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Synthesis and cytotoxicity of 7,8-dihalopyrido[1,2-*a*]benzimidazole-6,9-dione and its 1,2,3,4-tetrahydro analogue

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1. Experimental Procedures and Analytical Data

The melting points were determined with a Poly Therm A instrument with a heating rate of 3 °C and were not corrected. ^{1}H NMR spectra were recorded on a Bruker DRX500 instrument at a frequency of 500 MHz. ^{13}C NMR spectra were recorded at a frequency of 125 MHz using DMSO-d_6 as the solvent and internal standard. High-resolution mass spectra were recorded on a Bruker micrOTOF II instrument (Bruker Daltonics) with the electrospray ionization method (ESI) and MeCN as the solvent. The reduction of nitro hetarenes was carried out in a H-CUBE Pro reactor for flow hydrogenation (ThalesNano Nanotechnology Inc, Hungary). 1-(2-Nitro-4-chlorophenyl)pyridinium chloride **1** the chloride was obtained as reported [S1].

Method for the synthesis of 8-bromo-7-chloropyrido[1,2-*a*]benzimidazole-6,9-dione 6a and 8-bromo-7-chloro-1,2,3,4-tetrahydropyrido[1,2-*a*]benzimidazole-6,9-dione 6b

7-Chloropyrido[1,2-*a*]benzimidazole 2. To a solution of 1-(2-nitro-4-chlorophenyl)pyridinium chloride **1** (10 g, 39.6 mmol) in 90% propan-2-ol (90% aq., 100 ml) at 40 ºC was added a solution of $SnCl_2 \cdot H_2O$ (18.5 g, 81.2 mmol) in HCl (4% aq., 100 ml). After 5 min, the reaction mixture was treated with NH_4OH till pH 7–8 and extracted with several portions of hot chloroform (Σ = 300 ml). After distillation of chloroform, compound **2** was obtained. Yield 7.3 g (97%), mp 212–214°C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 7.03 t.d (1H, H^2, J_1 = 6.8, J_2 = 0.9 Hz), 7.37 d.d (1H, H^4, J_1 = 8.7, J_2 = 1.9 Hz), 7.60 d.d.d (1H, H^3, J_1 = 9.2, J_2 = 6.6, J_3 = 1.1 Hz), 7.68 d (1H, H^9, J = 9.2 Hz), 7.83 d (1H, H^6, J = 1.8 Hz), 8.33 d (1H, H^8, J = 8.7 Hz), 9.08 d (1H, H^1, J = 6.9 Hz). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 110.78, 113.38, 116.85, 118.07, 120.52, 127.19, 127.44, 129.70, 130.82, 144.74, 148.82. ESI-HRMS: *m/z* calcd for $C_{11}H_8ClN_2$ $[M^+ + H]^+$ 203.0377, found 203.0371

8-Bromo-7-chloropyrido[1,2-*a*]benzimidazole 3. A solution of NBS (6.8 g, 38.1 mmol) in conc. H_2SO_4 (50 ml) was slowly added dropwise at 30 ºC to a solution of 7-chloropyrido[1,2-*a*]-benzimidazole (7 g, 34.6 mmol) **2** in conc. H_2SO_4 (50 ml), and the mixture was stirred for 8 h. The reaction mixture was poured into water and neutralized with NH_4OH. The precipitate was filtered, washed on the filter with water (3×100 ml) and dried. Yield 9.05 g (93%), mp 268-270 °C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 9.10 (d, J = 6.9 Hz, 1H, H^1), 8.88 (s, 1H, H^9), 8.07 (s, 1H, H^6), 7.67 (d, J = 9.3 Hz, 1H, H^4), 7.63 (ddd, J_1 = 9.3 Hz, J_2 = 6.5 Hz, J_3 = 1.2 Hz, 1H, H^3), 7.07 (td, J_1 = 6.8 Hz, J_2 = 1.2 Hz, 1H, H^2). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 149.3 (C^{4a}), 143.9 (C^{5a}), 131.5 (C^3), 129.5 (C^7), 128.5 (C^{9a}), 127.5 (C^1), 119.7 (C^6), 117.1 (C^9), 117.0 (C^4), 112.0 (C^8), 111.2 (C^2). ESI-HRMS: *m/z* calcd for $C_{11}H_7BrClN_2$ $[M+H]^+$ 282.9451, found 282.9454.

8-Bromo-7-chloro-6-nitropyrido[1,2-*a*]benzimidazole 4.

To a solution of substrate **3** (9 g, 32 mmol) in conc. H_2SO_4 (80 ml), a solution of KNO_3 (3.55 g, 35.2 mmol) in conc. H_2SO_4 (30 ml) was added dropwise at 35°C within 0.5 h. The reaction mixture was stirred for 1.5 h and then poured onto ice and neutralized with NH_4OH. The precipitate that formed was filtered off, washed with several portions of water on the filter, and dried. Yield 9.8 g (94%), mp 268–271°C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 7.20 m (1H, H^2), 7.78 m (2H, $H^{3,4}$), 9.16 s (1H, H^9), 9.19 d (1H, H^1, J = 6.9 Hz). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 112.2; 113.1; 117.6; 120.3; 121.2; 128.5; 130.8; 134.2; 136.8; 139.0; 151.2. ESI-HRMS: *m/z* calcd for $C_{11}H_6BrClN_3O_2$ $[M+H]^+$ 325.9333. found 325.9331

8-Bromo-7-chloropyrido[1,2-*a*]benzimidazol-6-amine 5a. The cartridge CatCard THS 04115 containing 1% Pd/C catalyst was placed in the H-Cube Pro reactor, and using the pump the flow rate of the solvent of 1 ml min^{-1} was adjusted. Propan-2-ol was pumped through the reactor for 5 min to remove the air from the system. The substrate solution was prepared by the dissolution of nitro hetarene **4** (2 g, 6.1 mmol) in Pr^iOH (100 ml). The temperature of 60 °C and pressure of 20 bar were set on the H-Cube device. When stable conditions were established in the reactor, the inlet system was switched from the solvent to the substrate, and the substance was passed through the catalyst. After collecting the entire solution, the inlet valve was switched back to the solvent and the system was rinsed for 10 min. The reaction solution was passed through a column with activated carbon, and the most of the solvent was evaporated. After cooling, the formed precipitate was filtered off. Yield 1.62 g (89%), mp 230-232°C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 6.00 s (2H, NH_2), 6.99 t (1H, H^2, J = 6.7 Hz), 7.52 m (1H, H^3), 7.65 d (1H, H^4, J = 9.3 Hz), 7.99 s (1H, H^9), 8.94 d (1H, H^1, J = 6.9 Hz). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 103.8; 109.9; 111.5; 114.5; 117.5; 127.5; 127.8; 130.2; 133.1; 138.2; 147.2. ESI-HRMS: *m/z* calcd for $C_{11}H_8BrClN_3$ $[M+H]^+$ 295.9591, found 295.9588

8-Bromo-7-chloro-1,2,3,4-tetrahydropyrido[1,2-*a*]benzimidazol-6-amine 5b. The reduction of the nitro compound **4** was carried out at 80°C, similarly to the synthesis of amino hetarene **5a**, using a cartridge containing a 10% Pd/C catalyst (cartridge CatCard THS 01111). Yield 1.55 g (84%), mp 210–213°C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 1.87 m (2H, $H^{3,3}$ piperidine), 1.98 m (2H, $H^{2,2}$ piperidine), 2.94 t (2H, $H^{4,4}$ piperidine, J = 6.2 Hz), 3.97 t (2H, $H^{1,1}$ piperidine, J = 6.1 Hz), 5.61 (s, 2H, NH_2), 7.11 (s, 2H, H^9). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 20.7, 22.6, 25.4, 43.0, 102.5, 108.4, 115.1, 130.9, 134.2, 137.6, 150.9. ESI-HRMS: *m/z* calcd for $C_{11}H_{12}BrClN_3$ $[M+H]^+$ 299.9904, found 299.9896

Oxidation of heterocyclic amines 5a,b to *p*-quinones 6a,b.

