Supporting Information

New 1,2-dihydropyridine based fluorophores and their applications as fluorescent probes

Vellekkatt Jamsheena,[a] Rakesh K. Mishra,[a] Kollery S. Veena,[a] Suresh Sini,[b] Purushothaman Jayamurthy,[b]* and Ravi S. Lankalapalli[a]*

[a]Chemical Sciences and Technology Division, and Academy of Scientific and Innovative Research (AcSIR), CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram-695019, Kerala, India

[b]Agro-Processing and Technology Division, and Academy of Scientific and Innovative Research (AcSIR), CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram-695019, Kerala, India

*Corresponding author

E-mail: jayamurthy@live.com, ravishankar@niist.res.in

Table of Contents

Spectroscopic data

*(E)-Dimethyl-1-(4-methylbenzylideneamino)-2-para-tolyl-1,2-dihydropyridine-3,5-dicarboxylate (**1a**)*

R_f0.66 (CH_2Cl_2/hexane/EtOAc 2:7.8:0.2, developed four times); Yield 25%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.33 (d, J = 1 Hz, 1H), 7.94 (s, 1H), 7.66 (d, J = 1.5 Hz, 1H), 7.48 (d, J = 8 Hz, 2H), 7.34 (d, J = 8 Hz, 2H), 7.15 (d, J = 8 Hz, 2H), 7.08 (d, J = 8 Hz, 2H), 6.45 (s, 1H), 3.82 (s, 3H), 3.73 (s, 3H), 2.34 (s, 3H), 2.27 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.0, 165.9, 147.9, 142.9, 140.7, 138.3, 136.4, 130.9, 129.6, 129.5, 129.4, 127.4, 126.3, 117.0, 99.2, 58.3, 51.7, 51.4, 21.4, 21.1; HR-ESI-MS: *m/z* calcd for $C_{24}H_{25}N_2O_4$: 405.1814 $[M + H]^+$; found: 405.1821.

*(E)-Dimethyl-1-(4-methoxybenzylideneamino)-2-(4-methoxyphenyl)-1,2-dihydropyridine-3,5-dicarboxylate (**1b**)*

R_f 0.37 (CH_2Cl_2/hexane/EtOAc 2:7.8:0.2, developed four times); Yield 23%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.30 (s, 1H), 7.94 (s, 1H), 7.66 (d, J = 1 Hz, 1H), 7.54 (d, J = 8.5 Hz, 2H), 7.37 (d, J = 8 Hz, 2H), 6.88 (d, J = 9 Hz, 2H), 6.80 (d, J = 8.5 Hz, 2H), 6.43 (s, 1H), 3.81 (s, 6H), 3.74 (s, 3H), 3.73 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.0, 164.9, 160.4, 158.5, 146.6, 141.7, 130.7, 129.8, 128.0, 126.7, 125.3, 115.7, 113.2, 113.1, 97.8, 56.9, 54.4, 54.2, 50.7, 50.4; HR-ESI-MS: *m/z* calcd for $C_{24}H_{24}N_2O_6Na$: 459.1532 $[M + Na]^+$; found: 459.1531.

*(E)-Dimethyl-1-(4-methylbenzylideneamino)-2-methyl-1,2-dihydropyridine-3,5-dicarboxylate (**1c**)*

R_f 0.63 (EtOAc/hexane 1:9); Yield 60%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.03 (s, 2H), 7.64 (s, 1H), 7.60 (d, J = 8 Hz, 2H), 7.23 (d, J = 8 Hz, 2H), 5.58 (q, J = 6 Hz, 1H), 3.80 (s, 6H), 2.39 (s, 3H), 1.25 (d, J = 6 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.9, 146.8, 141.3, 140.7, 132.2, 130.9, 129.6, 127.4, 116.1, 99.6, 51.7, 51.3, 49.4, 21.5, 16.6; HR-ESI-MS: *m/z* calcd for $C_{18}H_{21}N_2O_4$: 329.1501 $[M + H]^+$; found: 329.1499.

*(E)-Dimethyl-1-(4-methoxybenzylideneamino)-2-methyl-1,2-dihydropyridine-3,5-dicarboxylate (**1d**)*

R_f 0.36 (CH_2Cl_2/hexane/EtOAc 2:7:1, developed four times); Yield 44%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.02, (s, 2H), 7.66 (d, J = 9 Hz, 2H), 7.65 (s, 1H), 6.95 (d, J = 8.5 Hz, 2H),

5.56 (q, J = 6 Hz, 1H), 3.86 (s, 3H), 3.80 (s, 3H), 3.79 (s, 3H), 1.25 (d, J = 6.5 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.9, 161.5, 146.8, 141.2, 132.3, 129.0, 126.4, 115.8, 114.4, 99.4, 55.4, 51.7, 51.3, 49.5, 16.7; HR-ESI-MS: *m/z* calcd for $C_{18}H_{21}N_2O_5$: 345.1450 $[M + H]^+$; found: 345.1454.

*(E)-Dimethyl-1-(ethylideneamino)-2-methyl-1,2-dihydropyridine-3,5-dicarboxylate (**1e**)*

R_f 0.53 (EtOAc/Hexane 2:8, developed twice); Yield 32%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.89 (s, 1H), 7.60 (d, J = 1 Hz, 1H), 7.48 (q, J = 5 Hz, 1H), 5.35 (q, J = 6 Hz, 1H), 3.77 (s, 3H), 3.76 (s, 3H), 2.07 (d, J = 5.5 Hz, 3H), 1.15 (d, J = 6 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.3, 166.2, 147.2, 142.3, 132.7, 115.7, 98.9, 52.0, 51.6, 49.4, 16.8, 14.4; HR-ESI-MS: *m/z* calcd for $C_{12}H_{16}N_2O_4Na$: 275.1008 $[M + Na]^+$; found: 275.1010.

*(E)-Dimethyl-1-(3,4,5-trimethoxybenzylideneamino)-2-methyl-1,2-dihydropyridine-3,5-dicarboxylate (**1f**)*

R_f=0.45 (EtOAc/hexane 2:8, developed four times); Yield 45%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.05 (s, 1H), 7.97 (s, 1H), 7.64 (d, J = 1.5 Hz, 1H), 6.96 (s, 2H), 5.58 (q, J = 6.5 Hz, 1H), 3.93 (s, 6H), 3.90 (s, 3H), 3.81 (s, 6H), 1.25 (d, J = 6 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.9, 153.6, 146.8, 140.7, 140.0, 132.1, 129.1, 116.3, 104.4, 99.9, 60.9, 56.2, 51.8, 51.4, 49.5, 16.5; HR-ESI-MS: *m/z* calcd for $C_{20}H_{25}N_2O_7$: 405.1662 $[M + H]^+$; found: 405.1669.

*(E)-Dimethyl-1-(4-(dimethylamino)benzylideneamino)-2-methyl-1,2-dihydropyridine-3,5-dicarboxylate (**1g**)*

R_f 0.32 (EtOAc/hexane 1:9, developed thrice); Yield 34%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.03 (s, 1H), 8.01 (s, 1H), 7.65 (s, 1H), 7.59 (d, J = 9 Hz, 2H), 6.71 (d, J = 9 Hz, 2H), 5.55 (q, J = 6.5 Hz, 1H), 3.79 (s, 3H), 3.78 (s, 3H), 3.04 (s, 6H), 1.25 (d, J = 6.5 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.1, 166.0, 151.9, 146.5, 142.8, 132.5, 129.0, 121.2, 114.9, 111.9, 98.5, 51.7, 51.2, 49.7, 40.2, 17.0; HR-ESI-MS: *m/z* calcd for $C_{19}H_{24}N_3O_4$: 358.1767 $[M + H]^+$; found: 358.1772.

