Supporting Information

Synthesis of Maleimide-Based Enediynes with Cyclopropane Moiety for Enhanced Cytotoxicity under Normoxic and Hypoxic Conditions

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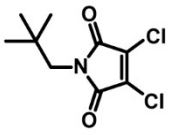
Materials

Toluene and tetrahydrofuran (THF) were dried using calcium hydride (CaH_2) and distilled before use. 3-[4,5-Dimethylthiazol-2-yl]2,5-diphenyltetrazolium-bromide (MTT) was obtained from Macklin. Annexin V-FITC/PI apoptosis detection kit, Alexa Fluor 555-labeled Donkey Anti-Rabbit IgG(H+L), phospho-histone H2AX rabbit monoclonal antibody were purchased from Beyotime Biotechnology. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were provided by BBI Life Sciences Corporation. Other reagents were purchased at commercial grade and used without further purification. Sonogashira reactions were performed with dry Schlenk techniques under an atmosphere of nitrogen.

Characterizations

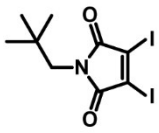
^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded in chloroform ($CDCl_3$) on a Ultra Shield 400 spectrometer (BRUKER BIOSPIN AG, AVANCE III 400) and referenced to Me_4Si. Mass spectra was recorded on a Micromass LCTTM mass spectrometer using the ESI method. Fluorescence spectra were recorded on a PerkinElmer LS-55. The electron paramagnetic resonance (EPR) measurements were performed with an X-band EMX-8/2.7C EPR spectrometer (Bruker, Germany). Fluorescence spectra were recorded in DCE (200 μM) on a PerkinElmer LS-55. Cytotoxicity assay was measured by microplate reader (Thermo Scientific). Fluorescence microscopy images were taken using a confocal laser scanning microscopy (CLSM, C1−Si, Nikon, Japan). Quantitative flow cytometry was recorded by a flow cytometer (Beckman).

Synthesis



3,4-Dichloro-1-(2,2-dimethyl-propyl)-pyrrole-2,5-dione (C2)

This compound was synthesized following the procedure described in our previous work[1, 2]. Briefly, dichloromaleic anhydride (7.95 g, 47.9 mmol) was dissolved in acetic acid (70 mL) with slow addition of neopentylamine (4.38 g, 50 mmol) at 0 °C, and then the solution was heated at 120 °C for 18 h. After removal of solvent, the crude residue was separated by column chromatography on silica gel (hexane/ethyl acetate = 9:1) to give the product (7.74 g, 75%).

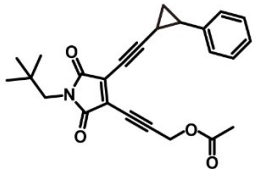


3,4-diiodo-1-(2,2-dimethyl-propyl)-pyrrole-2,5-dione (C3)

This compound was synthesized following the procedure described in our previous work[1, 2]. Briefly, a solution of sodium iodide (18.89 g, 125.9 mmol) and 3,4-dichloro-1-(2,2-dimethyl-propyl)-pyrrole2,5-dione (7.4 g, 31.5 mmol) in acetonitrile (80 mL) was heated at 90 °C for 24 h. After removal of solvent, the residue was purified by column chromatography on silica gel (hexane/ethyl acetate = 9:1) to give the product (12.11g, 92%).

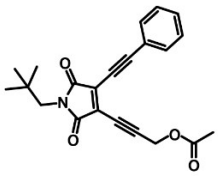
General procedure for the preparation of enediynes

Under a nitrogen atmosphere, compound C3 (209.5 mg, 0.5 mmol), NHC-$PdCl_2$-3-chloropyridine (35 mg, 0.05 mmol), CuI (38 mg, 0.2 mmol), and diisopropylethylamine (0.25 mL, 1.5 mmol) were successively added into a mixture of dry THF (2 mL) and toluene (4 mL). The terminal alkyne (0.75 mmol) in THF (0.6 mL) was then added dropwisely. The mixture was stirred at room temperature and monitored with TLC. After completion of the reaction, the mixture was purified through column chromatograph to give the desired compound.



3-(1-neopentyl-2,5-dioxo-4-(2-phenylcyclopropyl) ethynyl)-2,5-dihydro-1H-pyrrol-3-yl) prop-2-yn-1-yl acetate (EDY-A).

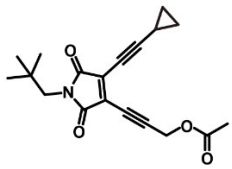
Isolated in 42% yield as yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29 (t, *J* = 7.6 Hz, 2H), 7.22 (t, *J* = 6.8 Hz, 1H), 7.12 (d, *J* = 7.2 Hz, 2H), 4.98 (s, 2H), 3.34 (s, 2H), 2.55 (ddd, *J* = 9.0, 6.5, 4.6 Hz, 1H), 2.12 (s, 3H), 1.86 (ddd, *J* = 8.7, 5.6, 4.6 Hz, 1H), 1.60 (dt, *J* = 8.9, 5.3 Hz, 1H), 1.54 (ddd, *J* = 8.7, 6.5, 4.9 Hz, 1H), 0.84 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.97, 166.79, 166.30, 138.42, 129.44, 127.57, 125.77, 125.06, 124.57, 115.14, 100.46, 75.33, 67.73, 51.50, 49.13, 32.53, 27.40, 26.87, 19.65, 18.15, 12.06. HRMS (ESI) m/z: calcd for $C_{25}H_{25}NO_4Na$ [M + Na]: 426.1681, found: 426.1682.



3-(1-neopentyl-2,5-dioxo-4-(phenylethynyl)-2,5-dihydro-1H-pyrrol-3-yl) prop-2-yn-1-yl acetate (EDY-B)

Isolated in 48% yield as yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (d, *J* = 7.1 Hz, 2H), 7.45 (t, *J* = 7.4 Hz, 1H), 7.40 (t, *J* = 7.4 Hz, 2H), 5.02 (s, 2H), 3.39 (s, 2H), 2.14 (s, 3H), 0.94 (s, 9H). ^{13}C NMR

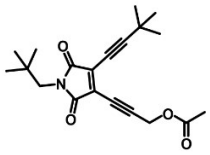
(100 MHz, $CDCl_3$) δ 168.99, 166.50, 166.21, 131.65, 129.56, 128.85, 127.59, 127.30, 125.27, 109.56, 101.62, 75.47, 72.56, 51.50, 49.23, 32.58, 26.89, 19.63. HRMS (ESI) m/z: calcd for $C_{22}H_{21}NO_4Na$ [M + Na]: 386.1368, found: 386.1364.



This compound was synthesized according to our previous report.[2]

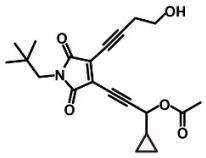
3-(4-(cyclopropylethynyl)-1-neopentyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)prop-2-yn-1-yl acetate (EDY-C)

Isolated in 45% yield as yellow oil. ^1H NMR (400 MHz, $CDCl_3$) δ 4.91 (s, 2H), 3.26 (s, 2H), 2.07 (s, 3H), 1.56 (ddd, *J* = 8.2, 5.1, 3.2 Hz, 1H), 1.02 – 0.92 (m, 4H), 0.83 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.98, 166.88, 166.32, 129.69, 124.30, 117.19, 100.14, 75.32, 66.49, 51.49, 49.09, 32.52, 26.86, 19.65, 9.49, 0.58, -1.03. HRMS (ESI) m/z: calcd for $C_{19}H_{21}NO_4Na$ [M + Na]: 350.1368, found: 350.1367.



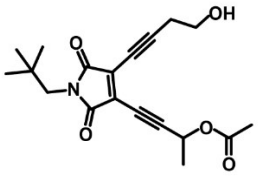
3-(4-(3,3-dimethylbut-1-yn-1-yl)-1-neopentyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl) prop-2-yn-1-yl acetate (EDY-D)

Isolated in 21% yield as yellow oil.^1H NMR (400 MHz, $CDCl_3$) δ 4.98 (s, 2H), 3.33 (s, 2H), 2.12 (s, 3H), 1.35 (s, 9H), 0.90 (s, 9H).^{13}C NMR (100 MHz, $CDCl_3$) δ 168.91, 166.73, 166.36, 129.81, 125.33, 120.68, 100.49, 75.29, 69.32, 51.42, 49.07, 32.50, 29.29, 28.17, 26.87, 19.60. HRMS (ESI) m/z: calcd for $C_{20}H_{25}NO_4Na$ [M + Na]: 366.1681, found: 366.1682.



1-cyclopropyl-3-(4-(4-hydroxybut-1-yn-1-yl)-1-neopentyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl) prop-2-yn-1-yl acetate (EDY-E).

Isolated in 35% yield as yellow oil. ^1H NMR (400 MHz, $CDCl_3$) δ 5.43 (d, *J* = 7.1 Hz, 1H), 3.86 (t, *J* = 5.8 Hz, 2H), 3.33 (s, 2H), 2.84 (t, *J* = 6.0 Hz, 2H), 2.13 (s, 3H), 1.42 – 1.34 (m, 1H), 0.90 (s, 9H), 0.70 – 0.52 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.20, 167.53, 167.37, 130.48, 127.22, 110.29, 103.82, 75.18, 72.94, 67.89, 60.33, 50.19, 33.54, 27.89, 21.02, 14.05, 3.78, 2.49, 1.02. HRMS (ESI) m/z: calcd for $C_{21}H_{25}NO_5Na$ [M + Na]: 394.1630, found: 394.1629.



This compound was synthesized according to our previous report[2].

3-[4-(4-hydroxy-but-1-ynyl)-1-isobutyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl]-1-methylprop-2-ynyl ester (EDY-F).

