Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

β-Galactosidase-activated theranostic for hepatic carcinoma therapy and imaging

Mrinmoy Maiti[a‡], Kai Kikuchi[b,c‡], K. K. Athul[d], Amandeep Kaur*[b,c] and Sankarprasad Bhuniya*[d]

[a]Department of Science, Amrita School of Engineering, Amrita Viswa Vidyapeetham, Coimbatore, India 641112

[b] School of Medical Sciences, Faculty of Medicine and Health, The University of Sydney NSW 2006, Australia. E-mail: a.kaur@sydney.edu.au

[c,]The University of Sydney Nano Institute, The University of Sydney NSW 2006, Australia.

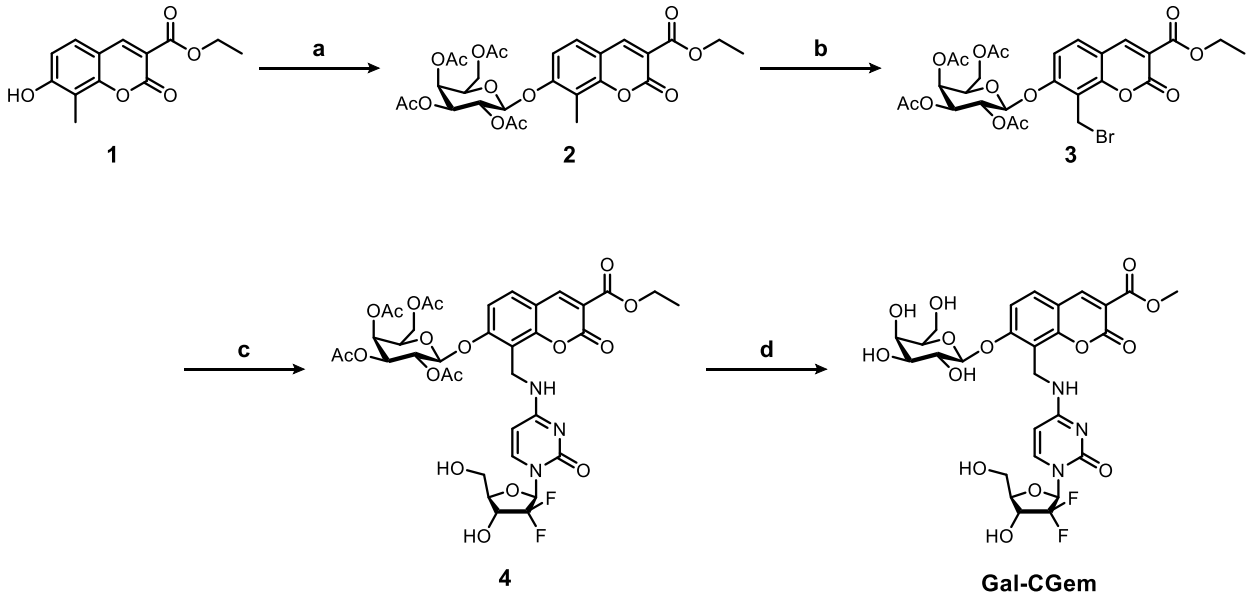
[d]Centre for Interdisciplinary Sciences, JIS Institute of Advanced Studies and Research, JIS University, Arch Waterfront, GP Block, Sector V, Kolkata, India 700091. Email: spbhuniya@jisiasr.org

EXPERIMENTAL SECTION

1.1. General information on materials, methods and instrumentations

2-Methylresorcinol (Avra, India), diethylmalonate (Avra, India), piperidine (Avra, India), phosphorous oxychloride (Avra, India), Acetobromo-a-D-galactose (Sigma Aldrich), silver(I) oxide (Sigma Aldrich), N-Bromo succinimide (TCI chemicals), AIBN (Avra, India),Potassium carbonate (Rankem), gemcitabine (Carbosynth limited) were purchased commercially and used without further purification. All the materials for UV/Vis and Fluorescence spectroscopy, DMSO (J.T. Baker), were purchased from commercial suppliers and were used without further purification. Flash column chromatography was performed using Silica gel (100-200 mesh) and Analytical thin layer chromatography was performed using silica gel 60 (pre-coated sheets with 0.25 mm thickness). Mass spectra were recorded on anion SpecHiResESI mass spectrometer. NMR spectra were collected on a 400MHz spectrometer (Bruker, Germany).

Synthetic scheme:



Scheme S1 a) Acetobromo- α-D-galactose, Ag_2O, rt, 48 h. b) NBS, AIBN, reflux, 6 h. c) Gemcitabine, K_2CO_3, acetonitrile, rt, 5 h. d) NaOMe, MeOH, 0°C, 1 h.

Compound 1 was synthesised by following procedure published by Kim and coworkers.[1]

Synthesis of compound **2**:

To a stirred solution of compound 3 (1.5 g, 4.028 mmol) in anhydrous acetonitrile (30 mL), acetobromo-D-galactose (2.48 g, 6.042 mmol), and silver (I) oxide (1.86 g, 8.057 mmol) were added under nitrogen atmosphere. The resulting reaction mixture was kept for stirred for 48 h at ambient temperature. After completion (TLC), the crude was filtered through celite, filtrate was washed with sat. $NaHCO_3$, extracted with ethyl acetate (100 mL), dried and concentrated, then purified by silica gel (100-200 mesh) column chromatography using 20-30 % ethyl acetate in hexane as eluent to obtain the desired product as white solid (1.8 g, 52% yield). ^{1}H NMR (400 MHz, DMSO-d_6); δ 8.63 (s, 1H); 7.85 (d, *J* = 8.76 Hz, 1H); 7.15 (d, *J* = 8.76 Hz, 1H); 5.62 (t, *J* = 4 Hz, 1H); 5.39 (s, 1H); 5.33 (d, *J* = 4.48 Hz, 2H); 4.53(s,1H); 4.31(m, *J* = 14.12 Hz, 2H); 4.15 (t, *J* = 7.08 Hz, 2H); 2.16 - 1.97(s, 15H); 1.33 (t, *J* = 7.12 Hz, 3H).^{13}C NMR (100 MHz, DMSO-d_6); δ 170.43, 163.14, 159.36, 156.54, 154.33, 149.57, 129.40, 115.03, 114.00, 113.55, 11.68, 97. 98, 71.18, 70.33, 68.60, 67.63, 61.73, 21.24, 14.56, 8.13 HRMS *m/z* ($M+Na^+$): calcd. 601.1636, found ($M+Na^+$): 601.1529.

Synthesis of compound **3**:

Compound **2** (1g, 1.73 mmol) was suspended in CCl_4 (30 mL), and then *N*-Bromosuccinimide (370.43 mg, 2.08 mmol) and AIBN (5.69 mg, 0.03mmol) were added. The mixture was refluxed for 6 h, under nitrogen atmosphere. After completion of reaction, the reaction mixture was cooled and then add water, extracted with DCM. The organic layer was washed with water, brine and dried over $MgSO_4$, concentrated under reduced pressure then purified by silica gel (100-200 mesh) column chromatography using 40-50% ethyl acetate in hexane as eluent to afford compound **3** (0.7 g, 61% yield) as a brown solid. ^{1}H NMR (400 MHz, DMSO-d_6); δ 8.74 (s, 1H); 7.96 (d, *J* = 9.56 Hz, 1H); 7.18 (d, *J* = 8.84 Hz, 1H); 5.64 (d, *J* = 6.96 Hz, 1H); 5.39 (s, 1H); 5.34 (d, *J* = 5.6 Hz, 1H); 5.32 (d, *J* = 8 Hz, 1H); 4.52 (s,1H); 4.52 (s, 2H); 4.16 (d, *J* = 7.52 Hz, 2H); 4.31 (q, *J* = 14.12, 2H); 2.16 -1.96 (s, 12 H), 1.32 (t, *J* = 7.12 Hz, 3 H). ^{13}C-NMR (100 MHz, DMSO-d6), δ 170.44, 163.07, 159.84, 156.48, 154.67, 149.58, 131.58, 117.63, 115.01, 113.59, 112.07, 98.14, 71.11, 70.34, 68.34, 67.61, 61.72, 51.08, 20.96, 14.56. HRMS *m/z* ($M+ Na^+$); calcd. 679.0641, found ($M+ Na^+$): 679.0633.

Synthesis of compound **4**:

To a solution of compound **3** (0.25g, 0.380 mmol) in Acetonitrile (10 mL) K_2CO_3 (157.66 mg, 1.14 mmol) was added, followed by addition of gemcitabine (120 mg, 0.456 mmol), in ice cold condition under argon atmosphere. The reaction mixture was then stirred at rt for 4 h. After completion of reaction, the reaction mixture was filtered and the filtrate was evaporated under

reduced pressure, added water and extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$, concentrated in vacuo, purified through silica gel(100 -200 mesh) column chromatography using 5- 10% MeOH in DCM (dichloromethane) as eluent to get desired compound **4** (0.08 g, 24 % yield) as white solid.^{1}H NMR(DMSO-d6, 400 MHz): δ (ppm) 8.67 (s, 1H), 7.87 (d, *J* = 8.8 Hz, 1H), 7.74 (s, 1H), 7.24 (s, 1H), 7.15 (d, *J* = 8.8 Hz, 1H), 6.25 (d, *J* = 6.4 Hz, 1H), 6.00 (t, *J* = 7.7 Hz, 1H), 5.80 (d, *J* = 8.2 Hz, 1H), 5.68 (d, *J* = 7.1 Hz, 1H), 5.43 – 5.38 (m, 1H), 5.38 – 5.20 (m, 4H), 5.03 (d, *J* = 15.2 Hz, 1H), 4.55 (t, *J* = 6.4 Hz, 1H), 4.26 (q, J = 7.1 Hz, 2H), 4.21 – 4.06 (m, 3 H), 3.80 (m, 2H), 3.67 – 3.57 (m, 1H), 2.17 (s, 3H), 2.06 – 1.98 (s, 9H), 1.29 (t, *J* = 7.1 Hz, 3H). ^{13} C NMR (100 MHz, DMSO-d6): δ 170.47, 163.01, 159.36, 156.33, 154.00, 151.53,150.47, 149.35, 146.92, 130.66, 121.50, 115.19, 114.33, 113.42, 107.70, 103.56, 94.65, 89.88, 70.61, 70.09, 68.72, 62.23, 61.74, 59.27, 21.00, 19.33, 14.57.HRMS *m/z* (M+ H): calcd. 840.2197, found: (M+ H): 840.2270.

