Supporting Information

A Dentritic β-Galactosidase-Responsive Folate-Monomethylauristatin E conjugate

Jérôme Alsarraf,[a] Elodie Péraudeau,[b, d] Pauline Poinot,[c] Isabelle Tranoy-Opalinski,[a] Jonathan Clarhaut,[a,d] Brigitte Renoux*[a] and Sébastien Papot*[a]

[a] *Université de Poitiers, UMR-CNRS 7285, Institut de Chimie des Milieux et des Matériaux de Poitiers, groupe « Systèmes Moléculaires Programmés », 4 rue Michel Brunet, TSA 51106, 86073 Poitiers, France.*

[b] *Université de Poitiers, CNRS ERL 7368, 1 rue Georges Bonnet, TSA 51106, 86073 Poitiers, France*.

[c] *Université de Poitiers, UMR-CNRS 7285, Institut de Chimie des Milieux et des Matériaux de Poitiers, Equipe Eau, Géochimie Organique, Santé (EGS), 4 rue Michel Brunet, TSA 51106, 86073 Poitiers, France*

[d] *CHU de Poitiers, 2 rue de la Milétrie, CS 90577, 86021 Poitiers, France*

**E-mail: sebastien.papot@univ-poitiers.fr; brigitte.renoux@univ-poitiers.fr*

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I. Chemistry Section

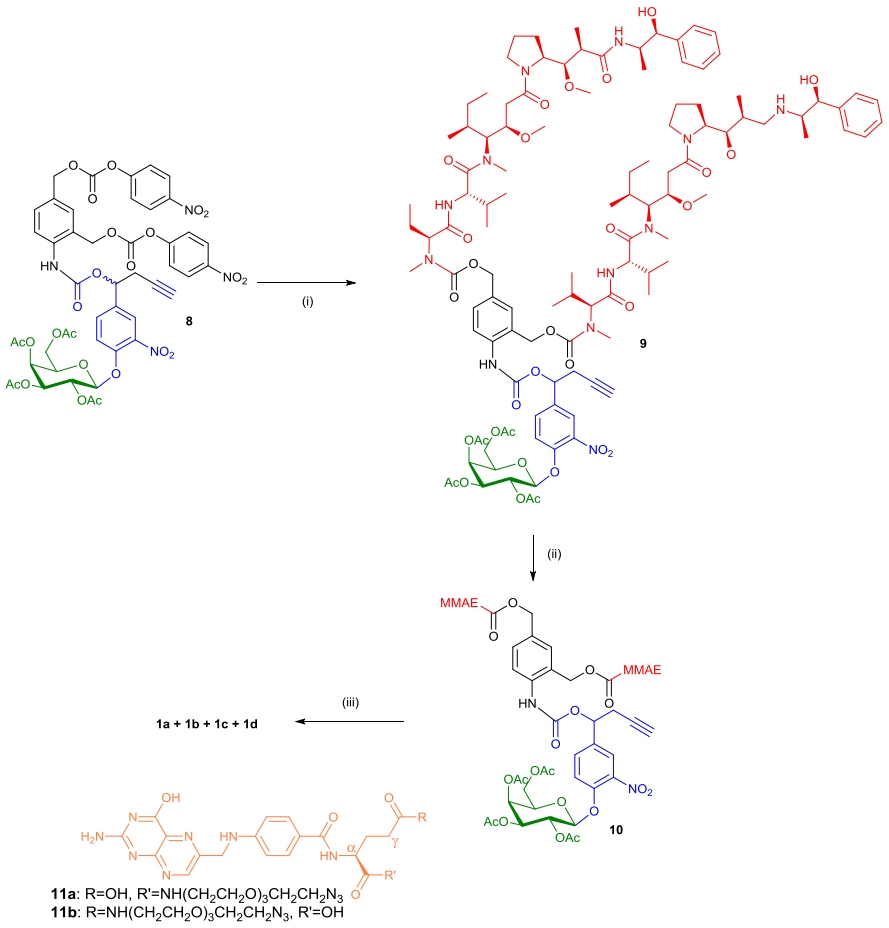
I.1. General experimental methods

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. 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. Standard abbreviations indicating multiplicity are used as follows: br = broad, s = singlet, d = doublet, t = triplet, m = multiplet. High resolution ESI mass spectrometry 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} with 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 1). 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) using a linear gradient composed of A (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). All chromatograms were recorded at 254 nm. HPLC/HRMS experiments were performed on an Accela UHPLC system coupled to a hybrid high resolution mass spectrometer (Q-Exactive^{TM}, Thermo Scientific). An Acclaim® C18 column (250x4.6 mm, 5 µm, 120 Å) at 30°C was used for chromatographic separation at a flow rate of 0.5 mL.min^{-1}. The column effluent was introduced into the electrospray ionisation source (ESI) of the mass spectrometer. Analyses were

performed in positive ion mode. The electrospray voltage was set at 4.0 kV. The capillary and heater temperatures were 275°C and 300°C respectively. The sheath, sweep and auxiliary gas (nitrogen) flow rates were set at 35, 10 and 20 (arbitrary units). Analysis of data was performed with Xcalibur™ software. Enzymatic hydrolysis was monitored by HPLC/HRMS using a linear gradient composed of A (0.1% formic acid in water) and B (0.1% formic acid in CH_3CN) (method 3). After injection of 20 µL of sample, compounds were separated through a linear gradient starting from 20% of B and reaching 100% of B within 30 min.

I.2. Synthetic overview of compound 1

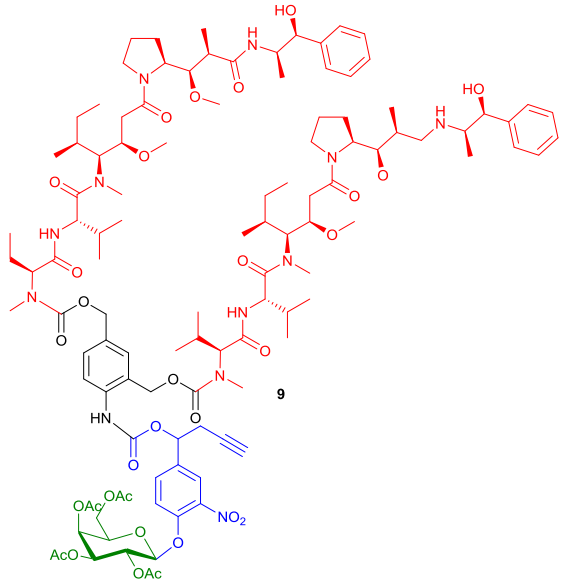
Compound **1** was prepared according to the following strategy:



Scheme 1 : *Reagents and conditions:* (i) MMAE, HOBt, DMF, RT, 24 h, 64%; (ii) LiOH-H_2O, MeOH, 0°C, 20 min, (iii) Azide **11**, $CuSO_4$, sodium ascorbate, DMSO/H_2O (9/1) , RT, 20 h, 62% (crude precipitate).

I.3. Synthetic procedures and characterization details

Preparation of compound 9



To a stirred solution of biscarbonate **8**[1] (15 mg, 0.0143 mmol) (mixture of two diastereoisomers due to the benzylic position) in DMF (0.3 mL) and pyridine (0.08 mL) was added MMAE (20.5 mg, 2 equiv.) and hydroxybenzotriazole (HOBt) (3.9 mg, 2 equiv.). The reaction was stirred 24 hours at room temperature and monitored by analytical RP-HPLC using method 1. The solution was concentrated under reduced pressure and the crude material was purified by column chromatography over silica gel (DCM/MeOH 5%) to afford **9** (20.3 mg, 64%).

^{1}H NMR of 9 as a mixture of two diastereoisomers (400 MHz, $CDCl_3$):

δ 8.70 (s, 0.4 H), 8.22 (m, 1H), 8.09 (br s, 4H), 8.04 (br s, 0.5H), 7.94 (br s, 1H), 7.77 (m, 2H), 7.52 (m, 0.5H), 7.37 (m, 9H), 7.17 (m, 0.5H), 6.79 (m, 0.5H), 6.54 (m, 1H), 5.98 (m, 1H), 5.56 (m, 1H), 5.48 (m, 1H), 5.15 (m, 7H), 4.97 (m, 0.5H), 4.68 (m, 7H), 4.10 (m, 9H), 3.87 (m, 2H), 3.62 (m, 7H), 3.35 (m, 7H), 2.95 (m, 8H), 2.5 (m, 6H), 2.08 (m, 23H), 1.88 (m, 4H), 1.60 (m, 3H), 1.32 (m, 20H), 0.9 (m, 39H).

