Supporting Information

An Enzyme-Responsive Molecular System programmed for the Double Release of Bioactive Molecules through an Intracellular Chemical Amplification Process

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I. Chemistry Section

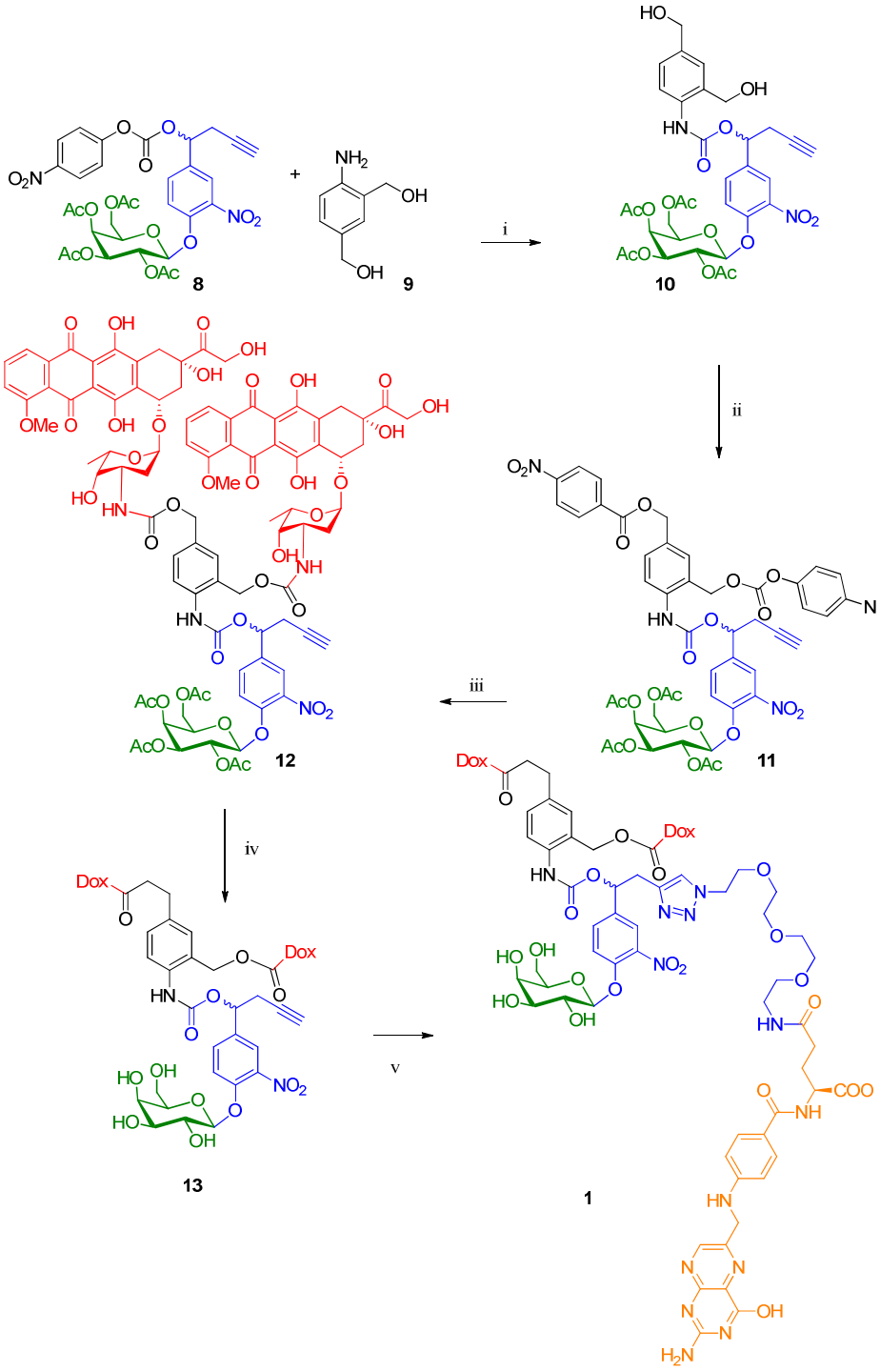
I.1. General experimental methods

All reactions were performed under a nitrogen atmosphere. 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 phosphomolibdic acid (3 g) in ethanol (100 mL) followed by heating with a heat gun. Flash columns chromatography were performed using MACHEREY-NAGEL silica gel 60 Å (15-40 µm) as the stationary phase. ^{1}H and ^{13}C NMR spectra were respectively recorded at 400 MHz and 100 MHz 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 (ppm) from low to high field and referenced to residual solvent. Coupling constants (*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 for all compounds and NMR data (500 MHz) for compound **1** were carried out by the CRMPO (Centre Régional de Mesures Physiques de l'Ouest), at the University of Rennes 1. 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}. Method 1 used a linear gradient composed of A (0.2% TFA in water) and B (CH_3CN) beginning with A/B = 80/20 v/v and reaching A/B = 0/100 v/v within 30 min. Method 2 used a linear gradient composed of A (0.1% acid formic in water) and B (CH_3CN)

beginning with A/B = 80/20 v/v and reaching A/B = 0/100 v/v within 30 min. All chromatograms were recorded at 254 nm. Semi-preparative RP-HPLC was performed with a VWR LaPrep system equipped with a spectrophotometer LaPrep P314 and a preparative pump LaPrep P110. Solvent flow 4 $mL.min^{-1}$ was applied to a semi-preparative column ACE® C18-AR (100x10 cm, 5 µm). Gradient eluent was composed of A (H_2O) and B (CH_3CN). Method: linear gradient beginning with A/B 80/20 v/v reaching A/B 0/100 v/v within 20 min. All chromatograms were recorded at 254 nm.

I.2. Synthetic overview of compound 1

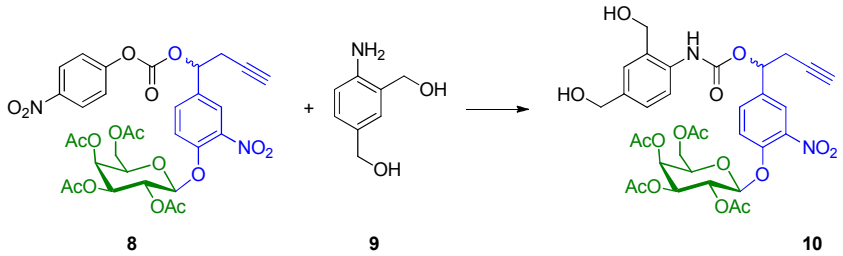
Compound **1** was prepared according to the following strategy:



Scheme S1. *Reagents and conditions:* (i) HOBt, DMF, 50°C, 3h, 98%; (ii) *para*-nitrophenyl chloroformate, pyridine, CH_2Cl_2, 0°C to RT, 3h, 89%; (iii) doxorubicin hydrochloride, Et_3N, HOBt, DMF, 3h, RT, 62%; (iv) MeONa/MeOH, -10°C, 5h, 81%; (v).Azide **14**, $CuSO_4$, sodium ascorbate, DMSO (+10% H_2O), RT, 4h30, 88% (crude precipitate).

I.3. Synthetic procedures and characterization details

Preparation of compound 10



8 **9** **10**

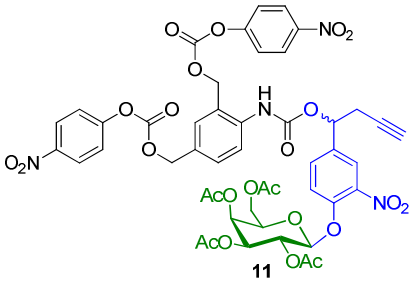
To a stirred solution of carbonate **8**[1] (100 mg, 0.142 mmol) in DMF (2 mL) was added aniline **9**[2] (43.6 mg, 2 equiv.) and hydroxybenzotriazole (HOBt) (19.2 mg, 1 equiv.). The mixture was stirred 3 hours at 50 °C. The solution was concentrated under reduced pressure and the crude material was purified by column chromatography over silica gel (gradient elution 50% to 75% ethyl acetate in petroleum ether) to afford **10** (100 mg, 0.139 mmol, 98%) as a colorless foam.

^{1}H NMR of 10 as a mixture of two diastereoisomers (400 MHz, $CDCl_3$): δ 8.19 (br s, 1H), 7.88 (s, 1H), 7.76 (d, *J* = 7.7, 1H), 7.58 (d, *J* = 8.6, 1H), 7.34 (d, *J* = 8.6, 1H), 7.18 (d, *J* = 8.3, 1H), 7.09 (s, 1H), 5.82 (t, *J* = 6.5, 1H), 5.51 (dd, *J* = 10.0, 8.0, 1H), 5.44 (d, *J* = 3.0, 1H), 5.09 (dd, *J* = 10.0, 3.0, 1H), 5.08 (d, *J* = 8.0, 1H), 4.61 (s, 2H), 4.53 (s, 2H), 4.25-4.06 (m, 3H), 3.15 (br s, 1H), 2.78 (m, 2H), 2.65 (br s, 1H), 2.17 (s, 3H), 2.10 (2s, 3H), 2.04 (m, 4H), 2.00 (s, 3H).

^{13}C NMR of 10 as a mixture of two diastereoisomers (100 MHz, $CDCl_3$): δ 170.4, 170.3, 170.2, 169.5, 152.6, 149.2, 141.0, 140.9, 136.4, 136.3, 135.1, 132.2, 132.0, 129.5, 127.6, 127.5, 123.4, 123.2, 121.0, 119.4, 100.5, 78.5, 72.9, 72.0, 71.4, 70.5, 67.8, 66.7, 64.4, 63.8, 61.3, 26.4, 20.6 (x4).

HRESI-MS: m/z 739.1961 (calcd. for $C_{33}H_{36}N_2O_{16}Na$ 739.19625 $[M+Na]^+$).

Preparation of compound 11



11

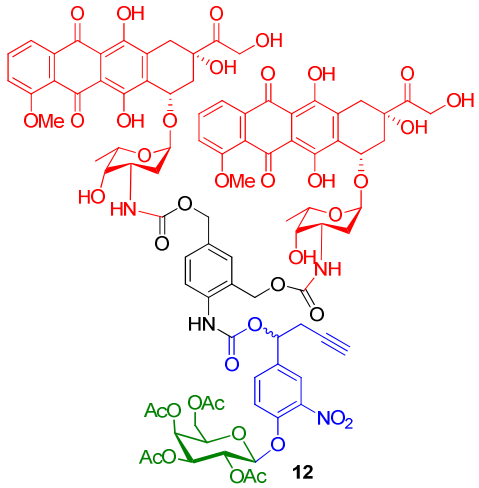
Anhydrous pyridine (151.3 µL, 1.87 mmol, 4 equiv.) was added dropwise to a cooled solution (0°C) of *p*-nitrophenyl chloroformate (376 mg, 1.87 mmol, 4 equiv.) in CH_2Cl_2 (3 mL). The mixture was stirred 20 minutes at 0°C. A solution of **10** (335 mg, 0.47 mmol, 1 equiv.) in CH_2Cl_2 (6 mL) was added and the mixture was stirred 1 hour at room temperature. The reaction was quenched with a saturated solution of NaCl and extracted three times with CH_2Cl_2. 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 (gradient elution: 0% to 1% MeOH in CH_2Cl_2) to give **11** (441 mg, 0.42 mmol, 89%) as a colorless foam.

