Supporting Information

A Self-Immolative Dendritic Glucuronide Prodrug of Doxorubicin

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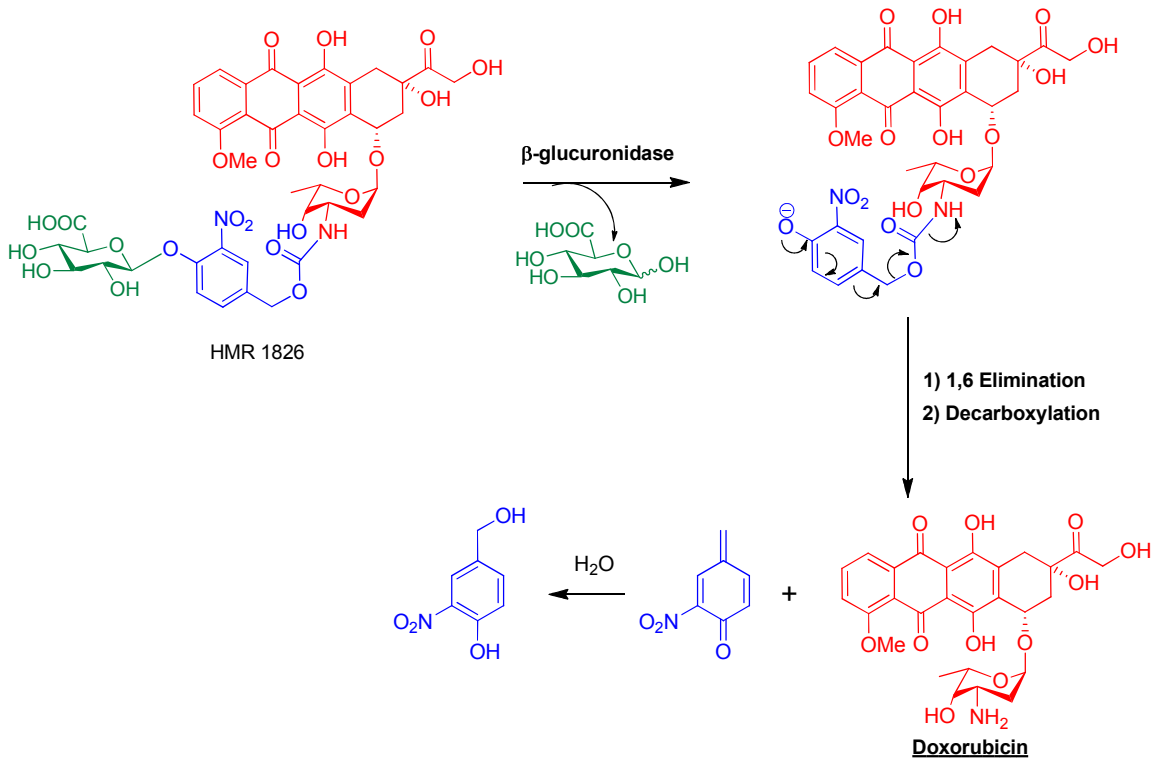
I. Chemistry Section

General experimental section

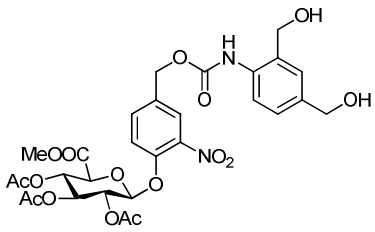
All reactions were performed under an atmosphere of N_2. Unless otherwise stated, solvents used were of HPLC quality. Chemicals were of analytical grade from commercial sources and were used without further purification. The reaction progress was monitored on precoated silica gel TLC plates MACHEREY-NAGEL ALUGRAM® SIL G/UV_{254}. (0.2 mm silica gel 60). Spots were visualized under 254 nm UV light and/or by dipping the TLC plate into a solution of 3 g of phosphomolibdic acid in 100 mL of ethanol followed by heating with a hot gun. Flash column chromatography was performed using MACHEREY-NAGEL silica gel 60 (15-40 µm) as the stationary phase. ^{1}H and ^{13}C NMR spectra were recorded either on a Bruker Avance DPX 300 instrument or on a Bruker 400 Avance III instrument, equipped with an ultra shielded magnet and a BBFO 5 mm broadband probe. Chemical shifts (δ) are reported in parts per million from high to low field and referenced to residual solvent. Coupling constant (*J*) are reported in hertz (Hz). Standard abbreviations indicating multiplicity are used as follows: br = broad, s = singlet, d = doublet, t = triplet, m = multiplet. Melting points were measured on a Büchi Melting Point B-545 instrument and are uncorrected. High resolution ESI mass spectrometry was carried out by the CRMPO (Centre Régional de Mesures Physiques de l'Ouest), at the University of Rennes 1. Low resolution ESI mass spectrometry was carried with a Waters instrument 3100 Mass Detector. Analytical RP-HPLC was carried out on a Dionex Ultimate 3000 system equipped with a UV/Visible variable wavelength detector and with a reverse-phase column chromatography Acclaim$^{(R)}$ (120, C18, 250x4.6 mm, 5 µm, 120 Å) at 30°C and 1mL.min^{-1}. Gradient eluent was composed of A (0.2% TFA in water) and B (CH_3CN). Preparative reverse-phase HPLC for **1** was performed with a VWR LaPrep system. Solvent flow of 30 mL.min^{-1} was applied to a semi-preparative column Merck Hibar® 125-25 (Purospher®STAR RP-18e 5 µm). Gradient eluent was composed of A (0.2% TFA in water) and B (CH_3CN). Method 1: linear gradient beginning with A/B = 70/30 v/v during 5 min., from A/B = 70/30 to A/B = 0/100 v/v within 15 min. and A/B = 0/100 during 5 min. Method 2: linear gradient beginning with A/B = 70/30 v/v during 5 min. and then from A/B = 70/30 to A/B = 30/70 v/v within 15 min. All chromatograms were recorded at 254 nm.

Structure of HMR 1826 and β-glucuronidase-catalysed drug release mechanism



Experimental procedures

Preparation of compound 6

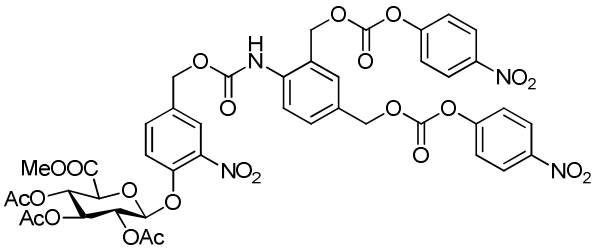


To a stirred solution of **4**[1] (549 mg, 0.85 mmol) in DMF (8 mL) was added **5**[2] (259 mg, 1.69 mmol) and HOBt (114 mg, 0.85 mmol). The mixture was stirred overnight at 50 °C. The solution was concentrated under reduced pressure and the crude material was purified by column chromatography over silica gel (petroleum ether/ethyl acetate: 60 to 100% ethyl acetate) to give **6** (393 mg, 0.59 mmol, 70%) as a colorless solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.97-2.00 (3x s, 9H), 3.63 (s, 3H), 3.86 (br s, 2H), 4.20 (d, 1H, *J* = 9.0 Hz), 4.36 (s, 2H), 4.41 (s, 2H), 5.00 (s, 2H) 5.15-5.26 (m, 4H), 6.93(s, 1H), 7.02 (d, 1H, *J* = 8.4 Hz), 7.24 (d, 1H, *J* = 8.5 Hz), 7.45 (d, 1H, *J* = 8.5 Hz), 7.58 (d, 1H, *J* = 8.5 Hz),

7.71 (s, 1H), 8.13 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.5, 20.5, 20.6, 53.1, 63.3, 64.1, 65.0, 68.7, 70.1, 71.1, 72.2, 99.4, 119.4, 121.1, 124.8, 127.3, 127.3, 130.0, 132.4, 133.8, 136.0, 136.4, 140.7, 148.7, 153.7, 166.9, 169.5, 169.6, 170.1; HRESI-MS: m/z 687.1644 (calcd. for **$C_{29}H_{32}N_2O_{16}Na$** 687.1644 $[M+Na]^+$); m.p. dec 71°C.