A solution of aminoarene **5a** or **5b** (5 mmol) in conc. H_2SO_4 (30 ml) was added to a solution of KNO_3 (4 g, 40 mmol) in conc. H_2SO_4 (30 ml). The reaction mixture was stirred at room temperature for 9 h, after which it was poured into ice and neutralized with NH_4OH. The precipitate was filtered off and washed with water.

8-Bromo-7-chloropyrido[1,2-*a*]benzimidazole-6,9-dione 6a. Yield 1.36 g (86%), mp 274–278°C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 7.47 t.d (1H, H^2, J_1 = 6.9, J_2 = 0.9 Hz), 7.80 m (1H, H^3), 8.02 d (1H, H^4, J = 9.1 Hz), 9.14 d (1H, H^1, J = 6.8 Hz). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 118.8; 119.8; 122.2; 128.4; 132.3; 137.6; 143.3; 144.3; 148.6; 167.7; 172.4. ESI-HRMS: *m/z* calcd for $C_{11}H_5BrClN_2O_2$ $[M+H]^+$ 310.9224, found 310.9221

8-Bromo-7-chloro-1,2,3,4-tetrahydropyrido[1,2-*a*]benzimidazole-6,9-dione 6b. Yield 1.39 g (88%), mp 202–205°C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 1.95–2.02 m (2H, H^{3,3}), 2.03–2.08 m (2H, H2^{,2}), 3.01 t (2H, H^{4,4}, J = 6.3 Hz), 4.29 t (2H, H^{1,1}, J = 6.2 Hz). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 19.6, 22.1, 25.0 (CH_2), 46.0 (NCH_2), 130.2, 135.6, 140.7, 143.5, 153.5, 168.8 (C=O), 171.2 (C=O). ESI-HRMS: *m/z* calcd for $C_{11}H_9BrClN_2O_2$ $[M+H]^+$ 314.9537, found 314.9531

2. Biological Assay Procedures and Results

2.1 Cell lines and cultivation

Human cell cultures of tumour origin A549 (lung adenocarcinoma cells), SH-SY5Y (neuroblastoma), Hep-2 (epidermoid carcinoma of the larynx), HeLa (cervical cancer) and MCF-7 (breast cancer) and normal origin HaCaT (keratinocyte cell line from adult human skin) provided by the Laboratory of Tumor Cell Genetics at the N. N. Blokhin Russian Cancer Research Institute, as well as by the Institute of Cytology of the Russian Academy of Sciences, were grown in DMEM (Gibco, Scotland, UK) containing fetal bovine serum (10% by volume) (ThermoFisher Scientific, Paisley, UK), L-glutamine (2 mM) (Gibco, Scotland, UK), and penicillin–streptomycin (1% by volume) (PanEco, Moscow, Russia). The cultivation was carried out at 37 °C in a humidified CO_2 atmosphere (5%).

2.2 Determination of cell viability

Cellular viability was determined by the MTT test [S2], as described in the paper [S3]. The method is based on the ability of living cells to restore MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble formazan, thus the amount of formazan produced reflects cell viability. Each cell line was seeded into 96-well plates ($1x10^4$ cells per well) and cultured for 24 h at 37 °C in a CO_2 atmosphere (5%). Incubation with the substances and Tamoxifen at selected concentrations (0.1–100 μM) was carried out for 24 h, then MTT solution (5 mg ml^{-1} in 0.9% NaCl) was added to each well, the cells were incubated for 2 h at 37°C. After the removal of culture medium, dimethyl sulfoxide was added into each well to dissolve the formazan. Using an EnVision plate analyzer (Perkin Elmer), the absorbance was determined at 540 nm. The concentration value causing 50% inhibition of cell population growth (IC_{50}) was determined from dose-response curves using GraphPad Prism 9.0 software. The selectivity index (SI) can be defined as the ratio of the toxic concentration of a sample to its effective bioactive concentration.

2.3 Rat Brain Homogenate and Liver Mitochondria

The experiments used male nonlinear rats weighing 200-220 g. All manipulations with animals were carried out in accordance with the decisions of the IPAC RAS Bioethics Commission.

To obtain a brain homogenate, decapitation of rats anesthetized in advance with CO_2 was performed using a guillotine. The brain was homogenized in a buffer containing KCl (120 mM) and HEPES (20 mM), pH = 7.4, at 4°C and centrifuged at 1500*g* to obtain a supernatant.

Rat liver mitochondria were isolated by the standard method of differential centrifugation using buffers of different composition. Rats were anesthetized with CO_2. The rats were anesthetized with CO_2, the abdominal cavity was opened. The liver was removed and placed in a beaker with ice-cold isolation buffer (mannitol 225 mM, sucrose 75 mM, HEPES 10 mM, EGTA 0.5 mM, EDTA·2K 0.5 mM, pH = 7.4). The liver was minced and washed with the same buffer. The resulting fraction was passed through a press, suspended in isolation buffer, homogenized in a Potter homogenizer (teflon: glass) at 900*g*. The resulting homogenate was centrifuged at 1500g. The fat-free supernatant was re-centrifuged at 10570*g*. The resulting pellet was resuspended in a small volume of buffer (mannitol 225 mM, sucrose 75 mM, HEPES 10 mM, EGTA 20 μM, pH = 7.4). The standard mitochondrial fraction contained from 80 to 100 mg ml^{-1} of protein in the sediment on the liver. The mitochondria were stored at 4°C until the future experimental procedures. Functional activity of the rat liver mitochondria remained constant during the next 4 hours.

The quantitative determination of protein was carried out according to the standard technique using the microbiuret method [S4]. The method is based on the reaction of proteins with copper sulfate in an alkaline medium. The final product of the reaction is blue-violet or red-violet complex compounds. The color intensity is proportional to the protein content in the solution. Biuret reagent (75 mg ml^{-1}) and sodium deoxycholate (0.5%) were added to all samples, mixed and left at 37°C for 15 min for color development. The stained solutions were colorimetric on a plate analyzer Victor 3 (Perkin Elmer, Germany) at 530 nm. The obtained values of optical density were used to calculate the protein in the studied samples.

2.4 TBA test

To study the effect of compounds on the process of lipid peroxidation (LPO) of rat brain homogenate we used a modified version of the TBA test according to the experimental scheme described in [S5]. The method is based on the reaction of 2-thiobarbituric acid with malonodialdehyde. The test compounds of the required concentration and the rat brain homogenate (2 mg ml^{-1}) were added to the plate according to the scheme. The prepared samples were incubated for 30 min at 37°C. After incubation, a reagent for TBA reactive products (thiobartituric and trichloroacetic acids) was added to each sample, shaken, and incubated for 90 min at 90°C. The plate was centrifuged at 5000*g*. The optical density of the supernatant was measured on a plate analyzer Victor 3 (Perkin Elmer, Germany) at 530 nm minus the measured background absorbance at 620 nm.