Procedure for the synthesis of 1,2-DHP **1h**, **1h′**and **2a**

*(E)-dimethyl-1-((4-(diethylamino)-2-hydroxybenzylidene)amino)-2-((2-(2-methoxyethoxy)ethoxy)methyl)-1,2-dihydropyridine-3,5-dicarboxylate (**1h**)*

To a solution of triethylene glycol monomethyl ether (7.92 mmol, 1 equiv) in CH_2Cl_2 at room temperature was added Dess-Martin periodinane (19.8 mmol, 2.5 equiv) in one portion. After one hour of stirring, the reaction mixture was filtered through a celite pad and washed with EtOAc. The resulting filtrate was concentrated under reduced pressure, and the ^{1}H NMR of the crude mixture confirmed the formation of the aldehyde. The crude aldehyde without further purification was treated with hydrazine hydrate (6.66 mmol, 3 equiv) in presence of trifluoroacetic acid (1.11 mmol, 0.5 equiv) and anhydrous $MgSO_4$ (22.2 mmol, 10 equiv) in CH_3CN (10 mL) solvent. After 15 min, dieneaminodioate (2.22 mmol, 1 equiv) and trifluoroacetic acid (1.11 mmol, 0.5 equiv) were added. The mixture was then allowed to stir for 8h at room temperature. After complete consumption of dieneaminodioate, as observed on TLC, the reaction mixture was quenched with saturated aqueous $NaHCO_3$ and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4, concentrated and the resulting residue was purified by column chromatography to afford the desired 1,2-DHP **1h**. R_f 0.40 (EtOAc/hexane 5:5); Yield 50%. ^{1}H NMR (500 MHz, MeOD): δ 8.51 (s, 1H), 8.06 (s, 1H), 7.70 (s, 1H), 7.27 (d, J = 8.5 Hz, 1H), 6.34 (d, J = 8.5 Hz, 1H), 6.18 (s, 1H), 5.68 (d, J = 6 Hz, 1H), 3.81 (s, 3H), 3.79 (s, 3H), 3.66-3.52 (m, 7H), 3.46-3.37 (m, 7H), 3.30 (s, 3H), 1.20 (d, J = 7 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.8, 165.7, 160.3, 151.0, 149.6, 144.4, 134.1, 133.4, 109.4, 105.9, 104.1, 99.4, 98.0, 71.8, 71.3, 71.0, 70.6, 70.5, 58.9, 54.5, 51.7, 51.3, 44.5, 12.6; HR-ESI-MS: *m/z* calcd for $C_{26}H_{37}N_3O_8Na$: 542.2478 $[M + Na]^+$; found: 542.2483.

*(E)-2-(((3,5-bis(methoxycarbonyl)-2-((2-(2-methoxyethoxy)ethoxy)methyl)pyridin-1(2H)-yl)imino)methyl)-5-(diethylamino)phenyl phosphate (**1h′**)*

To a stirred solution of 1,2-DHP **1h** (0.53 mmol, 1 equiv) and NaH (60% dispersion in oil, 0.79 mmol, 1.5 equiv) in THF (4 mL) at room temperature under nitrogen atmosphere was added diethyl chlorophosphate (0.79 mmol, 1.5 equiv). After 3h of stirring at room temperature, the reaction mixture was quenched with water and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography on silica

gel to afford the diethyl phosphate ester of 1,2-DHP **1h**. To a solution of this diethyl phosphate ester of 1,2-DHP **1h** (0.11 mmol, 1 equiv) in dry CH_2Cl_2 (3 mL) was added bromotrimethylsilane (1.08 mmol, 10 equiv) dropwise at room temperature. The reaction mixture was stirred overnight at room temperature under nitrogen atmosphere and quenched with MeOH (5 mL), stirring was continued for further 30 mins. The reaction mixture was then concentrated and purified by reversed-phase HPLC using H_2O/methanol to afford 1,2-DHP **1h′**. R_f 0.42 (MeOH/EtOAc 3:7); Yield 58% (over two steps). ^{1}H NMR (500 MHz, MeOD): δ 8.62 (s, 1H), 8.11 (s, 1H), 7.97 (d, J = 8.5 Hz, 1H), 7.69 (s, 1H), 7.13 (s, 1H), 6.87 (d, J = 8.5 Hz, 1H), 5.79 (t, J = 4 Hz, 1H), 3.81 (s, 3H), 3.79 (s, 3H), 3.63-3.52 (m, 14H), 3.30 (s, 3H), 1.20 (d, J = 7 Hz, 6H); ^{13}C NMR (125 MHz, MeOD): δ 166.3, 165.9, 153.0, 146.7, 140.3, 133.5, 127.4, 111.3, 99.0, 71.5, 70.9, 70.2, 70.1, 70.0, 57.6, 54.2, 50.8, 50.4, 48.2, 10.7; ^{31}P NMR (202 MHz, MeOD): δ -4.91; HR-ESI-MS: *m/z* calcd for $C_{26}H_{38}N_3O_{11}PNa$: 622.2142 $[M + Na]^+$; found: 622.2147.

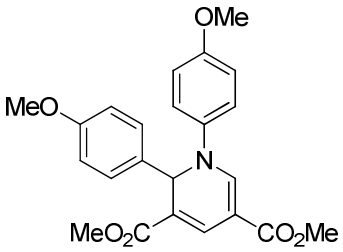
*(E)-5-Methyl-3-(4-nitrophenyl)-1-(4-(dimethylamino)benzylideneamino)-2-methyl-1,2-dihydropyridine-3,5-dicarboxylate (**2a**)*

To a solution of 1,2-DHP **1g** (0.08 mmol, 1 equiv) in MeOH (4 mL) was added 10% aqueous KOH (2.5 mL) and stirred at room temperature overnight. The reaction mixture was then evaporated, diluted with 20% aqueous $KHSO_4$ (4 mL), and extracted with $CHCl_3$/MeOH (7:1, 2 x 30 mL). The organic layer was dried over anhydrous Na_2SO_4, concentrated and the resulting residue was purified by column chromatography using $CHCl_3$/MeOH 98:2 to afford the mono-carboxylic acid product of 1,2-DHP **1g** (92%).

To a solution of mono-carboxylic acid (0.03 mmol, 1 equiv) in CH_2Cl_2 (1.5 mL) were added *p*-nitrophenol (0.03 mmol, 1.2 equiv), *N,N′*-dicyclohexylcarbodiimide (0.04 mmol, 1.5 equiv), and 4-dimethylaminopyridine (0.003 mmol, 0.1 equiv) at room temperature. After complete consumption of the starting material, the reaction mixture was quenched with saturated aqueous $NaHCO_3$ (5 mL) and extracted with CH_2Cl_2 (2 x 10 mL). The organic layer was washed thrice with saturated $NaHCO_3$. The combined organic layers were dried over anhydrous Na_2SO_4, concentrated and the resulting residue was purified by column chromatography using hexane/EtOAc 95:5 to afford 1,2-DHP **2a** as an orange red needle like crystalline product. R_f 0.57 (EtOAc/hexane 2:8, developed thrice); Yield 30%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.30 (d, J = 9 Hz, 2H), 8.14 (s, 1H), 8.06 (s, 1H), 7.97 (s, 1H), 7.62 (d, J = 9 Hz, 2H), 7.36 (d, J = 9 Hz, 2H), 6.73 (d, J = 9 Hz, 2H), 5.62 (q, J = 6.5 Hz, 1H), 3.83 (s, 3H),

3.06 (s, 6H), 1.35 (d, J = 6 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.8, 162.9, 155.9, 152.1, 147.2, 145.0, 144.2, 135.8, 129.2, 125.1, 122.4, 120.6, 112.1, 111.8, 98.7, 51.4, 49.9, 40.1, 14.1; HR-ESI-MS: *m/z* calcd for $C_{24}H_{25}N_4O_6$: 465.1774 $[M + H]^+$; found: 465.1780. CCDC 1054218 contains the crystallographic data.

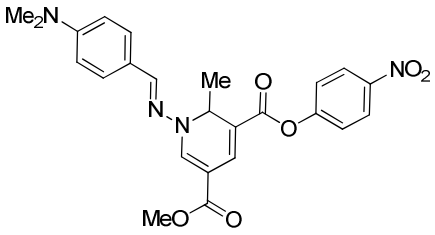
Photophysical properties of N-phenyl-1,2-DHP



N-phenyl-1,2-DHP

Table S1.

Absorption max. (λ_{abs})nm, (ε, M^{-1} cm^{-1})[	Emission max. (λ_{ems}),nm	Stokes shift ($\Delta\nu$),cm^{-1}	Emission quantum yields (Φ_F)
403 (8642)	524	5730	0.08



1,2-DHP **2a**

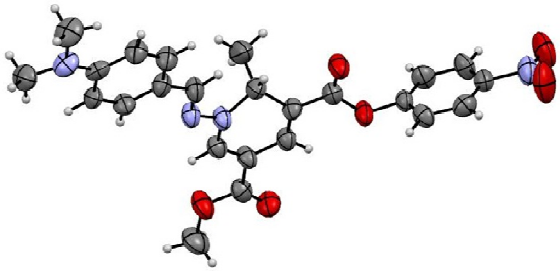


Figure S1. ORTEP diagram of 4-nitrophenyl ester of 1,2-DHP **2a** (CCDC No. 1054218)

