Supplementary information

Nitroaromatic-Based Triazene Prodrugs to Target the Hypoxic Microenvironment in Glioblastoma

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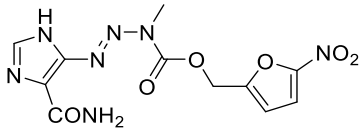
Table of Contents

Synthesis

General procedure for the synthesis of prodrug 8

3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-carbamoyl-1H-imidazol-5-yl)-3-methyltriazene (8)

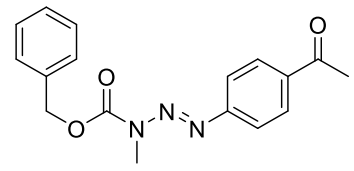


8

5-(3-methyltriazen-1-yl)imidazole-4-carboxamide was obtained in two steps following previously reported procedures.[1,2] To a solution of 5-nitrofurfuryl alcohol (0.63 mmol) in 1 mL of DMF at 0°C, was added CDI (112 mg, 0.69 mmol) and the reaction mixture was allowed to warm to room temperature and stirred for 1 h, until total consumption of starting material. Then, a solution of 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (0.63 mmol) previously activated with NaH 80% dispersion in mineral oil (28 mg, 0.94 mmol) in DMF (2 mL), was added to the activated alcohol at 0°C and the reaction mixture was allowed to warm to rt and stirred for 24 h, under nitrogen atmosphere. The reaction mixture was dissolved in AcOEt (10 mL) and washed with water (3 x 10 mL) and brine (10 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give the crude product, which was then crystallized from diethyl ether to afford **8** as a dark brown solid (50 mg, η = 25%); m.p. 180-182 °C; ^{1}H NMR (300 MHz, $(CD_3)_2SO$) δ 13.16 (s, 1H), 7.78 (bs, 1H), 7.77 (bs, 1H), 7.70 (d, *J* = 2.9 Hz, 1H), 7.24 (s, 1H), 7.04 (d, *J* = 3.1 Hz, 1H), 5.48 (s, 2H), 3.42 (s, 3H); ^{13}C NMR (75 MHz, $(CD_3)_2SO$) δ 159.9, 153.1, 152.6, 151.7, 146.1, 136.7, 121.1, 114.9, 113.6, 59.7, 30.7; HR-ESI(+)/MS: m/z calcd for $C_{11}H_{12}N_7O_6$ $[M + H]^+$: 338.0844; found 338.0847.

General procedure for the synthesis of the negative control 5

3-[benzyloxycarbonyl]-1-(4-acetylphenyl)-3-methyltriazene (5)



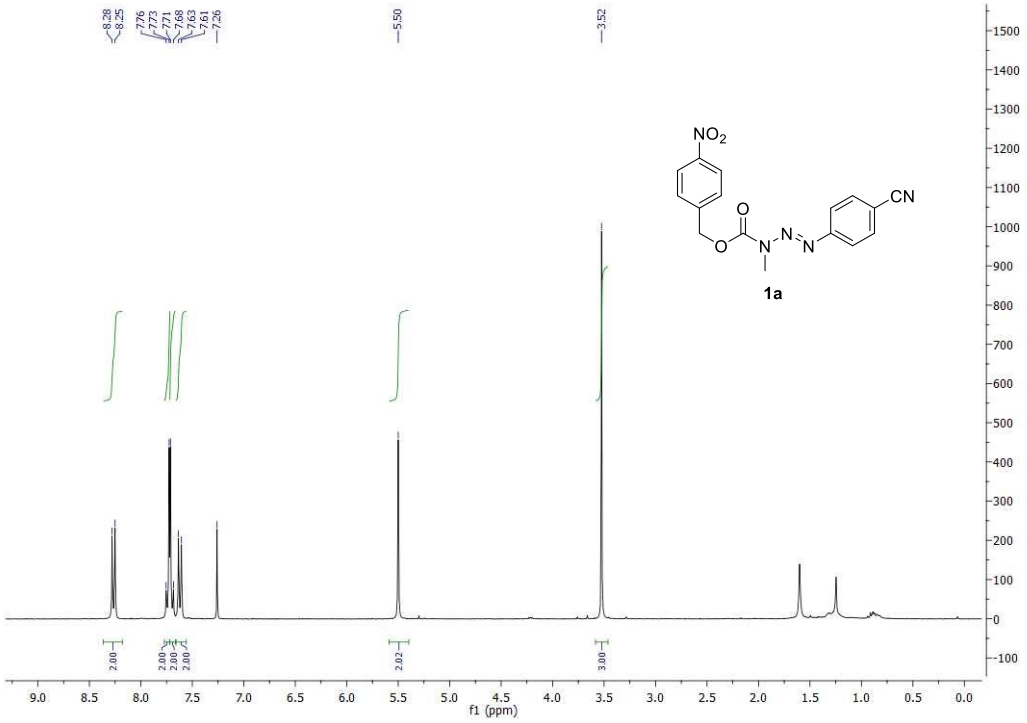
5

Method A was followed using benzyl chloroformate and 1-(4-acetylphenyl)-3-methyltriazene. The crude product was purified by column chromatography using DCM to afford **5** as an orange solid (53 mg, η = 30%); m.p. 80-83 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.02 (d, *J* = 8.7 Hz, 2H), 7.68 (d, *J* = 8.7 Hz, 2H), 7.48.7-7.35 (m, 5H), 5.42 (s, 2H), 3.51 (s, 3H), 2.62 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 197.4, 154.3, 152.2, 137.0, 135.6, 129.6, 128.8, 128.7, 128.4, 122.4, 69.0, 30.7, 26.8. HR-ESI(+)/MS: m/z calcd for $C_{17}H_{18}N_3O_3$ $[M + H]^+$: 312.1343; found 312.1349.

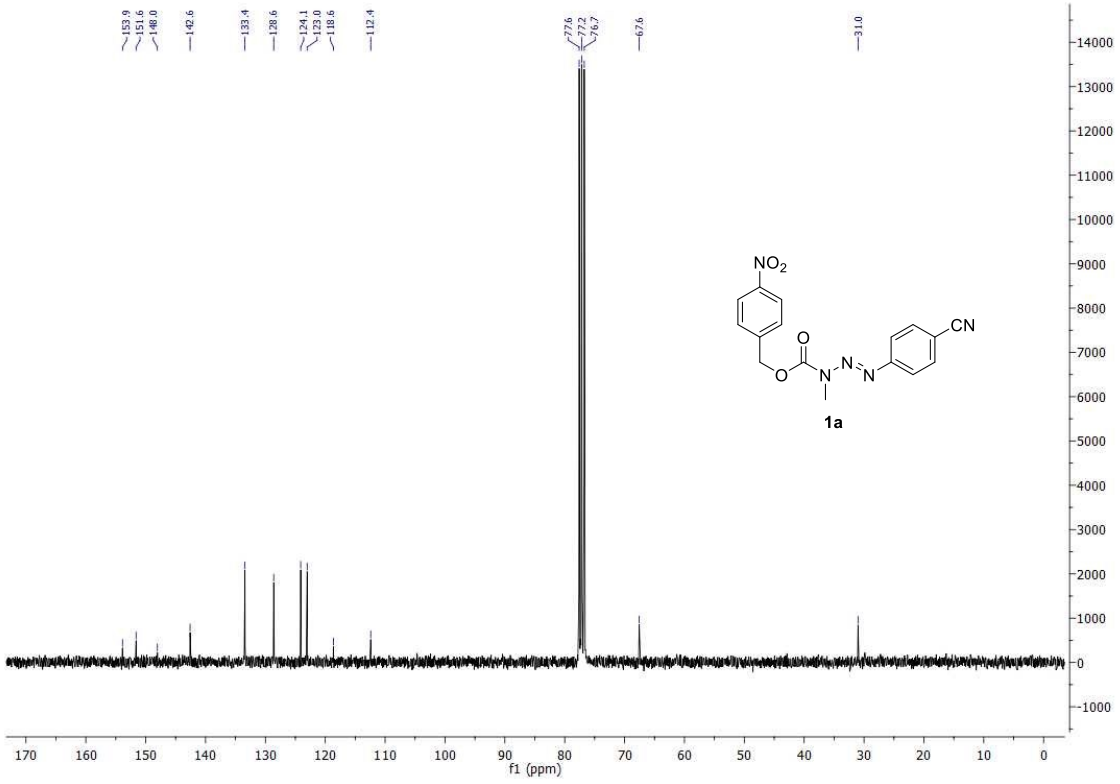
^{1}H, ^{13}C-NMR spectra for 1a-n, 5 and 8

3-[4-nitrobenzyloxycarbonyl]-1-(4-cyanophenyl)-3-methyltriazene (1a)

^{1}H NMR ($CDCl_3$, 300 MHz)

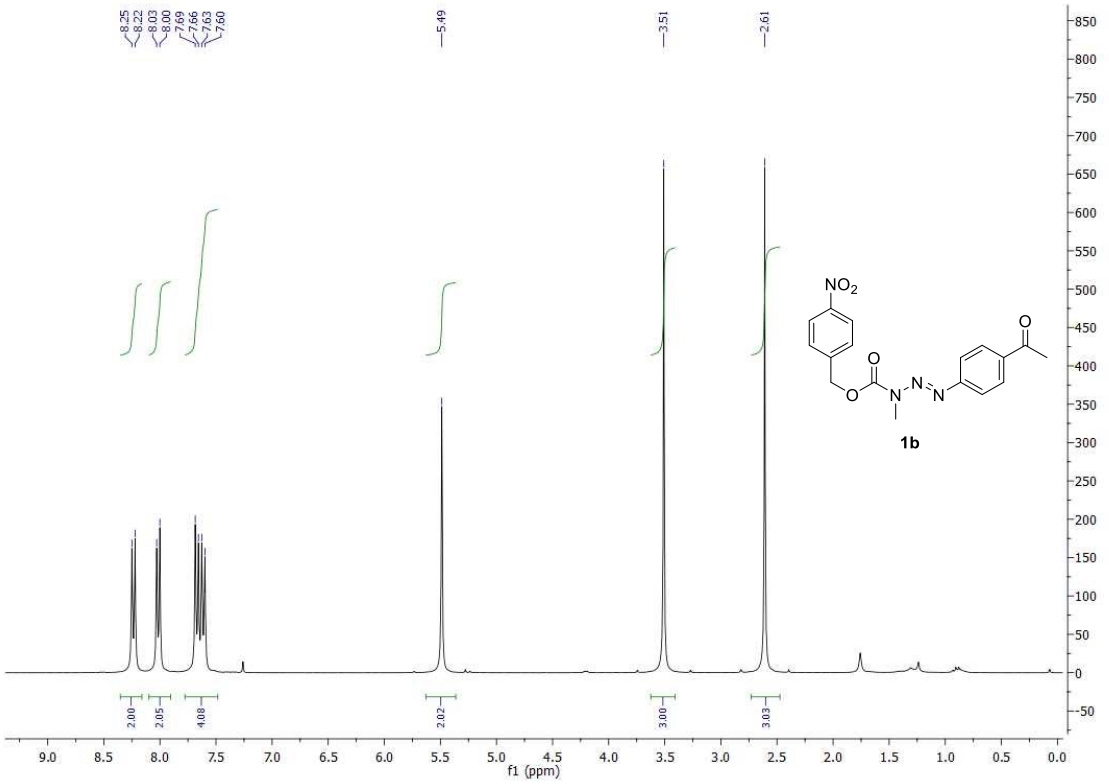


^{13}C NMR ($CDCl_3$, 75 MHz)

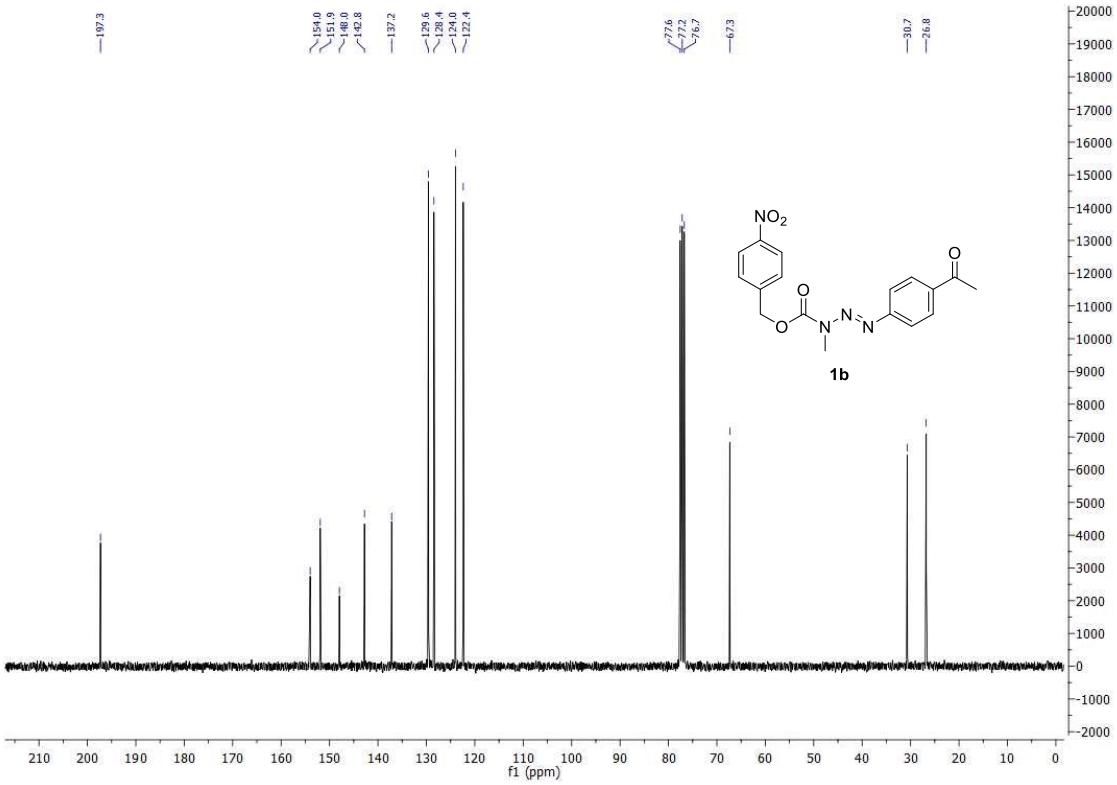


3-[4-nitrobenzyloxycarbonyl]-1-(4-acetylphenyl)-3-methyltriazene (1b)

^{1}H NMR ($CDCl_3$, 300 MHz)

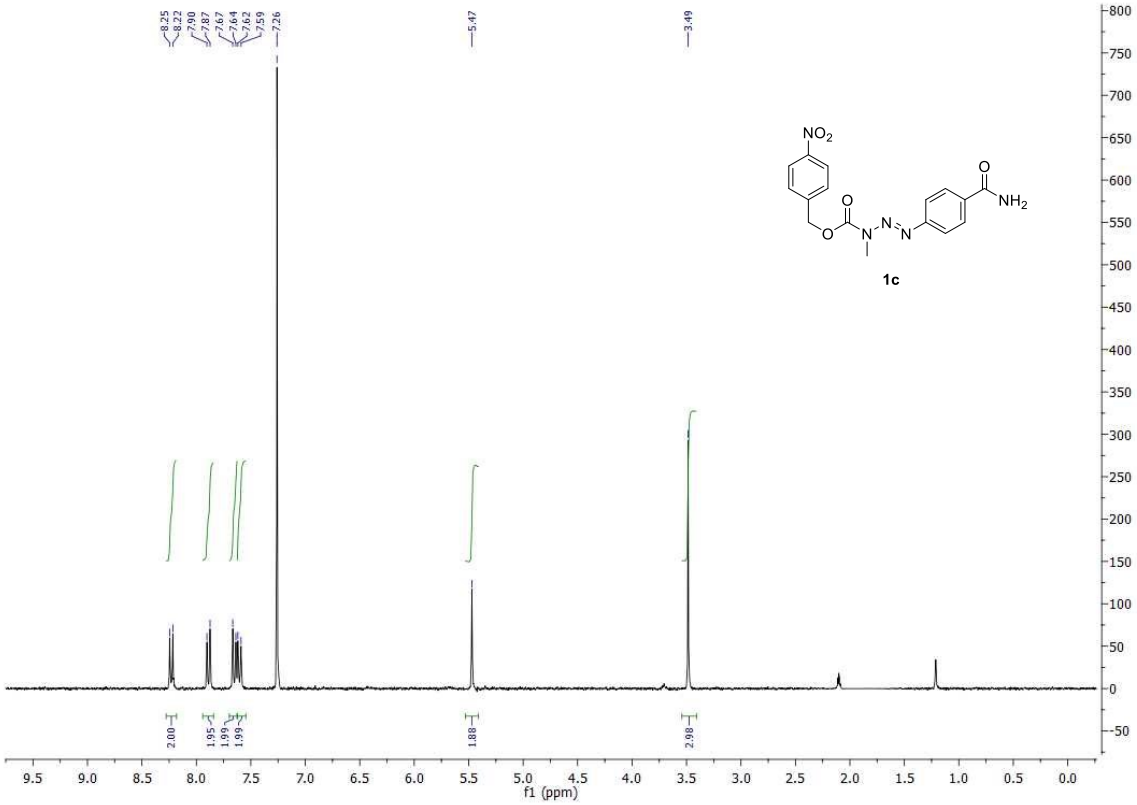


^{13}C NMR ($CDCl_3$, 75 MHz)

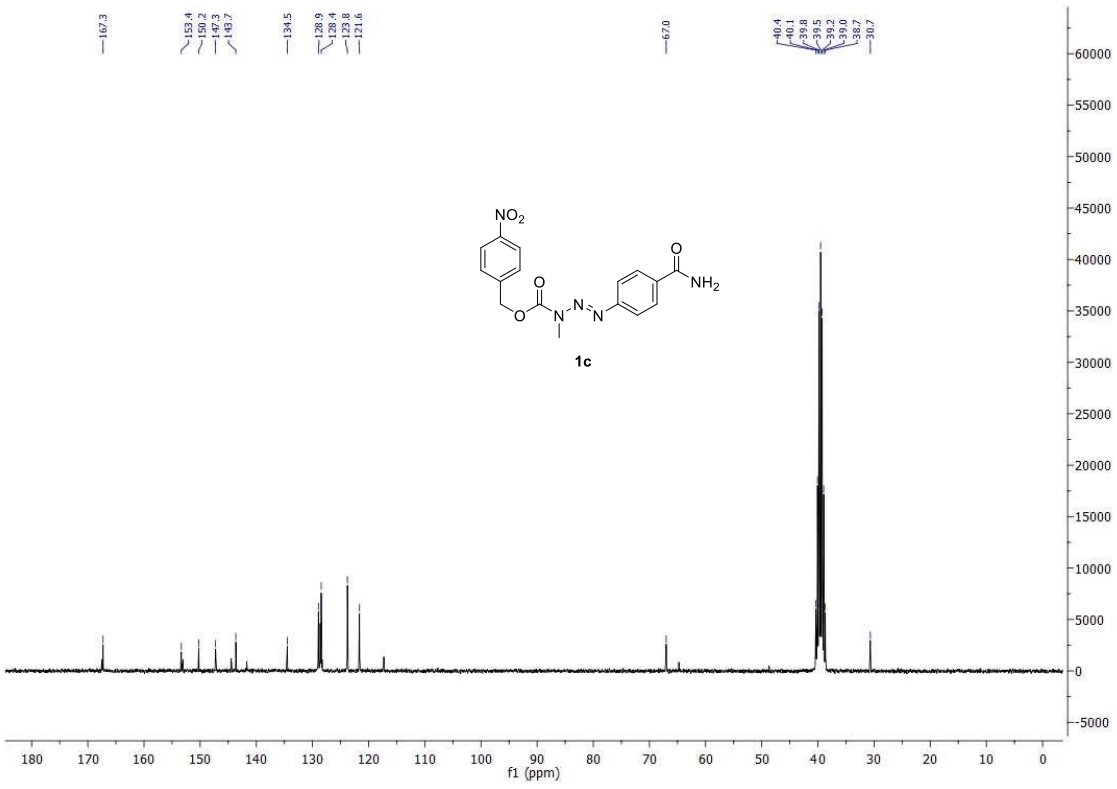


3-[4-nitrobenzyloxycarbonyl]-1-(4-carbamoylphenyl)-3-methyltriazene (1c)

^{1}H NMR ($CDCl_3$, 300 MHz)