2.5. Membrane potential of mitochondria

The transmembrane potential of rat liver mitochondria was measured using a potential-dependent indicator Safranin A [S6] at λ_{ex} = 485 nm, λ_{em} = 590 nm. The mitochondrial preparation was diluted in a buffer (mannitol - 225 mM, sucrose - 75 mM, HEPES - 10 mM, EGTA - 20 μM, pH = 7.4) at the rate of 0.5 mg of protein in 1 ml of mitochondrial buffer liver. Immediately before the start of the measurement, safranin A was added to the suspension at a final concentration of 5 μM. The plate with the studied samples was placed in a plate analyzer Victor 3 (Perkin Elmer, Germany). Mitochondria were energized by adding 5 mM potassium succinate and 1 μM rotenone to all samples. The test compounds were added against the background of energization. Induction mPTP was performed by adding 37 μM $CaCl_2$ to all samples.

3. References:

[S1] B. Lipke, *Z. Chem.*, 1970, **10**, 463. DOI:10.1002/zfch.19700101207

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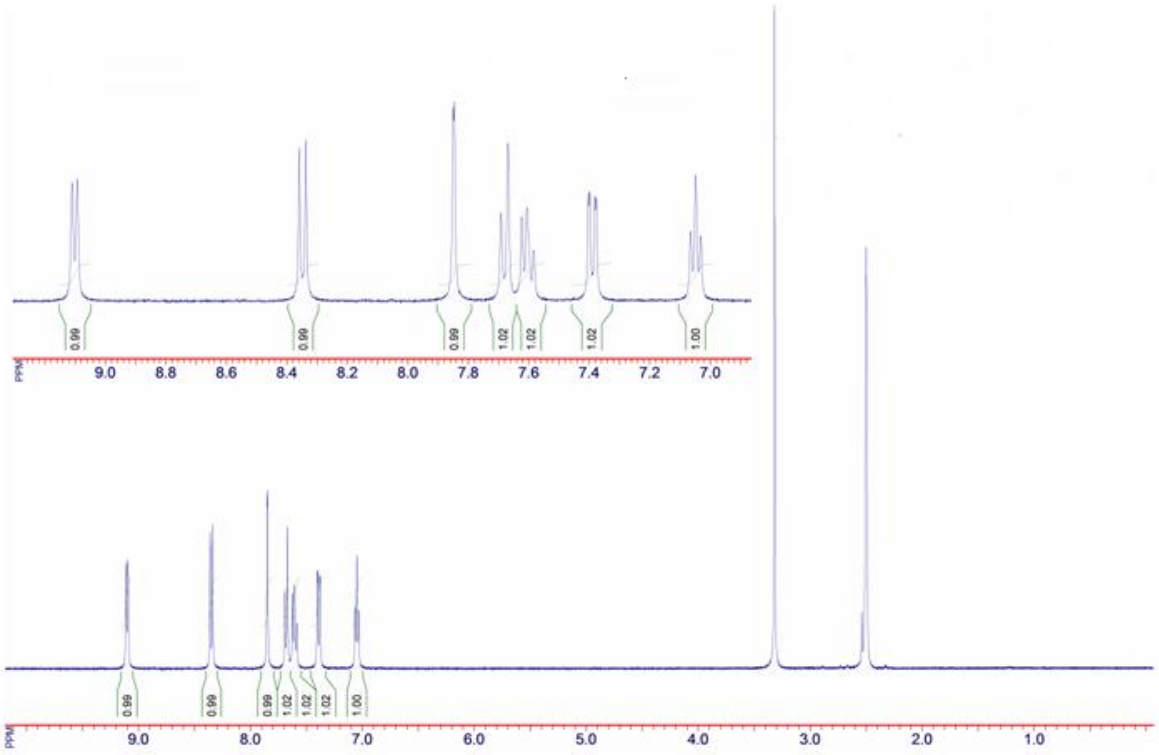
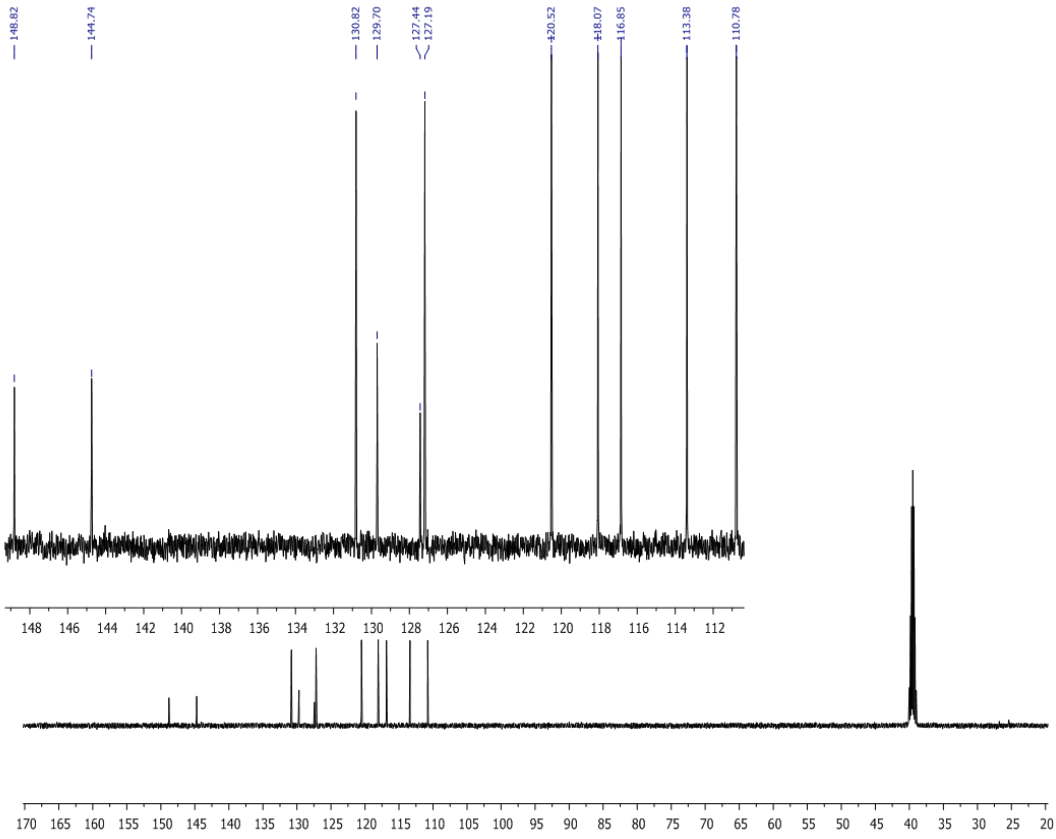
[S6] K. E. O. Åkerman and M. K. F. Wikstrom, *FEBS Lett.*, 1976, **68**, 191.

