Facilitating Myers-Saito Cyclization through Acid-Triggered Tautomerization for the Development of Maleimide-Base Antitumor Agents

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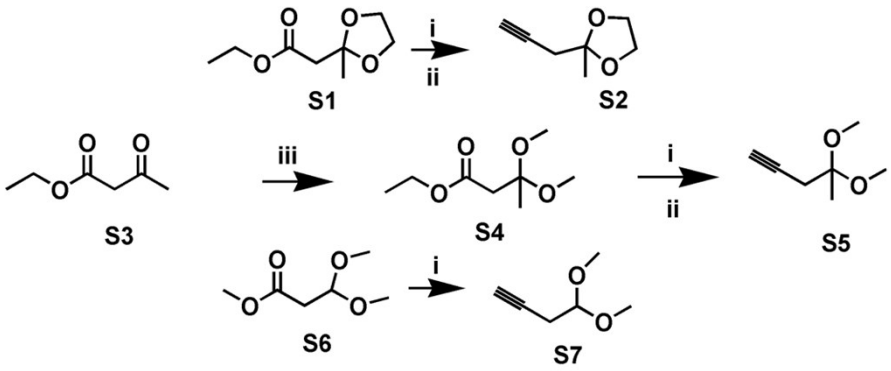
Materials

Toluene and tetrahydrofuran (THF) were dried over calcium hydride (CaH_2) and distilled before use. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, alexa fluorR 488 annexin V/dead cell apoptosis assay kit, ROS assay kit, phospho-histone H2A.X (Ser139) rabbit monoclonal antibody and Alexa Fluor 488-labeled Goat Anti-Rabbit IgG(H+L) were purchased from Beyotime Biotechnology. HeLa cancer cell lines were obtained from the Chinese Academy of Science Cell Bank for Type Culture Collection (Shanghai, China) and used for all of cell experiments. 3,4-Diiodo-N-maleimide was synthesized according to literature procedure with minor modification[1]. Other reagents were of commercial grade and used without further purification. Sonogashira reactions and amidation reactions were performed with dry Schlenk techniques under an atmosphere of nitrogen.

Characterizations

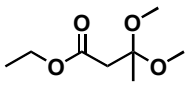
^{1}H NMR (400MHz) and ^{13}C NMR (101MHz) spectra were recorded in chloroform-d ($CDCl_3$) on a Bruker Avance III 400 NMR spectrometer. ^{1}H NMR (600MHz) and ^{13}C NMR (151MHz) spectra were recorded in $CDCl_3$ on a Bruker Avance III 600 NMR spectrometer. Chemical shifts were referenced to Me_4Si. Mass spectra were recorded on a Micromass LCTTM mass spectrometer using the ESI method. Fluorescence spectra were recorded in methanol (200 μM) on a PerkinElmer LS-55 (excited at 350 nm). EPR tests were performed with an X-band EMX-8/2.7C EPR spectrometer (Bruker, Germany). The settings of the spectrometer were as follows: sweep width, 150 G; time constant, 163.84 ms; conversion time, 40.96 ms; resolution, 1024 points; modulation frequency, 100.00 kHz; modulation amplitude, 1.00 G; and microwave power, 6.420 mW.

Synthesis



Scheme S1 Synthesis of compound S2, S5, S7. (i) DIBAL-H, dichloromethane, -78℃, 1 h. (ii) Bestmann–Ohira reagent, K_2CO_3, methanol, rt, 24 h. (iii) Trimethyl Orthoformate, $LiBF_4$, methanol, 65℃, 24h.

3,3-Dimethoxy-butyric acid ethyl ester (Compound S4)



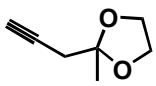
3,3-Dimethoxy-butyric acid ethyl ester (Compound S4) was synthesized according to a literature procedure with minor modification[2]. Trimethyl orthoformate (690 mg, 6.5 mmol) was added to a solution of 3-oxo-butyric acid ethyl ester (6.5 g, 50 mmol) and $LiBF_4$ (141 mg, 1.5 mmol) in anhydrous methanol (25 mL). The mixture was kept stirring at reflux for 24 h, and later quenched by adding saturated $NaHCO_3$ (100 mL) solution. The resulted mixture was extracted with ethyl acetate. The combined extracts were washed with saturated NaCl solution. The organic layer was dried (Na_2SO_4), and concentrated to give 7.2 g (83%) 3,3-Dimethoxy-butyric acid ethyl ester. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.09 (q, J = 7.2 Hz, 2H), 3.16 (s, 6H), 2.59 (s, 2H), 1.40 (s, 3H), 1.20 (t, J = 7.1 Hz, 3H).

Synthesis of compounds S2, S5, S7

General procedures: ester S1, S4, or S6 (40 mmol, 1 equiv.) was dissolved in dichloromethane (100 mL), and the solution was cooled to -78 ºC. DIBAL-H (48 mmol, 1 M solution in toluene, 1.2 equiv.) was added via syringe over 10 min, and the mixture was stirred for 4 h at -78 ºC. The reaction was monitored by TLC and determined to be complete after the ester was consumed. The mixture was quenched with anhydrous methanol (20 mL) at -78 ºC and allowed to warm to 0 ºC. More methanol (50 mL) was added followed by potassium carbonate (80 mmol, 2 equiv.) and dropwise addition of the Bestmann–Ohira reagent (48 mmol, 1.2 equiv.). The reaction mixture was stirred for 24 h at room temperature. Most of the solvent was removed, then ethyl acetate (400 mL) and Rochelle's salt (400 mL) were added. The mixture was stirred for 2 h and the organic layer was separated, washed with brine, and dried over

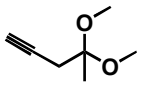
magnesium sulfate. The solvent was removed under reduced pressure. The crude material was purified by chromatographic column, eluted with 20-40% diethyl ether/pentane.

2-Methyl-2-prop-2-ynyl-[1,3]dioxolane (compound S2)[3]



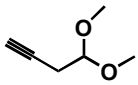
Light yellow oil, 3.27 g (65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.02 – 3.85 (m, 4H), 2.47 (d, J = 2.7 Hz, 2H), 2.00 (t, J = 2.7 Hz, 1H), 1.41 (s, 3H).

4,4-Dimethoxy-pent-1-yne (Compound S5)[4]



Yellow oil, 2.86 g (55%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.16 (s, 6H), 2.47 (d, J = 2.7 Hz, 2H), 1.98 (t, J = 2.7 Hz, 1H), 1.38 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 99.4, 78.8, 69.3, 47.5, 26.5, 20.5.

4,4-Dimethoxy-but-1-yne (Compound S7)[5]



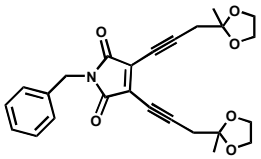
Colorless oil, 1.93 g (42.1%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.53 (t, J = 5.6 Hz, 1H), 3.36 (s, 6H), 2.51 (dd, J = 5.6, 2.7 Hz, 2H), 2.02 (t, J = 2.7 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 102.2, 79.2, 70.0, 53.4, 23.6.

Synthesis of Enediyne

General procedure:

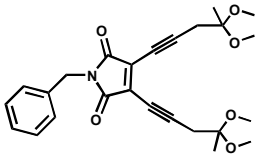
For symmetric enediyne: 3,4-diiodo-N-benzylmaleimide (263.4 mg, 0.6 mmol, 1 equiv.), CuI (45.7 mg, 40 %), $Pd(PPh_3)_2Cl_2$ (51.0 mg, 12.5 %), and K_2CO_3 (248.7 mg, 1.8 mmol) were successively added to a solvent mixture of dry THF (3 mL) and toluene (6 mL) under nitrogen atmosphere. Then, compound S2, S5 and S7 (3 equiv.) respective in THF (1 mL) was added dropwisely. The mixture was stirred at 40-50 ºC overnight. After the completion of the reaction as detected by TLC, the mixture was directly purified by column chromatography over alkaline silicate (eluted with 5-40% hexane/ethyl acetate) to yield the product.

1-Benzyl-3,4-bis-[3-(2-methyl-[1,3]dioxolan-2-yl)-prop-1-ynyl]-pyrrole-2,5-dione (EDY-A)



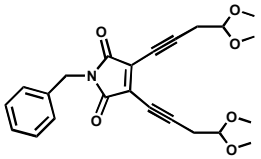
Isolated in 54% yield as brown oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.25 (m, 5H), 4.66 (s, 2H), 4.13 – 3.92 (m, 8H), 2.88 (s, 4H), 1.50 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.0, 134.7, 127.6, 127.5, 127.3, 126.9, 107.4, 106.3, 72.0, 64.4, 41.3, 31.0, 23.3. HR-MS (EI): m/z calcd. for $C_{25}H_{25}NO_6Na$ (M + Na): 458.1580; found: 458.1581.

1-Benzyl-3,4-bis-(4,4-dimethoxy-pent-1-ynyl)-pyrrole-2,5-dione (EDY-B)



Isolated in 46% yield as yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.26 (m, 5H), 4.67 (s, 2H), 3.23 (s, 12H), 2.88 (s, 4H), 1.48 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 167.0, 135.8, 128.7, 128.6, 127.9, 127.9, 107.0, 100.5, 73.2, 48.7, 42.3, 29.5, 21.8. HR-MS (EI): m/z calcd. for $C_{25}H_{29}NO_6Na$ (M + Na): 462.1893; found: 462.1891.