Isolated in 43% yield as yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.67 (q, J = 6.8 Hz, 1H), 3.86 (t, J = 5.5 Hz, 2H), 3.33 (s, 2H), 2.84 (t, J = 6.0 Hz, 2H), 2.11 (s, 3H), 1.60 (d, J = 6.8 Hz, 3H), 0.90 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.03, 166.51, 166.43, 129.27, 126.14, 109.22, 105.38, 73.50, 71.93, 59.65, 59.29, 49.15, 32.52, 26.85, 24.03, 19.94, 19.62. HRMS (ESI) m/z: calcd for $C_{19}H_{23}NO_5Na$ [M + Na]: 368.1474, found: 368.1475.

Experimental Section

The radical detection of EDY

A solution of PBN (100 mM) in DCE was prepared in advance, then six enediynes were added respectively to ensure their final concentration of 20 mM for enediyne. The solution of PBN (100 mM) without addition of enediyne was used as the control. Immediately, the prepared solutions (~150 μL) were transferred to capillary tubes before sealed, and placed at 37 ℃ for 12 h. The EPR spectra were recorded using an X-band EMX-8/2.7C EPR spectrometer (Bruker, Germany). DPBF was used to investigate the free radical-generating ability of EDY-A and EDY-B. The EDY-A or EDY-B solution was mixed with DPBF in DMSO and the final concentration of enediynes and DPBF corresponds to 25 μg/ml, 200 μg/ml respectively. Immediately after the treatment, 200 μL solution of the samples was transferred to a 96-well plate and subjected to a microplate reader to measure the absorption at 416 nm. At the predetermined time intervals, the absorption of different samples was recorded.

DNA Cleavage Assay

Freshly prepared enediyne compounds were dissolved in DMSO (8 μL) at a concentration of 20 mM respectively. Each enediyne solution was added to a solution of supercoiled plasmid pUC19 DNA (20 μg mL^{-1}) in TE buffer (pH 7.6, 8 μL). Control samples consisting a solution of supercoiled plasmid pUC19 DNA (20 μg mL^{-1}) in TE buffer (pH 7.6, 8 μL) were separately incubated with 8 μL DMSO. All the mixtures were incubated at 37 ℃ for 48 h. After incubation, each sample (10 μL) was mixed with a 6 × loading buffer (2 μL) and subjected to a 0.8% agarose gel electrophoresis at 90 V (101 mA) for 60 min, stained by DuRed, and then the gel was photographed on the UV transilluminator (FR-200A) and analyzed by scanning densitometry.

Cell Culture

Hela, MCF-7, HepG2, A549 cancer cells were obtained from the Chinese Academy of Science Cell

Bank for Type Culture Collection (Shanghai, China) and cultured in DMEM medium supplemented with 10% FBS and 1% antibiotics (penicillin–streptomycin, 10000 U/mL) in a humidified incubator at 37 ºC with 5% CO_2. Cells were cultured to ~50-70% confluency and harvested using 0.25% trypsin before further cell relevant experiments.

Cytotoxicity Assay

The cytotoxicity of enediynes in four kinds of cancer cell lines was determined via MTT assay. Briefly, cells were seeded in a 96-well plate at a density of 3000 cells per well in 100 μL of the cell culture medium and incubated overnight for adherence. Then the culture medium was removed and serial dilutions (1:1) of enediynes with fresh medium containing at most 0.2% DMSO as the supporting solvent were added to the wells, which provided a range of concentrations from 0.39 μM to 25 μM. For the hypoxia groups, the medium was replaced by fresh prepared solution containing 200 μM of DFO at 4 h before adding enediynes to induce hypoxia. Cells without sample treatment were used as a blank control. After incubation for 48 h, 10 μL of sterile filtered MTT stock solution (5 mg/mL) in PBS (pH 7.4) was added to each well and the cells were further incubated for 4 h at 37 ºC to allow the yellow dye to be transformed into blue crystals. Then, the medium was replaced with 150 μL of DMSO to dissolve the dark blue crystals. Finally, the optical density (OD) of each well was measured at 570 nm by microplate reader. The measurement was normalized using culture medium without cells. Cell viability (%) relative to control containing cell culture medium was calculated by $[OD]_{samples}/[OD]_{control}$.

Cellular uptake

The uptake and location of enediynes in Hela cells were examined with Confocal Laser Scanning Microscopy (CLSM). Hela cells were seeded in glass bottom confocal dishes at a density of 2×10^5 per well in 2 mL of DMEM and incubated overnight. After removal of culture medium, cells were then incubated with EDY-E (1 μM) or EDY-F (1 μM) in 2 mL of DMEM respectively. Hela cells without any drug incubation were performed as a blank control. After 16 h of incubation at 37 ºC, the cells were washed three times with PBS. Subsequently, the cells were fixed with 2.5% glutaraldehyde at room temperature for 10 min, and permeabilized with 0.5% Triton X-100 for another 10 min. After that, the cells were washed with PBS, then 400 μL of propidium iodide (PI) solution (15 μg/mL) was added and the cells were cultured at 37 °C for 10 min, followed by washing with PBS for three times, and finally visualized by CLSM.

Intracellular radical detection of EDY

A fluorescent probe 2,7-dichloro fluorescein diacetate (DCFH-DA) was used to detect the intracellular radical generation ability of EDY. Cells were cultured using the same procedure mentioned in cell internalization part. After cultured with EDY for 8 h, DCFH-DA were added (10 μM) and then placed in dark for 30 min at room temperature. Then, samples were washed for three times by PBS and subjected to fluorescence microscope.

γ-H2AX Foci Assay

HeLa cells were seeded in glass bottom confocal dishes at a density of 2×10^5 per well in 2 mL of DMEM and incubated overnight. Then the cells were incubated with EDY-E (1 μM) and EDY-F (1 μM) for 24 h, respectively. Cells without sample treatment were used as a blank control. At the end of incubation, the cells were washed, fixed and permeabilized as above mentioned. Next, cells were blocked by 8% BSA/PBS for 2 h at room temperature. Then, cells were stained with phospho-histone H2AX (Ser139) rabbit monoclonal antibody (1:200 dilution in 1% BSA/PBS) at room temperature for 2h. After the incubation with primary antibody incubation, Hela cells were labeled with secondary antibodies (Alexa Fluor 555-labeled Donkey Anti-Rabbit IgG(H+L), 1:1000 dilution, rt, 2 h. The nuclei were further stained with DAPI for 10 min. Finally, cells in each well were washed three times with PBS for 5 min and the γ-H2AX foci was imaged via CLSM.

Cell Cycle Arrest Assay

HeLa cells were seeded in 6-well plates at a density of 2×10^5 per well in 2 mL of DMEM and incubated overnight. After washing with PBS, the cells were incubated with different concentrations of EDY-E at 37 ºC for 24 h. Cells without sample treatment were used as a blank control. After that, the cells were collected by trypsinization, washed with cold PBS, and fixed with 70% ethanol overnight at 4 °C. Then the cells were washed, re-suspended in 400 μL PI staining solution (10 μg/mL PI and 0.1% Triton-X100 in PBS) after centrifugation (1000 r.p.m., 5 min) and incubated at 37 °C in the dark for 15 min. Finally, DNA content was measured by Flow Cytometry, the percentage of cells in each phase of the cell cycle was calculated using the Flow Jo software.

Cell Apoptosis Assay

HeLa cells were seeded in 6-well plates at a density of 2×10^5 per well in 2 mL of DMEM and incubated overnight. After washing with PBS, the cells were incubated with different concentrations of EDY-F at 37 ºC for 24 h. Cells without sample treatment were used as a blank control. At the end of incubation, the cells were collected by trypsinization, washed twice with cold PBS. Then the cells were re-suspended in 400 μL annexin binding buffer and stained with Annexin V-FITC (5 μL) and PI (5 μL) at room temperature for 15 min before analyzing by flow cytometry.

Molecular docking analysis

Molecular docking studies were carried out using AutoDock 4.2[3] to predict the preferred binding mode with ct-DNA. The geometries of enediynes were optimized using Gaussian 09[4]. The crystal structure of ct-DNA was obtained from the Protein Data Bank (PDB ID: 453D). Before docking analysis, polar hydrogen atoms and Gasteiger charges were added to the ct-DNA molecule. For docking calculations, the Lamarckian genetic algorithm (LGA) was employed in AutoDock 4.2[3].

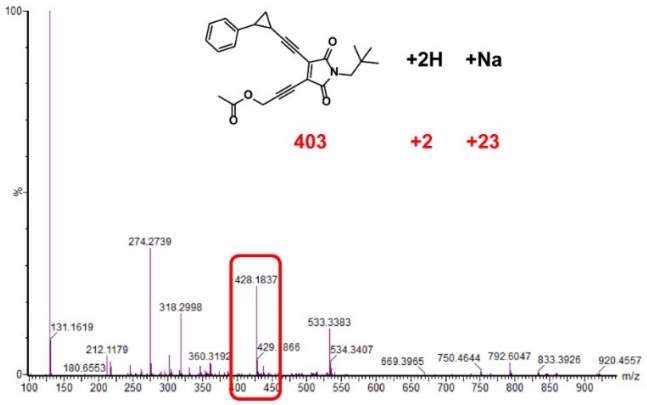


Figure S1 HR-MS spectrum of possible cyclization products (P1).

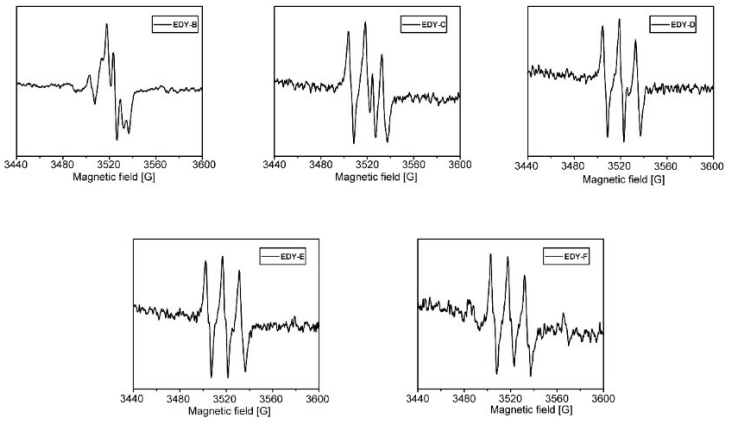


Figure S2 EPR curves of enediynes.

