Supporting Information

for

Cell-Permeable Esters of Diazeniumdiolate-Based Nitric Oxide Prodrugs

Harinath Chakrapani,* Anna E. Maciag, Michael L. Citro, Larry K. Keefer, and Joseph E. Saavedra*

Table of Contents

General

Nitric oxide gas was purchased from Matheson Gas Products (Montgomeryville, PA). Except as otherwise indicated, all starting materials were purchased from Aldrich Chemical Co. (Milwaukee, WI). NMR spectra were recorded on a Varian UNITY INOVA spectrometer operating at 400 MHz (^{1}H) and 100 MHz (^{13}C); chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane. Ultraviolet (UV) spectra were recorded on a Hewlett-Packard model 8451A diode array spectrophotometer. Elemental analyses were performed by Midwest Microlab (Indianapolis, IN). Reported procedures were used to prepare sodium 1-[*N*-(2-hydroxyethyl)-*N*-methylamino]diazen-1-ium-1,2-diolate[1] (the precursor to compounds 4 and 7), **2**[2] and **3**.[3] Diazomethane was prepared using a reported method.[4]

The human leukemia HL-60 cell line used in this study was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10 % fetal calf serum (Gemini Bio-Products, Sacramento, CA), 100 U/mL penicillin and 2 mM glutamine, at 37 °C and 5 % CO_2.

General Procedure for Diazeniumdiolation. Unless otherwise specified, the following procedure was used to prepare the diazeniumdiolate anions as their sodium salts. A methanolic (3 mL) solution of the amine was treated with an equimolar amount of sodium methoxide (25 % in methanol) in ether (9 mL). The resulting solution was charged with 50 psi of NO and stirred overnight. The solid precipitate that resulted was collected by filtration, washed with ether and dried under vacuum to afford the desired product.

General Procedure for Arylation of Diazeniumdiolate Anions. Unless otherwise specified, the following procedure was followed to synthesize the arylated diazeniumdiolates. The diazeniumdiolate salt was dissolved in ice cold 5 % aqueous sodium bicarbonate solution (10 mL), and this mixture was treated with a slurry of (di)fluorodinitroarene in *t*-BuOH (5 mL). A yellow precipitate resulted, which was collected by filtration and aqueous washing. Silica gel flash chromatography with mixtures of hexanes and ethyl acetate or recrystallization from ethanol afforded the desired product.

General Procedure for Esterification of Carboxylic Acids. The carboxylic acid was dissolved in methanol (2 mL) and diazomethane in ether (2 mL) was added. The mixture was stirred for 15 min and solvents were removed under reduced pressure. The remaining oil was dissolved in dichloromethane, and the resulting solution was washed with dilute HCl and then 5 % aqueous $NaHCO_3$. The organic layer was separated and dried ($MgSO_4$, Na_2SO_4), filtered, and concentrated under reduced pressure to form a yellow solid.

***O*^{2}-(2,4-Dinitrophenyl) 1-[*N*-(2-Hydroxyethyl)-*N*-methylamino]diazen-1-ium-1,2-diolate.** Starting from sodium 1-[*N*-(2-hydroxyethyl)-*N*-methylamino]diazen-1-ium-1,2-diolate (2.78 g, 17.6 mmol), *O*^{2}-(2,4-dinitrophenyl) 1-[*N*-(2-hydroxyethyl)-*N*-methylamino]diazen-1-ium-1,2-diolate (2.35 g, yield = 44 %) was isolated as a yellow solid: mp 67-68 °C; UV (EtOH) λ_{max} (ε) 306 nm (7.2 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 3.40 (s, 3H), 3.82 (t, *J* = 5.5 Hz, 2H), 3.93 (t, *J* = 5.5 Hz, 2H), 7.67 (d, *J* = 9.4 Hz, 1H), 8.46 (dd, *J* = 2.7, 9.4 Hz, 1H), 8.89 (d, *J* = 2.7 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 40.8, 53.6, 59.9, 117.4, 122.1, 129.2, 137.0, 142.2, 154.2. Anal. calcd for $C_9H_{11}N_5O_7$: C, 35.89; H, 3.68; N, 23.25. Found: C, 36.01; H, 3.80; N, 23.07.

***O*^{2}-(2,4-Dinitrophenyl) 1-[*N*-(Carboxymethyl)-*N*-methylamino]diazen-1-ium-1,2-diolate (4).** *O*^{2}-(2,4-Dinitrophenyl) 1-[*N*-(2-hydroxyethyl)-*N*-methylamino]diazen-1-ium-1,2-diolate (367mg, 1.22 mmol) in acetonitrile (2 mL) and water (3 mL) was treated with sodium periodate (1.05 g, 4.9 mmol) and $RuCl_3.H_2O$ (10 mg, 0.05 mmol). The reaction mixture was diluted with EtOAc (2 mL) and stirred overnight.[2] The dark reaction mixture was diluted with EtOAc (15 mL), and filtered through Celite. The filtrate was extracted with 5 % aqueous $NaHCO_3$, and washed with dichloromethane (DCM). The aqueous layer was acidified with dilute HCl and washed with DCM. The combined organic layer was separated, dried, and filtered; concentration of the organic layer under reduced pressure and silica gel flash chromatography of the crude product afforded **4** (263 mg, 68 % yield) as a yellow solid: mp 158-160 °C; UV (EtOH) λ_{max} (ε) 304 nm (15.0 mM^{-1} cm^{-1}); ^{1}H NMR (DMSO-d_6) δ 3.43 (s, 3H), 4.50 (s, 2H), 7.80 (d, *J* = 9.3 Hz, 1H), 8.54 (dd, *J* = 2.7, 9.3 Hz, 1H), 8.86 (d, *J* = 2.7 Hz, 1H), 13.30 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 40.4, 53.6, 117.6, 122.2, 130.2, 137.0, 142.2, 153.6, 169.6. Anal. calcd for $C_9H_9N_5O_8$: C, 34.29; H, 2.88; N, 22.22. Found: C, 34.65; H, 2.99; N, 21.87.