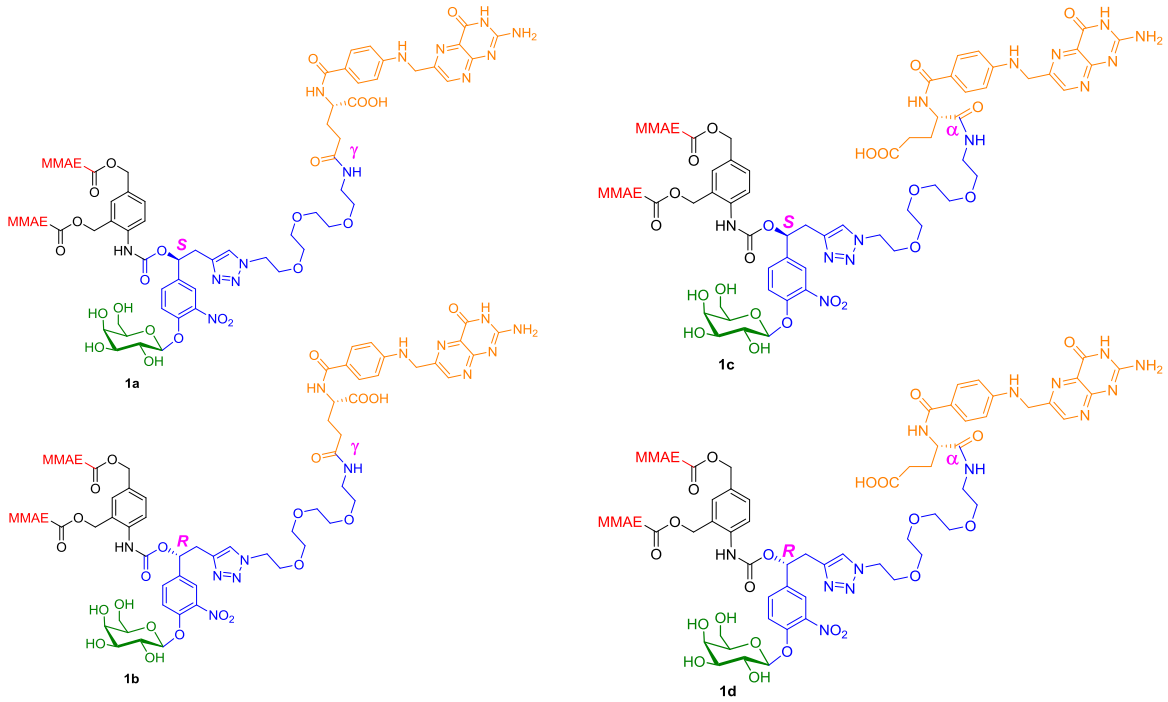
^{13}C NMR of 9 as a mixture of two diastereoisomers (100 MHz, $CDCl_3$):

δ 172.3, 172.2, 169.9, 169.8, 169.7, 169.5, 168.8, 168.7, 168.1, 156.0, 153.2, 148.0, 143.6, 140.0, 134 .9, 134.7, 132.1, 131.5, 127.7, 126.7, 126.4, 122.6, 117.6, 117.5, 98.5, 85.4, 79.6, 77.6, 76.9, 74.7, 73.9, 70.7, 69.9, 67.6, 67.0, 65.8, 63.2, 61.2, 60.9, 60.2, 58.6, 58.1, 57.1, 55.6, 54.9, 54.1, 49.7, 49.1,

47.2, 46.2, 43.70, 43.2, 37.1, 36.7, 35.1, 31.9, 31.8, 31.5, 31.3, 29.9, 29.7, 29.0, 26.70, 25.5, 25.3, 25.2, 24.3, 23.1, 20.5, 20.4, 20.3, 20.3, 20.2, 18.8, 18.7, 18.5, 18.3, 15.7, 15.6, 15.40, 15.2, 15.0, 10.3, 10.2.

HRESI-MS: m/z 2226.1595 (calcd. for $C_{113}H_{166}N_{12}O_{32}Na$ 2226.16233 $[M+Na]^+$). m/z 1124.5756 (calcd. for $C_{113}H_{166}N_{12}O_{32}Na_2$ 1124.57578 $[M+2Na]^{2+}$

Preparation of compound 1 as a mixture of four isomers



To a solution of **9** (13 mg, 0.0058 mmol) in MeOH (0.4 mL) cooled at 0°C, was added slowly a cooled solution of LiOH (0.051 mmol, 8.8 equiv.) in 0.4 mL of H_2O. Stirring was continued for 20 minutes at 0°C and the solution was neutralized with Amberlite® IRC 50 weakly acidic resin during 15 minutes and filtered through a pad of celite. The solvent were evaporated to dryness. The crude material (compound **10**, 12 mg, 0.0058 mmol) was dissolved in DMSO (0.3 mL) and H_2O (0.03 mL). Azide **11** (3.5 mg, 0.95 eq.), $CuSO_4$ (1.1 mg, 0.76 equiv.) and sodium ascorbate (2.3 mg, 2 equiv.) were then added and the resulting suspension was stirred at room temperature. The reaction was monitored by analytical RP-HPLC using method 1. Additional $CuSO_4$ (0.44 mg, 0.3 equiv.) and sodium ascorbate (0.55 mg, 0.6 equiv.) were added after 8 hours. Disappearance of starting material was observed by

HPLC after an additional 12 hours. Then a solution of ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA.2Na.2H_2O) (8.8 mg, 4 equiv.) in H_2O (0.3 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 and poured into a cold solution of Et_2O. The precipitate was filtrated and washed with MeOH to give the targeted compound **1** (9.6 mg, 62%) as a yellow powder.

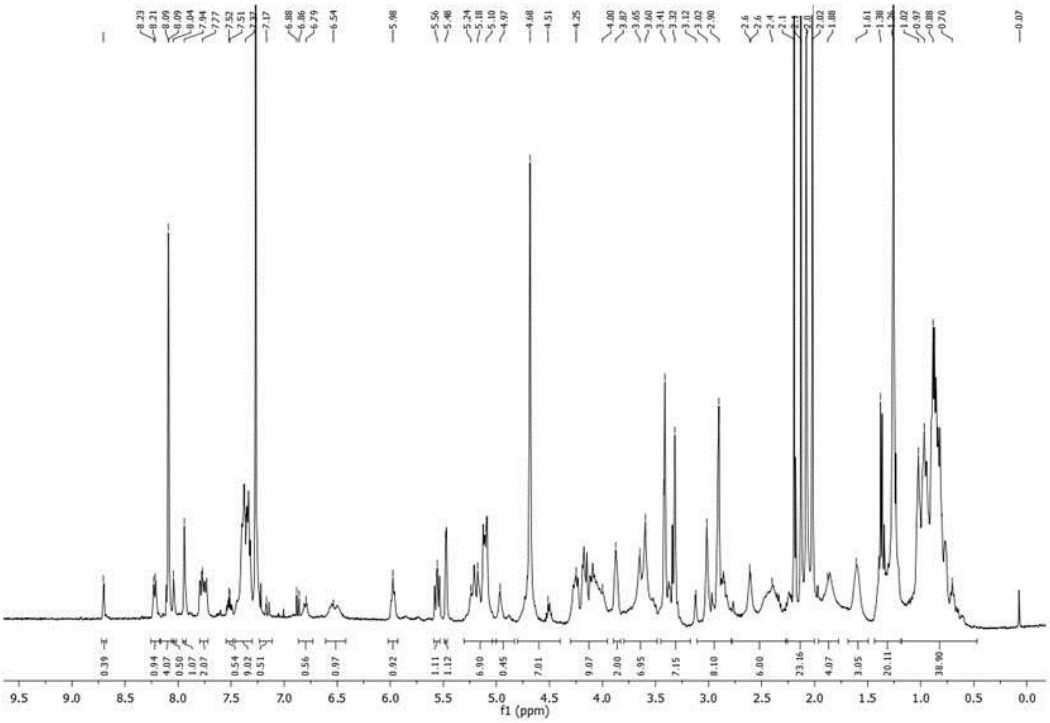
For biological evaluations, **1** was further purified by semi-preparative RP-HPLC (method 2). In this process, 4 mg were obtained with a purity >95%.

^{1}H NMR of 1 as a mixture of four diastereoisomers (400 MHz, DMSO-d_6): δ 9.28 (m, 1H), 8.62 (s, 1H), 8.32 (m, 1H), 8.08 (m, 1H), 7.84 (m, 4H), 7.64 (m, 4H), 7.28 (m, 12H), 6.91 (m, 2H), 6.62 (m, 2H), 5.96 (m, 1H), 5.37 (m, 2H), 5.01 (m, 4H), 4.66 (m, 3H), 4.45 (m, 9H), 4.27 (m, 1H), 3.98 (m, 4 H), 3.70 (m, 4H), 3.54 (m, 3H), 3.33 (m, masked CH_2-PEG, 50H), 3.18 (m, 11H), 2.91 (m, 10H), 2.53 (m, 18H), 2.23 (m, 2H), 2.11 (m, 3H), 1.95 (m, 3H), 1.75 (m, 4H), 1.49 (m, 5H), 1.22 (m, 12H), 0.99 (m, 9H), 0.79 (m, 32H).

