**Electronic supplementary materials** *Mendeleev Commun.*, 2022, **32**, 173–175

# Podophyllotoxin esters with alicyclic residues: an insight into the origin of microtubule-curling effect in cancer cells

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**1. Molecular modeling** Computer molecular modeling was performed using 3D model of the colchicine-binding site in α,β-tubulin (PDB ID: 1SA1). All compounds used for X-ray of the protein and all water molecules were previously excluded from the model (other molecules and ions at the interface of α- and β-subunits of the protein were maintained). Atomic charges of protein amino acids were assigned by standard Kollman method using AutoDock Tools 1.5.6. 2D structures of the lingands were converted to the 3D structures and were submitted to a conformational MMFF Amber ff14SB optimization using *Gasteiger charges in* USCF Chimera 1.15 program [S1]. Docking procedure was performed with AutoDock Vina 1.1.2 software [S2] (grid box 15.0Å×17.25Å ×15.0Å, grid center size x=118.881 Å, y=88.149 Å, z=7.522 Å, exhaustiveness = 16). Complexes with the best values of scoring functions were selected and visualized using CLC Drug Discovery Workbench (Limited mode, Version 4).

**Molecular dynamics simulations** were performed using CHARMM36 / CGenFF 4.4 force field [S3] from the GROMACS 2020.3 software [S4]. The initial models of the system were built using the Ligand Reader & Modeler and Solution Builder modules of the CHARMM-GUI web service [S5]. The protein molecule was inserted into a rectangular box with water in the TIP3P model; the distance from the protein to the box border was no less than 10 Å (the total box size was about 120 × 120 × 120 $Å^3$). Individual randomly selected water molecules were replaced with potassium and chlorine ions so that the electrical neutrality of the system and the total concentration of KCl were of the order of 0.15 M. For each system of the applied minimization by molecular mechanics (up to 5000 steps) at the central process, a preliminary check of the system is performed in for 125 ps at a temperature of 300 K at a constant volume using a v-rescale thermostat on an NVIDIA GeForce GTX 1080 GPU. The working simulation was performed on a GPU at a constant pressure of 1 atm and a temperature of 300 K using a v-rescale thermostat and a Parrinello – Rahman barostat. Hydrogen movements are limited using the LINCS algorithm. For the analysis and visualization of molecular docking models, the cpptraj software [S6] in the AmberTools 18 package [S7] and UCSF Chimera were used.

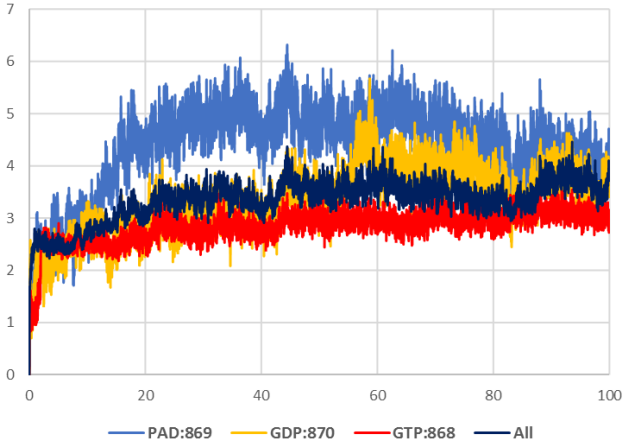
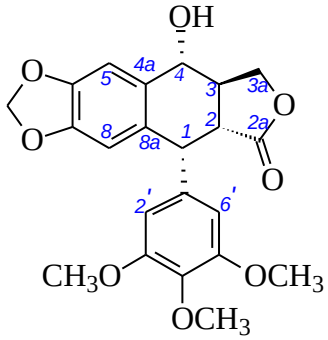



**Figure S1** RMSD of model of the complex with 4-*O*-(1-adamantylacetyl)-L-podophyllotoxin **2a** and graphs for each ligand of the protein.

## 2. Chemistry

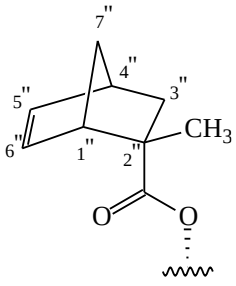
All reaction temperatures correspond to internal temperatures unless otherwise noted. Solvents for extraction and chromatography were technical grade and were purified by standard procedures prior to use. The carboxylic acids and other initial reagents were purchased at Sigma – Aldrich. Flash and column chromatography were performed on silica gel Acros (40-60 μm). Reaction control was carried out by thin-layer chromatography on ALUGRAM Xtra G/UV254 plates, using UV light for visualization. $^{1}$H and $^{13}$C NMR spectra were recorded on spectrometer Agilent 400-MR (400.0 MHz for $^{1}$H; 100.6 MHz for $^{13}$C) at room temperature; chemical shifts were measured with reference to the solvent ($CDCl_3$, $\delta_H$=7.27 ppm, $\delta_C$=77.0 ppm). Chemical shifts (δ) are given in ppm, spin-spin coupling constants (*J*) are reported in Hz; multiplicities are indicated by s (singlet), d (doublet), t (triplet), m (multiplet). Signals of atoms in podophyllotoxin fragment in NMR spectra were assigned according to [S8] using the following numbering of atoms in initial molecule [S9]:




Elemental analysis for compound **2e** was performed on CNH analyser "Carlo-Erba" ER-20. Electron impact mass spectra were obtained on a Bruker Autoflex II mass spectrometer with accelerating voltage of 20 kV.

**General procedure for the preparation of esters 2d,e,g,j,k.** To a solution of carboxylic acid in $CH_2Cl_2$ (10 ml) was added podophyllotoxin **1**, DCC (*N*,*N'*-dicyclohexylcarbodiimide) and catalytic amount (0.01 g) of 4-dimethylaminopyridine (DMAP). The mixture was stirred at room temperature for 12 h, then AcOH (5–10 μl) was added, and after 15 min the solvent was evaporated under reduced pressure. The residue was diluted in ethyl acetate (10–15 ml) and maintained at 0°C–4°C for 2–3 h. The precipitate of *N*,*N'*-dicyclohexylurea was filtered off, washed with ethyl acetate (2×5 ml). The filtrate was washed with brine (10 ml) and water (10 ml), dried over $Na_2SO_4$ and evaporated under reduced pressure. The residue was purified by column chromatography [EtOAc/petroleum ether (40–70°C) in a gradient mixture 1:8 – 1:5, unless indicated otherwise].

**4-*O*-{(2*SR*)-2-Methylbicyclo[2.2.1]hept-5-en-2-ylcarbonyl}-L-podophyllotoxin 2k** was synthesized by general procedure from 2-methyl-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid (0.023 g, 0.151 mmol), podophyllotoxin (0.048 g, 0.116 mmol) and DCC (0.031 g, 0.151 mmol). Yield (after column chromatography) 17% (0.011 g), white solid, m.p. 108–109°C.