4. NMR Spectral Data



^{1}H NMR spectrum of 7-chloropyrido[1,2-*a*]benzimidazole (**2**) (DMSO-d_6)



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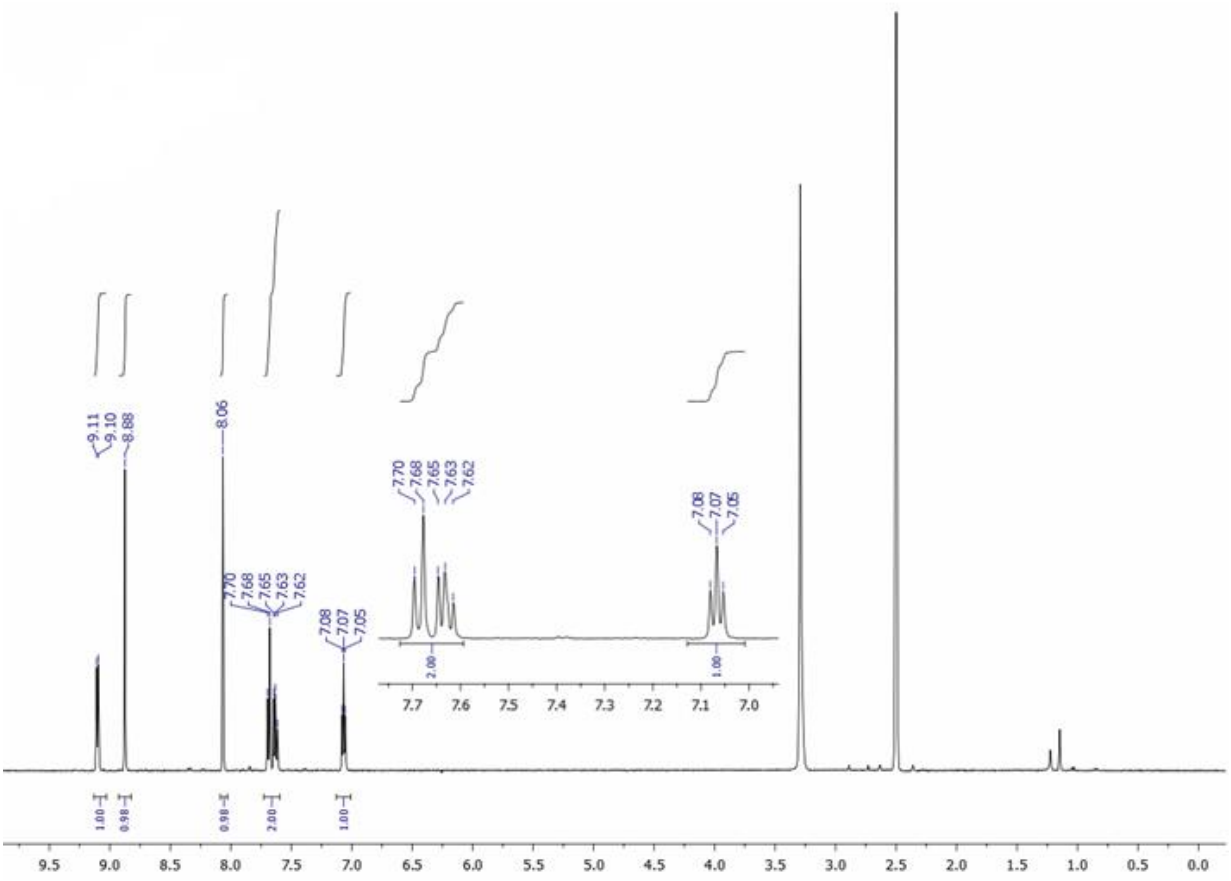

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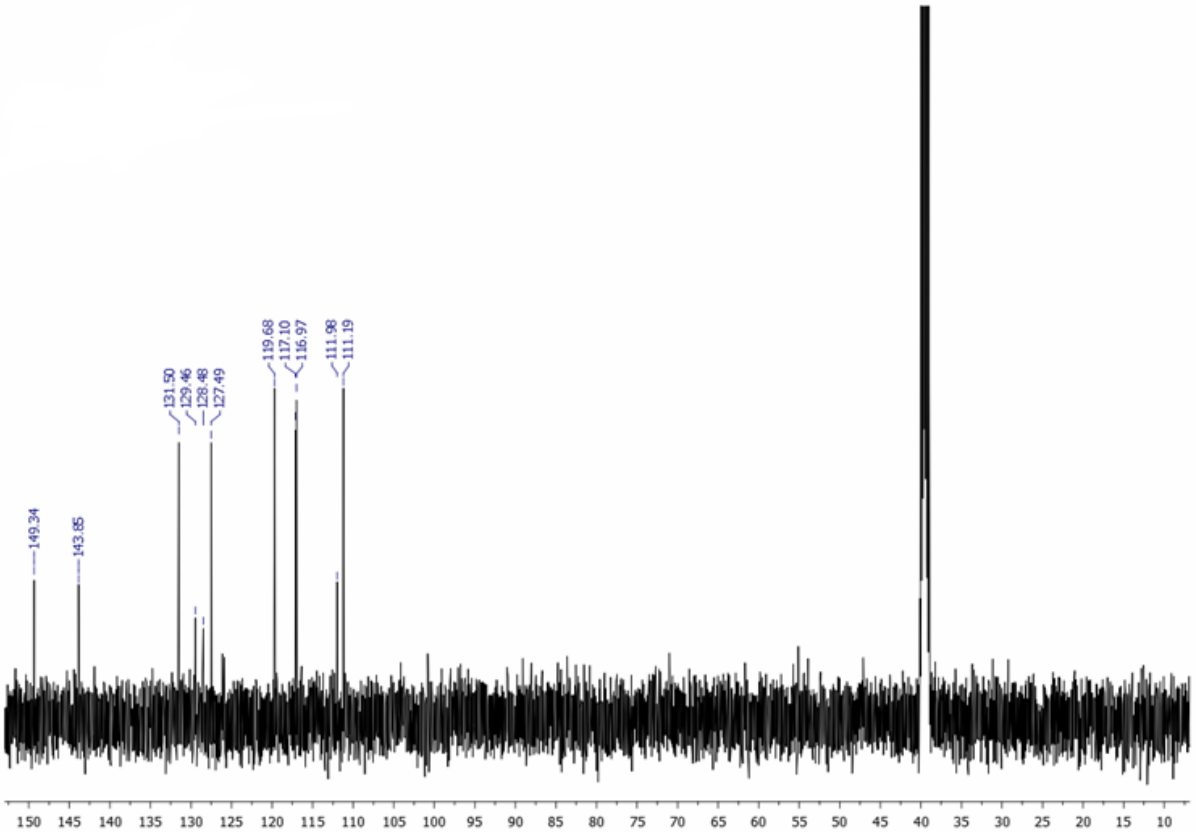