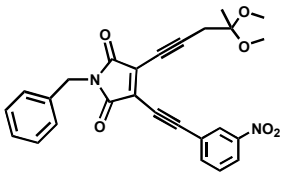
1-Benzyl-3,4-bis-(4,4-dimethoxy-but-1-ynyl)-pyrrole-2,5-dione (EDY-C)



Isolated in 34% yield as dark yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.25 (m, 5H), 4.66 (s, 2H), 4.63 (t, J = 5.6 Hz, 2H), 3.40 (s, 12H), 2.86 (d, J = 5.6 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.9, 135.7, 128.6, 128.5, 128.5, 127.9, 106.5, 102.0, 72.8, 53.8, 42.3, 25.9. HR-MS (EI): m/z calcd. for $C_{23}H_{25}NO_6Na$ (M + Na): 434.1580; found: 434.1579.

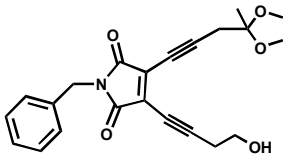
For asymmetric enediyne: 3,4-diiodo-N-benzylmaleimide (263.4 mg, 0.6 mmol 1 equiv.), CuI (45.7 mg, 40 %), $Pd(PPh_3)_2Cl_2$ (51.0 mg, 12.5 %), and K_2CO_3 (248.7 mg, 1.8 mmol) were successively added to a solvent mixture of dry THF (3 mL) and toluene (6 mL) under nitrogen atmosphere. Then, 1.5 equivalent of compound S5 or S7 with 1.5 equivalent of another alkyne (commercially available) in THF (1 mL) was added dropwisely. The mixture was stirred at 40-50 ºC overnight. After the completion of the reaction as detected by TLC, the mixture was directly purified by column chromatography over alkaline silicate (eluted with 5-40% hexane/ethyl acetate) to yield the product.

1-Benzyl-3-(4,4-dimethoxy-pent-1-ynyl)-4-(3-nitro-phenylethynyl)-pyrrole-2,5-dione (EDY-D)



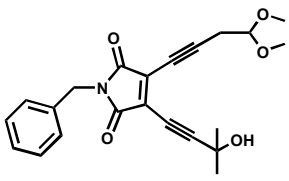
Isolated in 21% yield as yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.45 (s, 1H), 8.26 (d, J = 8.3 Hz, 1H), 7.88 (d, J = 7.7 Hz, 1H), 7.58 (t, J = 8.0 Hz, 1H), 7.39 – 7.27 (m, 5H), 4.72 (s, 2H), 3.26 (s, 6H), 2.95 (s, 2H), 1.54 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 165.5, 165.3, 147.2, 134.6, 129.2, 128.7, 127.7, 127.6, 127.1, 126.3, 126.2, 123.7, 122.2, 108.8, 103.9, 99.5, 80.6, 72.5, 59.4, 47.7, 41.5, 28.7, 20.8. HR-MS (EI): m/z calcd. for $C_{26}H_{21}N_2O_8$ (M-H): 457.1400; found: 457.1400.

1-Benzyl-3-(4,4-dimethoxy-pent-1-ynyl)-4-(4-hydroxy-but-1-ynyl)-pyrrole-2,5-dione (EDY-E)



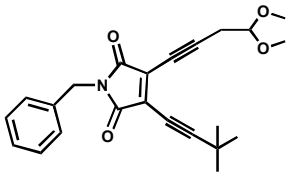
Isolated in 23% yield as brown oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.35 – 7.26 (m, 5H), 4.67 (s, 2H), 3.82 (t, J = 5.9 Hz, 2H), 3.24 (s, 6H), 2.89 (s, 2H), 2.80 (t, J = 6.0 Hz, 2H), 1.49 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.1, 165.9, 134.7, 127.8, 127.7, 127.7, 127.5, 127.0, 107.9, 106.3, 99.6, 72.3, 71.9, 59.3, 47.7, 41.3, 28.5, 24.0, 20.7. HR-MS (EI): m/z calcd. for $C_{22}H_{22}NO_5$ (M-H): 380.1498; found: 380.1498.

1-Benzyl-3-(4,4-dimethoxy-but-1-ynyl)-4-(3-hydroxy-3-methyl-but-1-ynyl)-pyrrole-2,5-dione (EDY-F)



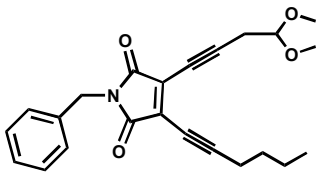
Isolated in 26% yield as light yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.35-7.25 (m, 5H), 4.63 (s, 2H), 4.61 (t, J = 5.6 Hz, 1H), 3.36 (s, 6H), 2.85 (d, *J* = 5.6 Hz, 2H), 1.57 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 165.8, 165.6, 134.6, 128.3, 127.7, 127.6, 127.4, 127.0, 113.2, 106.2, 100.7, 71.9, 71.5, 64.6, 52.5, 41.4, 29.8, 24.7. HR-MS (EI): m/z calcd. for $C_{22}H_{23}NO_5Na$ (M + Na): 404.1474; found: 404.1470

1-Benzyl-3-(4,4-dimethoxy-but-1-ynyl)-4-(3,3-dimethyl-but-1-ynyl)-pyrrole-2,5-dione (EDY-G)



Isolated in 21% yield as dark orange oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.35 – 7.26 (m, 5H), 4.66 (s, 2H), 4.62 (t, *J* = 5.5 Hz, 1H), 3.40 (s, 6H), 2.87 (d, *J* = 5.6 Hz, 2H), 1.33 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.2, 166.0, 134.8, 128.2, 127.7, 127.6, 126.9, 126.7, 119.2, 104.8, 101.1, 71.9, 69.3, 52.8, 52.5, 41.3, 29.4, 24.9. HR-MS (EI): m/z calcd. for $C_{23}H_{25}NO_4Na$ (M + Na): 402.1681; found: 402.1683

1-Benzyl-3-(4,4-dimethoxy-but-1-ynyl)-4-hex-1-ynyl-pyrrole-2,5-dione (EDY-H)



Isolated in 28% yield as dark brown oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.35-7.26 (m,*5H*), 4.66 (s, 2H), 4.63 (t, *J* = 5.6 Hz, 1H), 3.40 (s, 6H), 2.87 (d, *J* = 5.6 Hz, 2H), 2.54 (t, *J* = 7.1 Hz, 2H), 1.61 (p, *J* = 7.1 Hz, 2H), 1.47 (dq, *J* = 14.5, 7.3 Hz, 2H), 0.93 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.2, 166.1, 134.8, 128.2, 127.6, 127.6, 126.9, 126.8, 111.8, 104.8, 101.0, 71.8, 70.7, 52.8, 41.3, 29.0, 24.9, 20.9, 19.2, 12.5. HR-MS (EI): m/z calcd. for $C_{23}H_{24}NO_4$ (M-H): 378.1711; found: 378.1705.

EPR study of EDYs

PBS solutions of pH 5.5 and 7.5 containing TEMPO were prepared in advance. EDYs were dissolved in DMSO to a concentration of 10 mM. Samples were prepared and sealed into an EPR tube. For control, DMSO was added to PBS solution containing TEMPO to the same concentration of samples with EDY. The final concentrations for EDY and TEMPO were 1 mM and 0.10 mM, respectively. The tubes were placed at room temperature, and EPR spectra were recorded at 0 h and 24 h.

DNA-cleaving test

EDYs were dissolved in DMSO to a concentration of 20 mM and PUC19 plasmid DNA (200 ng/μL, in pH 8 TE solution) was used for these experiments. 1 μL TE solution with PUC19 plasmid DNA was added to 17 μL PBS solution (different pH), then EDYs in DMSO were added. Extra DMSO were added if it is necessary to maintain a total volume of 20 μL. For reactive species tests, an extra 2 μL PBS with different agents were added. Samples were placed at room temperature for 24 h. After incubation, each system (9 μL) was mixed with loading

buffer (1 μL) and subjected to 1% agarose gel electrophoresis at 90 V for 1 h, stained by Dured and then the gel was photographed on a UV transilluminator (FR-200A) and analyzed by scanning densitometry.

Cell viability

Cell viability tests were investigated by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. HeLa cells were cultured in complete Dulbecoo's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 ºC under a humidified atmosphere containing 5% CO_2. The cells were trypsinized until they reached 70% confluence in the tissue culture flasks with a buffered saline solution containing 0.25% trypsin and 0.03% EDTA. Cells were seeded into a 96-well plate (5000 cells and 100 μL cell culture medium per well). After 24 h of incubation, the culture medium was removed and the cells were then exposed to 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25 μM concentrations of EDY. The concentrated EDY prepared by dissolving EDY in DMSO to reach 10 mM and then diluted using cell culture medium to 25 μM. The culture medium (with 0.1% DMSO) was used as a blank control. After incubation for another 48 h, 10 mL 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 at 37 1C for 4 h to allow the yellow dye to transform into blue crystals. After removal of the supernatant MTT/medium, the purple MTT-formazan crystals were dissolved by adding 150 mL of DMSO to each well of the plate, and then the plate was shaken for 15 min. Finally, the optical density was measured using a microplate reader at a wavelength of 570 nm. The spectrophotometer was normalized using a culture medium without cells. Cell viability (%) related to control wells containing cell culture medium without HAPN suspension was calculated by $[OD]_{test}/[OD]_{control}$.