A

Compound	IC_{50} (μM)			
	Hela	MCF-7	HepG2	A549
EDY-A	7.93	7.58	8.11	6.85
EDY-B	> 25	> 25	~ 25	> 25

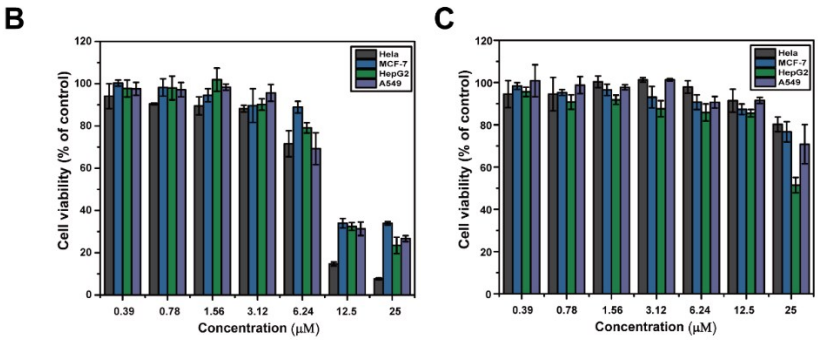


Figure S3 (A) The IC_{50} values of EDY-A and EDY-B against four kinds of cancer cell lines; Cells were incubated with a series of concentrations of EDY-A (B) or EDY-B (C) for 48 h and cell viability was determined and analysed by the MTT assay.

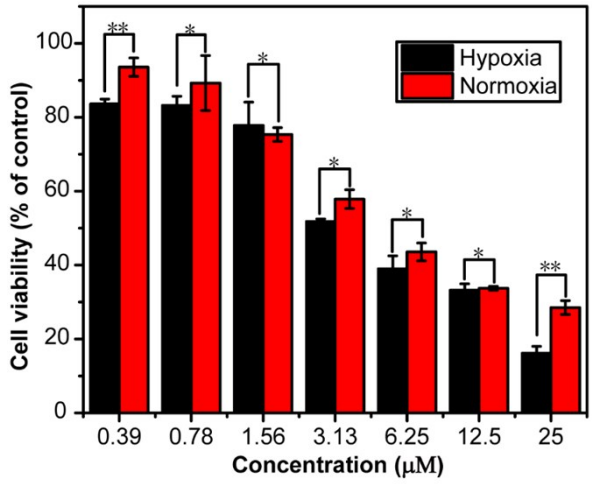


Figure S4 The cytotoxicity of enediynes against Hela cells under normoxic and hypoxic condition (DFO:200 μM).

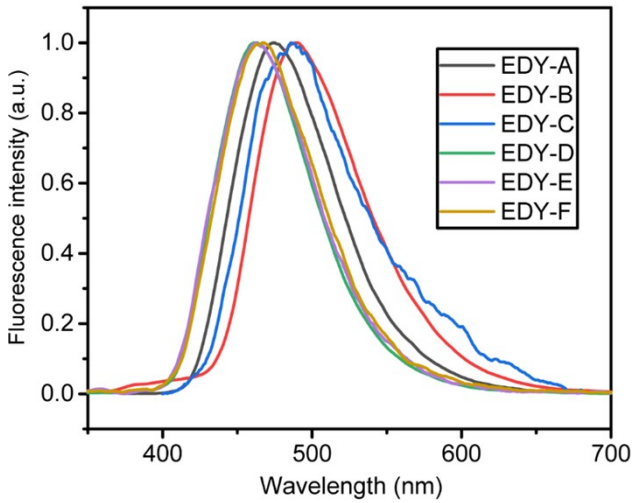


Figure S5 Fluorescence emission spectra of enediynes.

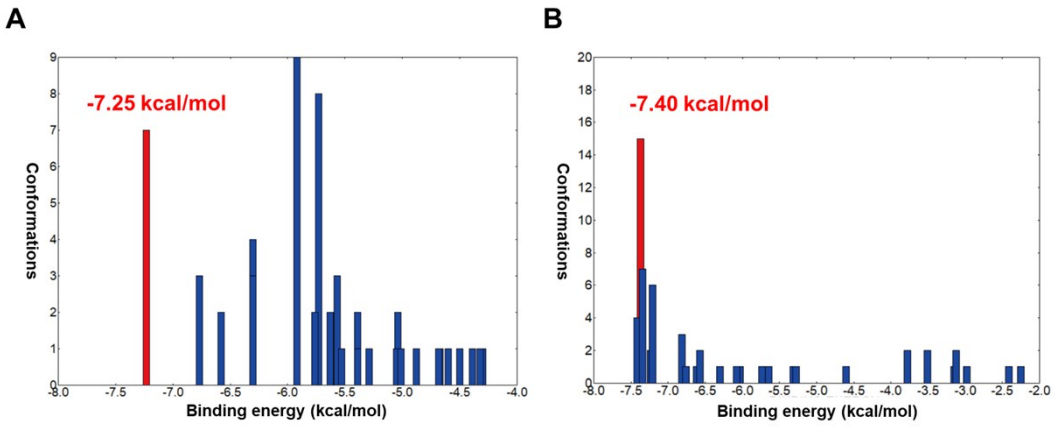


Figure S6 Cluster analysis of AutoDock runs of EDY-E (A) and EDY-F (B) with ct-DNA

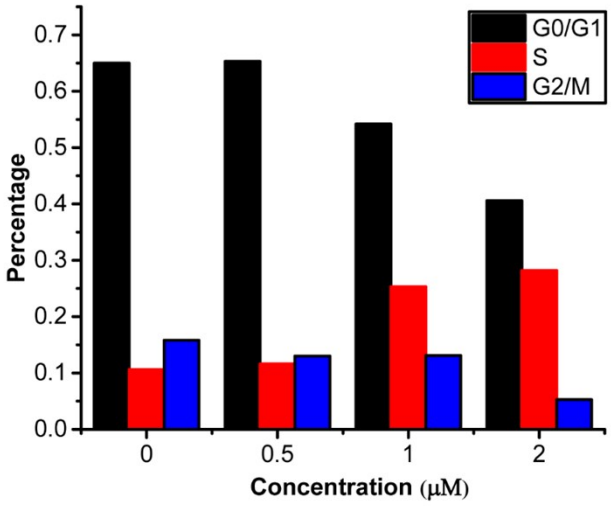


Figure S7 Concentration dependence of cell cycle distributions of Hela cells treated with EDY-E.

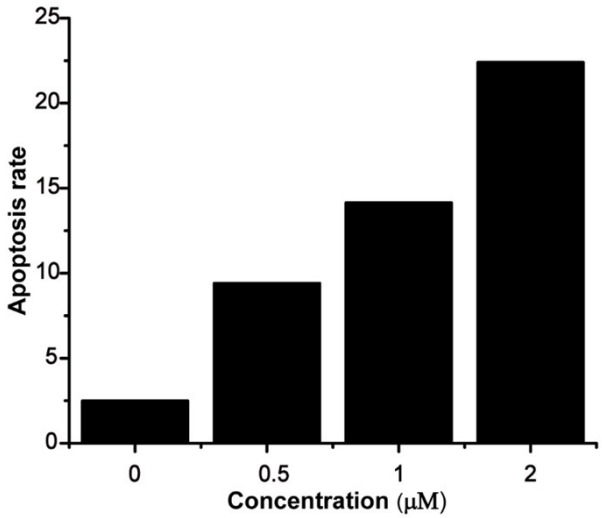


Figure S8 Concentration dependence of the apoptosis rate of Hela cells treated with EDY-E.