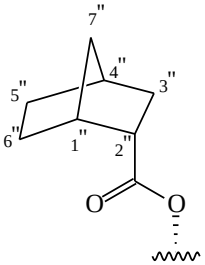



$^{1}$H NMR ($CDCl_3$, δ, ppm, Δδ, *J*/Hz, signals of the second diastereomer are given in square brackets): 1.50 [1.56] (3H, two s, Δδ = 2.2, $CH_3$), 1.51–1.54 [1.60–1.62] (2H, m, H7``), 1.92 [1.99] (1H, two dd, Δδ = 11.8, *J*= 2.7, 6.3, H3``), 2.78 (1H, m), 2.84 (1H, m, H1``), 2.89–2.95 (2H, m, H2, H3), 3.78 [3.78] (6H, two s, Δδ = 3.3, $OCH_3$), 3.83 (3H, s, O $CH_3$), 4.15 [4.19] (1H, two dd, Δδ = 10.3, *J* = 9.3, 3.6, H3a), 4.27 [4.30] (1H, two dd, Δδ = 3.9, *J*= 7.1, 9.2, H3a), 4.61 (1H, m, H1), 5.80 [5.83] (1H, two d, Δδ = 9.2, *J*= 3.8, H4), 5.99–6.01 (2H, m, *J* = 1.3, $OCH_2O$), 6.17 (1H, m, H6``), 6.23 (1H, m, H5``), 6.40 [6.41] (2H, two s, Δδ = 2.5, H2',6'), 6.55 (1H, s, H8), 6.72 [6.74] (1H, two s, Δδ = 9.2, H5).

$^{13}$C NMR ($CDCl_3$, δ): 22.68 [31.92] (C3), 26.41 [26.64] ($CH_3$), 29.65 [29.69] (C3``), 37.85 [37.91], 38.81 [38.84], 42.58 [42.65], 43.76 [43.78], 45.49 [47.08], 50.31 [50.37] (C2), 50.88 [50.92] (C1''),

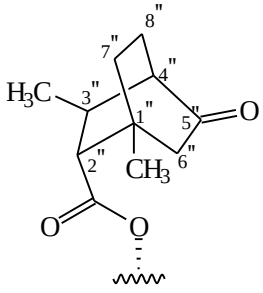
56.04 [56.07] (3',5'-$OCH_3$), 60.76 (4'-$OCH_3$), 71.41 [71.48] (C3a), 73.11 [73.32] (C4), 101.55 ($OCH_2O$), 106.89 [106.97] (C2', C6'), 107.96 [108.00] (C5), 109.69 (C8), 128.48 [128.52] (C4a), 132.28 [132.35] (C8a), 134.78 [134.82] (C4'), 134.73 [135.14] (C6''), 137.04 [137.11] (C1'), 138.41 [138.68] (C5''), 147.56 [147.58] (C6), 148.07 [148.09] (C7), 152.62 [152.65] (C3', C5'), 173.63 (C2a), 177.81 [177.90] (C=O).
MS (MALDI-TOF), *m/z*: 548 $[M]^+$, 571 $[M+Na]^+$, 587 $[M+K]^+$. Calculated for $C_{31}H_{32}O_9$: 548.58.

**4-*O*-{(2*SR*)-Bicyclo[2.2.1]heptan-2-ylcarbonyl}-L-podophyllotoxin 2l** was synthesized by general procedure from *endo*-bicyclo[2.2.1]heptane-2-carboxylic acid (0.022 g, 0.157 mmol), podophyllotoxin **1** (0.050 g, 0.121 mmol) and DCC (0.048 g, 0.233 mmol). Yield (after column chromatography) 58% (0.038 g), white solid, m.p. 128–130°C.




$^1$H NMR ($CDCl_3$, δ, ppm, Δδ, *J*/Hz, signals of the second diastereomer are given in square brackets): 1.18–1.33 (0.5H, m, H5'', H7''), 1.36–1.46 (3.5H, m, H3'', H7''), 1.53–1.75 (4H, m, H7'', H6'', H5''), 2.32 (1H, m, H4''), 2.51 [2.67] (1H, two m, Δδ = 66.1, H1''), 2.78–2.89 (1.5H, m, H2'', H3), 2.93 [2.93] (1.5H, two dd, Δδ = 2.4, *J* = 4.5, 14.6, H2, H3), 3.76 (6H, two s, $OCH_3$), 3.81 [3.81] (3H, two s, Δδ = 2.2, $OCH_3$), 4.21 [4.25] (1H, dd, Δδ = 9.8, *J* = 10.0, 5.8, H3a), 4.38 (1H, dd, *J*= 9.1, 7.5, H3a), 4.60 (1H, d, *J* = 4.5, H1), 5.83 [5.90] (1H, two d, Δδ = 26.0, *J* = 9.1, H4), 5.97 [5.98] (1H, two d, Δδ = 2.9, *J* = 1.3, $OCH_2O$), 5.99 [6.00] (1H, two d, Δδ = 2.9, *J* = 1.3, $OCH_2O$), 6.39 [6.40] (2H, two s, Δδ = 0.6, H2',6'), 6.53 [6.54] (1H, two s, Δδ = 2.4, H8), 6.75 [6.76] (1H, two s, Δδ = 8.0, H5).
$^{13}$C NMR ($CDCl_3$, δ): 24.98 [25.03] (C6``), 29.02 [29.11] (C5``), 31.80 [31.89] (C3``), 36.85 [36.90] (C4``), 38.85 [38.77] (C3), 40.12 [40.13] (C7``), 40.52 [40.55] (C1``), 43.69 (C1), 45.50 [45.55] (C2), 46.02 [46.13] (C2``), 56.03 [56.09] (3',5'-$OCH_3$), 60.70 (4'-$OCH_3$), 71.50 [71.56] (C3a), 73.27 [73.48] (C4), 101.49 [101.52] ($OCH_2O$), 106.68 [107.07] (C5), 107.96 [108.05] (C2', C6'), 109.58 [109.66] (C8), 128.53 [128.57] (C4a), 132.22 [132.26] (C8a), 134.77 [134.84] (C4'), 137.00 [137.07] (C1'), 147.51 (C6), 147.98 [148.01] (C7), 152.56 (C3', C5'), 173.61 [173.68] (C=O), 175.45 (C2a).
MS (MALDI-TOF), *m/z*: 536 $[M]^+$. Calculated for $C_{30}H_{32}O_9$: 536.57.

**4-*O*-{(1*SR*,2*SR*,3*RS*,4*SR*)-1,3-Dimethyl-5-oxobicyclo[2.2.2]octan-2-ylcarbonyl}-L-podophyllotoxin 2g** was synthesized by general procedure from 1,3-dimethyl-5-oxobicyclo[2.2.2]octane-2-carboxylic acid (0.036 g, 0.184 mmol), podophyllotoxin **1** (0.050 g, 0.121 mmol) and DCC (0.037 g, 0.180 mmol). Yield (after chromatography) 52% (0.037 g), white solid, m.p. 130–131°C.