^{1}H NMR of 11 as a mixture of two diastereoisomers (400 MHz, $CDCl_3$): δ 8.24 (m, 4H), 7.88 (s, 1H), 7.81 (m, 1H), 7.65 (br s, 1H), 7.58 (dd, *J* = 8.7, 2.1, 1H), 7.51 (m, 1H), 7.49 (dd, *J* = 8.3, 1.8, 1H), 7.35 (m, 5H), 5.86 (t, *J* = 6.4, 1H), 5.54 (dd, *J* = 10.0, 8.0, 1H), 5.46 (d, *J* = 3.4, 1H), 5.31 (s, 2H), 5.26 (s, 2H), 5.10 (m, 2H), 4.27-4.08 (m, 3H), 2.81 (m, 2H), 2.17 (s, 3H), 2.10 (2s, 3H), 2.04 (m, 4H), 2.00 (s, 3H).

^{13}C NMR of 11 as a mixture of two diastereoisomers (100 MHz, $CDCl_3$): δ 170.3, 170.2, 170.1, 169.4, 155.4, 155.1, 152.9, 152.6, 152.4, 149.3, 145.6, 145.4, 141.1 (x2), 137.0, 134.8 (x2), 132.1, 131.7, 131.1, 131.0, 125.3 (x4), 123.6, 123.2, 121.8 (x4), 119.5, 100.5, 78.3, 73.3, 72.0, 71.4, 70.5, 70.0, 67.8, 67.5, 66.7, 61.3, 26.4, 20.6 (x4).

HRESI-MS: m/z 1069.2081 (calcd. for $C_{47}H_{42}N_4O_{24}Na$ 1069.20812 $[M+Na]^+$);

Preparation of compound 12



To a solution of doxorubicin hydrochloride (119.7 mg, 0.206 mmol, 2 equiv.) in DMF (1mL) was added triethylamine (28.7 µL, 0.206 mmol, 2 equiv.) and the mixture was stirred 20 minutes at room temperature. HOBt (27.8 mg, 0.206 mmol, 2 equiv.) and a solution of **11** (108 mg, 0.103 mmol) in DMF (1 mL) were added and stirring was pursued for 3 hours at room temperature. The reaction was quenched with a saturated solution of NaCl and extracted four times with CH_2Cl_2 and one time with ethyl acetate. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure (CAUTION: temperature of the bath water $\leq$ 30°C). The resulting crude material was purified by column chromatography over silica gel (gradient elution: 0% to 4% MeOH in CH_2Cl_2) to give **12** (119 mg, 0.064 mmol, 62%) as red powder.

^{1}H NMR of 12 as a mixture of two diastereoisomers (400 MHz, DMSO-*d6*): δ 13.92-13.89 (2s, 2H), 13.17-13.16 (2s, 2H), 9.20 (br s, 1H), 7.93 (s, 1H), 7.86-7.78 (m, 4H), 7.73 (d, *J* = 8.7, 1H), 7.56 (m, 2H), 7.39 (dd, *J* = 8.8, 2.6, 1H), 7.26 (m, 2H), 7.19 (d, *J* = 8.4, 1H), 6.91 (d, *J* = 7.8, 1H), 6.82 (d, *J* = 7.8, 1H), 5,76 (m, 1H), 5.58 (d, *J* = 7.2, 1H), 5.42-5.36 (m, 3H), 5.24 (m, 4H), 4.87 (m, 8H), 4.69 (m, 2H), 4.57 (2s, 4H), 4.46 (t, *J* = 6.1, 1H), 4.11 (m, 4H), 3.92

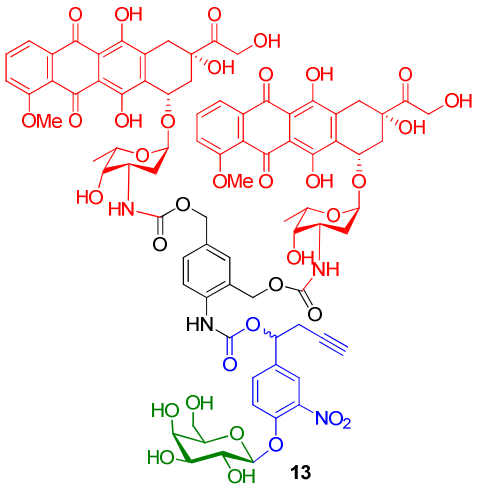
(2s, 6H), 3.69 (m, 2H), 3.44 (m, 2H), 2.97-2.81 (m, 6H), 2.15-1.95 (m, 17H), 1.82 (m, 2H), 1.52 (m, 2H), 1.12 (d, *J* = 6.1, 6H).

^{13}C NMR of 12 as a mixture of two diastereoisomers (100 MHz, DMSO-*d6*): δ 213.8 (x2), 186.3 (x2), 186.1 (x2), 169.9 (x2), 169.5, 168.9, 160.7 (x2), 156.1 (x2), 155.2, 154.4 (x2), 153.2, 147.9, 140.0, 136.0 (x2), 135.5 (x2), 134.9, 134.6, 134.5, 133.9, 132.0, 127.6 (x2), 124.7, 122.6, 119.9 (x2), 119.6, 118.8 (x2), 117.5, 110.5 (x2), 100.3 (x2), 98.5, 79.7, 75.0 (x2), 73.8 (x2), 72.2, 70.8, 69.9, 69.8, 67.9 (x4), 67.6, 67.1, 66.6 (x2), 64.7, 63.7 (x2), 61.4 (x2), 61.2, 56.5 (x2), 47.1 (x2), 36.6 (x2), 32.1 (x2), 29.7 (x2), 25.4, 20.4 (x4), 17.0 (x2).

HRESI-MS: m/z 1877.5048 (calcd. for $C_{89}H_{90}N_4O_{40}Na$ 1877.5029 $[M+Na]^+$).

m.p. dec. 215°C.

Preparation of compound 13



To a solution of **12** (156 mg, 84 µmol) in MeOH (10 mL) and CH_2Cl_2 (6 mL) cooled at -15°C, was added slowly MeONa (72.6 mg, 16 equiv.). Stirring was continued for 5 hours at -10°C and the solution was neutralized with Amberlite IRC 50 during 15 minutes and filtered through a pad of cotton. MeOH was then evaporated and the crude material was purified by

column chromatography over silica gel (gradient elution: 0% to 10% MeOH in CH_2Cl_2) to give **13** (115 mg, 68 μmol, 81%) as a red powder.

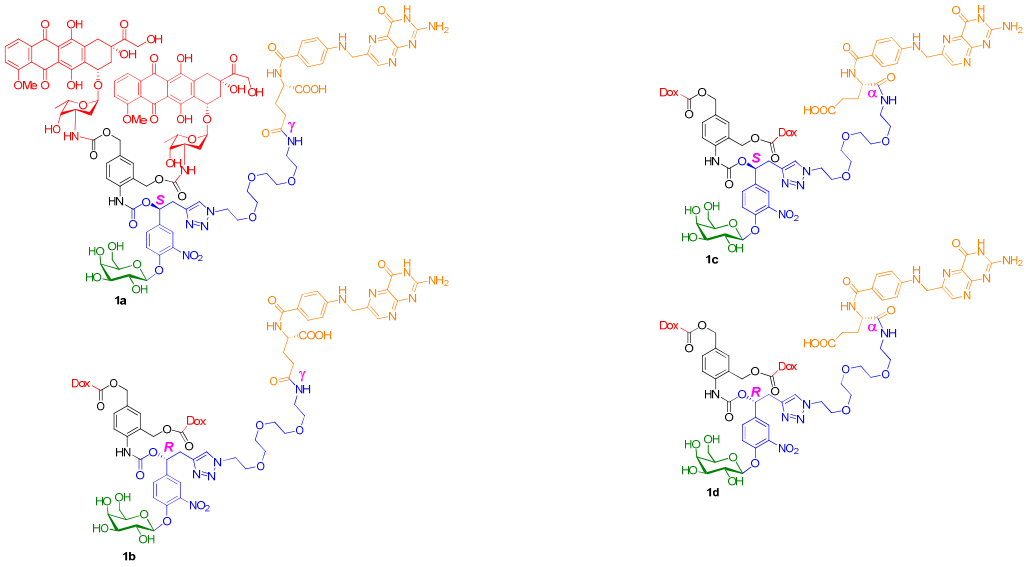
^{1}H NMR of 13 as a mixture of two diastereoisomers (400 MHz, DMSO-*d6*) δ 13.90-13.88 (2s, 2H), 13.15 (s, 2H), 9.08 (br s, 1H), 7.925-7.75 (m, 5H), 7.63 (m, 2H), 7.54 (m, 1H), 7.43 (m, 1H), 7.34 (m, 1H), 7.25 (s, 1H), 7.20 (d, *J* = 8.1, 1H), 6.93 (d, *J* = 8.1, 1H), 6.82 (d, *J* = 8.1, 1H), 5.54 (m, 2H), 5.40 (m, 2H), 5.32 (m, 2H), 5.20 (m, 2H), 5.06 (m, 2H), 4.78 (m, 8H), 4.70 (m, 2H), 4.57 (m, 3H), 4.13 (m, 3H), 3.91 (m, 6H), 3.64 (m, 6H), 3.37 (m, 7H masked by water signal), 2.91 (m, 4H), 2.22-2.02 (m, 4H), 1.81 (m, 2H), 1.51 (m, 2H), 1.12 (m, 6H).

^{13}C NMR of 13 as a mixture of two diastereoisomers (100 MHz, DMSO-*d6*): δ 213.7 (x2), 186.2 (x2), 186.0 (x2), 169.0, 160.6 (x2), 156.0 (x2), 155.2 (x2), 154.4 (x2), 153.9, 148.6, 139.8, 136.0 (x2), 135.4 (x2), 135.0 (x2), 134.4, 133.9, 130.7, 127.8 (x2), 124.3, 119.8, 118.8 (x2), 116.8, 110.6 (x2), 110.4 (x2), 109.9 (x2), 100.3 (x2), 99.7, 75.6, 75.1, 74.9, 72.7, 71.1, 69.7 (x2), 67.9, 66.6, 64.8 (x2), 63.7, 61.4 (x2), 56.4 (x4), 52.0 (x2), 47.1 (x2), 36.5 (x2), 32.0, 31.5, 29.7, 29.4, 29.0, 17.0 (x2).