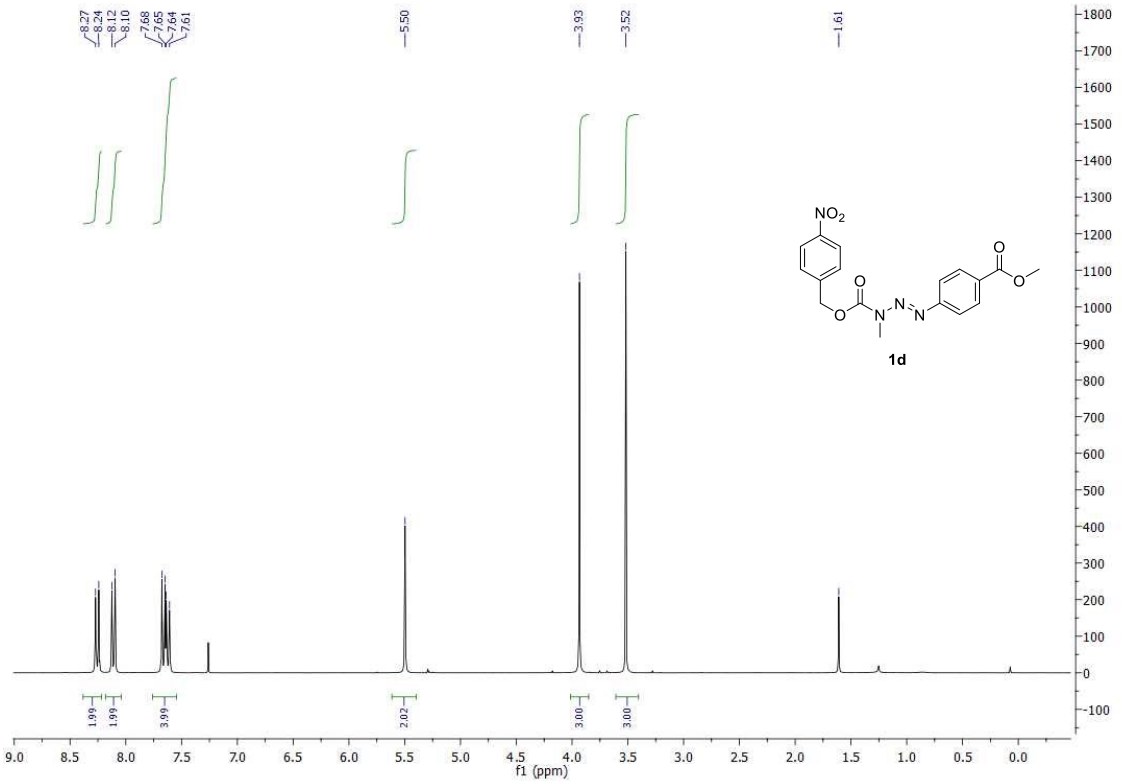


^{13}C NMR ($(CD_3)_2SO$, 75 MHz)

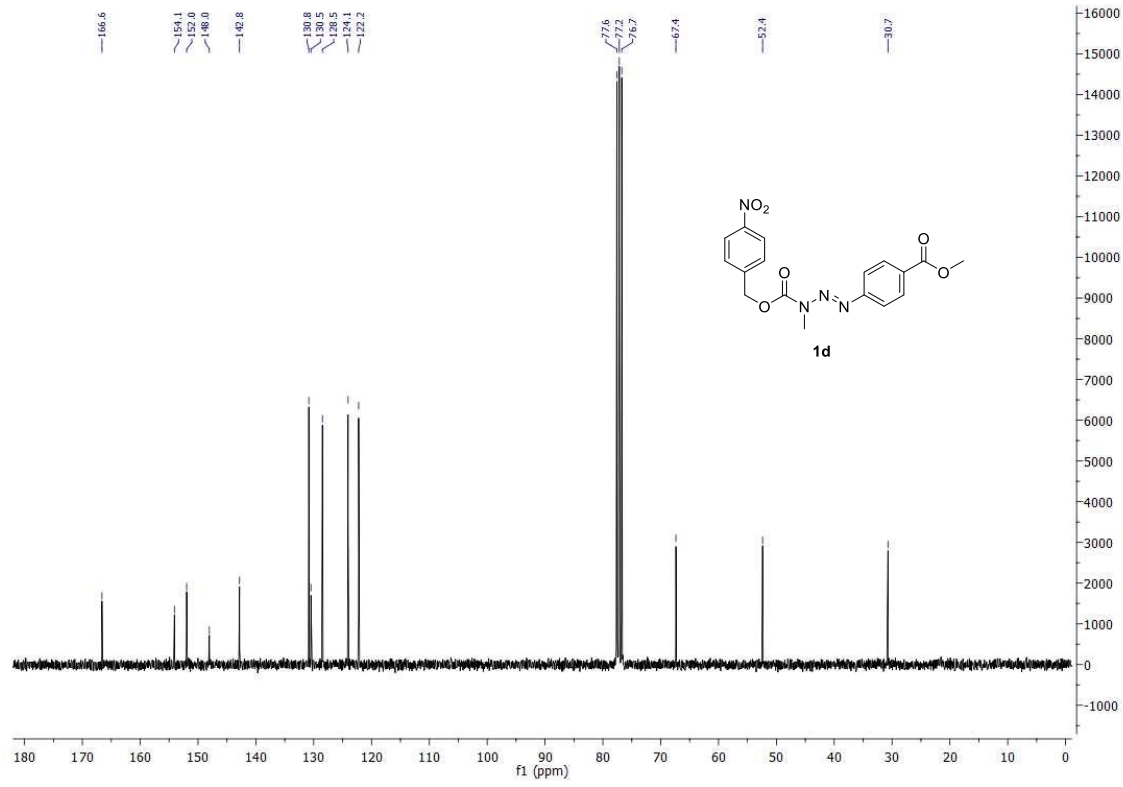


3-[4-nitrobenzyloxycarbonyl]-1-(4-methoxycarbonylphenyl)-3-methyltriazene (1d)

^{1}H NMR ($CDCl_3$, 300 MHz)

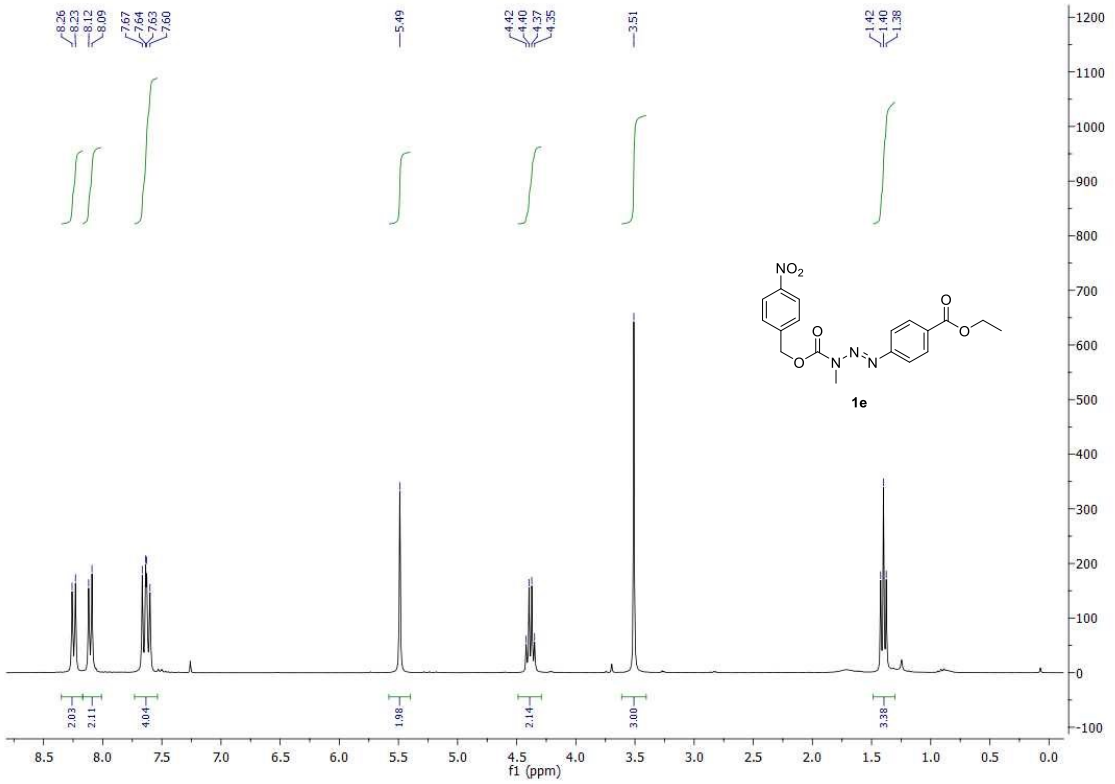


^{13}C NMR ($CDCl_3$, 75 MHz)

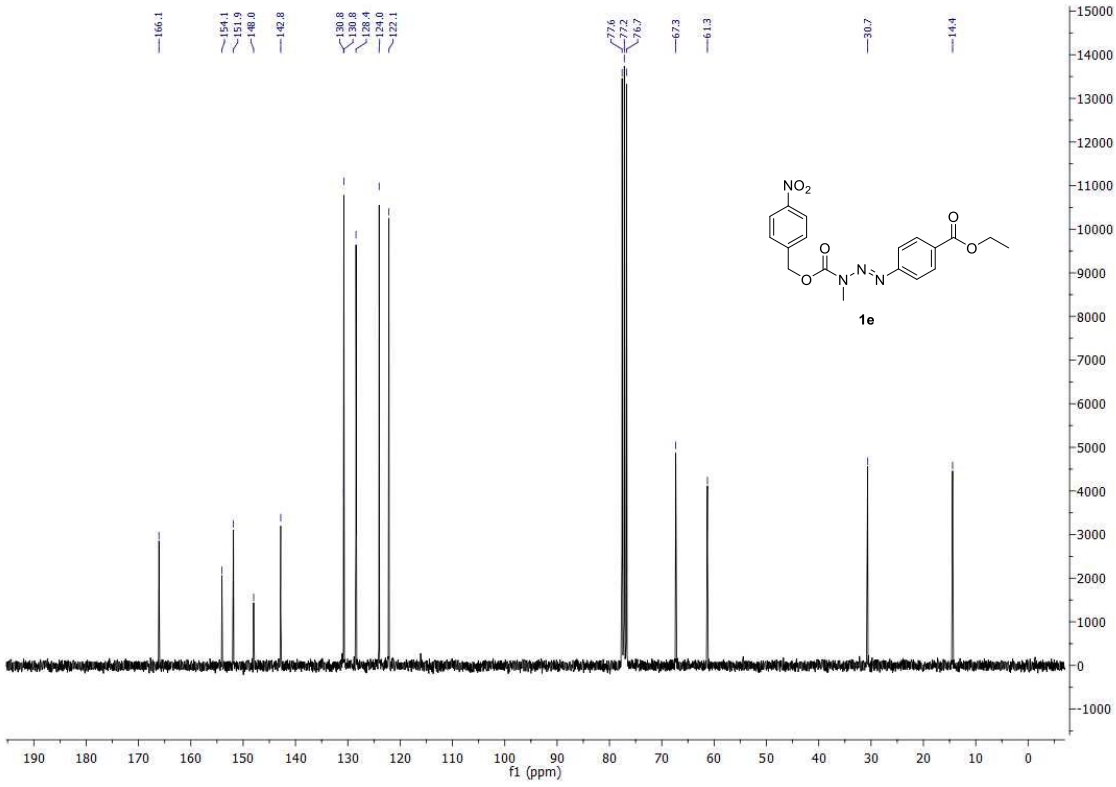


3-[4-nitrobenzyloxycarbonyl]-1-(4-ethoxycarbonylphenyl)-3-methyltriazene (1e)

^{1}H NMR ($CDCl_3$, 300 MHz)

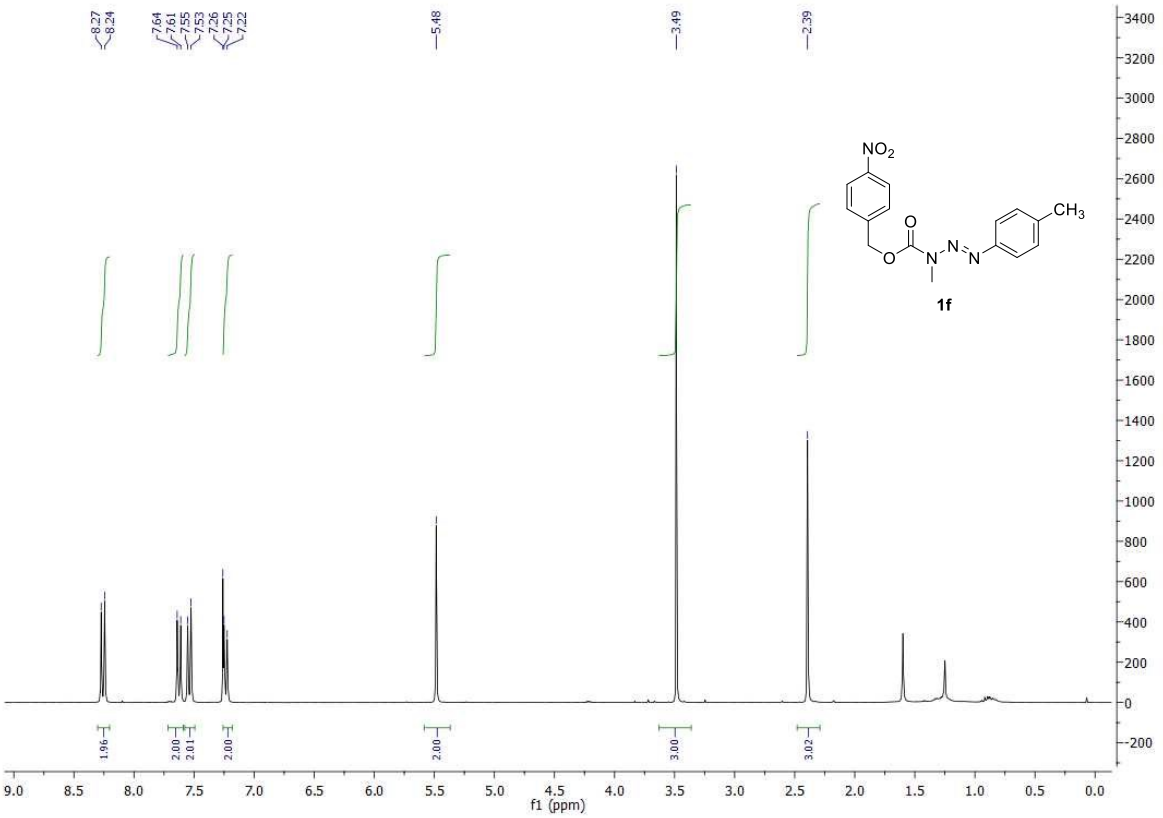


^{13}C NMR ($CDCl_3$, 75 MHz)

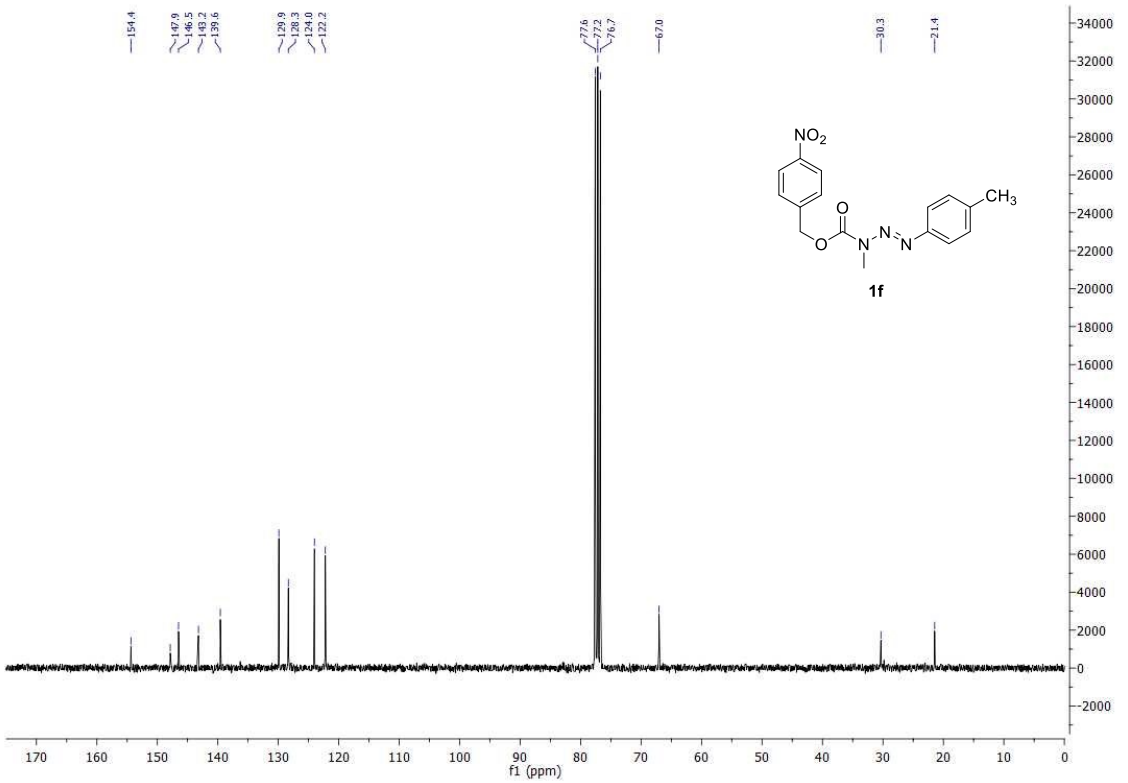


3-[4-nitrobenzyloxycarbonyl]-1-(4-tolyl)-3-methyltriazene (1f)

^{1}H NMR ($CDCl_3$, 300 MHz)

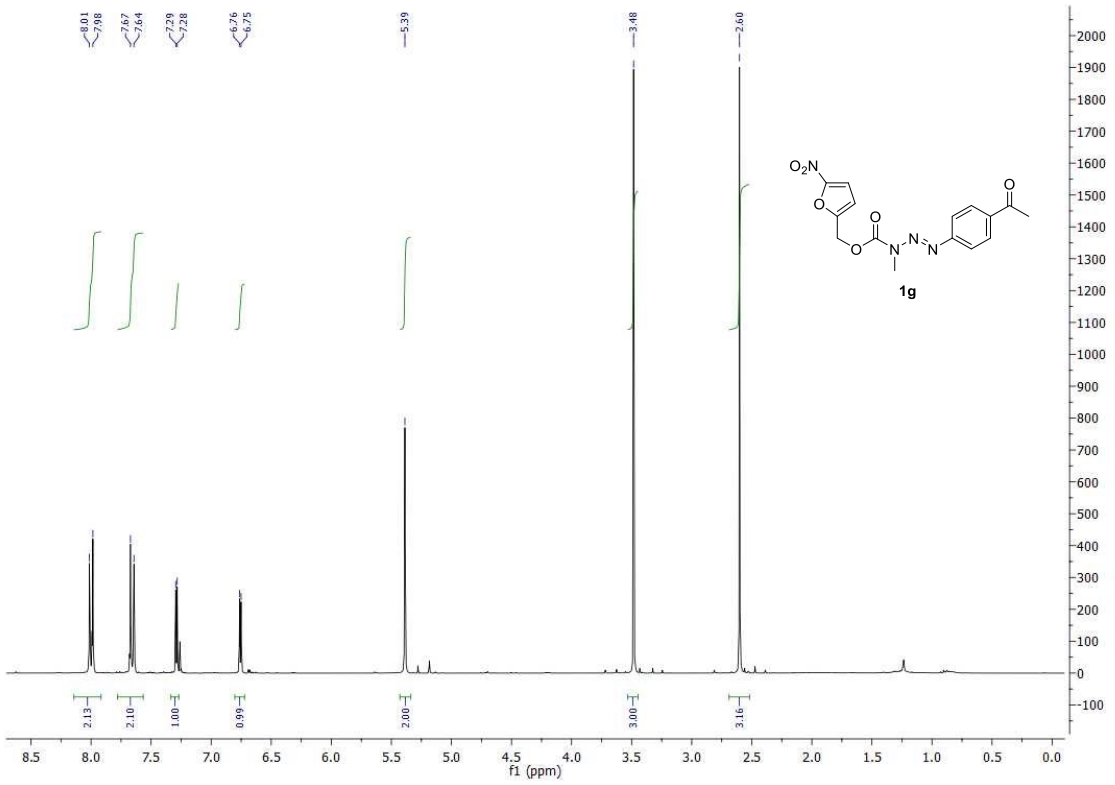


^{13}C NMR ($CDCl_3$, 75 MHz)

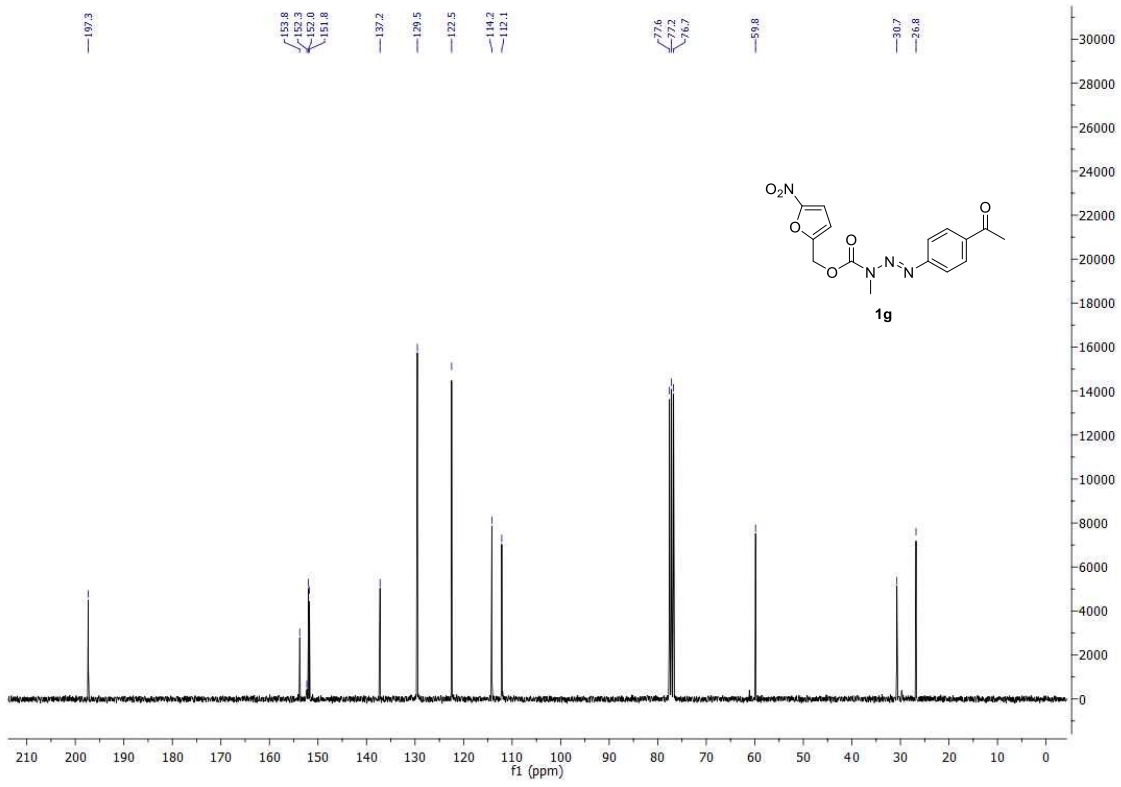


3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-acetylphenyl)-3-methyltriazene (1g)