$^1$H NMR ($CDCl_3$, δ, ppm, Δδ, *J*/Hz, signals of the second diastereomer are given in square brackets): 1.10 (3H, s, $CH_3$), 1.12 [1.13] (3H, d, Δδ = 2.4, *J*=6.8, $CH_3$), 1.45–1.55 (2H, m), 1.71–1.80 (1H, m), 1.87 [1.88] (1H, two d, *J*=19.0), 1.95–2.01 (1H, m), 2.12–2.19 (2H, m), 2.25–2.40 (1H, m), 2.73–2.86 (2H, m), 2.91 [2.94] (1H, two dd, Δδ = 2.1, *J*=4.5, 14.6, H2), 3.75 [3.76] (6H, two c, Δδ = 2.9, $OCH_3$), 3.80 [3.81] (3H, two c, Δδ = 1.1, $OCH_3$), 4.21 (1H, dd, *J*=9.7, H3a), 4.31 [4.32] (1H, two dd, Δδ = 2.1, *J*=7.2, 9.3, H3a), 4.59 (1H, d, *J*=4.3, H1), 5.89 [5.91] (1H, two d, Δδ = 11.2, *J*=9.1, H4), 5.98 (1H, d, *J*=1.2, $OCH_2O$), 6.00 (1H, d, *J*=1.2, $OCH_2O$), 6.38 [6.39] (2H, two c, Δδ = 2.1, H2`,6`), 6.54 (1H, s, H8), 6.69 (1H, s, H5).
$^{13}$C NMR ($CDCl_3$, δ): 18.04 [18.25] ($CH_3$), 17.63 [17.67] ($CH_3$), 24.47 [24.51], 32.64 [33.07], 34.31 [34.37], 36.19 [36.33], 38.76 [39.00], 43.71, 45.30, 45.50 [45.53] (C6``), 48.16 [48.23] (C2``), 55.20 [55.38] (C4''), 56.16 (3',5'-$OCH_3$), 60.75 (4'-$OCH_3$), 71.32 [71.35] (C3a), 73.97 [74.09] (C4), 101.64 ($OCH_2O$), 106.73 [106.77] (C5), 108.04 [108.09] (C2', C6'), 109.86 (C8), 127.83 [127.95] (C4a), 132.43 [132.46] (C8a), 134.63 (C4`), 137.20 (C1`), 147.66 (C7), 148.24 (C6), 152.67 [152.69] (C3', C5'), 173.38 (C=O), 175.30 [175.38] (C2a), 214.25 (C5``).
MS (MALDI-TOF), *m/z*: 593 $[M]^+$. Calculated for $C_{33}H_{36}O_{10}$: 592.63.

**4-*O*-(3-Bromoadamantan-1-ylacetyl)-L-podophyllotoxin 2d** was synthesized by general procedure from 3-bromoadamantane-1-acetic acid (0.040 g, 0.146 mmol), podophyllotoxin **1** (0.046 g, 0.111 mmol) and DCC (0.046 g, 0.223 mmol). Yield (after column chromatography) 52.5% (0.039 g), white solid, m.p. 202–203°C.
$^1$H NMR ($CDCl_3$, δ, ppm, Δδ, *J*/Hz): 1.66–1.70 (6H, m, Ad), 2.19–2.32 (10H, m, Ad+C$H_2$COO), 2.77–2.87 (1H, m, H3), 2.93 (1H, dd, H2, *J*=4.3, 14.5), 3.76 (6H, c, $OCH_3$), 3.81 (3H, c, $OCH_3$), 4.22 (1H, m, H3a), 4.37 (1H, dd, H3a, *J*=7.0, 9.4), 4.61 (1H, d, H1, *J*=4.7 Hz), 5.89 (1H, d, H4, *J*=9.4), 5.99 (1H, d, *J*=1.2 Hz, $OCH_2O$), 6.0 (1H, d, *J*=1.2 Hz, $OCH_2O$), 6.39 (2H, c, H2',6'), 6.54 (1 H, c, H8), 6.77 (1 H, c, H5).
$^{13}$C NMR ($CDCl_3$, δ): 32.12, 34.54, 37.35, 38.77, 40.22, 40.48, 43.68 (C1), 45.57 (C2), 47.48 (C10), 48.12, 53.63, 56.13 (3',5'-$OCH_3$), 60.72 (4'-$OCH_3$), 64.12 (CBr), 71.41 (C3a), 73.59 (C4), 101.59 ($OCH_2O$), 107.05 (C5), 108.05 (C2', C6'), 109.72 (C8), 128.15 (C4a), 132.36, 134.73, 137.14, 147.58 (C6), 148.13 (C7), 152.62 (C3', C5'), 171.39 (C=O), 173.52 (C2a).
MS (MALDI-TOF), *m/z*: 668 $[M]^+$. Calculated for $C_{34}H_{37}O_9Br$: 669.56.

**4-*O*-(Cyclohexylacetyl)-L-podophyllotoxin 2e** was synthesized by general procedure from cyclohexylacetic acid (0.022 g, 0.154 mmol), podophyllotoxin **1** (0.050 g, 0.121 mmol) and DCC (0.040 g, 0.194 mmol). Yield (after chromatography) 57% (0.037 g), white solid, m.p. 101–103°C.
$^1$H NMR ($CDCl_3$, δ, ppm, Δδ, *J*/Hz): 0.99–1.05 (2H, m), 1.14–1.30 (3H, m), 1.66–1.75 (5H, m), 1.79–1.84 (1H, m), 2.30–2.32 (2H, d, *J*=7.0, C$H_2C_6H_{11}$), 2.82 (1H, m, H3), 2.93 (1H, dd, *J*=4.3, 14.5, H2), 3.76 (6H, s, $OCH_3$), 3.81 (3H, s, $OCH_3$), 4.22 (1H, dd, *J*=9.8, H3a), 4.36 (1H, dd, *J*=7.4, 9.0, H3a), 4.60 (1H, d, *J*=4.3, H1), 5.88 (1H, d, *J*=9.0, H4), 5.98 (1H, s, $OCH_2O$), 6.00 (1H, s, $OCH_2O$), 6.39 (2H, c, H2`,6`), 6.54 (1H, s, H8), 6.75 (1H, s, H5).
$^{13}$C NMR ($CDCl_3$, δ): 25.91, 25.98, 33.00, 34.98, 38.79, 42.12, 43.70, 45.53, 56.09 (3',5'-$OCH_3$), 60.71 (4'-$OCH_3$), 71.42 (C3a), 73.31 (C4), 101.54 ($OCH_2O$), 106.98 (C5), 108.05 (C2', C6'), 109.66 (C8), 128.38 (C4a), 132.38, 134.79, 137.09, 147.52 (C6), 148.05 (C7), 152.59 (C3', C5'), 173.49 (COO), 173.61 (C2a).
MS (MALDI-TOF), *m/z*: 538 $[M]^+$. Calculated for $C_{30}H_{34}O_9$: 538.59.
Anal. calcd. for, %: $C_{30}H_{34}O_9$, %: C 66.90; H 6.36. Found, %: C 66.64; H 6.38.

## 3. Biological tests

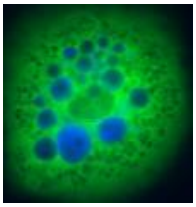
**Cell culture**. A549 human lung epithelial carcinoma cells (CCL-185™) were cultured with Dulbecco´s Modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic penicillin/streptomycin at 37 °C under a 5% $CO_2$ humidified atmosphere.

***MTT Cytotoxicity Assay.*** The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide, Roth GmbH, Karlsruhe, Germany) quantitative colorimetric assay was used to measure the cytotoxicity, viability and metabolic activity [S10]. The A549 cells were seeded in 96-well plates at a density of 8000 cells per well. Stock solutions of test compounds were prepared in DMSO at concentration 20 mM. Cells were treated with selected compounds at 1–50000 nM (8 wells for each concentration) for 24 h. DMSO (0.5%) was used as a negative control. Optical density was measured at 550 nm with 690 nm reference filter using a EL808 Ultra Microplate Reader (BioTek Instruments, Winooski, USA). Experiments for all compounds were repeated 3–6 times and $EC_{50}$ values were determined by sigmoid curve fitting using Excel-based software.

***Proliferation assay.*** A549 cells were incubated with 2–10000 nM of each tested compound during 24 and 48 hours. 0.5% DMSO was used as a control. After culturing the cells were re-suspended in PBS and counted directly by phase-contract microscopy using hemocytometer.