***O*^{2}-(2,4-Dinitrophenyl) 1-[(2-Methoxycarbonyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate] (5).** Product was recrystallized from ether:dichloromethane; yield = 94 %: mp 95-99 °C; UV (EtOH) λ_{max} (ε) 304 nm (14.0 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 2.12-2.23 (m, 3H), 2.36-2.44 (m, 1H), 3.79 (s, 3H), 3.87-3.93 (m, 1H), 4.02-4.07 (m, 1H), 4.79 (dd, *J* = 3.5, 8.4 Hz, 1H), 7.60 (d, *J* = 9.0 Hz, 1H), 8.44 (dd, *J* = 2.7, 9.2 Hz, 1H), 8.89 (d, *J* = 2.7 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 22.4, 28.2, 50.6, 52.8, 61.9, 103.9, 125.6, 131.0, 154.6, 171.0. Anal. calcd for $C_{12}H_{13}N_5O_8$: C, 40.57; H, 3.69; N, 19.71. Found: C, 40.54; H, 3.74; N, 19.59.

2,4-Dinitrophenyl-1,5-*bis*{[(2-Methoxycarbonyl)pyrrolidin-1-yl]diazen-1-ium-1-ol-2-ato]}benzene (6). Product was recrystallized from ether:dichloromethane: yield = 61 %; UV (EtOH) λ_{max} (ε) 294 nm (24.5 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 2.12-2.22 (m, 6H), 2.38-2.44 (m, 2H), 3.77 (s, 6H), 3.90-3.97 (m, 2H), 4.04-4.10 (m, 2H), 4.76 (dd, *J* = 3.2 Hz, 8.6 Hz 2H), 7.50 (s, 1H), 8.87 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 22.4, 28.2, 50.6, 52.8, 61.9, 103.9, 125.6, 131.0, 154.6, 170.9. Anal. calcd for $C_{18}H_{22}N_8O_{18}$: C, 39.86; H, 4.09; N, 20.66. Found: C, 39.51; H, 4.03; N, 20.37.

***O*^{2}-(2,4-Dinitrophenyl) 1-[(*N*-(Methoxycarbonyl)-*N*-methylamino]diazen-1-ium-1,2-diolate (7).** Product was recrystallized from ether:dichloromethane: yield = 86 %; mp 103-104 °C; UV (EtOH) λ_{max} (ε) 301 nm (14.7 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 3.49 (s, 3H), 3.79 (s, 3H), 4.40 (s, 2H), 7.64 (d, *J* = 9.2 Hz, 1H), 8.46 (dd, *J* = 2.4, 9.2 Hz, 1H), 8.88 (d, *J* = 2.4 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 40.6, 52.6, 53.1, 110.0, 117.3, 122.2, 129.2, 142.1, 154.3, 167.8. Anal. calcd for $C_{10}H_{11}N_5O_8$: C, 36.48; H, 3.37; N, 21.27. Found: C, 36.33; H, 3.37; N, 21.42.

***O*^{2}-(2,4-Dinitrophenyl) 1-[(4-Methoxycarbonyl)piperidin-1-yl)]diazen-1-ium-1,2-diolate (8).** Methyl isonipecotate was diazeniumdiolated under the standard diazeniumdiolation conditions with NaOMe/MeOH. A white precipitate was formed, which was washed with ether and dried under vaccum. This powder (0.5 g, 1.3 mmol) was used without further purification in the arylation step to afford the desired product **8** (0.18g, yield = 38 %) as a yellow solid: UV (EtOH) λ_{max} (ε) 304 nm (21.0 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 1.98-2.05 (m, 2H), 2.11-2.16 (m, 2H), 2.54 (septet, *J* = 4.4 Hz, 1H), 3.26-3.32 (m, 2H), 3.73 (s, 3H), 4.07-4.12 (m, 2H), 7.67 (d, *J* = 9.2 Hz, 1H), 8.46 (dd, *J* = 2.4, 9.2 Hz, 1H), 8.88 (d, *J* = 2.4 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 26.4, 39.3, 49.8, 52.0, 117.5, 122.1, 129.0, 142.2, 153.9, 173.8. Anal. calcd for $C_{13}H_{15}N_5O_6$: C, 42.28; H, 4.09; N, 18.96. Found: C, 41.98; H, 4.15; N, 18.72.

***O*^{2}-(2,4-Dinitrophenyl) 1-[(4-Ethoxycarbonyl)piperidin-1-yl)]diazen-1-ium-1,2-diolate (9).** Ethyl isonipecotate was diazeniumdiolated under the standard diazeniumdiolation conditions, except that $NaOSiMe_3$ in THF was used as the base.[5] A white precipitate was formed, a portion of which (0.75 g, 3.1 mmol) was used without further purification in the arylation step to afford the desired product **9** (0.35 g, yield = 29 %) as a yellow solid: UV (DMSO) λ_{max} (ε) 308 nm (13.0 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 1.28 (t, *J* = 7.2 Hz, 3H), 1.95-2.05 (m, 2H), 2.05-2.16 (m, 2H), 2.52 (septet, *J* = 4.4 Hz, 1H), 3.27-3.33 (m, 2H), 4.07-4.12 (m, 2H), 4.18 (q, *J* = 7.2 Hz, 2H), 7.67 (d, *J* = 9.2 Hz, 1H), 8.46 (dd, *J* = 2.8, 9.2 Hz, 1H), 8.88 (d, *J* = 2.4 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 14.2, 26.4, 39.4, 49.9, 60.9, 117.7, 122.1, 129.1, 137.2, 142.2, 153.9, 173.3. Anal. calcd for $C_{14}H_{17}N_5O_8 \cdot 0.25H_2O$ C, 43.30; H, 4.68; N, 18.04. Found: C, 43.04; H, 4.37; N, 17.70.