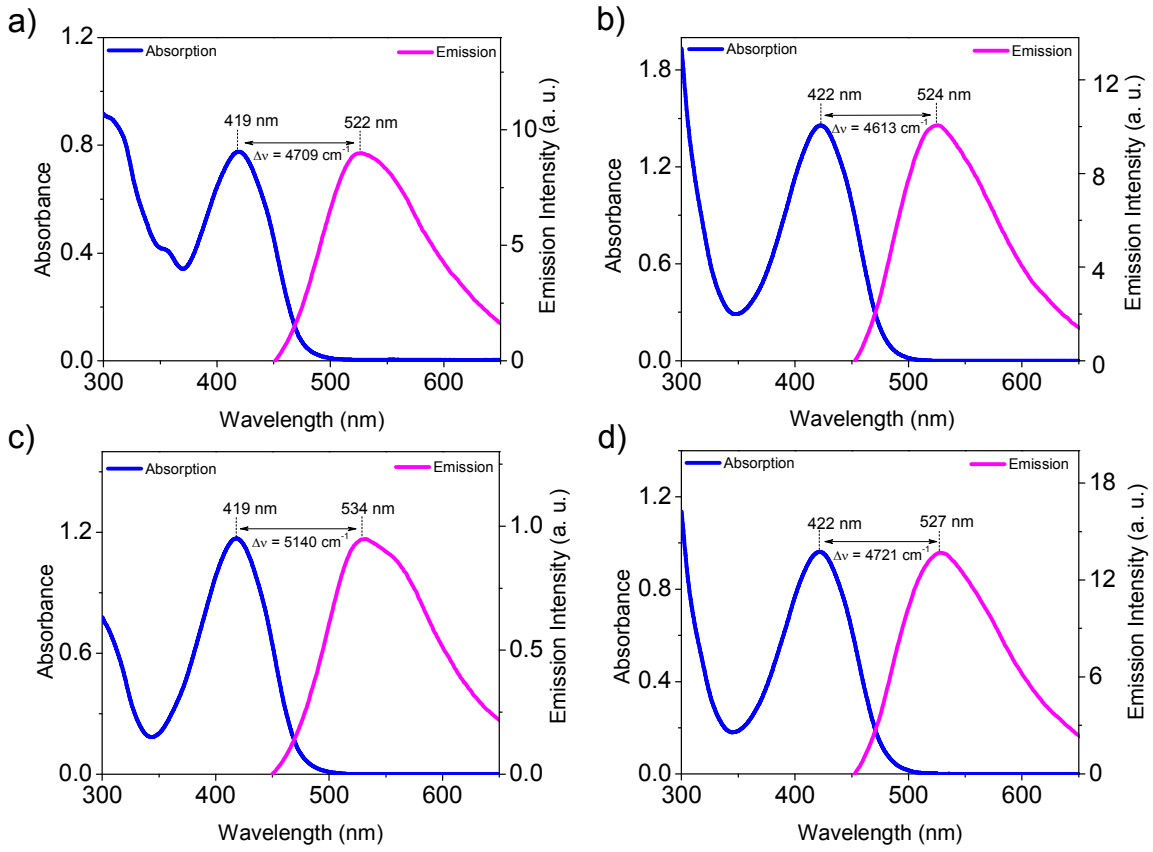


Figure S2. Absorption and emission (λ_{ex} = 430 nm) spectra of 1,2-DHPs in MeOH at room temperature. a) **1a**, b) **1b**, c) **1c**, and d) **1d**.

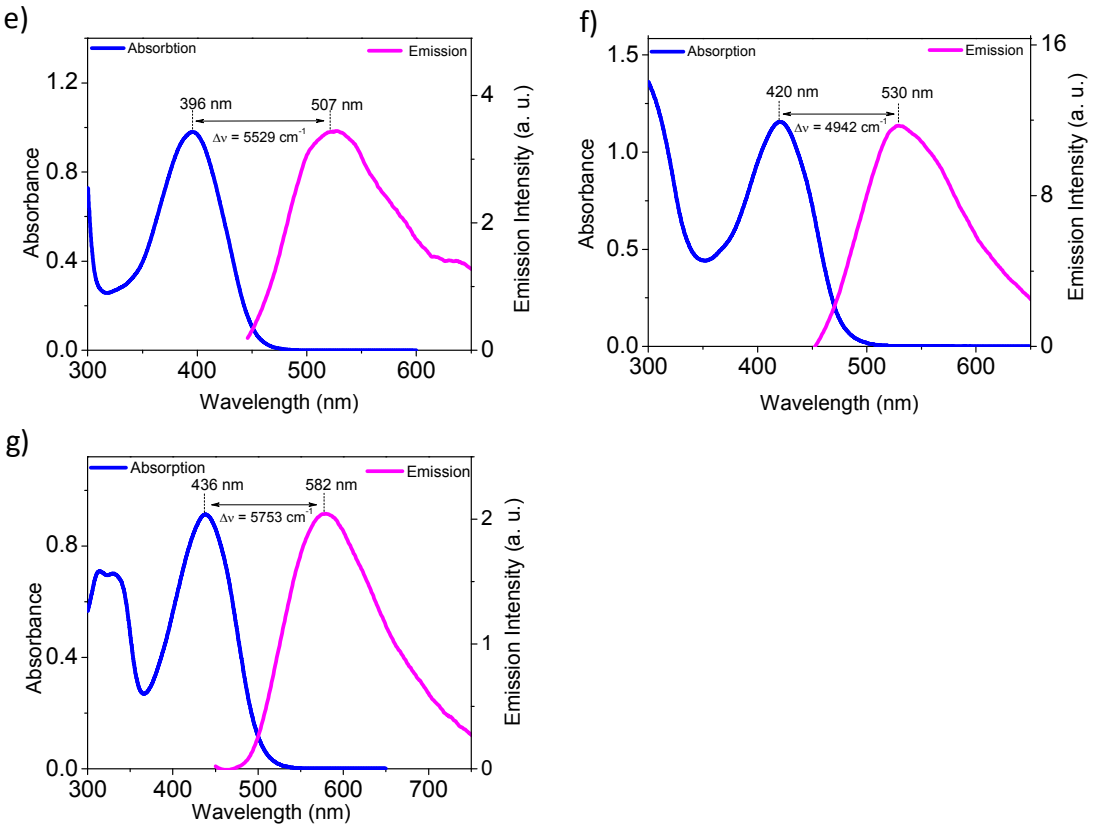


Figure S3. Absorption and emission (λ_{ex} = 430 nm) spectra of 1,2-DHPs in MeOH at room temperature. e) **1e**, f) **1f**, and g) **1g**.

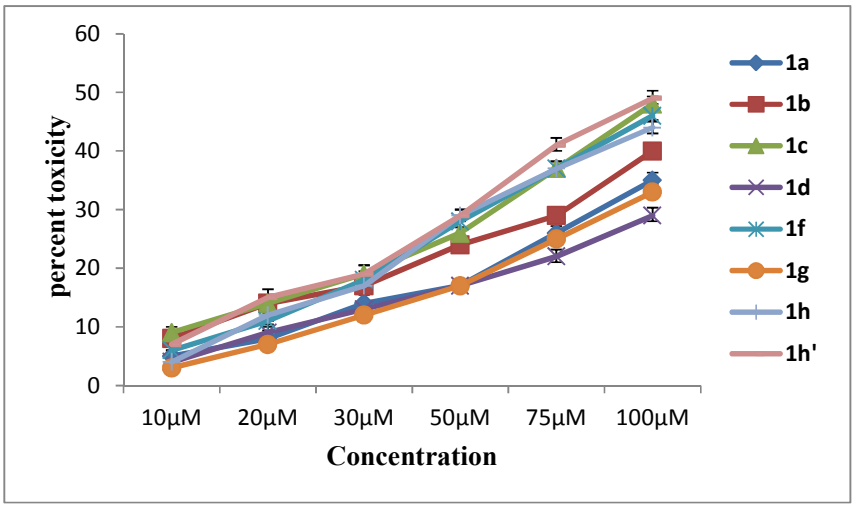


Figure S4. Cytotoxicity assessed by MTT assay in HeLa cells. Different concentrations of **1a**, **1b**, **1c**, **1d**, **1f**, **1g**, **1h** and **1h′** were evaluated. Values are the mean ± SD of three different experiments.

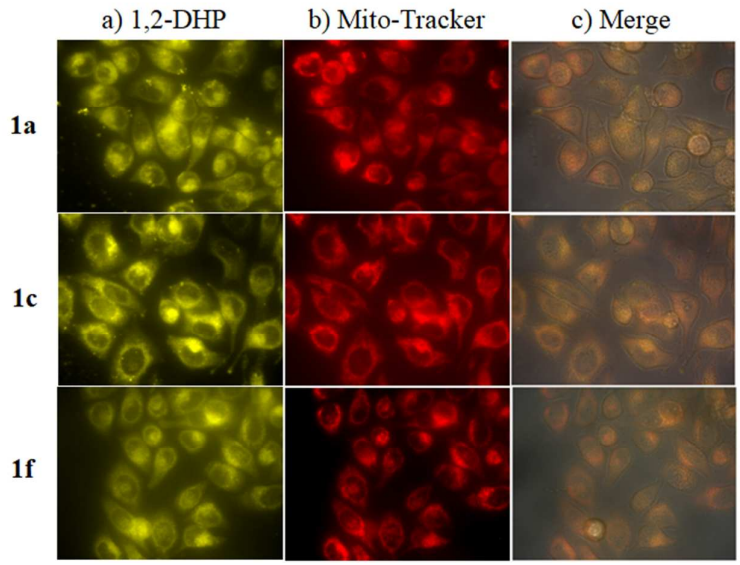


Figure S5. Fluorescent images of HeLa cells a) treated with 1,2-DHPs **1a**, **1c** and **1f** (30 μM) for 10 min, b) MitoTracker red CMXRos (CMXRos, 50 nM) for 30 min, c) merged image of (a) and (b) with bright field image (60 X magnification). Excitation wavelength: 440 nm (for 1,2-DHP) and 540 nm (for CMXRos) and Emission wavelength: 515 nm (for 1,2-DHP) and 645 nm (for CMXRos). Pearson's correlation coefficients were obtained as 0.84, 0.87 and 0.89 for 1,2-DHP **1a**, **1c** and **1f**, respectively.