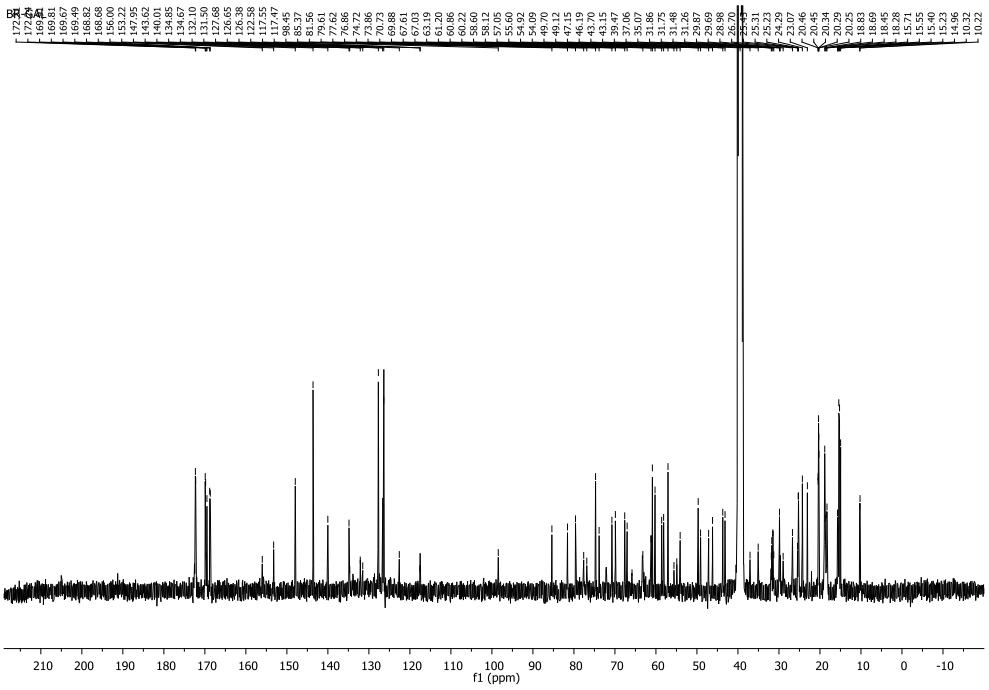
HRESI-MS: m/z 2697.3740 (calcd. for $C_{132}H_{191}N_{23}O_{36}Na$ 2697.37253 [M-2H+Na]).

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

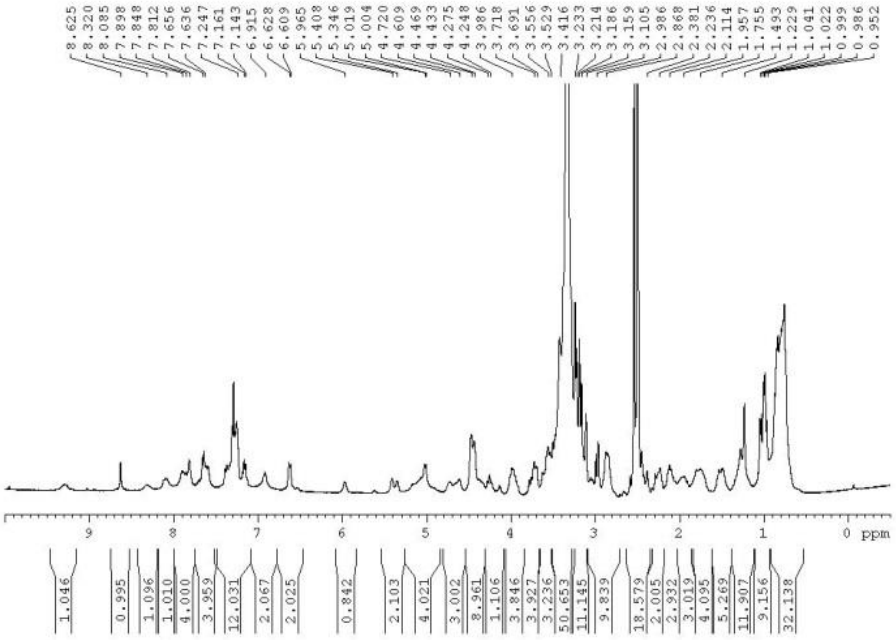
*^{1}H NMR spectrum (400 MHz, 298 K, $CDCl_3$) of **9***



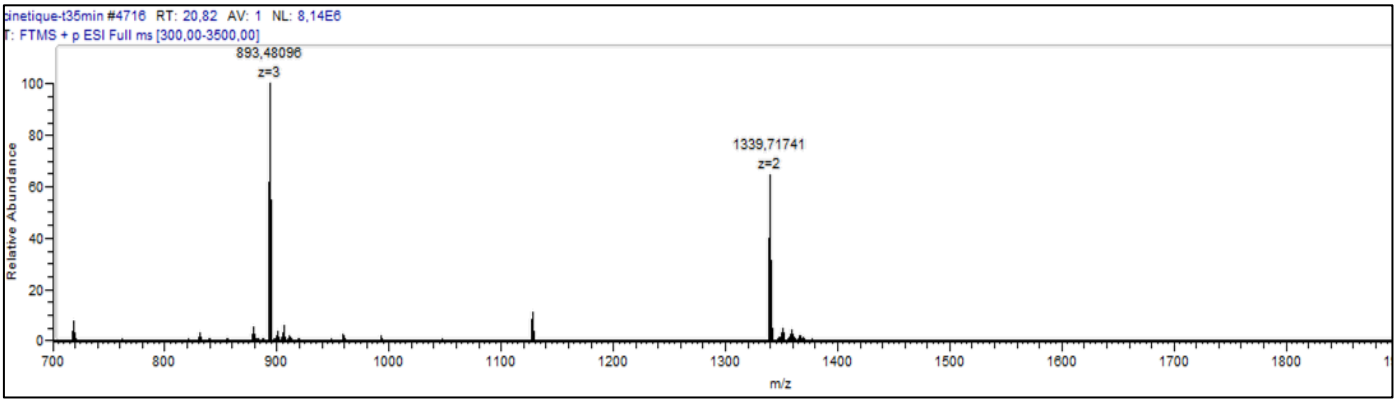
*^{13}C NMR spectrum (100 MHz, 298 K, $CDCl_3$) of **9***



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



*HMRS spectrum of **1***

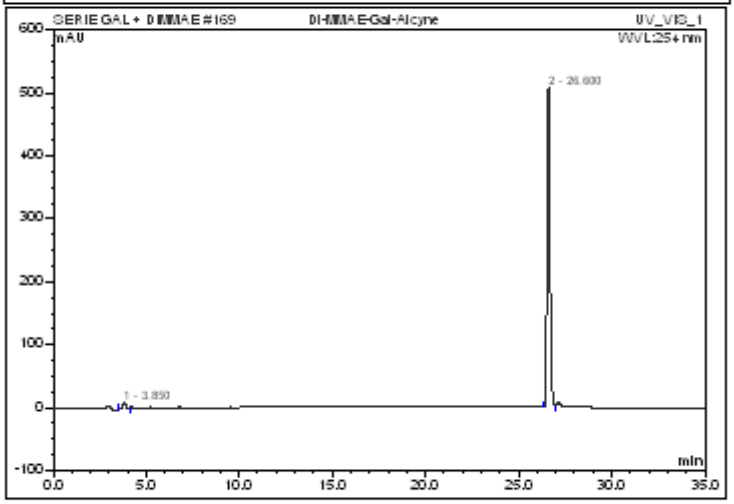


I.5. HPLC chromatograms

Compound 9

169 SERIE GAL + DIMMAE

Sample Name:	DI-MMAE-Gal-Alcyne	*Injection Volume:*	20.0
Vial Number:	762	*Channel:*	UV_VIS_1
Sample Type:	unknown	*Wavelength:*	254
Control Program:	m s275	*Bandwidth:*	n.a.
Quantif. Method:	CH994	*Dilution Factor:*	1.0000
Recording Time:	30/10/2014 10:11	*Sample Weight:*	1.0000
Run Time (min):	35.00	*Sample Amount:*	1.0000

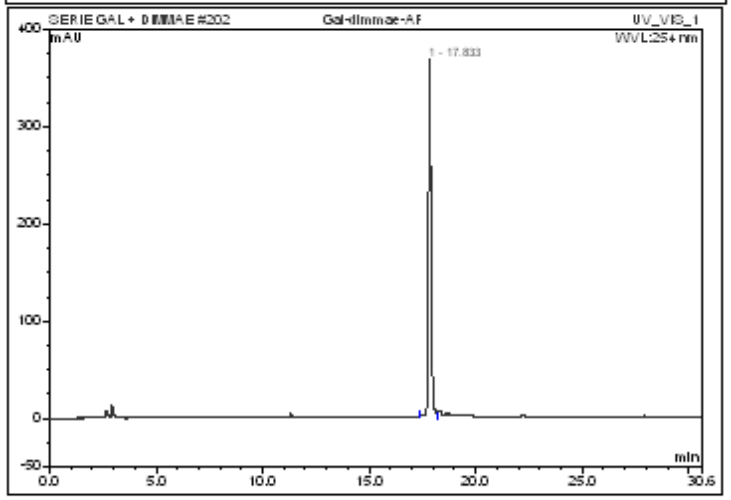


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	3.85	n.a.	9.412	1.746	2.07	n.a.	BMB
2	26.60	n.a.	504.528	82.506	97.93	n.a.	BMB
Total:			513.940	84.251	100.00	0.000	