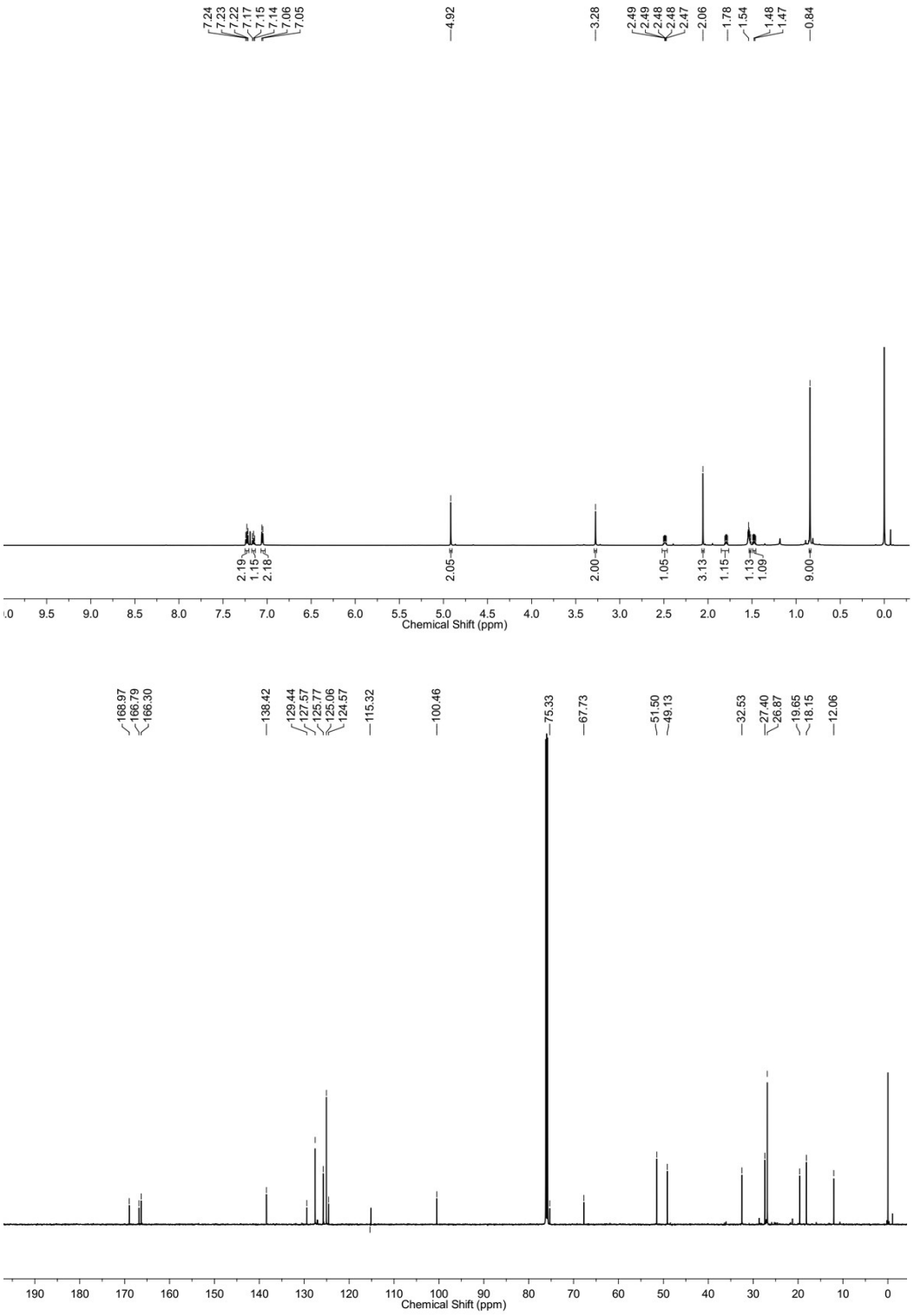


Figure **S9** ^{1}H NMR and ^{13}C NMR spectra of EDY-A

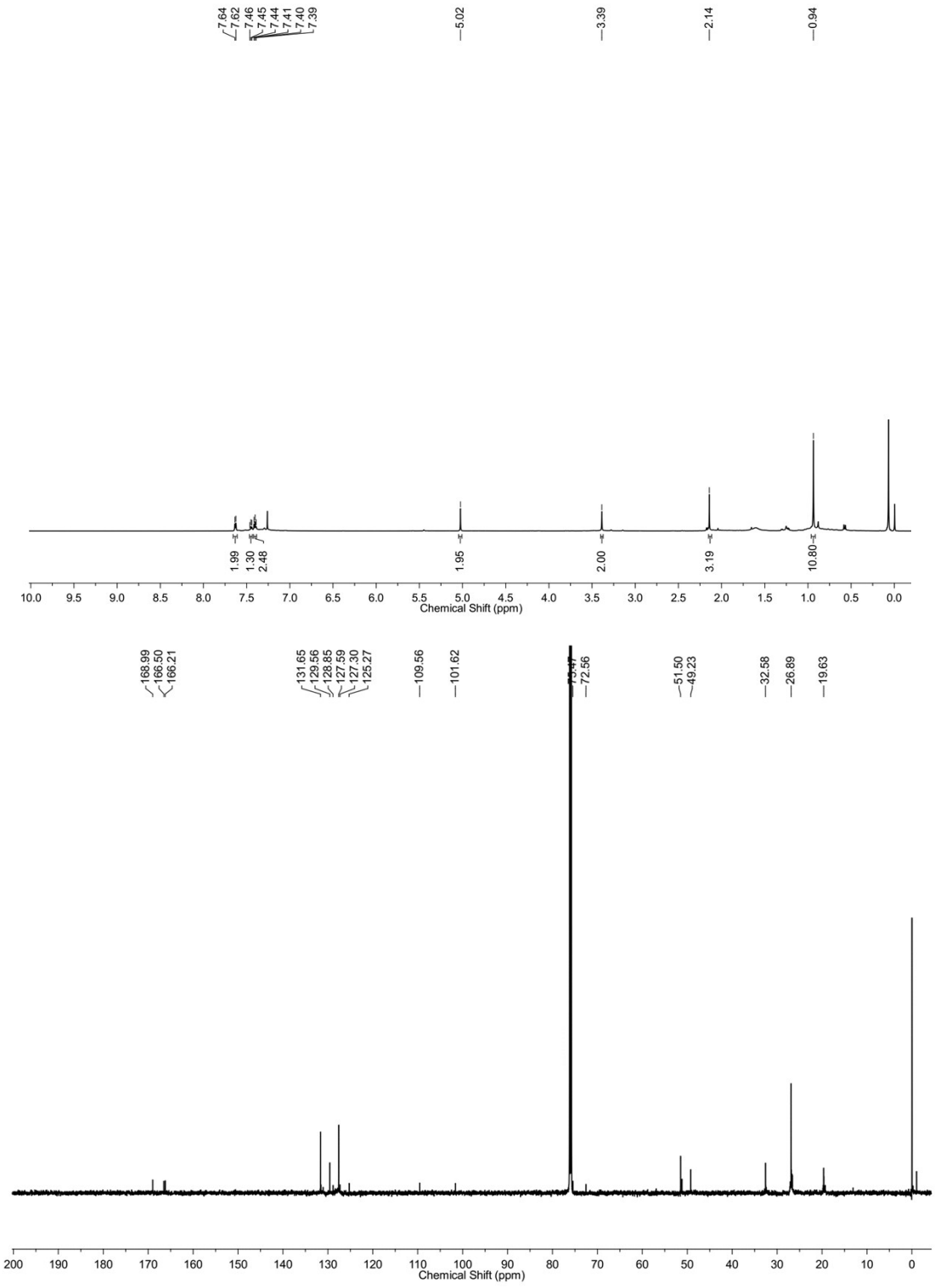


Figure S10 ^{1}H NMR and ^{13}C NMR spectra of EDY-B

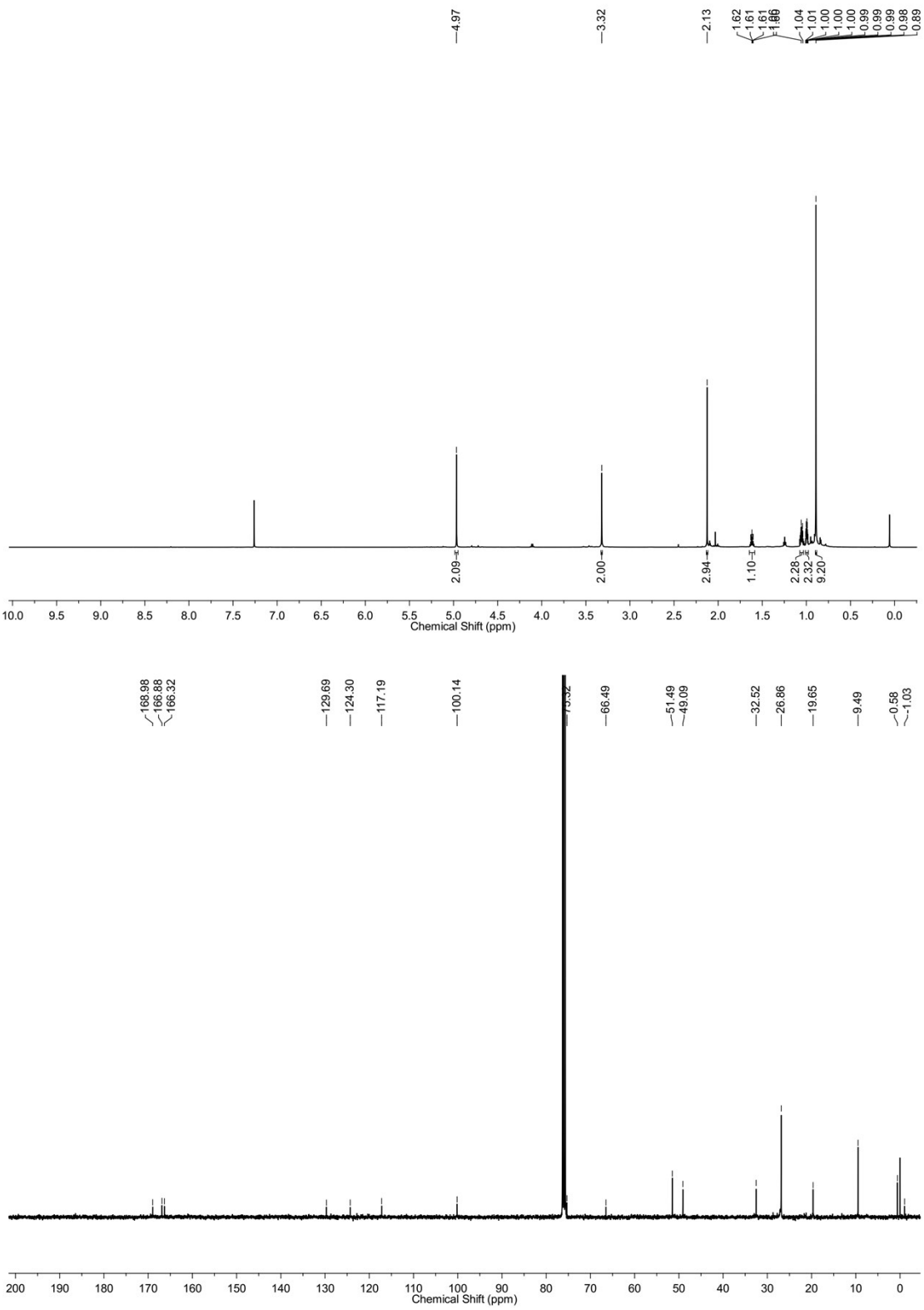


Figure S11 ^{1}H NMR and ^{13}C NMR spectra of EDY-C

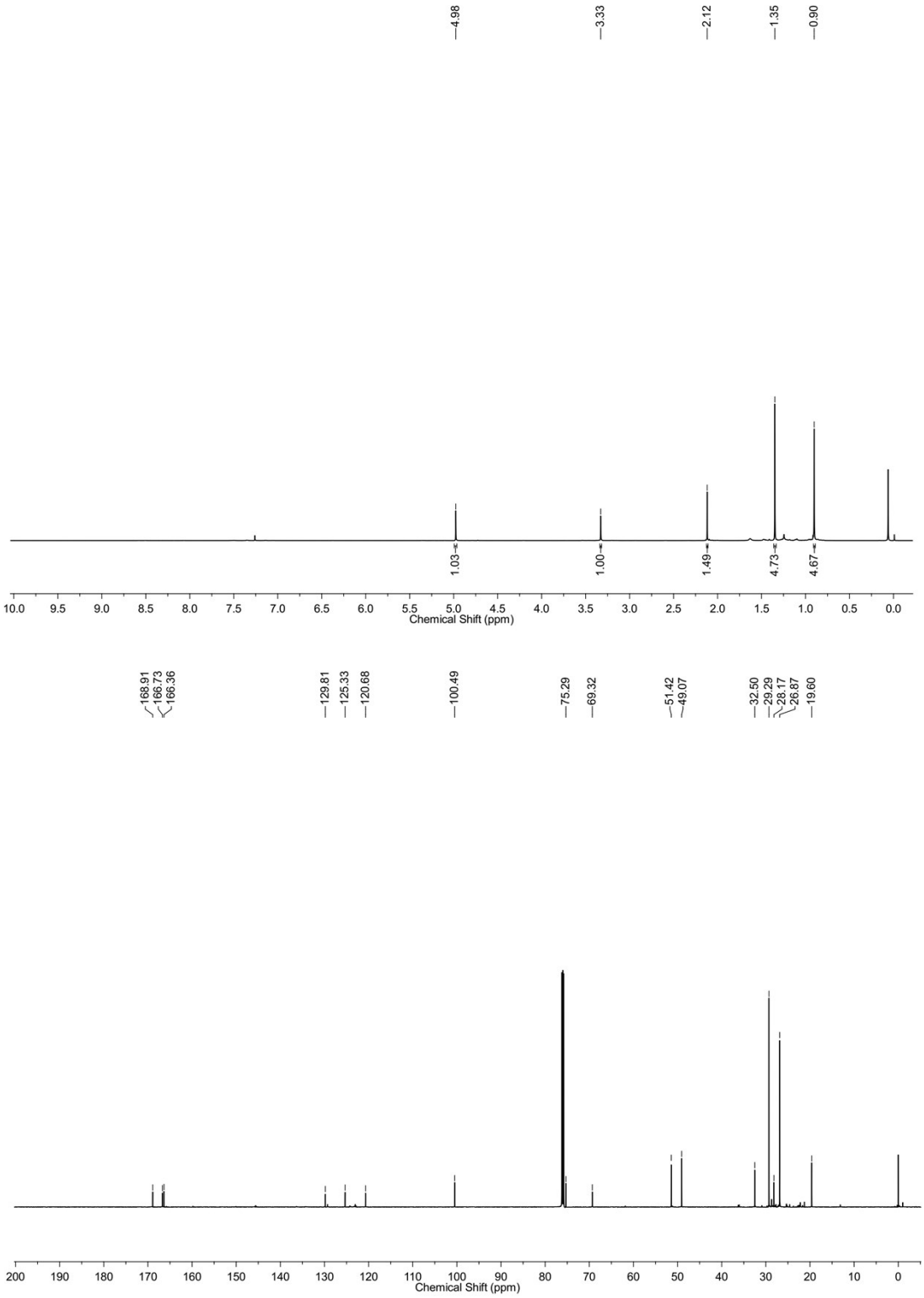


Figure S12 ^{1}H NMR and ^{13}C NMR spectra of EDY-D

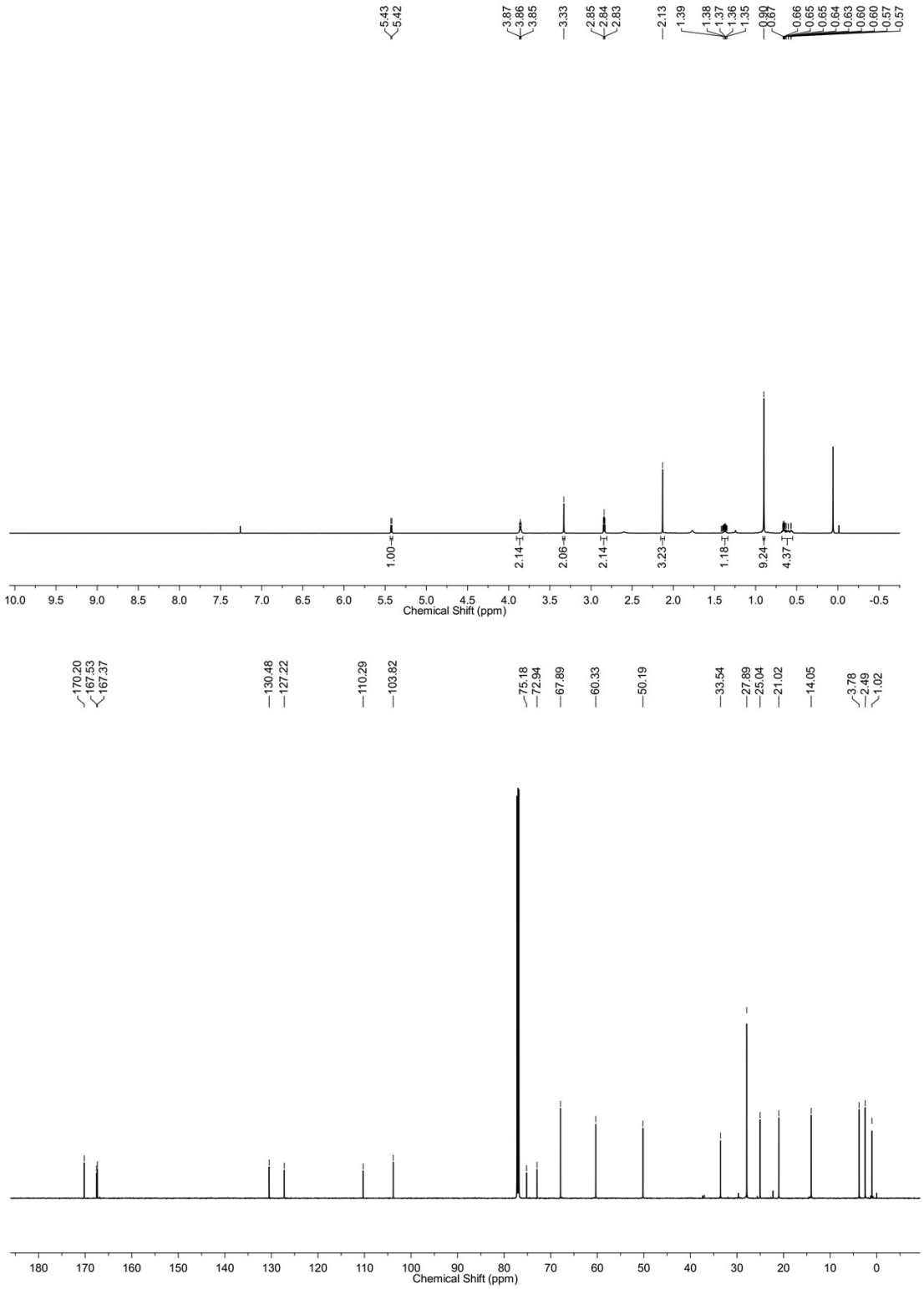


Figure S13 ^{1}H NMR and ^{13}C NMR spectra of EDY-E