Synthesis of **Gal-CGem**:

Compound **4** (0.100 g, 0.119 mmol) was dissolved in methanol (10 mL) sodium methoxide (0.064 g, 11.9 mmol) was added at 0 °C under an inert atmosphere. The mixture was stirred till completion (1h). Cation exchange resin amberlite ir120 (0.1 g) was added and mixture was further stirred for 30 min. The mixture was filtered, concentrated under reduced pressure and washed with diethyl ether in hexane to obtain **Gal-CGem** as off white solid (0.065 g, 77 % yield).
^{1}H NMR (DMSO-d6, 400 MHz):δ (ppm) 8.74 (s, 1H), 7.85 (d, *J* = 8.4 Hz, 1H),7.83 (s, 1 H), 7.26 (d, *J* = 8.4 Hz , 1H), 7.23 (d, *J* = 8.0 Hz), 6.27 (d, *J* = 6.4 Hz, 1H), 6.01 (t, *J* = 8.0 Hz, 1H), 5.94 (d, *J* = 8.0 Hz, 1 H), 5.46 – 5.42 (m, 1H), 5.27 (d, *J* = 5.2 Hz, 1H), 5.18 – 5.14 (m, 1H), 4.94 (d, *J* = 5.6 Hz, 1H), 4.85 (d, *J* = 7.6 Hz, 1H), 4.72 (s, 1H), 4.59 (d, *J* = 4.0 Hz, 1H), 4.29 (d, *J* = 7.2 Hz, 1H), 4.21 - 4.13 (m, 2H), 3.81 – 3.76 (m, 2H), 3.74 – 3.70 (m, 4H), 3.68 – 3.57 (m, 4H), 3.47 – 3.43 (m, 2H).^{13}C NMR (100 MHz, DMSO-d_6); δ 166.09, 163.71, 161.41, 157.29, 155.98, 154.45, 150.52, 141.25, 131.16, 123.39, 121.49, 114.08, 113.07, 107.70, 103.38, 101.78, 95.08, 81.00, 76.56, 73.92, 70.78, 69.00, 60.92, 59.38, 52.77, 49.05, 35.88. HRMS *m/z* (M+ H): calcd. 658.1618; found (M+ H): 658.1693 & *m/z* (M+ Na^+): calcd. 680.1618, found (M+Na^+): 680.1513.

Synthesis of **CGem**

In a sealed tube compound 1(0.5g, 2.016 mmol) in acetonitrile (10 mL) methyl iodide (1.43 g, 10.08 mmol) and potassium carbonate (0.834 g, 6.048 mmol) were added and the mixture was stirred at 70° c for 6h. After checking TLC, the reaction mixture was filtered, and the filtrate was evaporated under reduced pressure, then add water and extracted with ethyl acetate. The organic layer was dried to obtained a crude. The crude was suspended in CCl_4 (10 mL), and then *N*-bromosuccinimide (254 mg,1.43 mmol) and AIBN (4.69 mg, 0.028mmol) were added. The mixture was refluxed for 6 h, under nitrogen atmosphere. After completion of reaction, the reaction mixture was cooled and then add water, extracted with DCM. The organic layer was washed with

water, brine and dried over $MgSO_4$, concentrated under reduced pressure then purified by silica gel (100-200 mesh) column chromatography using 10-20% ethyl acetate in hexane as eluent to get crude compound**.** Without further analysis the compound was taken for next step. In a round bottom flask crude compound (0.15g, 0.439 mmol) in 10 ml acetonitrile (ACN) was added K_2CO_3(0.121g, 0.879 mmol), followed by addition of gemcitabine (0.115g, 0.439 mmol), in ice cold condition. The reaction mixture was then stirred at rt for 4h. After completion of reaction, the reaction mixture was filtered and the filtrate was evaporated under reduced pressure, then add water and extracted with dichloromethane (DCM). The organic layer was washed with brine, dried over $MgSO_4$, concentrated in vacuo; purified through silica gel(100-200 mesh) column chromatography using 0-5% MeOH in DCM as eluent to get desired compound **CGem** (0.06g, 27 % yield) as white solid. ^{1}H-NMR (400 MHz, DMSO-d_6); 8.71(s, 1H); 7.87 (d, *J* = 8.8 Hz, 1H); 7.56 (s, 1H); 7.16 (d, *J* =8.64 Hz, 1H); 6.05(d, *J* = 7.44 Hz,2H); 5.28(d, *J* = 4.88 Hz,2H); 4.30(q, *J* = 14.2 Hz, 2H); 4.17 (t, *J* = 5 Hz, 2H); 3.64 (s, 2H); 3.86 (s, 3H); 1.31(t, *J* = 7.12, 3H).^{13}C-NMR (100 MHz, DMSO-d_6), 177.25, 170.85, 167.57, 160.83, 159.08, 154.70, 139.43, 136.91, 133.90, 125.54, 118.44, 116.76, 115.53, 114.14, 99.79, 89.37, 85.61, 74.08, 66.19, 64.15, 61.86, 32.41, 19.37.

Photophysical studies

All reagents and solvents used for fluorescence spectroscopy were commercial and used without further purification. Except under special case, the following methods provide all of measurements for fluorescence spectroscopy. Absorption spectra were recorded on an UV-1800 spectrophotometer (Shimadzu), and fluorescence spectra were recorded using an RF-6000 fluorescence spectrofluorometer (Shimadzu) with a 3000 μL volume of 1 cm standard quartz cell.

Screening with biological enzymes and analytes

Cell culture

HepG2, HeLa and HFF-1 cells were maintained at 37 ˚C in 5% carbon dioxide. Cells were cultured in Advanced Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific) supplemented with 2% foetal calf serum (FCS, Thermo Fisher Scientific) and 2.5 mM L-glutamine (Sigma-Aldrich). Fluorobrite DMEM (DMEM, Thermo Fisher Scientific) was supplemented with 2% FCS and 2.5 mM L-glutamine, unless otherwise stated. Stock solutions of **Gal-CGem**, **CGem** and Gemcitabine were made in DMSO and were freshly dissolved and used within a week. For treatments, the DMSO stock was diluted into DMEM or DMEM at the appropriate concentrations and then added to cells.

MTT cytotoxicity assay

The cytotoxic evaluation of **Gal-CGem**, **CGem** and Gemcitabine in HepG2, HeLa and HFF-1 cells was carried out using the MTT (3-[4, 5 dimethylthiazol 2 yl] 2, 5 diphenyltetrazolium bromide) assay. HepG2, HeLa and HFF-1 cells were seeded in clear 96 well plates in DMEM (100 µL) at a density of 5000 cells/well and allowed to adhere overnight. Stock solutions of probes were diluted to various concentrations (1-4 µM) in DMEM, containing 1% DMSO. 100 µL of DMEM containing vehicle control (1% DMSO), probe solution was added to wells in triplicate. The cells were incubated for 72 h, before the addition of MTT reagent. After a further 3 h incubation, the absorbance of each well was measured at 590 nm in a Perkin EnSpire Plate Reader. Values were normalised to controls containing DMEM only and the data is presented as the average percentage viability of at least triplicate values from a single experiment. A dose-response curve was fitted and IC_{50} values representing the drug concentration required to elicit a 50 % growth inhibition compared to vehicle control were calculated in GraphPad Prism software (GraphPad PRISM v.8, La Jolla, CA, USA)".

Confocal microscopy

For all imaging experiments, 10,000 cells were seeded into 35 mm glass bottom dishes (MatTek) and allowed to adhere overnight. Images were obtained at 37 ˚C in a 5% CO2 atmosphere on Leica SP5 II confocal and multi-photon microscope equipped with a LUCPLFLN 40X air objective lens (NA = 0.60) and a UPLSAPO 63X water-immersion objective lens (NA = 1.20), and 458 and 633 nm lasers. Samples were excited by a multi-photon 820 nm laser. Unless otherwise stated, images were processed using FIJI software. For image acquisition, the media was removed from the cells and replaced with DMEM supplemented with desired concentrations of the **Gal-CGem** or **CGem** (1.0 mL), following the incubation for specified time points, the media was removed, cells washed with PBS (3×1 mL) and resuspended in Fluorobrite DMEM (1.0 mL).

Evaluation of β-Gal activity

β-Gal activity was evaluated in 96-well black plates (CORNING) using PBS (pH 7.4) and a FluoReporter lacZ/Galactosidase Quantitation Kit (Thermo Fisher Scientific), in accordance with the manufacturer's instructions. First, cell lysate samples (1 mg/mL protein concentration, 10 µL/well) were added to triplicate wells. To this a 1 mM solution of 3-carboxyumbelliferyl β-D-galactopyranoside in PBS (pH 7.4) was added to the wells. To serve as a reference standard 0.1 mM of 7-hydroxycoumarin-3-carboxylic acid in PBS (100 µL/well) was added to triplicate wells. To measure the β-Gal activity at pH 7.4, the plates were incubated for 30 min (5% CO_2, 37 °C). and the fluorescence intensity (Ex/Em: 390/460 nm) was measured using a Perkin EnSpire Plate Reader. The intensity of each sample was normalised against that of the reference standard.

Annexin V-FITC PI Assay.

For all imaging experiments, 10,000 cells were seeded into 35 mm glass bottom dishes (MatTek) and allowed to adhere overnight. Cells were treated with vehicle control and **Gal-CGem** (20 μM, 6 h) Cells were washed once in PBS, then once in 1X Binding Buffer and detached using accutase to avoid the use of EDTA and EDTA-containing solutions. Cells were resuspended in 1X Binding Buffer at 1 x 106 cells/mL. To 100 μL of cell suspension, 5 μL of Annexin V-FITC was added and the cells were incubated for 10-15 minutes at room temperature. Cells were washed in 1X Binding Buffer and resuspended in 200 μL of 1X Binding Buffer. 5 μL of Propidium Iodide Staining Solution (cat. 00-6990) was added and cells were analysed by flow cytometry within 4 hours, at 2-8°C in the dark. Data analysis was performed using FlowJo software.

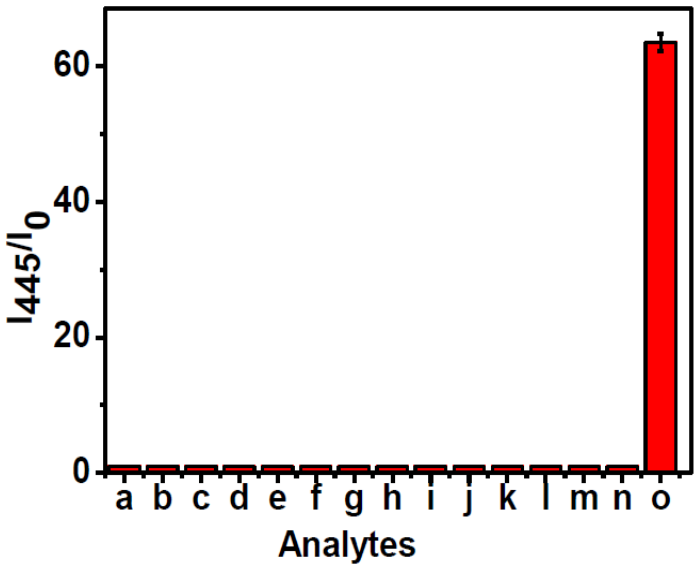


Fig. S1 Fluorescence response of **Gal-CGem**(5 μM) in various biological analytes (200 μM) in PBS buffer (pH=7.4;0.1% DMSO).a:KCl, b:$CaCl_2$, c:vitamin C (1 mM), d:$FeSO_4$, e:cystein, f:$NaNO_2$, g: $MgSO_4$, h: $Cu(OAc)_2$, i:$Zn(OAC)_2$, j:$NH_4(OAC)_2$, k:H_2O_2, l:$FeCl_3$, m:Tyrosinase, n:GSH(5mM), o:β-gal. Bars represents the comparative fluorescence changes at λ_{em} = 450 nm in presence of analytes. Each spectrum was acquired 30 min after addition of analytes at 37°C.