***Immunofluorescence staining of cellular microtubules and nuclei***. A549 Cells were cultured in 12-well plates on small glass coverslips (11 mm diameter) at a density of 20000 cells per coverslip. Cells were incubated with tested compounds at concentrations of 2, 10 and 100 μM for 24 and 48 hours; 0.5 % DMSO served as a negative control. The cells were fixed and stained as described in [S11]. Fixed cells were labelled for tubulin with mouse monoclonal antibody against α-tubulin at a dilution of 1:300 (Sigma, St. Louis. USA), followed by incubation of Alexa Fluor488 labelled goat anti-mouse IgG at a dilution of 1:300 (Invitrogen, Germany). In order to analyze the compound effect on the apoptosis induction effect, the cell nuclei were stained with the highest no. 33258 (Sigma, St. Louis. USA) at concentration 5 μg $ml^{-1}$. Images of all samples were acquired with a Nikon Diaphot 300 inverted microscope (Nikon GmbH, Düsseldorf, Germany) equipped with a cooled charge-couple device camera system (SenSys; Photometrics, Munich, Germany).

***Apoptotic index*.** Apoptotic index was defined as a percentage that refers to the indicated cell deaths in comparison to all cells [S12]. A549 cells were incubated with 2 μM of each tested compound during 24 and 48 hours. 0.5% DMSO was used as a control. After incubation the cells were fixed and stained with Hoechst. Since a main feature of apoptotic cell is a fragmentation of the nucleus [S13], the number of cells containing micronuclei (see example for cpd. **2e**, after 24 h at the picture) related the total 200 cells was counted using fluorescent microscopy.



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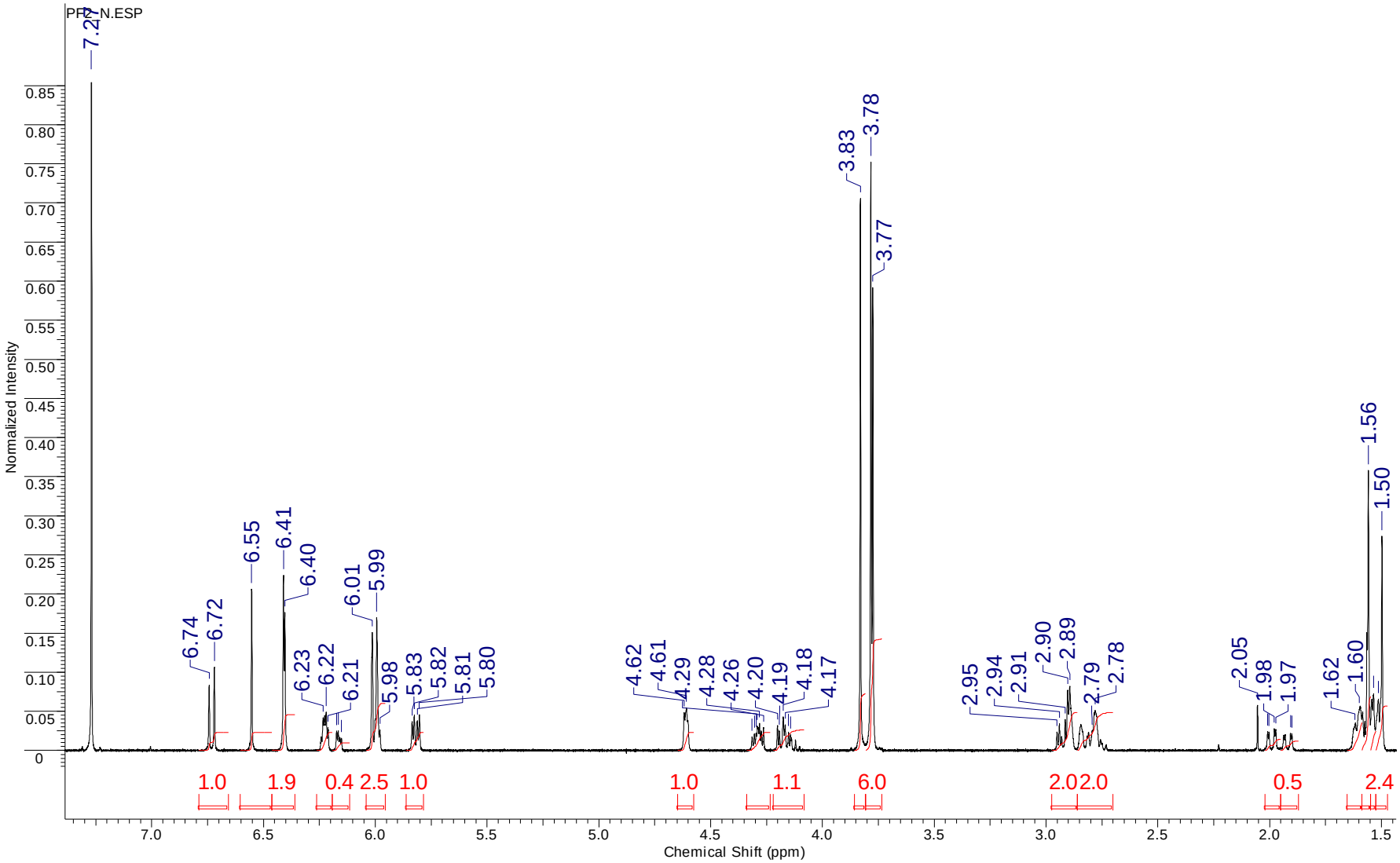
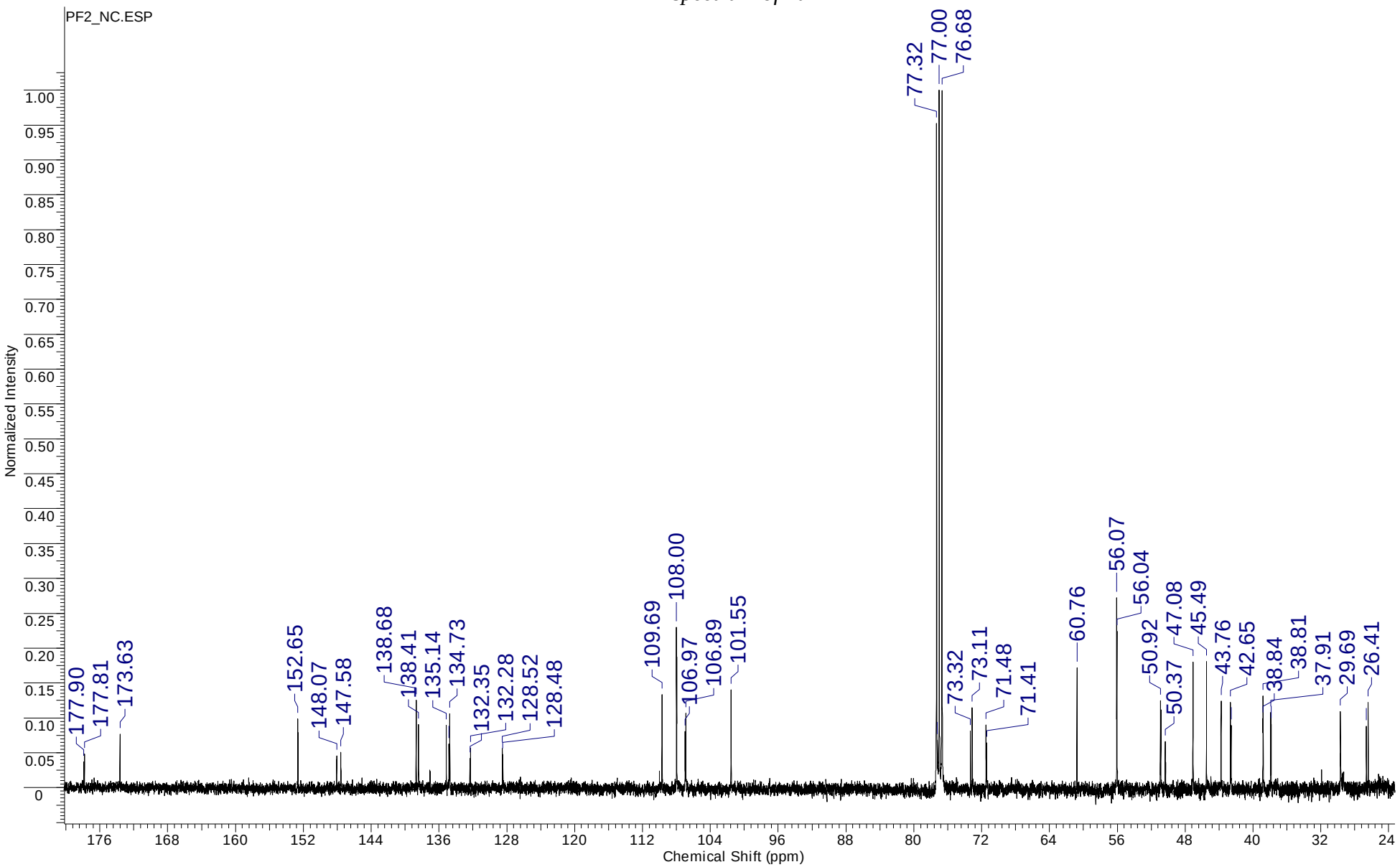
## $^{1}$H and $^{13}$C NMR spectra of representative compounds