Cell internalization

The cellular internalization was observed using HeLa cells as a model cell line with fluorescence confocal microscopy. In brief, HeLa cells were seeded in glass-bottom dishes at 2.5×10^5 cells per well in 2 mL of DMEM and incubated for 24 h, followed by removal of the culture medium and addition of EDY solution (2 mL DMEM medium) at the planned concentration. The control group was HeLa cells incubated without any additives. After incubation at 37 ºC for 8 h or 16 h, the culture medium was removed, and the cells were washed with PBS two times. Subsequently, the cells were fixed with 2.5% glutaraldehyde at room temperature for 20 min, and the slides were rinsed with PBS three times. After permeated by 0.5% Triton solution for 10 min, 400 μL solution with propidium iodide (15 μg/mL) and 3,3-dioctadecyloxacarbocyanine perchlorate (10 μM) were added and the cells were cultured at 37 ºC for another 15 min. The slides were rinsed with cold PBS three times. The resulting slides were mounted and observed using a LEICA TCS SP8 fluorescence microscope.

In vitro DNA Damage and ROS Detection

DNA Damage:

Cells were cultured using the same procedure mentioned in cell internalization part. After fixation and permeation, cells were blocked by 8% BSA/PBS for 2 h at room temperature. Then, Cells were stained with phospho-histone H2A.X (Ser139) rabbit monoclonal antibody (1:200 dilution in 1% BSA/PBS, at 4 ºC, overnight) and labeled with secondary antibodies (Alexa Fluor® 488 dye, 1:1000 dilution, RT, 2 h). To visualize DNA, cells were further stained with PI. Cells were washed three times with PBS after all incubations (5 min each), after which samples were subjected to fluorescence microscope.

ROS Detection:

ROS Assay Kit (Beyotime) with fluorescent probe 2, 7-dichloro fluorescein diacetate (DCFH-DA) was used to detect the intracellular ROS levels. Cells were cultured using the same procedure mentioned in cell internalization part. Negative and positive control were prepared by adding DMSO and ROSUP. After cultured with EDY for 6 h, DCFH-DA were added (10 μM) placed in dark for 30 min at room temperature. Then, samples were washed for three times by PBS and subjected to fluorescence microscope.

Flow cytometry analysis of cell cycle and Apoptosis

HeLa cells were seeded in 6-well plates at 5×105 cells per well in 2 mL of complete DMEM and cultured for 24 h. The cells were treated with EDY at planned concentration for 24 h. HeLa cells without the treatment were used as a control. Cells were harvested, rinsed in PBS, re-suspended and fixed in 70% ethanol. Then the pellets were suspended in 1 mL of propidium iodide solution containing 20 mg/mL of PI, 0.2 mg/mL RNase, and 0.1% Trition X-100. Cell samples were incubated at room temperature in the dark for at least 30 min and analyzed by a flow cytometer (BDFACSCalibur, USA).

HeLa cells were seeded in 6-well plates at 5×10^5 cells per well in 2 mL of complete DMEM and cultured for 24 h. The cells were treated with EDY at planned concentration for 24 h. HeLa cells without the treatment were used as a control. For quantitative measurements of apoptosis, the treated cells were harvested and washed twice with ice-cold PBS, and stained with FITC-A and PI according to the manufacturer's instructions. sample were analyzed by flow cytometry (BDFACSCalibur, USA).

Table S1 DNA cleaving ability of EDYs (the amount of Form II DNA) under pH 5.5 and 7.5**.**

	pH 5.5	pH 7.5	Enhancement
EDY-A	**100.00%**	**85.21%**	**17.35%**
EDY-B	**99.08%**	**9.41%**	**963.08%**
EDY-C	**99.76%**	**37.61%**	**166.25%**
EDY-D	**20.22%**	**8.66%**	**133.84%**
EDY-E	**70.17%**	**9.83%**	**613.61%**
EDY-F	**97.68%**	**8.41%**	**1061.52%**
EDY-G	**20.62%**	**8.03%**	**156.84%**

Table S2 IC_{50} values for EDYs against Hela cells determined by MTT assay.

EDY	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
IC_{50} (μM)	**22.2**	**1.4**	**4.56**	**3.66**	**1.65**	**3.08**	**2.19**	**2.38**

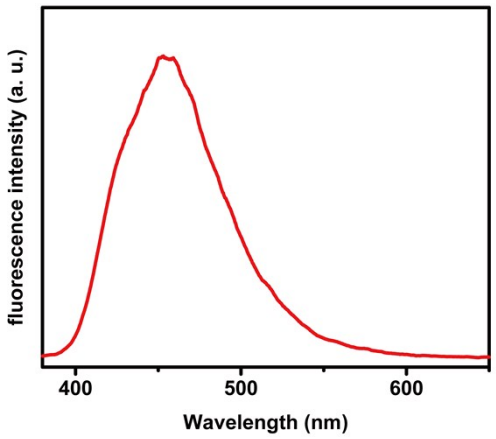


Figure S1 Fluorescence emission spectrum of EDY-B in methanol (λ_{ex} = 365 nm).

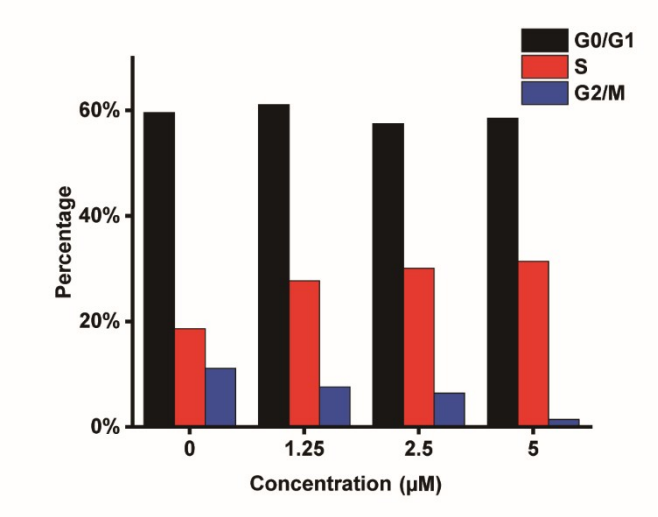


Figure S2 Concentration dependence of cell cycle distribution of Hela cells treated with **EDY-B.**

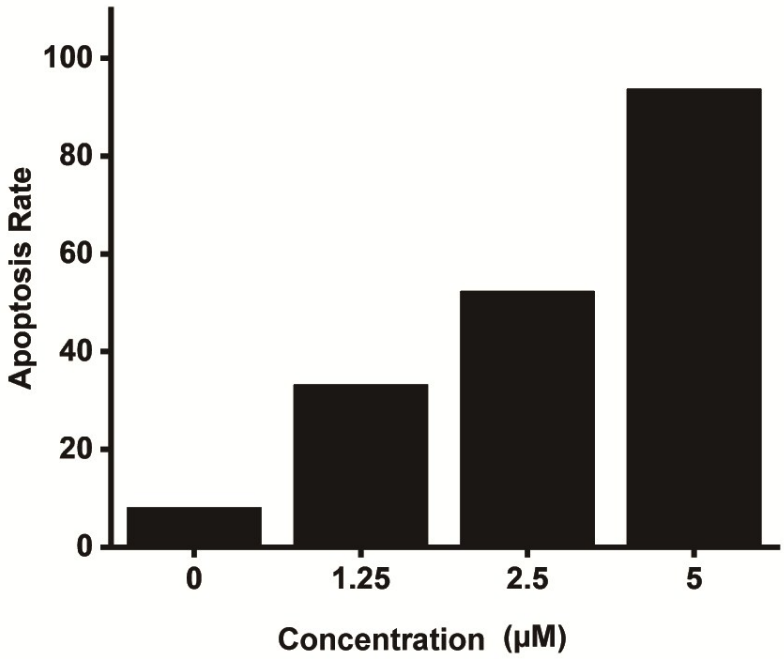


Figure S3 Concentration dependence of the apoptosis rate of Hela cells treated with **EDY-B.**