^{1}H NMR ($CDCl_3$, 300 MHz)

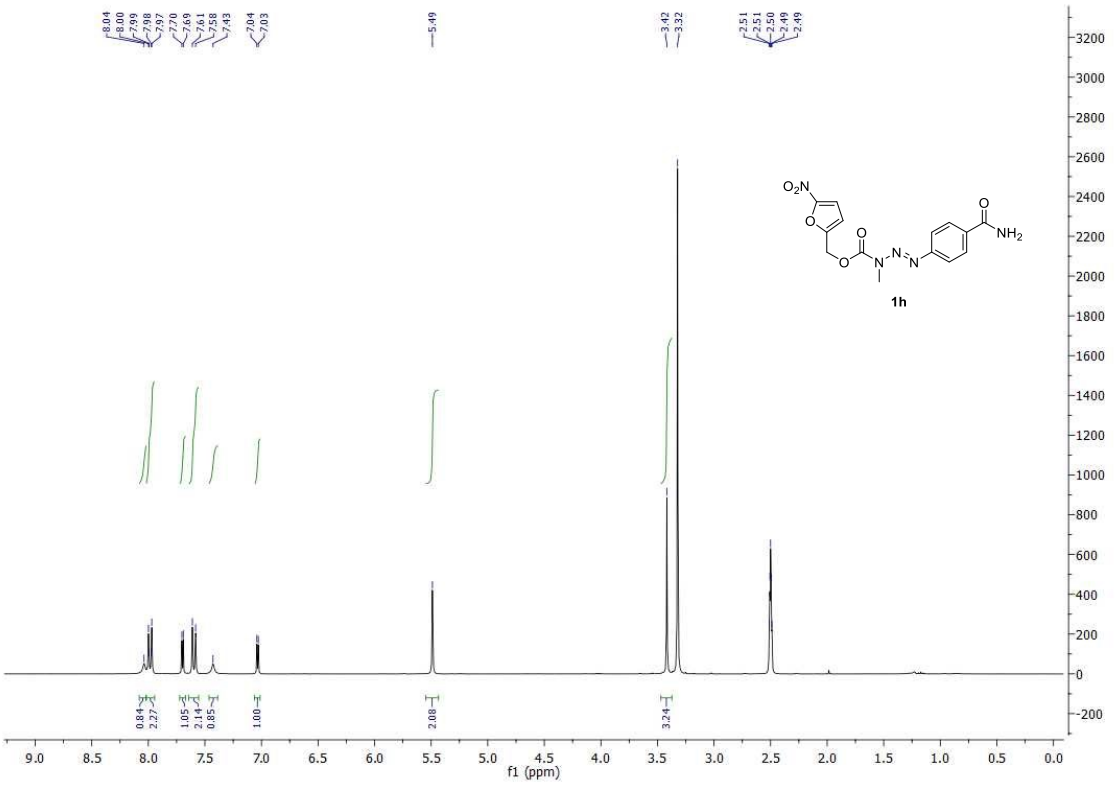


^{13}C NMR ($CDCl_3$, 75 MHz)

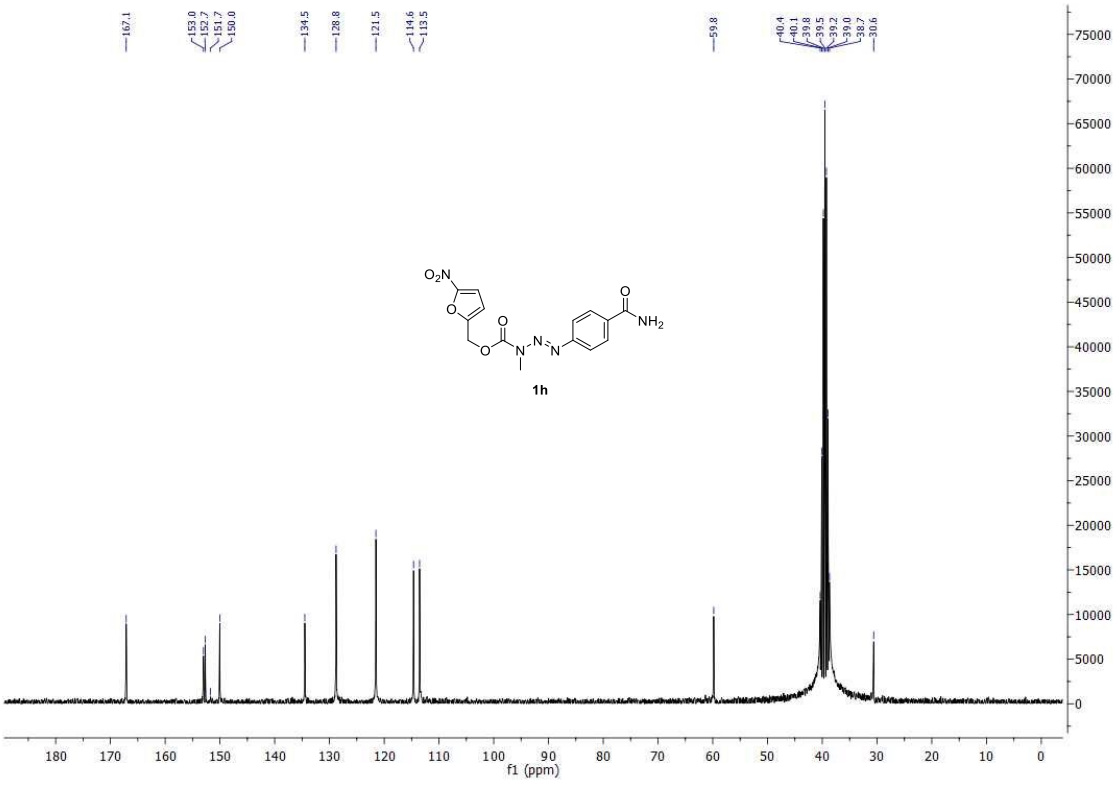


3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-carbamoylphenyl)-3-methyltriazene (1h)

^{1}H NMR ($(CD_3)_2SO$, 300 MHz)

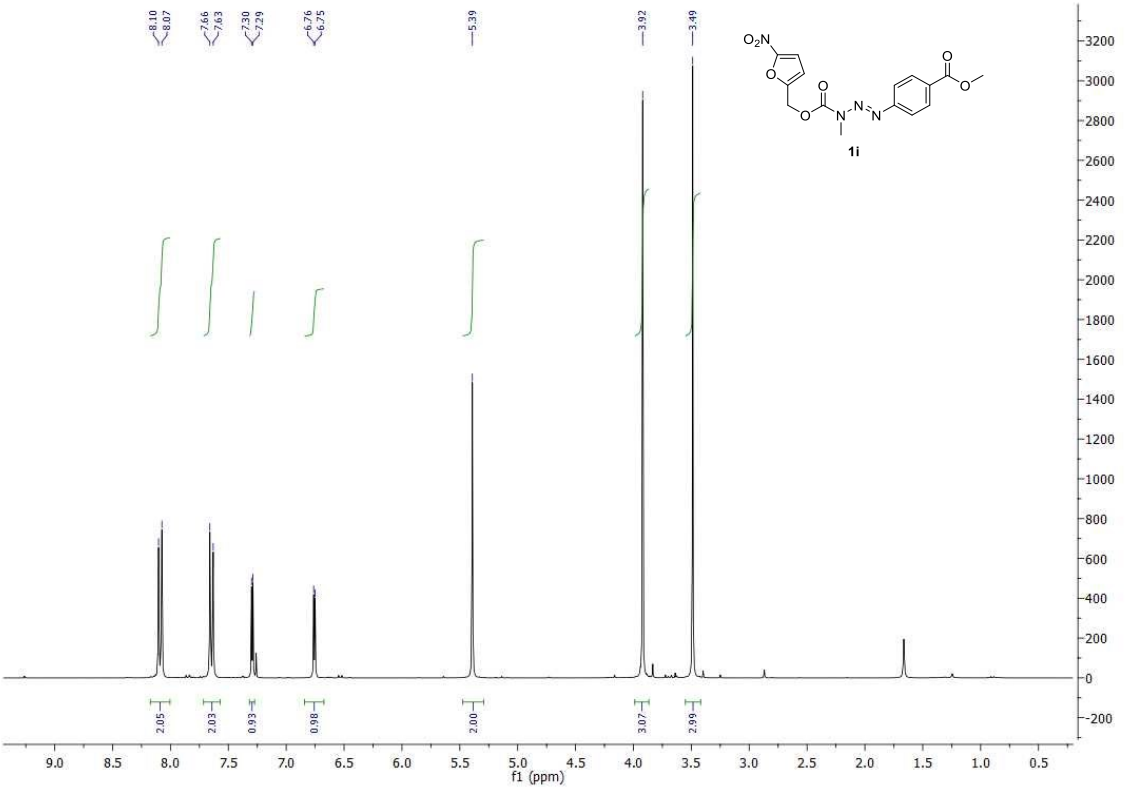


^{13}C NMR ($(CD_3)_2SO$, 75 MHz)

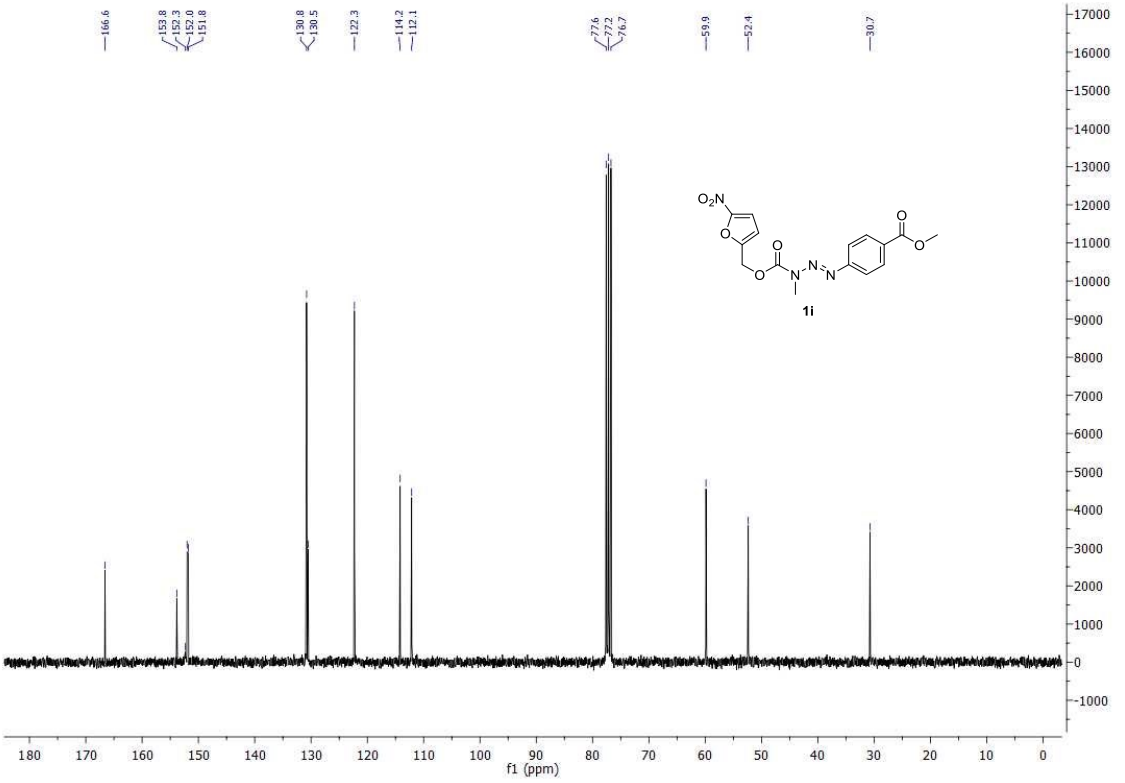


3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-methoxycarbonylphenyl)-3-methyltriazene (1i)

^{1}H NMR ($CDCl_3$, 300 MHz)

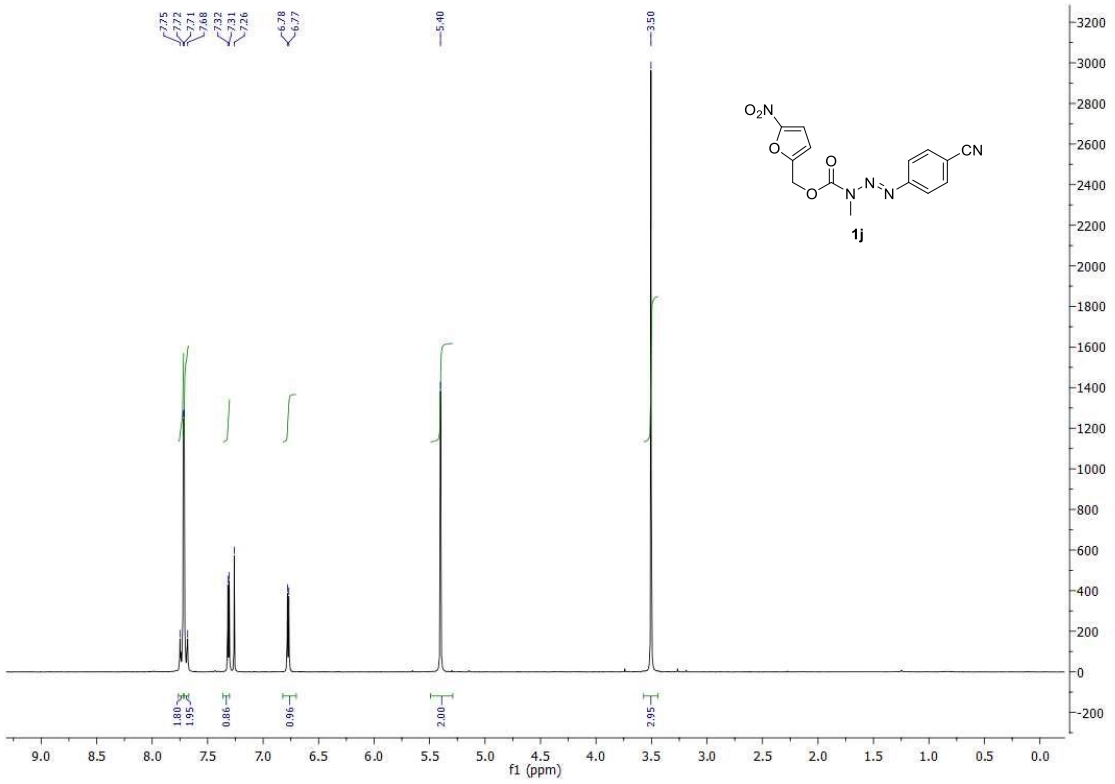


^{13}C NMR ($CDCl_3$, 75 MHz)

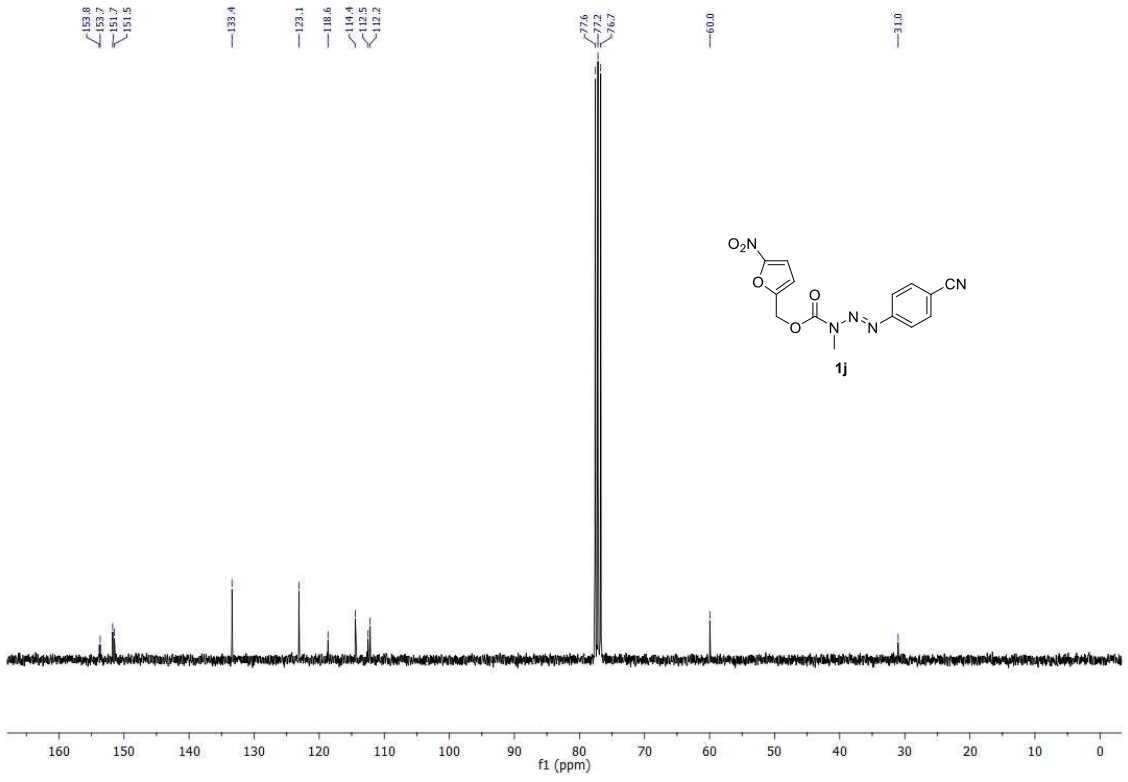


3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-cyanophenyl)-3-methyltriazene (1j)

^{1}H NMR ($CDCl_3$, 300 MHz)

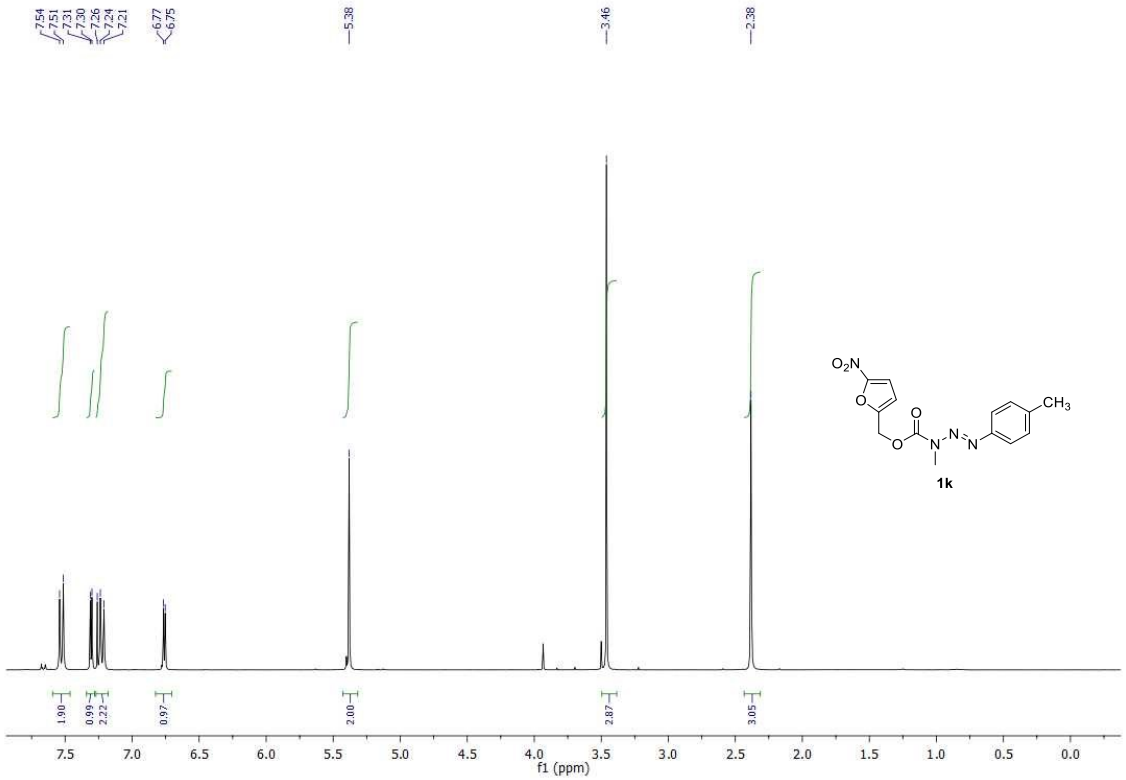


^{13}C NMR ($CDCl_3$, 75 MHz)