***O*^{2}-(2,4-Dinitrophenyl) 1-[(3-Methoxycarbonyl)piperidin-1-yl)]diazen-1-ium-1,2-diolate (10).** Methyl nipecotate was converted to **10** using the same procedure as **8** (0.48 g, yield = 30 %): UV (EtOH) λ_{max} (ε) 306 nm (12.1 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 1.93-1.96 (m, 1H), 2.07-2.11 (m, 1H), 2.81 (tt, *J* = 4.0, 10.4 Hz, 1H), 3.16-3.23 (m, 2H), 3.38 (dd, *J* = 10.4, 12.0 Hz, 3H), 3.75 (s, 3H), 4.08 (dt, *J* = 4.0, 12.0 Hz, 1H), 7.67 (d, *J* = 9.2 Hz, 1H), 8.46 (dd, *J* = 2.4, 9.2 Hz, 1H), 8.88 (d, *J* = 2.4 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 22.7, 26.1, 40.1, 51.0, 51.9, 52.2, 117.6, 122.1, 129.1, 137.1, 142.2, 153.8, 172.4. Anal. calcd for $C_{13}H_{15}N_5O_6$: C, 42.28; H, 4.09; N, 18.96. Found: C, 42.33; H, 4.06; N, 18.82.

Gluathione-activated NO Release

Calibration of the Sievers Nitric Oxide Analyzer (NOA), model 280i (Instruments Business Group, Boulder, CO) was performed by injection of various volumes of known concentrations of NO/He (50 ppm, 500 ppm and 5 %) certified standards into the reaction chamber and recording the peaks. Samples and reaction chambers were incubated at 37 °C and the contents were sparged with argon and swept into the chemiluminescence detector. Data were recorded using Agilent Chemstation software and processed using Microsoft Excel. Approximately 3.5 mL of pH 7.4 buffer containing GSH (3.6 mM) and diethylenetriaminepentaacetic acid (DTPA, 50 μM) was placed into the reaction chamber of the NOA and then sparged for several minutes with argon. A DMSO solution (10 mM) of the analyte (50-100 μL) was injected into the reaction chamber and nitric oxide release was recorded. Total amount of NO released was determined by measuring the area under the curve.

Intracellular NO Release

The intracellular level of nitric oxide after diazeniumdiolate prodrug treatment was quantified using the NO-sensitive fluorophore 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate; Invitrogen, Carlsbad, CA). HL-60 cells were loaded with 2.5 μM DAF-FM diacetate in Hanks' balanced salt solution (HBSS) at 37 °C and 5 % CO_2. After 30 min of incubation the cells were collected by centrifugation, rinsed with HBSS to remove excess of probe, and resuspended in fresh HBSS. The compounds were added to the cells at 5 μM final concentration. After 40 min incubation the fluorescence of the benzotriazole derivative formed on DAF-FM's reaction with aerobic NO was analyzed using a Perkin Elmer LS50B luminescence spectrometer with the excitation source at 495 nm and emission at 515 nm. The mean value of three independent experiments is reported.

Cell Culture and Cytotoxicity Assays

The CellTiter 96 non-radioactive cell proliferation assay (MTT assay, Promega, Madison, WI), performed according to the manufacturer's protocol, was used to measure cell growth. Cells were seeded in 96-well plates at a density of 10^4 per well and allowed to grow for 24 h before addition of the drugs. Diazeniumdiolate prodrugs were prepared as 10 mM stock solutions in DMSO (Sigma, St. Louis, MO) and diluted with pH 7.4 phosphate buffered saline (PBS) to form stock solutions of desired concentrations, which were then added to 100 μL of the culture medium. Cell viability was determined 72 h after the commencement of the assay. Each compound concentration was represented in six repeats, and the screening was performed as at least two independent experiments.