NMR Spectra for EDYs

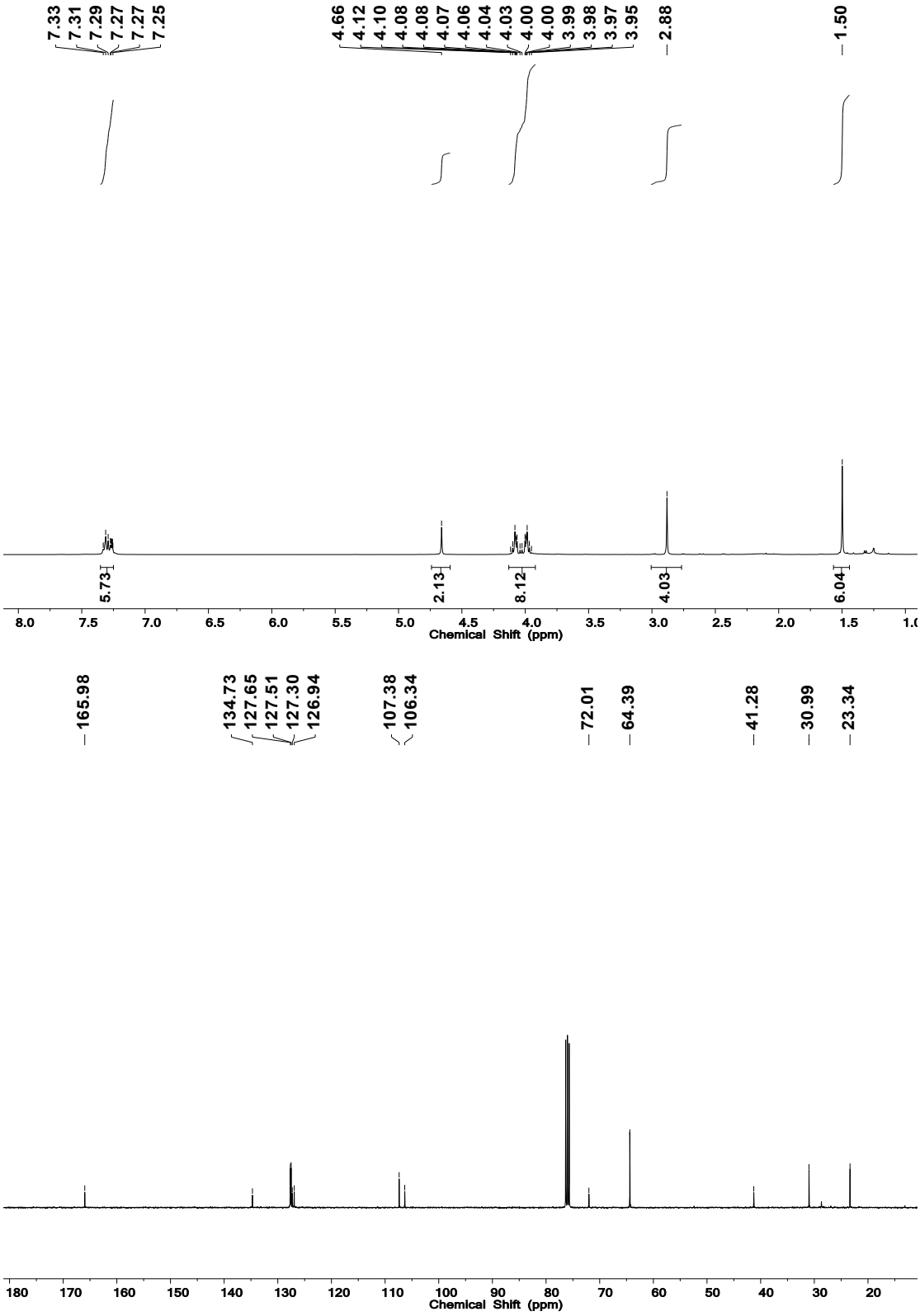


Figure S4 ^{1}H NMR and ^{13}C NMR spectra of **EDY-A**

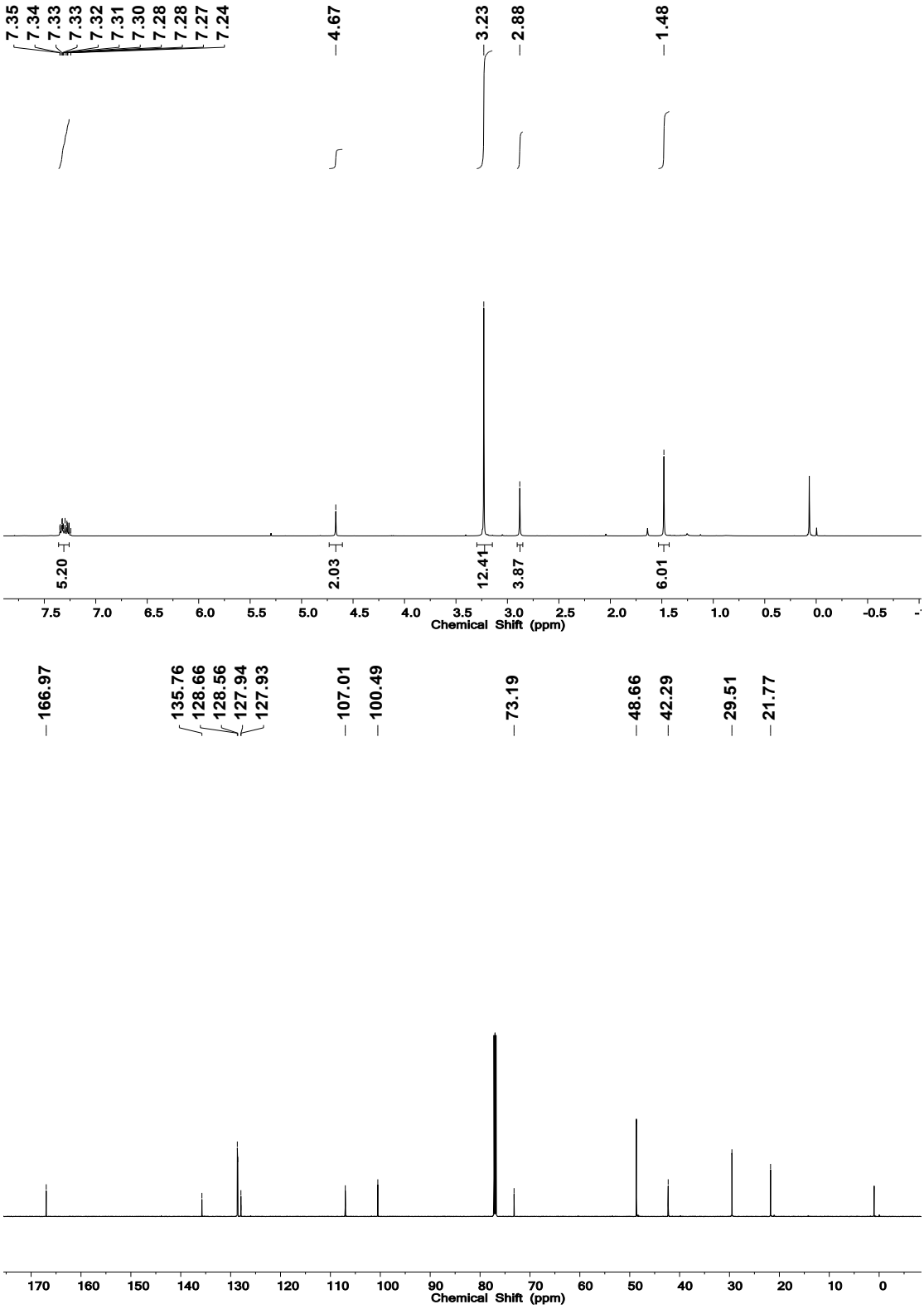


Figure S5 ^{1}H NMR and ^{13}C NMR spectra of **EDY-B**

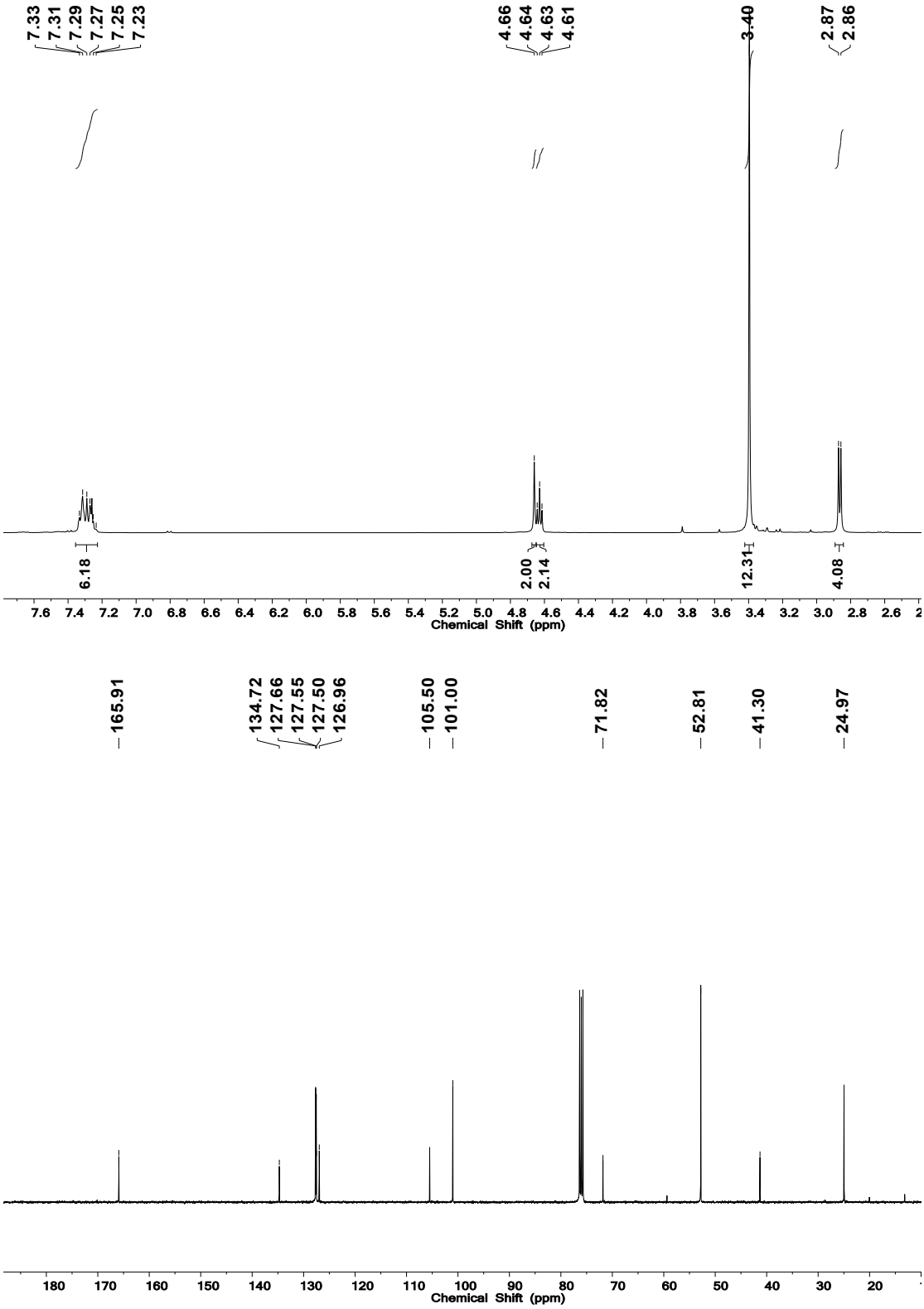


Figure S6 ^{1}H NMR and ^{13}C NMR spectra of **EDY-C**

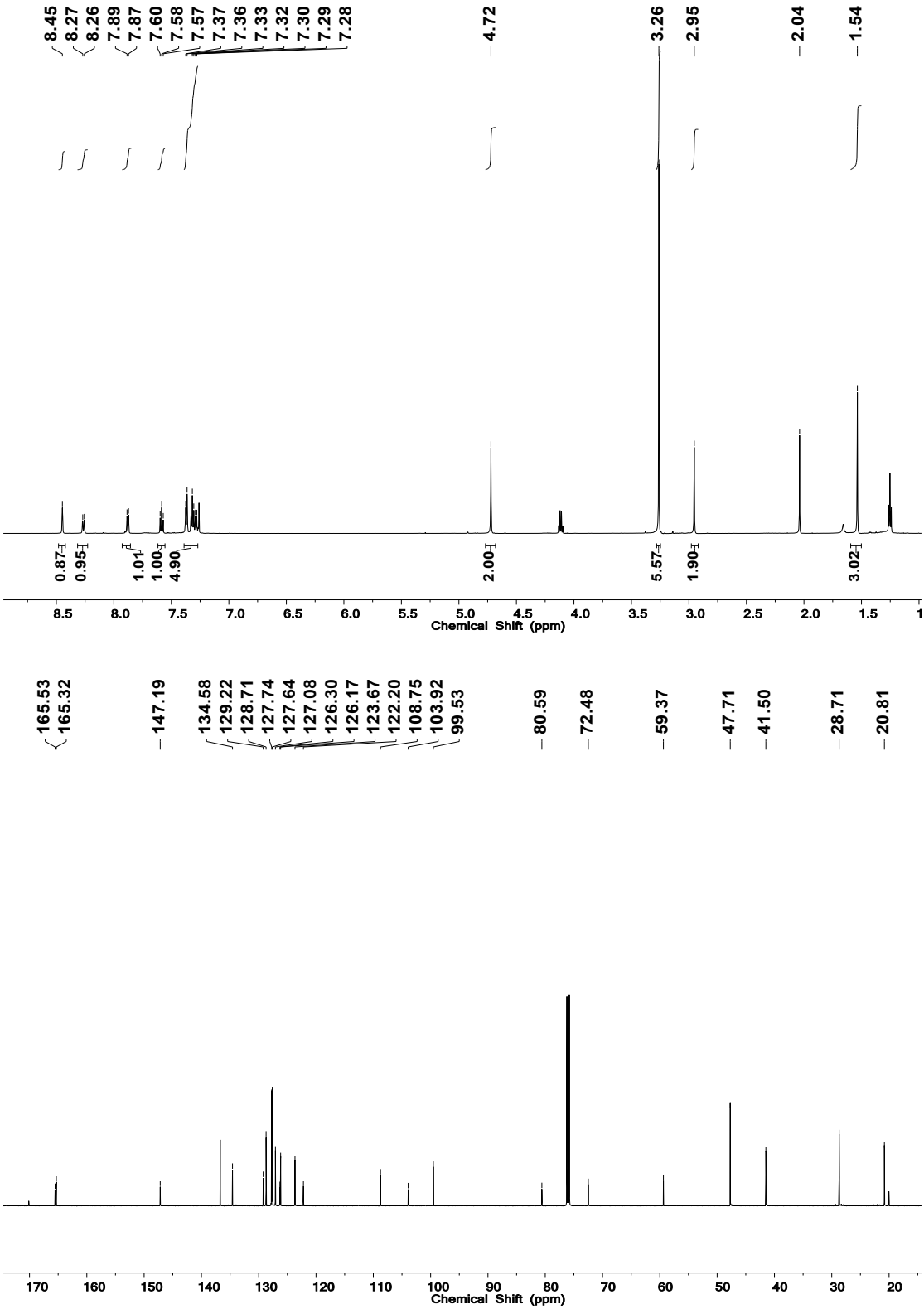


Figure S7 ^{1}H NMR and ^{13}C NMR spectra of **EDY-D**

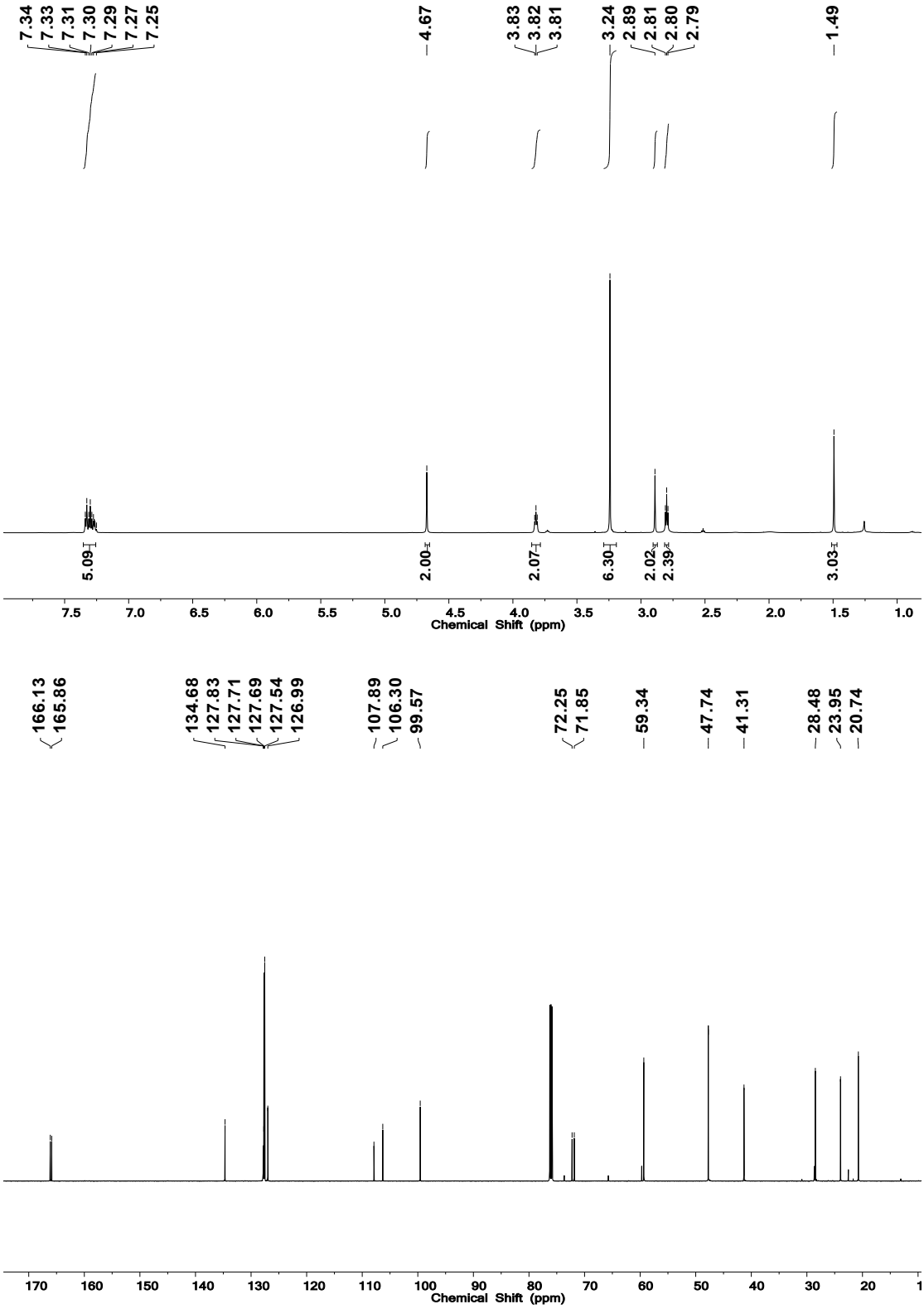


Figure S8 ^{1}H NMR and ^{13}C NMR spectra of **EDY-E**

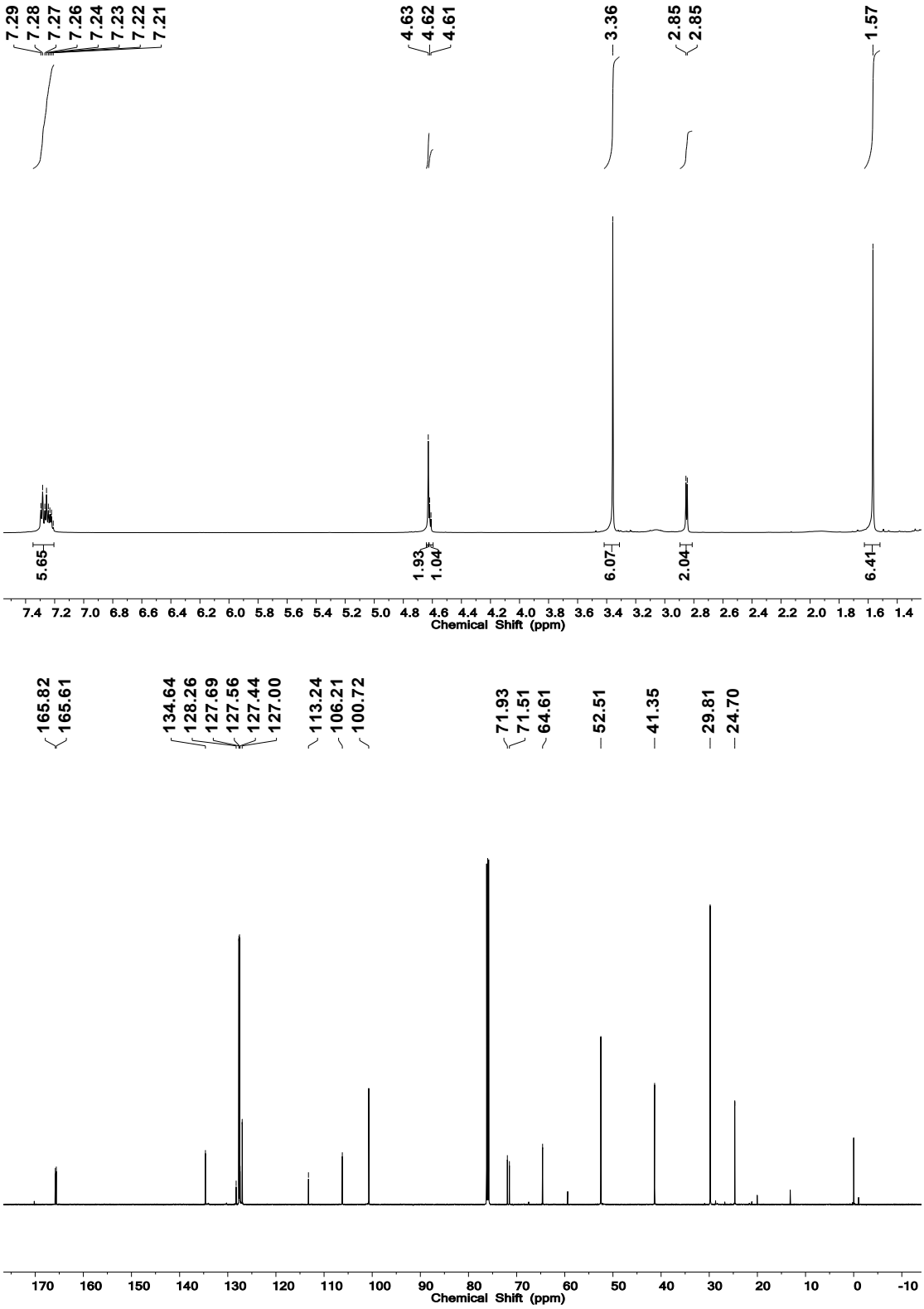


Figure S9 ^{1}H NMR and ^{13}C NMR spectra of **EDY-F**

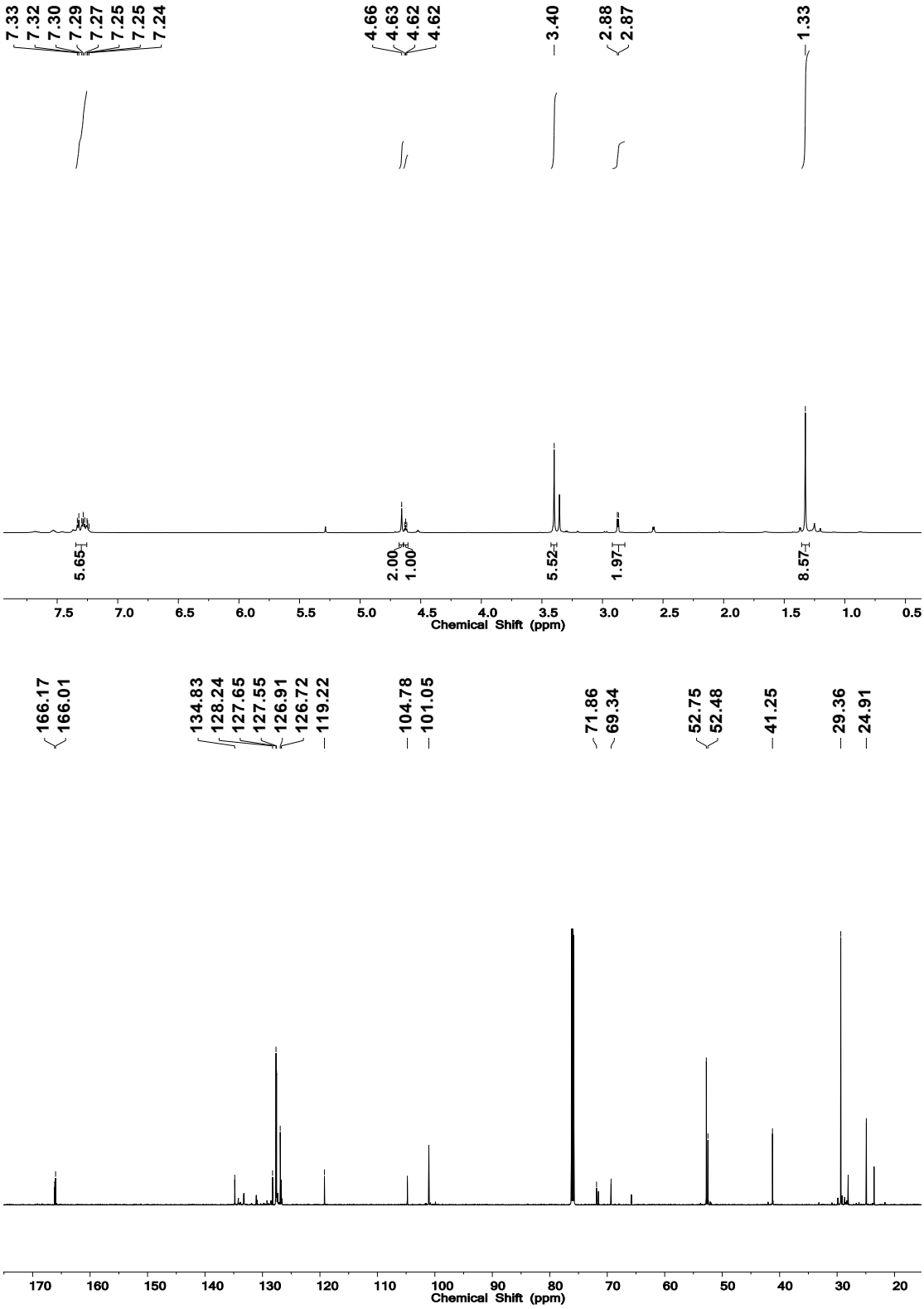


Figure S10 ^{1}H NMR and ^{13}C NMR spectra of **EDY-G**

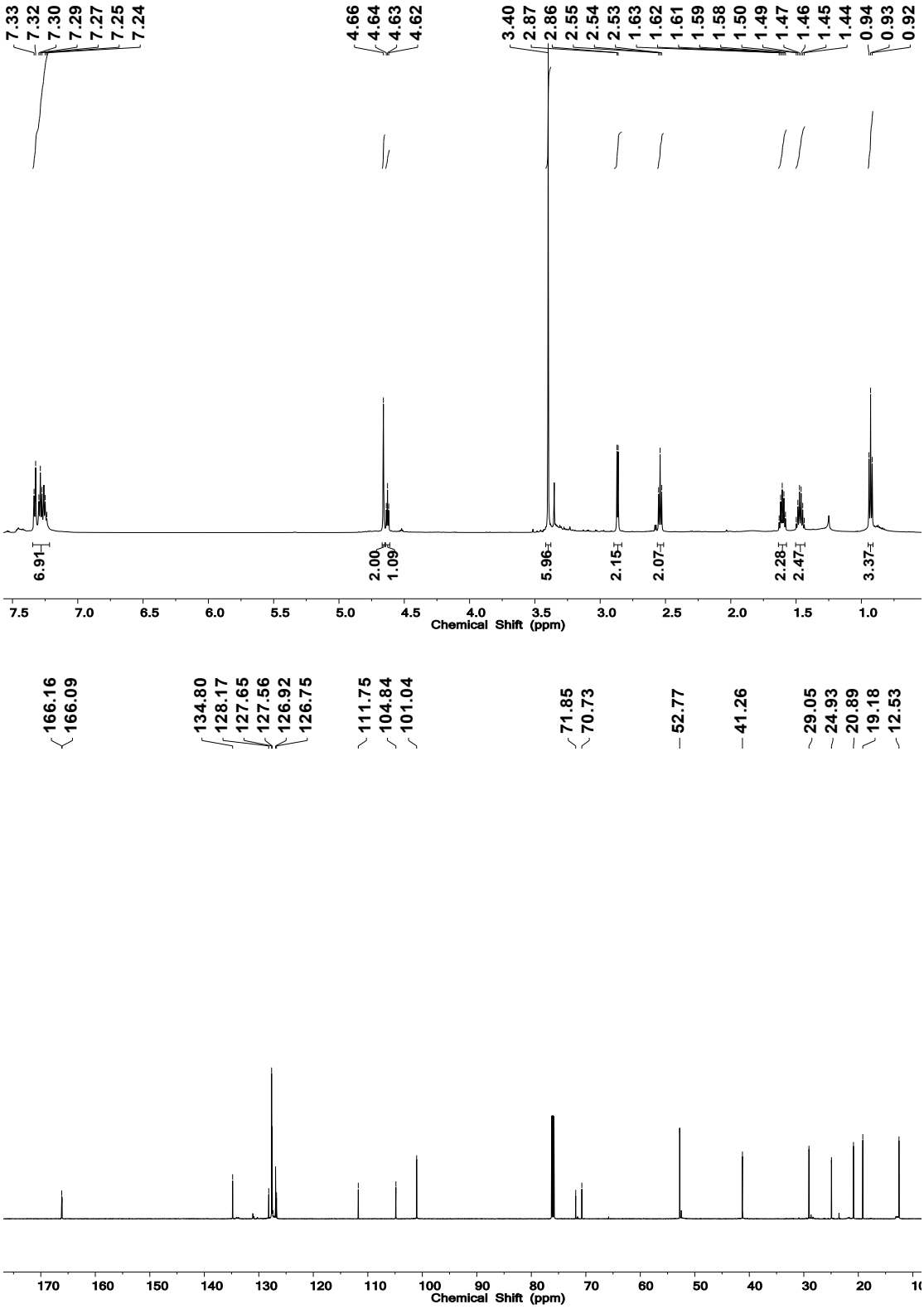


Figure S11 ^{1}H NMR and ^{13}C NMR spectra of **EDY-H**

Coordinates for the optimized local minima and transition states

All structures are optimized with (U)B3LYP/6-31G* with Grimme's dispersion correction.

Structure Ⅰ

C	-3.52824	-1.31077	0.23128
C	-2.13215	-0.79338	-0.00784
C	-2.20885	0.55338	-0.22794