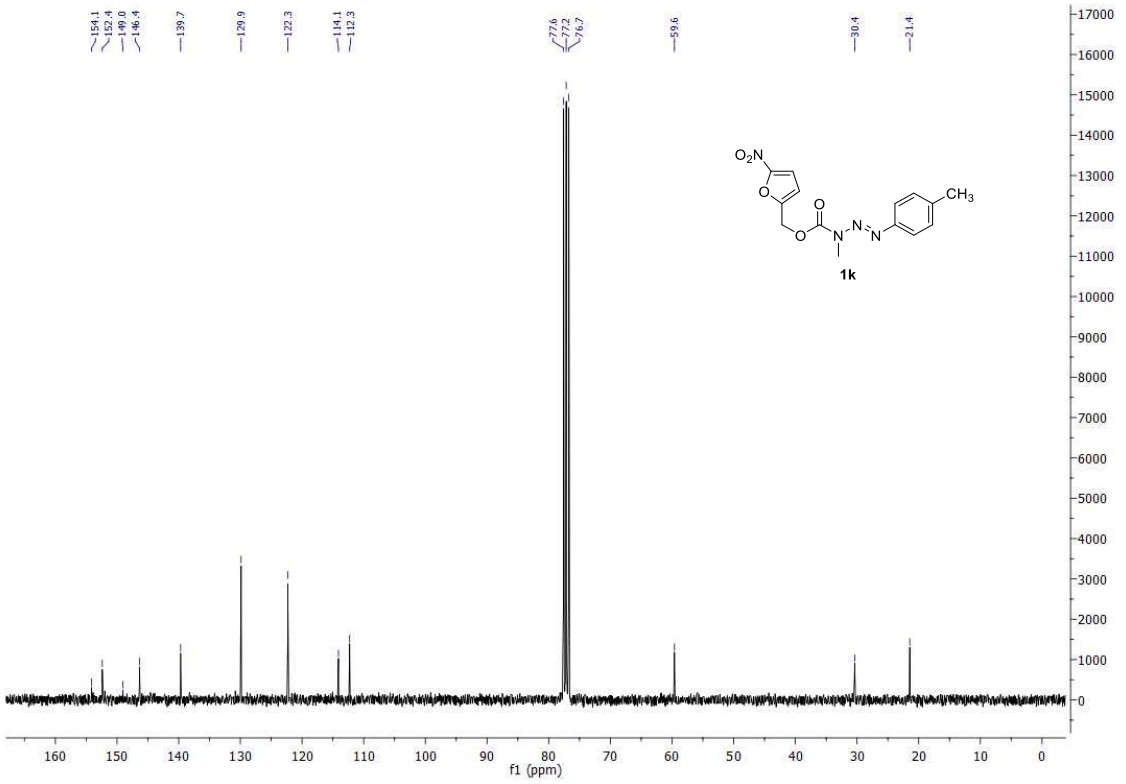


3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-tolyl)-3-methyltriazene (1k)

^{1}H NMR ($CDCl_3$, 300 MHz)

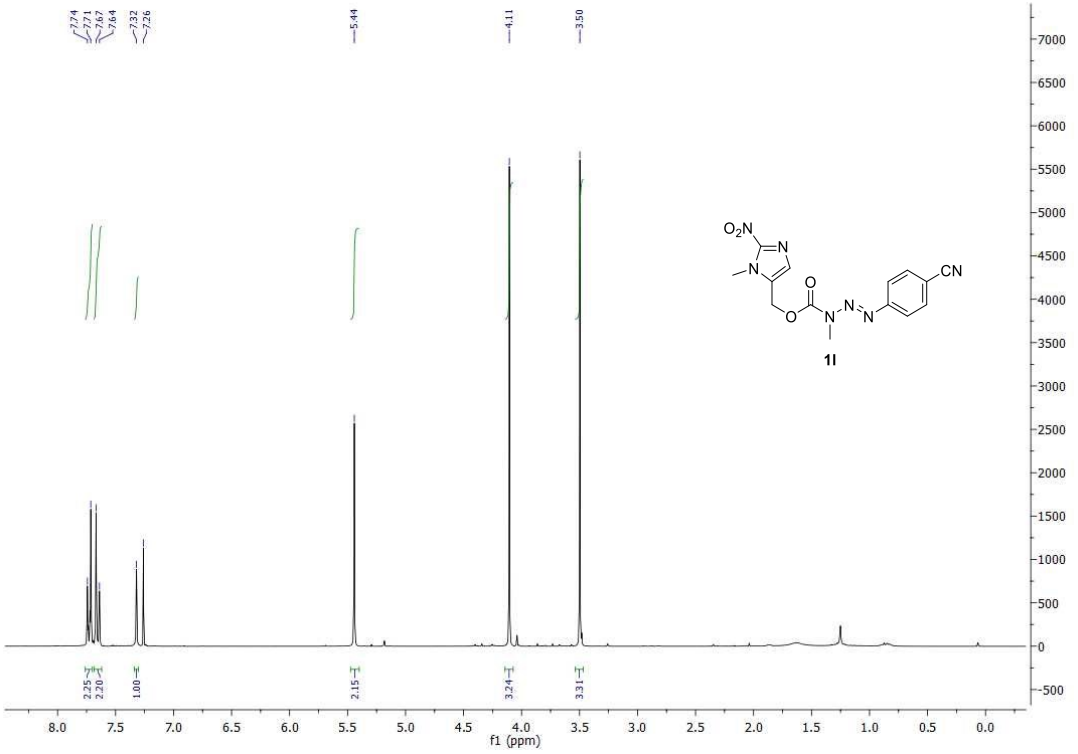


^{13}C NMR ($CDCl_3$, 75 MHz)

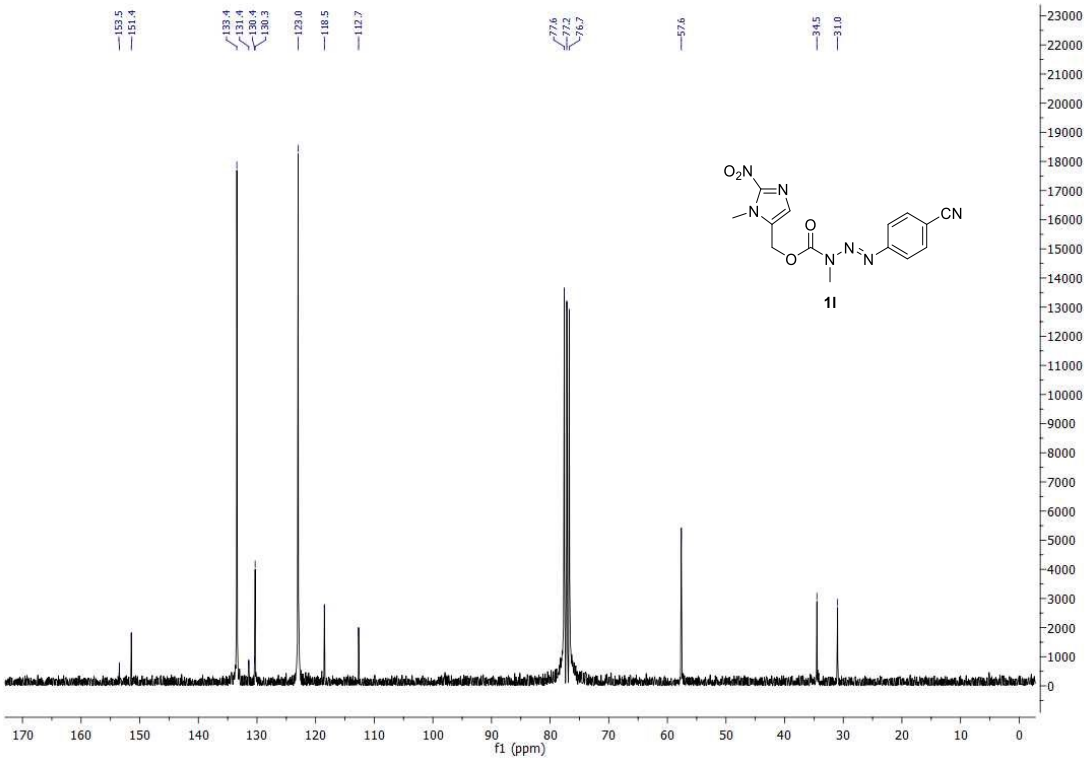


3-[(1-methyl-2-nitro-1H-imidazol-5-yl)methyloxycarbonyl]-1-(4-cyanophenyl)-3-methyltriazene (1l)

^{1}H NMR ($CDCl_3$, 300 MHz)

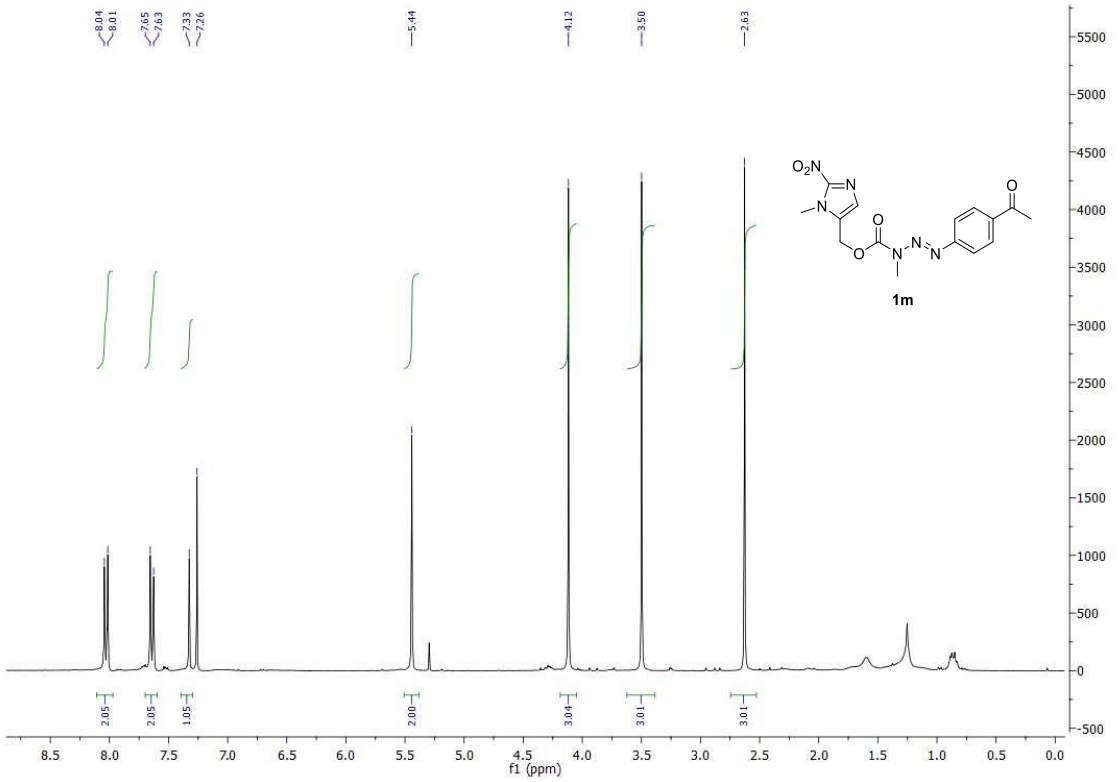


^{13}C NMR ($CDCl_3$, 75 MHz)



3-[(1-methyl-2-nitro-1H-imidazol-5-yl)methyloxycarbonyl]-1-(4-acetylphenyl)-3-methyltriazene (1m)

^{1}H NMR ($CDCl_3$, 300 MHz)



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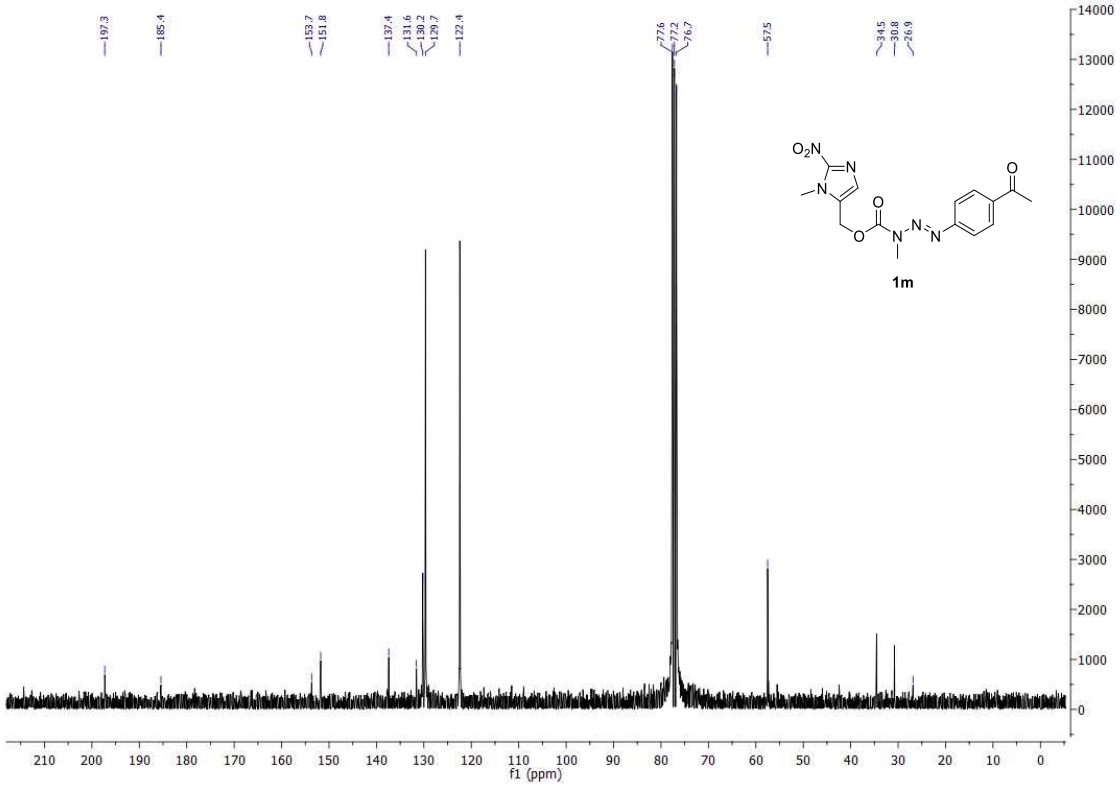
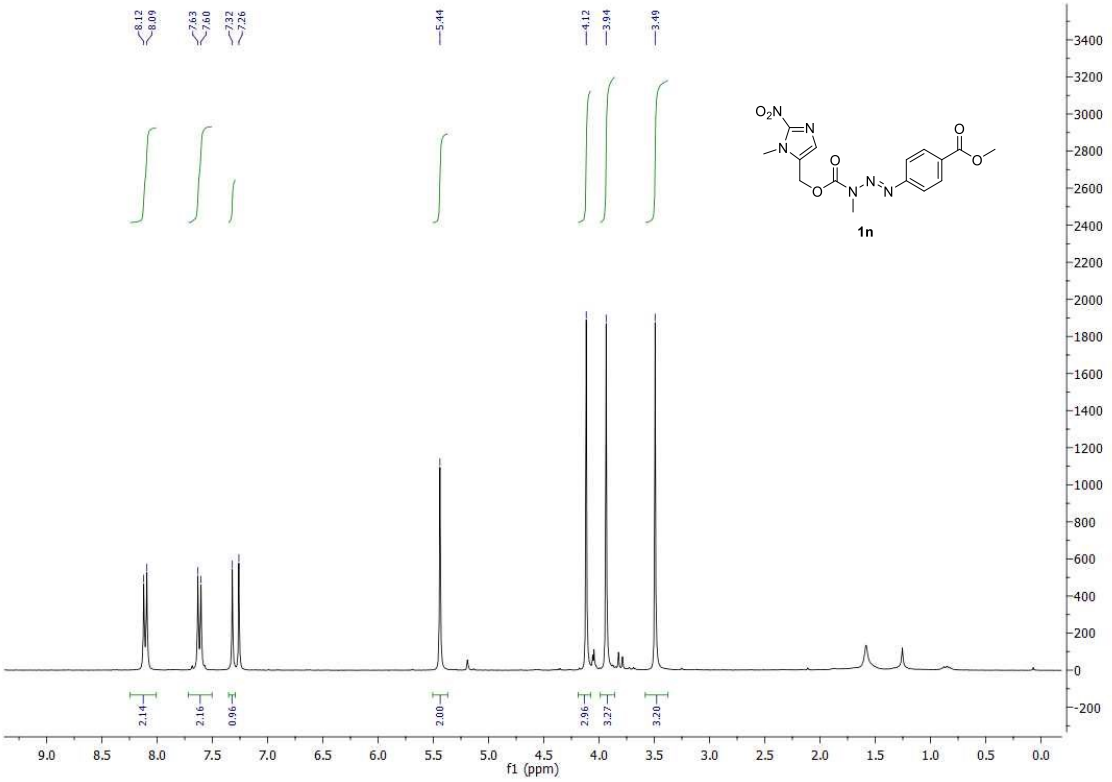
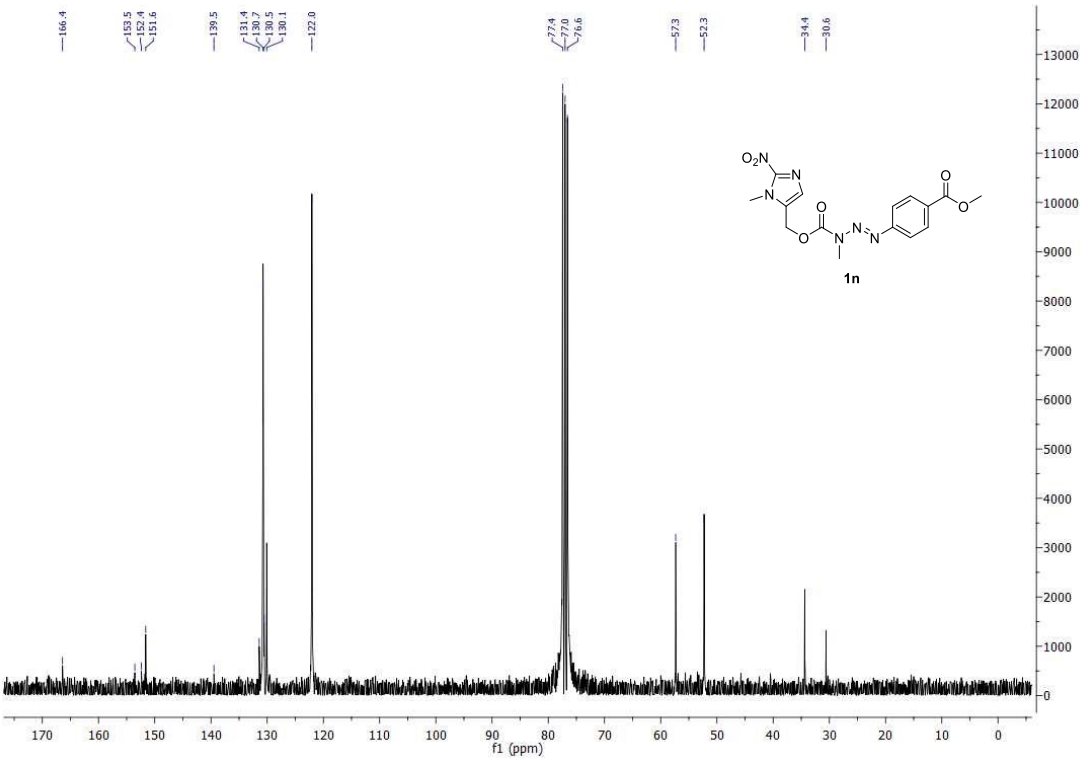


3-[(1-methyl-2-nitro-1H-imidazol-5-yl)methyloxycarbonyl]-1-(4-methoxycarbonylphenyl)-3-methyltriazene (1n)

^{1}H NMR ($CDCl_3$, 300 MHz)

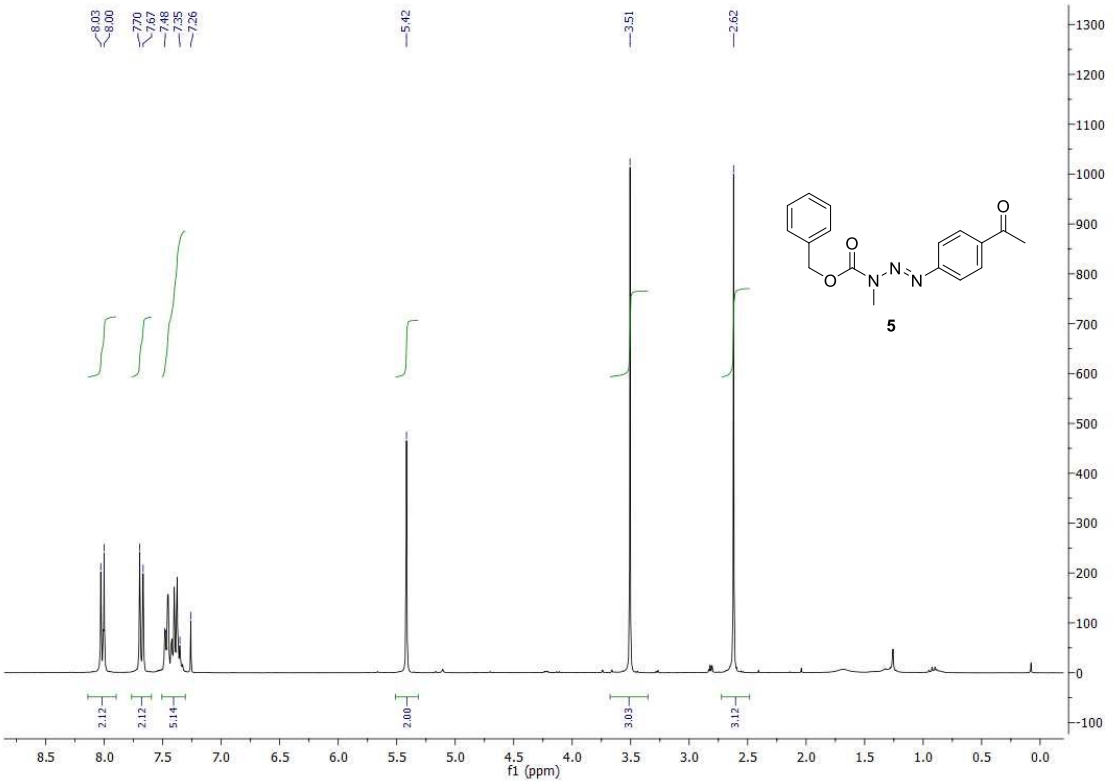


^{13}C NMR ($CDCl_3$, 75 MHz)