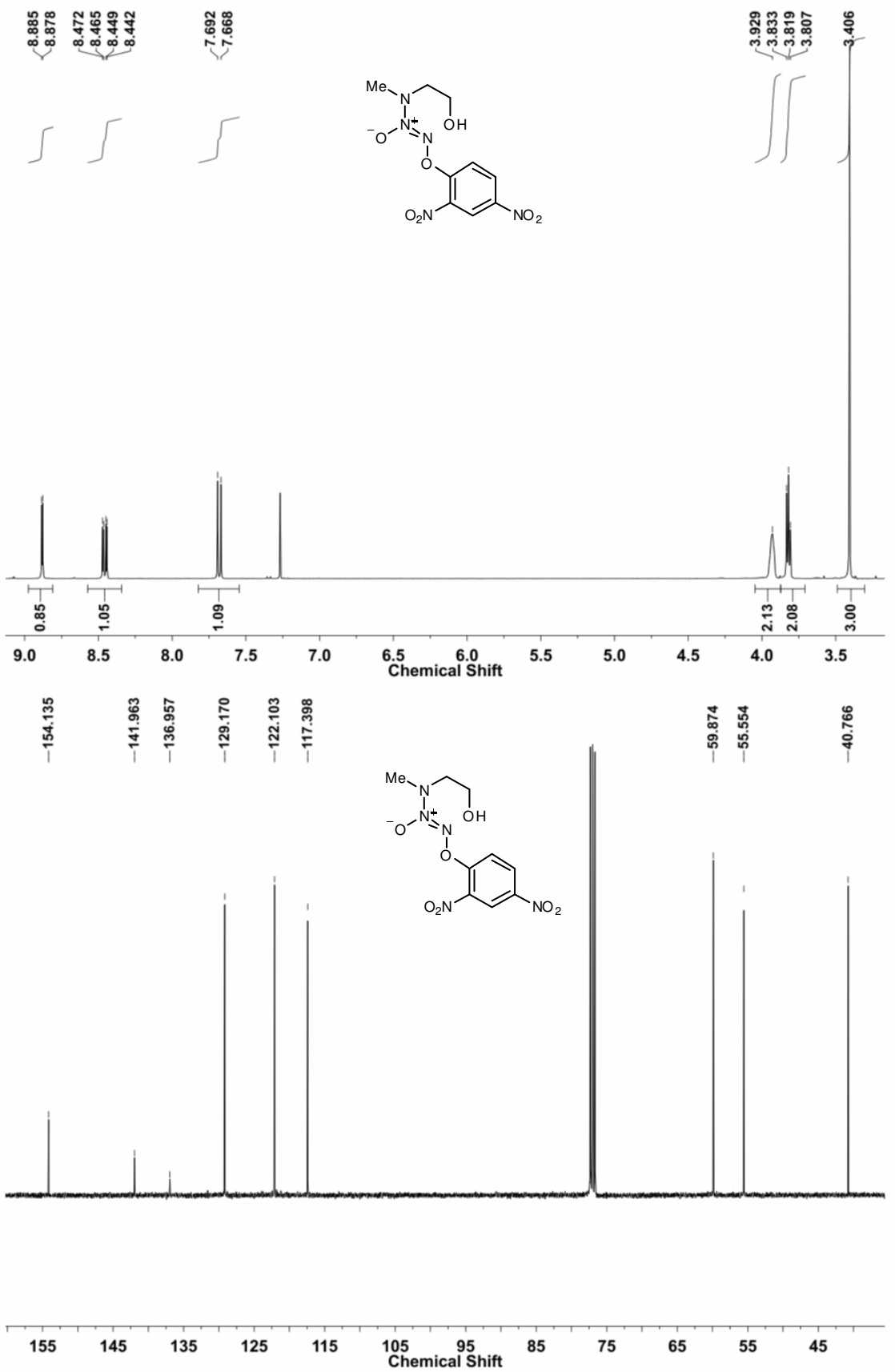

Figure S1. ^{1}H and ^{13}C NMR spectra of O^{2}-(2,4-dinitrophenyl) 1-[*N*-(2-hydroxyethyl)-*N*-methylamino]diazen-1-ium-1,2-diolate.

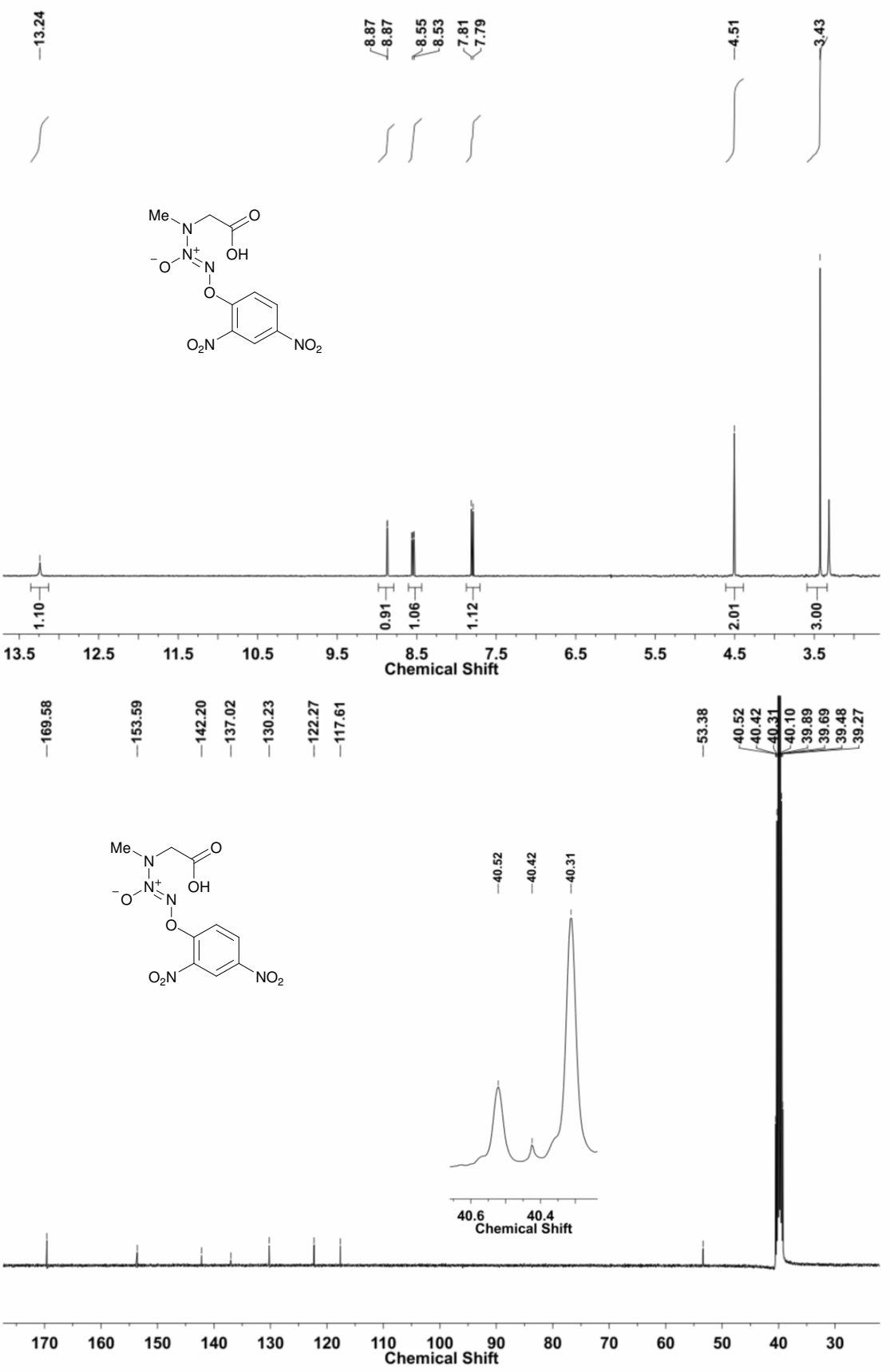

Figure S2. ^{1}H and ^{13}C NMR spectra of **4**.