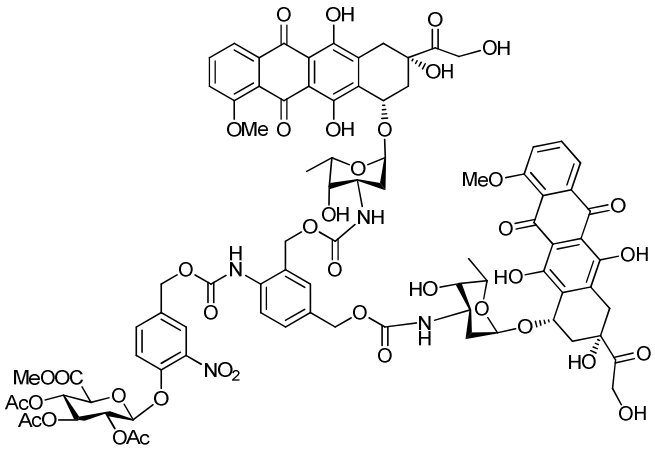
Preparation of compound 7



Anhydrous pyridine (304 µL, 3.76 mmol) was added dropwise to a cooled solution (0°C) of *p*-nitrophenyl chloroformate (759 mg, 3.76 mmol) in CH_2Cl_2 (8 mL). The mixture was stirred for 20 minutes at 0°C. A solution of **6** (833 mg, 1.25 mmol) in CH_2Cl_2 (11 mL) was added and the mixture was stirred for 1 hour at room temperature. The reaction was quenched with a saturated solution of NaCl and extracted with CH_2Cl_2 (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The resulting crude material was purified by column chromatography over silica gel (petroleum ether/ethyl acetate: 40 to 75% ethyl acetate) to give **7** (1.22 mg, 1.23 mmol, 98%) as a colorless solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ 2.07-2.12 (3x s, 9H), 3.74 (s, 3H), 4.23-4.26 (m, 1H), 5.20-5.36 (m, 10H), 7.33-7.41 (m, 5H), 7.50-7.60 (m, 3H), 7.68 (br s, 1H), 7.86-7.91 (m, 2H), 8.24-8.27 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.5, 20.5, 20.6, 53.1, 65.4, 67.6, 68.6, 70.0, 70.1, 70.9, 72.5, 99.5, 115.6, 119.8, 121.7, 121.8 (x2), 123.3, 124.9, 125.2, 125.3 (x2), 125.4, 126.1, 130.8, 131.1, 131.9, 132.2, 133.7, 137.3, 141.1, 145.4, 145.6, 148.9, 152.4, 152.9, 153.4, 155.1, 155.4, 166.8, 169.3, 169.4, 170.0; HRESI-MS: m/z 1017.1766 (calcd. for **$C_{43}H_{38}N_4O_{24}Na$** 1017.17682 $[M+Na]^+$); m.p. dec. 78°C.

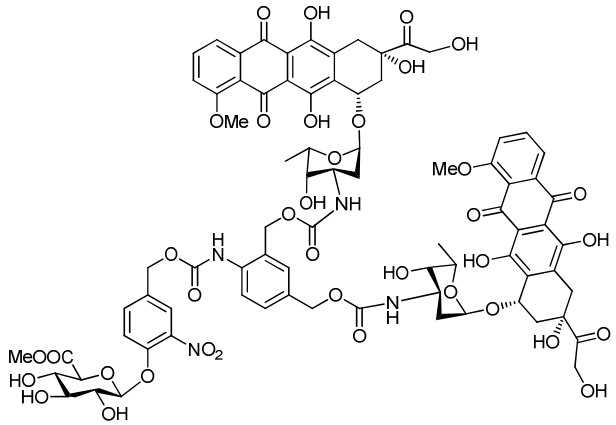
Preparation of compound 8



To a solution of doxorubicin hydrochloride (175 mg, 0.30 mmol) in DMF (1.5 mL) was added triethylamine (42 µL, 0.30 mmol). The mixture was stirred for 30 minutes at room temperature before adding HOBt (41 mg, 0.30 mmol) and a solution of **7** (150 mg, 0.15 mmol) in DMF (1 mL) and stirring was continued for 3 hours. The reaction was quenched with a saturated solution of NaCl and extracted with CH_2Cl_2 (4x) and ethyl acetate (1x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure (CAUTION: Do not heat the water bath). The resulting crude material was purified by column chromatography over silica gel (CH_2Cl_2/MeOH: 0 to 7% MeOH) to give **8** (308 mg, 0.17 mmol, 57%) as red powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.24–1.29 (m, 6H), 1.82 (m, 6H), 2.05-2.28 (m, 14H), 2.81-2.88 (m, 2H), 3.10-3.20 (m, 4H), 3.62-3.79 (m, 6H), 4.01-4.25 (m, 10H), 4.57-5.42 (m, 21H), 7.100 (m, 2H), 7.25-7.34 (m, 2H, masked by $CHCl_3$ residual signal), 7.42-7.53 (m, 3H), 7.72-7.90 (m, 6H), 13.07 (br s, 2H), 13.85 (br s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 16.8 (x2), 20.5, 20.6, 21.0, 29.7 (x2), 30.0 (x2), 33.7 (x2), 35.6 (x2), 47.1 (x2), 47.2 (x2), 53.1, 56.5 (x2), 60.4 (x2), 65.4 (x2), 67.4 (x2), 68.7, 69.4, 70.2, 71.1, 72.4, 99.6, 100.9 (x2), 111.2 (x2), 111.4 (x2), 114.4, 118.5, 119.8, 124.8 (x2), 132.6 (x2), 133.4 (x2), 133.6 (x2), 134.1 (x2), 134.2 (x2), 135.1 (x2), 135.8, 135.9, 140.9, 144.1 (x2), 148.6 (x2), 153.6 (x2), 155.4(x2), 155.6 (x2), 155.9, 156.1 (x2), 160.9, 166.7, 167.7, 169.3, 169.4, 170.0, 171.2, 186.3 (x2), 186.7 (x2), 213.7 (x2); HRESI, MS: m/z 1825.4709 (calcd. for **$C_{85}H_{86}N_4O_{40}Na$** 1825.47105 $[M+Na]^+$); m.p. dec. 208°C.

Preparation of compound 9

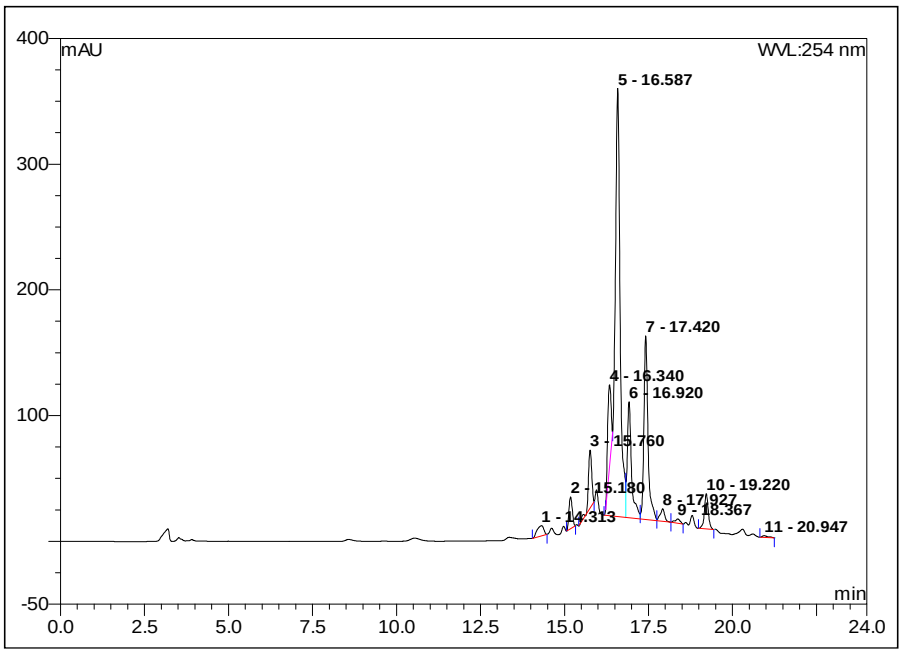


To a solution of **8** (198 mg, 0.11 mmol) in MeOH (10 mL) cooled at -10°C, was added a solution of MeONa 0.1M in MeOH (14.3 mL, 1.43 mmol). Stirring was continued for 3 hours at -10°C and the solution was neutralized with Amberlite IRC 50 over 20 minutes and filtered through a pad of cotton. After removing the volatiles under reduced pressure, the crude material was purified by column chromatography over silica gel (CH_2Cl_2/MeOH: 0 to 8% MeOH) to give **9** (128 mg, 0.089 mmol, 81%) as a red powder.