HRESI-MS: m/z 1709.4601 (calcd. for $C_{81}H_{82}N_4O_{36}Na$ 1709.4601 $[M+Na]^+$).

m.p. dec. 185°C.

Preparation of compound 1 as a mixture of four isomers



To a solution of alkyne derivative **13** (30 mg, 18 µmol) and azide **4a,b**[1] (11.4 mg, 1 equiv.) in DMSO (1 mL) containing 10% of water, L-ascorbic acid sodium salt (7 mg, 2 equiv.) and $CuSO_4$ (2.7 mg, 0.6 equiv.) were added. The solution was stirred at room temperature and the reaction was monitored by analytical RP-HPLC using method 1. A portion of $CuSO_4$ (1.3 mg, 0.3 equiv.) was added after one hour. Then, the mixture was stirred for an extra hour before adding another portion of azide **4a,b** (5.7 mg, 0.5 equiv.) with $CuSO_4$ (1.3 mg, 0.3 equiv.) and L-ascorbic acid sodium salt (3.5 mg, 1 equiv.). Disappearance of azide **4a,b** was observed by HPLC after 4h30. Then a solution of ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA.2Na.2H_2O) (25 mg, 4 equiv.) in 0.2 M, pH 7 phosphate buffer (1 mL) was added dropwise at 0°C and the solution was stirred at room temperature during 2 hours for complete decomplexation of copper. The reaction mixture was diluted with MeOH (2 mL) and poured into a cold solution of Et_2O. The red precipitate was filtered and washed with MeOH and H_2O to give the targeted compound **1** (33 mg, 88%) as a red powder. Compound **1** was obtained as a mixture of four isomers (**1a**, **1b**, **1c** and **1d**). After precipitation, the purity of **1** was >80% and ^{1}H NMR and ^{13}C NMR data were recorded without further purification.

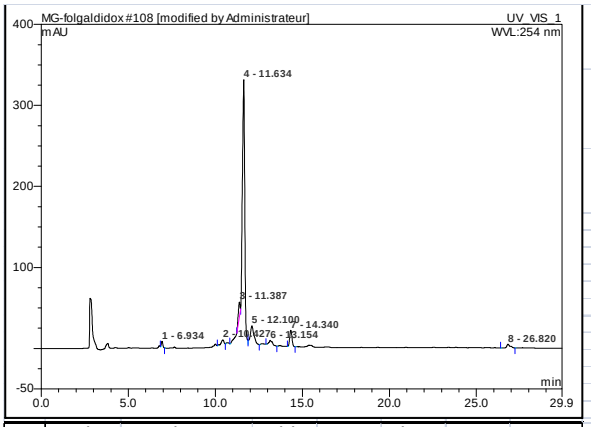
For biological evaluations, **1** was further purified by semi-preparative RP-HPLC. Crude sample was dissolved in DMSO for injection (1 mL). **1** was obtained as a mixture of four isomers (**1a**, **1b**, **1c** and **1d**) with a purity of 94%. In the course of this process 1.6 mg of **1** were recovered from 3 mg of starting material.

^{1}H NMR of 1 as a mixture of four diastereoisomers (500 MHz, DMSO-*d6*) δ 13.94 (bs, 2H), 13.21 (bs, 2H), 8.63 (s, 1H), 7.85-7.76 (m, 8H), 7.67-7.56 (m, 5H), 7.37 (d, *J* = 8.1, 1H), 7.26 (m, 2H), 7.18 (d, *J* = 8.1, 1H), 6.91 (m, 3H), 6.80 (m, 1H), 6.64 (m, 2H), 5.91 (m, 1H), 5.45 (m, 2H), 5.23 (m, 3H), 5.02-4.85 (m, 10H), 4.72 (m, 3H), 4.58 (m, 5H), 4.48 (m, 2H), 4.40-4.34 (m, 3H), 4.14 (m, 3H), 3.93 (7H), 3.69 (m, 6H), 3.63 (m, 2H), 3.55 (m, 4H), 3.34 (m, 15H masked by water signal), 2.96 (m, 4H), 2.24-2.09 (m, 6H), 2.02-1.95 (m, 2H), 1.91-1.81 (m, 3H), 1.57 (m, 2H), 1.13-1.12 (2s, 6H).

^{13}C NMR of 1 as a mixture of four diastereoisomers (125 MHz, DMSO-*d6*): δ 213.6, 213.5, 186.3, 186.1, 174.1, 171.7, 166.1, 160.8, 160.6, 156.0, 155.1, 154.3, 153.2, 151.7, 150.6, 149.1, 148.5, 148.4, 141.7, 139.6, 136.0, 134.6, 134.5, 133.9, 133.6, 131.9, 128.9, 127.8, 127.5, 123.3, 122.5, 121.3, 119.9, 119.5, 118.8, 116.8, 111.1, 111.0, 110.6, 110.5, 100.9, 100.2, 100.1, 75.7, 74.9 (x2), 73.6, 73.5, 73.2, 69.9, 69.7, 69.4, 69.1, 68.9, 68.8, 68.7, 67.9, 66.5, 63.5, 60.2, 56.4, 52.6, 49.2, 47.0, 45.8, 38.4, 36.6, 32.0, 31.9, 30.6, 29.6, 27.0, 16.9 (x2).

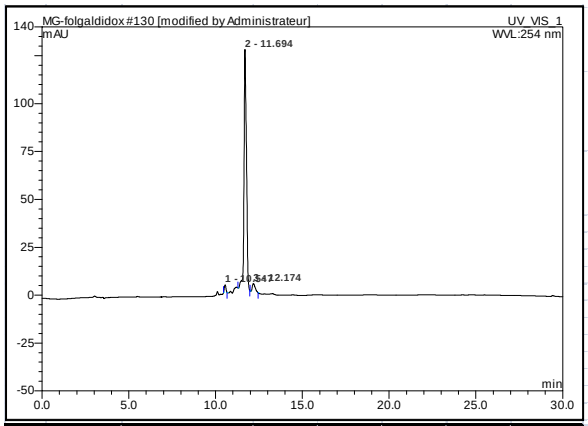
HRESI-MS: m/z (z = 2) 1162.8616 (calcd. for $C_{108}H_{115}N_{15}O_{44}$ 1162.86166 $[M-2H]^{2-}$).

HPLC chromatograms (method 1):



No.	Ret.Time	Peak Name	Height	Area	Rel.Area	Amount	Type
	min		mAU	mAU*min	%		
1	6,93	n.a.	7,295	0,706	0,91	n.a.	BMB
2	10,43	n.a.	5,575	0,898	1,16	n.a.	BMB
3	11,39	n.a.	16,654	2,058	2,65	n.a.	Ru
4	11,63	n.a.	326,410	63,123	81,18	n.a.	BM
5	12,10	n.a.	23,019	5,663	7,28	n.a.	MB
6	13,15	n.a.	5,698	1,284	1,65	n.a.	BMB
7	14,34	n.a.	19,961	3,072	3,95	n.a.	BMB
8	26,82	n.a.	4,476	0,947	1,22	n.a.	BMB
Total:			409,089	77,753	100,00	0,000	

HPLC chromatogram of **1** after precipitation (r.t. 11.63 min.)



No.	Ret.Time	Peak Name	Height	Area	Rel.Area	Amount	Type
	min		mAU	mAU*min	%		
1	10,55	n.a.	3,970	0,422	1,96	n.a.	BMB*
2	11,69	n.a.	125,210	20,356	94,45	n.a.	BMB*
3	12,17	n.a.	4,235	0,775	3,60	n.a.	BMB*
Total:			133,414	21,553	100,00	0,000	

HPLC chromatogram of **1** after semi-preparative purification (r.t. 11.69 min.)

I.4. Stability and enzymatic cleavage

Targeting device **1** (0.05 mg, 0.021 µmol) was incubated at 37°C either in 20 mM phosphate buffer at pH 7.0 (1 mL) containing 2% of DMSO or in cell culture medium supplemented by 10% fetal bovine serum (Lonza). Stability was monitored by analytical HPLC using method 1. HPLC analysis showed no detectable degradation of compound **1** during 24 hours under these conditions.

Enzymatic hydrolysis was carried out with commercial β-galactosidase from *Escherichia coli* E.C. 3.2.1.23 (768 units/mg protein (biuret), suspension in 50% glycerol, 10 mM Tris buffer salts and 10 mM magnesium chloride, pH 7.3). The targeting device **1** (0.05 mg, 0.021 µmol) was incubated at 37°C with the enzyme (40 $U.\mu mol^{-1}$) in a solution of 20 mM phosphate buffer at pH 7.0 (1 mL) containing 2% of DMSO. Hydrolysis was monitored by analytical HPLC using method 2.

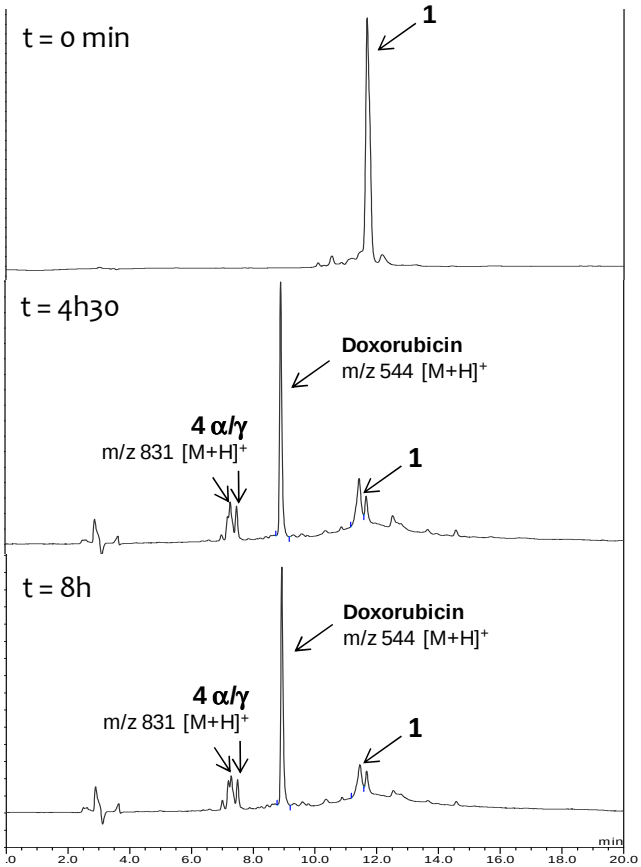


Figure S1. HPLC chromatograms of enzymatic hydrolysis of prodrug **1** with β-galactosidase