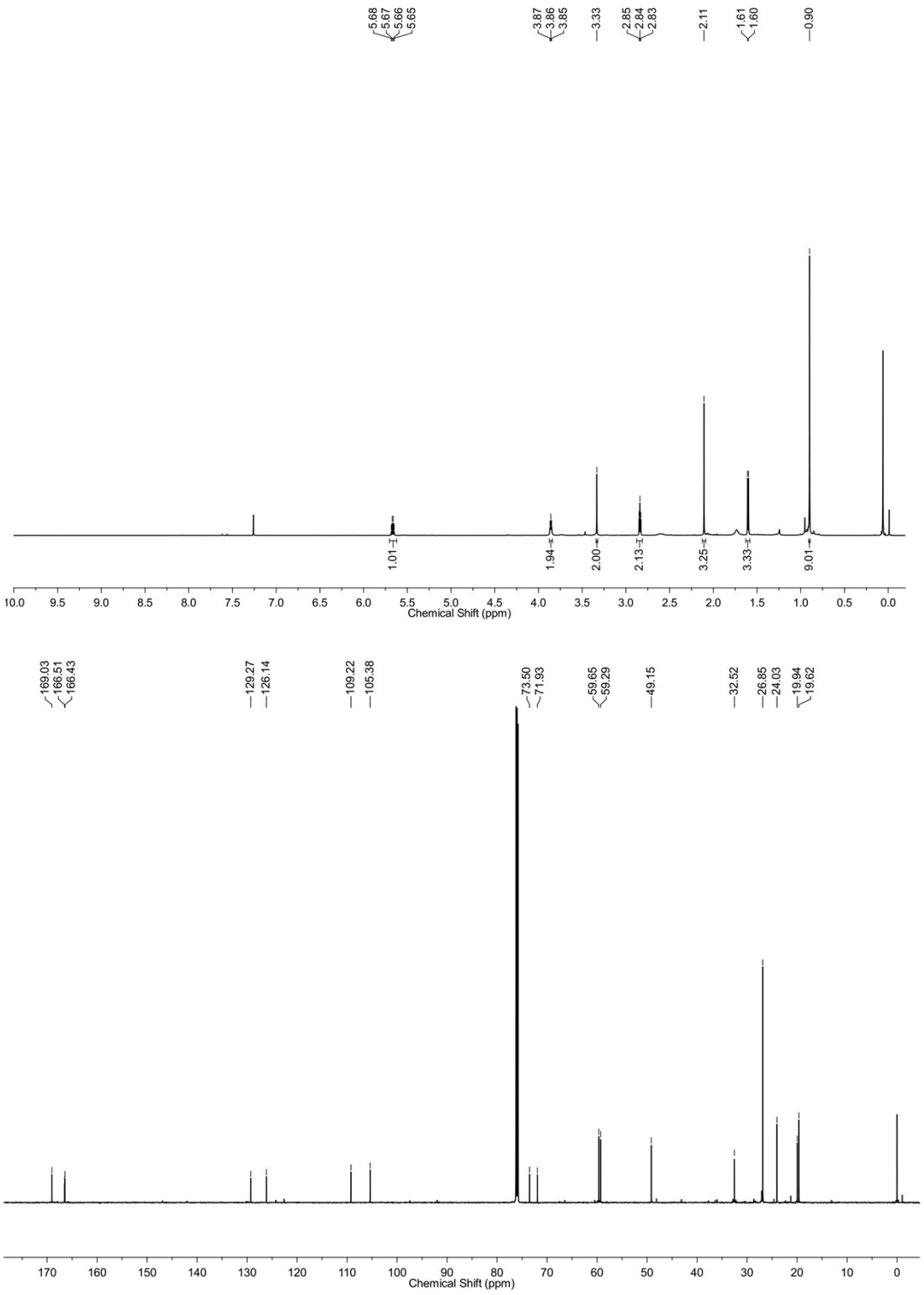


Figure S14 ^{1}H NMR and ^{13}C NMR spectra of EDY-F

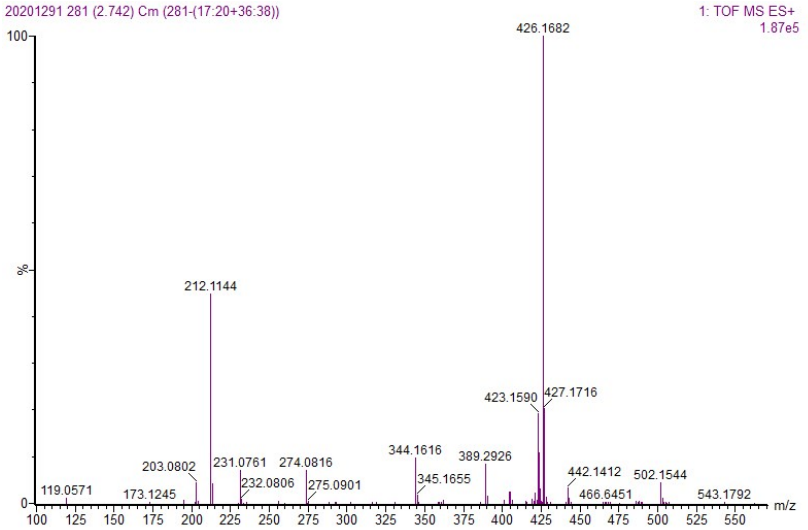


Figure S15 HR-MS spectrum of EDY-A

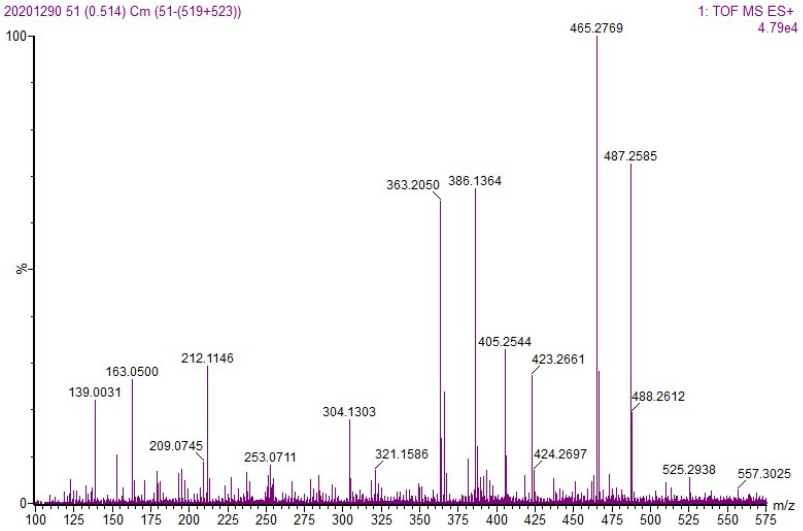


Figure S16 HR-MS spectrum of EDY-B

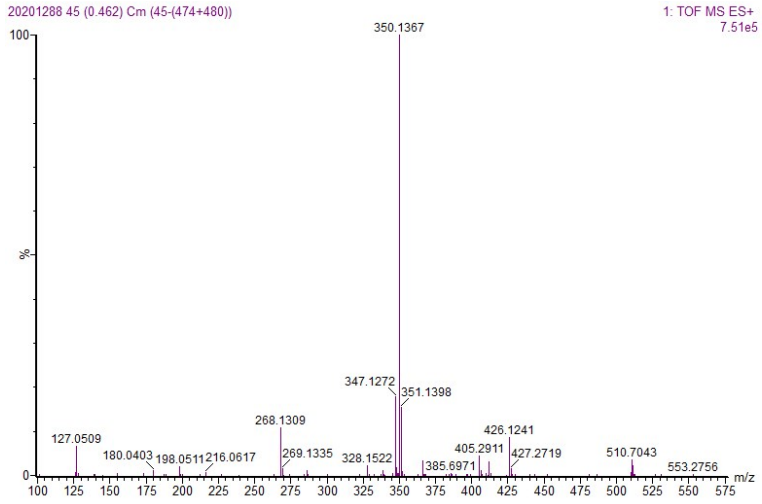


Figure S17 HR-MS spectrum of EDY-C

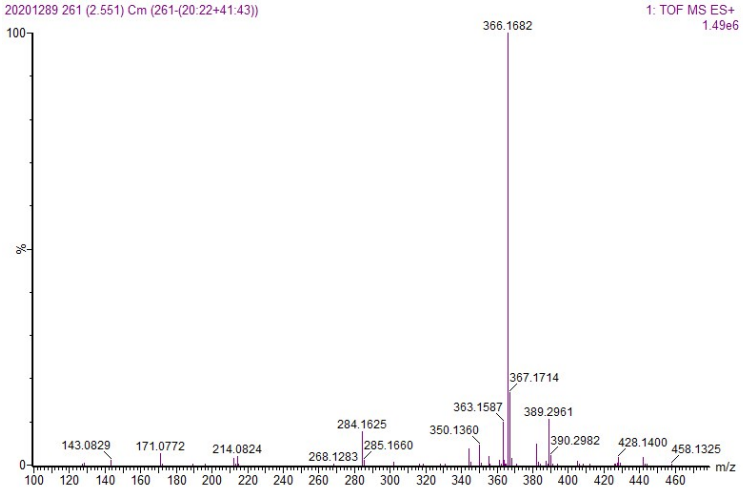


Figure S18 HR-MS spectrum of EDY-D

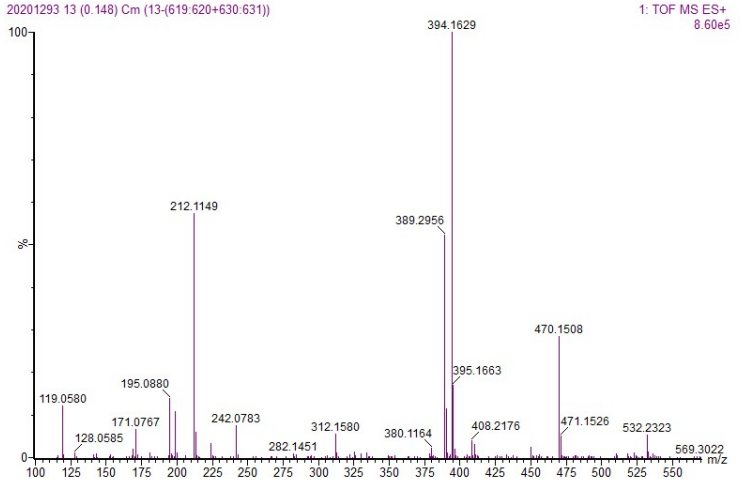


Figure S19 HR-MS spectrum of EDY-E