Compound 1

202 SERIE GAL + DIMMAE

Sample Name:	Gal-dimmae-AF	*Injection Volume:*	20.0
Vial Number:	824	*Channel:*	UV_VIS_1
Sample Type:	unknown	*Wavelength:*	254
Control Program:	m s275	*Bandwidth:*	n.a.
Quantif. Method:	CH994	*Dilution Factor:*	1.0000
Recording Time:	8/1/2015 8:45	*Sample Weight:*	1.0000
Run Time (min):	30.58	*Sample Amount:*	1.0000



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	17.83	n.a.	365.073	50.179	100.00	n.a.	BMB
Total:			365.073	50.179	100.00	0.000	

I.6. Stability and Enzymatic cleavage

Targeting device **1** (0.1 mg) was incubated at 37°C in phosphate buffer at pH 7.0 (1 mL). Stability in phosphate buffer and enzymatic cleavage were monitored by HPLC-HRMS using method 3. No detectable degradation of compound **1** was observed during 24 hours under these conditions. Enzymatic hydrolysis was carried out with commercial β-galactosidase from *Escherichia coli* (766 units/mg protein, suspension in 50% glycerol, 40 U.µmol^{-1}) (Figure 1).

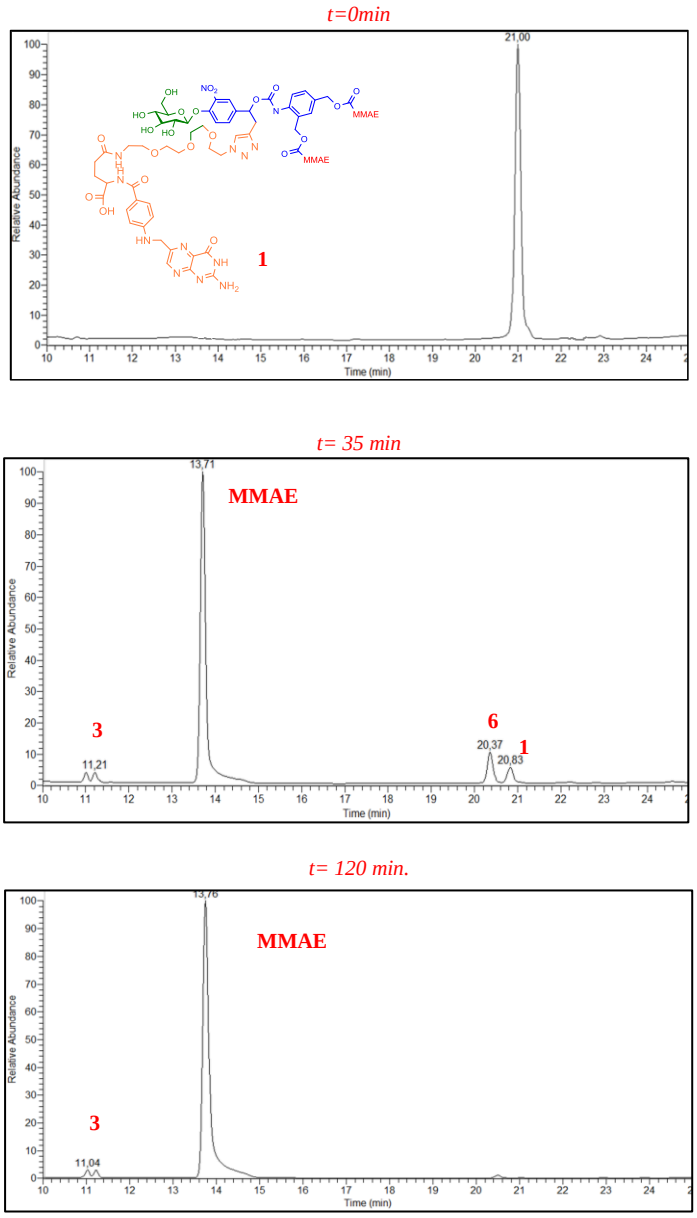
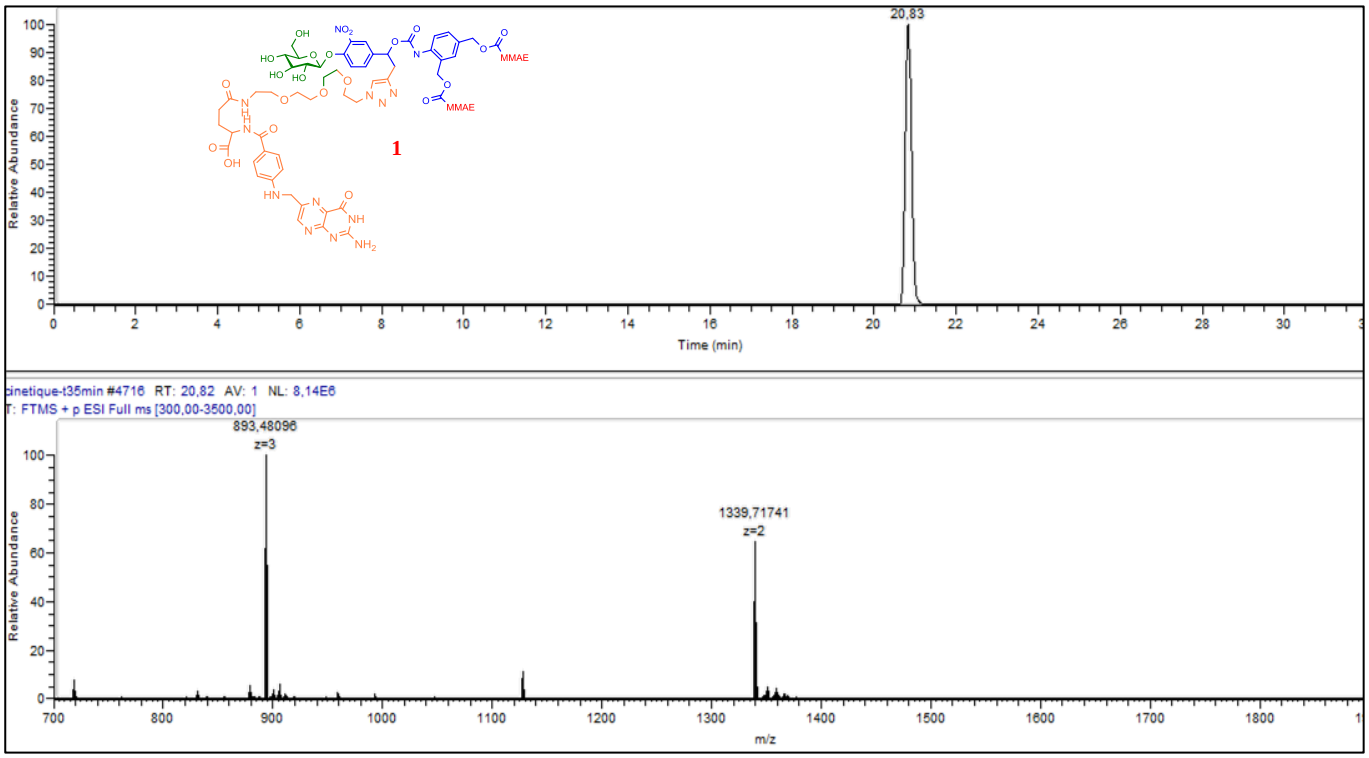
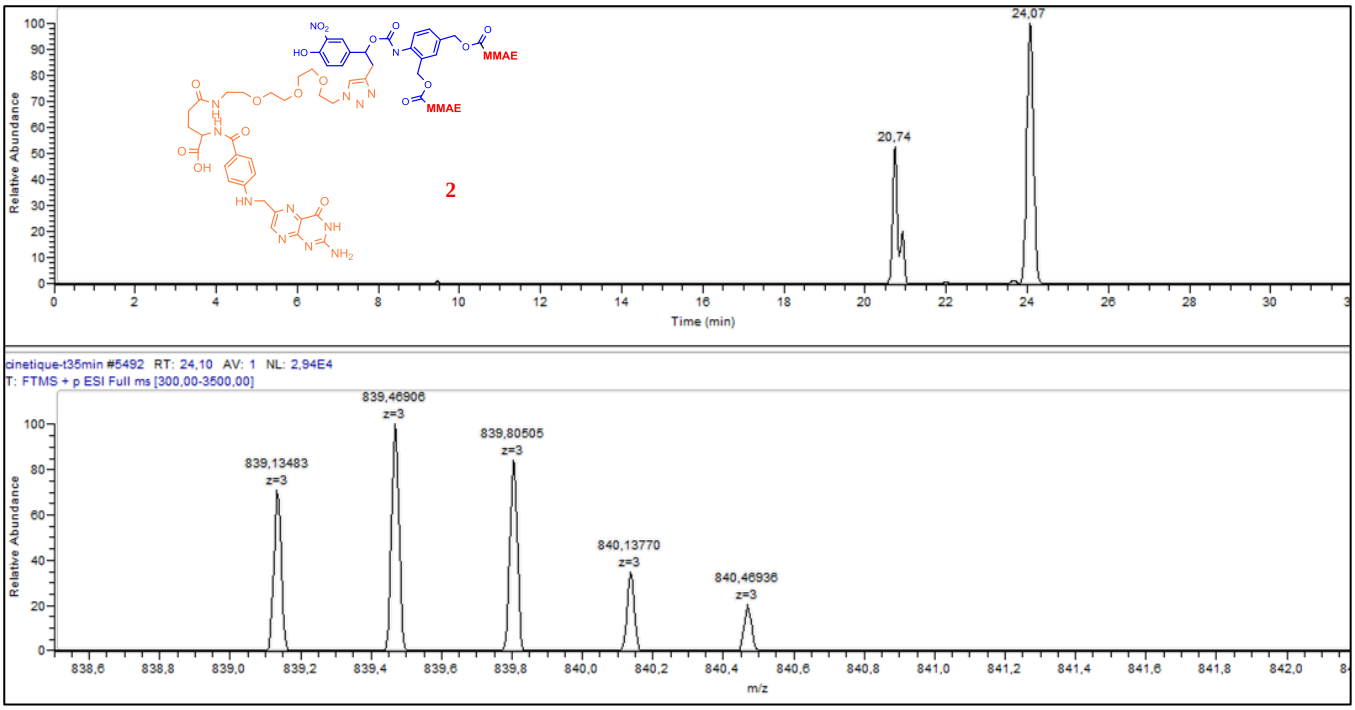