^{1}H NMR (300 MHz, $CDCl_3$ + 10% CD_3OD-*d4*): δ 1.12-1.21 (m, 6H), 1.72-2.29 (m, 4H), 3.17-3.35 (m, 8H, masked by CD_3OH residual signal), 3.51-3.97 (m, 27H), 4.66-5.85 (m, 9H), 7.16-7.19 (m, 2H), 7.26-7.33 (m, 6H, masked by $CHCl_3$ residual signal), 7.41-7.90 (m, 5H); LRESI-SM: $[M+Na]^+$= 1699.62; m.p. dec. 156°C.

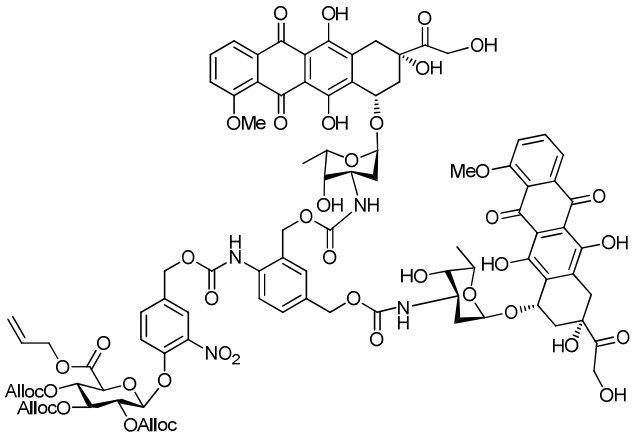
Experimental conditions attempted for the hydrolysis of ester 9

To a solution of **9** (11.9, 27, 29 or 32 μmol) in water/THF v/v (4, 9, 10 or 11 mL) was added an aqueous solution of NaOH 2N (2 or 3 équiv.) at -5°C or -15°C. The mixture was stirred for 15, 30 min, 2 or 3 hours. In all cases, the reaction led to a complex mixture (see HPLC analysis).



HPLC analysis of the crude mixture of ester 9 hydrolysis conducted in the presence of NaOH.

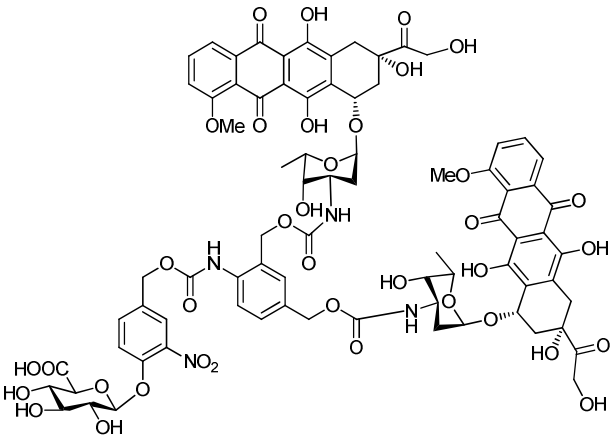
Preparation of compound 11



To a solution of doxorubicin hydrochloride (148.2 mg, 0.25 mmol) in DMF (1mL) was added triethylamine (35.5 µL, 0.25 mmol). The mixture was stirred for 30 minutes at room temperature before adding HOBt (34.5 mg, 0.25 mmol) and a solution of **10**[3] (145 mg, 0.127 mmol) in DMF (1 mL) and stirring was continued for 3 hours. The reaction was quenched with a saturated solution of NaCl and extracted with CH_2Cl_2 (4x) and ethyl acetate (1x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure (CAUTION: Do not heat the water bath). The resulting crude material was purified by column chromatography over silica gel (CH_2Cl_2/MeOH: 0 to 5% MeOH) to give **11** (144 mg. 0.074 mmol, 58%) as red powder.

^{1}H NMR(400 MHz, DMSO-*d6*): δ 1.13 (d, 6H, *J* = 6.0 Hz), 1.23 (br s, 2H), 1.51-1.54 (m, 2H), 1.80-1.83 (m, 2H), 1.99-2.22 (m, 4H), 2.73-2.93 (m, 4H), 3.44 (m, 2H) : 3.69 (m, 2H), 3.93 (2x s, 6H), 4.15 (d, 2H, *J* = 6.0 Hz), 4.54-4.72 (m, 13H), 4.84-4.94 (m, 8H), 5.00-5.45 (m, 17H), 5.80-5.94 (m, 5H), 6.83 (d, 1H, *J* = 7.7 Hz), 6.93 (d, 1H, *J* = 7.7 Hz), 7.21 (d, 1H, *J* = 8.3 Hz), 7.27 (s, 1H), 7.36 (d, 1H, *J* = 8.3 Hz), 7.43 (d, 1H, *J* = 8.7 Hz), 7.57 (d, 2H, *J* = 7.6 Hz), 7.70 (d, 1H, *J* = 8.7 Hz), 7.81-7.87 (m, 4H), 7.93 (s, 1H), 9,11 (br s, 1H), 13.18-13.20 (2x s, 2H), 13.91-13.94 (2x s, 2H); ^{13}C RMN (100 MHz, DMSO-*d6*): δ 17.0 (x2), 29.7 (x2), 32.1 (x2), 36.6 (x2), 47.1 (x2), 56.5, 63.7 (x2), 64.3 (x2), 66.0 (x2), 66.6 (x2), 67.9 (x2), 68.4 (x2), 68.6, 68.6, 68.8, 70.7, 72.2, 73.7, 74.9, 97.4, 100.3 (x2), 110.6 (x4), 117.4, 118.0, 118.4, 118.6, 118.7 (x2), 118.8 (x2), 119.6 (x2), 119.9, 124.4 (x2), 131.5 (x2), 131.6 (x2), 131.7 (x2), 131.7, 132.4 (x2), 133.9 (x2), 134.5, 135.0, 135.5, 136.0 (x2), 139.9 (x2), 147.6 (x2), 153.1 (x2), 153.2 (x2), 153.6, 153.9 (x2), 153.9, 154.4, 155.2, 155.2, 156.1 (x2), 160.7, 162.3, 165.8 (x2), 186.1 (x2), 186.4 (x2), 213.7 (x2); HRESI-MS: m/z 1977.5180 (calcd. for **$C_{93}H_{94}N_4O_{43}Na$** 1977.5184 **[**M+Na]$^{+}$); m.p. dec. 146°C.

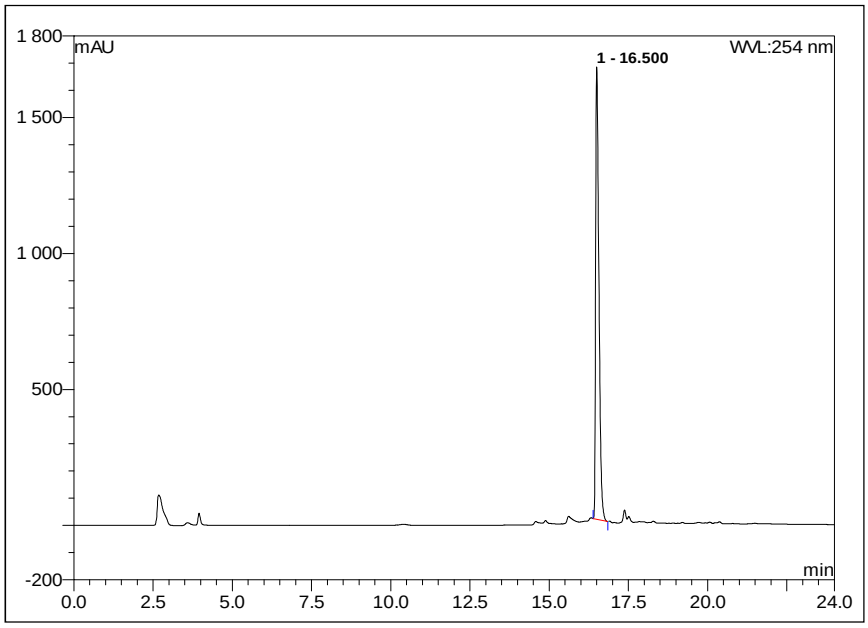
Preparation of prodrug 1



To a solution of compound **11** (23 mg, 12 μmol) in MeOH/CH_2Cl_2 (10/90; 1mL) was added Tetrakis(triphenylphosphine)palladium(0) (3 mg, 2.5 μmol) and aniline (4.6 μL, 50 μmol).3 Th mixture was stirred at room temperature and the reaction was monitored by analytical HPLC using Method 1 (retention time for **1**: 16.5 min). Completion of the reaction was accomplished after 24 hours and solvents were removed under reduced pressure (CAUTION: do not heat the water bath). The crude material was triturated with CH_2Cl_2 and the resulting solid was collected by filtration and rinsed with CH_2Cl_2 to afford **1** as a red powder (16 mg,

10 µmol, 84%) with 82% purity. For biological evaluations, **1** was further purified up to 97% purity by preparative reverse-phase HPLC using Method 2.