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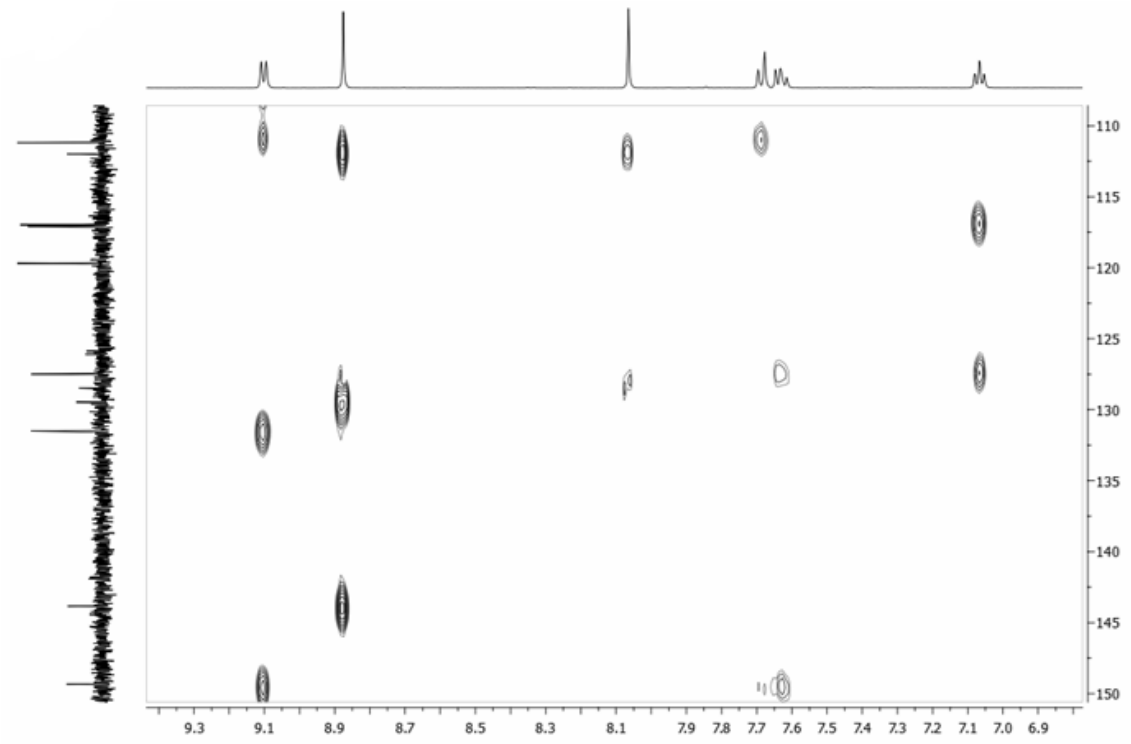
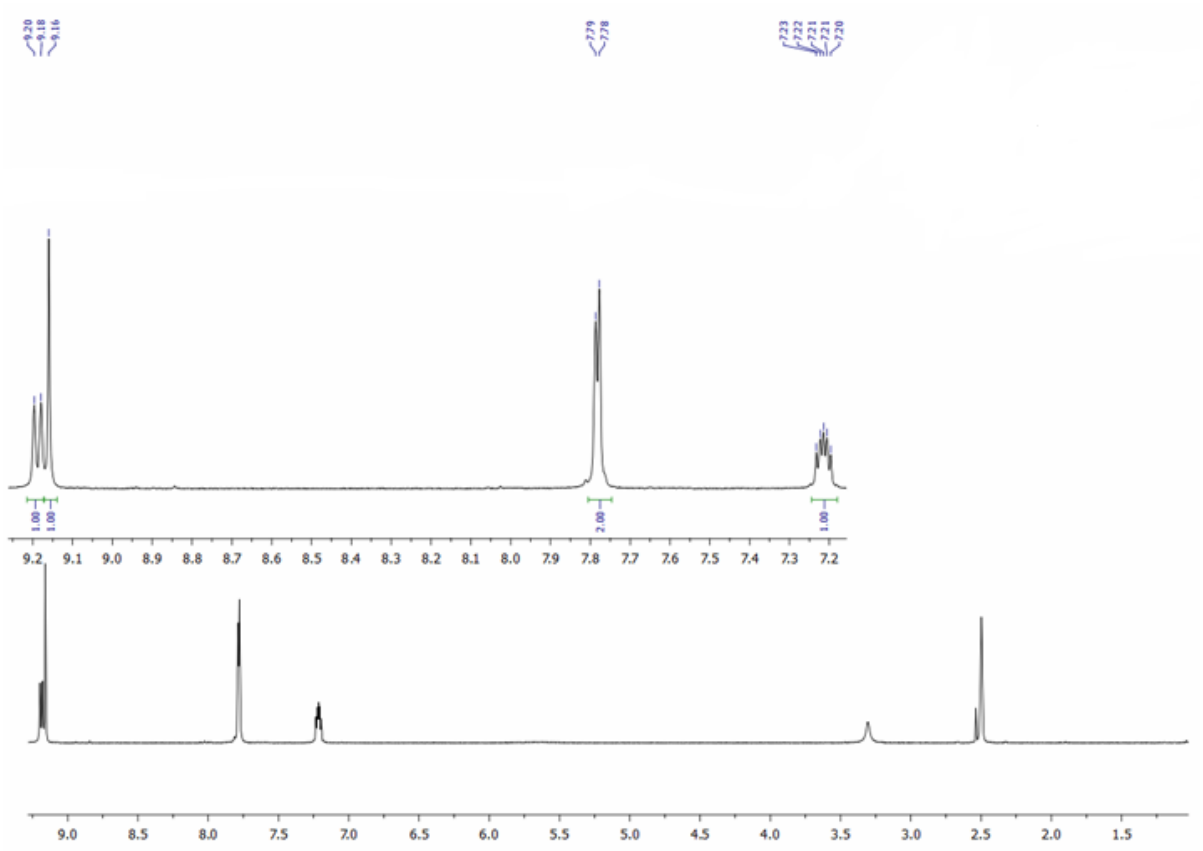


Fragment of ^{1}H-^{13}C HMBC spectrum of 8-bromo-7-chloropyrido[1,2-*a*]benzimidazole (**3**) (DMSO-d_6)