Figure 1: Enzymatic hydrolysis by HPLC/HRMS (method 3)
t = 0, 35 and 120 minutes.

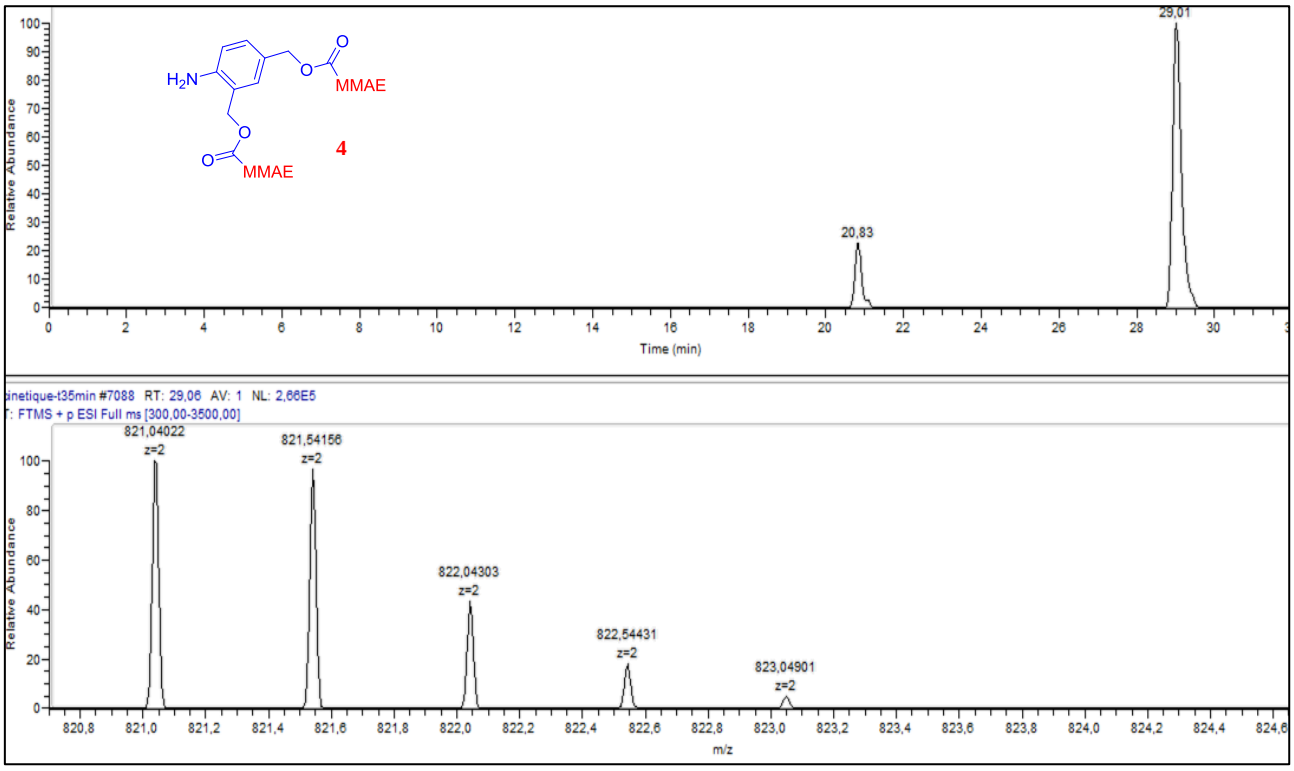
Retention time: 20.83min



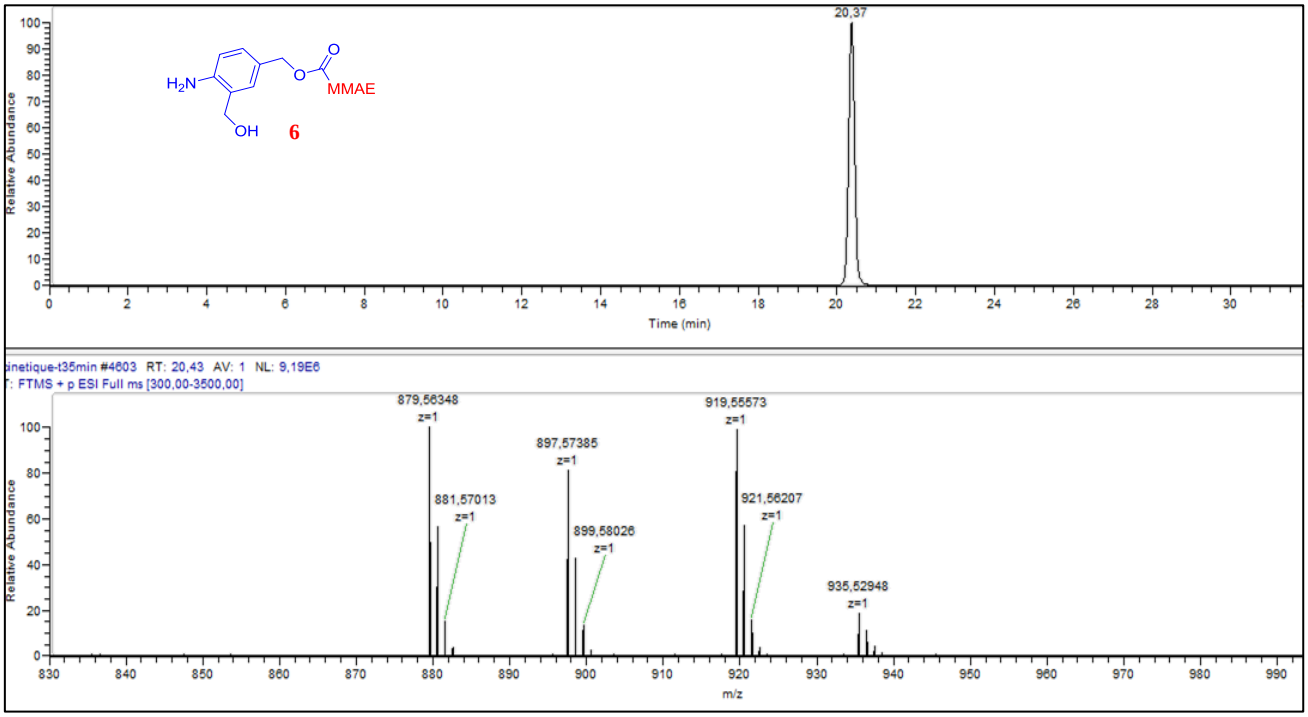
Retention time: 24.07 min



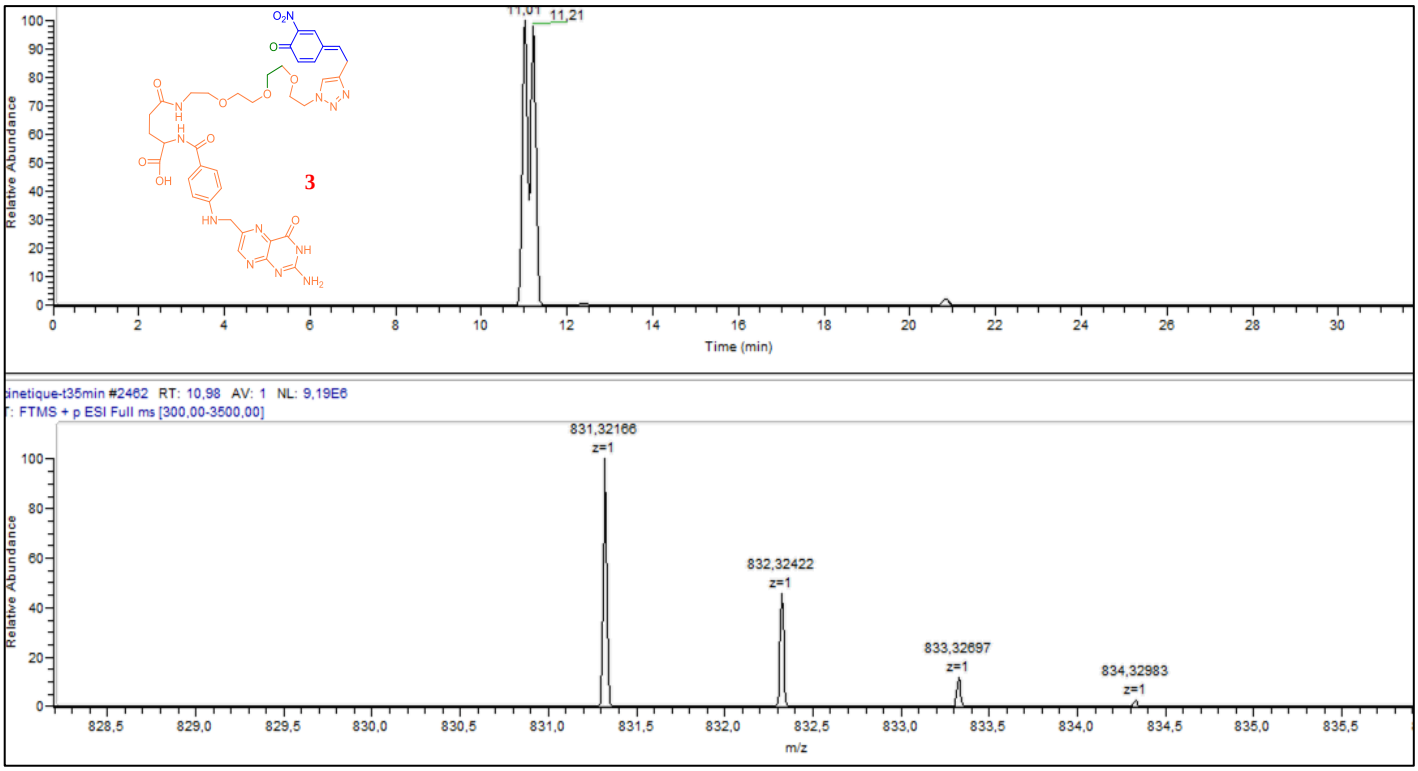
Retention time: 29.01 min



Retention time: 20.37 min



Retention times: 11.01 and 11.21 min