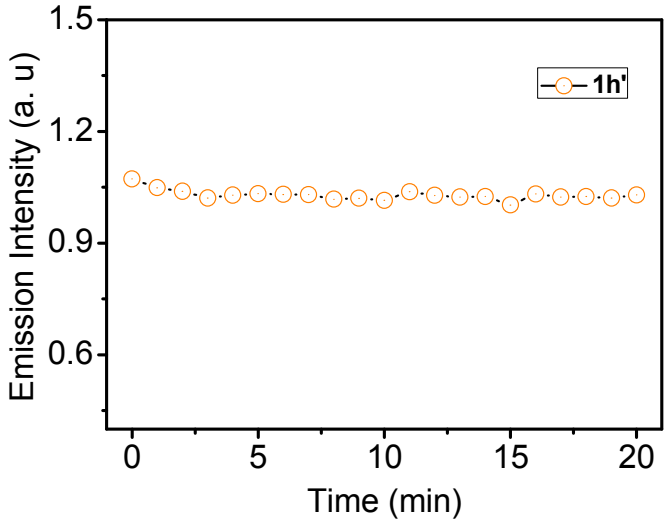


Figure S6. Photostability of 1,2-DHP **1h′**.

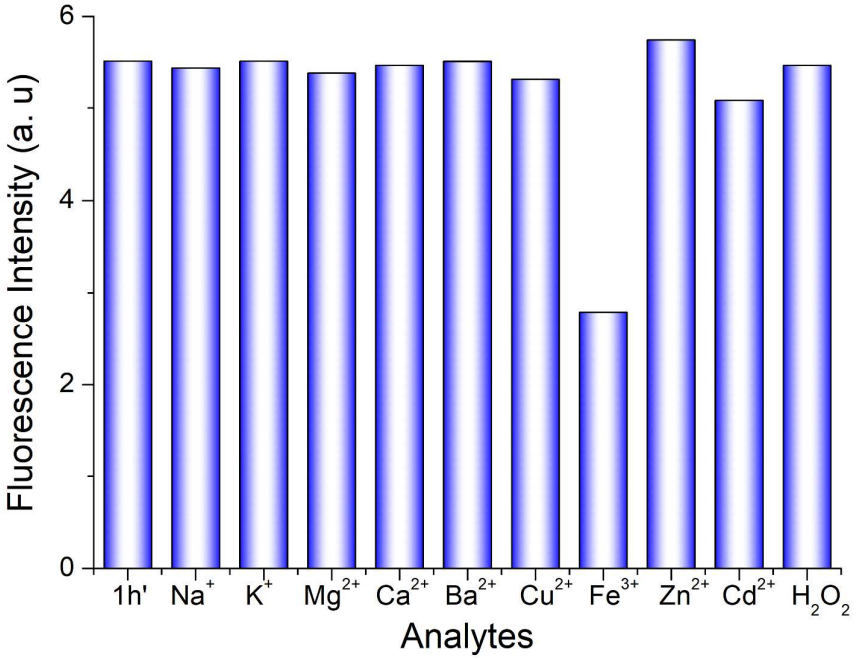


Figure S7. Changes in the fluorescence intensity of 1,2-DHP **1h′** (10 μM in aqueous solution at pH 7.4) in presence various biologically important metal ions (100 μM) and reactive oxygen species H_2O_2 (10 μM).

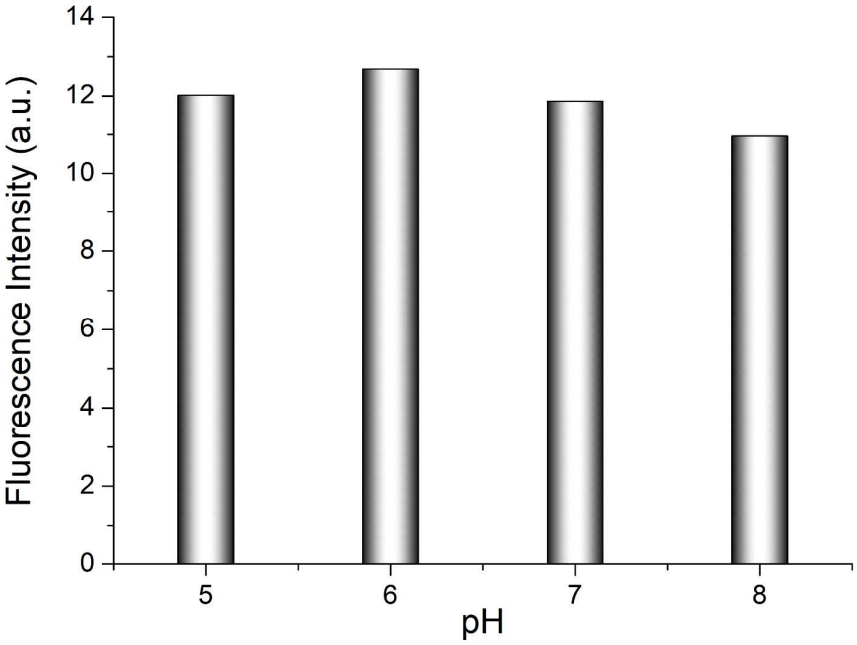


Figure S8. Changes in the fluorescence intensity of 1,2-DHP **1h′**(10 μM) at different pH (5 to 8).