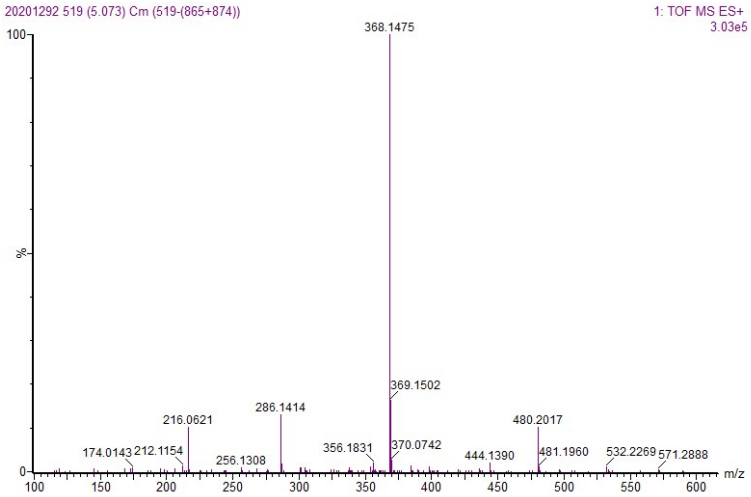


Figure S20 HR-MS spectrum of EDY-F

Computational Details

Below is a list of the calculated electronic energies without zero-point correction and Gibbs free energies in atomic units at (U)B3LYP/6-31G(d) as well as the Cartesian coordinates of all the optimized structures. The nature of the stationary points was characterized by vibrational frequency analysis. The structure of **H2** is similar to that of **H2-1** (Fig. 2) except the lack of 1,4-CHD.