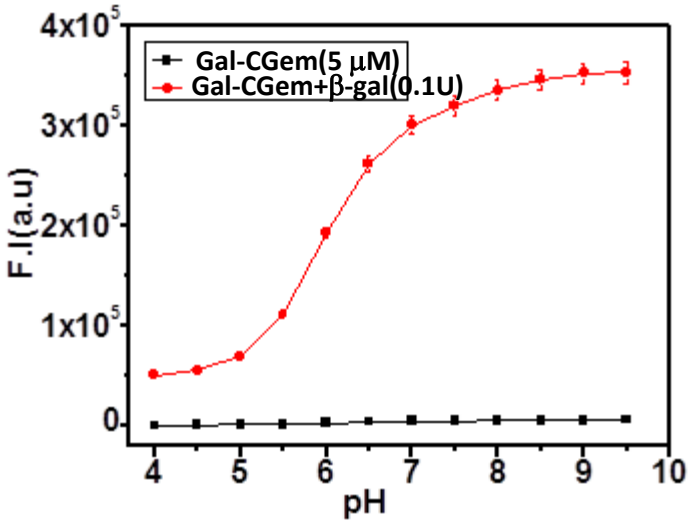


Fig. S2 Fluorescence changes of **Gal-CGem**(5 μM) in variation of pH in absence and presence of β-gal (0.1U) in PBS buffer (0.1% DMSO). **Gal-CGem** was incubated with β-gal for 30 min at 37°C. A 400 nm excitation wavelength was used with the excitation and emission slit widths set at 5 nm.

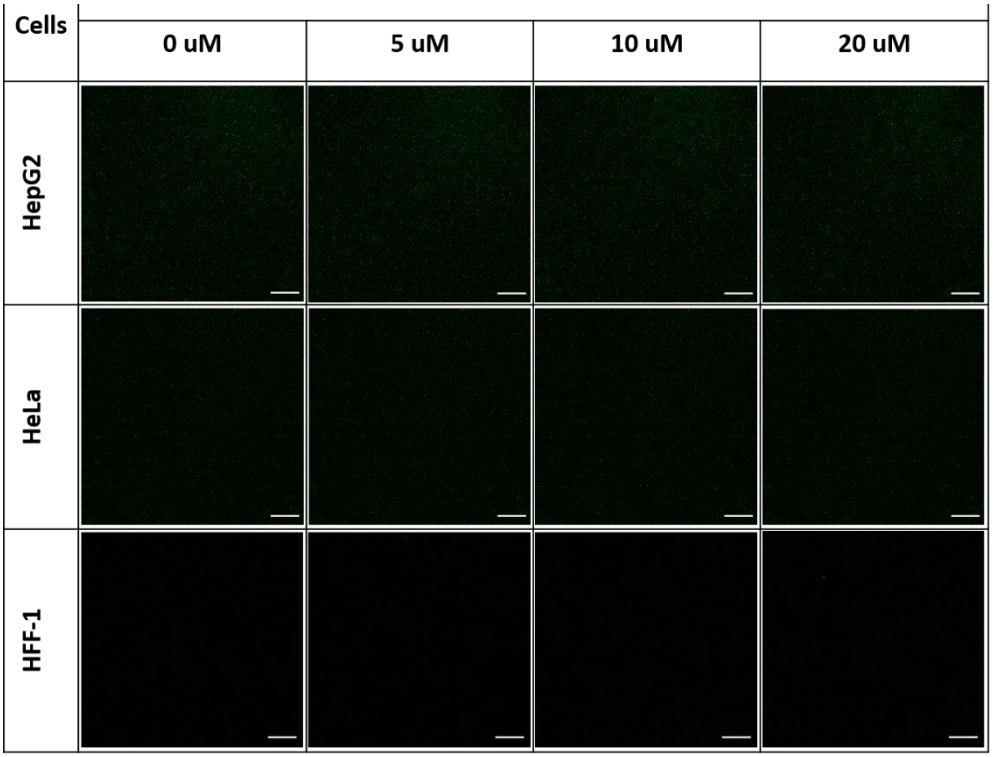


Fig. S3 Fluorescence imaging of **CGem**. Multiphoton confocal microscopy images of HepG2, HeLa and HFF-1 cell lines treated with vehicle control or **CGem** (5-20 μM, 12 h). Images were acquired upon two-photon excitation at 820 nm, and emission was collected 420-500 nm. Scale bar represents 20 μm.

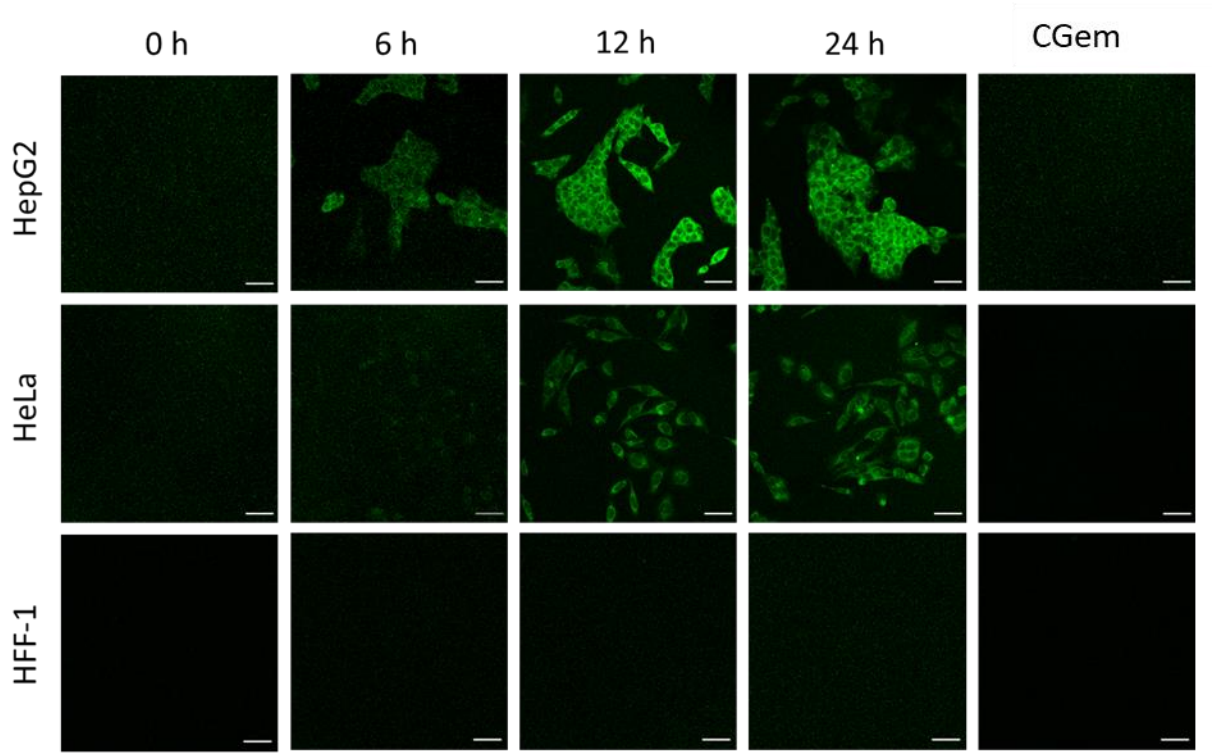


Fig. S4 Time-course fluorescence imaging of **Gal-CGem**. Multiphoton confocal microscopy images of HepG2, HeLa and HFF-1 cell lines treated with vehicle control or **Gal-CGem** (20 μM) for 0 – 24 h. Images were acquired upon two-photon excitation at 820 nm, and emission was collected 420-500 nm. Scale bar represents 20 μm

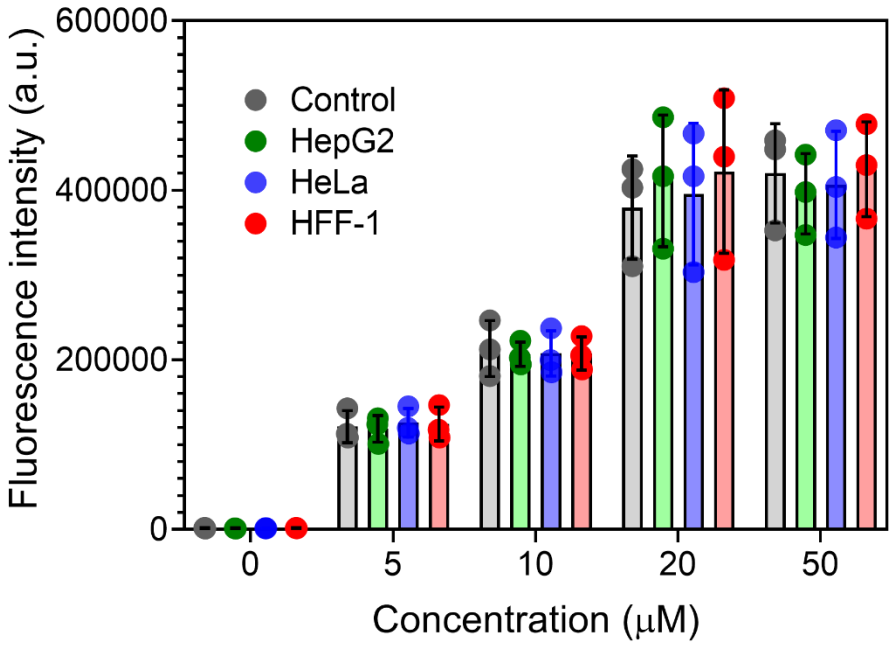


Fig. S5 Fluorescence intensity measurements of lysates of HepG2, HeLa and HFF-1 cells treated with **Gal-CGem** (0 – 50 μM). Cell lysates were incubated with β-gal at 37°C. A 400 nm excitation wavelength was used with the excitation and emission slit widths set at 5 nm.

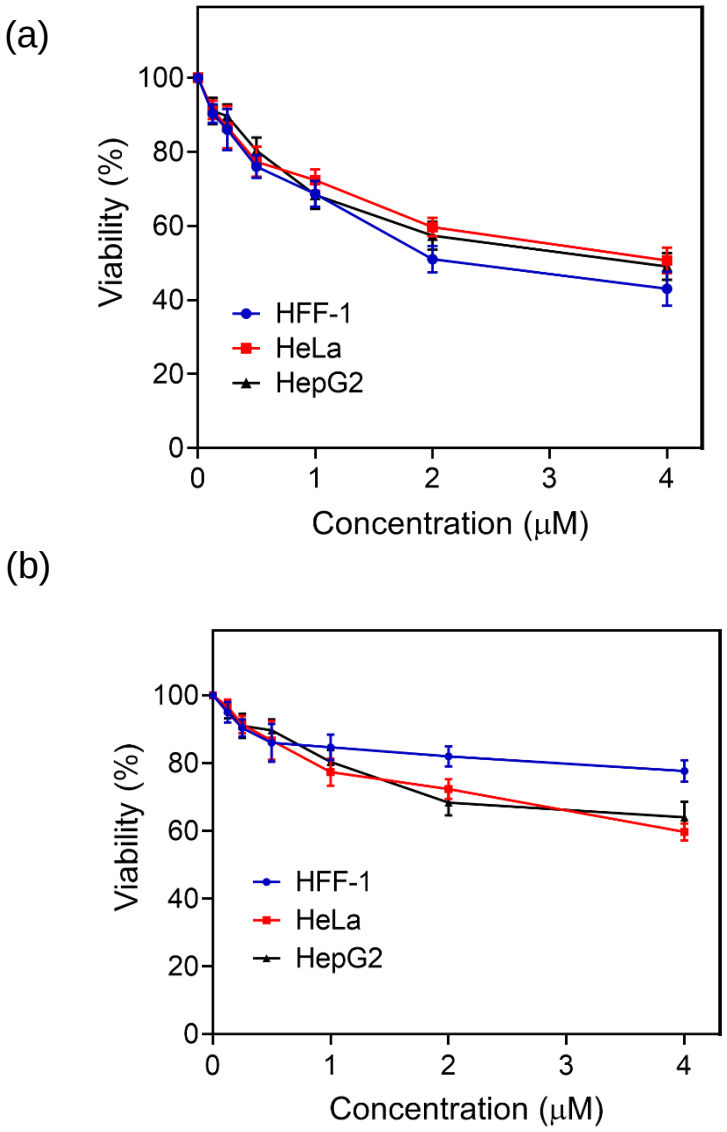


Fig. S6 Cytotoxicity assays of (a) Gemcitabine and (b) **CGem**) in HepG2, HeLa and HFF-1 cell lines treated with vehicle control or **Gal-CGem** (0 – 4 μM, 72 h). Cell viability was determined using the MTT assay.