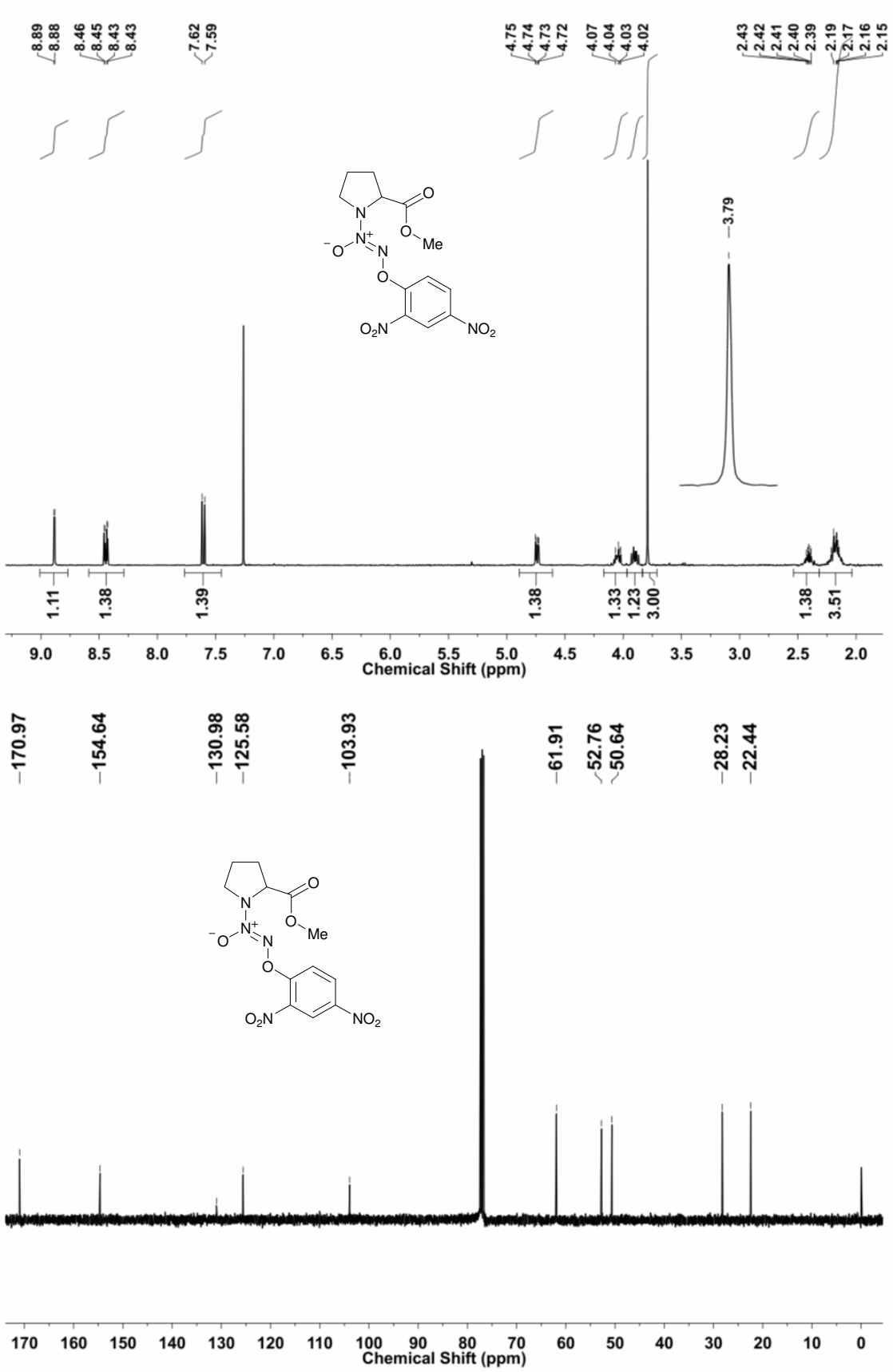

Figure S3. ^{1}H and ^{13}C NMR spectra of **5**.