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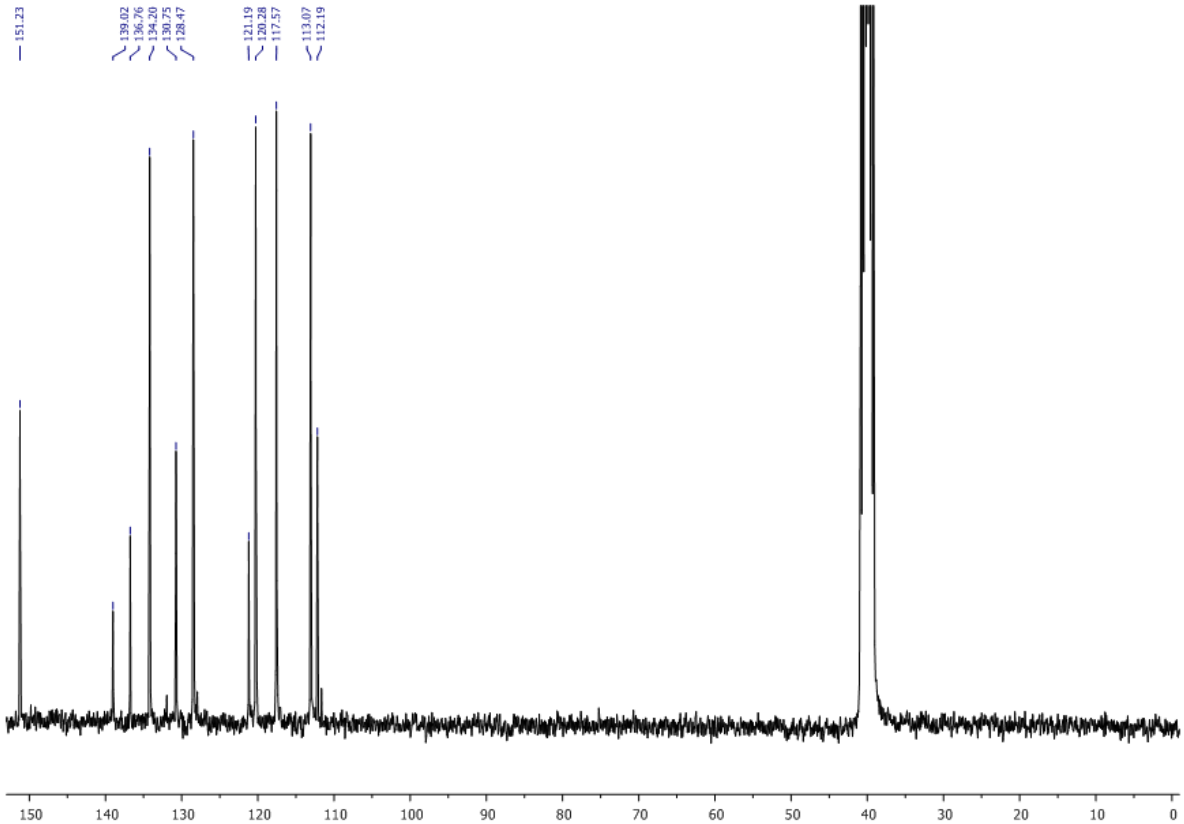
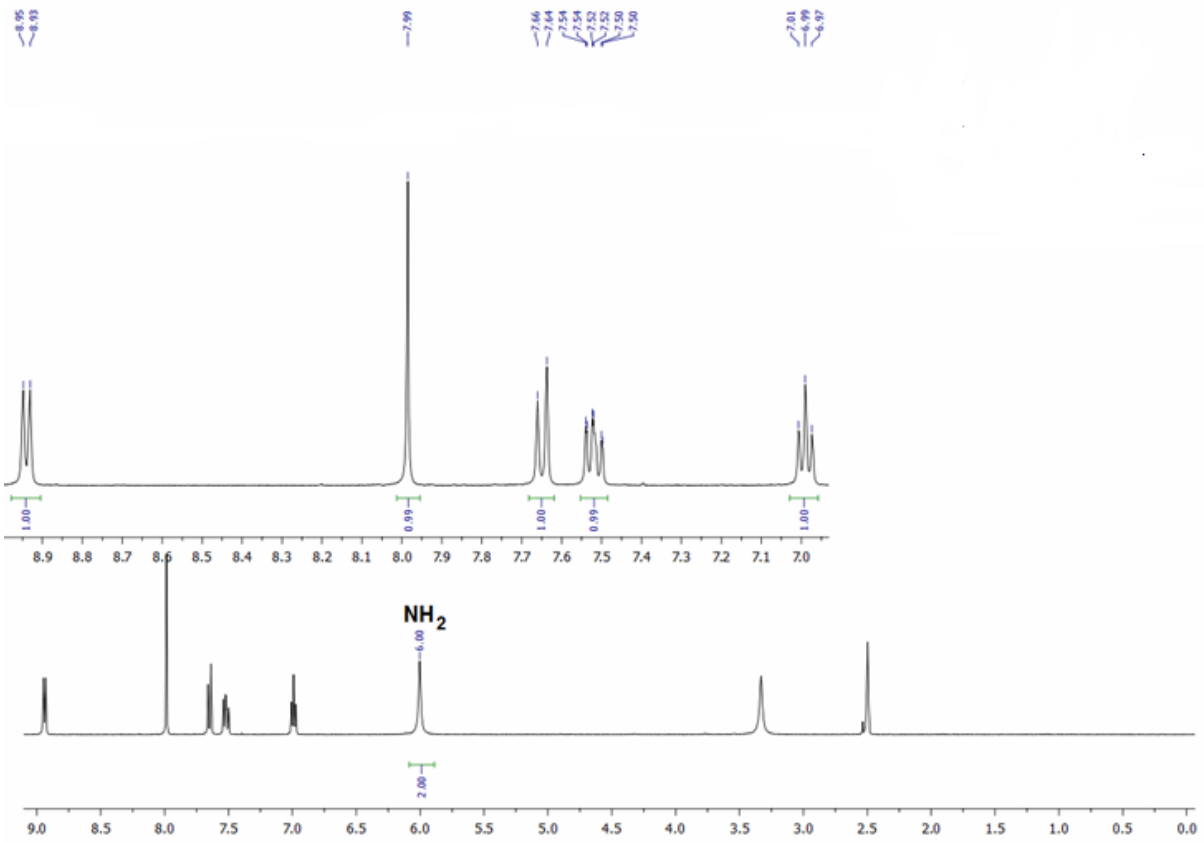


^{13}C NMR spectrum of 8-bromo-7-chloro-6-nitropyrido[1,2-*a*]benzimidazole (**4**) (DMSO-d_6)