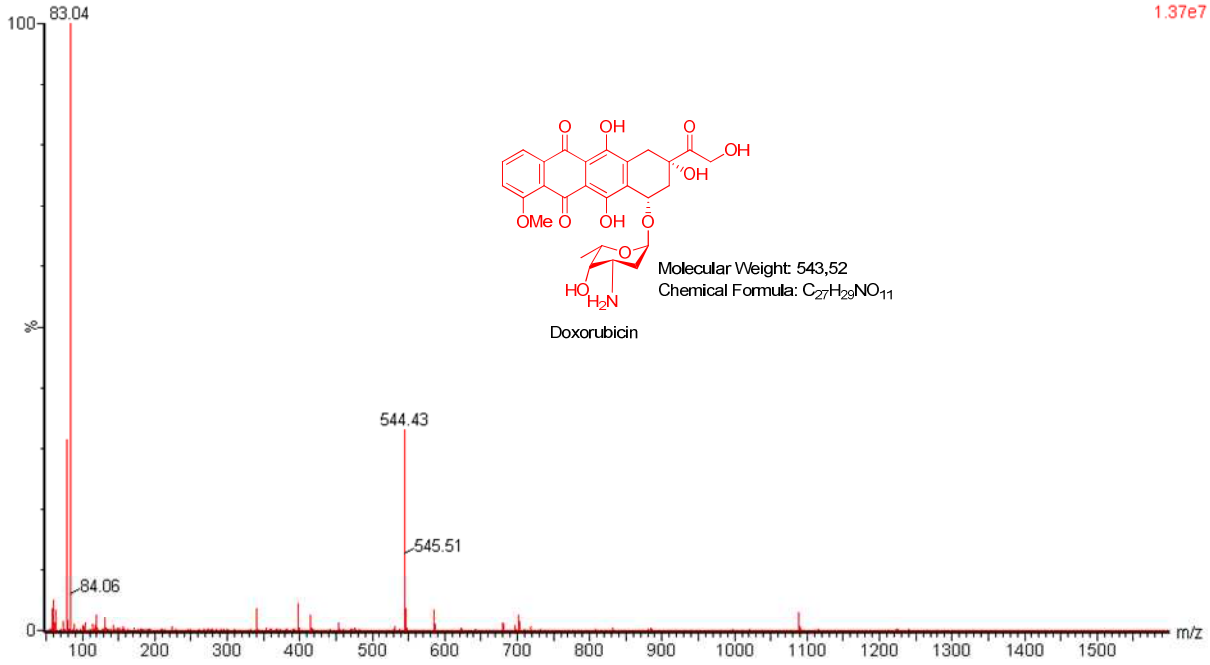


Figure S2. LRESI-SM spectrum of doxorubicin

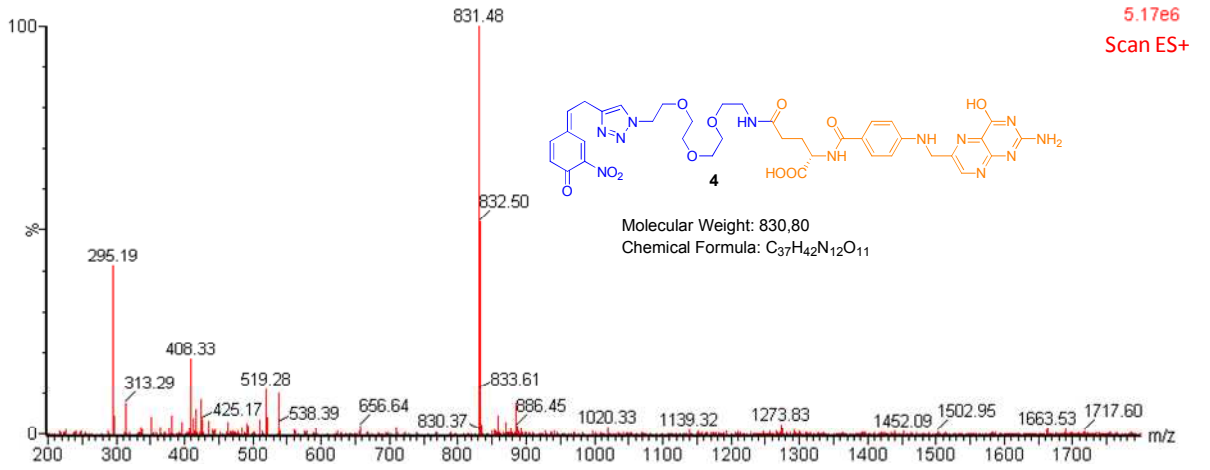
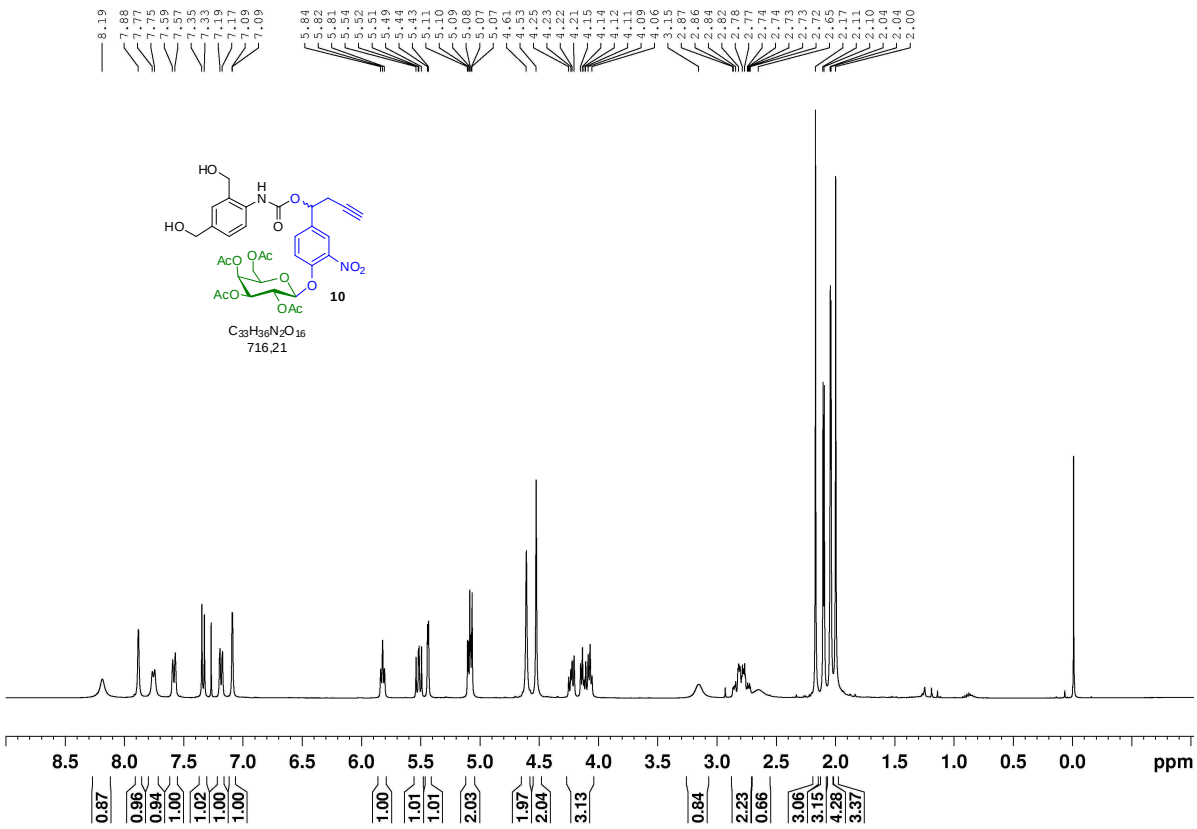
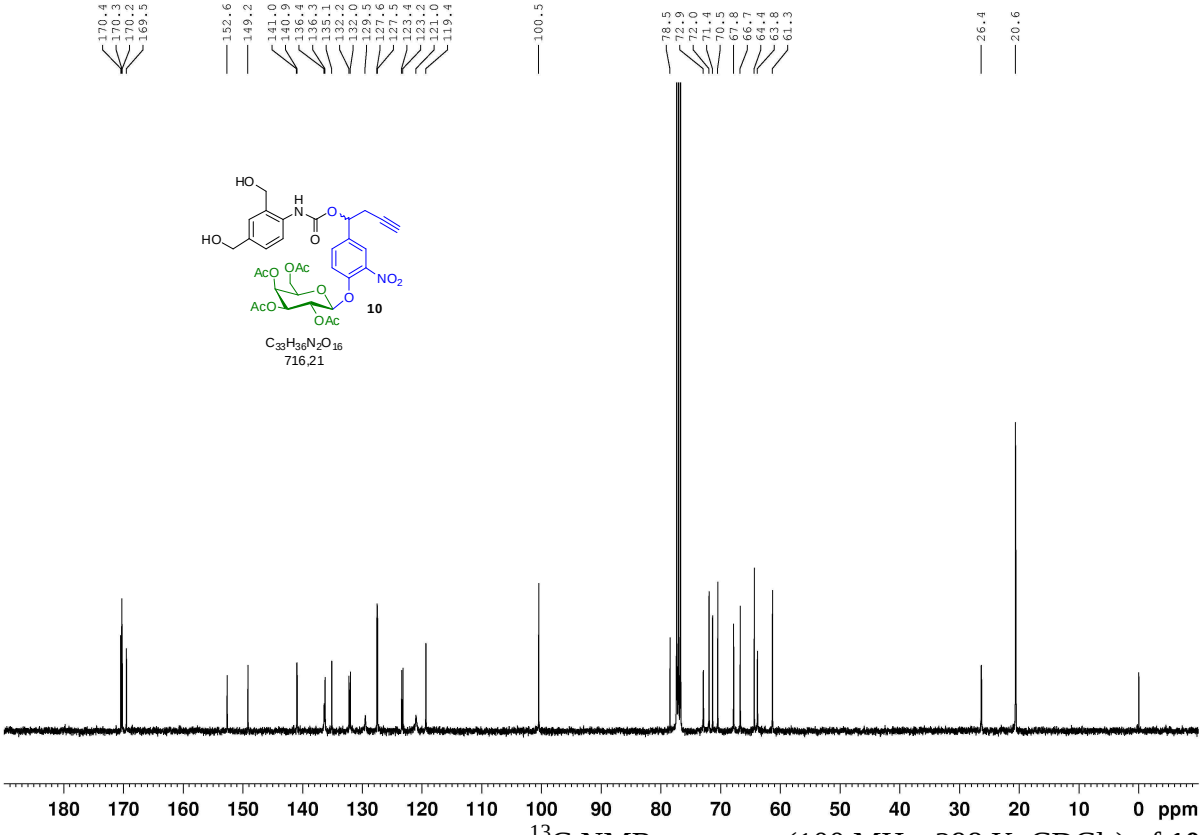