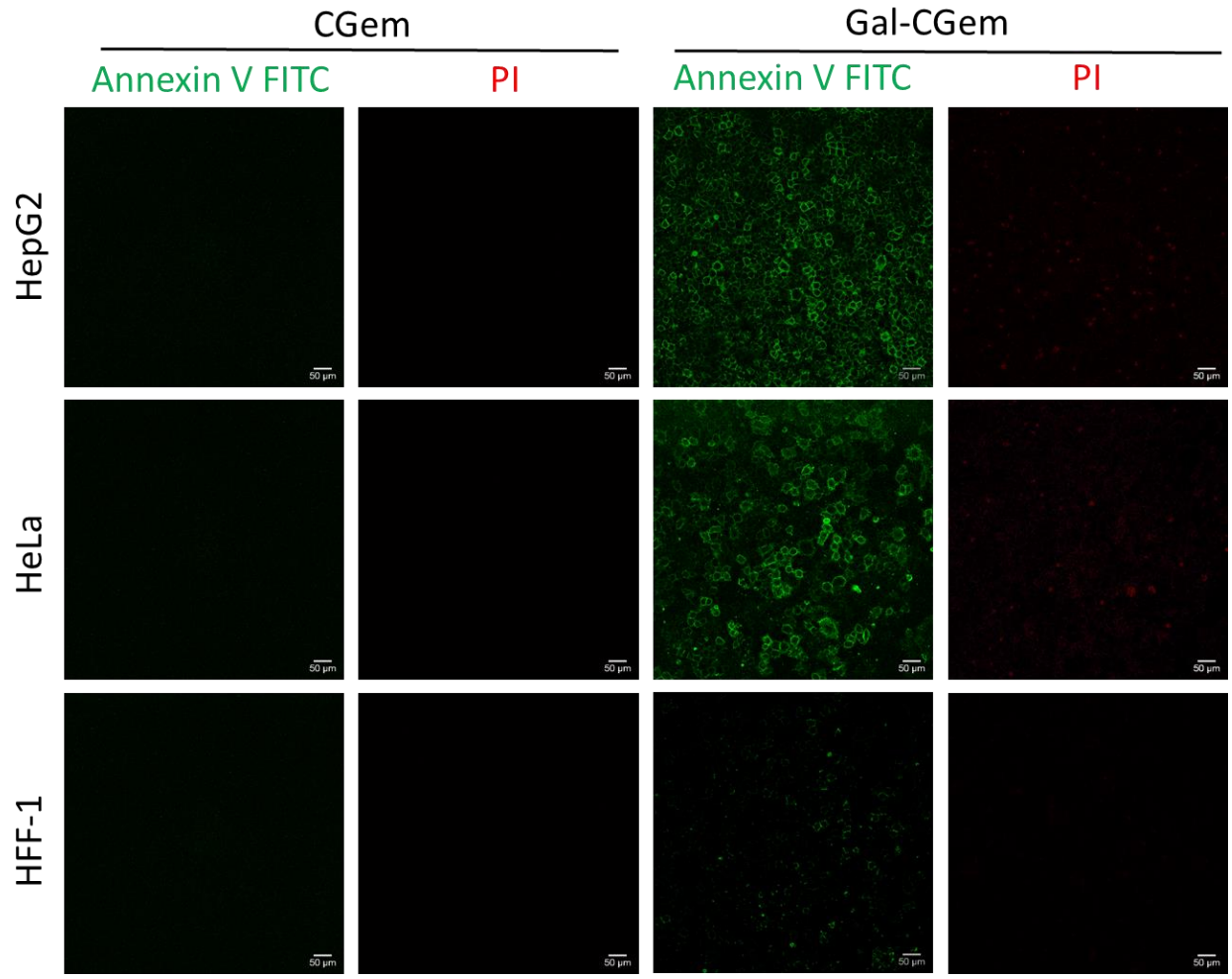


Fig. S7 Annexin V apoptosis assay of **Gal-CGem**. Multiphoton confocal microscopy images of HepG2, HeLa and HFF-1 cell lines treated with CGem (20 µM, 12 h) or Gal-CGem (20 µM, 12 h) and stained with a mixture of Annexin V-FITC (λex = 488 nm, λem = 500-540 nm) and PI (λex = 561nm, λem = 600-630 nm. Scale bar represents 20 µm

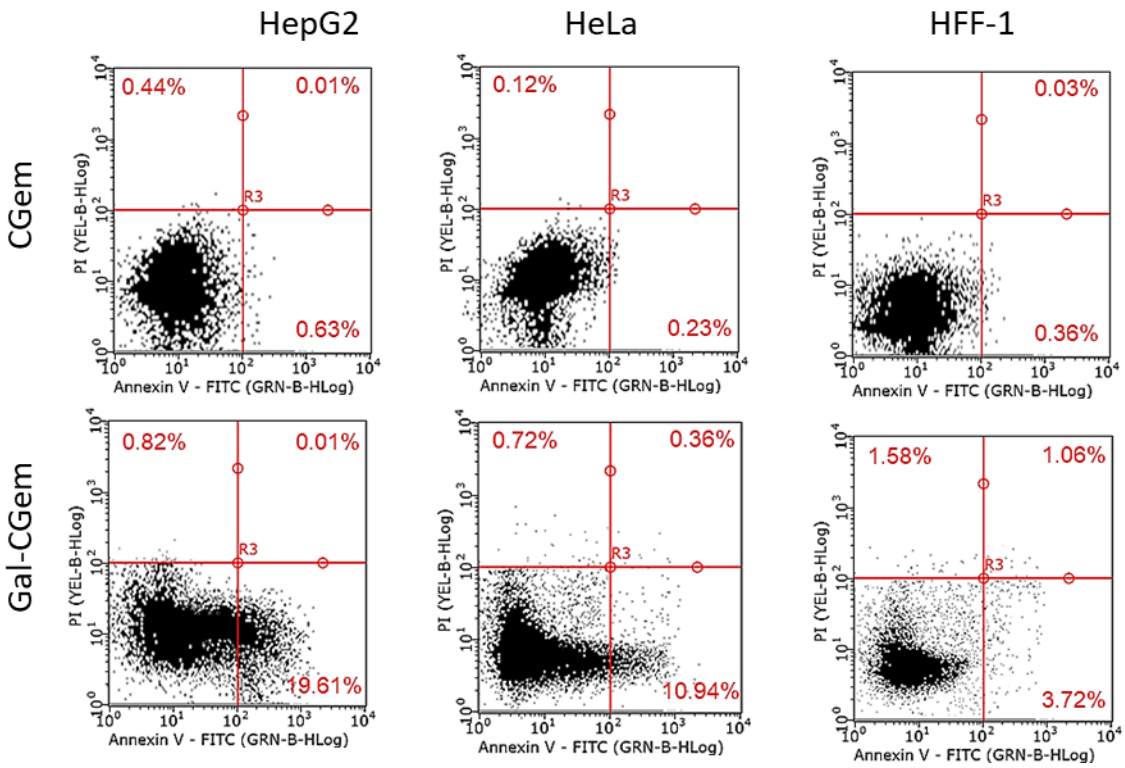


Fig. S8 Flow cytometry dot plots of Annexin V apoptosis assay of HepG2, HeLa and HFF-1 cell lines treated with CGem (20 μM, 12 h) or Gal-CGem (20 μM, 12 h) and stained with a mixture of Annexin V-FITC (λ_{ex} = 488 nm, λ_{em} = 500-540 nm) and PI (λ_{ex} = 561nm, λ_{em} = 600-630 nm).