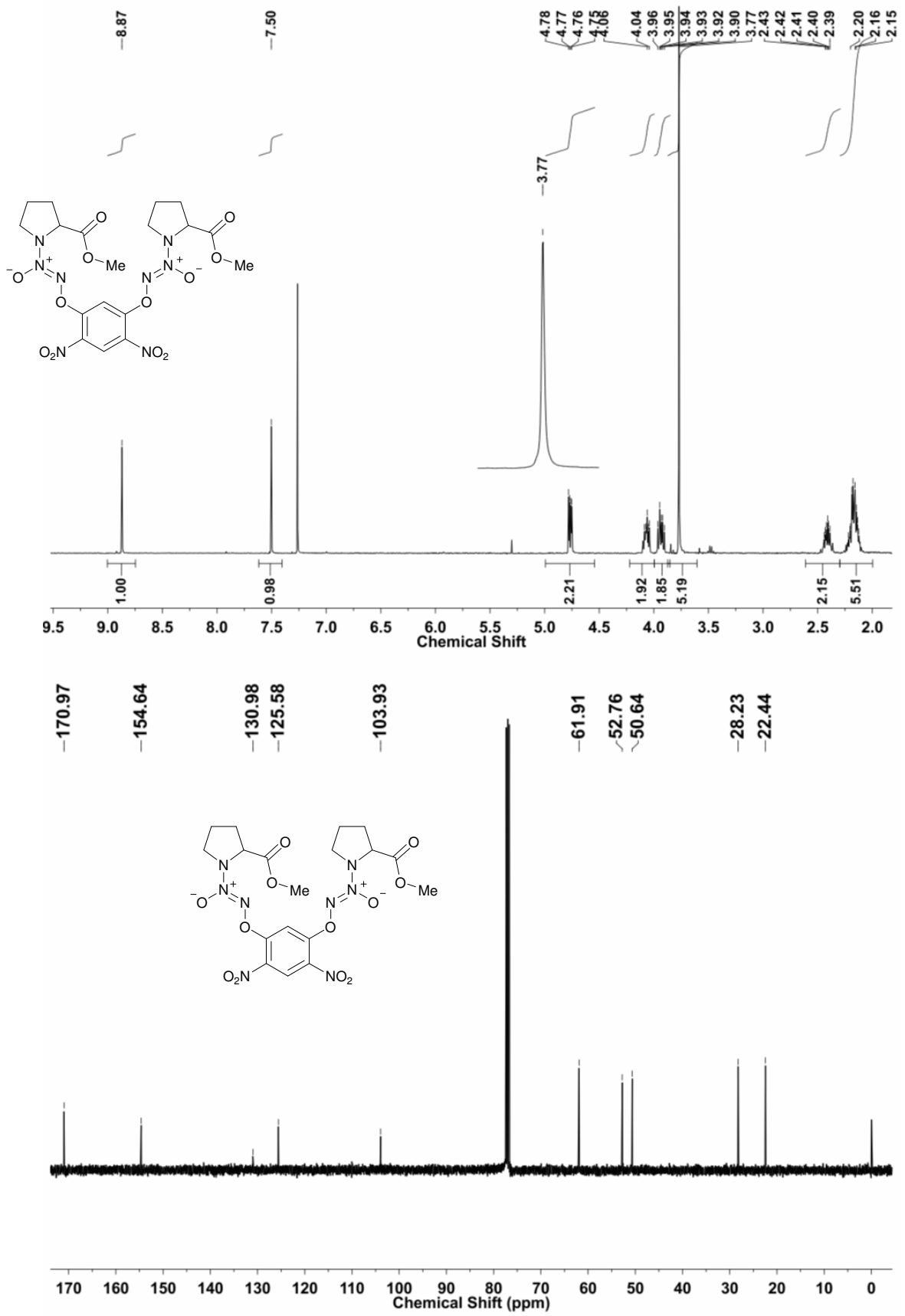

Figure S4. ^{1}H and ^{13}C NMR spectra of **6**.

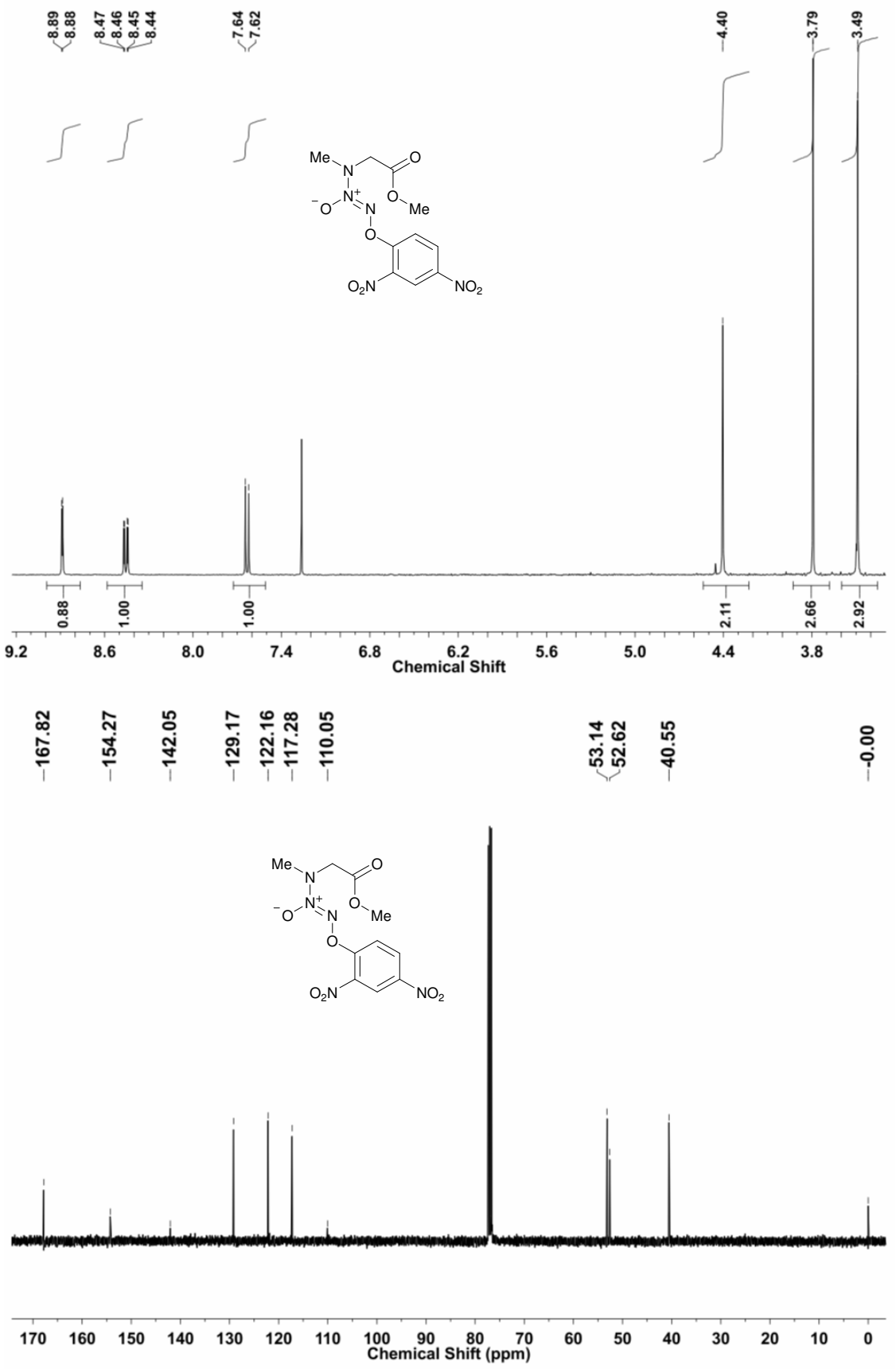

Figure S5. ^{1}H and ^{13}C NMR spectra of **7**.