Figure S3. LRESI-SM spectrum of intermediate **4**

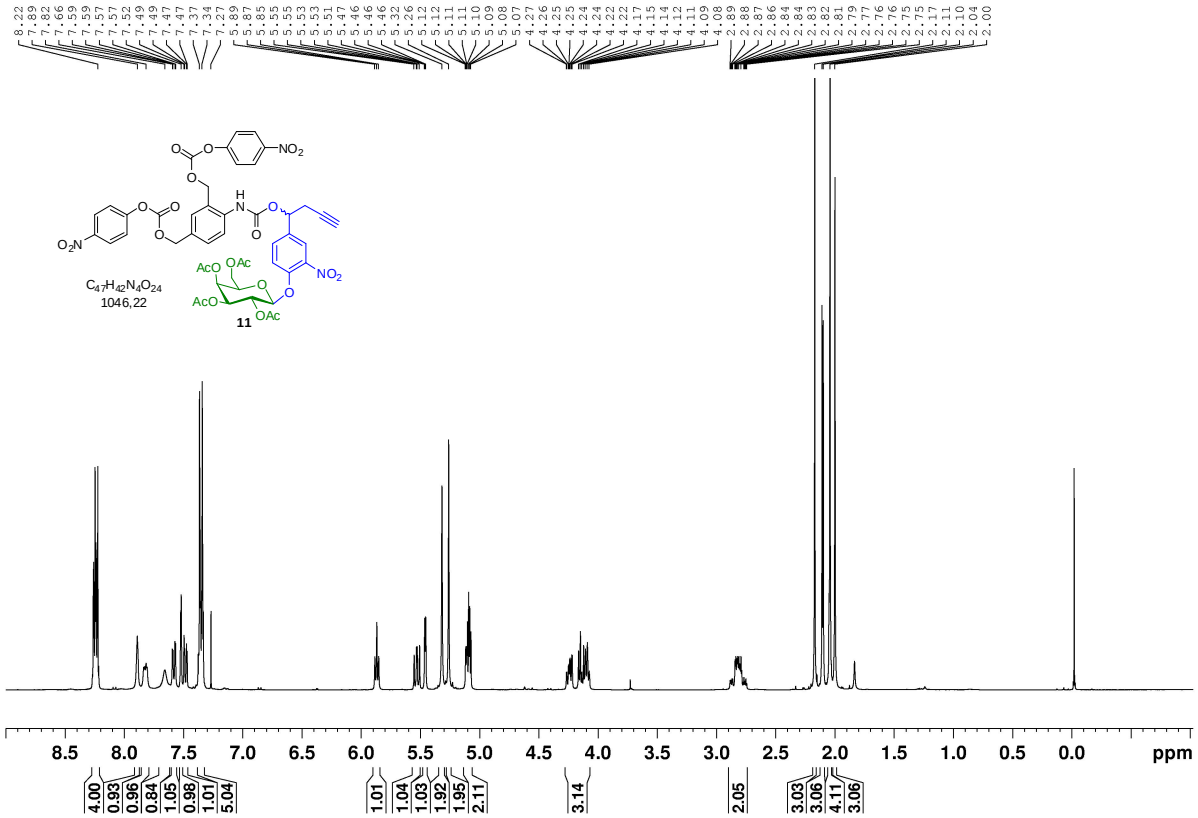
I.5. ^{1}H NMR and ^{13}C NMR plots



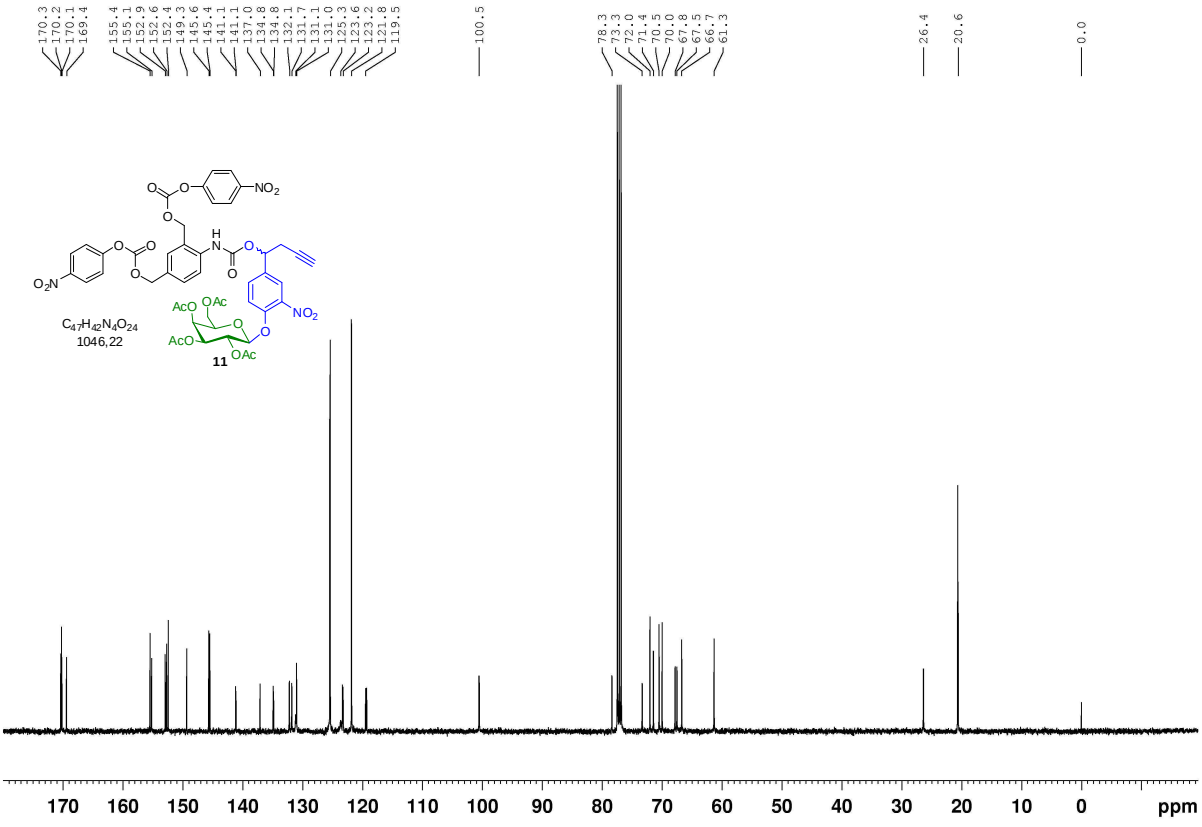
^{1}H NMR spectrum (400 MHz, 298 K, $CDCl_3$) of **10**.



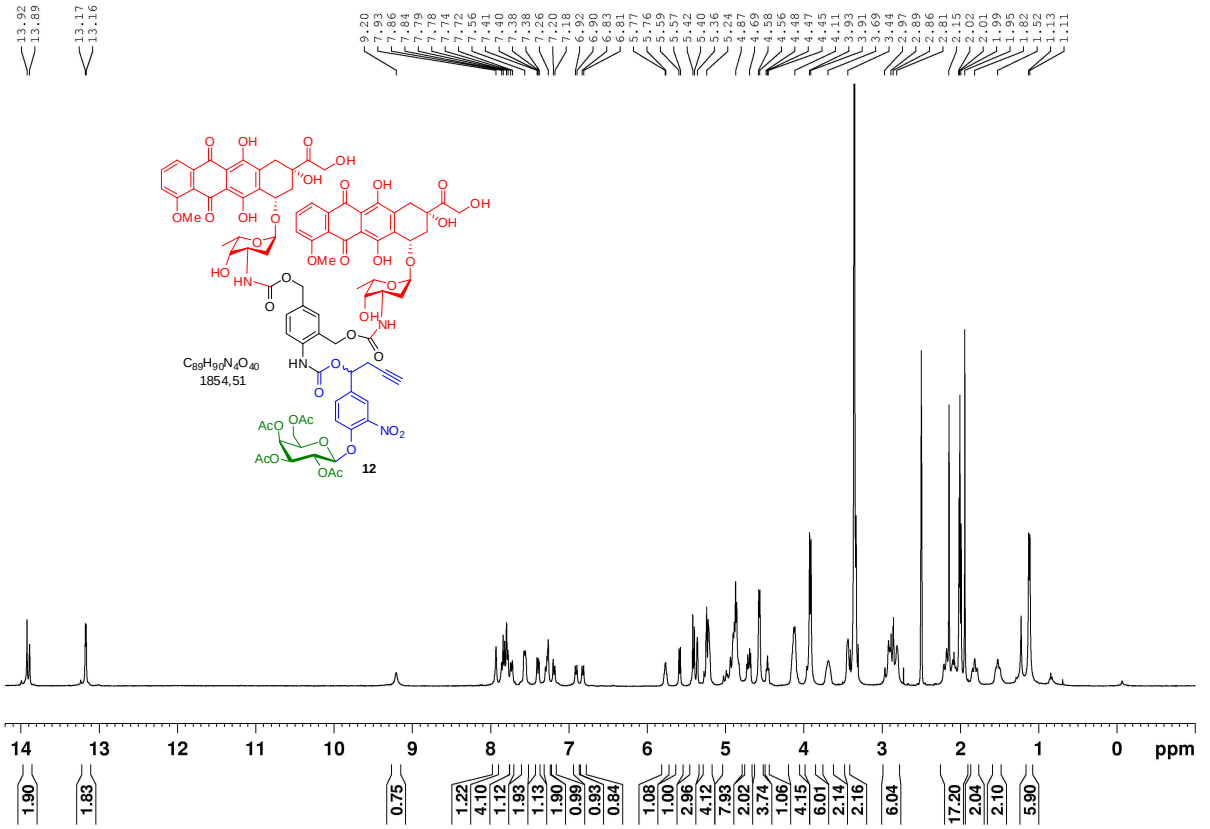
^{13}C NMR spectrum (100 MHz, 298 K, $CDCl_3$) of **10**.