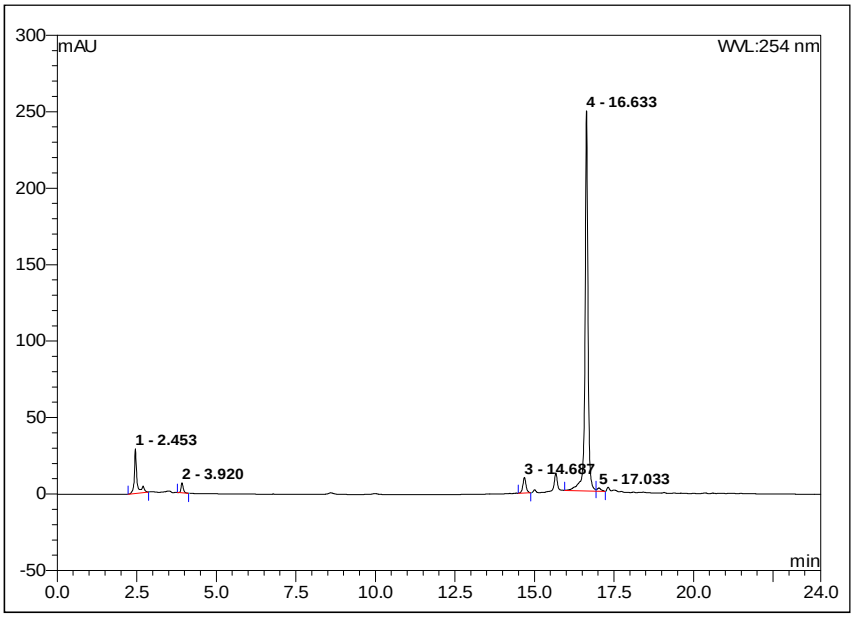
^{1}H NMR(400 MHz, DMSO-*d6*): δ 1.11 (d, 6H, *J* = 6.0 Hz), 1.51-1.53 (m, 2H), 1.83 (m, 2H), 2.09-2.22 (m, 4H), 2.88-3.01 (m, 5H), 3.27-3.44 (m, 10H, masked by HOD residual signal), 3.65-3.71 (m, 3H), 3.93-3.98 (m, 6H), 4.15 (d, 2H, *J* = 6.0 Hz), 4.58 (s, 3H), 4.73-5.22 (m, 12H), 5.37 (br s, 1H), 5.46 (br s, 1H), 6.86 (d, 1H, *J* = 8.0 Hz), 6.94 (d, 1H, *J* = 8.0 Hz), 7.00-7.94 (m, 14H), 9.10 (br s, 1H), 13.96 (s, 2H), 14.03 (s, 2H); HRESI-MS: m/z 1661.4264 (calcd. for **$C_{78}H_{77}N_4O_{37}$** 1661.42722 **[**M-H]$^-$). m.p. dec. 142°C.



HPLC analysis of prodrug 1 after purification by preparative reverse-phase HPLC

Stability

Compound **1** (0.1 mg, 0.060 µmol) was incubated at 37°C in 20 mM phosphate buffer at pH 7.0 (1 mL). Stability in phosphate buffer was monitored by analytical HPLC using Method 1. HPLC analysis showed no detectable degradation of compound **1** during 10 days under these conditions.

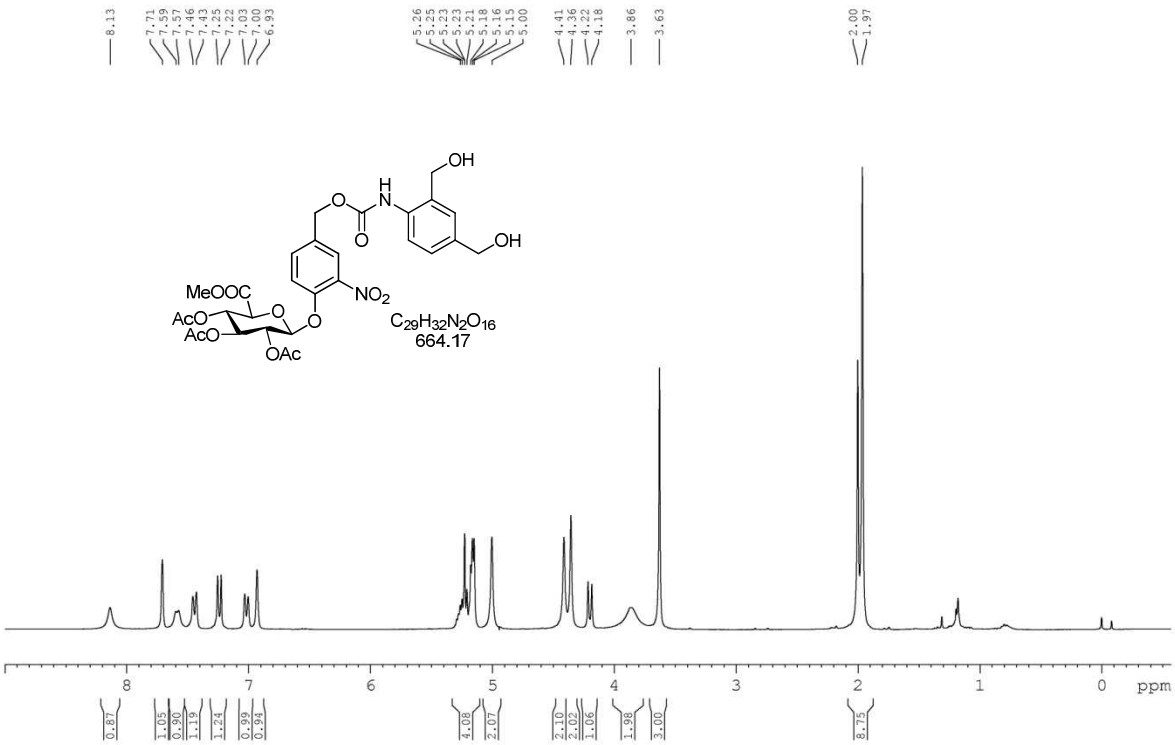


HPLC analysis of compound 1 after 10 days at 37°C in 20 mM phosphate buffer at pH 7.0.