C	-3.65337	0.96742	-0.13441
N	-4.36997	-0.20196	0.13905
C	-5.81006	-0.26583	0.30548
H	-6.19275	0.75462	0.25269
H	-6.26062	-0.87239	-0.48589
H	-6.05705	-0.71011	1.27362
O	-4.12665	2.07662	-0.26258
O	-3.88323	-2.44714	0.46117
C	-1.13595	1.42272	-0.48438
C	-0.9627	-1.56683	-0.00592
C	-0.13865	2.08238	-0.69273
C	0.13033	-2.09354	-0.03679
C	1.11091	2.80496	-0.88282
H	1.56752	2.56299	-1.84879
H	0.92816	3.89064	-0.87453
C	1.4988	-2.58261	-0.1189
H	1.80698	-3.04913	0.82412
H	1.59558	-3.34395	-0.90342
C	2.49552	-1.43579	-0.41527
C	2.19688	-0.68134	-1.70728
H	2.21762	-1.36671	-2.56018
H	1.21297	-0.20956	-1.64782
H	2.95078	0.09752	-1.85045
O	2.48567	-0.50642	0.67573
O	3.78857	-2.02131	-0.44269
C	3.79096	-0.47267	1.26081
H	4.04245	0.56627	1.48504
H	3.80927	-1.07494	2.17948
C	4.67027	-1.07996	0.17039
H	5.54151	-1.62276	0.5446
H	4.99448	-0.31447	-0.54789
C	2.16956	2.52231	0.21188
C	1.66848	2.39689	1.62968
H	0.86711	3.1125	1.84169

H	1.25558	1.38812	1.74391
H	2.49691	2.5289	2.32864
O	3.341	2.43227	-0.0941
O	0.6084	8.23424	0.20655
P	0.60896	7.27479	-0.96443