H1

E (UB3LYP) = -1166.06924178A.U.
G (298.15 K) = -1165.791350 A.U.
No imaginary frequency

1	C	3.3588880	1.0291170	-0.1429570
2	C	2.4928160	-0.0624550	-0.1558200
3	C	1.1011370	0.0438620	-0.4591360
4	C	1.5843260	2.4231880	-0.6828400
5	C	2.9440710	2.3264310	-0.4438130
6	C	4.7381420	0.5511180	0.1780240
7	C	3.2990920	-1.2743630	0.1651180
8	H	3.6415070	3.1557100	-0.4998930
9	O	2.9574550	-2.4408750	0.2632580
10	O	5.7627690	1.1968060	0.2743730
11	N	4.6176110	-0.8309170	0.3539680
12	C	5.7159810	-1.7158370	0.6911950
13	H	5.8531210	-2.4677230	-0.0916000
14	H	6.6148360	-1.1038980	0.7820210
15	H	5.5144260	-2.2275410	1.6368200
16	C	0.6677950	1.4061190	-0.6762100
17	O	-0.6742190	1.7230620	-0.8724280
18	C	-1.4838590	1.7970080	0.2502170
19	C	-2.8389200	2.3158280	-0.1435120
20	H	-2.7335490	3.2746270	-0.6604120
21	H	-3.3207540	1.6124790	-0.8289470
22	H	-3.4564910	2.4218510	0.7468750
23	O	-1.1123520	1.4820070	1.3502400
24	C	0.2807850	-1.1013850	-0.4896260
25	H	0.7411730	-2.0131030	-0.1195540
26	C	-1.0994390	-1.2088080	-0.9350750
27	C	-1.7195680	-2.5772400	-1.1118940
28	C	-2.1548520	-1.7856690	0.0792350
29	H	-1.4951430	-0.4155750	-1.5590840
30	H	-1.0809250	-3.4386860	-0.9328380
31	H	-2.4132990	-2.7079710	-1.9376600
32	H	-1.7143540	-2.0791010	1.0282050
33	C	-3.4757140	-1.1171350	0.2046060

34	C	-3.8514840	-0.5814380	1.4470990
35	C	-4.3551870	-0.9624110	-0.8792020
36	C	-5.0688170	0.0795430	1.6041450
37	H	-3.1713840	-0.6692090	2.2898550
38	C	-5.5704780	-0.2945920	-0.7245810
39	H	-4.0944690	-1.3660660	-1.8535730
40	C	-5.9345840	0.2280390	0.5183400
41	H	-5.3382860	0.4838130	2.5763100
42	H	-6.2367560	-0.1888620	-1.5767640
43	H	-6.8829430	0.7441560	0.6390510

TS1

E (UB3LYP) = -1166.06190990 A.U.
G (298.15 K) = -1165.784341 A.U.
One imaginary frequency

1	C	3.2576839	1.1549517	-0.0171736
2	C	2.4502059	0.0332617	-0.2185076
3	C	1.0605969	0.1126307	-0.4992946
4	C	1.4094809	2.5145777	-0.3252936
5	C	2.7697399	2.4570397	-0.0853656
6	C	4.6600129	0.7062837	0.2335104
7	C	3.3233509	-1.1693313	-0.0903106
8	H	3.4146869	3.3220937	0.0284494
9	O	3.0489169	-2.3540433	-0.1807466
10	O	5.6482789	1.3808117	0.4455274
11	N	4.6155079	-0.6904923	0.1774654
12	C	5.7608349	-1.5574063	0.3764534
13	H	5.9408869	-2.1616743	-0.5176276
14	H	6.6240739	-0.9205173	0.5760964
15	H	5.5865239	-2.2281553	1.2228704
16	C	0.5555589	1.4570137	-0.5090616
17	O	-0.7976491	1.7228297	-0.7117706
18	C	-1.6224521	1.6930487	0.4004284
19	C	-2.9988341	2.1608667	0.0155394
20	H	-2.9376311	3.1396347	-0.4697866
21	H	-3.4444071	1.4582137	-0.6950776
22	H	-3.6249151	2.2074287	0.9050444
23	O	-1.2496151	1.3379527	1.4877454
24	C	0.2923629	-1.0765333	-0.7105106
25	H	0.8098539	-1.9983703	-0.4598976
26	C	-1.0119181	-1.1917963	-1.1945166
27	C	-1.7017661	-2.5148033	-1.2987606
28	C	-2.2509621	-2.0200123	-0.0113076

29	H	-1.5359161	-0.3331403	-1.5942706
30	H	-1.0451811	-3.3822353	-1.2202976
31	H	-2.4046151	-2.6210053	-2.1247626
32	H	-1.6887411	-2.2681223	0.8823384
33	C	-3.5147651	-1.3424433	0.1734774
34	C	-3.8626421	-0.8707243	1.4611014
35	C	-4.4336821	-1.1234793	-0.8794586
36	C	-5.0751141	-0.2299883	1.6868024
37	H	-3.1578121	-1.0009963	2.2774494
38	C	-5.6430021	-0.4739463	-0.6484546
39	H	-4.2026491	-1.4707223	-1.8822116
40	C	-5.9745341	-0.0256413	0.6343104
41	H	-5.3184001	0.1199117	2.6864934
42	H	-6.3349051	-0.3215813	-1.4727236
43	H	-6.9207491	0.4776917	0.8109454

H2

E (UB3LYP) = - -1166.07355277A.U.
G (298.15 K) = -1165.797391 A.U.
No imaginary frequency

1	C	3.5370710	1.0085640	-0.2096480
2	C	2.6470250	-0.0663990	-0.1201150
3	C	1.2531560	0.0743670	-0.2327550
4	C	1.7368450	2.4349340	-0.4930200
5	C	3.1088000	2.3163610	-0.4134700
6	C	4.9317440	0.4973760	-0.0632960
7	C	3.4627860	-1.3018460	0.0979130
8	H	3.8046150	3.1453470	-0.4941260
9	O	3.1089830	-2.4575850	0.2427170
10	O	5.9762820	1.1166460	-0.0900620
11	N	4.8012830	-0.8852440	0.1171650
12	C	5.9144900	-1.7939850	0.3170380
13	H	5.9011830	-2.5787080	-0.4445190
14	H	6.8332880	-1.2107020	0.2390960
15	H	5.8524720	-2.2619580	1.3040740
16	C	0.8121600	1.4186750	-0.4087330
17	O	-0.5363820	1.7370100	-0.5258020
18	C	-1.2842490	1.7861780	0.6423160
19	C	-2.7003100	2.1725210	0.3146000
20	H	-2.7142260	3.1791340	-0.1167070
21	H	-3.1218980	1.4863080	-0.4251420
22	H	-3.3023170	2.1437650	1.2221970
23	O	-0.8213990	1.5561540	1.7274670

24	C	0.3692450	-1.0900020	-0.1277510
25	H	0.8035180	-1.9404770	0.3909940
26	C	-0.8652200	-1.2043000	-0.6448600
27	C	-1.7671050	-2.3938350	-0.4128470
28	C	-2.8368300	-1.9479240	0.5478560
29	H	-1.3071800	-0.3866470	-1.2064400
30	H	-1.1976190	-3.2264250	0.0136150
31	H	-2.1977280	-2.7417920	-1.3612480
32	H	-2.6212760	-2.0775800	1.6057340
33	C	-3.9794660	-1.1890800	0.1997350
34	C	-4.8320760	-0.6767810	1.2219830
35	C	-4.3206140	-0.8736920	-1.1488800
36	C	-5.9363980	0.1029500	0.9166030
37	H	-4.5940910	-0.9001940	2.2590680
38	C	-5.4285730	-0.0901930	-1.4426560
39	H	-3.7019180	-1.2457670	-1.9598390
40	C	-6.2441850	0.4078960	-0.4176090
41	H	-6.5645910	0.4833140	1.7178890
42	H	-5.6629680	0.1383440	-2.4792130
43	H	-7.1078950	1.0222230	-0.6545540

H2-1

E (UB3LYP) = -1399.50320338A.U.
G (298.15 K) = -1399.118927 A.U.
No imaginary frequency

1	C	4.6053530	-0.1532980	0.0634070
2	C	3.2491190	-0.2940320	-0.2638680
3	C	2.3456160	0.7798650	-0.1755780
4	C	4.2575470	2.0775390	0.5843790
5	C	5.1649700	1.0429180	0.4999680
6	C	5.3086280	-1.4448500	-0.1853790
7	C	3.0640020	-1.7023610	-0.7501800
8	H	6.2213590	1.1272910	0.7342580
9	O	2.0713940	-2.2807740	-1.1490640
10	O	6.4810790	-1.7161060	-0.0173120
11	N	4.3260100	-2.3104980	-0.6720170
12	C	4.5625910	-3.6909000	-1.0506860
13	H	4.3317130	-3.8393310	-2.1095630
14	H	5.6156930	-3.9070090	-0.8643850
15	H	3.9310070	-4.3587220	-0.4578560
16	C	2.9201480	2.0038030	0.2768440
17	O	2.1387630	3.1537920	0.3629200
18	C	-4.1795580	-0.4442390	-2.8320710