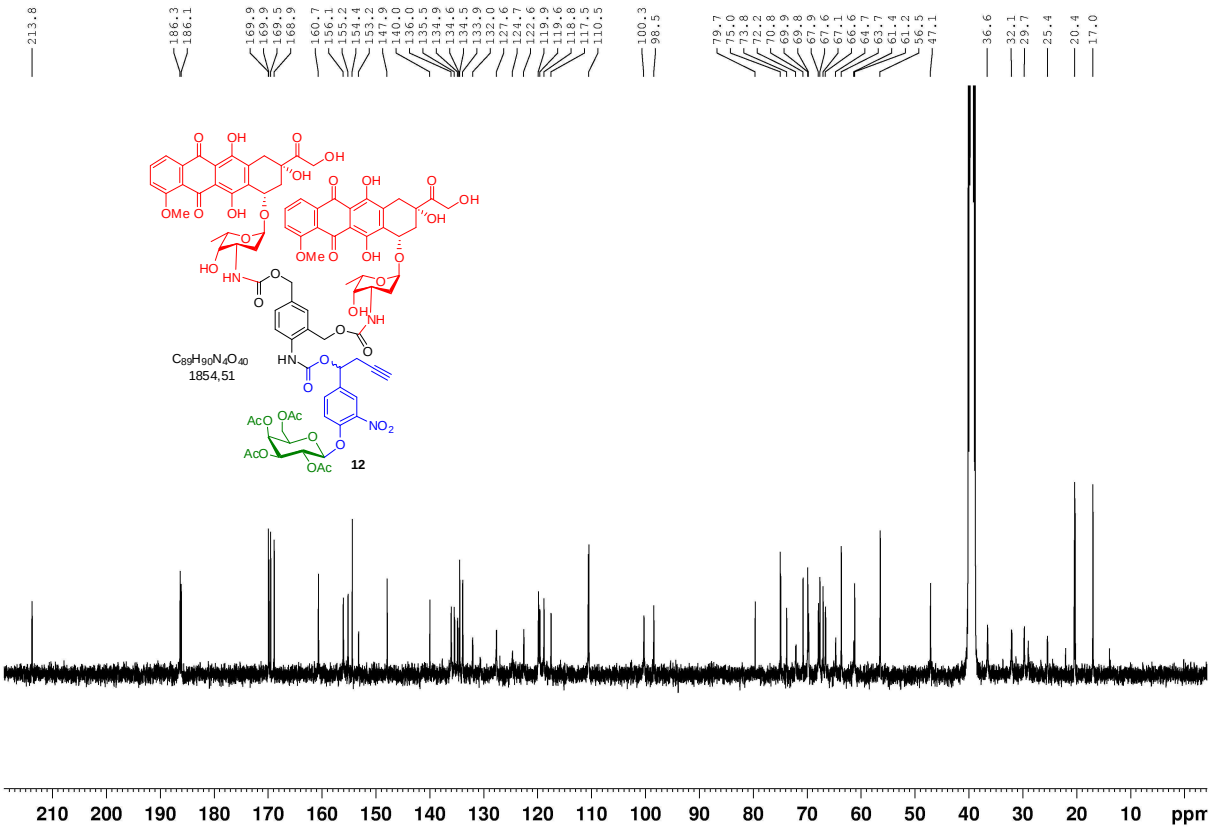
^{1}H NMR spectrum (400 MHz, 298 K, $CDCl_3$) of **11**.



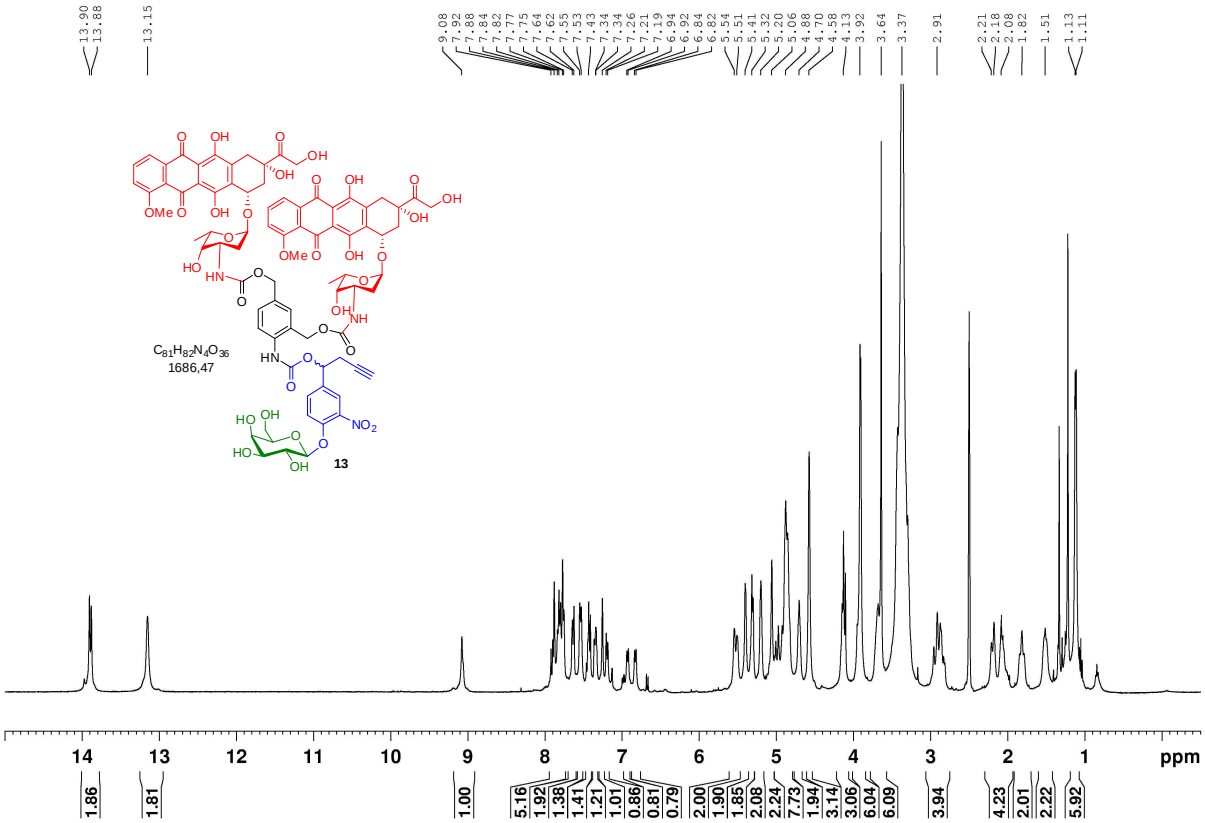
^{13}C NMR spectrum (100 MHz, 298 K, $CDCl_3$) of **11**.



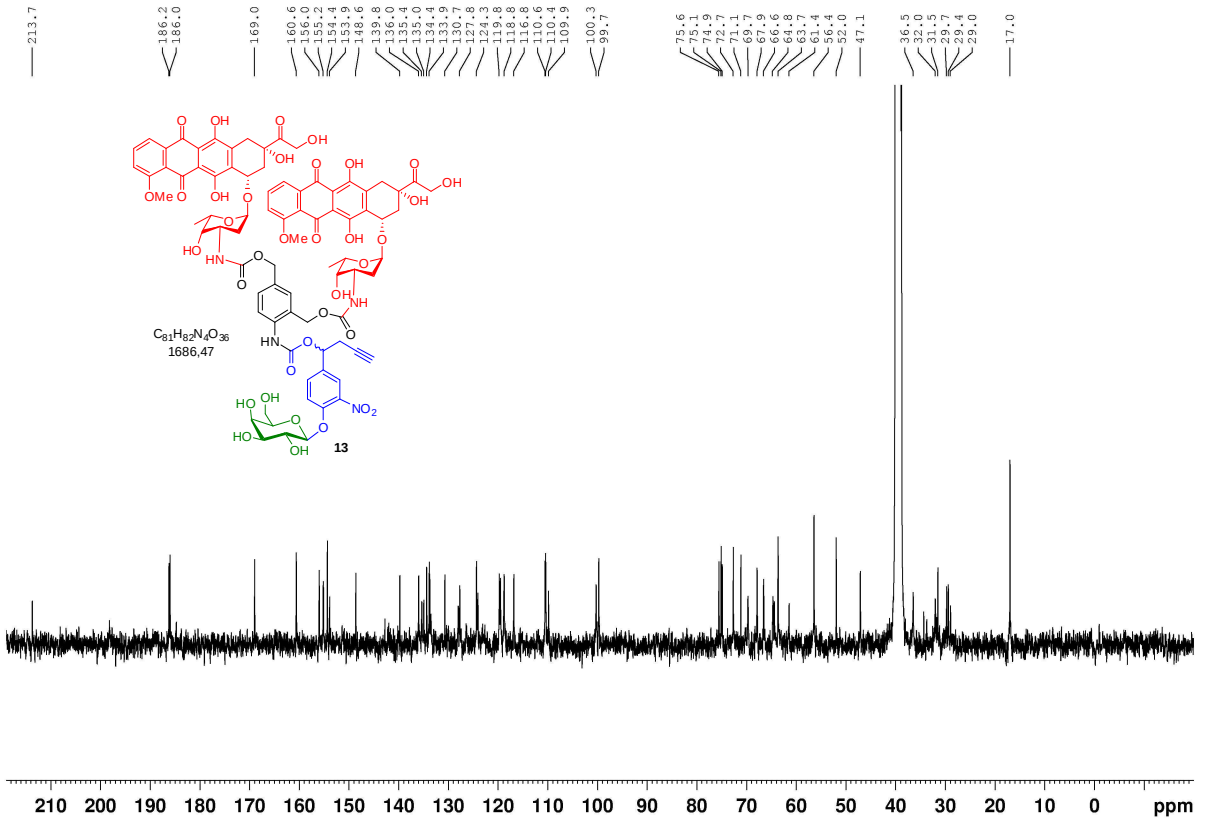
^{1}H NMR spectrum (400 MHz, 298 K, DMSO-d_6) of **12**.



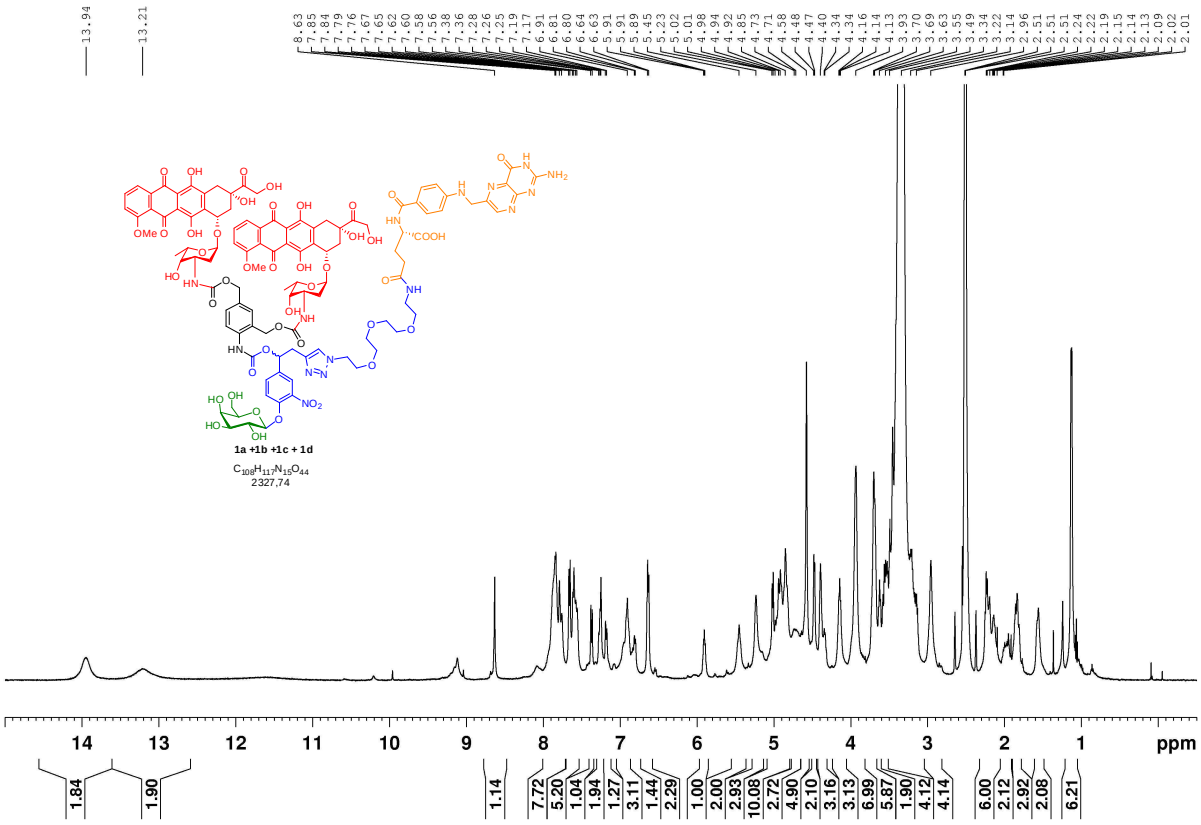
^{13}C NMR spectrum (100 MHz, 298 K, DMSO-d_6) of **12**.