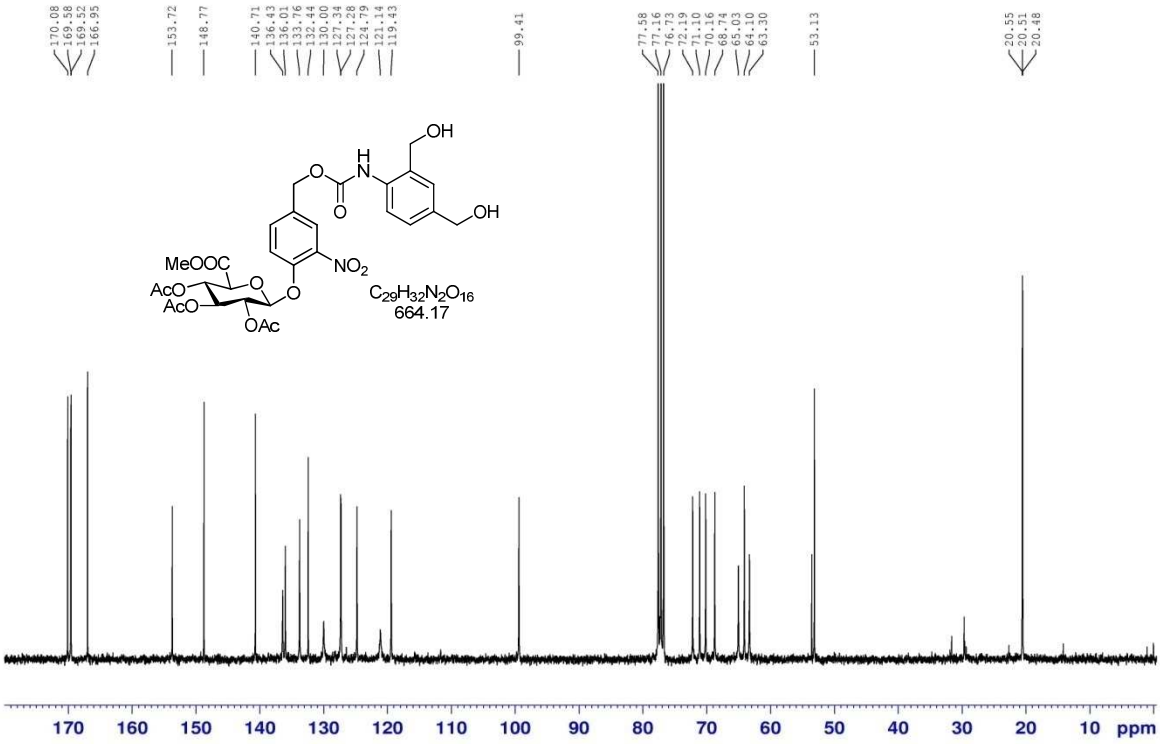
Enzymatic hydrolysis

Enzymatic hydrolysis was carried out with commercial β-glucuronidase from *Escherichia coli* (aqueous glycerol solution, 20,000,000-60,000,000 units/g protein, pH 6.8 (biuret)). Compound **1** (0.1 mg, 0.060 µmol) was incubated with the enzyme (133 U) at 37°C in a solution of phosphate buffer (20 mM, pH 7)/DMSO 99/1 (1 mL). Hydrolysis was monitored by analytical HPLC using Method 1.

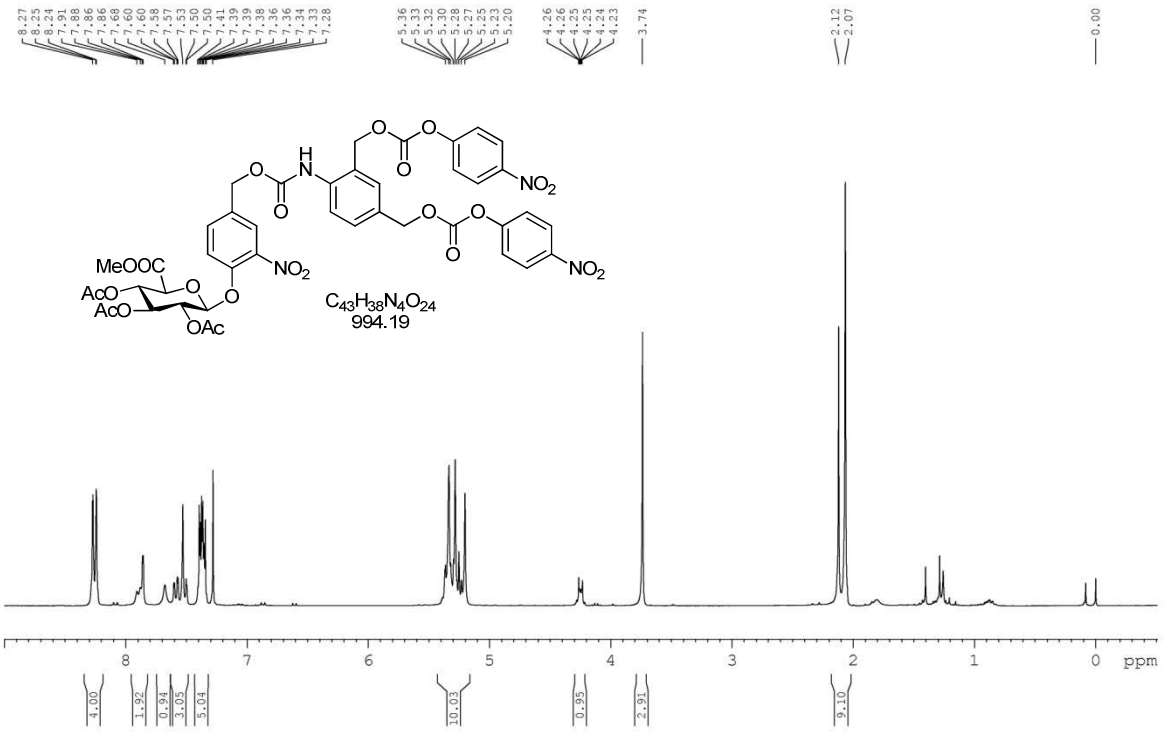
^{1}H NMR and ^{13}C NMR spectra



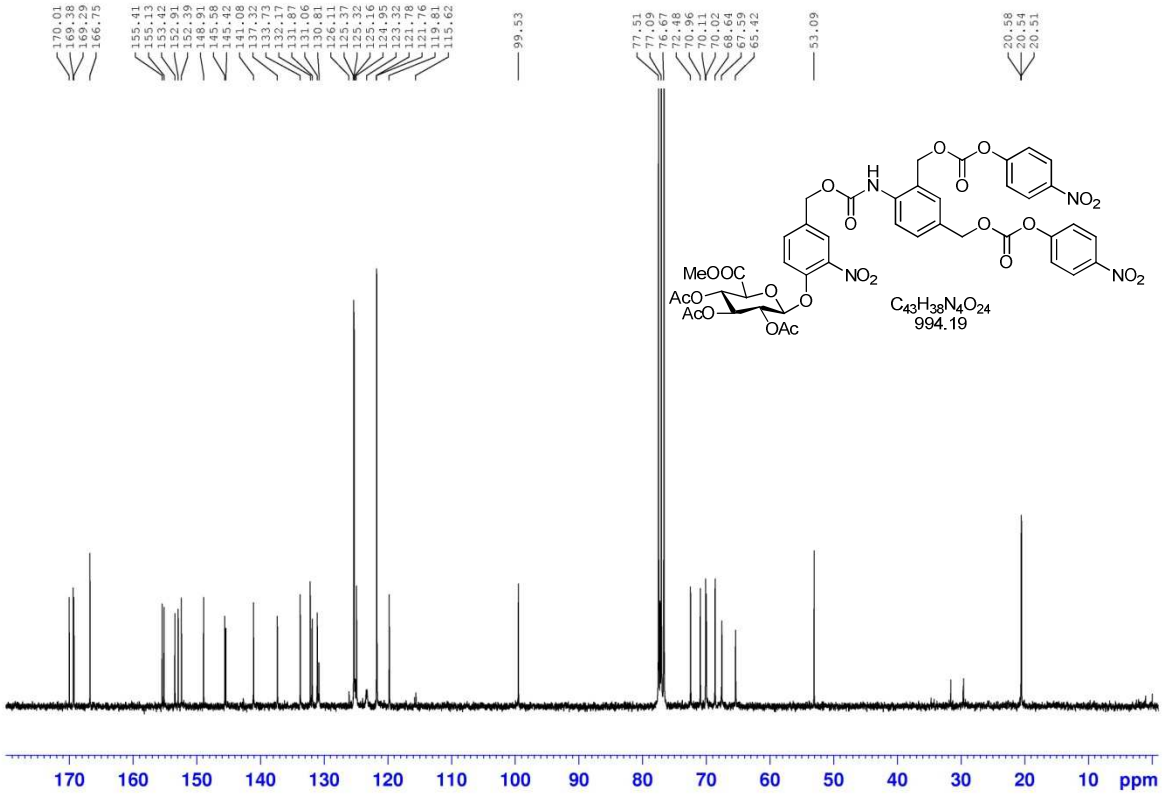
^{1}H NMR spectrum of **6**,
300 MHz, 298 K, $CDCl_3$.