19	C	-3.4031780	0.6886700	-3.4523030
20	C	-3.5524440	1.9844380	-2.6972710
21	C	-4.2871750	2.1110480	-1.5890000
22	C	-5.0587790	0.9768920	-0.9664230
23	C	-4.9167800	-0.3152090	-1.7262610
24	H	-4.1162350	-1.4099970	-3.3316680
25	H	-2.3357680	0.4167350	-3.5153080
26	H	-3.0217480	2.8472820	-3.0979150
27	H	-4.3520350	3.0776600	-1.0909740
28	H	-5.4432660	-1.1763100	-1.3201740
29	H	-3.7142390	0.8284820	-4.5009260
30	H	-6.1244880	1.2496090	-0.8883290
31	C	1.2222030	3.2383720	1.3933750
32	C	0.4265850	4.5097040	1.2524440
33	H	1.0952690	5.3680590	1.1398710
34	H	-0.1918020	4.4532440	0.3496490
35	H	-0.2114670	4.6348770	2.1275870
36	O	1.0954430	2.3976130	2.2439370
37	C	0.9166770	0.7708820	-0.4965010
38	H	0.5259250	1.7245630	-0.8481790
39	C	0.0535830	-0.2367360	-0.3006410
40	H	0.3966450	-1.1877760	0.0911050
41	C	-1.4326720	-0.0894360	-0.4975480
42	H	-1.6500260	0.8348080	-1.0502310
43	H	-1.8258860	-0.9144410	-1.0992610
44	C	-2.0921350	-0.0251940	0.8508890
45	H	-4.7308450	0.8262160	0.0736860
46	C	-2.9868460	-0.9649060	1.4130120
47	C	-3.4218550	-2.1425140	0.7358780
48	C	-3.5148170	-0.7375300	2.7201700
49	C	-4.3250360	-3.0149070	1.3255620
50	H	-3.0470690	-2.3574360	-0.2586250
51	C	-4.4178040	-1.6154020	3.2980020
52	H	-3.1952800	0.1489960	3.2630120
53	C	-4.8339630	-2.7626540	2.6069110
54	H	-4.6384640	-3.9048750	0.7854620
55	H	-4.8029720	-1.4128500	4.2941270
56	H	-5.5410400	-3.4506730	3.0614340
57	H	-1.7943470	0.8191170	1.4707000

TS2

E (UB3LYP) = -1399.48133643 A.U.
G (298.15 K) = -1399.098162 A.U.

One imaginary frequency

1	C	4.9602661	0.2777609	-0.2333193
2	C	3.6349441	0.0560319	-0.6320163
3	C	2.6553821	1.0590139	-0.5571673
4	C	4.4254551	2.4586769	0.3479537
5	C	5.4096381	1.4950149	0.2681387
6	C	5.7600351	-0.9558711	-0.4870963
7	C	3.5638691	-1.3453271	-1.1609843
8	H	6.4457081	1.6476459	0.5533147
9	O	2.6266061	-1.9743001	-1.6127203
10	O	6.9395111	-1.1543761	-0.2724923
11	N	4.8607631	-1.8699551	-1.0434673
12	C	5.2062331	-3.2215941	-1.4409883
13	H	5.0199041	-3.3622791	-2.5096343
14	H	6.2655311	-3.3672961	-1.2240163
15	H	4.6054271	-3.9454741	-0.8827323
16	C	3.1124681	2.2994859	-0.0245543
17	O	2.2348421	3.3777459	0.0817317
18	C	-4.5550689	-1.4247111	-1.7146813
19	C	-4.3018939	-0.5861491	-2.9412383
20	C	-4.2613469	0.8901619	-2.6430043
21	C	-4.2533909	1.3776099	-1.3900723
22	C	-4.2368139	0.5017619	-0.2038843
23	C	-4.5514189	-0.9141571	-0.4706903
24	H	-4.7569539	-2.4826361	-1.8706333
25	H	-3.3526989	-0.8960461	-3.4167123
26	H	-4.2440839	1.5680229	-3.4942373
27	H	-4.2241099	2.4533439	-1.2258083
28	H	-4.7359989	-1.5590191	0.3851447
29	H	-5.0674259	-0.7955831	-3.7065263
30	H	-4.7147759	0.9186819	0.6877497
31	C	1.2576311	3.3287689	1.0542757
32	C	0.3555021	4.5277369	0.9196097
33	H	0.9446511	5.4470529	0.8568947
34	H	-0.2220979	4.4425309	-0.0078063
35	H	-0.3237989	4.5656959	1.7716467
36	O	1.1550741	2.4391439	1.8587517
37	C	1.2469041	0.9519379	-0.9417443
38	H	0.8064951	1.8692089	-1.3312113
39	C	0.4447031	-0.0964671	-0.7050013
40	H	0.8453641	-0.9989871	-0.2557023
41	C	-1.0496709	-0.0275851	-0.8487383
42	H	-1.3450239	0.7471379	-1.5654633
43	H	-1.4631759	-0.9767461	-1.2062873

44	C	-1.6063129	0.3241739	0.5291237
45	H	-2.9990459	0.4856469	0.2184527
46	C	-1.5602699	-0.6510991	1.6212517
47	C	-1.8280359	-2.0212911	1.4113577
48	C	-1.2967499	-0.2223791	2.9396017
49	C	-1.8342549	-2.9187721	2.4761517
50	H	-2.0440929	-2.3786851	0.4090157
51	C	-1.3016429	-1.1228421	4.0005667
52	H	-1.0541519	0.8233059	3.1122357
53	C	-1.5734139	-2.4763651	3.7764167
54	H	-2.0430759	-3.9696281	2.2915727
55	H	-1.0831269	-0.7711711	5.0056957
56	H	-1.5761749	-3.1794441	4.6049217
57	H	-1.3228109	1.3329349	0.8390537

H3

E (UB3LYP) = -1399.53337891 A.U.
G (298.15 K) = -1399.144376 A.U.
No imaginary frequencies

1	C	3.7215440	-0.7876240	0.4404560
2	C	2.4708050	-0.6669270	-0.1822590
3	C	2.0352880	0.5368320	-0.7584080
4	C	4.1773510	1.4333360	-0.0375470
5	C	4.6288120	0.2622340	0.5345930
6	C	3.9010960	-2.1922310	0.9097990
7	C	1.8328480	-2.0219220	-0.1444240
8	H	5.5964810	0.1413010	1.0109610
9	O	0.7648860	-2.4049070	-0.5835380
10	O	4.8438570	-2.6834470	1.4971910
11	N	2.7347980	-2.8610360	0.5231700
12	C	2.4735270	-4.2667470	0.7697750
13	H	2.3302340	-4.7969690	-0.1760580
14	H	3.3360410	-4.6734910	1.2998210
15	H	1.5712620	-4.3835270	1.3772670
16	C	2.9641110	1.6119350	-0.6575310
17	O	2.6534420	2.8479740	-1.2210690
18	C	-2.3240980	-2.4934690	-0.0902450
19	C	-2.2124860	-2.8310180	-1.5498890
20	C	-3.4504000	-2.5012260	-2.3348500
21	C	-4.5602020	-1.9585590	-1.7462520
22	C	-4.5931160	-1.6677390	-0.3579680
23	C	-3.4515710	-1.9386720	0.4442870
24	H	-1.4523050	-2.6902770	0.5254460

25	H	-1.3307590	-2.3173240	-1.9733650
26	H	-3.4430200	-2.7114010	-3.4019780
27	H	-5.4368740	-1.7391740	-2.3528370
28	H	-3.4786520	-1.6788520	1.4993780
29	H	-1.9559900	-3.9015040	-1.6719580
30	H	-5.4798310	-1.2300880	0.0889410
31	C	1.7521090	3.6441710	-0.5453410
32	C	1.4601150	4.8802400	-1.3534230
33	H	2.3904190	5.3572240	-1.6748020
34	H	0.9049600	4.5984320	-2.2552590
35	H	0.8622410	5.5687330	-0.7558520
36	O	1.2776390	3.3534730	0.5221600
37	C	0.7523840	0.7937160	-1.4217480
38	H	0.8029960	1.4959900	-2.2534420
39	C	-0.4508970	0.3690650	-1.0099770
40	H	-0.5321030	-0.2820310	-0.1458620
41	C	-1.7373290	0.8506460	-1.6156710
42	H	-1.5722670	1.1492450	-2.6590310
43	H	-2.4824840	0.0484100	-1.6110470
44	C	-2.3089710	2.0773010	-0.8445380
45	H	-3.1945130	2.4336920	-1.3850590
46	C	-2.6727350	1.7782750	0.5935390
47	C	-3.9806140	1.4161000	0.9367680
48	C	-1.7010400	1.8229660	1.6043200
49	C	-4.3140400	1.0925810	2.2529370
50	H	-4.7415860	1.3745840	0.1613420
51	C	-2.0292130	1.4910740	2.9195390
52	H	-0.6862430	2.1242360	1.3580800
53	C	-3.3359730	1.1222520	3.2487760
54	H	-5.3361450	0.8154300	2.4993570
55	H	-1.2635580	1.5295810	3.6902890
56	H	-3.5917710	0.8696750	4.2745780
57	H	-1.5625350	2.8810870	-0.8773580

References:

1. M. Zhang, B. Li, H. Chen, H. Lu, H. Ma, X. Cheng, W. Wang, Y. Wang, Y. Ding and A. Hu, *J. Org. Chem.*, 2020, **85**, 9808-9819.
2. H. Chen, B. Li, M. Zhang, H. Lu, Y. Wang, W. Wang, Y. Ding and A. Hu, *ChemistrySelect*, 2020, **5**, 7069-7075.
3. G. M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell and A. J. Olson, *J Comput Chem*, 2009, **30**, 2785-2791.
4. M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K.

Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, Ö. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski and D. J. Fox, *Journal*, 2009.