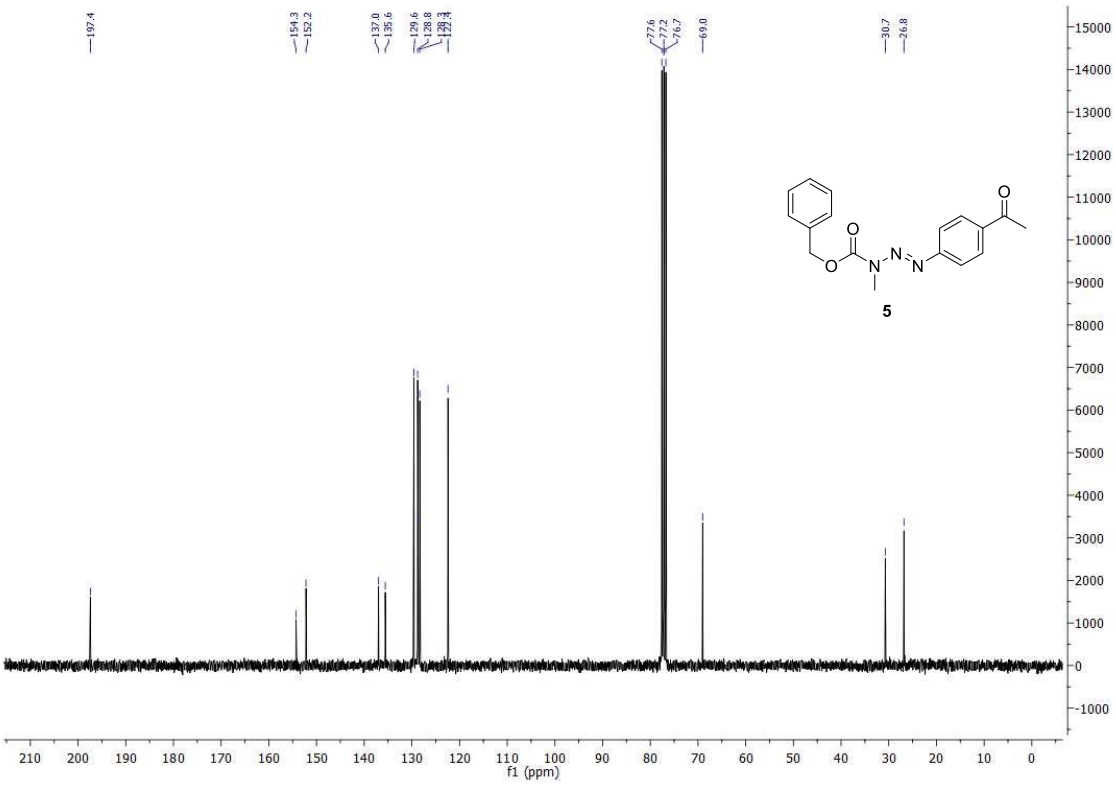


3-[benzyloxycarbonyl]-1-(4-acetylphenyl)-3-methyltriazene (5)

^{1}H NMR ($CDCl_3$, 300 MHz)

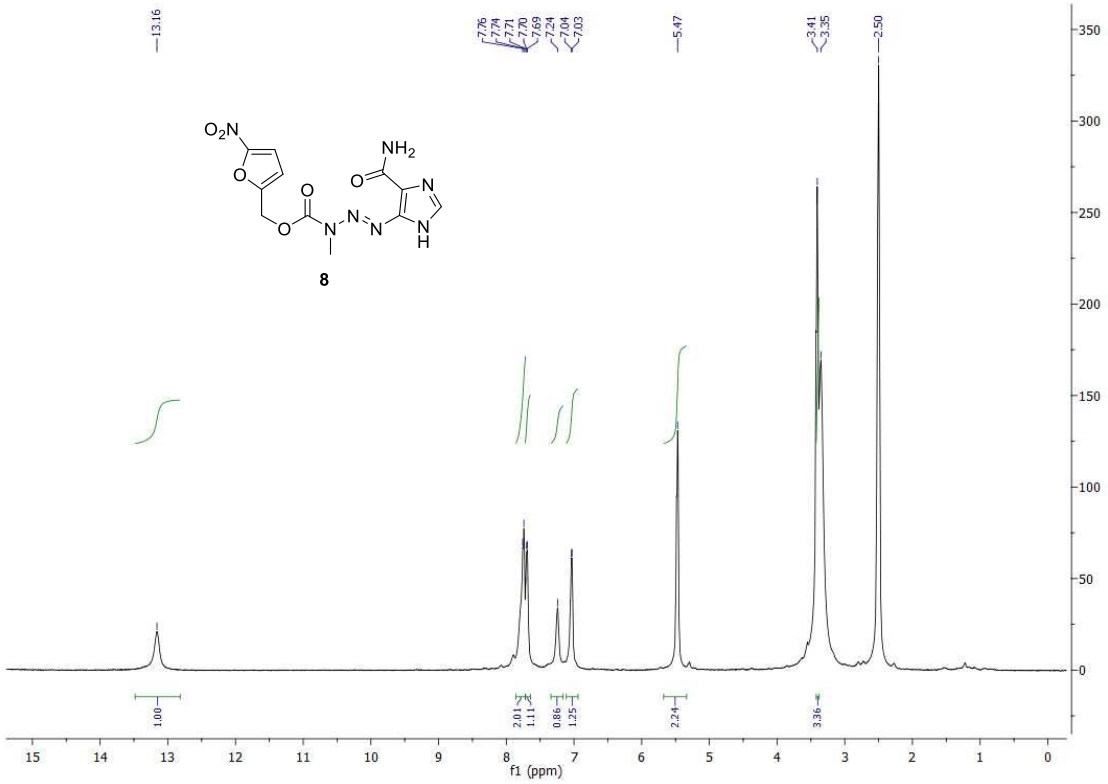


^{13}C NMR ($CDCl_3$, 75 MHz)

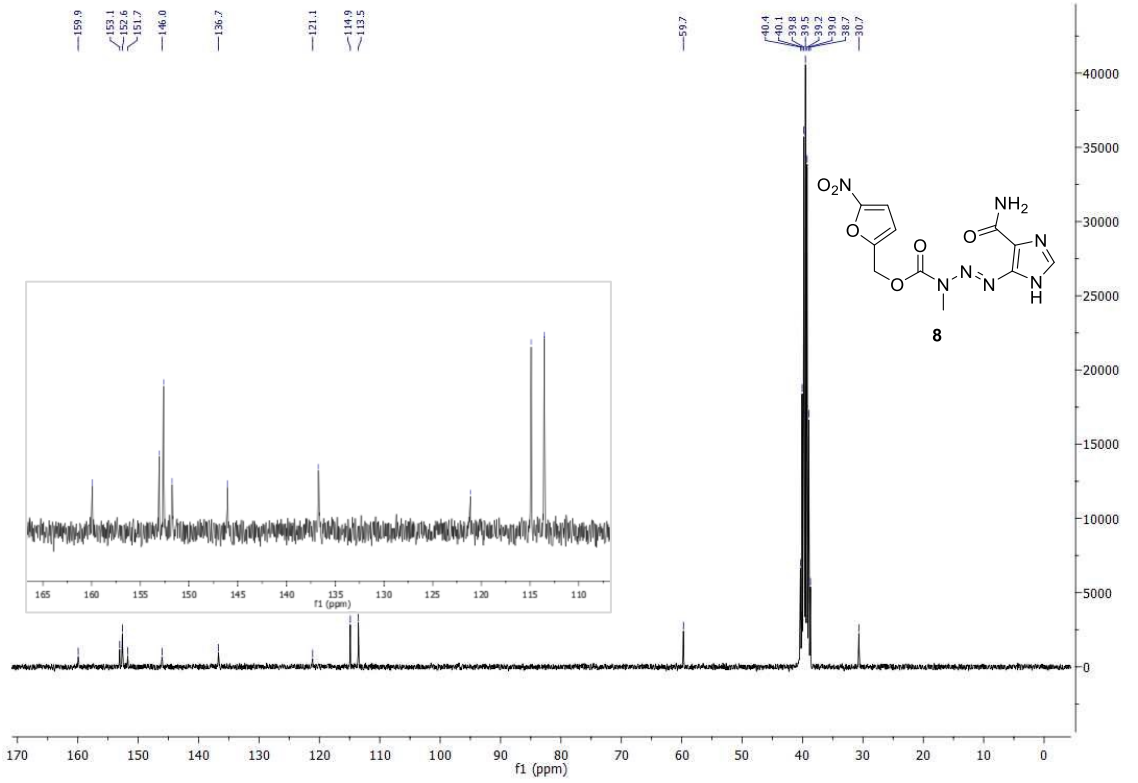


3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-carbamoyl-1H-imidazol-5-yl)-3-methyltriazene (8)

^{1}H NMR ($(CD_3)_2SO$, 300 MHz)



^{13}C NMR ($(CD_3)_2SO$, 75 MHz)



CNS MPO scores

Table S1 - CNS MPO scores of compounds **1a-n**, **8** and **TMZ**. cLogP values were calculated with ALOGPS2.1 software.[3] TPSA and pKa values were determined using Marvin Sketch 21.18 (ChemAxon). CNS MPO scores were calculated using the method reported by Wager et al.[4]

Compd.	MW	T0 MW	Log D	T0 LogD	Log P_{calc}	T0 cLogP	HBD	T0 HBD	TPSA	T0 TPSA	pKa	T0 pKa	MPO
1a	339.31	1	3.18	0.41	3.18	0.91	0	1	121.19	0	-2.15	1	4.3
1b	356.34	1	3.62	0.19	3.62	0.69	0	1	114.47	0.18	-2.48	1	4.1
1c	357.33	1	2.92	0.54	2.92	1	2	0.5	140.49	0	-0.43	1	4.0
1d	372.34	0.91	3.79	0.11	3.79	0.61	0	1	123.7	0	-2.82	1	3.6
1e	386.36	0.81	4.27	0	4.27	0.37	0	1	123.7	0	-2.82	1	3.2
1f	328.33	1	4.42	0	4.42	0.29	0	1	97.40	0.75	-0.98	1	4.0
1g	346.30	1	2.80	0.6	2.80	1	0	1	127.61	0	-2.43	1	4.6
1h	347.29	1	1.92	1	1.92	1	2	0.5	153.63	0	-0.43	1	4.5
1i	362.3	0.98	2.81	0.6	2.81	1	0	1	136.84	0	-2.71	1	4.6
1j	329.27	1	2.88	0.56	2.88	1	0	1	134.33	0	-2.12	1	4.6
1k	318.29	1	3.16	0.42	3.16	0.92	0	1	110.54	0.32	-0.97	1	4.7
1l	343.3	1	2.29	0.86	2.29	1	0	1	139.01	0	-0.39	1	4.9
1m	360.33	1	2.35	0.83	2.35	1	0	1	132.29	0	-0.39	1	4.8
1n	376.33	0.88	2.54	0.73	2.54	1	0	1	141.52	0	-0.4	1	4.6
8	337.25	1	0.13	1	0.51	1	3	0.17	182.31	0	1.69	1	4.2
TMZ	194.15	1	-1.00	1	-1.00	1	2	0.5	105.94	0.47	-3.57	1	5.0

Chemical reduction

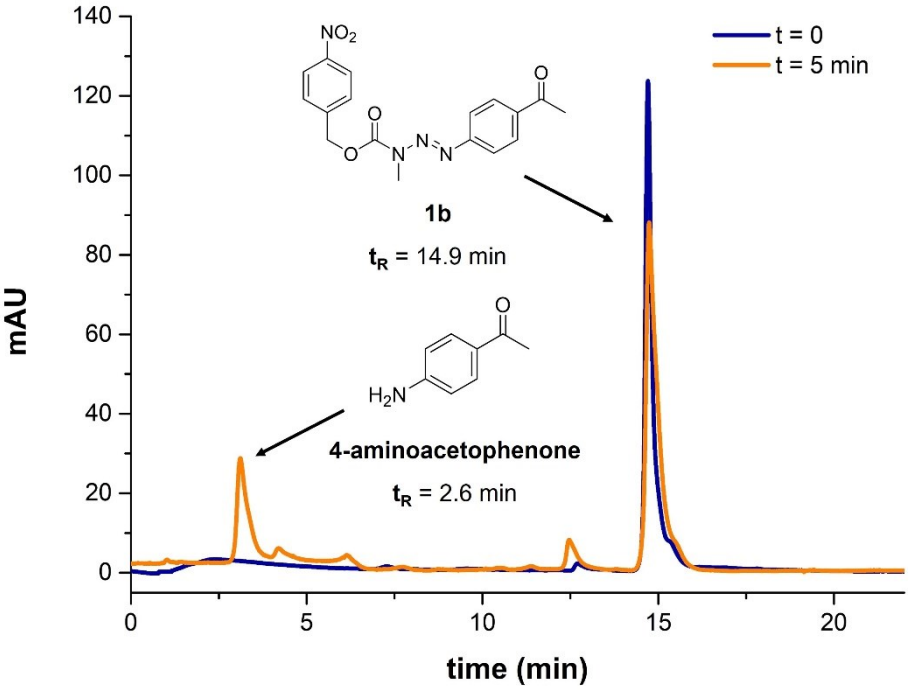


Figure S1 - HPLC traces of the chemical reduction assay of prodrug **1b**. t = 0 aliquot represents the control experiment and was taken before the addition of zinc dust.

Nitroreductase assays

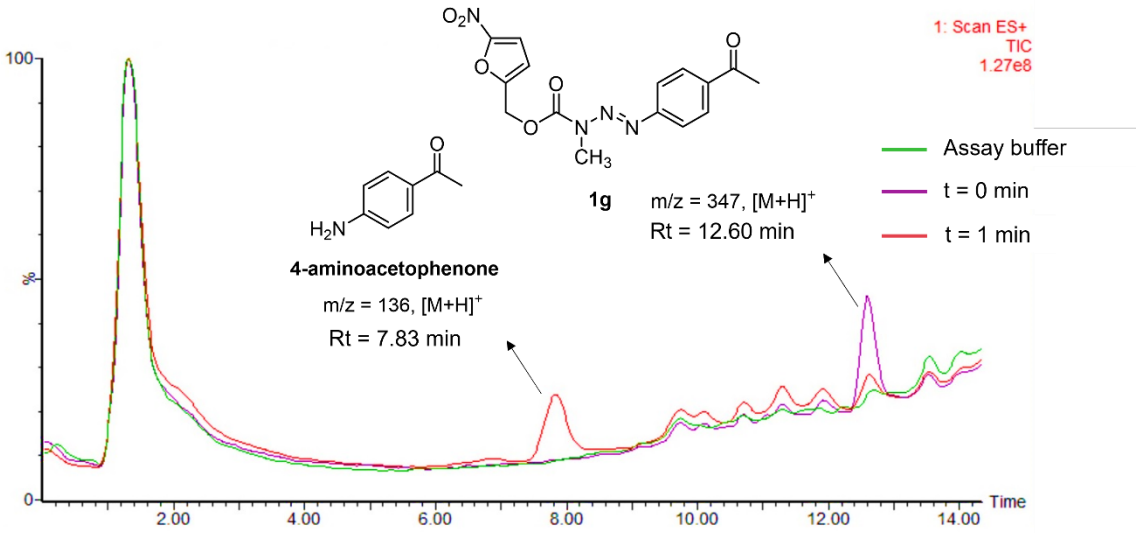


Figure S2 – Total ion count (TIC) mass spectrometry chromatograms of NTR-mediated bioreduction of prodrug **1g**. At t = 0 min, the aliquot was taken before NTR addition.

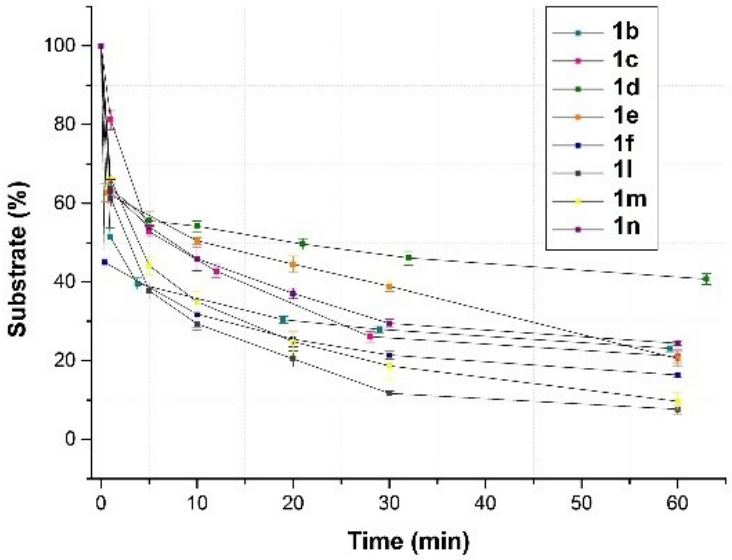


Figure S3 – Time course of the enzymatic reaction for prodrugs **1b-f** and **1l-n** over 60 minutes. Conditions: Prodrug (10 µM), NTR (10 µg mL^{-1}), NADH (500 µM) in PBS (0.01 M, pH = 7.4, 20% DMSO) at 37 °C.

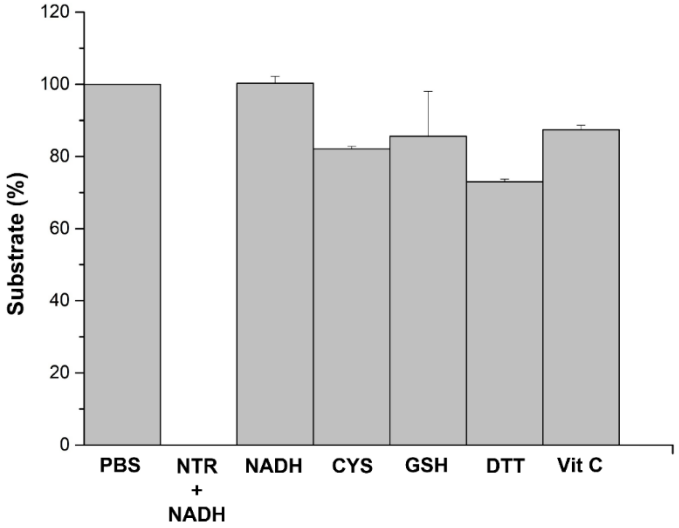


Figure S4 – Percentage of prodrug remaining after the incubation of **1j** with various biological reductant species. All measurements were performed in PBS (0.01 M, pH 7.4) with 20% DMSO. Concentrations: NTR (10 µg/mL), NADH (100 µM), GSH (1 mM), Cys (1 mM), DTT (1 mM), ascorbic acid (1 mM).

Biological evaluation

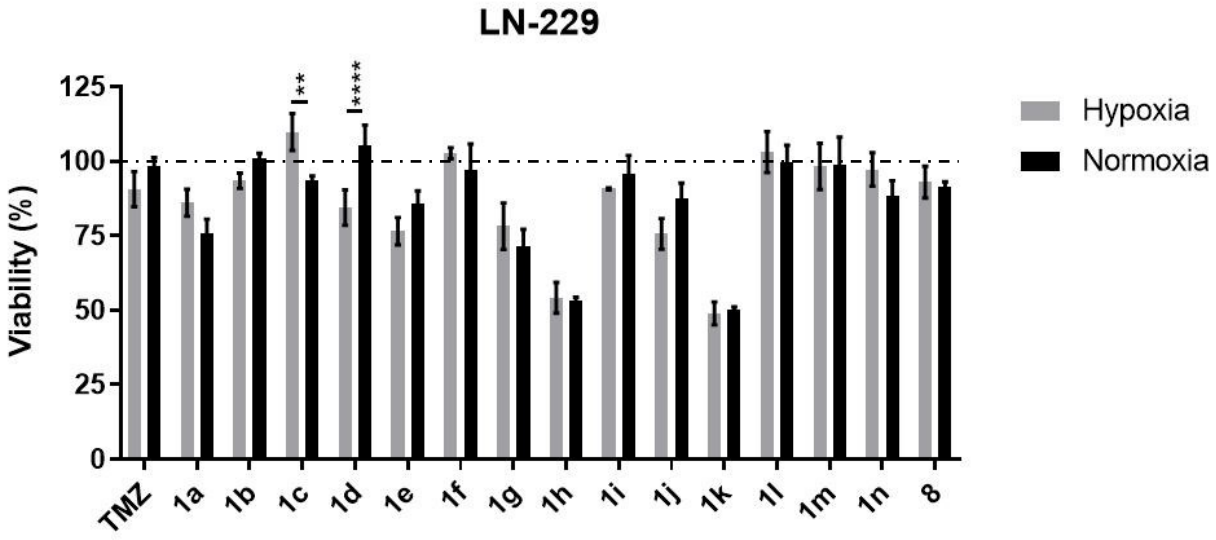


Figure S5. Cellular viability (%) in hypoxia and normoxia conditions obtained for LN-229 cells after exposure to 100 µM of temozolomide (TMZ) or compounds **1a-n** and **8**. 0.1 % (v/v) DMSO was used as vehicle control. Results presented as mean ± SD of two independent experiments. The statistical analysis was performed with One-way ANOVA followed by Dunnet's multiple comparisons test, where results were compared to DMSO. **p-value < 0.01; ****p-value < 0.0001 – GraphPad Prism®7 (GraphPad Software, San Diego, CA, USA).