Figure S9. ^{1}H and ^{13}C NMR spectrum of 1,2-DHP **1a**

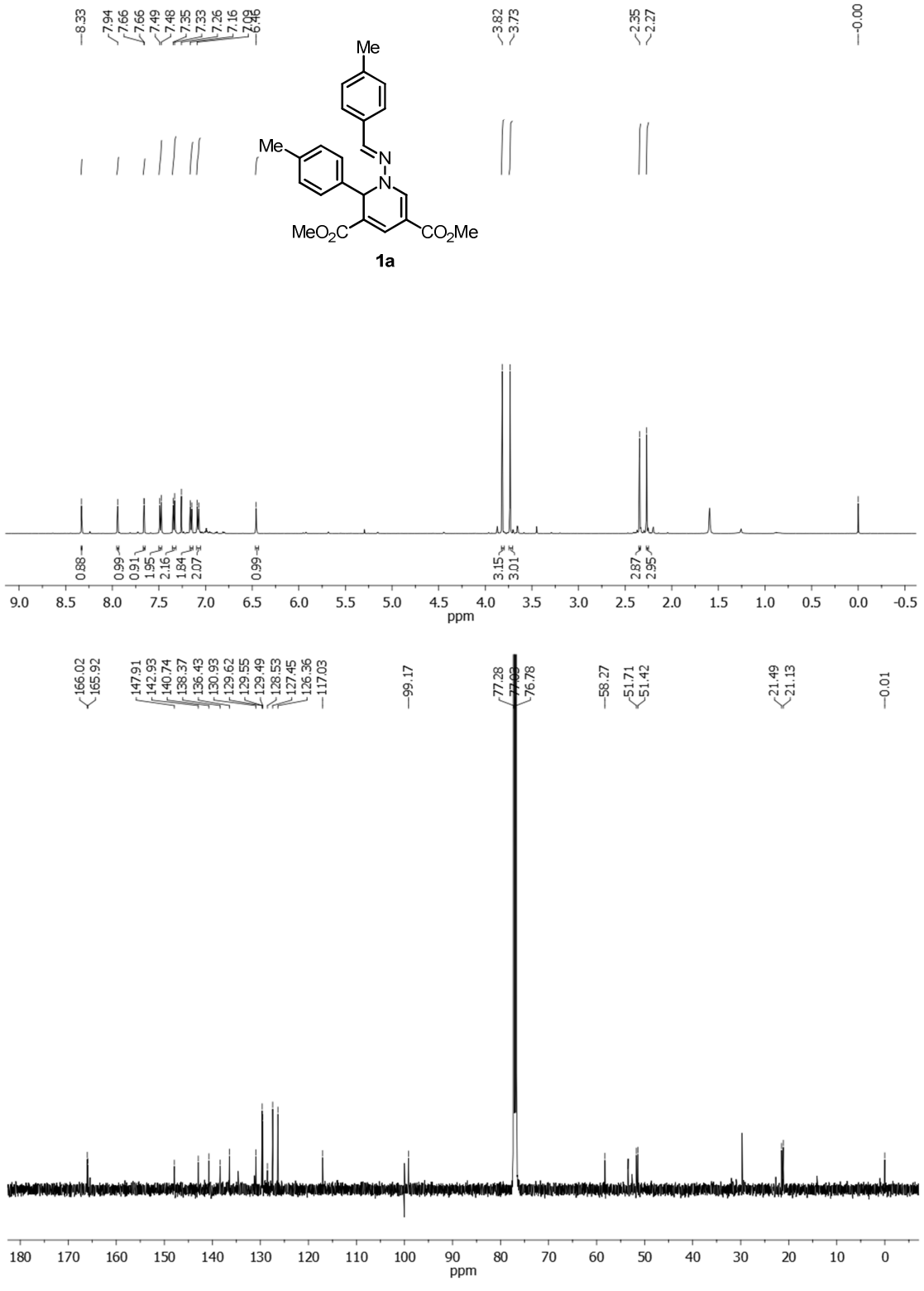


Figure S10. ^{1}H and ^{13}C NMR spectrum of 1,2-DHP **1b**

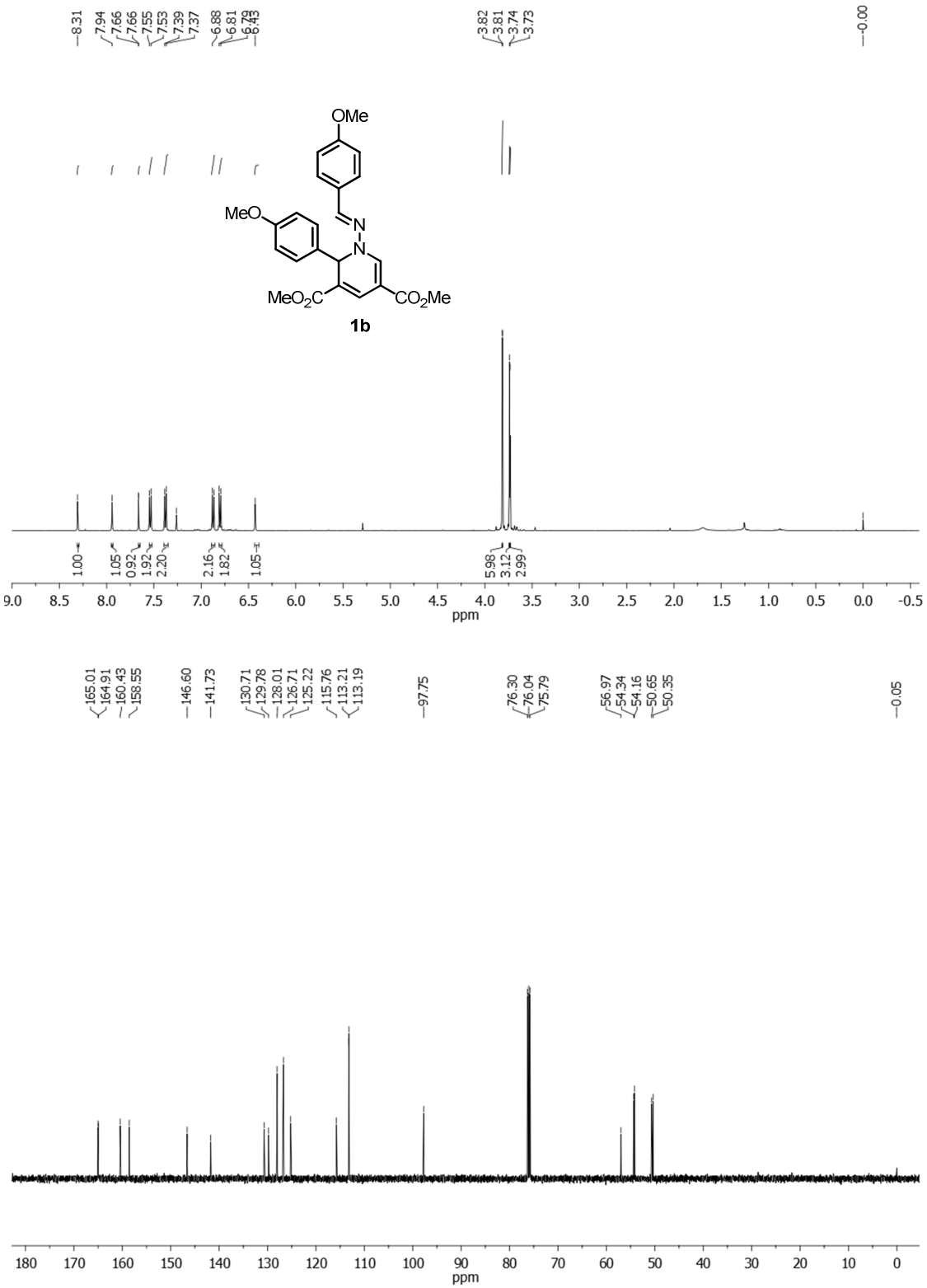


Figure S11. ^{1}H and ^{13}C NMR spectrum of 1,2-DHP **1c**