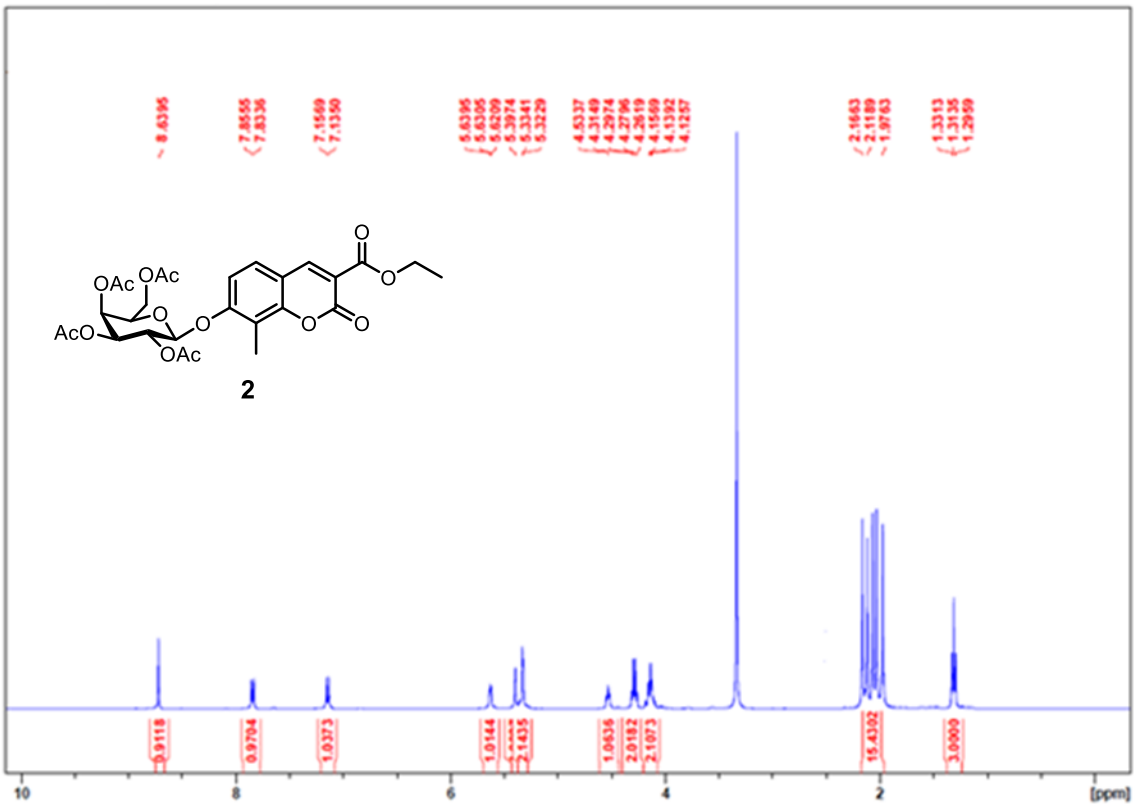


Fig. S9 ^{1}H NMR compound **2** in DMSO-d_6

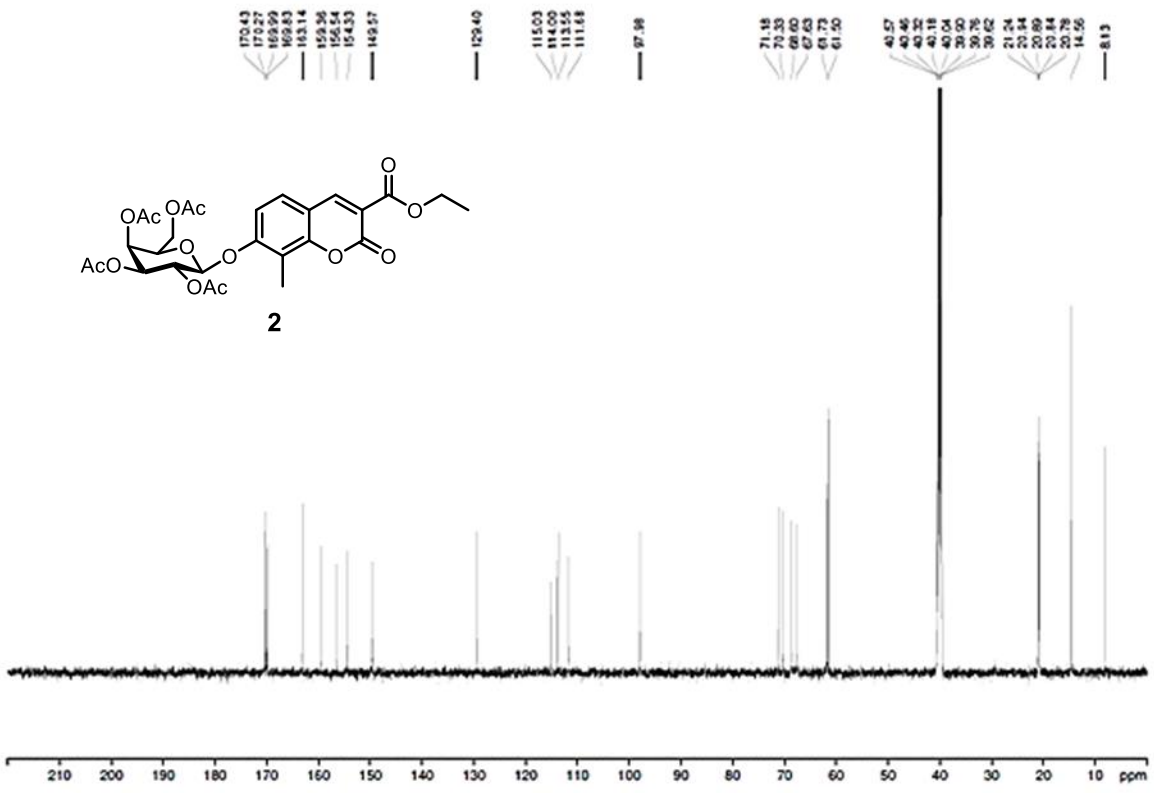


Fig. S10 ^{13}C NMR of compound **2** in DMSO-d_6

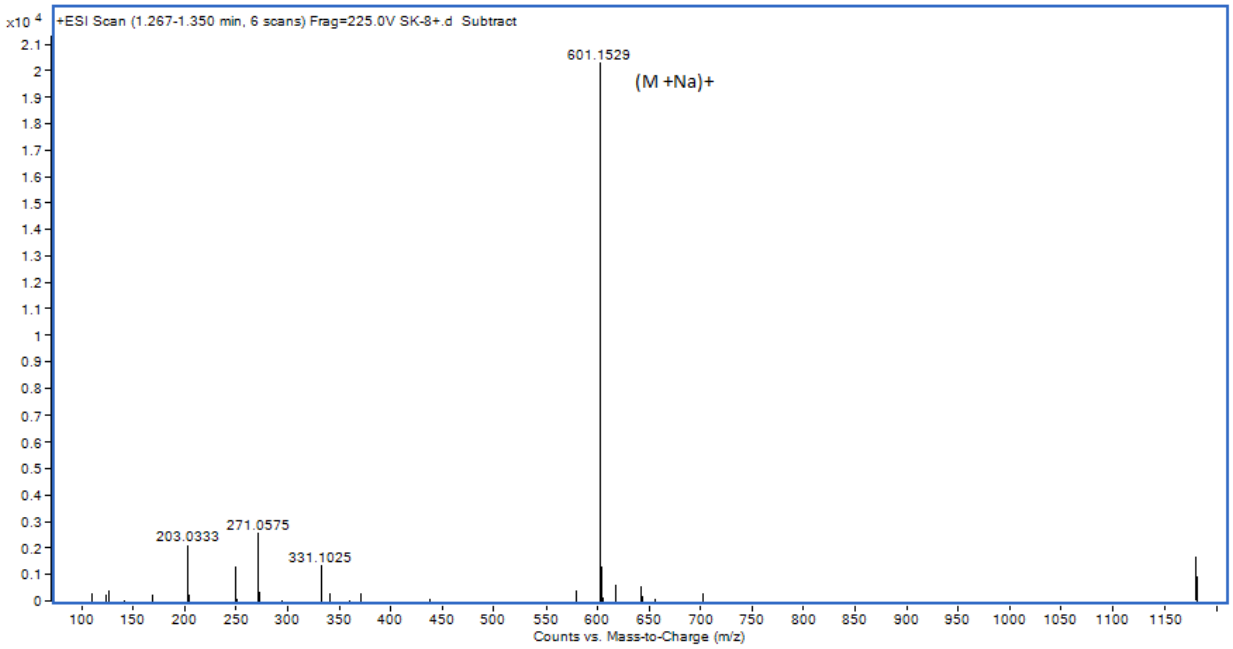


Fig. S11 HRMS of Compound **2**

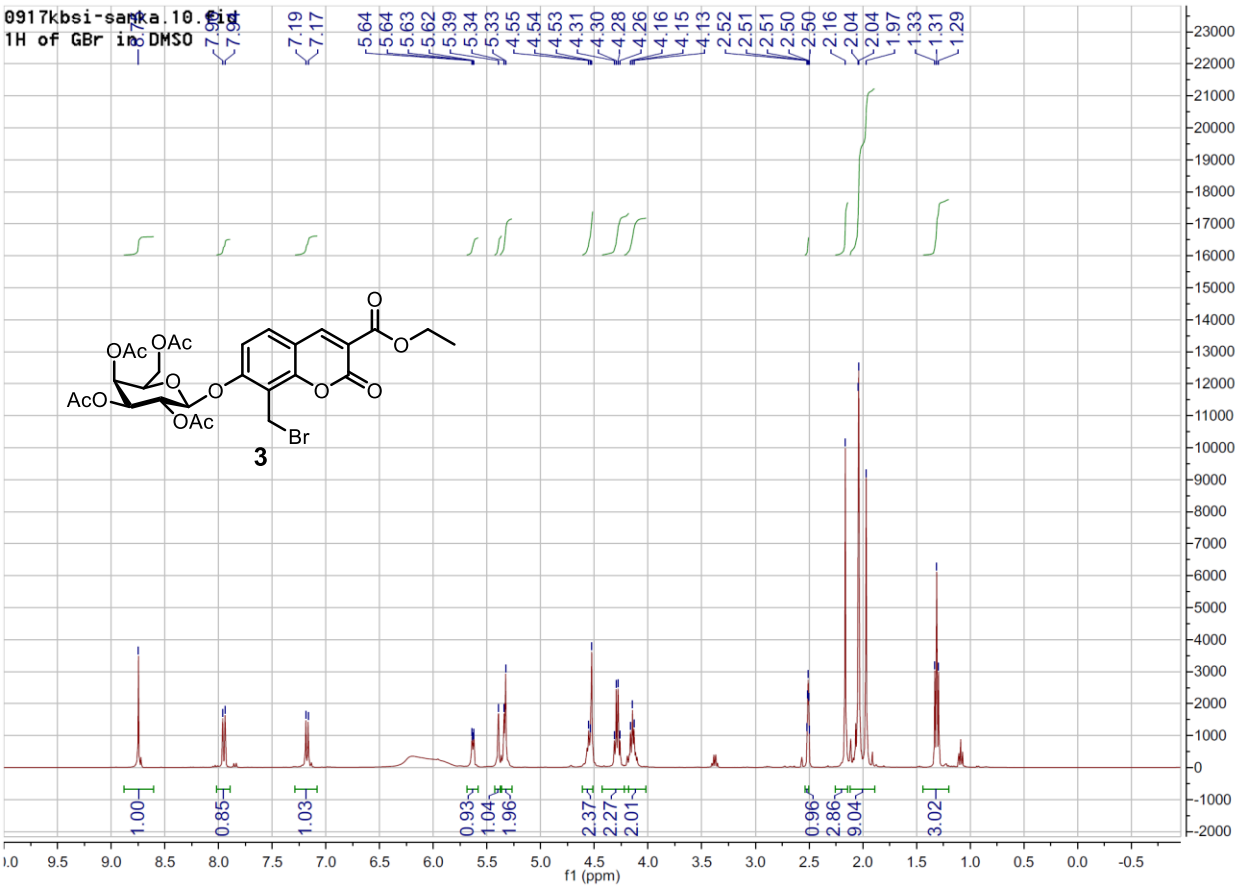


Fig. S12 [1] H NMR Compound **3** in DMSO-d_6.