O	0.60961	5.78297	-0.86009
O	-0.69012	7.73752	-1.92903
H	-1.00653	8.53997	-1.48631
O	1.90782	7.73863	-1.92877
H	2.22311	8.54167	-1.48632

Structure TS1

C	-3.52824	-1.31077	0.23128
C	-2.13215	-0.79338	-0.00784
C	-2.20885	0.55338	-0.22794
C	-3.65337	0.96742	-0.13441
N	-4.36997	-0.20196	0.13905
C	-5.81006	-0.26583	0.30548
H	-6.19275	0.75462	0.25269
H	-6.26062	-0.87239	-0.48589
H	-6.05705	-0.71011	1.27362
O	-4.12665	2.07662	-0.26258
O	-3.88323	-2.44714	0.46117
C	-1.13595	1.42272	-0.48438
C	-0.9627	-1.56683	-0.00592
C	-0.13865	2.08238	-0.69273
C	0.13033	-2.09354	-0.03679
C	1.11091	2.80496	-0.88282
H	1.56752	2.56299	-1.84879
H	0.87852	4.18549	-0.87228
C	1.4988	-2.58261	-0.1189
H	1.80698	-3.04913	0.82412
H	1.59558	-3.34395	-0.90342
C	2.49552	-1.43579	-0.41527
C	2.19688	-0.68134	-1.70728
H	2.21762	-1.36671	-2.56018
H	1.21297	-0.20956	-1.64782
H	2.95078	0.09752	-1.85045
O	2.48567	-0.50642	0.67573
O	3.78857	-2.02131	-0.44269
C	3.79096	-0.47267	1.26081
H	4.04245	0.56627	1.48504
H	3.80927	-1.07494	2.17948

C	4.67027	-1.07996	0.17039
H	5.54151	-1.62276	0.5446