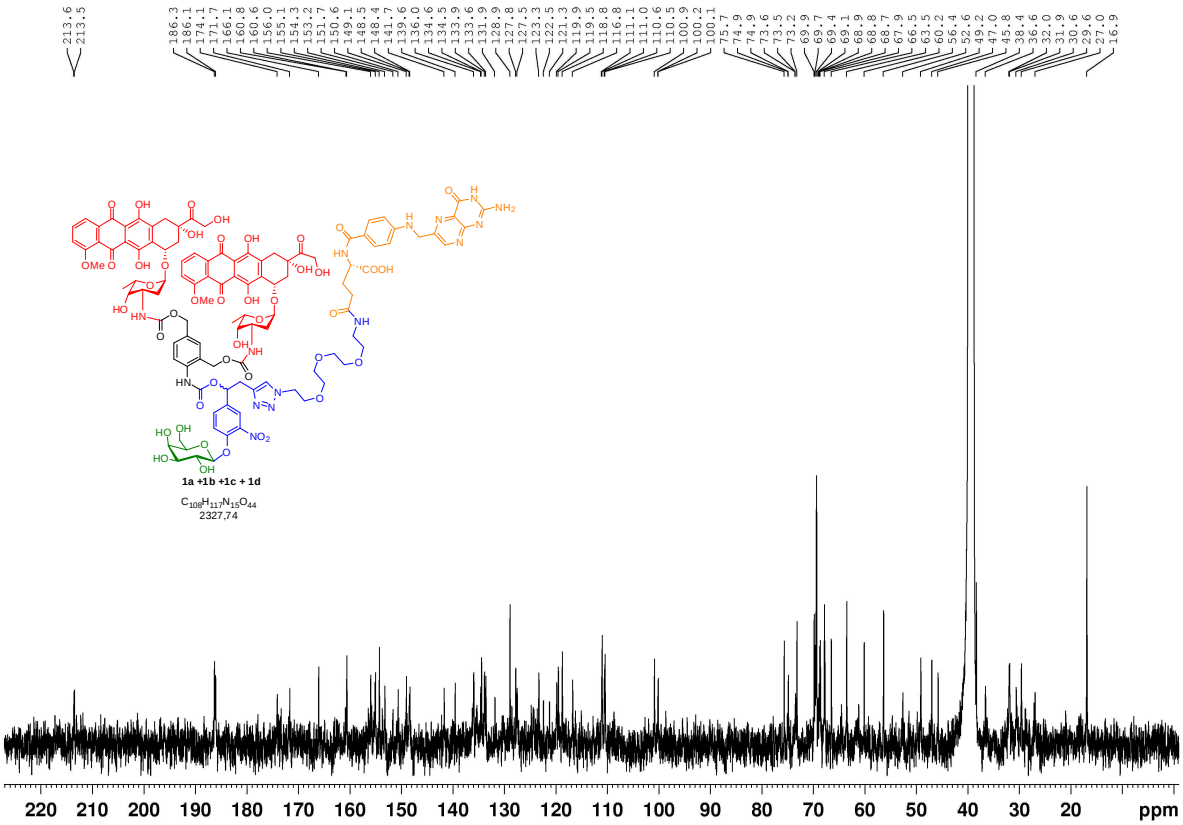
^{1}H NMR spectrum (400 MHz, 298 K, DMSO-d_6) of **13**.



^{13}C NMR spectrum (100 MHz, 298 K, DMSO-d_6) of **13**.



^{1}H NMR spectrum (500 MHz, 298 K, DMSO-d_6) of **1**.



^{13}C NMR spectrum (125 MHz, 298 K, DMSO-d_6) of **1**.

II. Biological Section

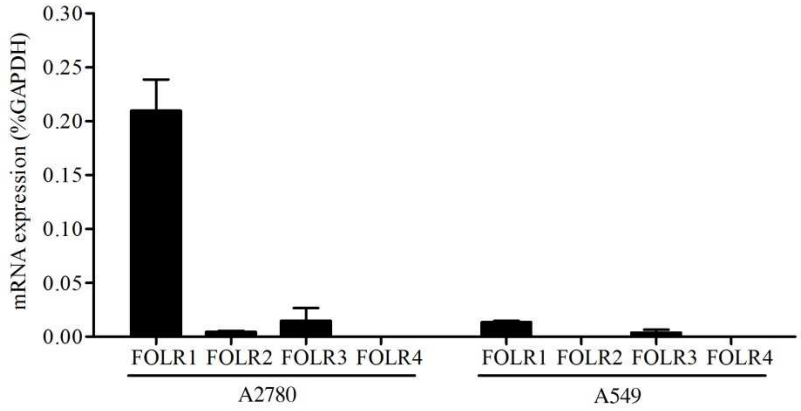
II.1. Cell culture

A2780 (human ovarian carcinoma) and A549 (human lung carcinoma) cells were grown in RPMI 1640 (Invitrogen) supplemented by 10% fetal bovine serum (Lonza) and 1% penicillin/streptomycin in a humidified incubator at 37°C and 5% CO_2. For experiments, cells were transfered in RPMI 1640 without folic acid medium (Invitrogen) supplemented by 10% fetal bovine serum (Lonza) and 1% penicillin/streptomycin in a humidified incubator at 37°C and 5% CO_2.

II.2. Real-Time quantitative RT-PCR

Two micrograms of total RNA isolated using SV Total RNA isolation kit (Promega) were converted in cDNA with SuperScript II (Invitrogen) in accordance with the instructions of the manufacturer. FOLR1, FOLR2, FOLR3 and FOLR4 gene expressions were assessed relative to glyceraldehyde-3-phosphate dehydrogenase GADPH by quantitative real-time PCR with the GeneAmp 7000 Sequence Detection System and SYBRGreen chemistry (Applied Biosystems). Human GAPDH, FOLR1, FOLR2, FOLR3 and FOLR4 sequence primers are shown in the table below. Sensitivity and specificity of each primer couple were checked. Experiments were performed 6 times in triplicates.

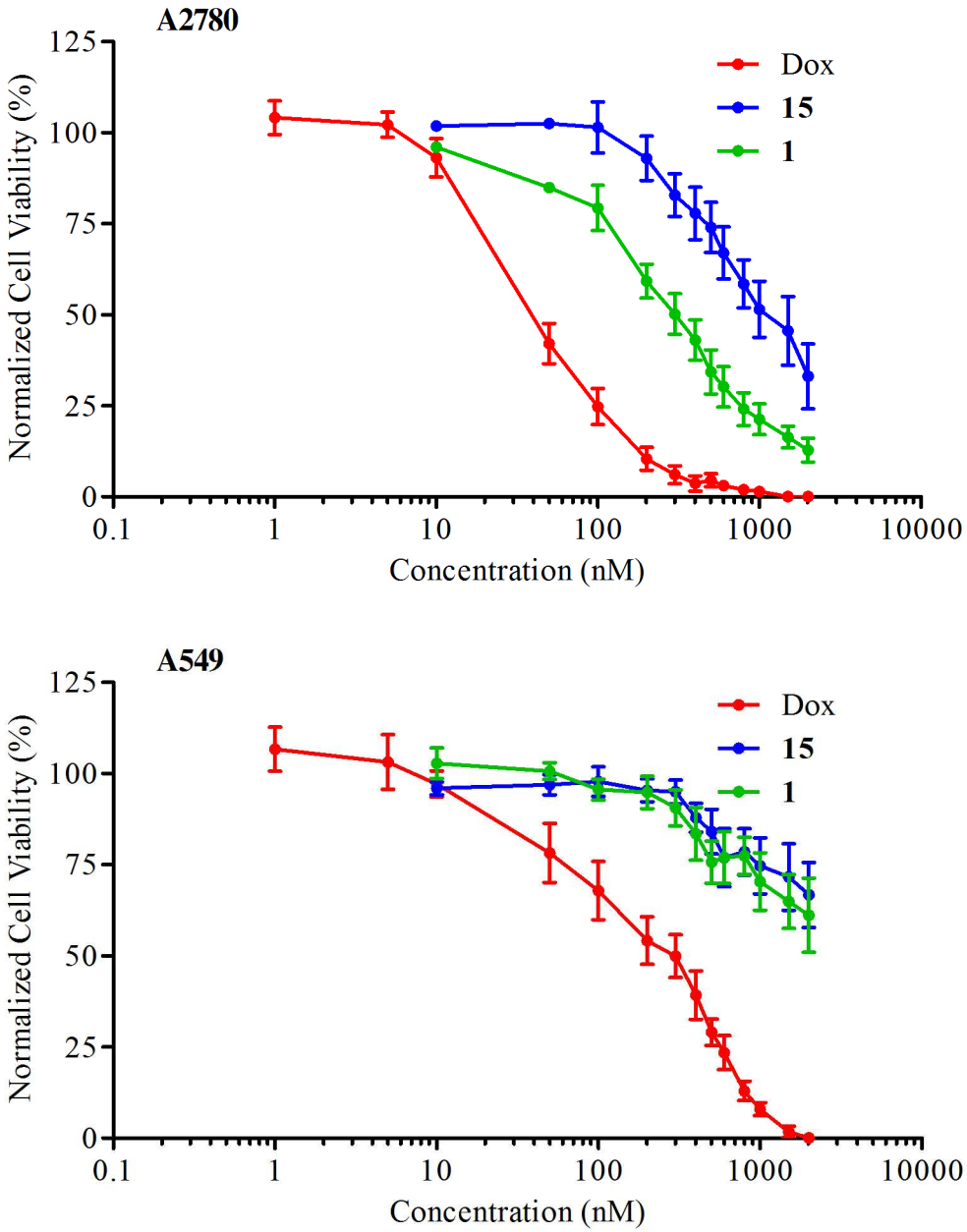
	Forward (5'>3')	***Reverse (5'>3')***
GADPH	*TGCACCACCAACTGCTTAGC*	*GGCATGGACTGTGGTCATGAG*
FOLR1	*AGCACCACAAGGAAAAGCCAGG*	*GTGCCATCTCTCCACAGTGGTT*
FOLR2	*CCACTTCATCCAGGACACCTGT*	*CATCCAGGAAGCGTTCTTTGCG*
FOLR3	*CTACACCTGCAAAAGCAACTGGC*	*GGAAGTAGGACTCAAAGGTGCTG*
FOLR4	*TGTCGGAAGCACTTCATCCAGG*	*CGGCACATTCACAACTCGCTCT*