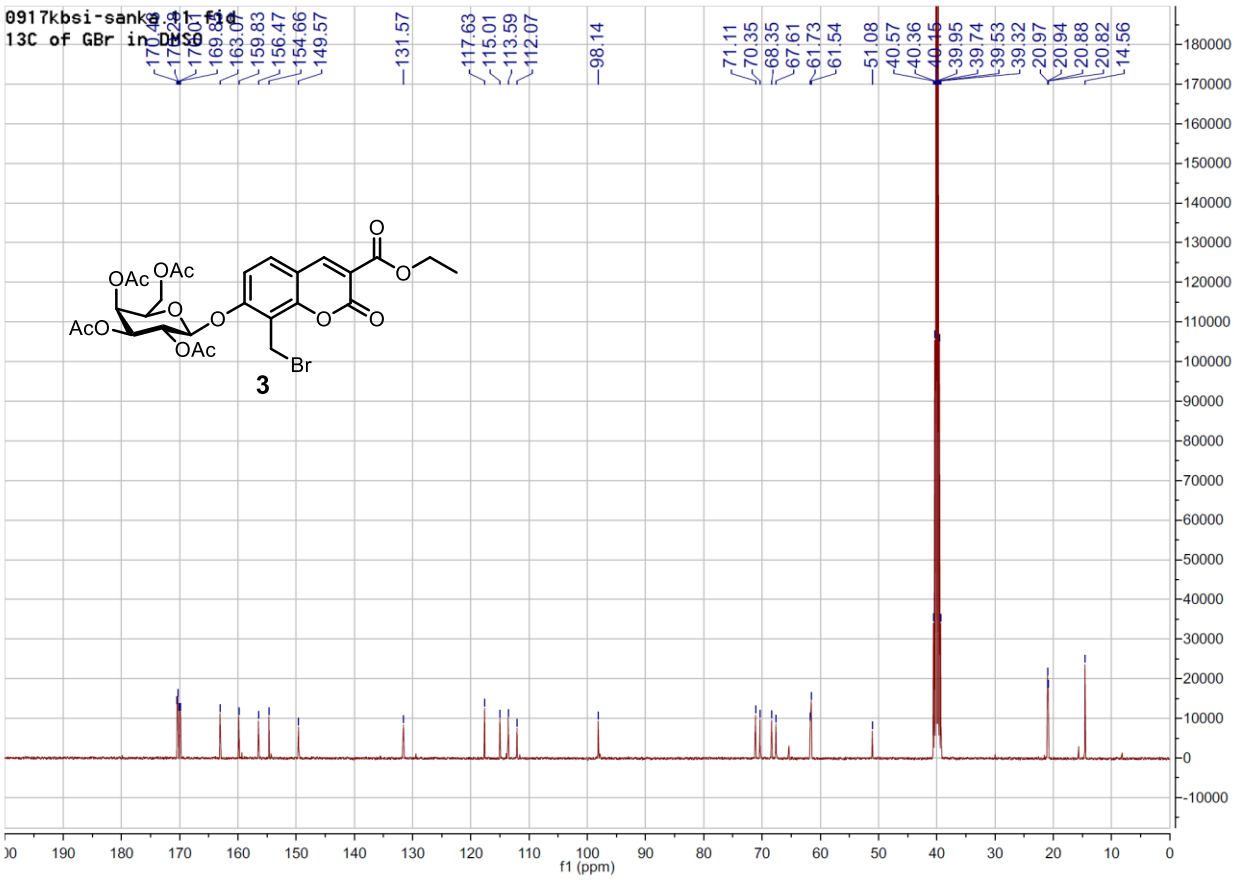


Fig. S13 ^{13}C NMR of Compound **3** in DMSO-d_6

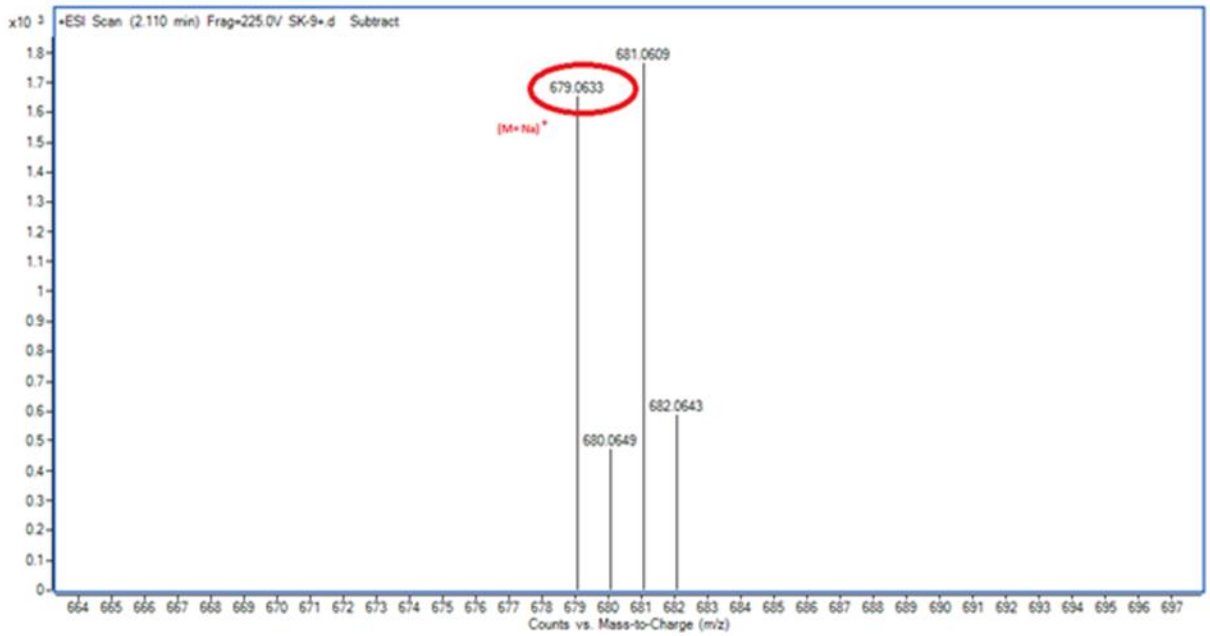


Fig. S14 HRMS of Compound **3**

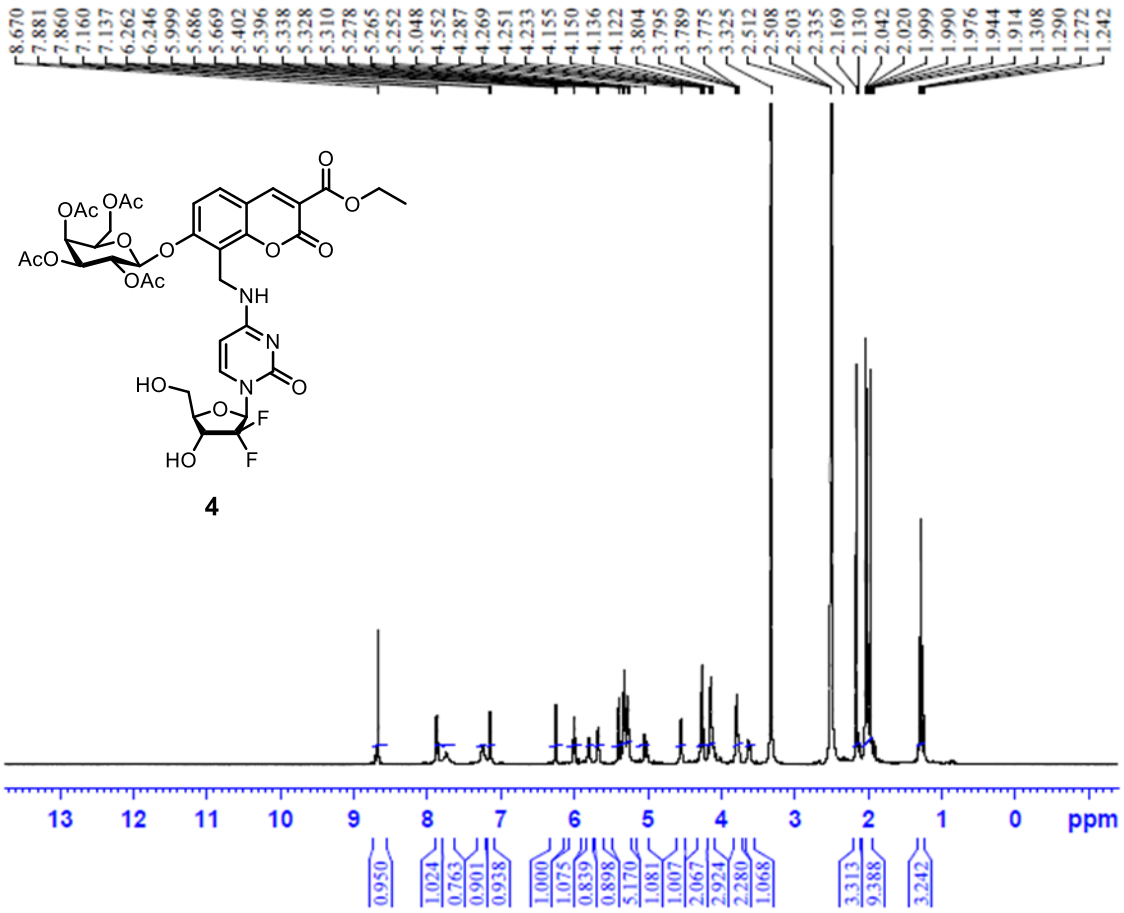


Fig. S15 [1] H NMR Compound **4** in DMSO-d_6.

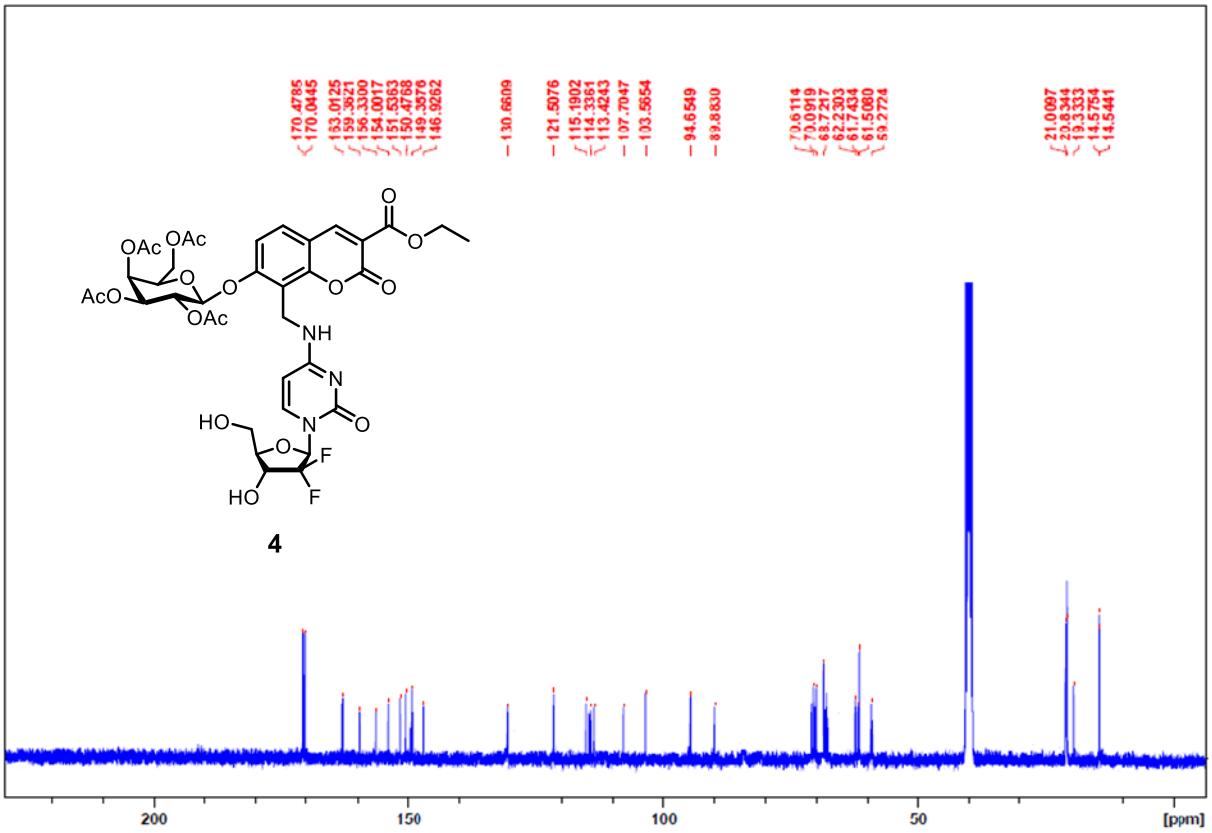


Fig. S16 ^{13}C NMR of Compound **4** in DMSO-d_6.