Retention time: 13.71min.

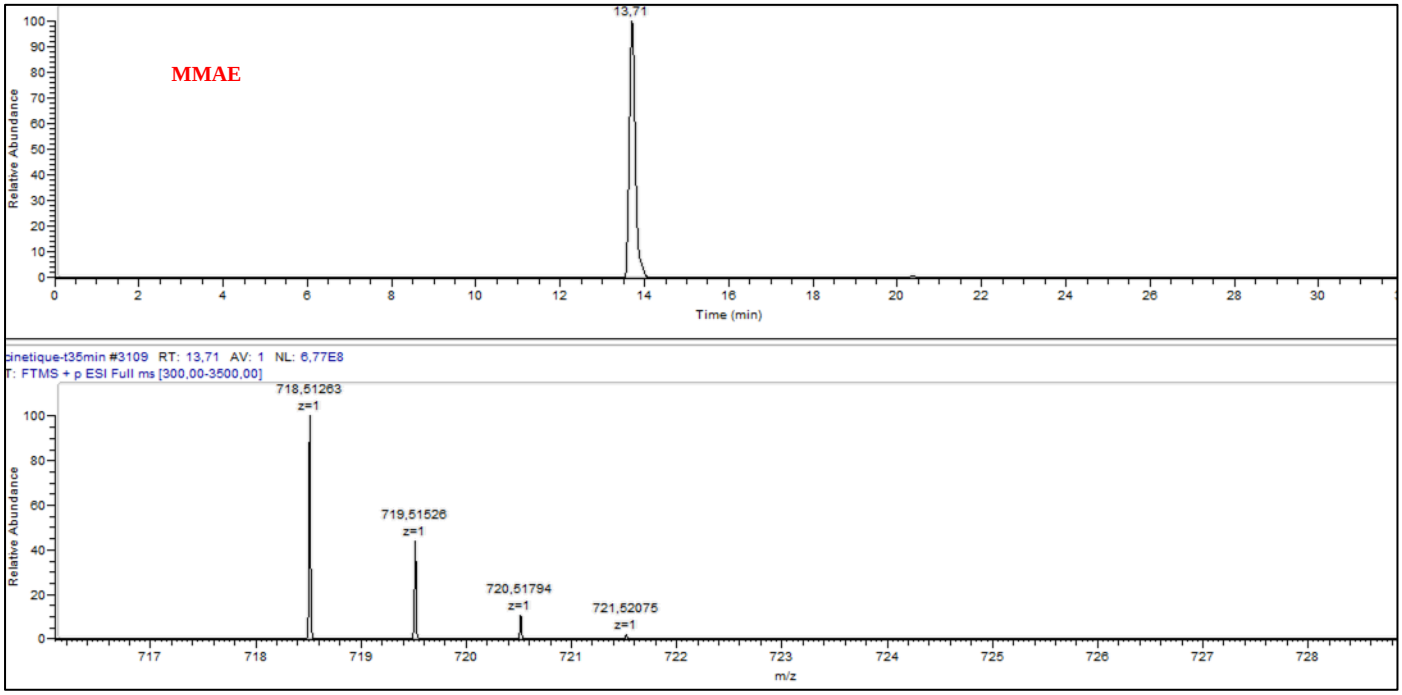
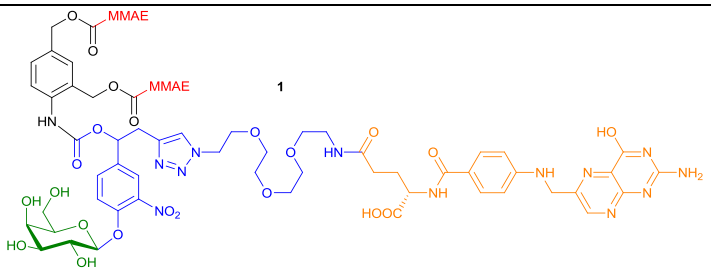
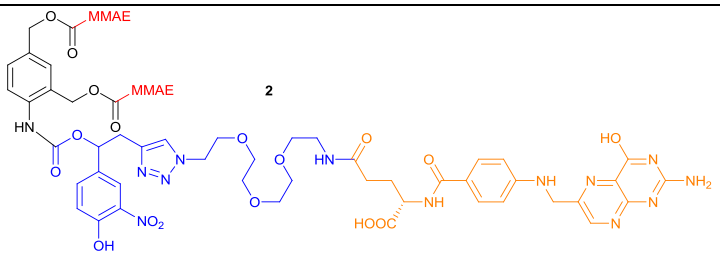
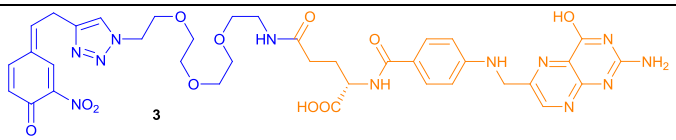
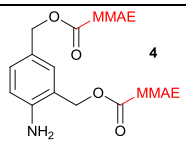
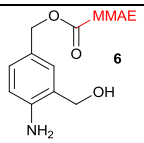
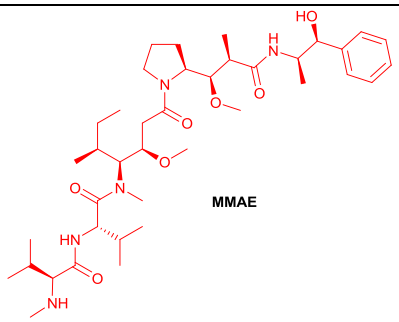


Figure 2: Chromatograms of enzymatic cleavage: t = 35 min.