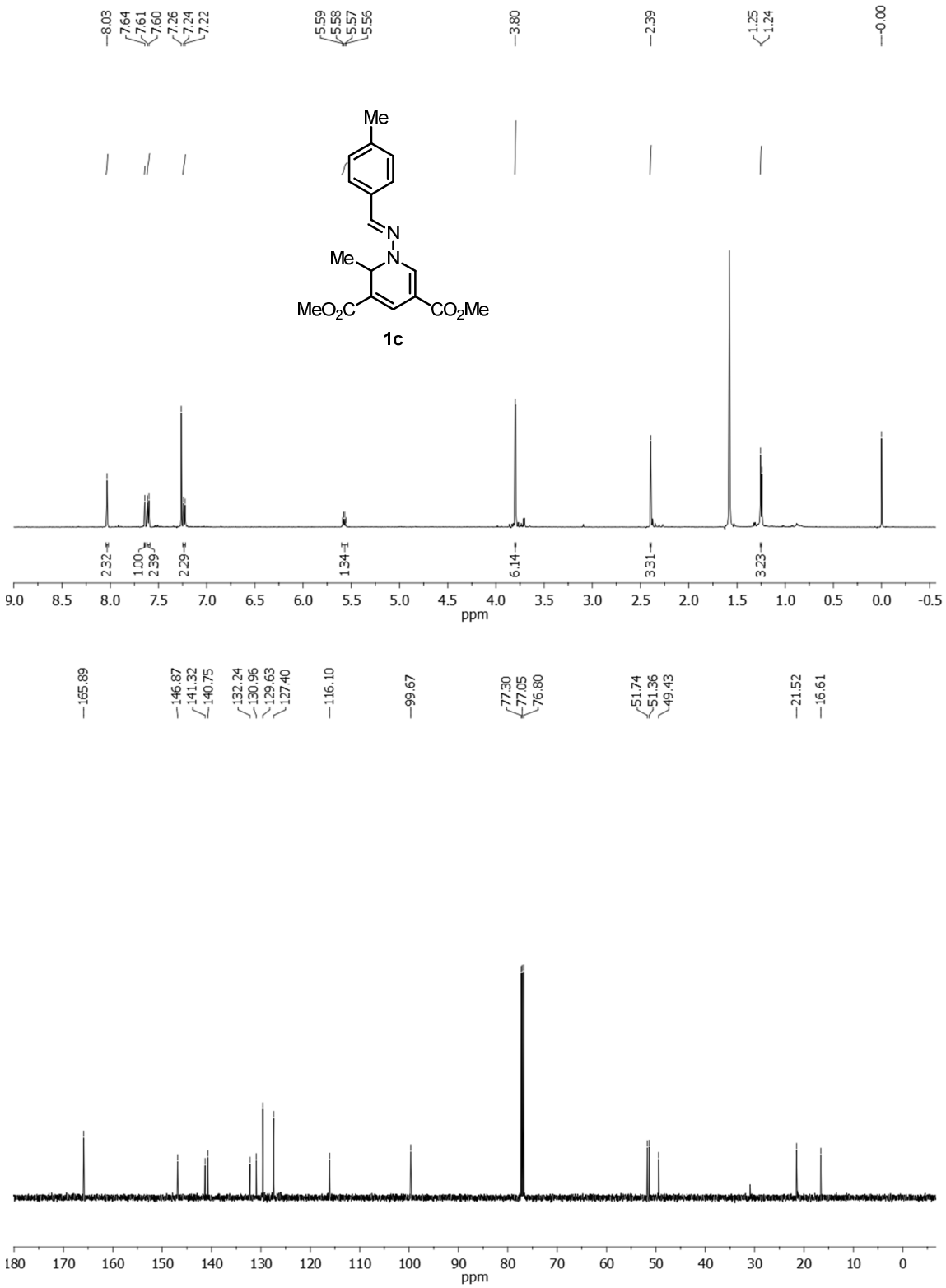


Figure S12. ^{1}H and ^{13}C NMR spectrum of 1,2-DHP **1d**

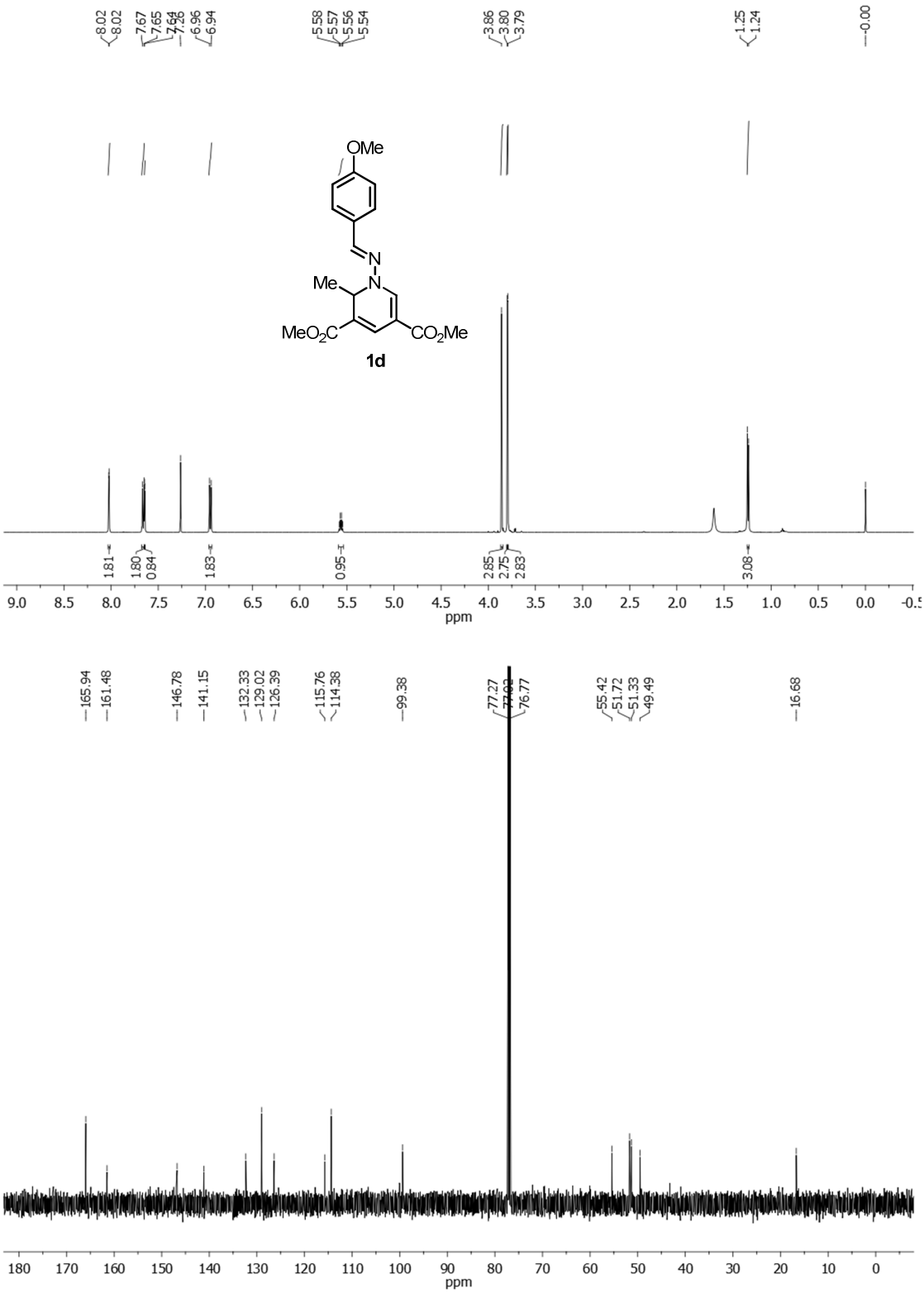


Figure S13. ^{1}H and ^{13}C NMR spectrum of 1,2-DHP **1e**

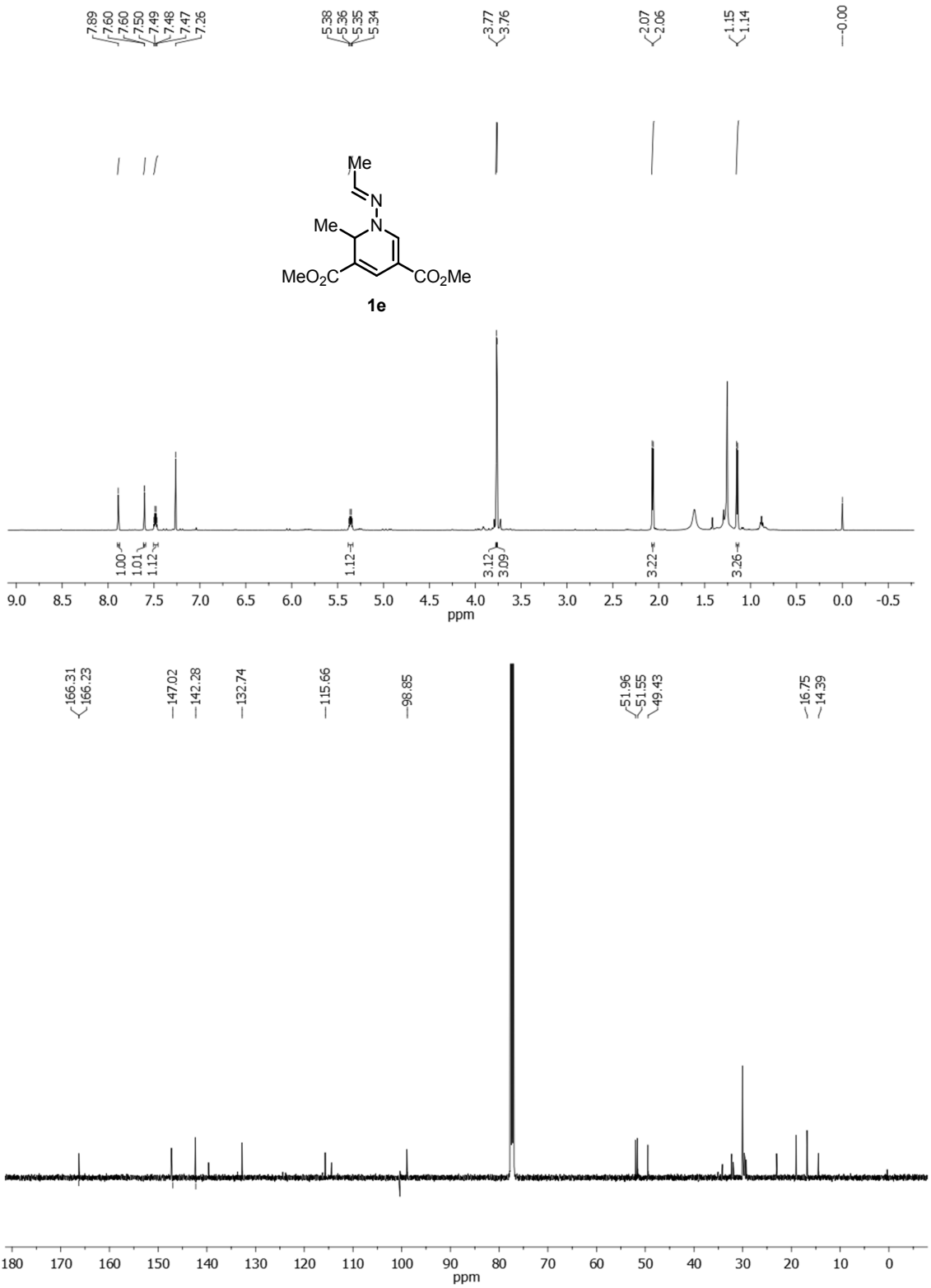