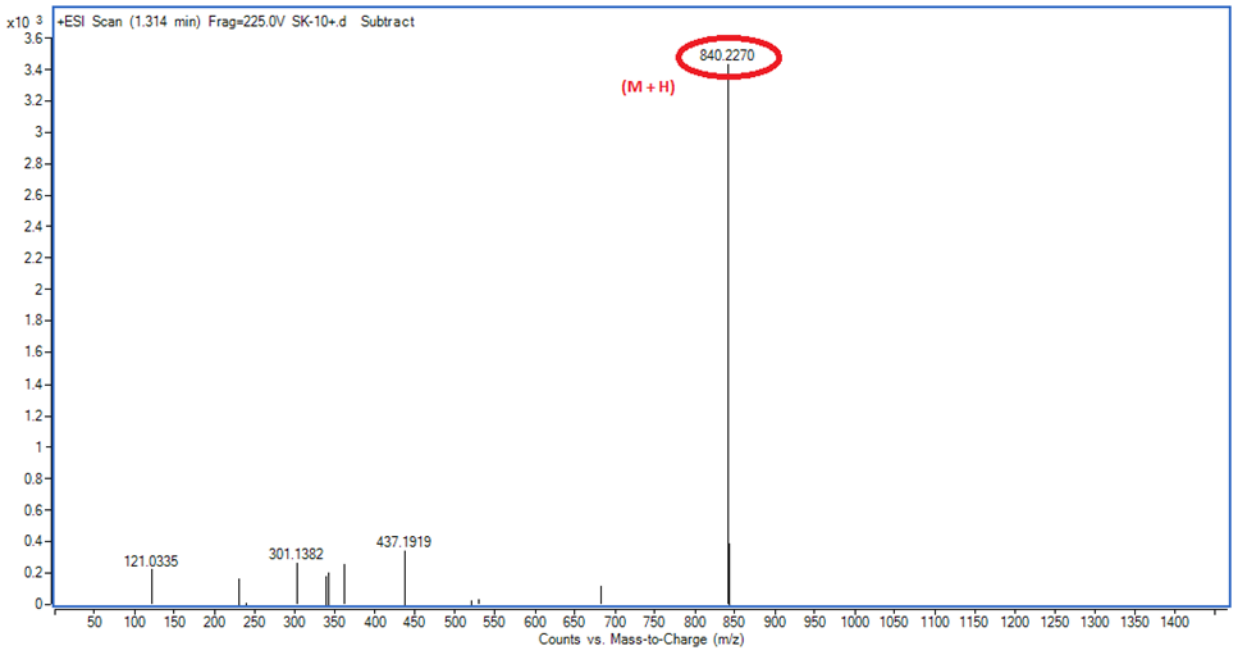


Fig. S17 HRMS of Compound **4**

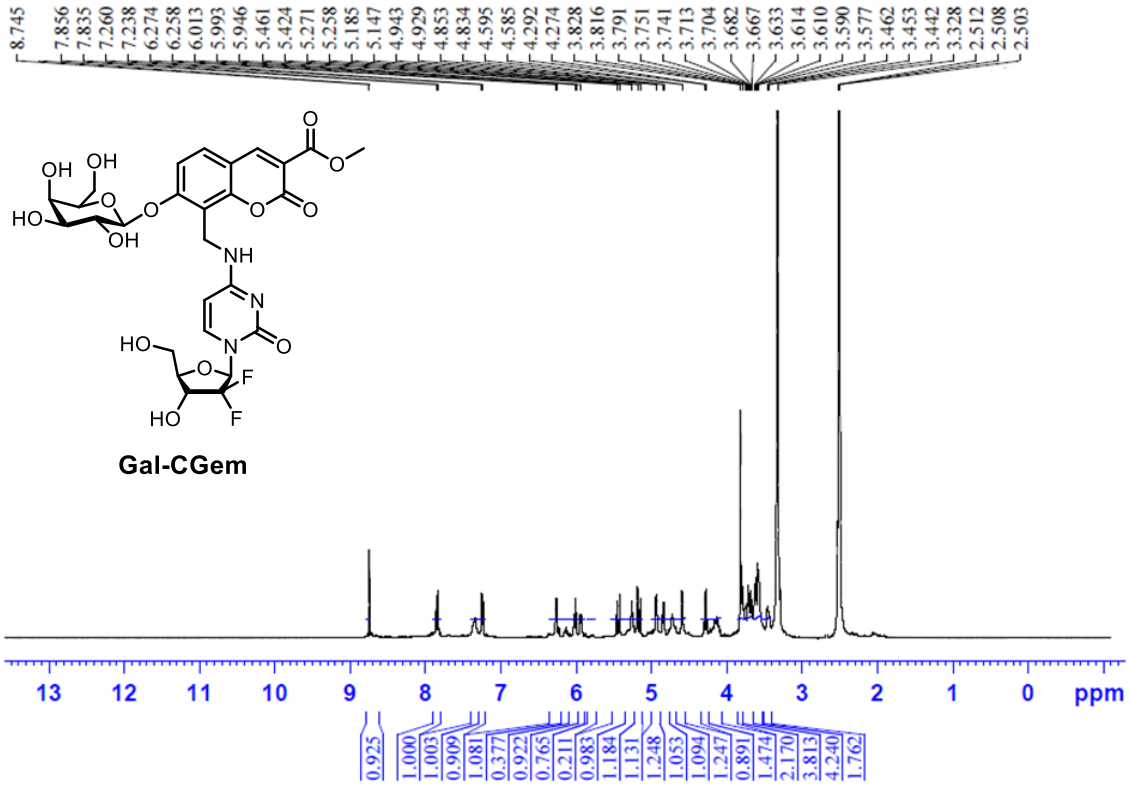


Fig. S18 [1] H NMR **Gal-CGem** in DMSO-d_6.

