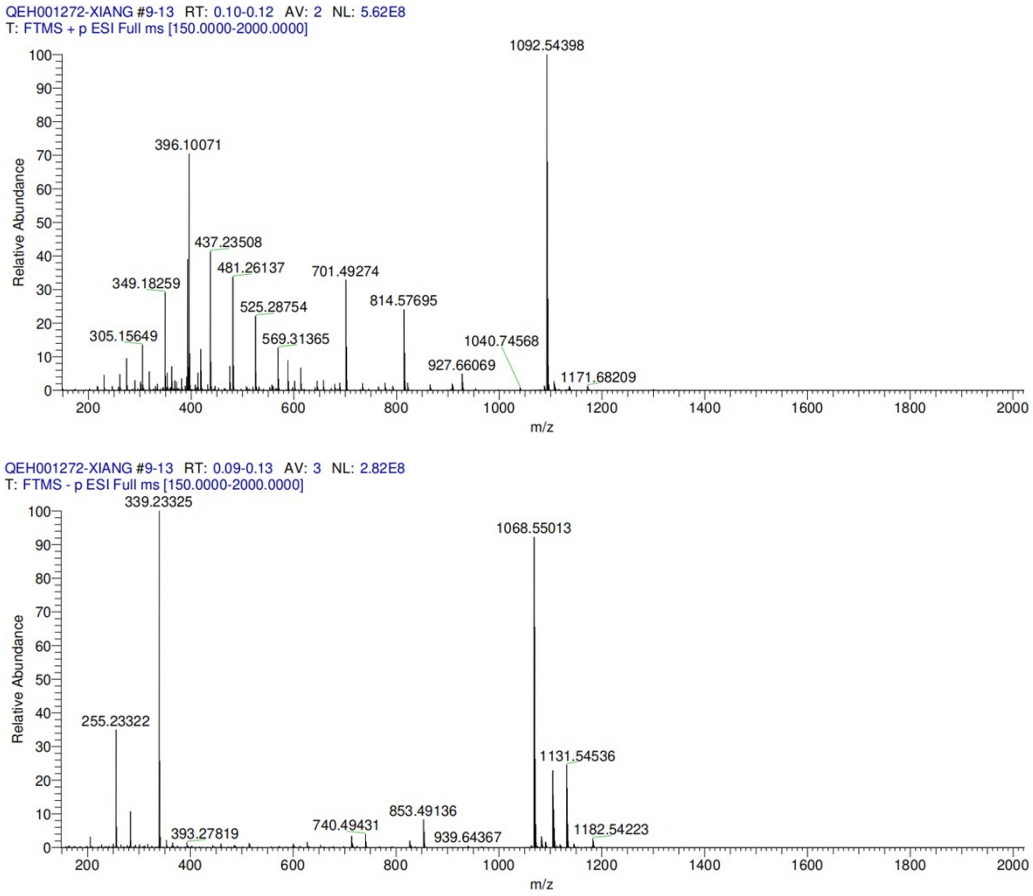


Figure S11. HRMS spectrum of **Glu-Cum-CA**.

Table S1. Drug-loading and drug encapsulation efficiency of Sugar-Cum-CA-PEG-DTX NPs

Tested samples	Mass of NPs [a] (mg)	Mass of DTX [b] (mg)	DL[c] (wt%)	EE [d] (wt%)
Glu-Cum-CA-PEG	23	-	-	-
Glu-Cum-CA-PEG-DTX	41	7.34	91.00%	18.00%
Man-Cum-CA-PEG	29	-	-	-
Man-Cum-CA-PEG-DTX	39	7.06	87.48%	18.10%
Gal-Cum-CA-PEG	29	-	-	-
Gal-Cum-CA-PEG-DTX	34	6.44	79.80%	18.94%

[a] Based on lyophilized sample mass. [b] Determined from HPLC analysis of the lyophilized NP sample. [c] Drug loading rate. [d] Drug encapsulation efficiency.

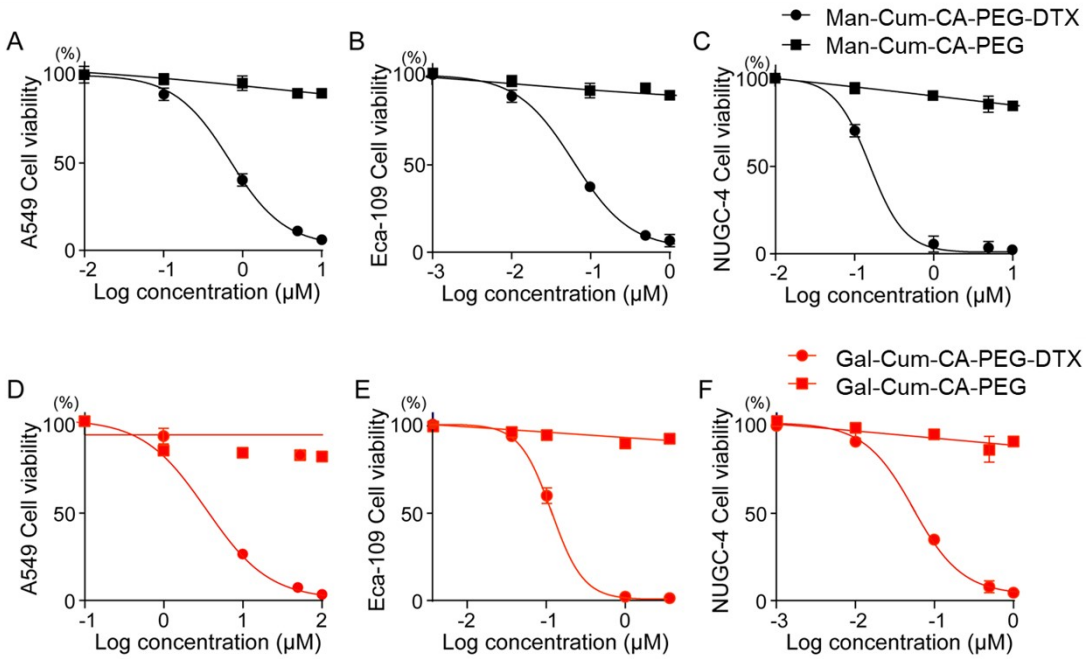


Figure S12. Anti-proliferative effects of Man-Cum-CA-PEG-DTX (A) and Gal-Cum-CA-PEG-DTX NPs (B) toward A549, Eca-109 and NUGC-4 three human cancer cell lines. From left to right: Dose response proliferation curves for A549 cells, Eca-109 cells and NUGC-4 cells. Data treated with free-DTX was shown in the main text. Data represent the mean ± SD of at least three replicates. $P < 0.001$ for each testing result was obtained compared with the corresponding Man- and Gal-Cum-CA-PEG groups.

4. Reference

1. a) V. Yarlagadda, M. M. Konai, G. B. Manjunath, C. Ghosh, J. Haldar, *J. Antibiot.* 2015, **68**, 302; b) R. Liu, H. Li, X. Gao, Q. Mi, H. Zhao, Q. Gao, *Biochem. Biophys. Res. Commun.*, 2017, **487**, 34.

2. a) S, Liu, W. Song, Y. Cui, Z. Sun, Z. Huang, Q. Gao, *Chem. Commun.,* 2021, **57**, 5530; b) M. Wu, H. Li, R. Liu, X. Gao, M. Zhang, P. Liu, Z. Fu, J. Yang, D. Zhang-Negrerie, Q. Gao, *Eur. J. Med. Chem.*, 2016, **110**, 32.