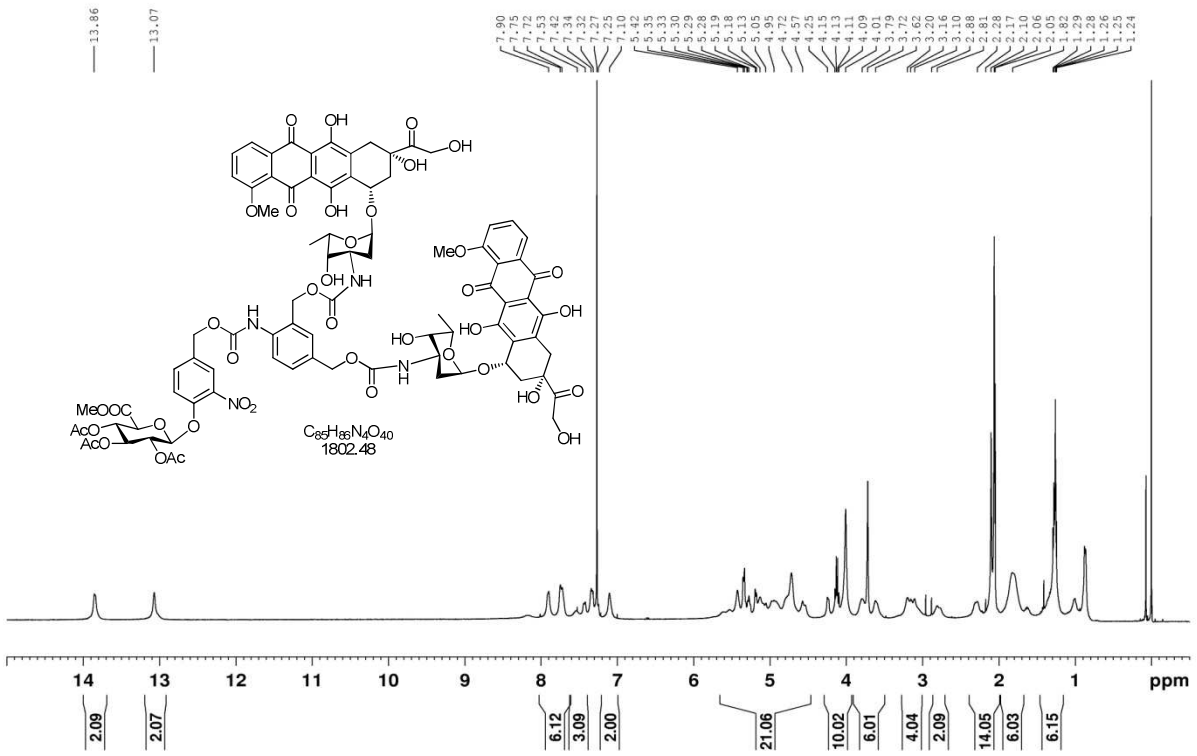
^{13}C NMR spectrum of **6**,
75 MHz, 298 K, $CDCl_3$.



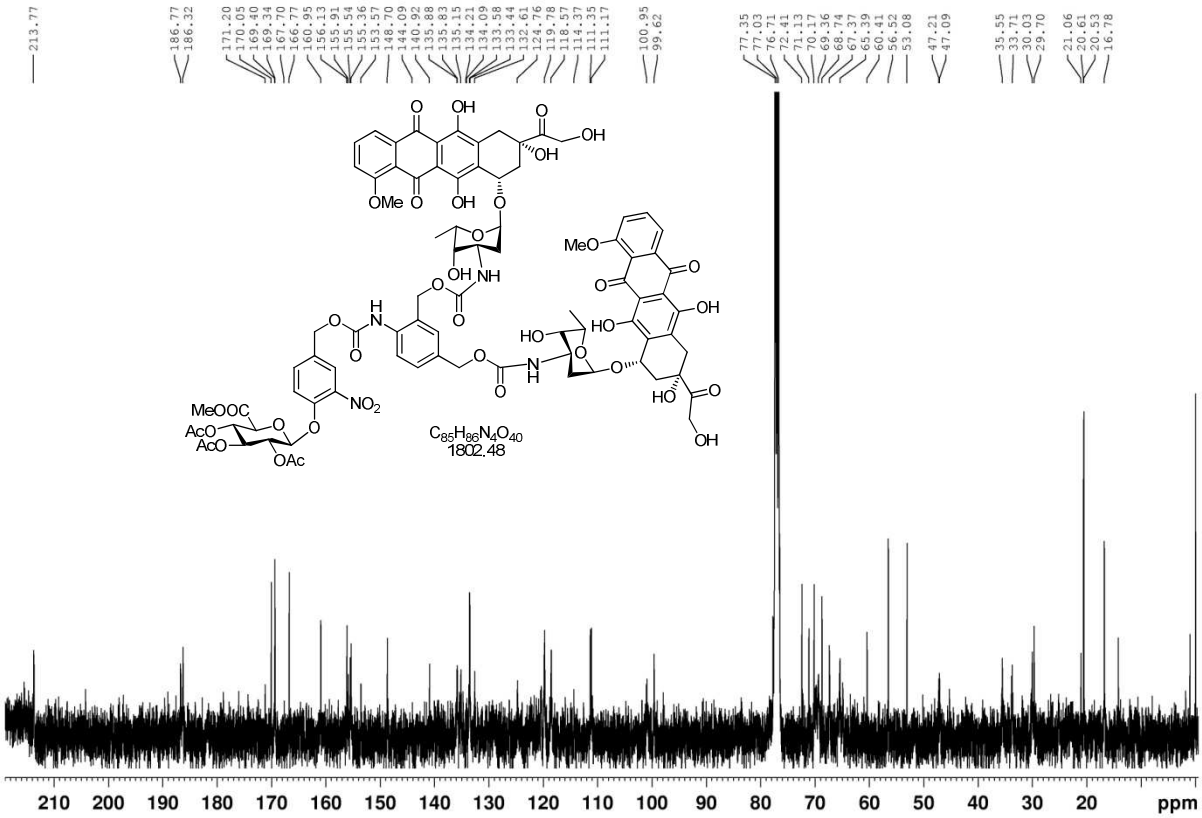
^{1}H NMR spectrum of **7**,
300 MHz, 298 K, $CDCl_3$.



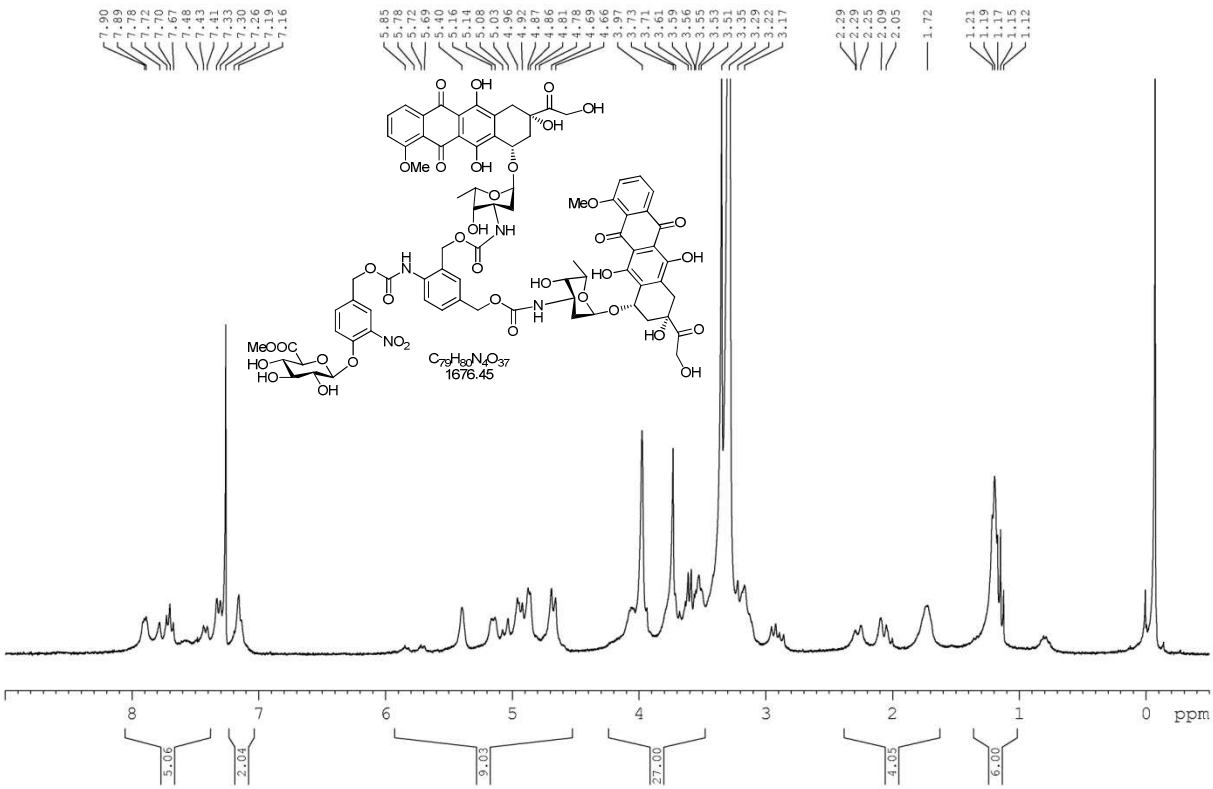
^{13}C NMR spectrum of **7**,
75 MHz, 298 K, $CDCl_3$.