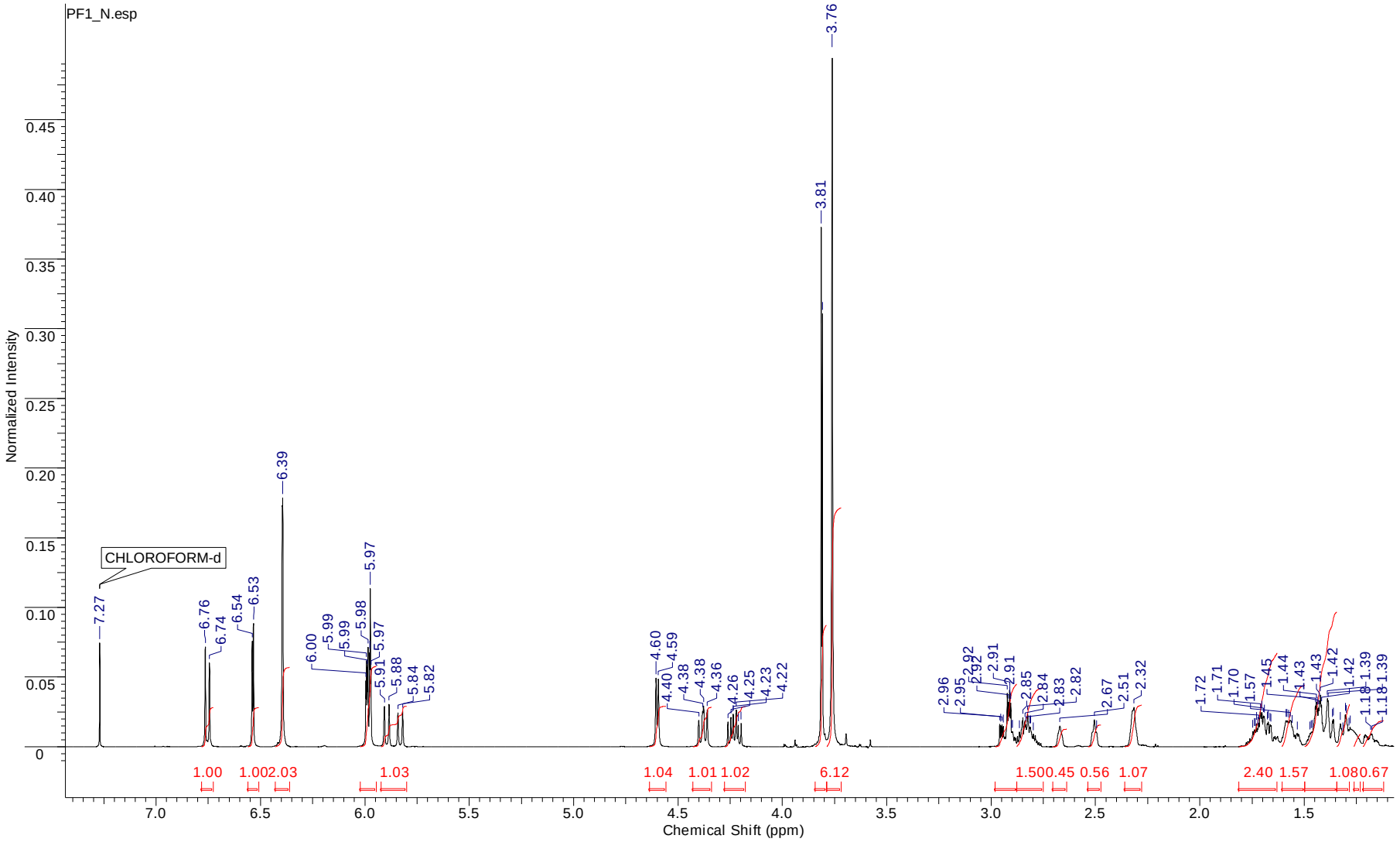
*$^{1}$H NMR spectrum of **2a***



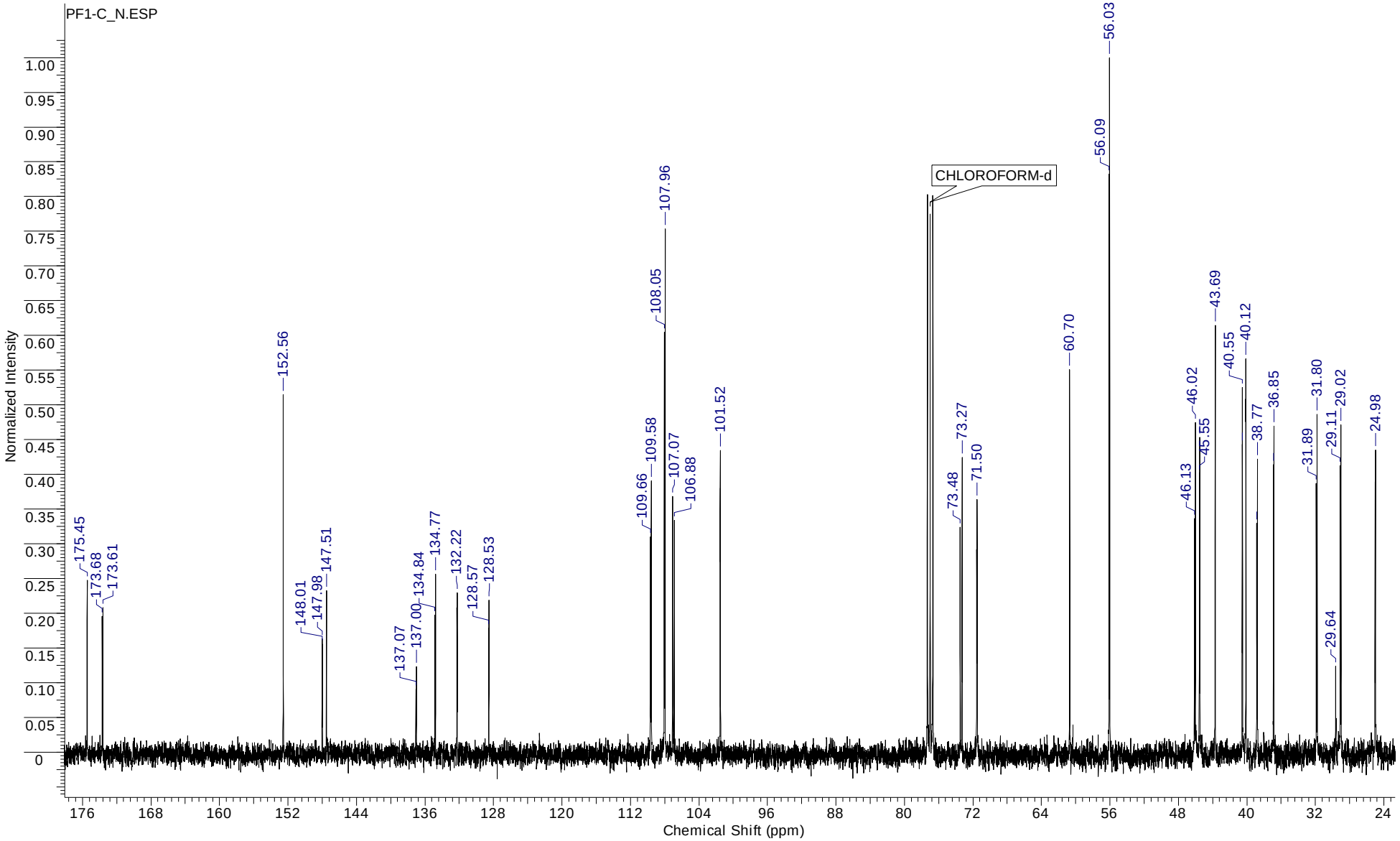
*$^{1}H$ NMR spectrum of **2a***



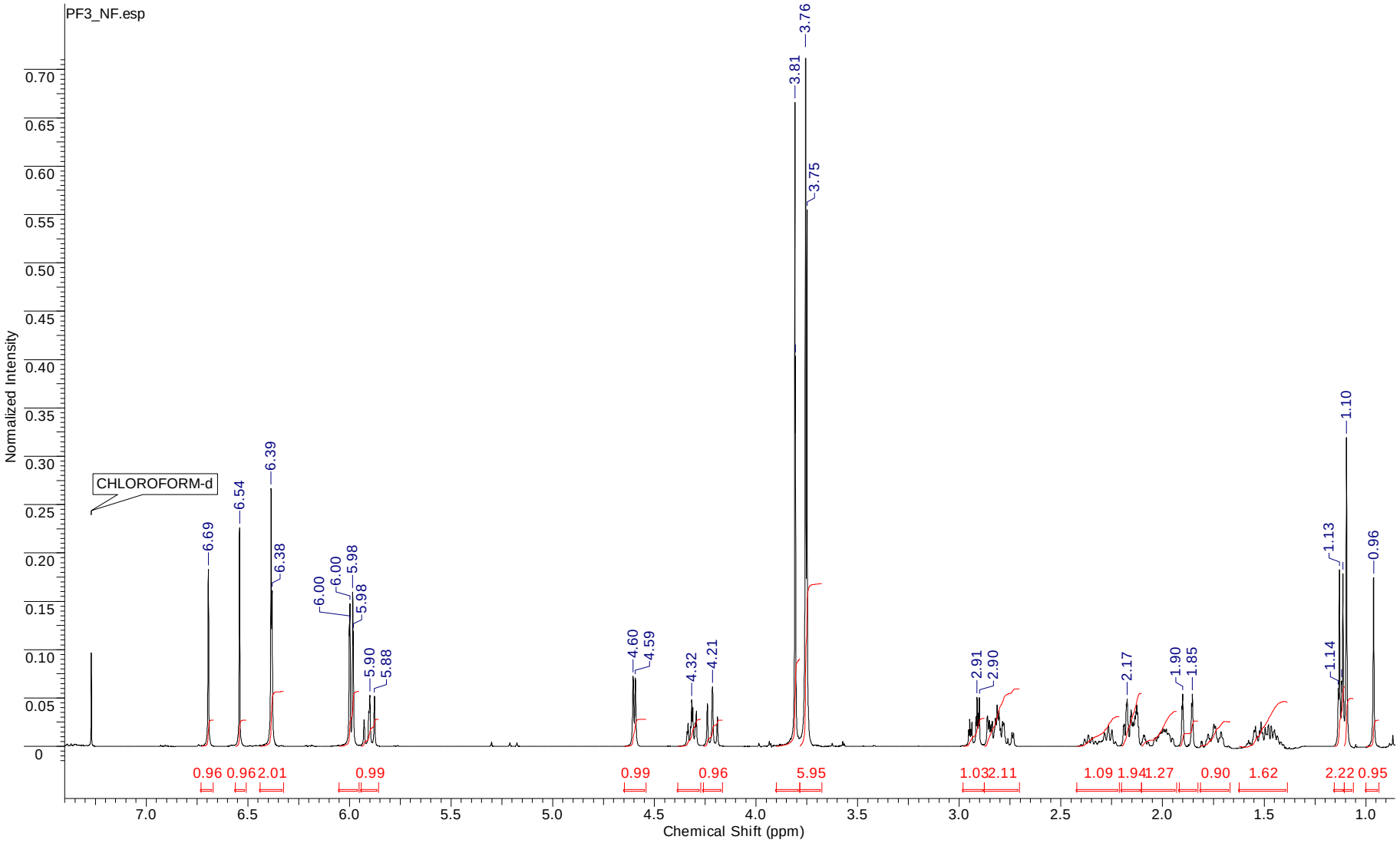
*$^{1}H$ NMR spectrum of **2b***