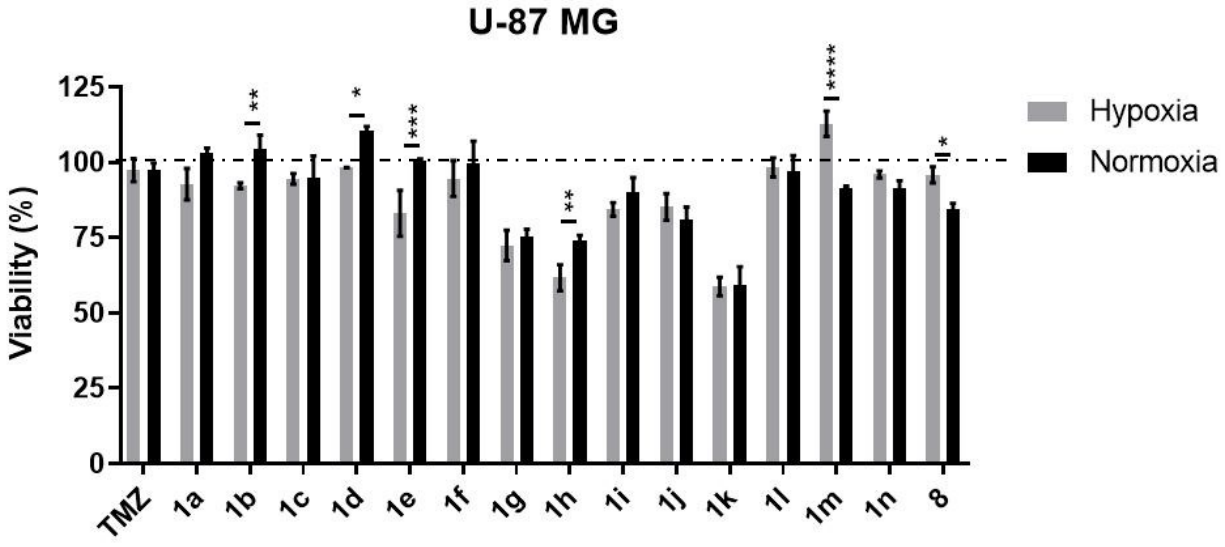


Figure S6. Cellular viability (%) in hypoxia and normoxia conditions obtained for U-87 MG cells after exposure to 100 µM of temozolomide (TMZ) or compounds **1a-n** and **8**. 0.1 % (v/v) DMSO was used as vehicle control. Results presented as mean ± SD of two independent experiments. The statistical analysis was performed with One-way ANOVA followed by Dunnet's multiple comparisons test, where results were compared to DMSO. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001; ****p-value < 0.0001 – GraphPad Prism®7 (GraphPad Software, San Diego, CA, USA).

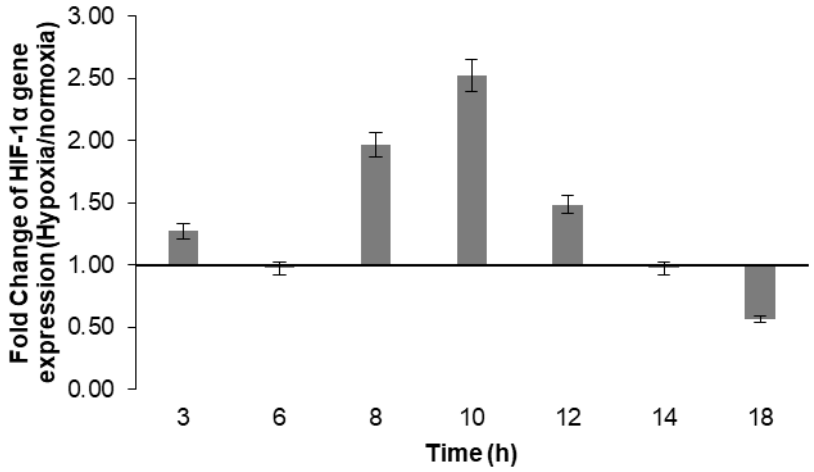


Figure S7 – *HIF-1α* gene expression (fold change) in hypoxic conditions (normalized to normoxic). Gene expression was measured over time (3-18h) in hypoxic or normoxic conditions and values calculated using $2^{-\Delta\Delta Ct}$ method. Results are the mean ± SD of at least two independent biological experiments.

Methods for *HIF-1α* gene expression

RNA Extraction

For RNA extraction assays, after the protocol described in the hypoxia chamber model, medium was removed, and cells were lysed with 300 µL of NZYol (NZYtech, Lisbon, Portugal). After 5 min incubation at room temperature, 150 µL of chloroform (≥99.9%, Merck, Darmstadt, Germany) was added and was vigorously shaken. Afterwards, a

centrifugation at 12,000× *g* for 5 min was performed, and the RNA from the upper aqueous phase was precipitated by adding 500 µL of isopropanol (≥99.5%, Merck, Darmstadt, Germany). After a 10 min incubation at room temperature, the solution was centrifuged at 12,000× *g* for 10 min at 4 °C. The pellet was resuspended in 500 µL of 75% (*v/v*) ethanol (≥99.8%, Merck, Darmstadt, Germany) and centrifuged at 7500× *g* for 5 min at 4 °C. The precipitated RNA was air dried for 5–10 min, resuspended in DEPC-treated water, and incubated in a heat block at 60 °C for 10 min. The quality and concentration of isolated RNA was assessed using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity of each sample was evaluated by denaturing agarose gel electrophoresis (0.8% (*w/v*) agarose), and samples were stored at −80 °C. Gel images were captured using the Gel Doc XR system and Quantity One 1-D analysis software (Bio-Rad Laboratories, Hercules, CA, USA).

RNA Purification

For RNA purification, 1 µg RNA was incubated with 1 µL NZY DNase I (NZYtech, Lisbon, Portugal) at 37 °C for 20 min. Then, 200 µL absolute ethanol (Merck, Darmstadt, Germany) was added, and samples were stored at −20 °C overnight. On the following day, a centrifugation at 15,000× *g* for 15 min at 4 °C was performed, precipitated RNA was suspended in 100 µL of 75% (*v/v*) ethanol, and further centrifuged at 15,000× *g* for 15 min at 4 °C. The pellet was air dried and afterwards was hydrated with DEPC-treated water and incubated in a heat block at 60 °C for 10 min. The quality and concentration of isolated RNA was assessed using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and purified RNA was stored at −80 °C.

cDNA Synthesis

The cDNA was synthesized from 100 ng of RNA using an NZY M-MuLV First-Strand cDNA Synthesis Kit (NZYtech, Lisbon, Portugal) for a final volume of 10 µL. Samples were incubated in DNA Engine® Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) at 25 °C for 10 min, 37 °C for 50 min, and 85 °C for 5 min. Afterwards, 0.5 µL of

RNase H (NZYtech, Lisbon, Portugal) was added and samples were stirred in a vortex and incubated in a heat block at 37 °C for 20 min. The synthetized cDNA was stored at −20 °C for further analysis.

RT-qPCR

The *HIF-1α* gene expression was analyzed by quantitative reverse transcription polymerase chain reaction (RT-qPCR) using the synthetized cDNA as a template. The 18S ribosomal RNA (*18S*) gene was used as an endogenous control. Relative gene expression levels were quantified based on the threshold cycle ($2^{-\Delta\Delta CT}$) method[5],

where:

$\Delta\Delta CT = (CT_{HIF\text{-}1\alpha} - CT_{18S})\text{sample} - (CT_{HIF\text{-}1\alpha} - CT_{18S})\text{calibrator}$

was used to analyze gene expression obtained using the hypoxia chamber model, the reaction mixture for RT-qPCR was prepared according to the manufacturer's instructions, and RT-qPCR was performed in a Rotor-Gene™ 6000 (Corbett Life Science, Cambridge, UK).

For hypoxia inducible factor 1 α (*HIF-1α* gene), the 20 µL reaction mixture was composed of 10 µL NZY qPCR Green Master Mix (NZYtech, Lisbon, Portugal), 2 µL cDNA, and 0.2 µM of forward and reverse gene-specific primers for *18S* and *HIF-1α* (STAB VIDA, Setúbal, Portugal), depicted below. The conditions included an initial denaturation at 95 °C for 2 min, followed by 10 cycles of amplification consisting of denaturation at 95 °C for 20 s, annealing at 50 °C for 10 s, and extension at 72 °C for 20 s and by 30 cycles amplification consisting of denaturation at 95 °C for 20 s, annealing at 58 °C for 10 s, and extension at 72 °C for 20 s.

Forward and Reverse Primers

HIF1α F– 5' TTG ATG GGA TAT GAG CCA GA–3'

HIF1α R 5'– TGT CCT GTG ACT TGT CC– 3'

18S F 5'– GTA ACC CGT TGA ACC CCA TT–3'

18S R 5'– CCA TCC AAT CGG TAG CG–3'

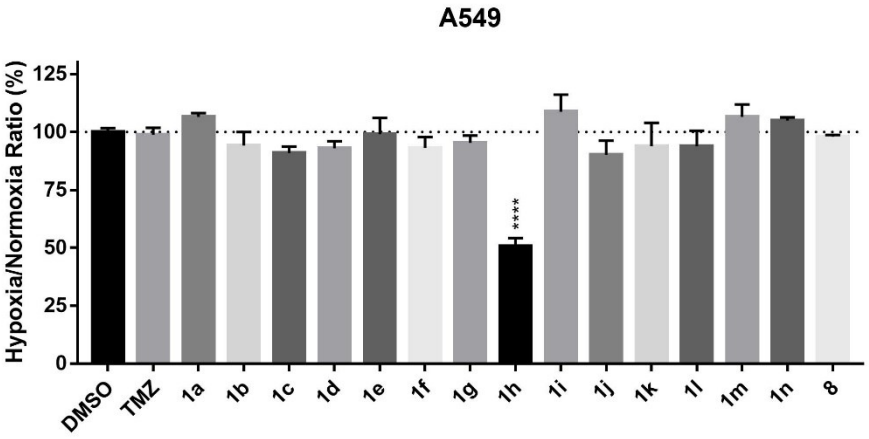


Figure S8 – Cellular viability ratio between hypoxia and normoxia conditions (%) obtained for A549 cells after exposure to 100 µM of temozolomide (TMZ) or the synthetized compounds **1a-n** and **8**. Results presented as mean ± SD of two independent experiments. The statistical analysis was performed with One-way ANOVA followed by Dunnet's multiple comparisons test, where results were compared to DMSO. ****p-value < 0.0001 - GraphPad Prism®7 (GraphPad Software, San Diego, CA, USA).

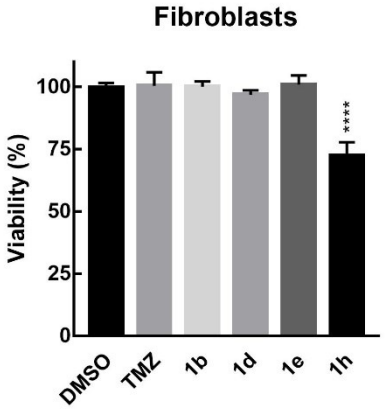


Figure S9 – Cellular viability (%) obtained for fibroblasts in normoxia conditions after exposure to 100 µM of temozolomide (TMZ) or prodrugs **1b**, **1d**, **1e** or **1h**. 0.1% (v/v) DMSO was used as vehicle control. Results presented as mean ± SD of two independent experiments. The statistical analysis was performed with One-way ANOVA followed by Dunnet's multiple comparisons test, where results were compared to DMSO. ****p-value < 0.0001 – GraphPad Prism®7 (GraphPad Software, San Diego, CA, USA).

Stability in cell culture medium

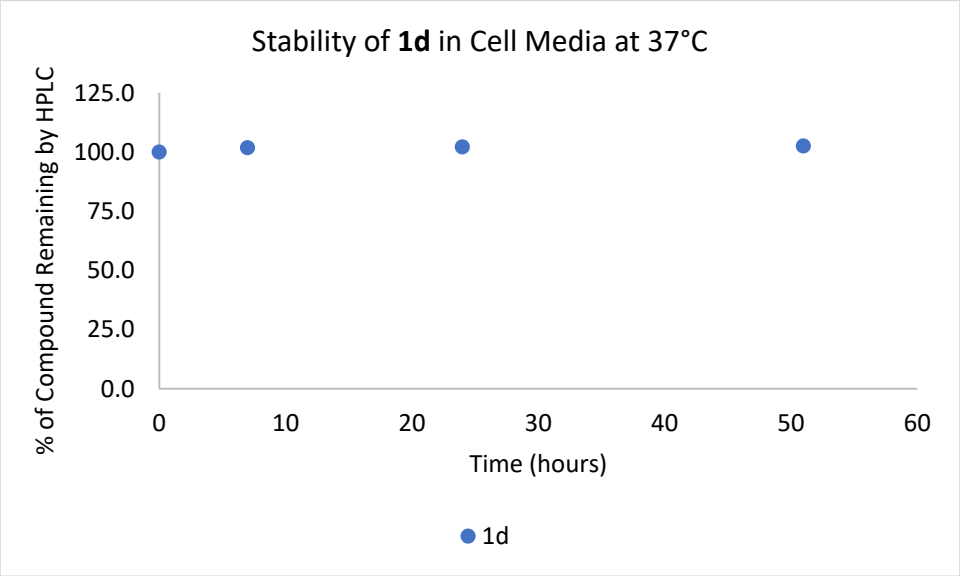
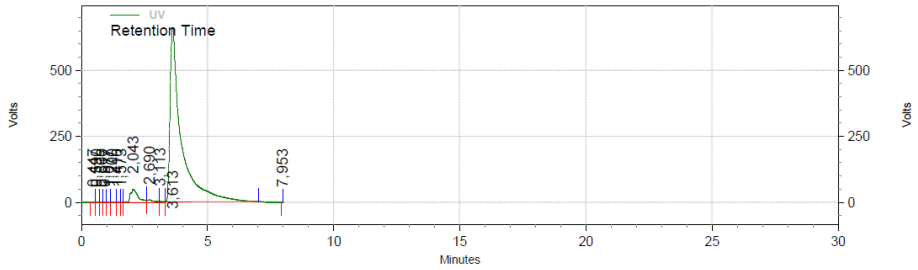


Figure S10 – Stability of compound **1d** in cell culture medium (DMEM supplemented with 10% FBS (v/v) and 1% (v/v) antibiotic–antimycotic solution) at 37°C, during 48h.

Time (h)	Percentage of remaining compound (%)
0	100,0
7	101,8
24	102,1
51	102,5

Chromatograms - 1d

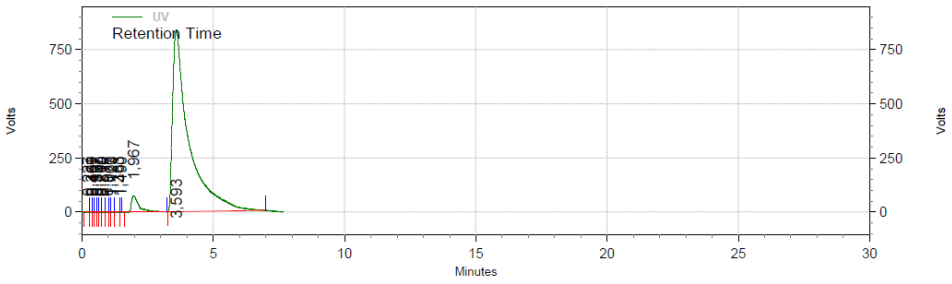
t = 0



UV Results

Retention Time	Area	Area %	Height	Height %
0,447	589	0,00	90	0,00
0,590	664	0,00	115	0,00
0,800	390	0,00	74	0,00
0,917	190	0,00	52	0,00
1,077	978	0,00	145	0,01
1,200	2331	0,00	211	0,01
1,410	610	0,00	97	0,00
1,573	237	0,00	69	0,00
2,043	4065774	4,74	196652	6,81
2,690	725425	0,85	36856	1,28
3,113	148809	0,17	12290	0,43
3,613	80862641	94,24	2642408	91,46
7,953	53	0,00	39	0,00
Totals	85808691	100,00	2889098	100,00

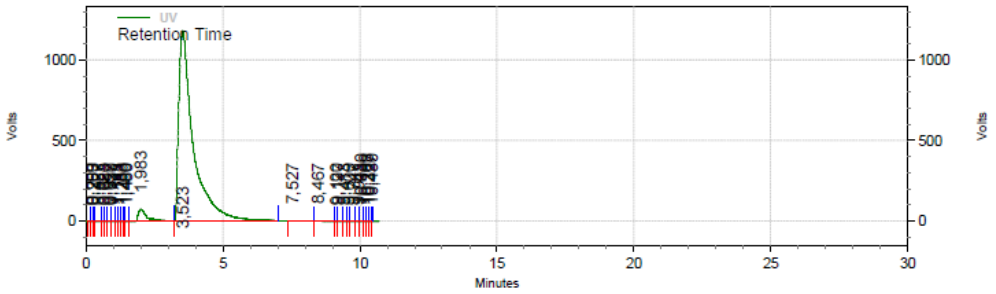
t = 7h



UV Results

Retention Time	Area	Area %	Height	Height %
0,227	559	0,00	83	0,00
0,360	220	0,00	74	0,00
0,407	262	0,00	66	0,00
0,537	216	0,00	60	0,00
0,597	158	0,00	69	0,00
0,700	117	0,00	54	0,00
0,810	345	0,00	70	0,00
0,977	223	0,00	43	0,00
1,060	212	0,00	67	0,00
1,123	321	0,00	73	0,00
1,383	612	0,00	66	0,00
1,490	148	0,00	56	0,00
1,967	6065367	4,05	300477	8,22
3,593	143872258	95,95	3354463	91,76
Totals	149941018	100,00	3655721	100,00

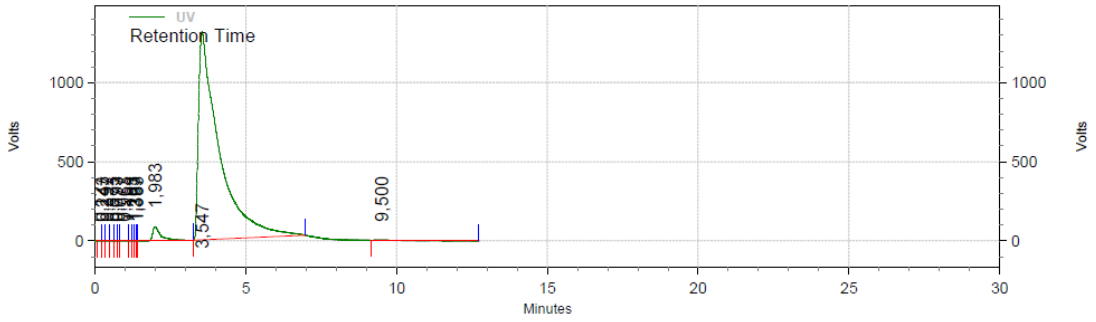
t = 24h



UV Results

Retention Time	Area	Area %	Height	Height %
0,050	456	0,00	100	0,00
0,200	383	0,00	82	0,00
0,253	203	0,00	65	0,00
0,427	761	0,00	95	0,00
0,623	297	0,00	71	0,00
0,680	451	0,00	101	0,00
0,857	424	0,00	77	0,00
0,930	589	0,00	99	0,00
1,113	369	0,00	107	0,00
1,183	446	0,00	86	0,00
1,283	225	0,00	61	0,00
1,400	187	0,00	59	0,00
1,480	189	0,00	67	0,00
1,983	6451998	3,57	295335	5,89
3,523	174192118	96,26	4711528	93,90
7,527	218061	0,12	4682	0,09
8,467	79038	0,04	3231	0,06
9,120	1659	0,00	281	0,01
9,197	2574	0,00	272	0,01
9,413	1304	0,00	179	0,00
9,600	647	0,00	125	0,00
9,747	1116	0,00	133	0,00
9,943	936	0,00	115	0,00
10,030	782	0,00	126	0,00
10,190	403	0,00	112	0,00
10,263	474	0,00	99	0,00
10,327	403	0,00	88	0,00
10,430	4	0,00	0	0,00
Totals	180956497	100,00	5017376	100,00

t = 51h



UV Results

Retention Time	Area	Area %	Height	Height %
0,127	577	0,00	85	0,00
0,243	374	0,00	98	0,00
0,397	435	0,00	78	0,00
0,573	271	0,00	60	0,00
0,693	196	0,00	69	0,00
0,777	292	0,00	71	0,00
0,963	929	0,00	115	0,00
1,193	232	0,00	65	0,00
1,263	150	0,00	54	0,00
1,337	102	0,00	40	0,00
1,383	70	0,00	36	0,00
1,983	7296955	2,81	353034	6,27
3,547	250536249	96,63	5264142	93,42
9,500	1425846	0,55	17014	0,30
Totals	259262678	100,00	5634961	100,00

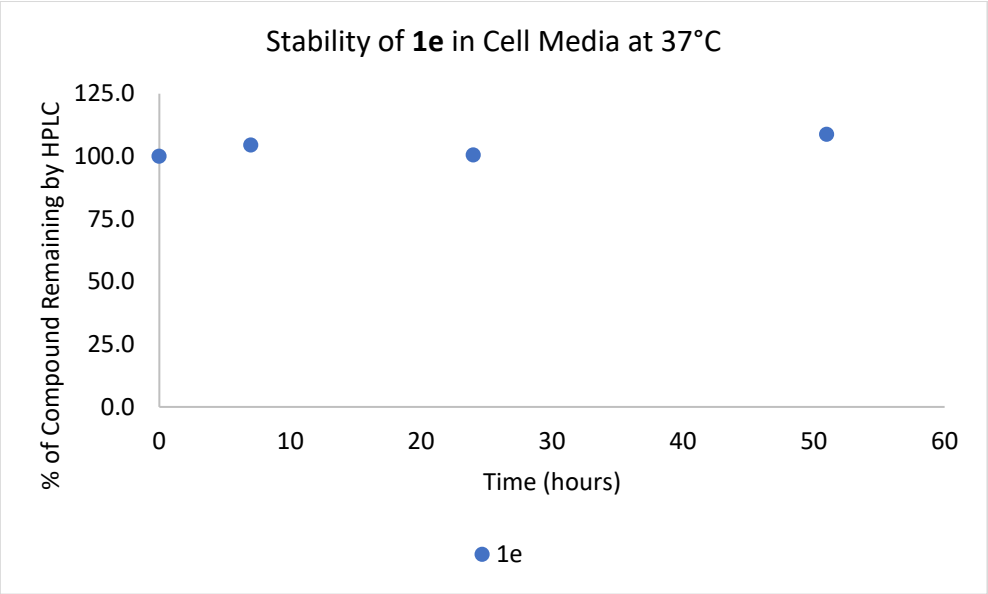
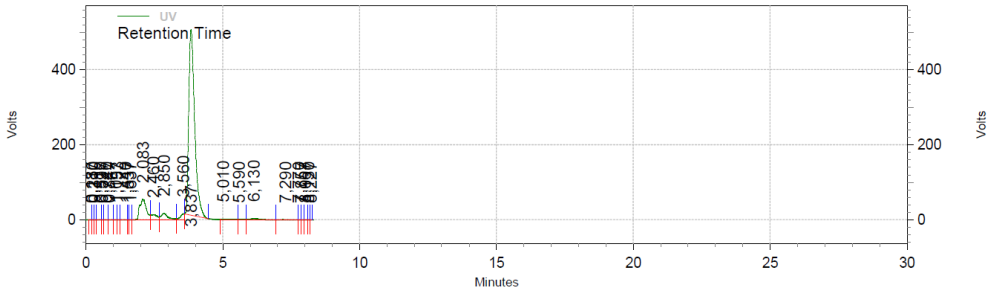


Figure S11 – Stability of compound **1e** in cell culture medium (DMEM supplemented with 10% FBS (v/v) and 1% (v/v) antibiotic–antimycotic solution) at 37°C, during 48h.