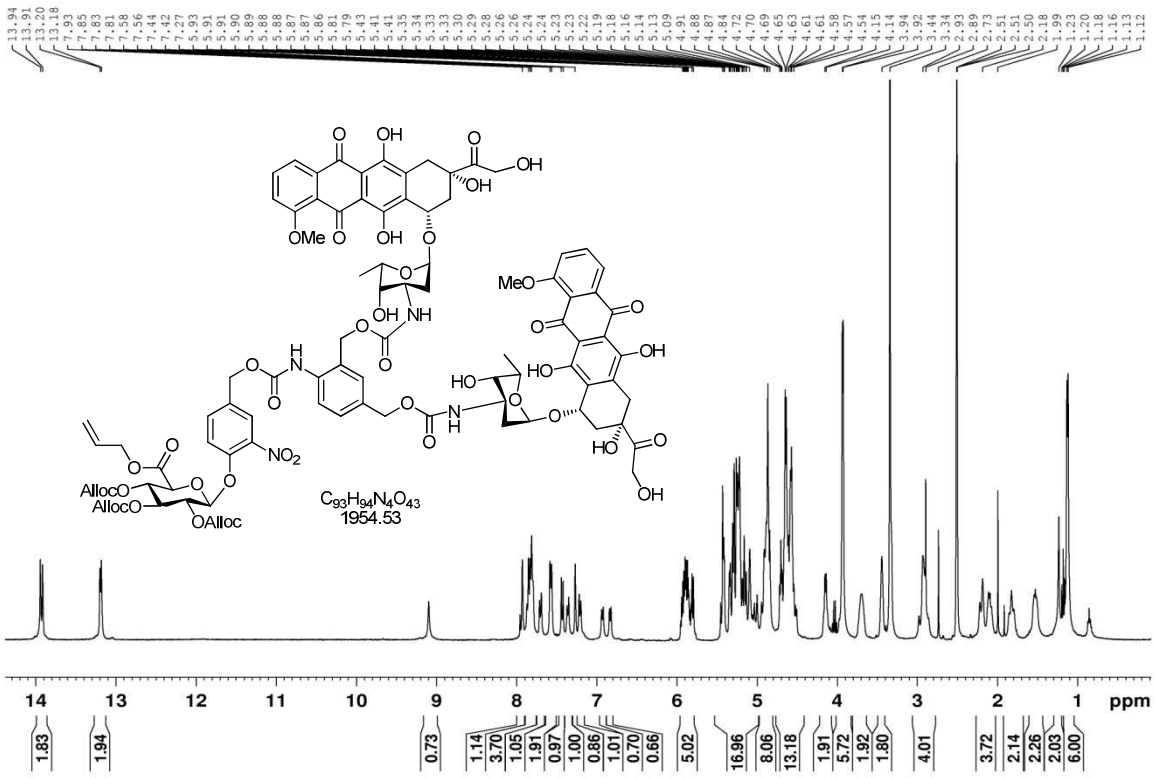
^{1}H NMR spectrum of **8**,
400 MHz, 298 K, $CDCl_3$.



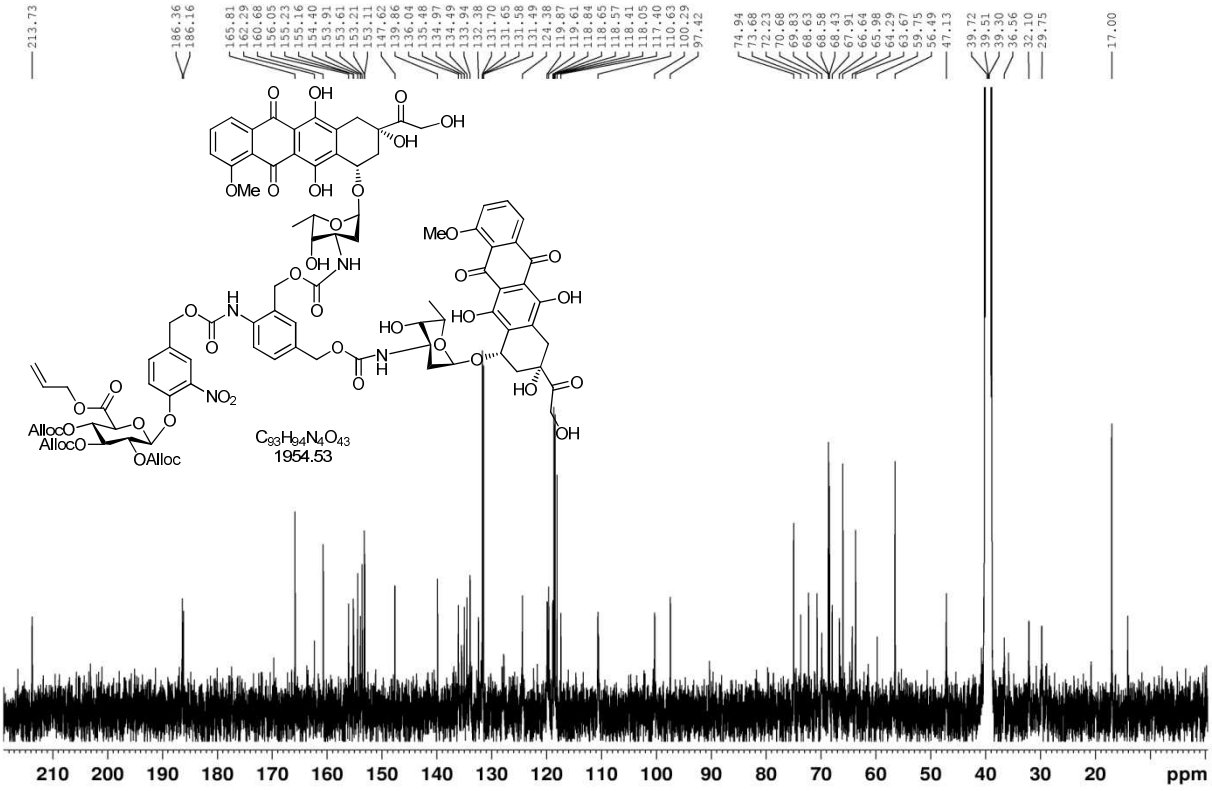
^{13}C NMR spectrum of **8**,
100 MHz, 298 K, $CDCl_3$.



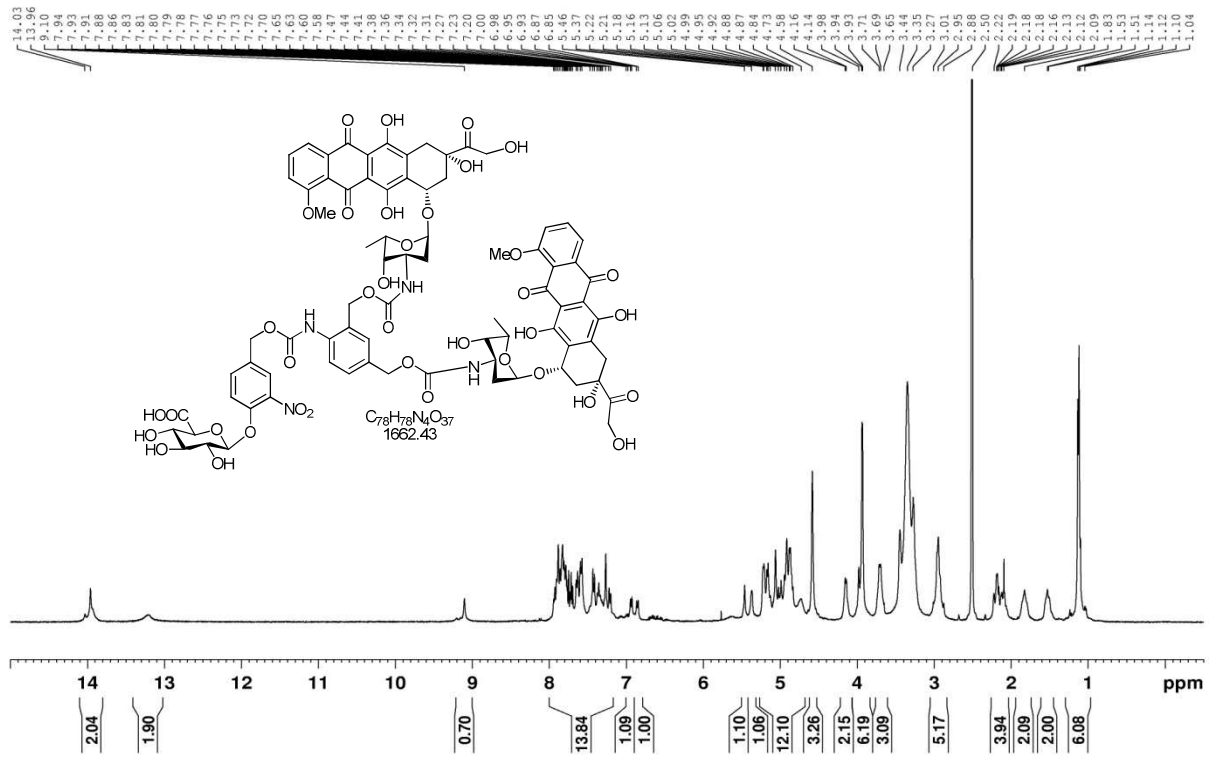
^{1}H NMR spectrum of **9**,
300 MHz, 298 K, $CDCl_3$+10% MeOD-*d4*.