I.7. Table

Structure	Retention time (min)	Exact mass ($g.mol^{-1}$)	m/z
	20.83	2676.3979	893.4810 $[M + 3H]^{3+}$
	24.07	2514.3450	839.1348 $[M + 3H]^{3+}$
	11.01-11.21	830.3096	831.3217 $[M + H]^{+}$
	29.01	1640.0456	821.0402 $[M + 2H]^{2+}$
	20.37	896.5623	897.5739 $[M + H]^{+}$
	13.71	717.5040	718.5126 $[M + H]^{+}$

Retention times were recorded with method 3

II. Biological Section

II.1. Materials and Methods

Cell Culture

HeLa (human cervix adenocarcinoma), A2780 and SKOV-3 (human ovarian carcinoma) cells were maintained in RPMI 1640 (Invitrogen) supplemented by 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (Lonza) in a humidified incubator at 37°C and 5 %CO_2.

Cell viability

The Cell Proliferation Kit II (XTT; Roche) was used to measure cell viability. For this assay, cells were seeded in 96-well plate at a density of 2×10^3 cells/well in RPMI 1640 depleted in folic acid and supplemented by 10% FBS and 1% Penicillin/Streptomycin. After 24 h, cells were exposed to the different compounds at the indicated concentration. After 3 days of treatment, 25 µL of the XTT labeling mixture were added per well. After additional 4 h of incubation, absorbance was determined at 490 nm. Experiments were performed between 5 and 6 times in triplicate (Figure 3). Data were analyzed with GraphPad software. For each compound, inhibitory concentration values (IC_{50}) were determined by the software and reported Table 1.

Relative quantification of MMAE in A2780 cells

The relative quantity of MMAE was determined in A2780 treated with **1** or **12**. Non treated cells were used as controls. Two replicates were performed per condition. 1×10^6 A2780 cells, grown in RPMI 1640 depleted in folic acid and supplemented by 10% fetal bovine serum and 1% Penicillin/Streptomycin, were treated with 100 nM of **1** or **12** for 24 h. Culture medium was removed and cells were collected with Versene (Lonza), pelleted by centrifugation, lysed using a micropestle in 0.5 mL of 0.3 M sodium acetate and centrifuged for 5 min at 1500×g. The supernatant was transferred in 1 mL of cold ethanol and incubated 1 h at -20°C. After centrifugation at 17000×g and 4°C for 20 min, 0.5 mL of acetonitrile-methanol (2:1)(v:v) was added to the supernatant and incubated 1 h at -20°C. The sample was then centrifuged for 5 minutes at 3000xg. The supernatant was transferred to 2 mL microcentrifuge tubes and centrifuged again at 17 000×g and 4°C for 5 min.

Supernatants were analyzed by HPLC/HRMS. One milliliter of sample was injected and desalted by the mean of on-line trap columns at a flow rate of 0.5 $mL.min^{-1}$ for 2 min with water-0.1% formic acid as the loading eluent. Trapped analytes were then back flushed onto the analytical column and separated using method 3.

Target selected ion monitoring data dependent-MS/MS (t-SIM-ddMS/MS) (ESI+) was used to relatively quantify MMAE in A2780 cells. Targeted MS parameters were optimized at resolution 70000 for precursor ion and 17500 for product ions, AGC target 105 (precursor ion) and 2.10^5 (product ions), max IT 100 ms (precursor ion) and 50 ms (product ions), MSX count 1, and isolation window 2.0 m/z. The normalized collision energy was set at 35%. The precursor ion selected for MMAE identification was $[M+H]^+$ m/z 718.509. The product ions m/z 86.097, 134.096 and 154.123 were selected for verification (Figure 4). Peaks integration and MS spectra acquisition was performed with Thermo Xcalibur™ Qualitative Browser. A mass tolerance of 10 ppm was applied for the extraction of target product ions. The relative area of MMAE peak was determined and MMAE ratio in A2780 treated with **1** and **12** was calculated (Figure 5).

II.2. Results

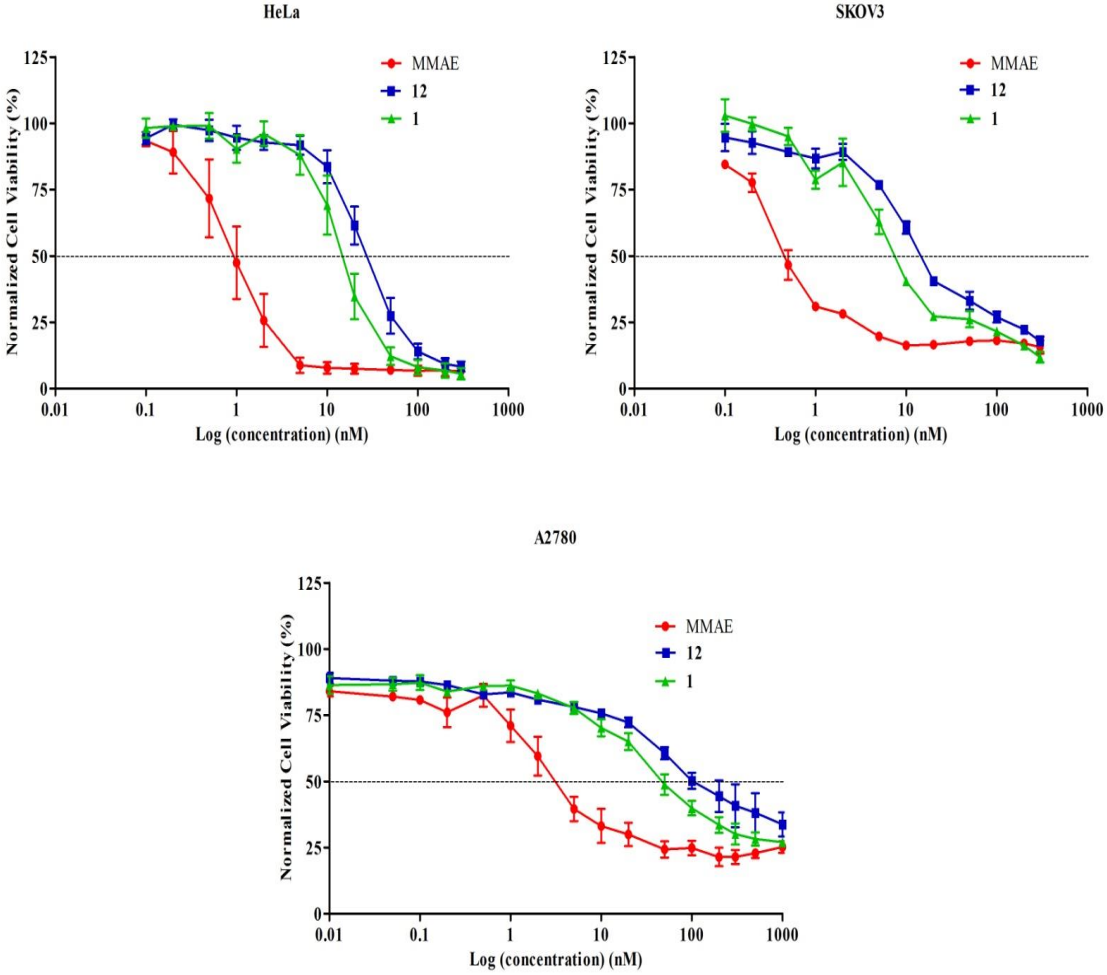


Figure 3: Cell viability of cancer cells after 3 days of treatment with different concentrations of MMAE, compounds **1** and **12**.

	Folate receptor expression (Relative to GAPDH)	IC50 values (nM)			**12/1** ratio
		MMAE	**12**	**1**	
HeLa	23.7%	1.12±0.41	29.36±5.77	16.3±3.76	**1.80**
SKOV-3	3.37%	0.64±0.08	20.16±1.45	9.62±1.12	**2.10**
A2780	0.29%	6.67±3.54	248.23±87.09	64.51±14.13	**3.85**

Table 1: IC_{50} values (nM) of MMAE, prodrugs **1** and **12** after 3 days of treatment. Folate receptor expression of KB cells (human oral squamous carcinoma) was 99.5% (relative to GAPDH).

a.

b.