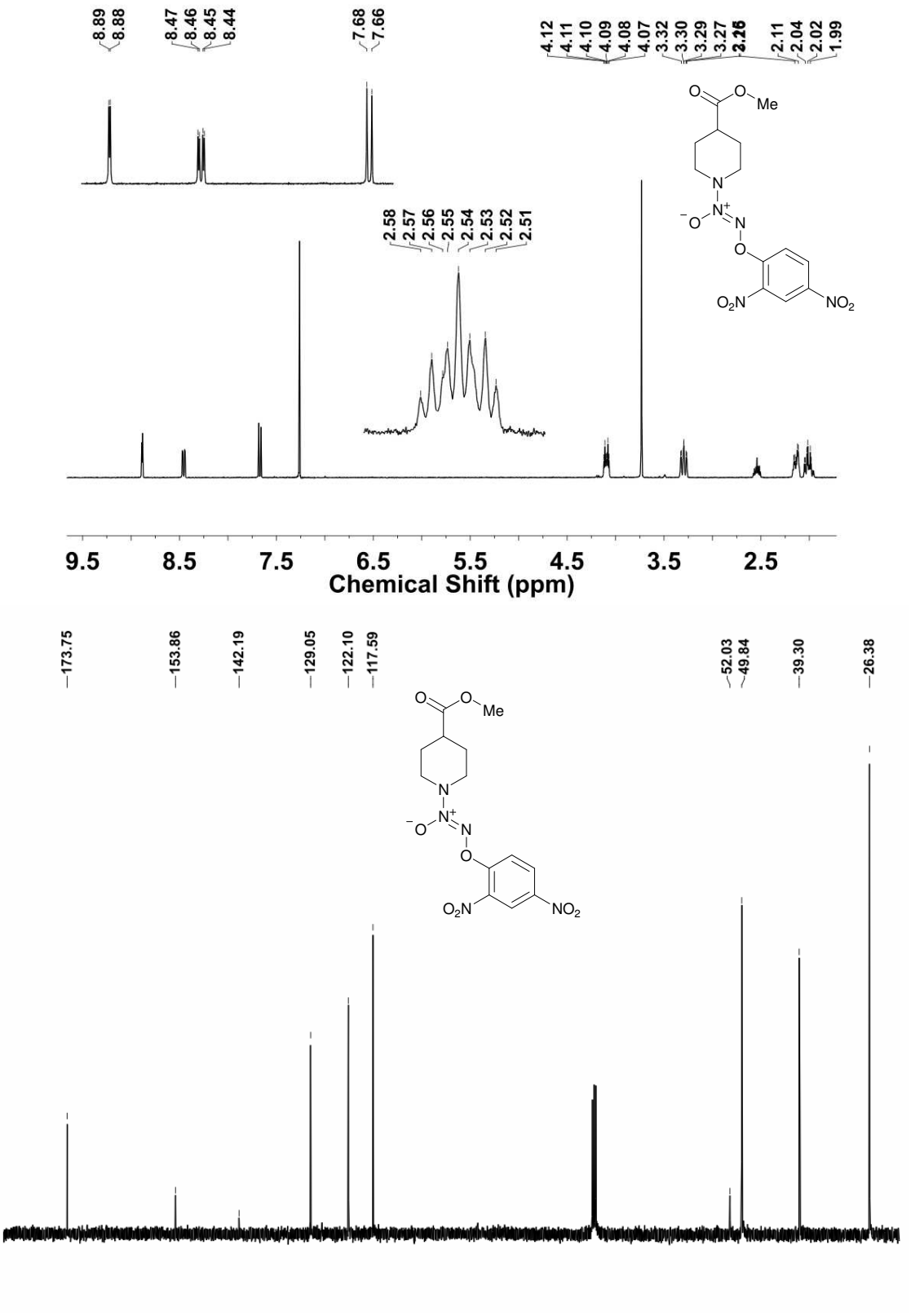

Figure S6. ^{1}H and ^{13}C NMR spectra of **8**.