Figure S14. ^{1}H and ^{13}C NMR spectrum of 1,2-DHP **1f**

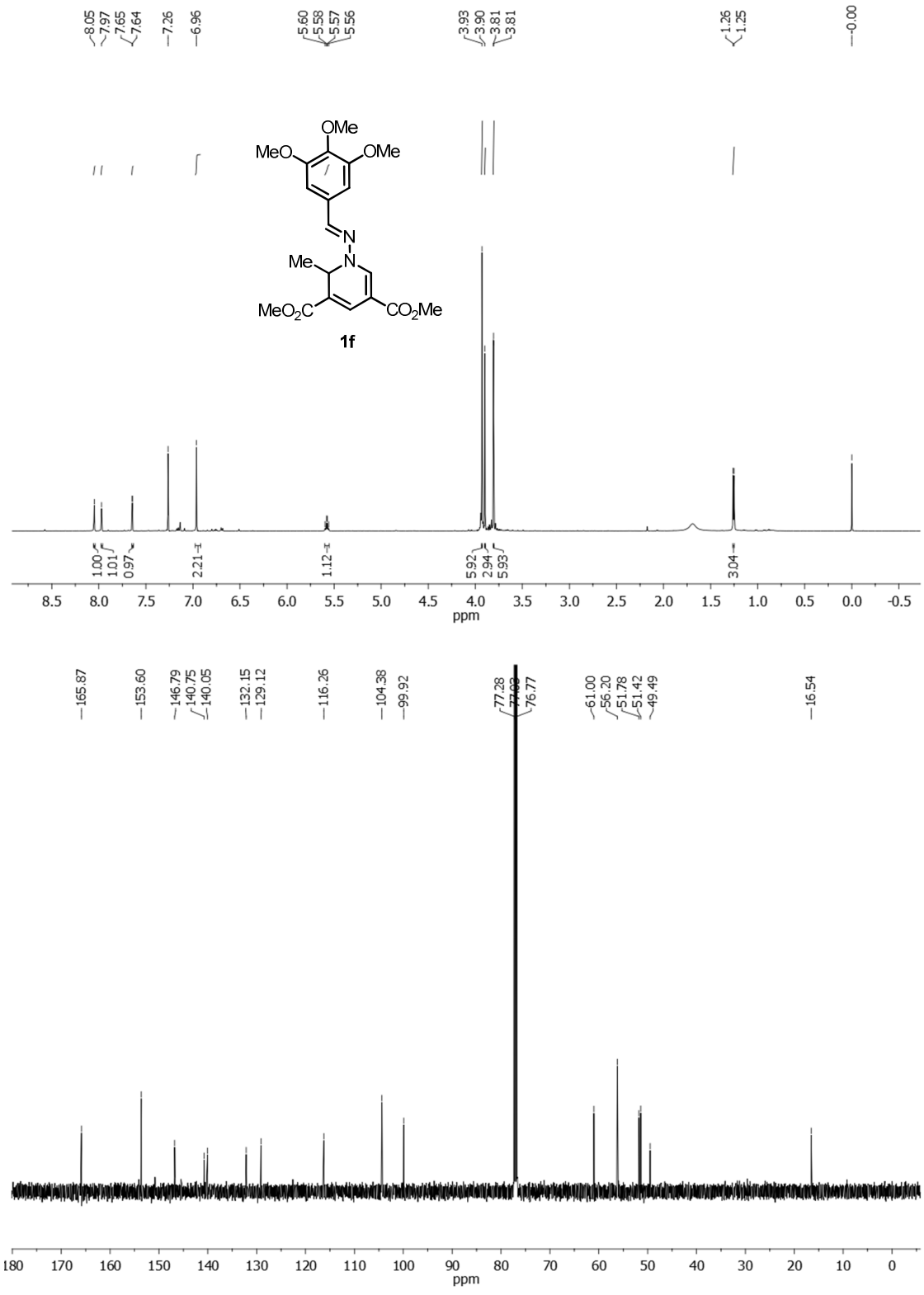


Figure S15. ^{1}H and ^{13}C NMR spectrum of 1,2-DHP **1g**

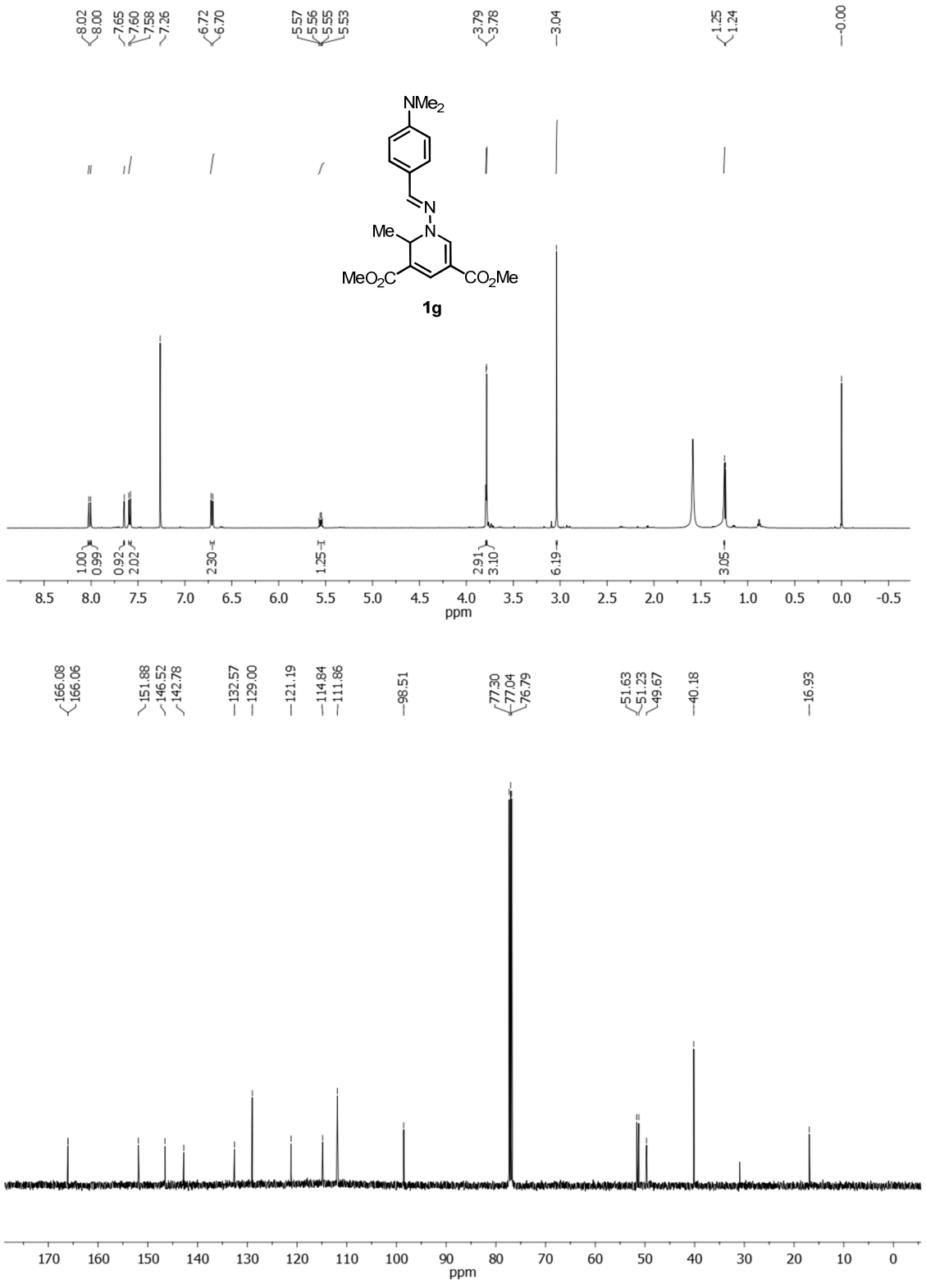


Figure S16. ^{1}H and ^{13}C NMR spectrum of 1,2-DHP **1h**

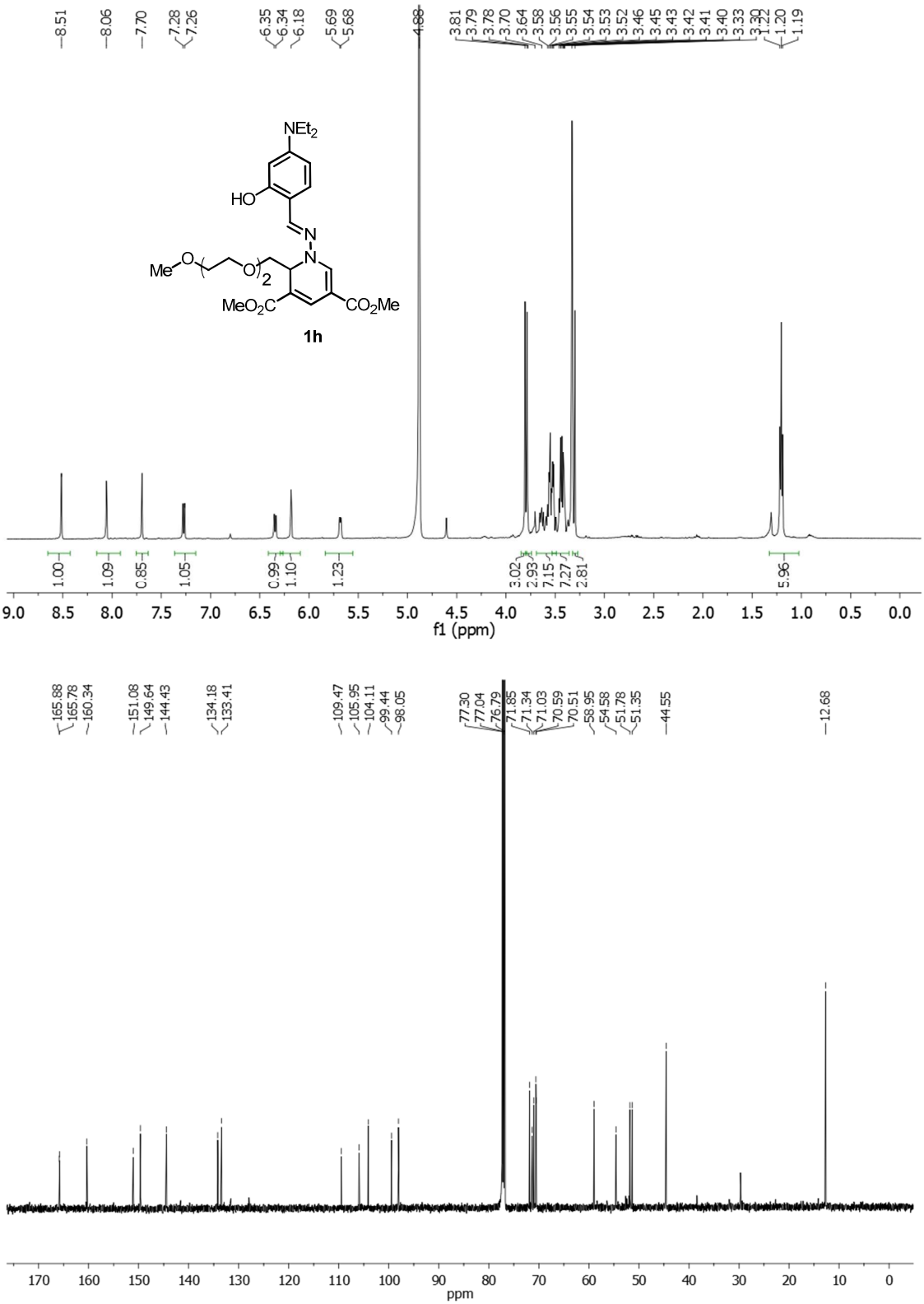