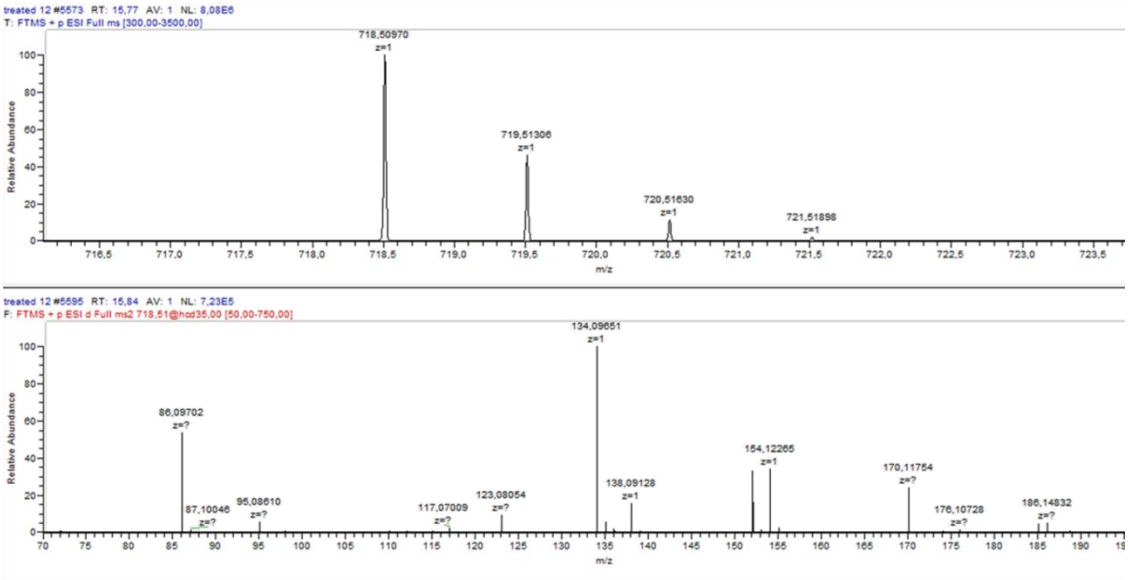
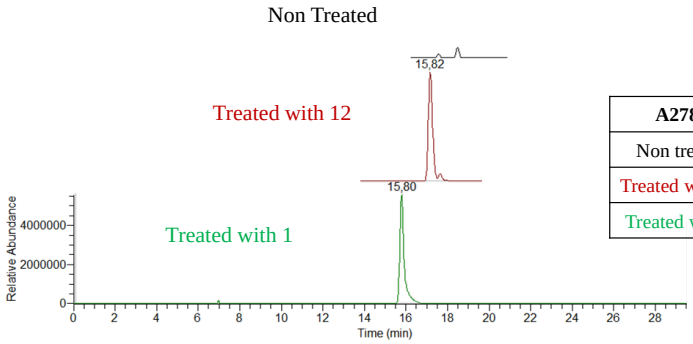


Figure 4: a. Mass spectrum of MMAE. b. MS^2 spectrum of the precursor ion ($[M + H]^+$ m/z 718.509).



A2780	Relative Area	Ratio 1/12
Non treated	/	
Treated with 12	13934519	4.14
Treated with 1	57705018	

Figure 5: Relative quantification of MMAE in A2780 cells. Illustrative chromatograms and mean relative areas.

III. Investigation of the β-galactosidase-catalysed process of drug release

In order to confirm that the release of the drug was due to the galactoside trigger activation by lysosomal β-galactosidase, we designed the non-cleavable glucuronide analogue **13** illustrated in **Figure 6**. Compound **13** was prepared using the same synthetic strategy than that employed for the preparation of the galactoside **1** and obtained as a mixture of 4 isomers.

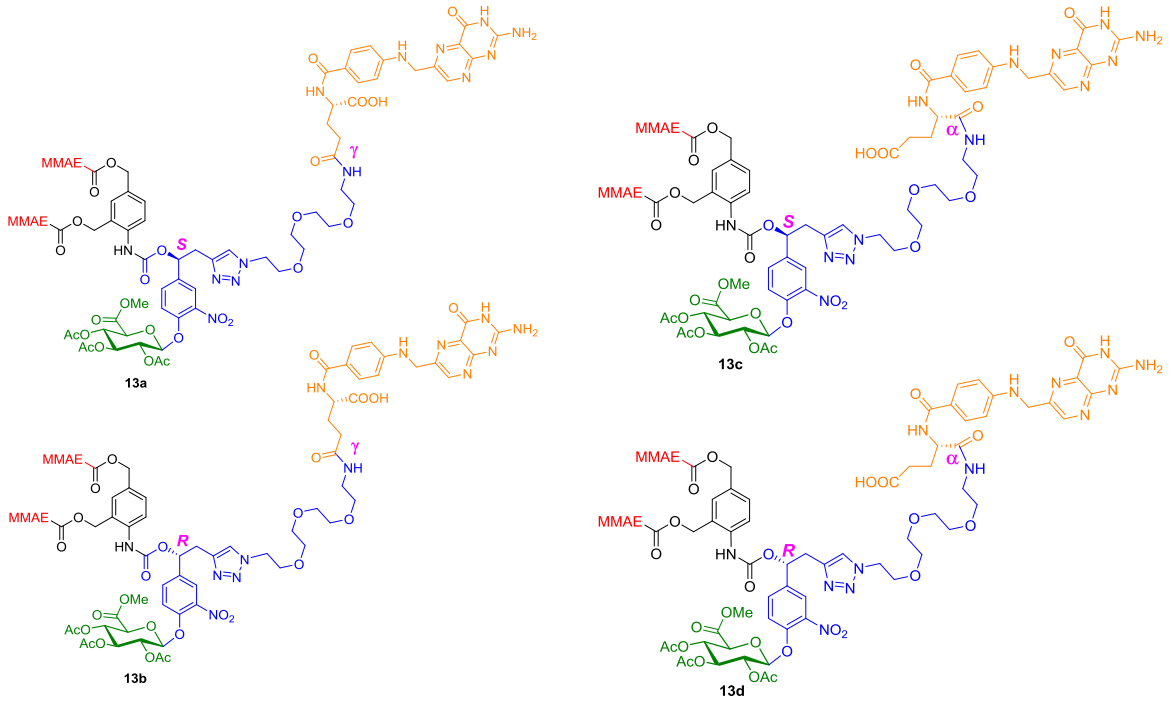


Figure 6: Structure of the non-cleavable glucuronide **13** obtained as a mixture of 4 isomers.

The antiproliferative activity of **13** was evaluated on A2780 cancer cells. As shown on **Figure 7**, these cells were not sensitive at all to the dimer **13** until the highest tested dose of 1 µM. Since compound **1** is highly toxic for A2780 cells (IC_{50} = 64.51 nM), this result clearly demonstrates the role of the galactoside trigger in the intracellular activation process of drug release.

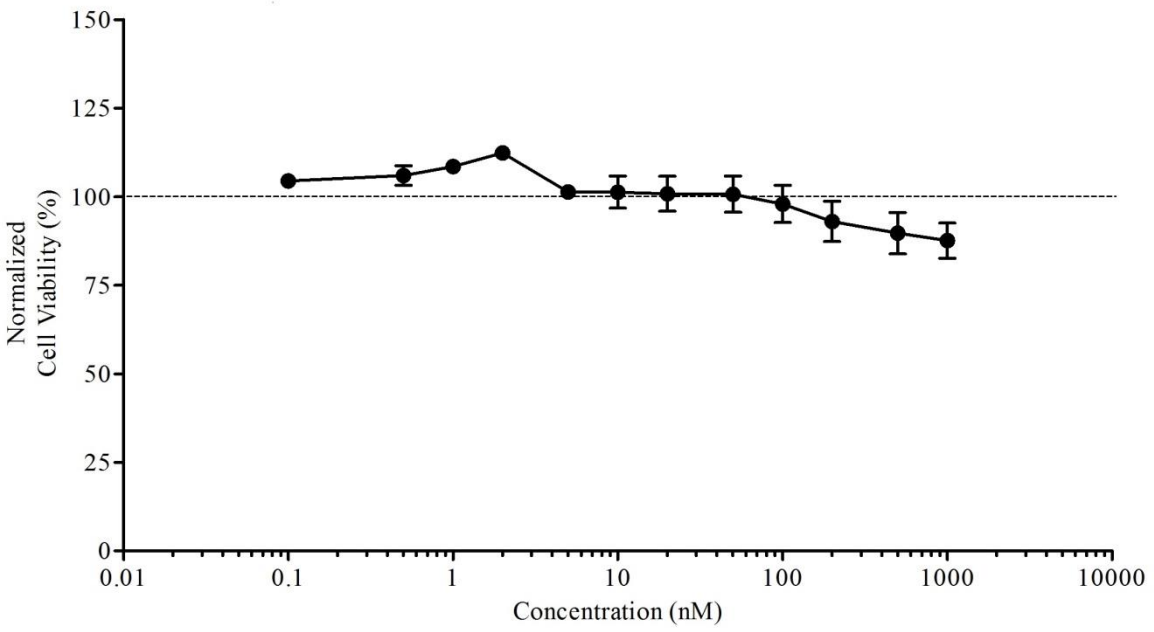


Figure 7: Cell viability of A2780 cells after 3 days of treatment with different concentrations of compound **13**.

III. References

[1] M. Grinda, T. Legigan, J. Clarhaut., E. Péraudeau, I. Tranoy-Opalinski, B. Renoux, M. Thomas, F. Guilhot and S. Papot, *Org. Biomol. Chem.*, 2013, **11**, 7129-7133.