II.3. 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 spectrophotometry. Assays were carried out essentially as described by the manufacturer. Briefly, $1x10^3$ A549 cells/well or $2x10^3$ A2780 cells/well were plated in a 96-well plate. Cells were cultured for 24 h before adding the compounds at the indicated concentration in the culture media. After 4 days of treatment, 25 µl of the XTT labeling mixture were added per well. Cells were then incubated for additional 4 h at 37°C

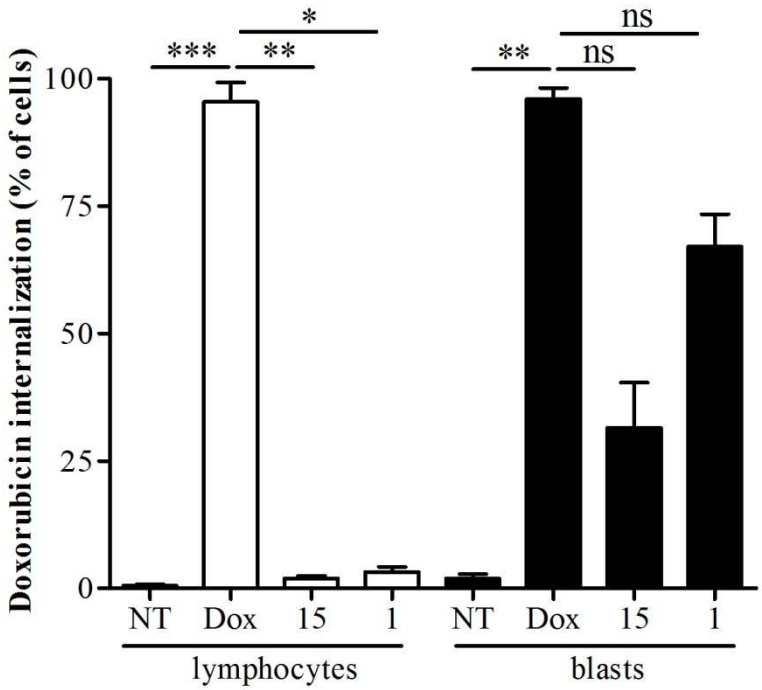
and 5% CO_2 before determination of the absorbance at 490 nm. IC_{50} values were graphically determined. Experiments were performed between 7 to 11 times.



II.4. Mononuclear cell preparation and flow cytometry assay

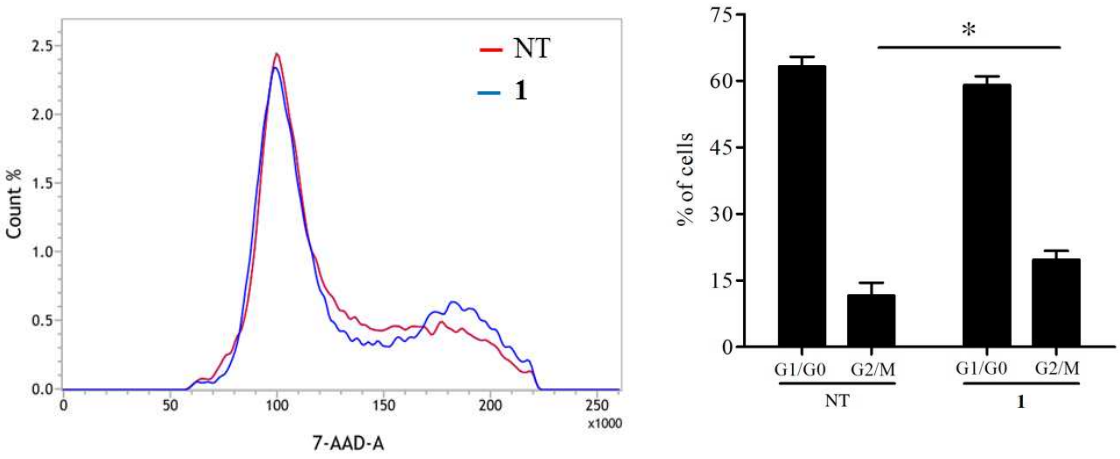
Blood and bone marrow samples were obtained from 6 AML patients with a high FOLR expression level. All patients gave a written informed consent in accordance with the Declaration of Helsinki and the protocol was accepted by the IRB of the CIC 0802. All these samples were collected at diagnostic, before treatment initiation. Bone marrow or blood cells

of 6 AML patients were diluted in serum-free medium and layered over Ficoll-Hypaque (Sigma-Aldrich) solution. After 20 min centrifugation at 2500 rpm without brakes, mononuclear cells are removed from the interface and washed twice with PBS before dilution in folate-free RPMI-1640 supplemented by 10% human serum for assay. $5x10^5$ cells were transferred to V-bottomed plates and incubated 3 h with indicated compounds at 10µM. Cells were then washed twice in ice-cold PBS-4% BSA and stained for 30 min at 4°C with CD3-FITC and CD34-APC (BD Biosciences). After two additional washes, cells were suspended in 7-aminoactinomycinD-containing PBS, a cell viability marker (BD Biosciences). Live cells initially gated by forward and side scatter were analyzed for CD3 and CD34 expression and doxorubicin internalization using FACS CantoII machine and FACS DivaII software (BD Biosciences). Anti-isotype antibodies (BD Biosciences) were used in parallel, for specificity control. One-way ANOVA and Dunns post-tests were performed for statistical analysis. Doxorubicin treatments were used as reference.



II.5. Cell cycle analysis

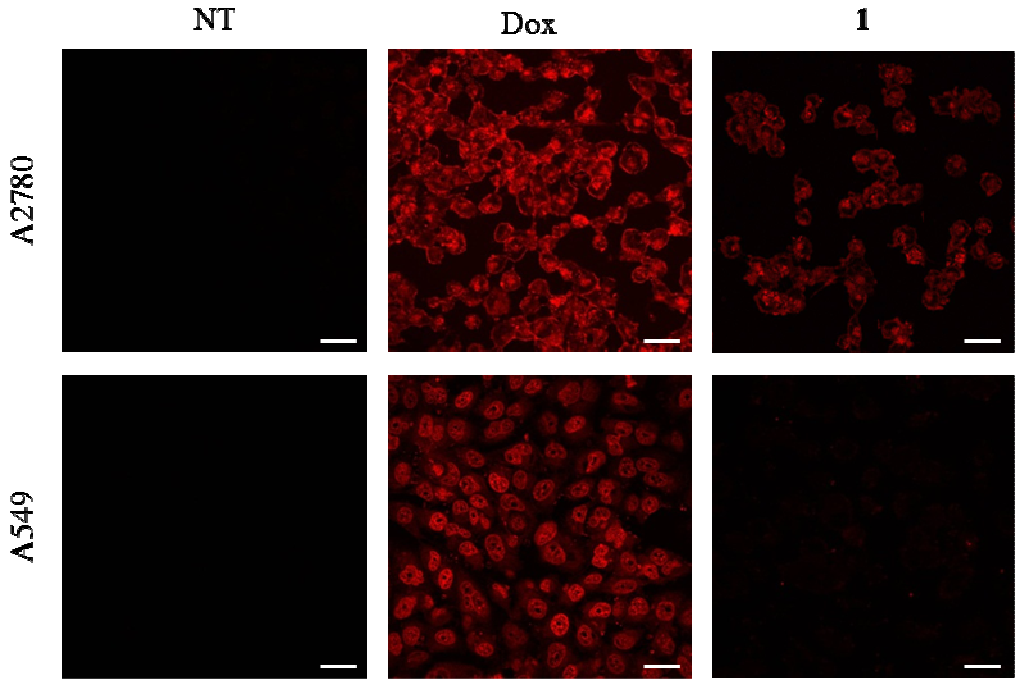
A2780 cells were seeded in RPMI 1640 without folic acid medium supplemented with 10% fetal bovine serum at a density of 2.5×10^5 cells per 60mm diameter dishes. After 24 h of growth, cells were treated with 0.5 µM of **1** for additional 4 days. Cells were then washed twice with PBS and collected by trypsinization. After two washes with PBS, cells were fixed in 70% EtOH for 2 h 30 at 4°C. Cells were then resuspended in PBS and incubated with 100 µg/ml RNAse A (Sigma) for 30min at 37°C. 10µg/ml of 7-AAD (BD Bioscience) were added and cells were analyzed by flow cytometry (FACS Verse BD Bioscience) for total DNA content based on 7-amino-actinomycin D fluorescence detected in the far red range of the spectrum (650 nm long-pass filter). Thirty thousand cells were analyzed per condition. Representative of three independent experiments. Two-way ANOVA and Bonferroni post-tests was performed for statistical analysis



II.6. Doxorubicin internalization analysis by confocal microscopy

A2780 and A549 cells were plated at a density of 2×10^5 or 1×10^5 cells/well respectively in 12-well plate on glass coverslips in RPMI 1640 without folic acid medium supplemented with 10% fetal bovine. After 24 h, cells were treated with 10 µM doxorubicin or **1** for additional 1 h or 3 h respectively. Cells were then washed twice with PBS, fixed 20 min with 3.7% formaldehyde and washed twice again with PBS. Samples were mounted in Mowiol medium

prior to observation with a confocal microscope (FV 1000, Olympus IX-81). The excitation wavelength of 488nm was used for acquisition of in red channel (590 nm) for doxorubicin auto-fluorescence. Images are representative of 2 independent experiments. Scale bar: 50 µm.



III. References

[1] a) M. Thomas, J. Clarhaut, P.-O. Strale, I. Tranoy-Opalinski, J. Roche, S. Papot, *ChemMedChem* **2011**, *6*, 1006-1010; b) T. legigan, J. Clarhaut, I. Tranoy-Opalinski, A. Monvoisin, B. Renoux, M. Thomas, A. Le Pape, S. Lerondel, S. Papot, *Angew. Chem. Int. Ed.* **2012**, *46*, 11606-11610.

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