Figure S17. ^{1}H and ^{13}C NMR spectrum of 1,2-DHP **1h′**

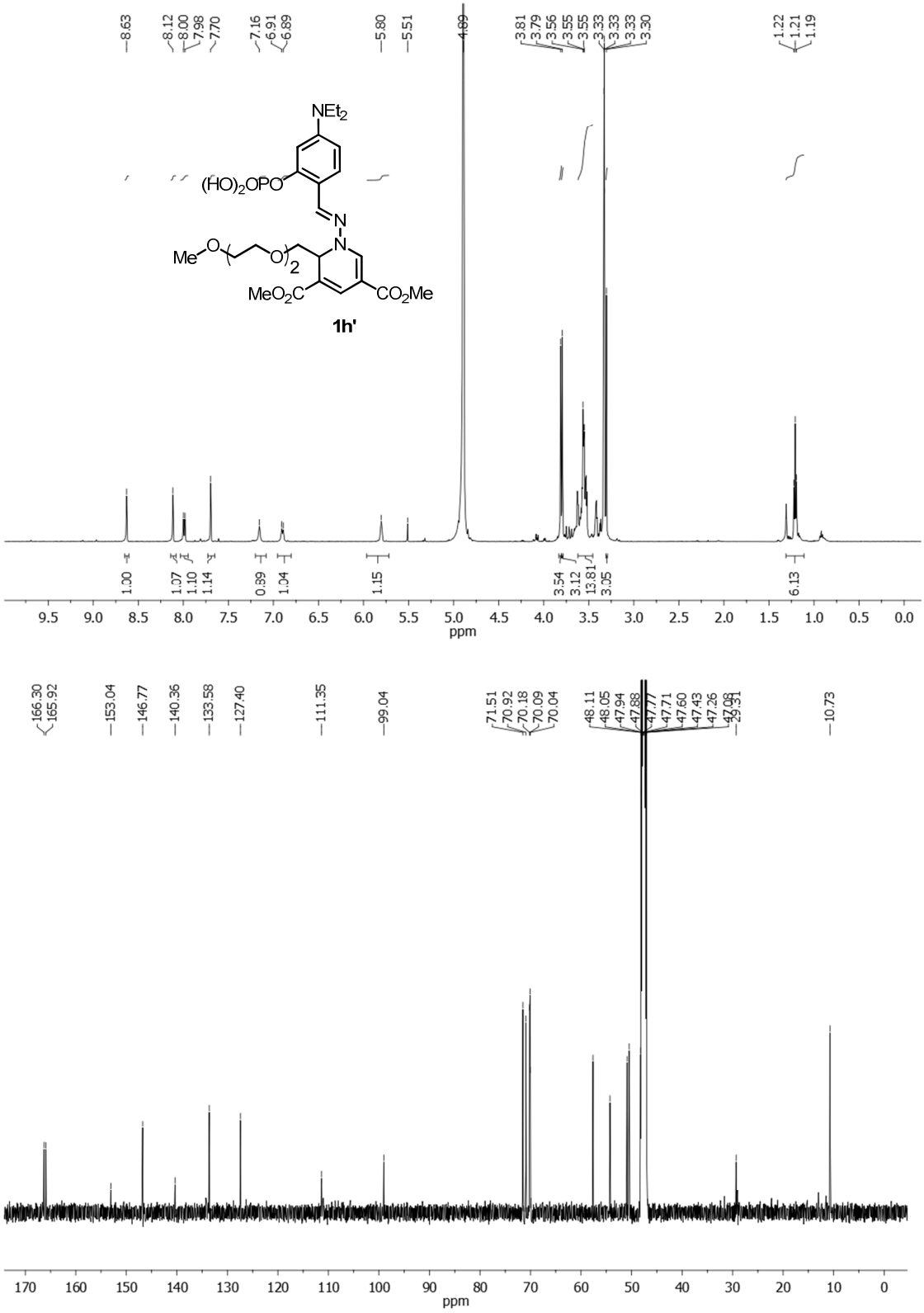


Figure S18. ^{31}P NMR spectrum of 1,2-DHP **1h′**

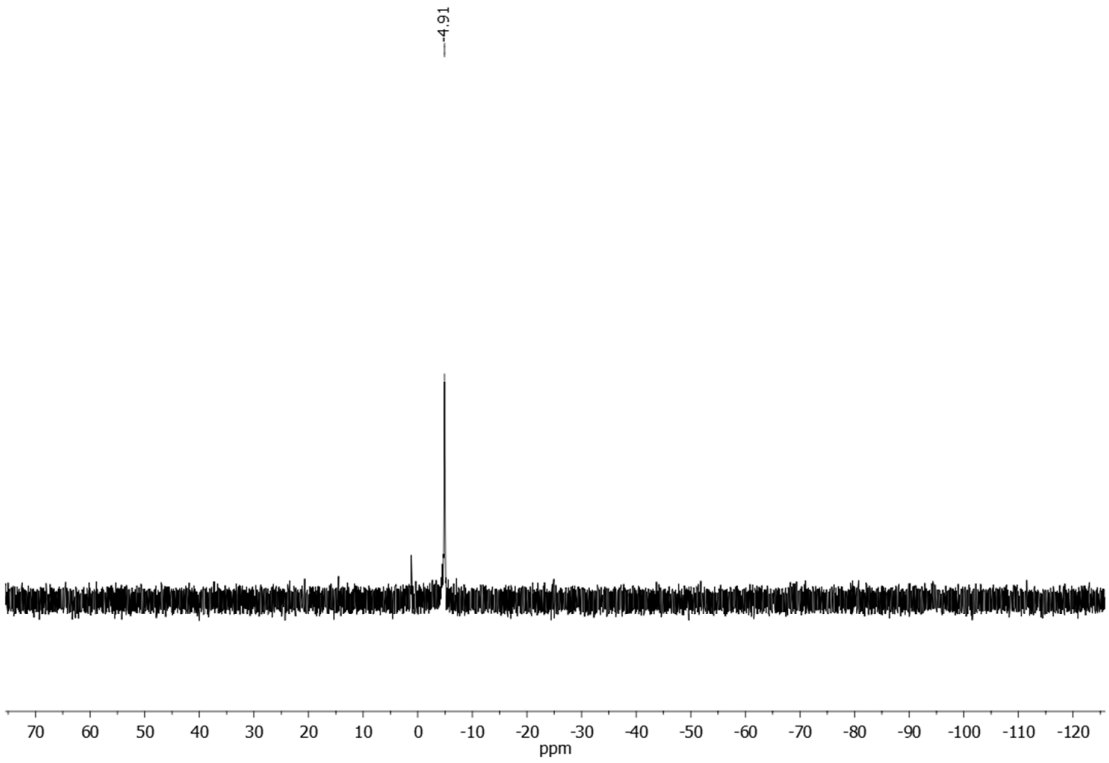


Figure S19. ^{1}H and ^{13}C NMR spectrum of 1,2-DHP **2a**