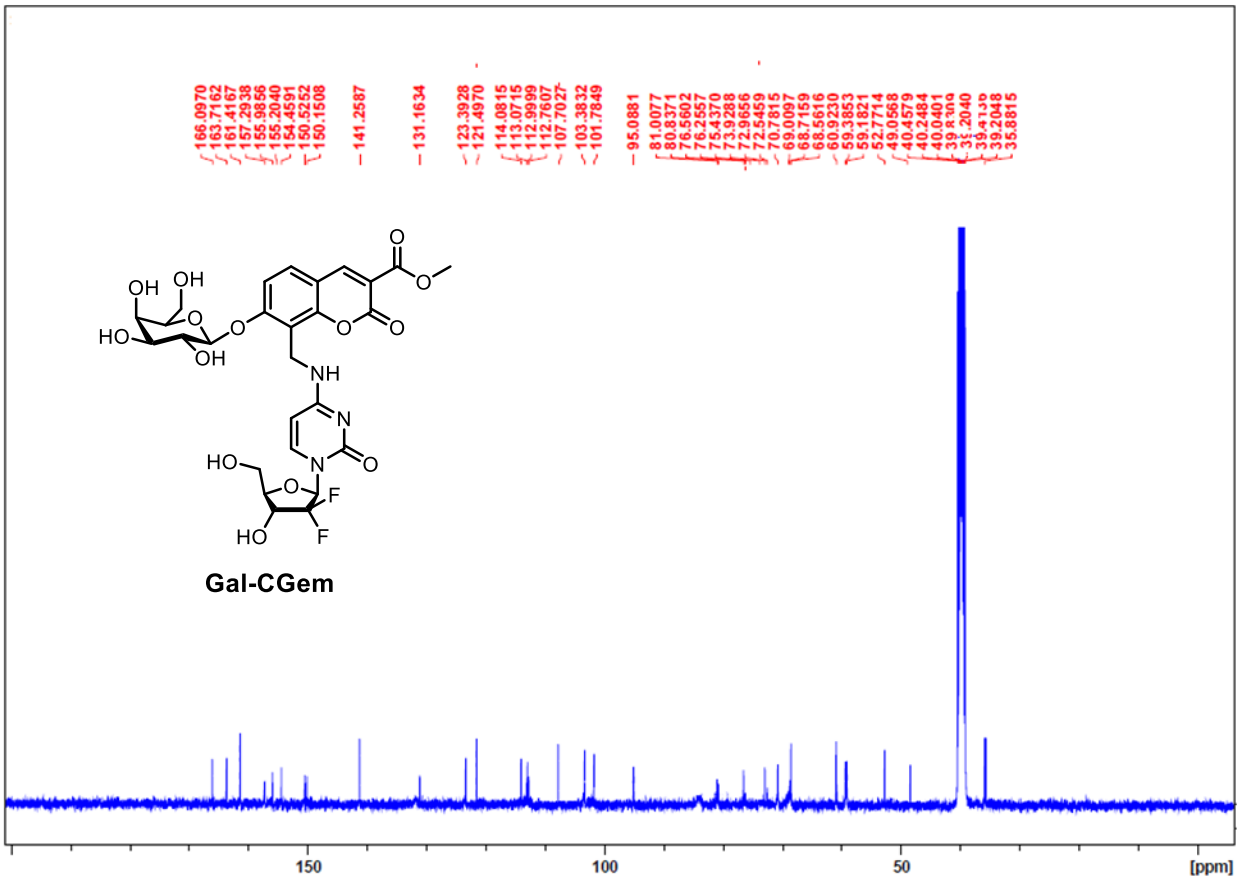


Fig. S19 ^{13}C NMR of **Gal-CGem** in DMSO-d_6

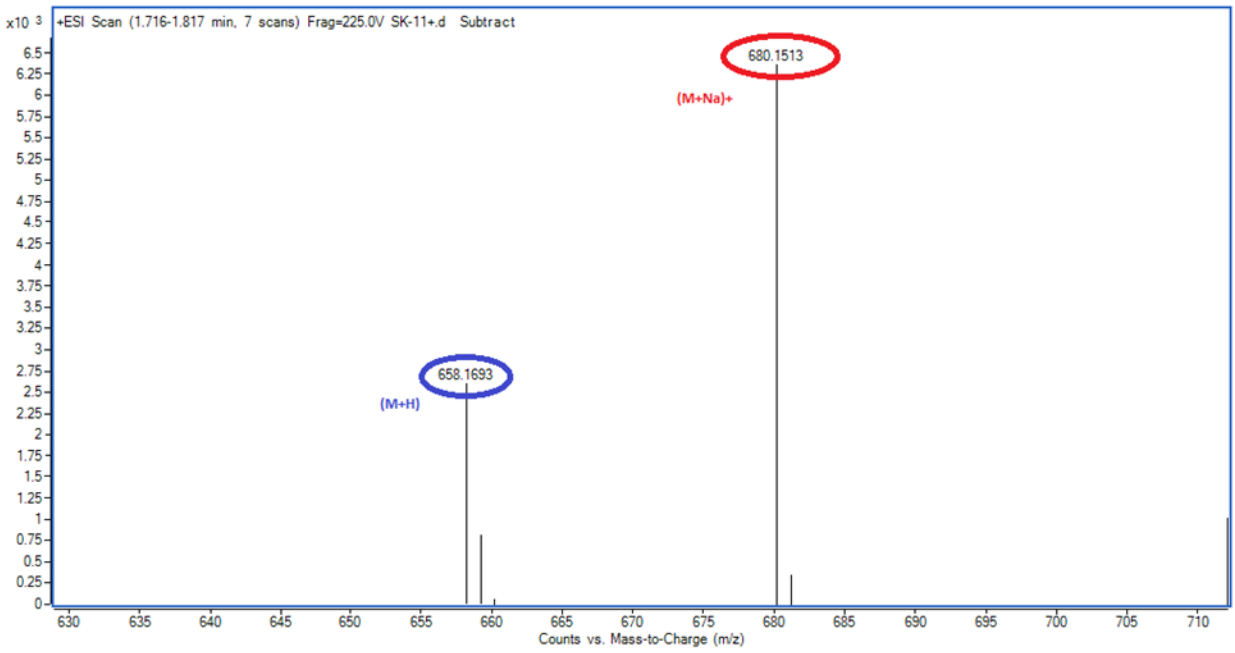


Fig. S20 HRMS of **Gal-CGem**

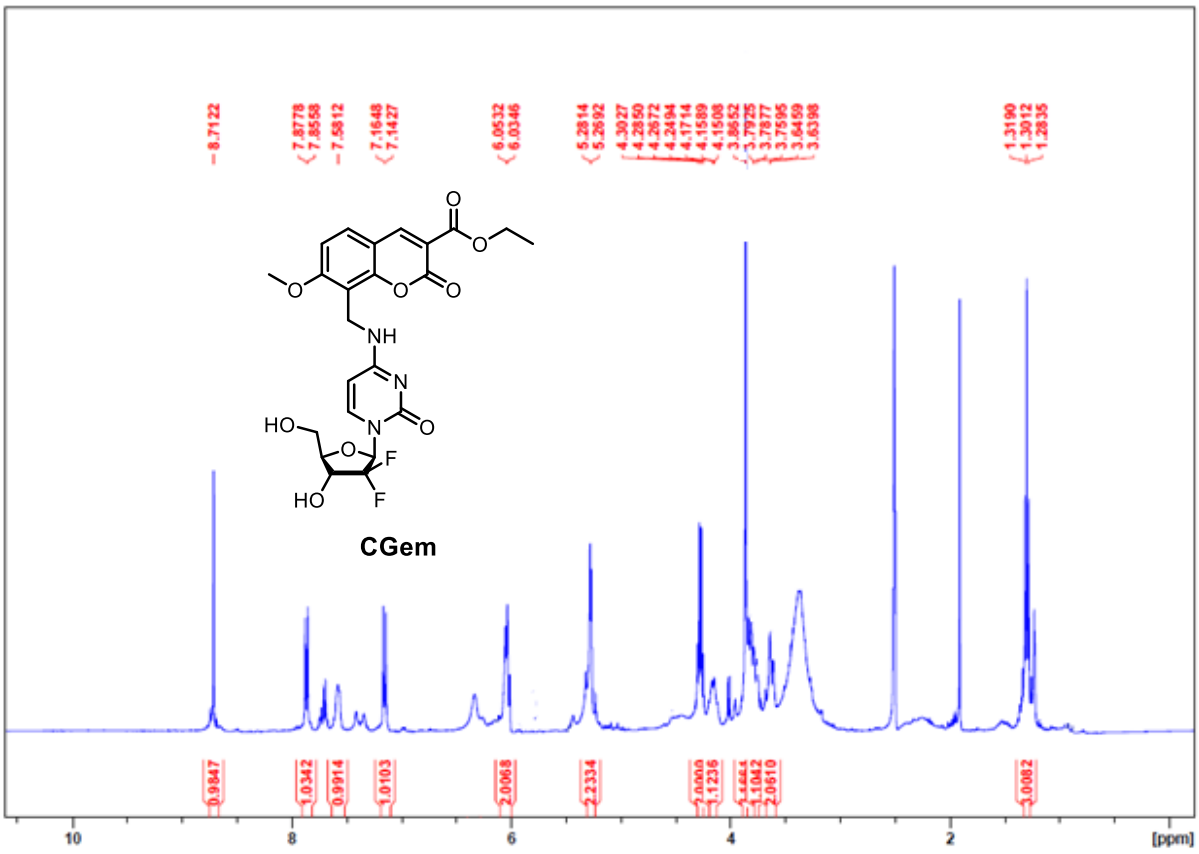


Fig. S21 ^{1}H- NMR of **CGem** in DMSO

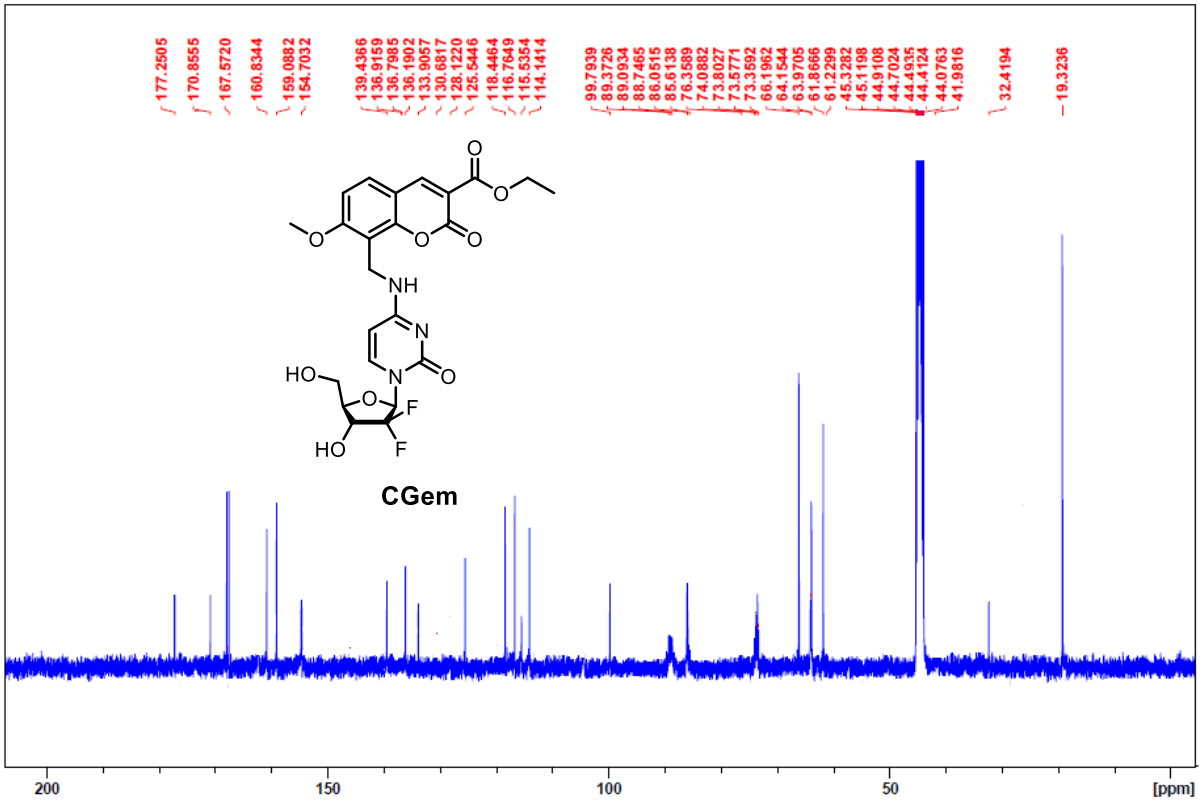


Fig. S22 ^{13}C NMR of **CGem** in DMSO-d_6

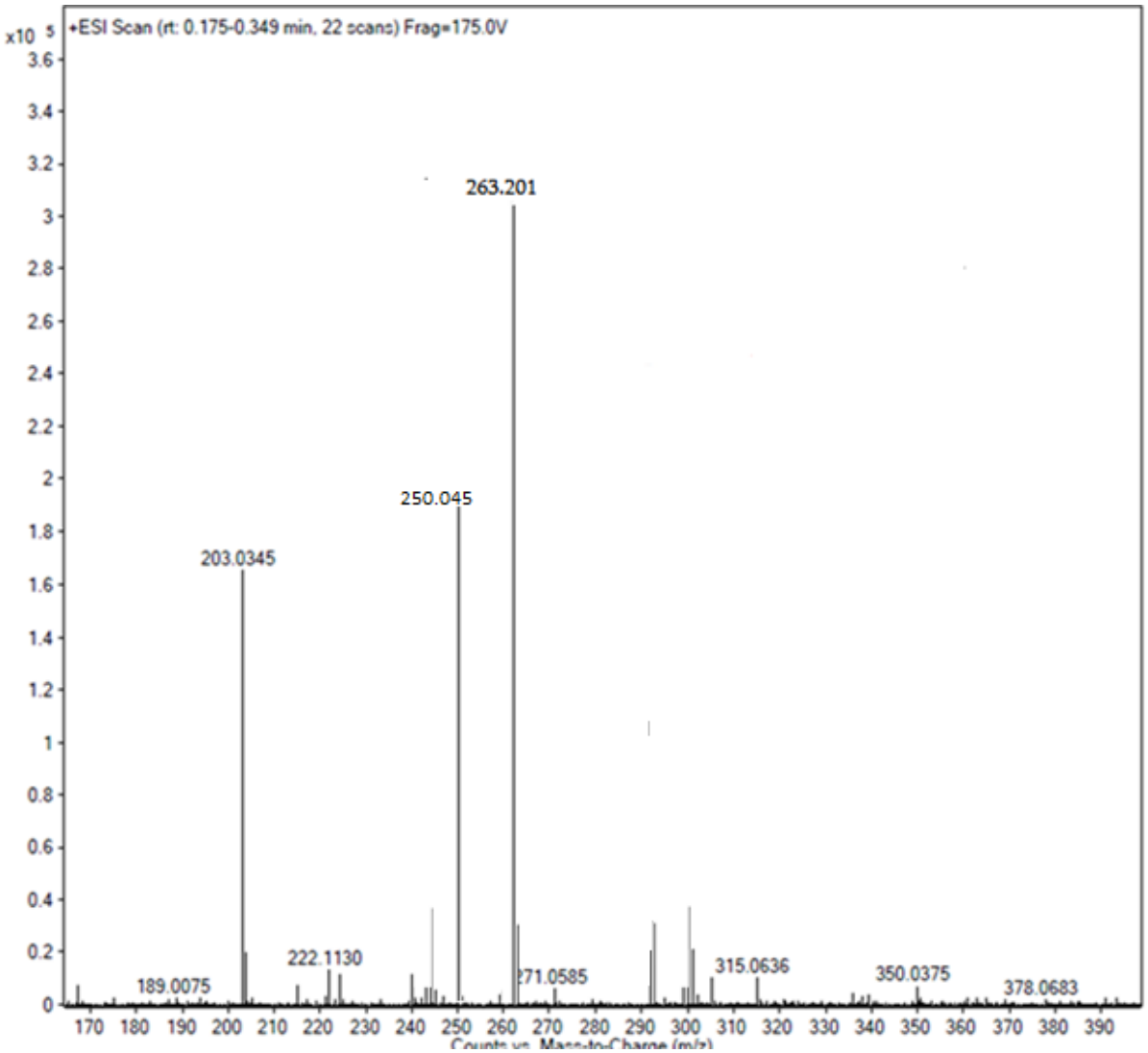


Fig. S23 HRMS of **Gal-CGem** treated with β-galactosidase

References:

S1. A. Podder, M. Won, S. Kim, P. V., M. Maiti, Z. Yang, J. Quc, S. Bhuniya, J.S. Kim, *Sens. Actuators B: Chem.*, 2018, **268**, 195–204.