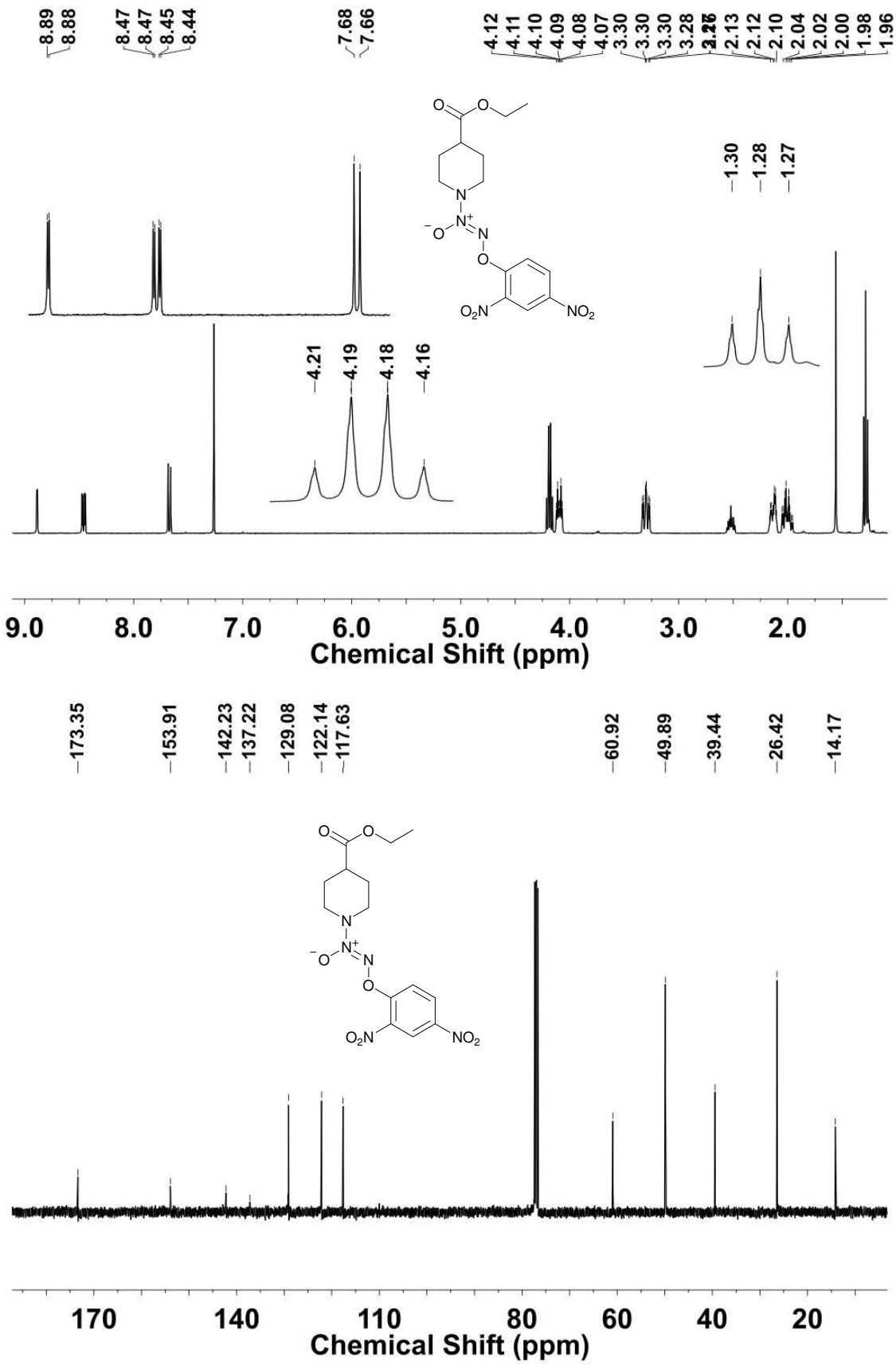

Figure S7. ^{1}H and ^{13}C NMR spectra of **9**.

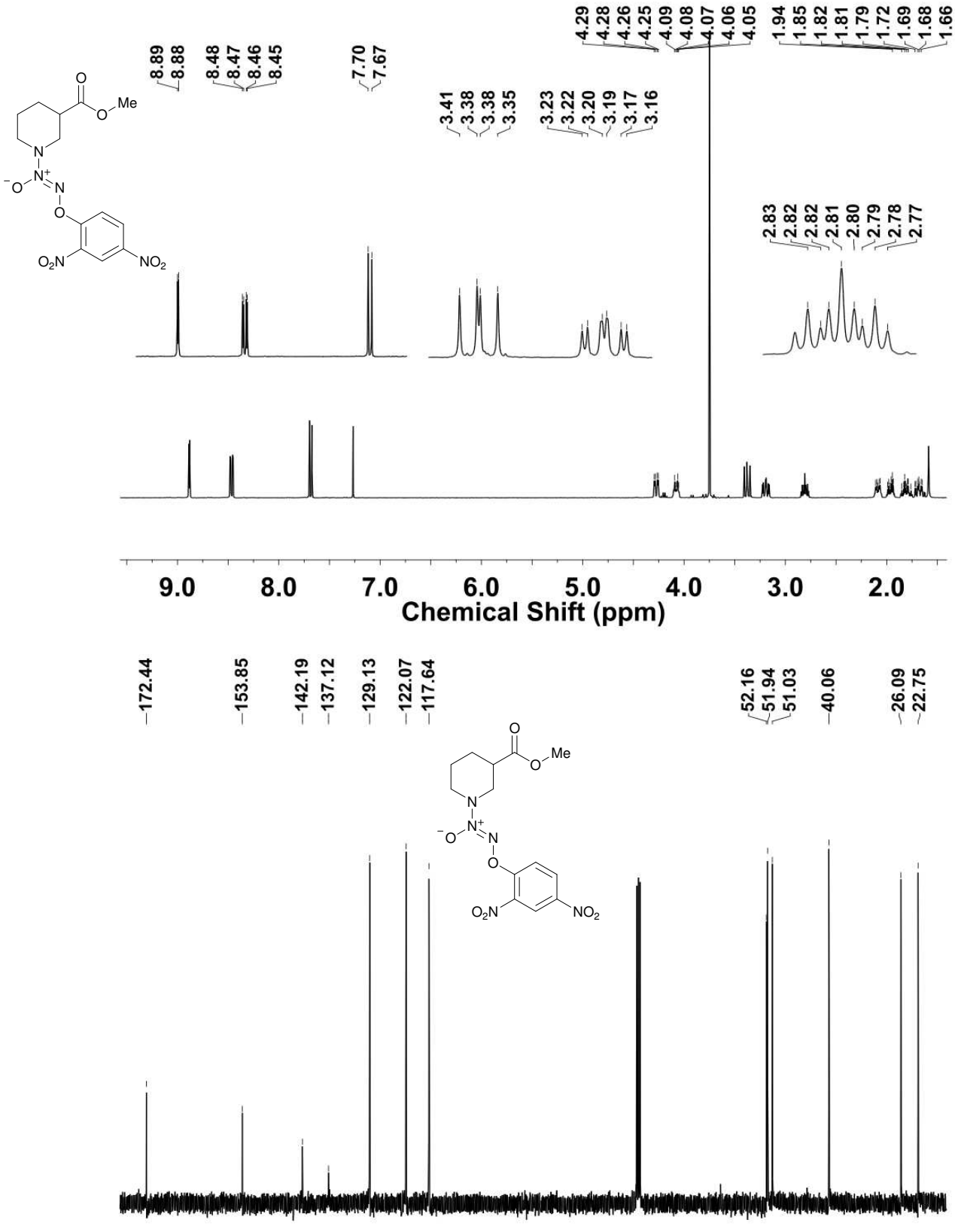

Figure S8. ^{1}H and ^{13}C NMR spectra of **10**.

References

1. Nguyen, J.-T.; Velazquez, C. A.; Knaus, E. E. *Bioorg. Med. Chem.* **2005**, *13*, 1725-1738.
2. Chakrapani, H.; Showalter, B. M.; Kong, L.; Keefer, L. K.; Saavedra, J. E. *Org. Lett.* **2007**, *9*, 3409-3412.
3. Andrei, D.; Maciag, A. E.; Chakrapani, H.; Citro, M. L.; Keefer, L. K.; Saavedra, J. E. **2008** (submitted to *Journal of Medicinal Chemistry*).
4. Black, T. H. *Adrichimica Acta* **1983**, *16*, 3-10.
5. Reynolds, M. M.; Zhou, Z.; Oh, B. K.; Meyerhoff, M. E. *Org. Lett.* **2005**, *7*, 2813-2816.