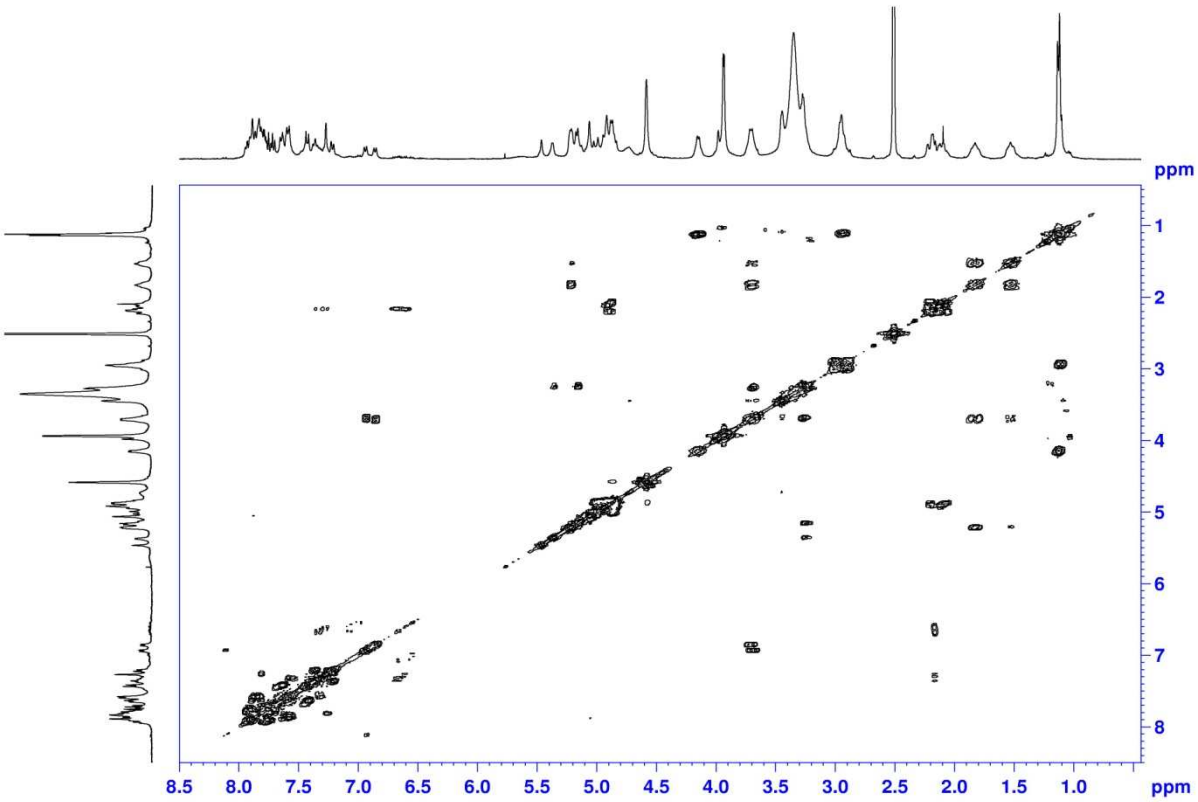
^{1}H NMR spectrum of **11**,
400 MHz, 298 K, DMSO-*d6*.



^{13}C NMR spectrum of **11**,
100 MHz, 298 K, DMSO-*d6*.

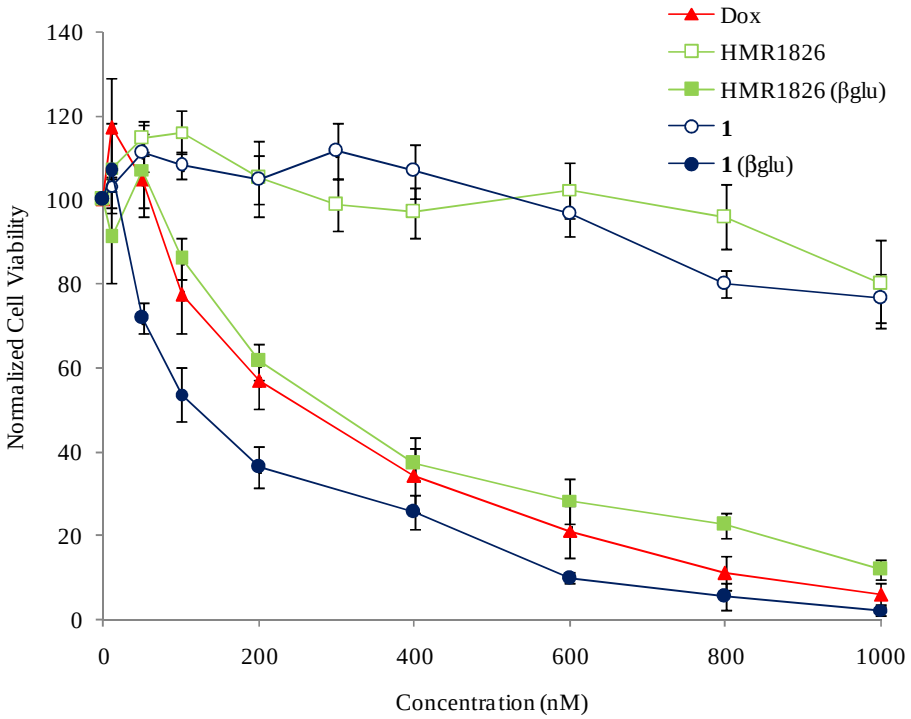


^{1}H NMR spectrum of **1**,
400 MHz, 298 K, DMSO-*d6*.



COSY NMR spectrum of **1**,
400 MHz, 298 K, DMSO-*d6*.

II. Biological Section



Cell viability of H661 cells treated 3 days with indicated compounds (from 10 to 1000 nM) compared to untreated cells. IC_{50} values are 250, 280 and 110 nM for doxorubicin, HMR 1826 and prodrug **1** respectively. Graphs represent mean +/- SEM of four independent experiments performed in triplicate.

Cell culture

H661 (non-small cells lung cancer) cells were grown in RPMI medium supplemented by 10% Fetal Bovine Serum and 1% penicillin/streptomycin in 5% CO_2 humidified atmosphere at 37°C. When indicated, cells were incubated with 40 U/ml β-glucuronidase.

Cell viability

The Cell Proliferation Kit II (XTT, Roche) was used to assess cell viability. This assay is based on the cleavage of XTT by metabolic active cells resulting in the production of an orange formazan dye quantified by spetrometry. Assays were carried out as described by the manufacturer. Briefly, 3.10^3 cells/well were plated in 100 µl of media in a 96-well plate. Cells were cultured 24 h before adding the compound at the indicated concentration in the culture media. After 3 days of treatment, 50 µl of XTT labeling mixture were added per well. Cells were further incubated for additional 3 h at 37°C before determination of absorbance at 480

nm. IC_{50} values were graphically determined using cell viability graphs. The compounds were first dissolved in DMSO at a 5 mM concentration and then diluted with the cell culture medium at the indicated concentration.

III. References

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[2] R. Erez, D. Shabat, *Org Biomol Chem* 2008, ***6***, 2669-2672.
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