Time (h)	Percentage of remaining compound (%)
0	100,0
7	104,4
24	100,5
51	108,8

Chromatograms – 1e

t = 0

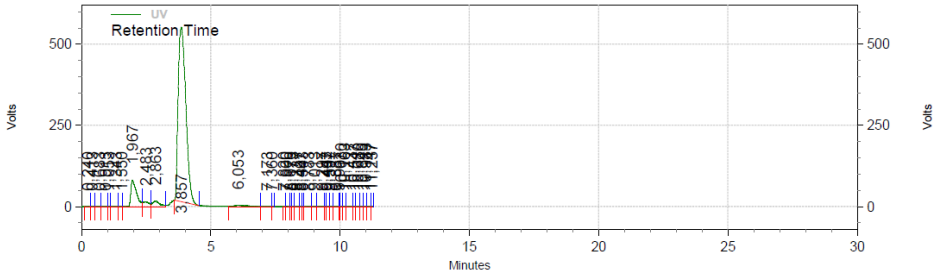


UV Results

Retention Time	Area	Area %	Height	Height %
0,187	466	0,00	103	0,00
0,230	228	0,00	74	0,00
0,310	175	0,00	41	0,00
0,520	363	0,00	73	0,00
0,597	178	0,00	60	0,00
0,740	701	0,00	98	0,00
0,847	717	0,00	89	0,00
1,097	497	0,00	92	0,00
1,153	200	0,00	69	0,00
1,440	1097	0,00	99	0,00
1,537	134	0,00	74	0,00
1,637	443	0,00	106	0,00
2,083	3674179	9,88	215967	8,97
2,460	914786	2,46	54198	2,25
2,850	1235978	3,32	68036	2,83
3,560	661131	1,78	64521	2,68
3,837	30215838	81,24	1981710	82,31
5,010	136806	0,37	4304	0,18
5,590	48269	0,13	2769	0,12
6,130	280723	0,75	13845	0,58
7,290	17388	0,05	641	0,03
7,770	793	0,00	160	0,01
7,857	554	0,00	114	0,00
8,007	625	0,00	108	0,00
8,130	273	0,00	82	0,00
8,227	196	0,00	55	0,00

Totals	37192738	100,00	2407488	100,00

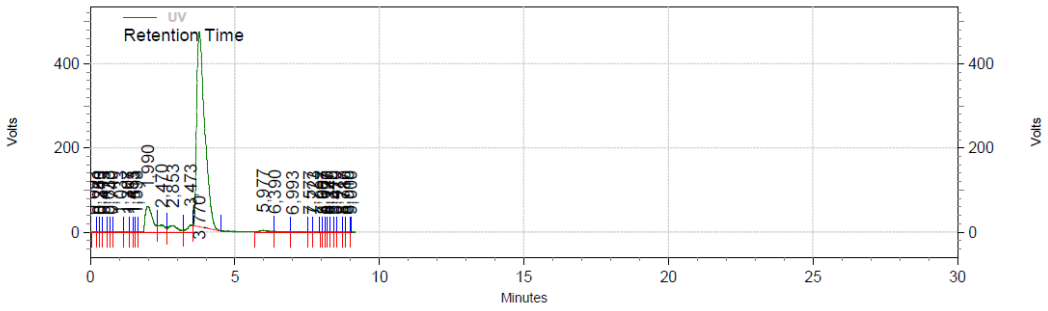
t = 7h



UV Results

Retention Time	Area	Area %	Height	Height %
0,240	575	0,00	78	0,00
0,413	454	0,00	62	0,00
0,683	617	0,00	101	0,00
0,913	510	0,00	69	0,00
1,053	277	0,00	75	0,00
1,343	541	0,00	97	0,00
1,550	246	0,00	64	0,00
1,967	4979796	9,53	328378	12,51
2,483	1017522	1,95	58982	2,25
2,863	1374954	2,63	69863	2,66
3,857	44346369	84,85	2148666	81,84
6,053	526001	1,01	16590	0,63
7,173	4934	0,01	303	0,01
7,360	409	0,00	121	0,00
7,820	151	0,00	53	0,00
7,900	320	0,00	47	0,00
8,070	124	0,00	53	0,00
8,173	180	0,00	58	0,00
8,337	407	0,00	68	0,00
8,467	115	0,00	40	0,00
8,543	159	0,00	46	0,00
8,783	1155	0,00	99	0,00
9,013	1187	0,00	145	0,01
9,297	1829	0,00	139	0,01
9,443	496	0,00	121	0,00
9,497	630	0,00	126	0,00
9,597	582	0,00	89	0,00
9,837	873	0,00	123	0,00
9,967	191	0,00	97	0,00
10,030	294	0,00	83	0,00
10,163	319	0,00	71	0,00
10,437	825	0,00	116	0,00
10,543	522	0,00	110	0,00
10,680	594	0,00	107	0,00
10,840	410	0,00	88	0,00
10,983	394	0,00	93	0,00
11,147	509	0,00	80	0,00
11,237	335	0,00	89	0,00
Totals	52265806	100,00	2625590	100,00

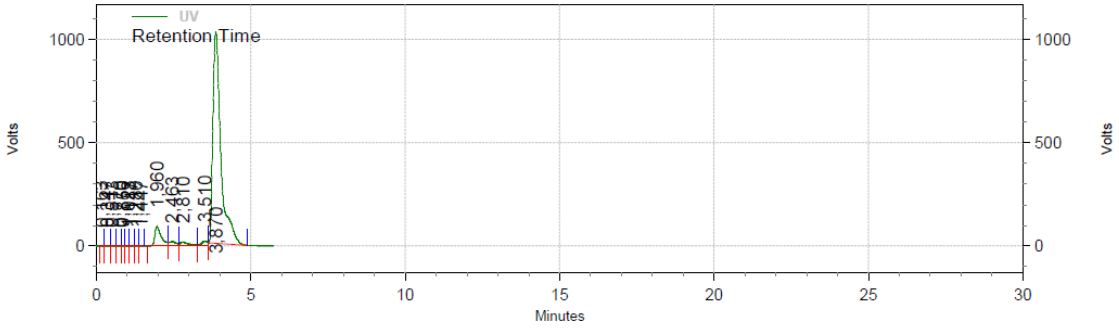
t = 24h



UV Results

Retention Time	Area	Area %	Height	Height %
0,093	616	0,00	80	0,00
0,280	260	0,00	69	0,00
0,343	264	0,00	69	0,00
0,473	639	0,00	88	0,00
0,633	284	0,00	75	0,00
0,740	216	0,00	74	0,00
1,033	677	0,00	68	0,00
1,307	433	0,00	79	0,00
1,453	317	0,00	60	0,00
1,513	70	0,00	47	0,00
1,593	272	0,00	78	0,00
1,990	4235242	9,65	245711	10,63
2,470	1143485	2,61	64287	2,78
2,853	1352782	3,08	61302	2,65
3,473	821595	1,87	66428	2,87
3,770	35831571	81,67	1850834	80,05
5,977	362367	0,83	15623	0,68
6,390	100873	0,23	5364	0,23
6,993	18661	0,04	801	0,03
7,577	1586	0,00	230	0,01
7,723	1056	0,00	149	0,01
7,997	113	0,00	66	0,00
8,067	131	0,00	50	0,00
8,170	229	0,00	67	0,00
8,227	205	0,00	57	0,00
8,340	118	0,00	38	0,00
8,470	184	0,00	62	0,00
8,717	388	0,00	73	0,00
8,780	177	0,00	69	0,00
8,910	550	0,00	89	0,00
9,000	100	0,00	53	0,00
Totals	43875461	100,00	2312140	100,00

t = 51h



UV Results

Retention Time	Area	Area %	Height	Height %
0,163	171	0,00	43	0,00
0,347	509	0,00	76	0,00
0,513	534	0,00	86	0,00
0,770	324	0,00	59	0,00
0,850	316	0,00	73	0,00
1,023	356	0,00	72	0,00
1,117	355	0,00	64	0,00
1,280	378	0,00	87	0,00
1,447	563	0,00	80	0,00
1,960	5433210	6,53	374851	7,95
2,463	1417788	1,70	79993	1,70
2,810	1575188	1,89	73664	1,56
3,510	1250138	1,50	87896	1,86
3,870	73515772	88,36	4098476	86,91
Totals	83195602	100,00	4715520	100,00

Autophagy

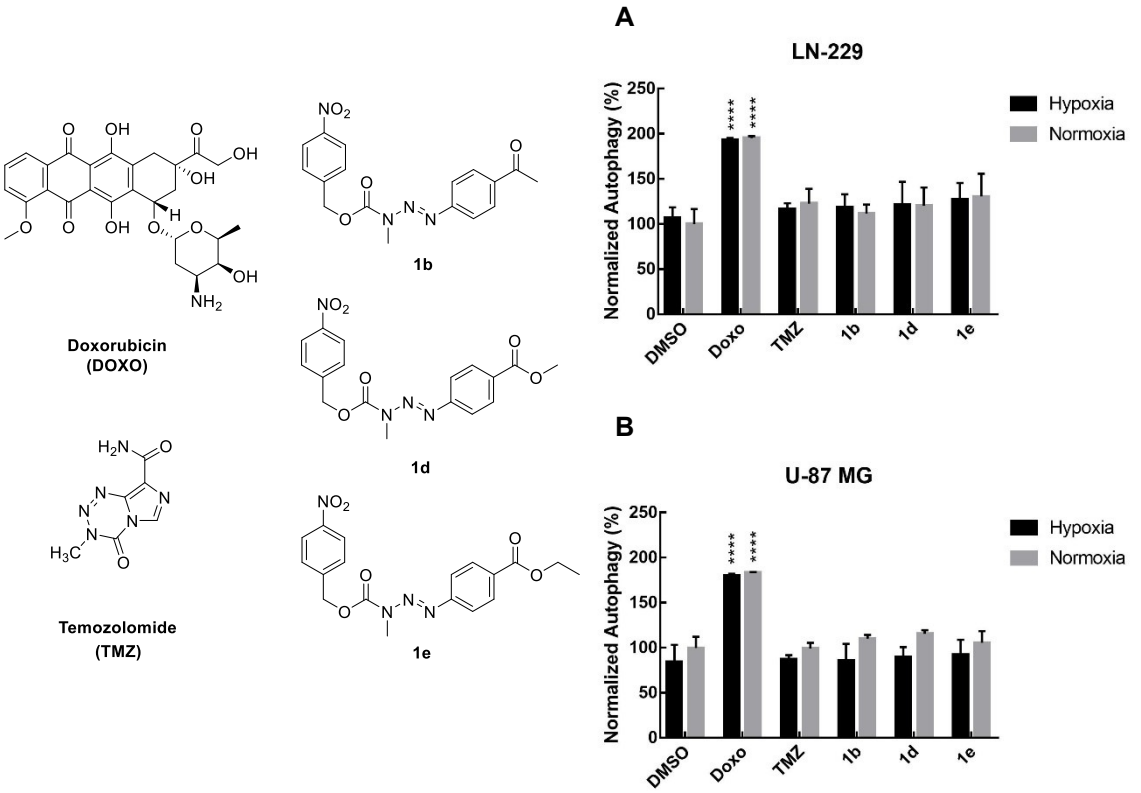


Figure S12 – Induction of autophagy in LN-229 (**A**) or U87-MG (**B**) cell lines after 10 h incubation with 100 µM of temozolomide (TMZ) or prodrugs **1b**, **1d** or **1e** in hypoxia or normoxia. 0.1% (v/v) DMSO was used as vehicle control and doxorubicin was used as a positive control at 40 µM for LN-229 and 100 µM for U87-MG cells. All data was normalized to DMSO in normoxia. Results presented as mean ± SD of two independent experiments. The statistical analysis was performed with Two-way ANOVA followed by Tukey's multiple comparisons test, where results were compared to DMSO in hypoxia or normoxia, respectively. ****p-value < 0.0001 – GraphPad Prism®7 (GraphPad Software, San Diego, CA, USA).

Table S2 – HPLC elution gradients used for chromatographic separation of prodrugs **1b-n**, **8** and negative control **5**.

Method A			**Method B**			**Method C**			**Method D**		
time (min)	**% water**	**% ACN**	**time (min)**	**% water**	**% ACN**	**time (min)**	**% water**	**% ACN**	**time (min)**	**% water**	**% ACN**
0	80	20	0	90	10	0	65	35	0	55	45
5	65	35	5	75	25	5	45	55	5	35	65
10	45	55	10	45	55	10	35	65	10	20	80
15	35	65	15	35	65	15	25	75	15	15	85
20	25	75	20	25	75	20	15	85	20	10	90
1b, **1g**, **1j**, **1l**, **1m**, **1n**, **8**, **5**			**1c**, **1h**			**1d**, **1i**			**1e**, **1f**, **1k**		
Flow rate: 1 mL/min											

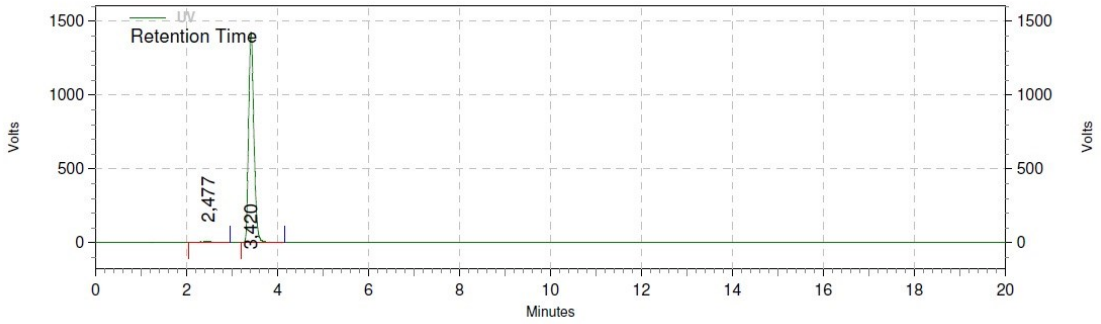
Table S3 – LC-MS gradient elution conditions for prodrug **1g**.

Time (min)	% H_2O (0.5% HCOOH)	%ACN
0	98	2
2	98	2
12	2	98
20	2	98
20.1	98	2
30	98	2

HPLC purity data

3-[4-nitrobenzyloxycarbonyl]-1-(4-cyanophenyl)-3-methyltriazene (1a)

Isocratic elution – water : acetonitrile (20:80)

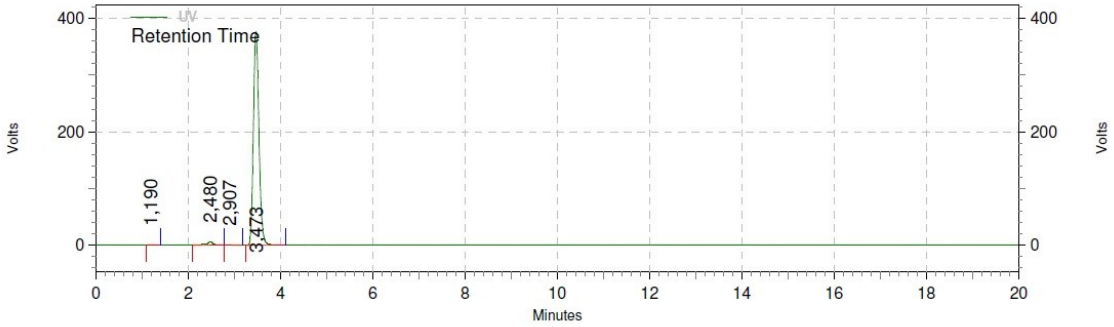


UV Results

Retention Time	Area	Area %	Height	Height %
2,477	497937	1,07	33633	0,59
3,420	46169447	98,93	5704222	99,41
Totals	46667384	100,00	5737855	100,00

3-[4-nitrobenzyloxycarbonyl]-1-(4-acetylphenyl)-3-methyltriazene (1b)

Isocratic elution – water : acetonitrile (20:80)

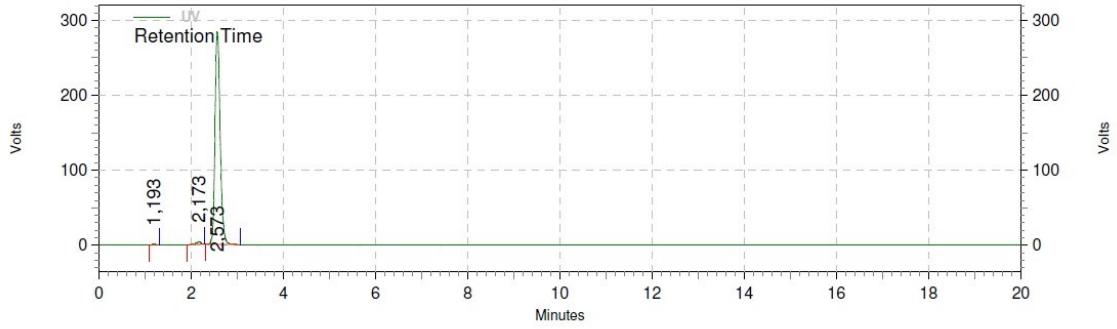


UV Results

Retention Time	Area	Area %	Height	Height %
1,190	21958	0,17	4110	0,27
2,480	229588	1,79	24055	1,57
2,907	11595	0,09	1427	0,09
3,473	12552438	97,95	1504826	98,07
Totals	12815579	100,00	1534418	100,00

3-[4-nitrobenzyloxycarbonyl]-1-(4-carbamoylphenyl)-3-methyltriazene (1c)

Isocratic elution – water : acetonitrile (20:80)

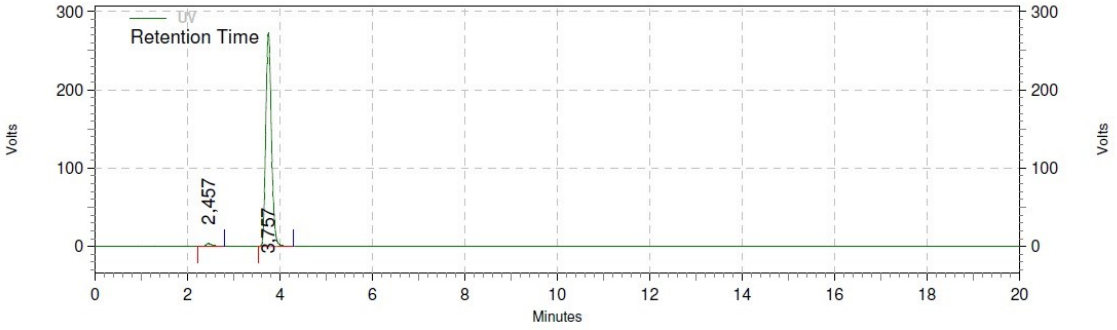


UV Results

Retention Time	Area	Area %	Height	Height %
1,193	23690	0,27	4746	0,41
2,173	115789	1,30	13675	1,19
2,573	8794571	98,44	1134410	98,40
Totals	8934050	100,00	1152831	100,00