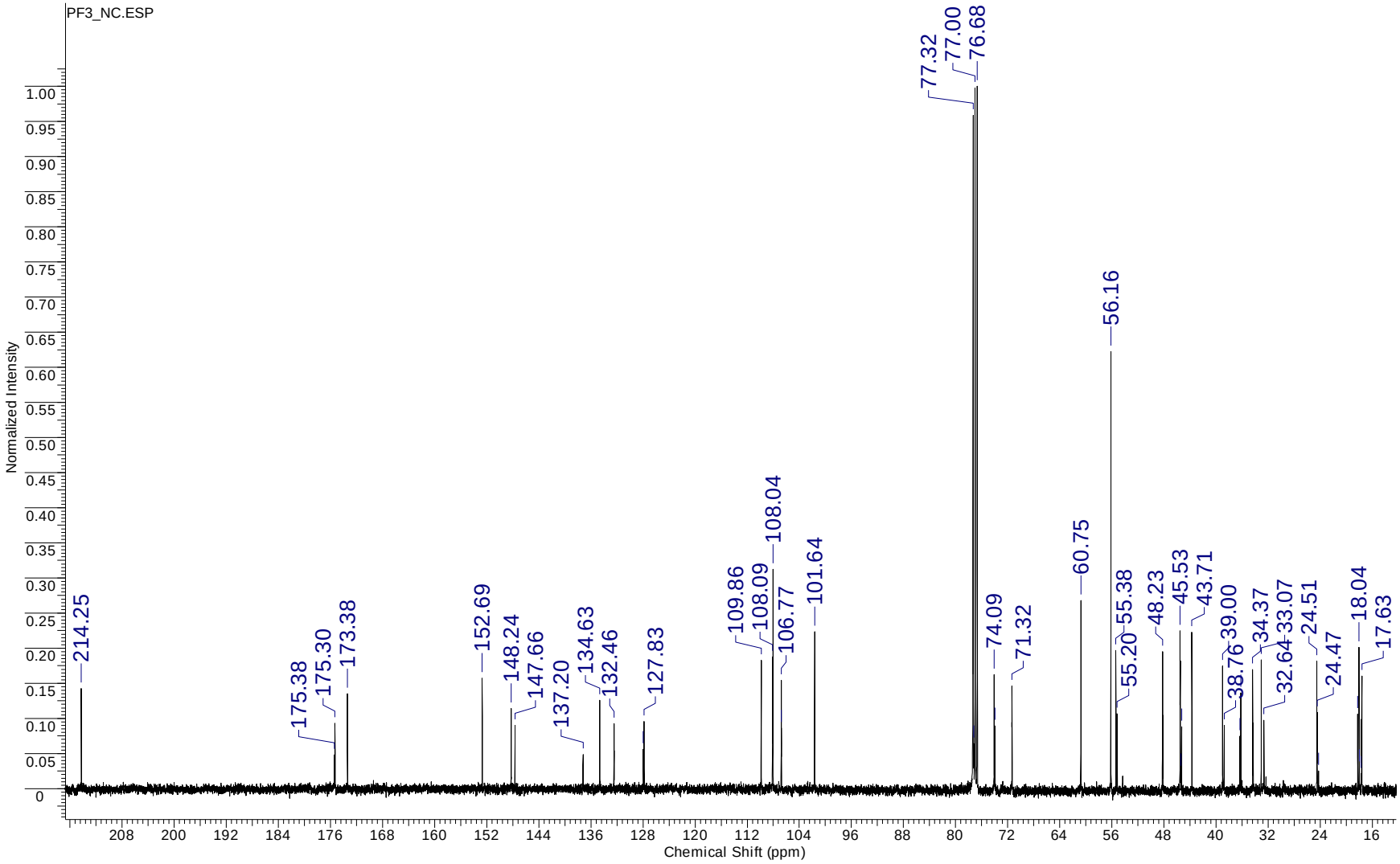
*$^{13}C$ NMR spectrum of **2b***



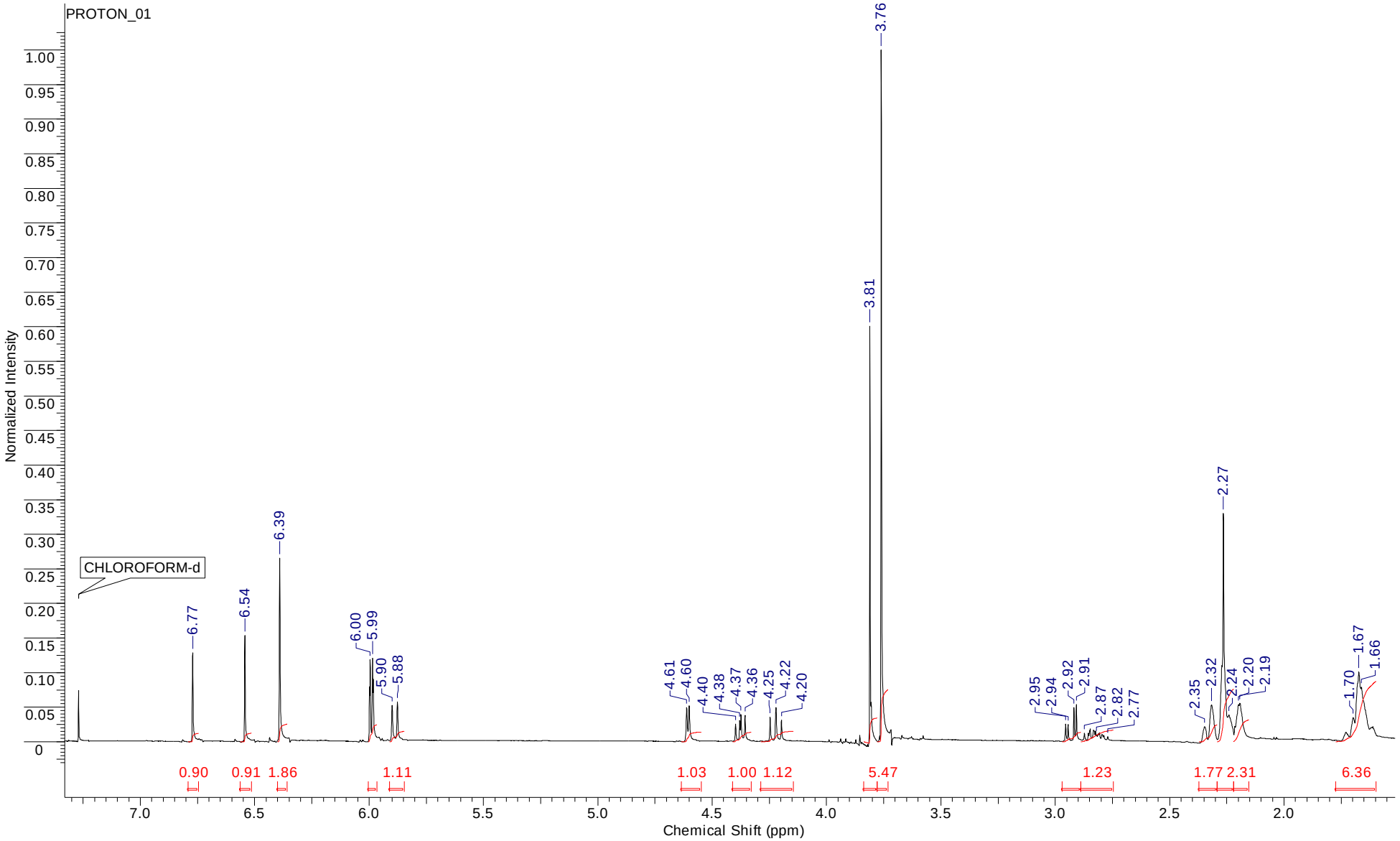
*$^{1}H$ NMR spectrum of **2c***



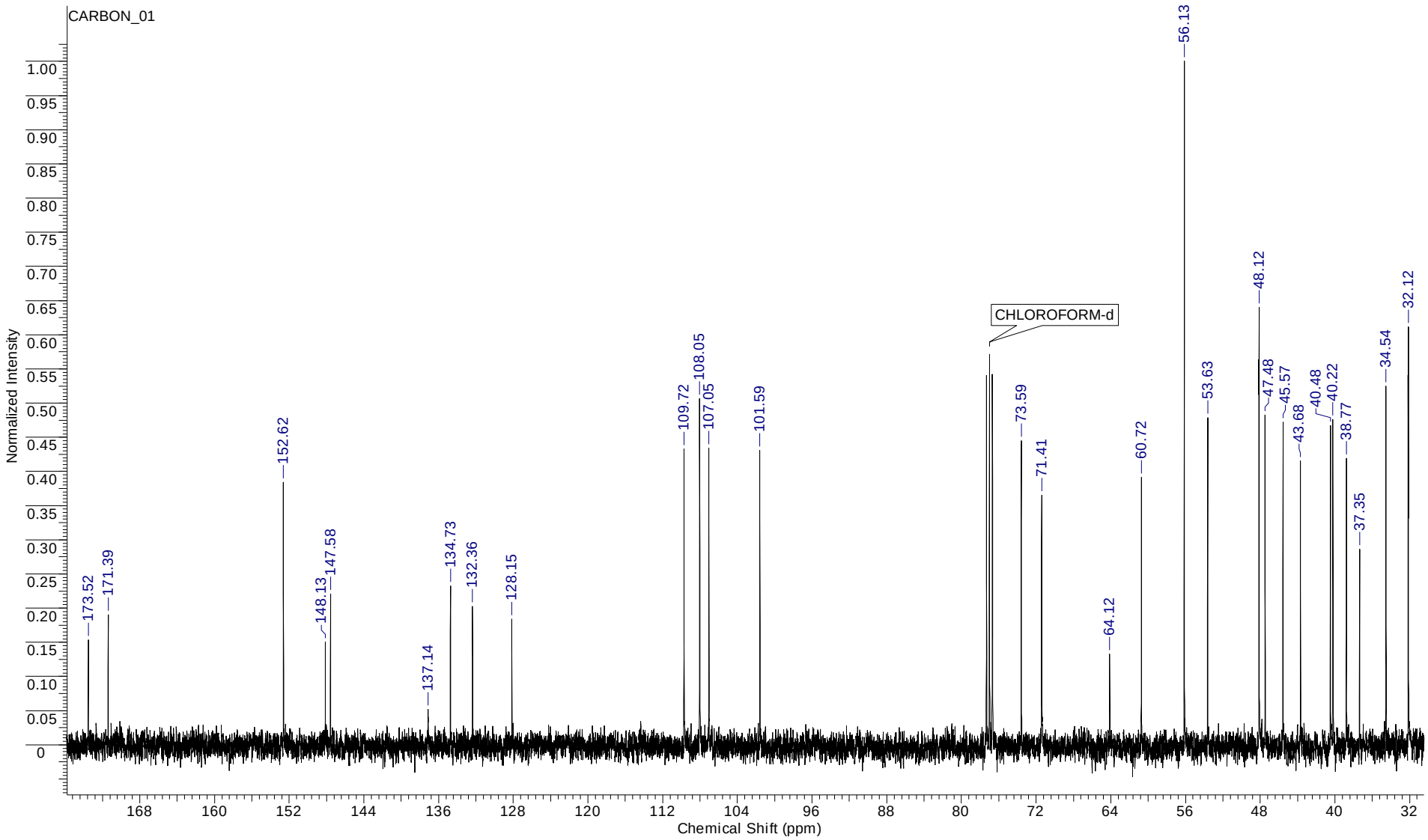
*$^{13}C$ NMR spectrum of **2c***



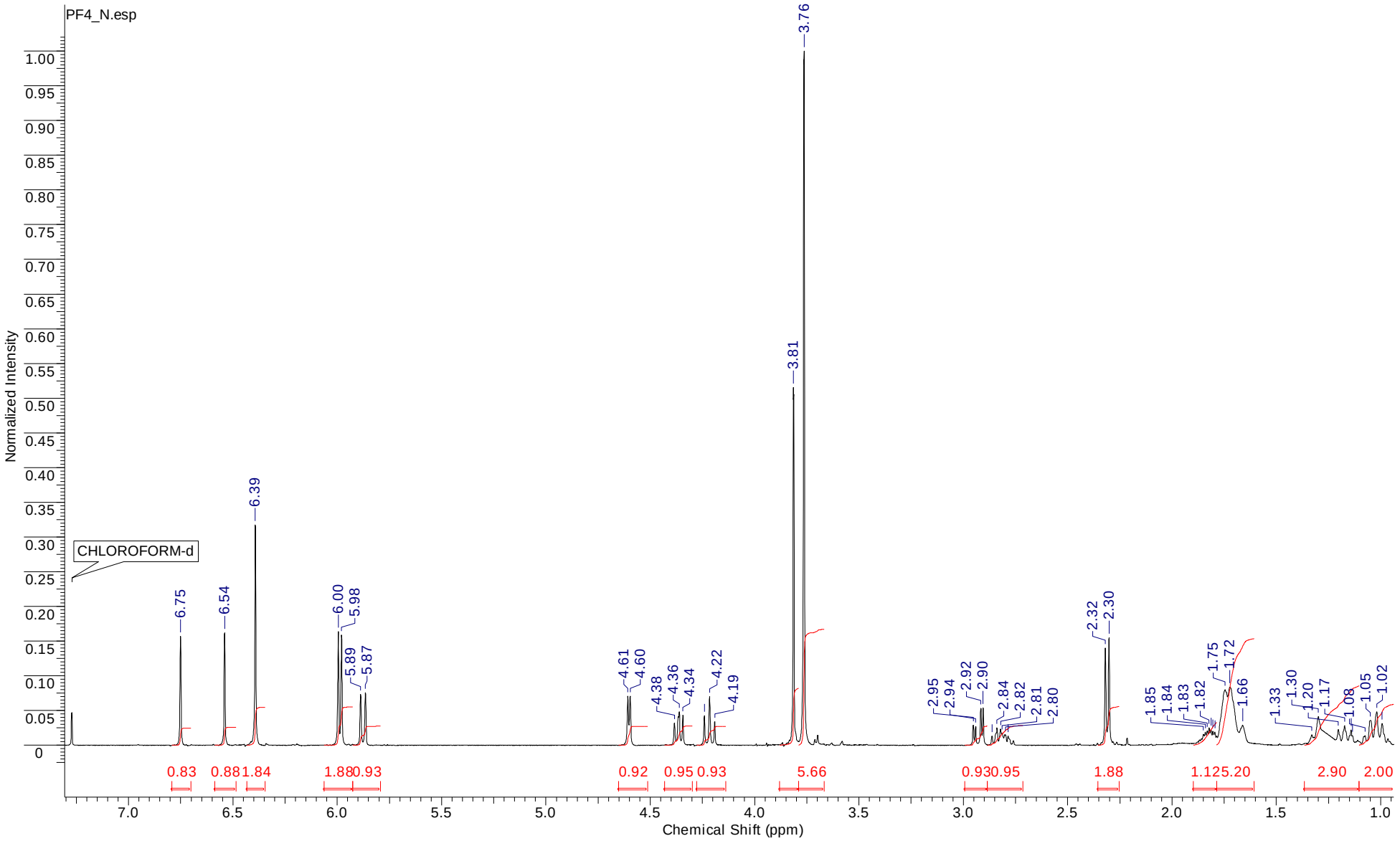
*$^{1}H$ NMR spectrum of **2d***



*$^{13}$C NMR spectrum of **2d***




*$^{1}$H NMR spectrum of **2e***


PF4_N.esp
Normalized Intensity
Chemical Shift (ppm)
CHLOROFORM-d
6.75
6.54
6.39
6.00
5.98
5.89
5.87
4.61
4.60
4.38
4.36
4.34
4.22
4.19
3.81
3.76
2.95
2.94
2.92
2.90
2.84
2.82
2.81
2.80
2.32
2.30
1.85
1.84
1.83
1.82
1.75
1.72
1.66
1.33
1.30
1.20
1.17
1.08
1.05
1.02
0.83
0.88
1.84
1.88
0.93
0.92
0.95
0.93
5.66
0.93
0.95
1.88
1.12
5.20
2.90
2.00

*$^{13}C$ NMR spectrum of **2e***