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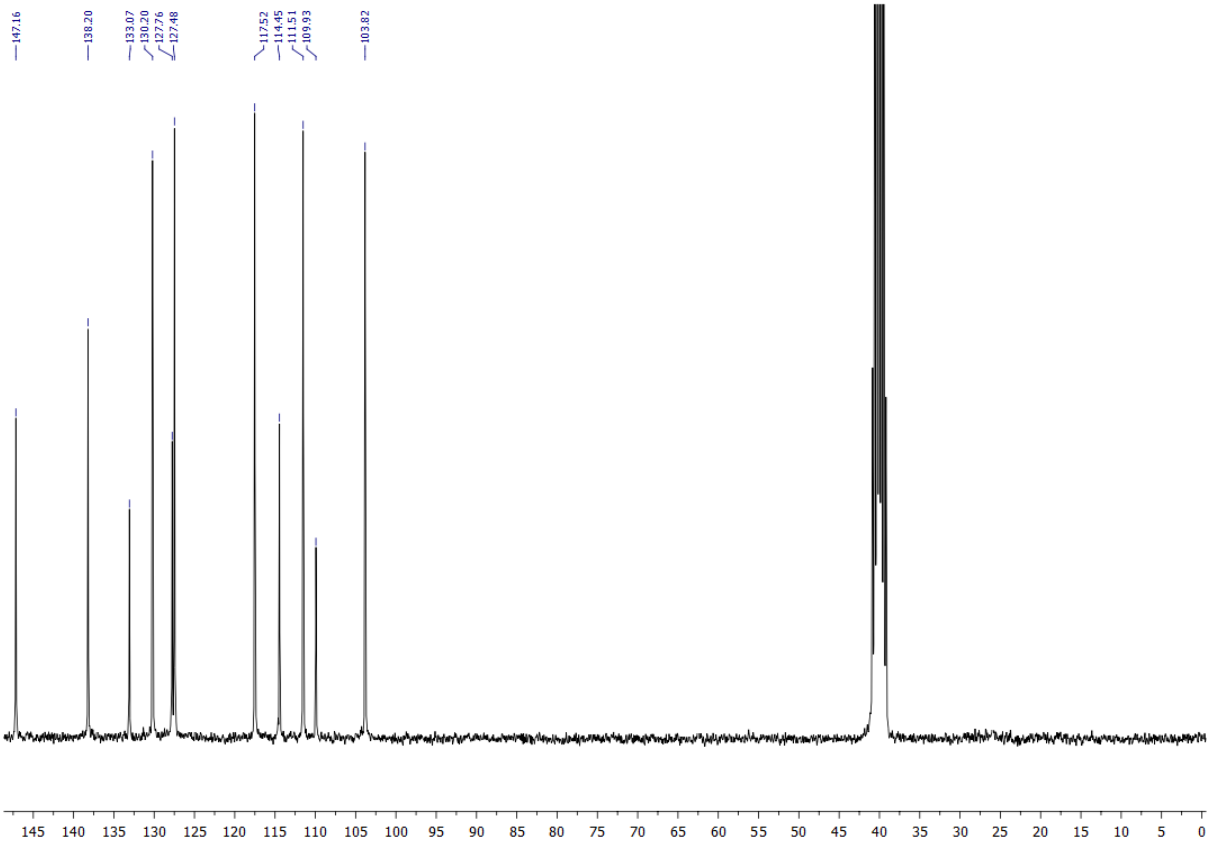
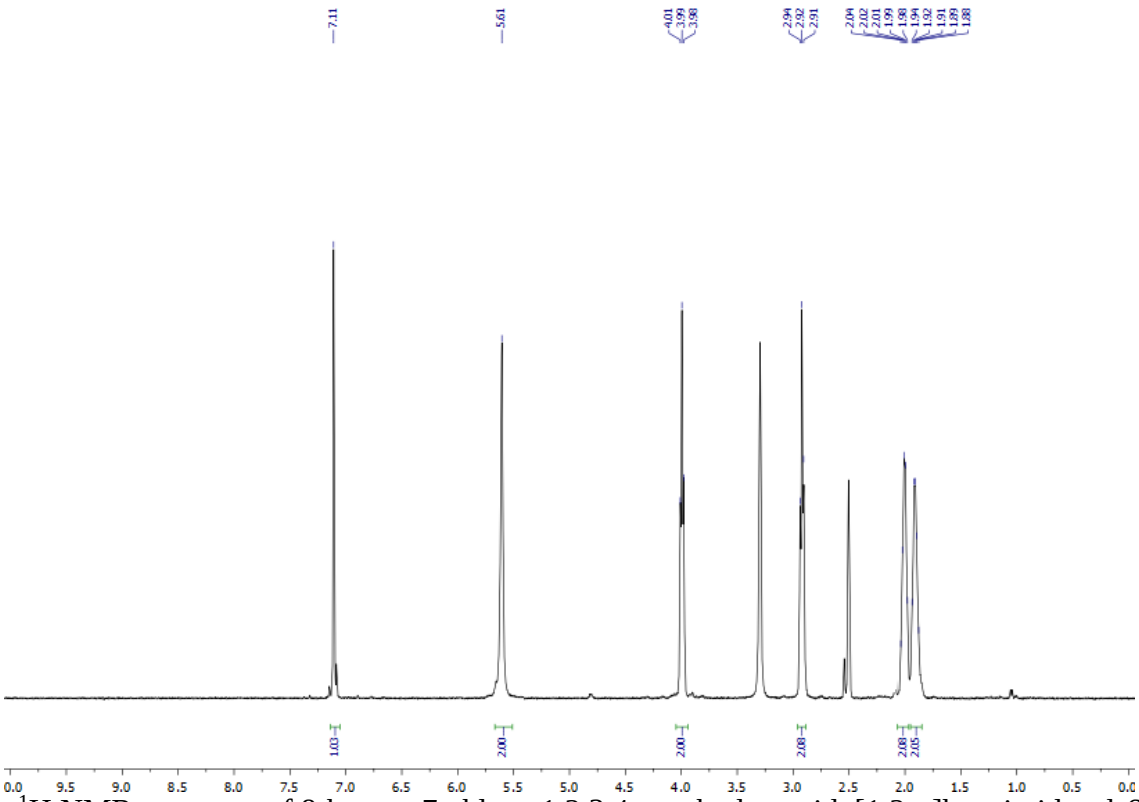
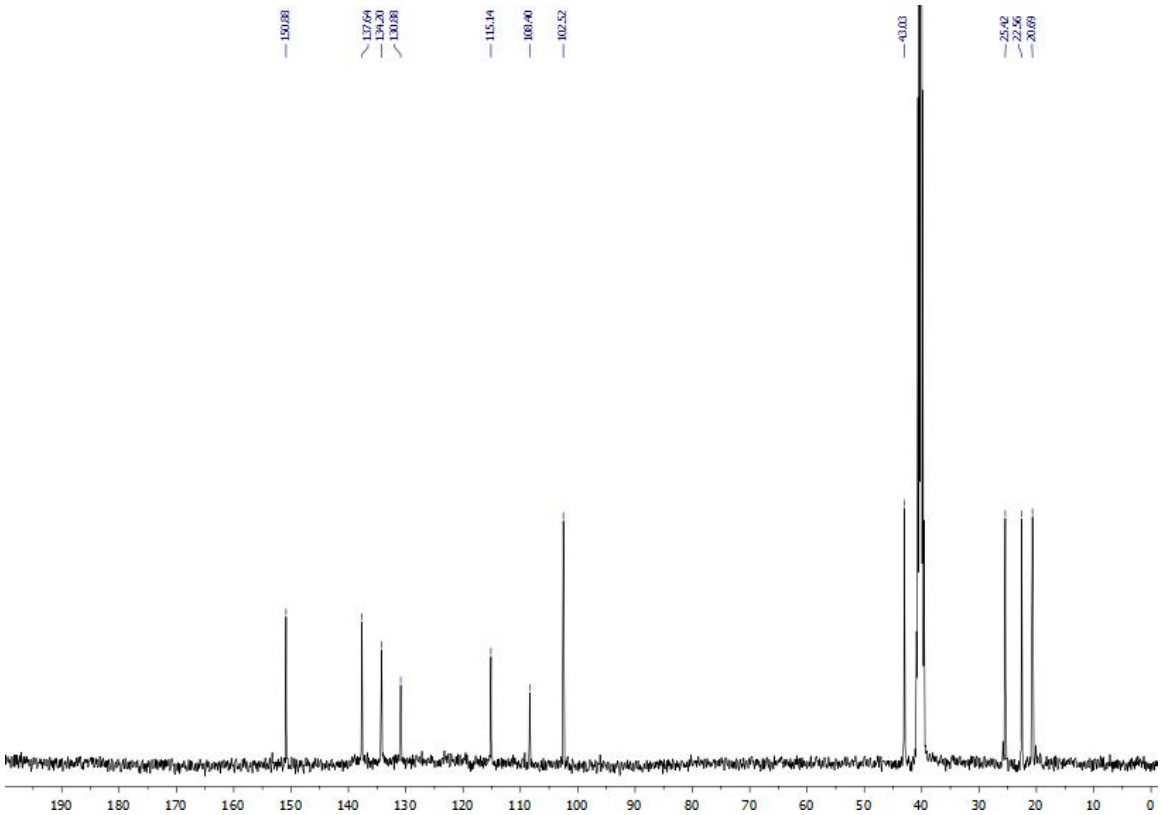