H	4.99448	-0.31447	-0.54789
C	2.16956	2.52231	0.21188
C	1.66848	2.39689	1.62968
H	0.86711	3.1125	1.84169
H	1.25558	1.38812	1.74391
H	2.49691	2.5289	2.32864
O	3.341	2.43227	-0.0941
O	0.65986	7.92855	0.20422
P	0.66042	6.9691	-0.96676
O	0.66107	5.47728	-0.86242
O	-0.63866	7.43183	-1.93136
H	-0.95507	8.23428	-1.48865
O	1.95928	7.43294	-1.9311
H	2.27457	8.23598	-1.48866

Structure Ⅱ

C	3.82051	-2.65066	0.36485
C	2.83301	-1.58179	0.1385
C	1.61665	-2.1661	-0.21174
C	1.82032	-3.66961	-0.21318
N	3.14792	-3.87357	0.13842
C	3.77739	-5.16698	0.26233
H	3.0243	-5.92242	0.0288
H	4.62066	-5.2546	-0.43139
H	4.15338	-5.31834	1.28001
O	1.01384	-4.54529	-0.46761
O	4.99823	-2.58965	0.684
C	0.40306	-1.61224	-0.52923
C	3.13003	-0.21557	0.2857
C	-0.70236	-1.13986	-0.82378
C	3.38549	0.96439	0.42075
C	-1.93217	-0.61204	-1.138
H	-2.74736	-1.28957	-1.38876
H	-3.14122	-0.49997	0.65762
C	3.65158	2.39242	0.54926
H	2.7546	2.93902	0.86984
H	4.42784	2.58074	1.3034
C	4.11969	3.04468	-0.76734
C	5.39605	2.43048	-1.32965
H	6.22531	2.59276	-0.63354
H	5.25656	1.35802	-1.48226