3-[4-nitrobenzyloxycarbonyl]-1-(4-methoxycarbonylphenyl)-3-methyltriazene (1d)

Isocratic elution – water : acetonitrile (20:80)

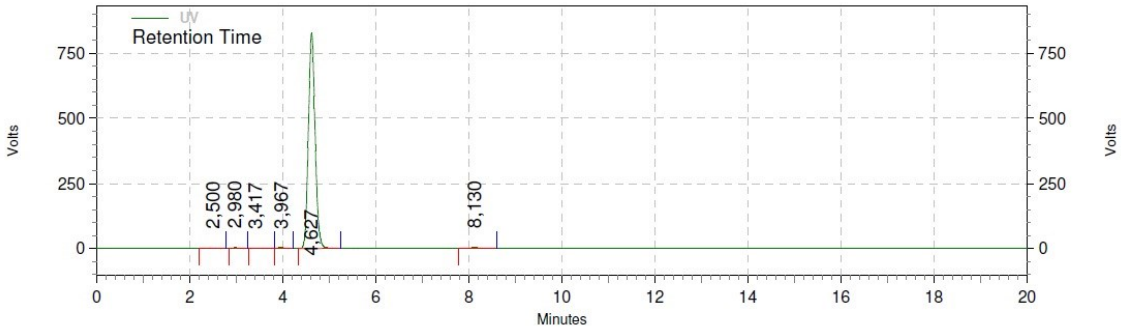


UV Results

Retention Time	Area	Area %	Height	Height %
2,457	127949	1,36	13993	1,27
3,757	9295609	98,64	1090453	98,73
Totals	9423558	100,00	1104446	100,00

3-[4-nitrobenzyloxycarbonyl]-1-(4-ethoxycarbonylphenyl)-3-methyltriazene (1e)

Isocratic elution – water : acetonitrile (20:80)

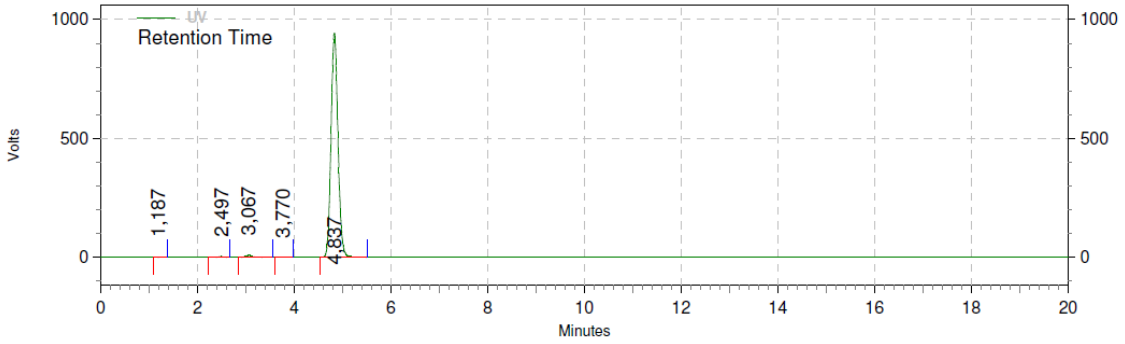


UV Results

Retention Time	Area	Area %	Height	Height %
2,500	85915	0,25	10197	0,30
2,980	90983	0,27	12032	0,36
3,417	41285	0,12	3859	0,11
3,967	125808	0,37	16017	0,48
4,627	33328835	98,38	3309654	98,32
8,130	205779	0,61	14451	0,43
Totals	33878605	100,00	3366210	100,00

3-[4-nitrobenzyloxycarbonyl]-1-(4-tolyl)-3-methyltriazene (1f)

Isocratic elution – water : acetonitrile (20:80)

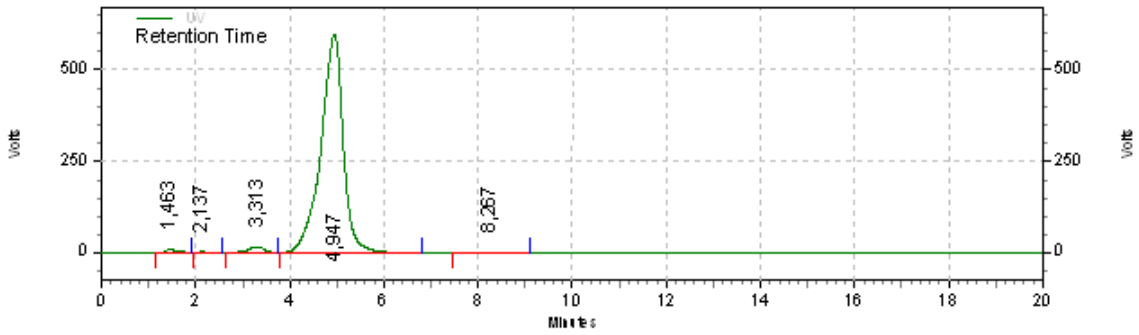


UV Results

Retention Time	Area	Area %	Height	Height %
1,187	11542	0,03	2178	0,06
2,497	88119	0,23	12803	0,34
3,067	327267	0,86	35499	0,93
3,770	16402	0,04	1756	0,05
4,837	37608991	98,83	3766665	98,63
Totals	38052321	100,00	3818901	100,00

3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-acetylphenyl)-3-methyltriazene (1g)

Isocratic elution – water : acetonitrile (50:50)

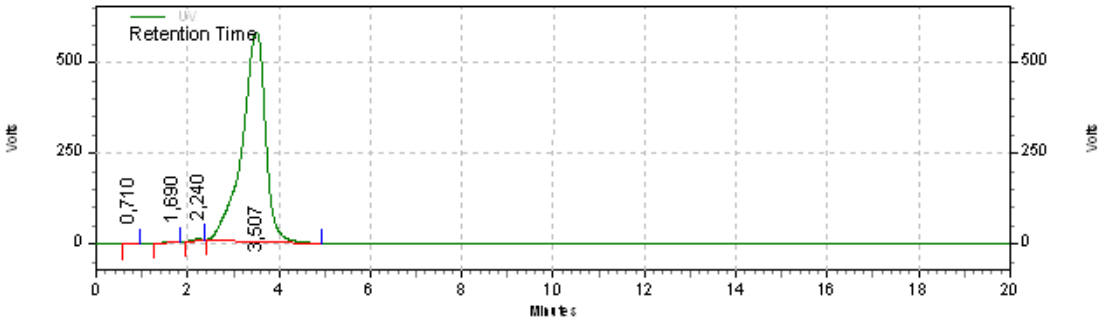


UV Results

Retention Time	Area	Area %	Height	Height %
1,463	784070	0,97	49648	1,98
2,137	149607	0,18	9701	0,39
3,313	1614753	1,99	65564	2,61
4,947	78297859	96,64	2379035	94,84
8,267	173825	0,21	4503	0,18
Totals	81020114	100,00	2508451	100,00

3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-carbamoylphenyl)-3-methyltriazene (1h)

Isocratic elution – water : acetonitrile (60:40)

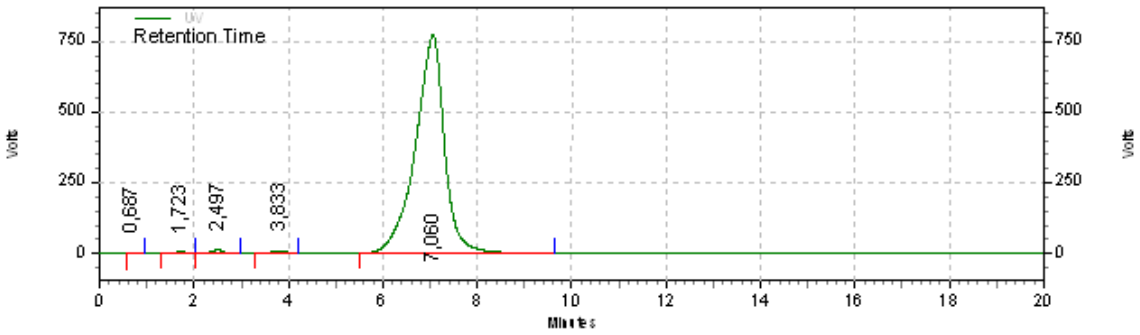


UV Results

Retention Time	Area	Area %	Height	Height %
0,710	92650	0,11	8808	0,38
1,690	109820	0,13	7744	0,33
2,240	197270	0,24	13807	0,59
3,507	81514713	99,51	2300214	98,70
Totals	81914453	100,00	2330573	100,00

3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-methoxycarbonylphenyl)-3-methyltriazene (1i)

Isocratic elution – water : acetonitrile (50:50)

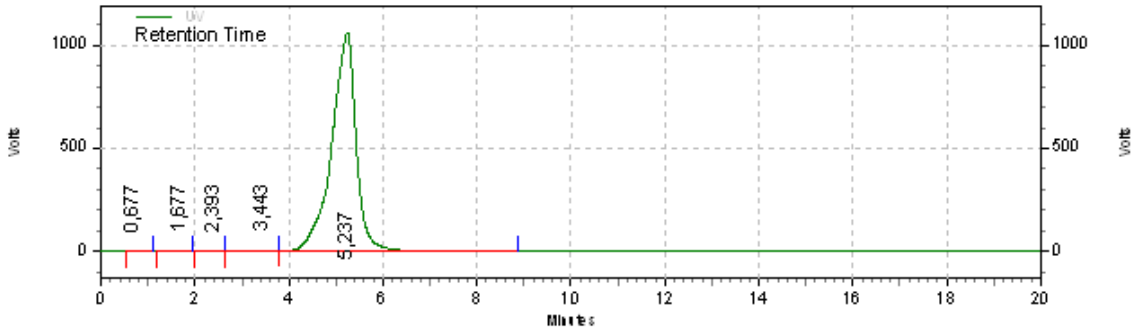


UV Results

Retention Time	Area	Area %	Height	Height %
0,687	69476	0,05	8125	0,25
1,723	362956	0,27	22722	0,71
2,497	830104	0,62	50329	1,57
3,833	635814	0,47	32933	1,03
7,060	132554229	98,59	3098519	96,45
Totals	134452579	100,00	3212628	100,00

3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-cyanophenyl)-3-methyltriazene (1j)

Isocratic elution – water : acetonitrile (50:50)

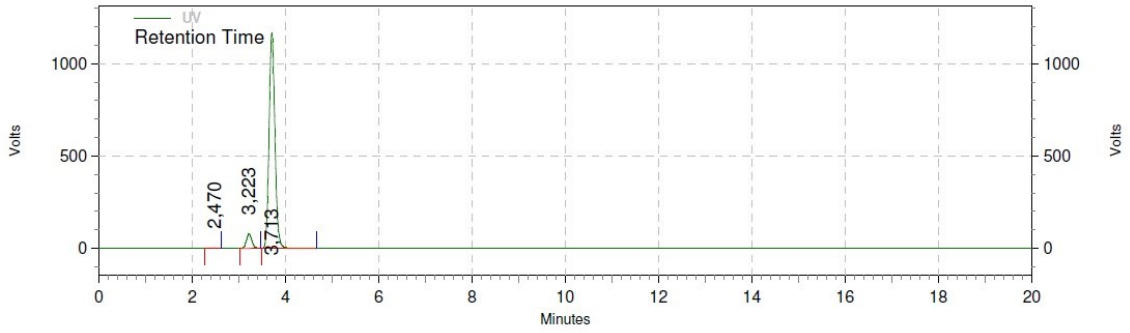


UV Results

Retention Time	Area	Area %	Height	Height %
0,677	58755	0,04	4708	0,11
1,677	112940	0,07	8037	0,19
2,393	27465	0,02	1517	0,04
3,443	297355	0,19	10152	0,24
5,237	153713847	99,68	4240838	99,43
Totals	154210362	100,00	4265252	100,00

3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-tolyl)-3-methyltriazene (1k)

Isocratic elution – water : acetonitrile (20:80)

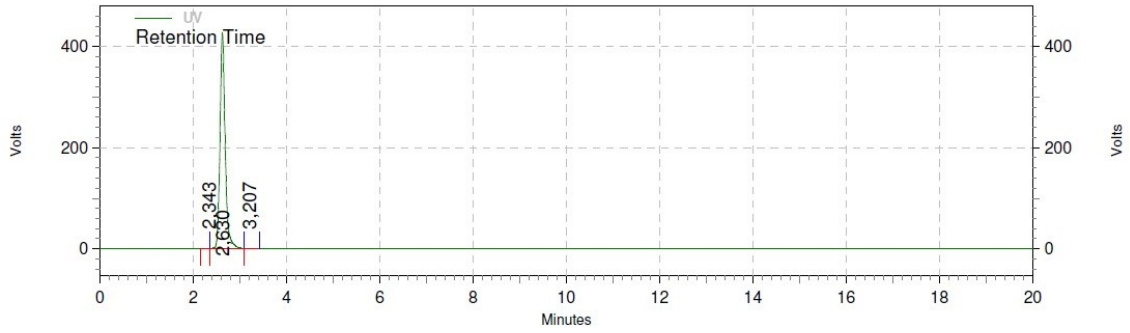


UV Results

Retention Time	Area	Area %	Height	Height %
2,470	74230	0,17	10988	0,22
3,223	2583848	5,94	315652	6,33
3,713	40816762	93,89	4662803	93,45
Totals	43474840	100,00	4989443	100,00

3-[(1-methyl-2-nitro-1H-imidazol-5-yl)methyloxycarbonyl]-1-(4-cyanophenyl)-3-methyltriazene (1l)

Isocratic elution – water : acetonitrile (20:80)

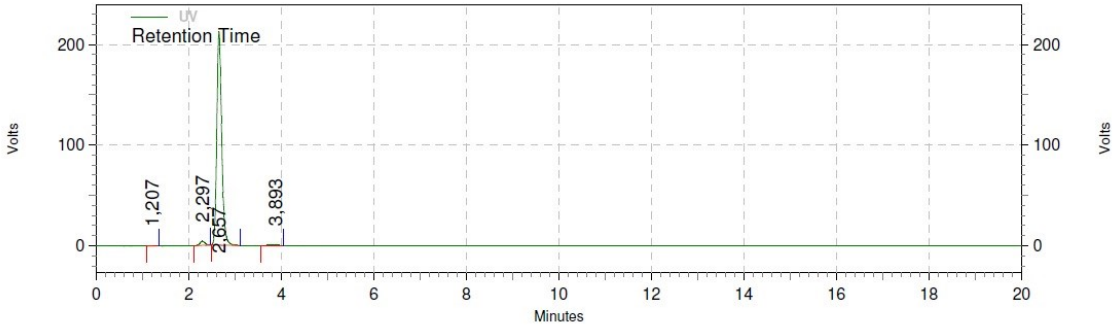


UV Results

Retention Time	Area	Area %	Height	Height %
2,343	40753	0,31	4126	0,24
2,630	13176399	99,36	1705529	99,49
3,207	43910	0,33	4580	0,27
Totals	13261062	100,00	1714235	100,00

3-[(1-methyl-2-nitro-1H-imidazol-5-yl)methyloxycarbonyl]-1-(4-acetylphenyl)-3-methyltriazene (1m)

Isocratic elution – water : acetonitrile (20:80)

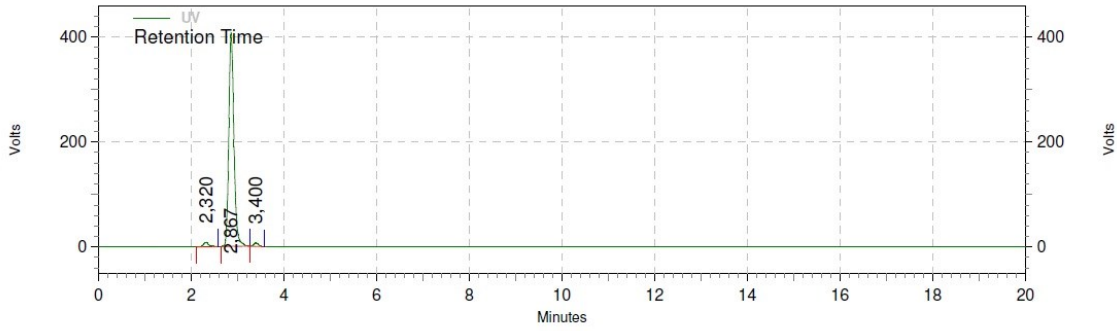


UV Results

Retention Time	Area	Area %	Height	Height %
1,207	9890	0,15	1790	0,21
2,297	127881	2,00	17322	1,99
2,657	6197007	96,97	847605	97,40
3,893	55852	0,87	3509	0,40
Totals	6390630	100,00	870226	100,00

3-[(1-methyl-2-nitro-1H-imidazol-5-yl)methyloxycarbonyl]-1-(4-methoxycarbonylphenyl)-3-methyltriazene (1n)

Isocratic elution – water : acetonitrile (20:80)

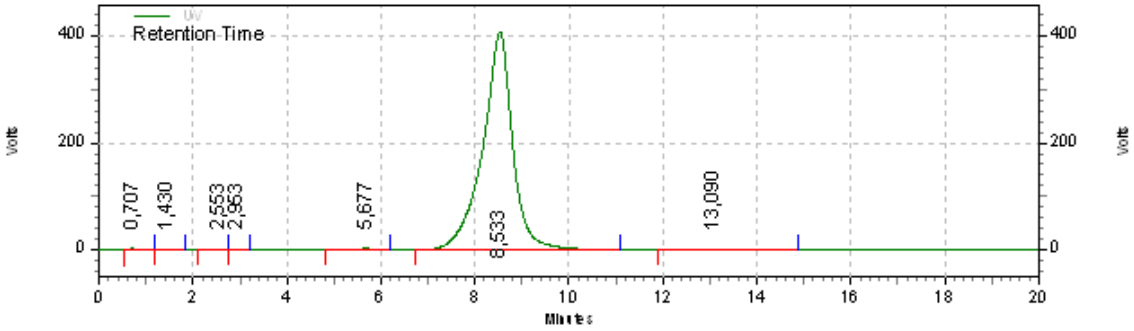


UV Results

Retention Time	Area	Area %	Height	Height %
2,320	248740	1,94	34517	2,05
2,867	12402027	96,70	1625908	96,47
3,400	174342	1,36	24900	1,48
Totals	12825109	100,00	1685325	100,00

3-[benzyloxycarbonyl]-1-(4-acetylphenyl)-3-methyltriazene (5)

Isocratic elution – water : acetonitrile (50:50)



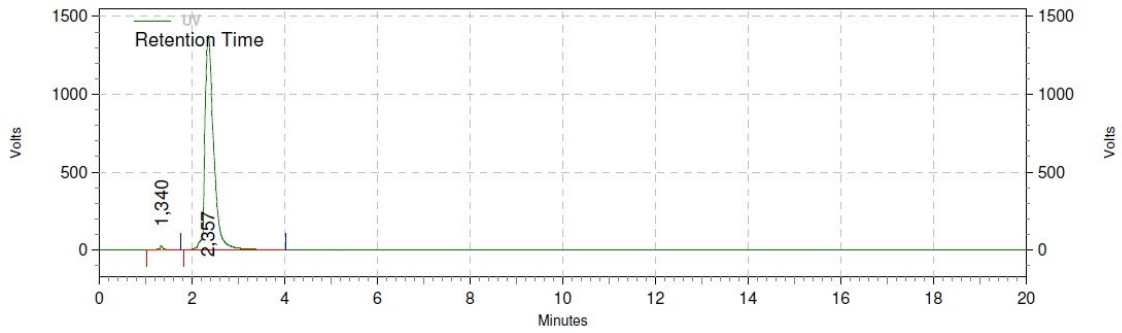
UV Results

Retention Time	Area	Area %	Height	Height %
0,707	159810	0,22	10401	0,63
1,430	58704	0,08	4367	0,26
2,553	13320	0,02	904	0,05
2,953	23409	0,03	1719	0,10
5,677	325831	0,45	10380	0,63
8,533	72021483	98,75	1624082	98,05
13,090	328684	0,45	4509	0,27

Totals	72931241	100,00	1656362	100,00

3-[(5-nitrofuran-2-yl)methyloxycarbonyl]-1-(4-carbamoyl-1H-imidazol-5-yl)-3-methyltriazene (8)

Isocratic elution – water : acetonitrile (20:80)



UV Results

Retention Time	Area	Area %	Height	Height %
1,340	690773	0,95	116617	2,08
2,357	72072915	99,05	5497532	97,92

Totals	72763688	100,00	5614149	100,00

LC-MS conditions

Prodrugs were analysed on an Alliance 2695 separation module HPLC system (Waters, Dublin, Ireland) coupled to a 2996 Photodiode Array Detector and a Micromass Quattro Micro tripe quadrupole (TQ) (Waters, Dublin, Ireland). Aliquots (20 µL) of the samples were analysed following the gradient elution described in Table S3, at a flow rate of 0.3 mL/min, over 30 min. The chromatographic separation procedure was carried out using a Sunfire C18 (2.1 x 100 mm, 5 µm) in a thermostated oven at 35 ºC. DAD was used to scan wavelength absorption from 210 to 780 nm. Mass spectrometry detection was performed using an electrospray ionisation (ESI) source operating at 120 °C, applying a capillary voltage of 3.0 kV and a cone voltage of 30 V. Data was processed using MassLynx software.

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