H	5.64237	2.90263	-2.28494
O	3.0462	2.96559	-1.6999
O	4.35152	4.44725	-0.5328
C	2.99426	4.2106	-2.38223
H	3.71085	4.24036	-3.21653
H	1.98261	4.34518	-2.77314
C	3.38435	5.18571	-1.27395
H	2.51613	5.43767	-0.64625
H	3.84777	6.1101	-1.63439
C	-2.16783	0.78609	-1.3261
C	-1.00803	1.75554	-1.2128
H	-1.39951	2.76614	-1.07021
H	-0.32426	1.50192	-0.39954
H	-0.42072	1.73697	-2.13967
O	-3.30461	1.2543	-1.60592
O	-6.38407	0.30414	1.31605
P	-5.3383	-0.47738	0.61279
O	-3.87457	-0.19483	1.23578
O	-5.588	-2.08778	0.76204
H	-6.18424	-2.20856	1.51751
O	-5.18028	-0.37644	-0.95997
H	-4.48333	0.31231	-1.27272

Structure TS2

C	0.14297	3.31663	-0.60052
C	-0.07822	1.83628	-0.55141
C	1.12728	1.22141	-0.33967
C	2.17647	2.29433	-0.24702
N	1.51696	3.51212	-0.39786
C	2.16178	4.80704	-0.41524
H	3.21734	4.65219	-0.18488
H	2.06302	5.27739	-1.3996
H	1.70834	5.46483	0.33232
O	3.38148	2.17873	-0.09567
O	-0.65512	4.21454	-0.78768
C	-1.33745	1.22122	-0.6608
C	0.98221	-1.17508	-0.79493
C	-2.37578	0.59744	-0.72737
C	0.67742	-2.27952	-1.47203
H	1.37889	-2.64777	-2.2217
C	-3.59523	-0.19733	-0.75505
H	-4.34995	0.26093	-1.40667
H	-3.36933	-1.19516	-1.14584

C	-4.23712	-0.38818	0.64208
C	-3.29545	-0.99357	1.66652
H	-2.87239	-1.92342	1.28217
H	-2.4744	-0.30119	1.86832
H	-3.84848	-1.17938	2.5921
O	-4.73381	0.85124	1.15167
O	-5.38864	-1.23126	0.43641
C	-6.1122	0.91824	0.8234
H	-6.60654	1.58425	1.53673
H	-6.26867	1.30307	-0.19715
C	-6.53109	-0.5462	0.92975
H	-7.39644	-0.81128	0.31279
H	-6.73682	-0.82087	1.97614
C	-0.58358	-3.01769	-1.30403
C	-0.69971	-4.29613	-2.12993
H	-0.58049	-4.07759	-3.19894
H	0.09515	-5.00102	-1.85499
H	-1.6748	-4.75606	-1.95525
O	-1.50491	-2.66358	-0.57388
C	1.43266	-0.16647	-0.12292
H	2.25955	-0.43354	0.7875
O	5.73891	-1.58347	1.75612
P	4.49988	-1.33494	0.97419
O	3.25212	-0.73739	1.68206
O	4.72231	-0.386	-0.35215
H	4.35657	0.50011	-0.16273
O	4.02695	-2.73806	0.24473
H	3.1384	-2.60883	-0.12593

Structure Ⅲ

C	0.14297	3.31663	-0.60052
C	-0.07822	1.83628	-0.55141
C	1.12728	1.22141	-0.33967
C	2.17647	2.29433	-0.24702
N	1.51696	3.51212	-0.39786
C	2.16178	4.80704	-0.41524
H	3.21734	4.65219	-0.18488
H	2.06302	5.27739	-1.3996
H	1.70834	5.46483	0.33232
O	3.38148	2.17873	-0.09567
O	-0.65512	4.21454	-0.78768
C	-1.33745	1.22122	-0.6608
C	0.98221	-1.17508	-0.79493

C	-2.37578	0.59744	-0.72737
C	0.67742	-2.27952	-1.47203
H	1.37889	-2.64777	-2.2217
C	-3.59523	-0.19733	-0.75505
H	-4.34995	0.26093	-1.40667
H	-3.36933	-1.19516	-1.14584
C	-4.23712	-0.38818	0.64208
C	-3.29545	-0.99357	1.66652
H	-2.87239	-1.92342	1.28217
H	-2.4744	-0.30119	1.86832
H	-3.84848	-1.17938	2.5921
O	-4.73381	0.85124	1.15167
O	-5.38864	-1.23126	0.43641
C	-6.1122	0.91824	0.8234
H	-6.60654	1.58425	1.53673
H	-6.26867	1.30307	-0.19715
C	-6.53109	-0.5462	0.92975
H	-7.39644	-0.81128	0.31279
H	-6.73682	-0.82087	1.97614
C	-0.58358	-3.01769	-1.30403
C	-0.69971	-4.29613	-2.12993
H	-0.58049	-4.07759	-3.19894
H	0.09515	-5.00102	-1.85499
H	-1.6748	-4.75606	-1.95525
O	-1.50491	-2.66358	-0.57388
O	5.79732	-1.6018	1.81407
P	4.55829	-1.35327	1.03214
O	3.31053	-0.75571	1.74
O	4.78072	-0.40432	-0.2942
H	4.41498	0.48178	-0.10478
O	4.08536	-2.75638	0.30267
H	3.19681	-2.62716	-0.06798
C	1.43266	-0.16647	-0.12292
H	2.1181	-0.53648	0.61067

Structure TS3

C	-2.7625	-1.89254	-0.22072
C	-1.61149	-0.9518	-0.35353
C	-2.07685	0.35482	-0.10087
C	-3.53753	0.2759	0.20113
N	-3.86316	-1.08396	0.11476
C	-5.19366	-1.61849	0.33902
H	-5.85044	-0.78043	0.57692

H	-5.55027	-2.13191	-0.55855
H	-5.1794	-2.33164	1.16824
O	-4.30603	1.17804	0.46804
O	-2.80454	-3.09676	-0.35857
C	0.05017	1.49619	-0.48499
C	0.73729	-0.20805	-0.83116
C	0.94366	2.59942	-0.57864
H	1.89209	2.44081	-1.07926
C	2.18186	-0.54201	-1.11601
H	2.22111	-1.40327	-1.78865
H	2.71759	0.27074	-1.61154
C	2.98147	-0.94155	0.15308
C	3.08326	0.15403	1.20314
H	3.5381	1.05228	0.77636
H	2.09307	0.40604	1.59182
H	3.7052	-0.20513	2.02714
O	2.39922	-2.08937	0.76164
O	4.28421	-1.32415	-0.3004
C	3.11873	-3.2262	0.28791
H	3.0287	-4.02155	1.0317
H	2.71578	-3.58043	-0.67217
C	4.52873	-2.66253	0.13187
H	5.13227	-3.16978	-0.62631
H	5.06807	-2.66934	1.08993
C	0.85548	3.97112	-0.07452
C	-0.24152	4.44092	0.86694
H	-0.5058	3.68429	1.61247
H	0.10871	5.34687	1.36592
H	-1.15041	4.69512	0.30706
O	1.74829	4.75484	-0.41098
C	-1.25913	1.45488	-0.18751
H	-1.68475	2.43654	-0.03336
C	-0.29046	-1.0861	-0.68163

Structure Ⅳ

C	-2.22005	-2.29258	-0.44486
C	-1.38623	-1.00873	-0.54158
C	-2.24173	0.05106	-0.58261
C	-3.67568	-0.48935	-0.51468
N	-3.65037	-1.96563	-0.67575
C	-4.55944	-2.66384	0.24457
H	-5.56692	-2.36141	0.04854
H	-4.46989	-3.7204	0.10115

H	-4.30322	-2.41793	1.25392
O	-4.70943	0.20744	-0.34313
O	-1.76482	-3.44032	-0.20191
C	-0.39494	1.57505	-0.68854
C	0.49699	0.47014	-0.64576
C	0.16281	3.00804	-0.77266
H	0.34095	3.45763	-1.72714
C	2.01768	0.71023	-0.6837
H	2.35308	0.71305	-1.69977
H	2.24115	1.65398	-0.23171
C	2.73403	-0.41205	0.09022
C	2.23257	-0.42531	1.54623
H	2.43108	0.52293	2.00048
H	1.1793	-0.61339	1.55901
H	2.7389	-1.19467	2.09085
O	2.47141	-1.73157	-0.48852
O	4.17552	-0.1661	0.00868
C	3.65602	-1.96021	-1.21486
H	3.78929	-2.98679	-1.48557
H	3.68814	-1.37452	-2.10975
C	4.67045	-1.46695	-0.20569
H	5.67553	-1.47704	-0.57257
H	4.6383	-2.05262	0.68922
C	0.46417	3.79395	0.51697
C	1.85779	3.70337	1.16599
H	2.28564	2.74456	0.95978
H	2.48846	4.46908	0.76494
H	1.76756	3.83354	2.2242
O	-0.42835	4.51016	1.04043
C	-1.78986	1.36487	-0.65378
H	-2.47515	2.18616	-0.68112
C	-0.00482	-0.84643	-0.56817

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