^{13}C NMR spectrum of 8-bromo-7-chloropyrido[1,2-*a*]benzimidazol-6-amine (**5a**) (DMSO-d_6)



^{1}H NMR spectrum of 8-bromo-7-chloro-1,2,3,4-tetrahydropyrido[1,2-*a*]benzimidazol-6-amine (**5b**) (DMSO-d_6)



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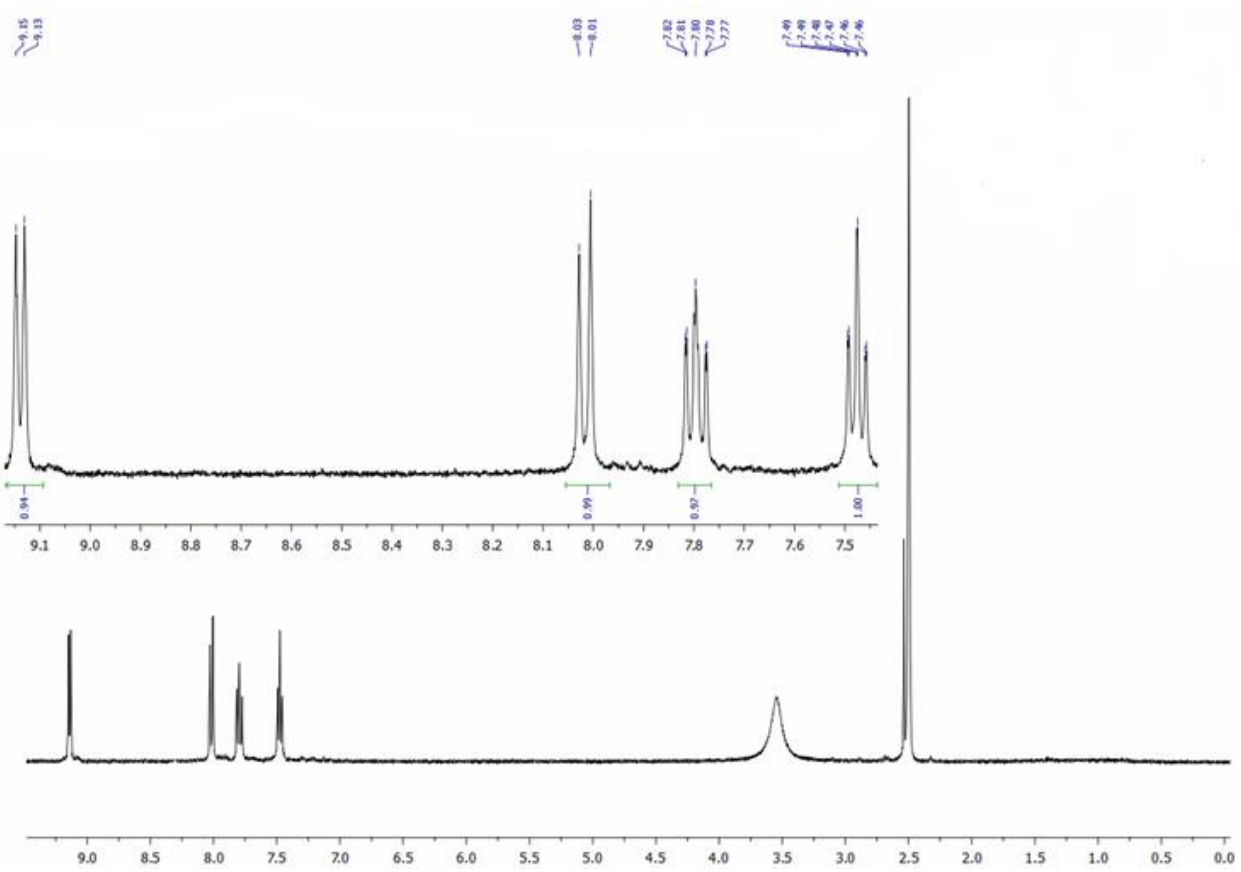

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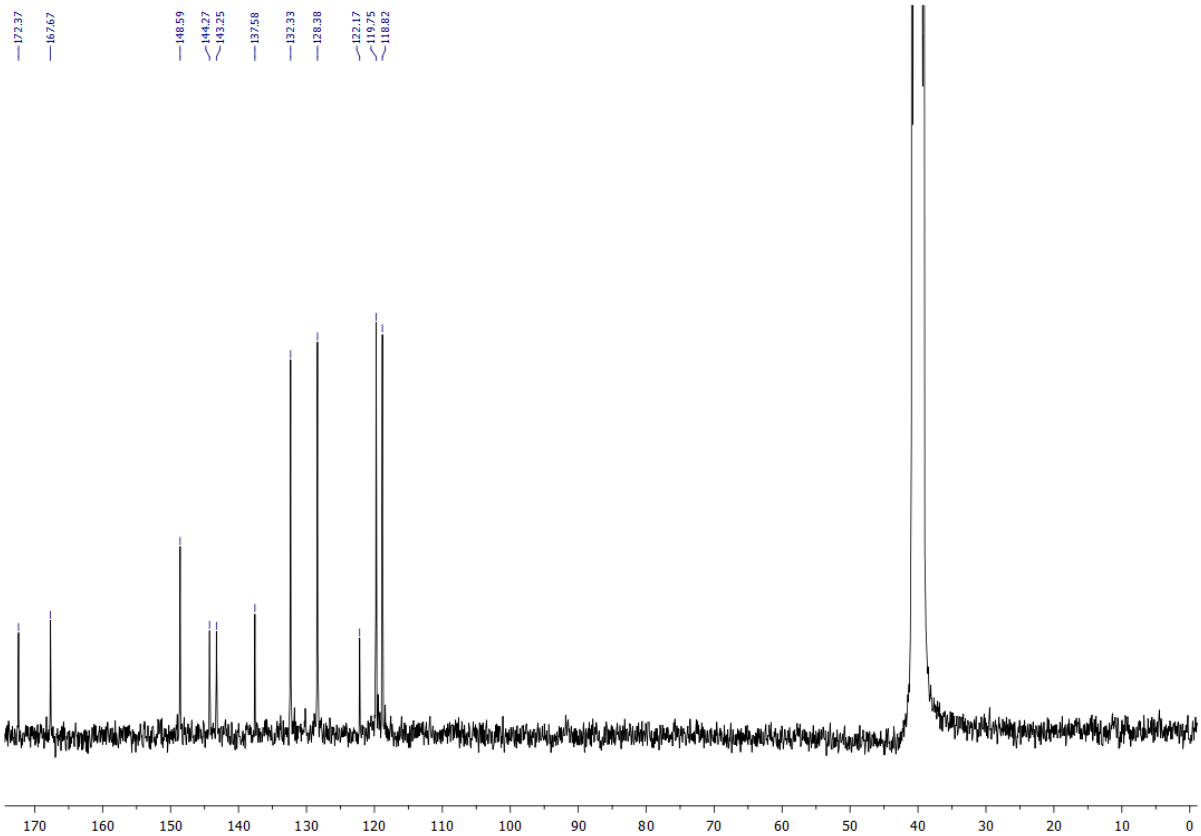

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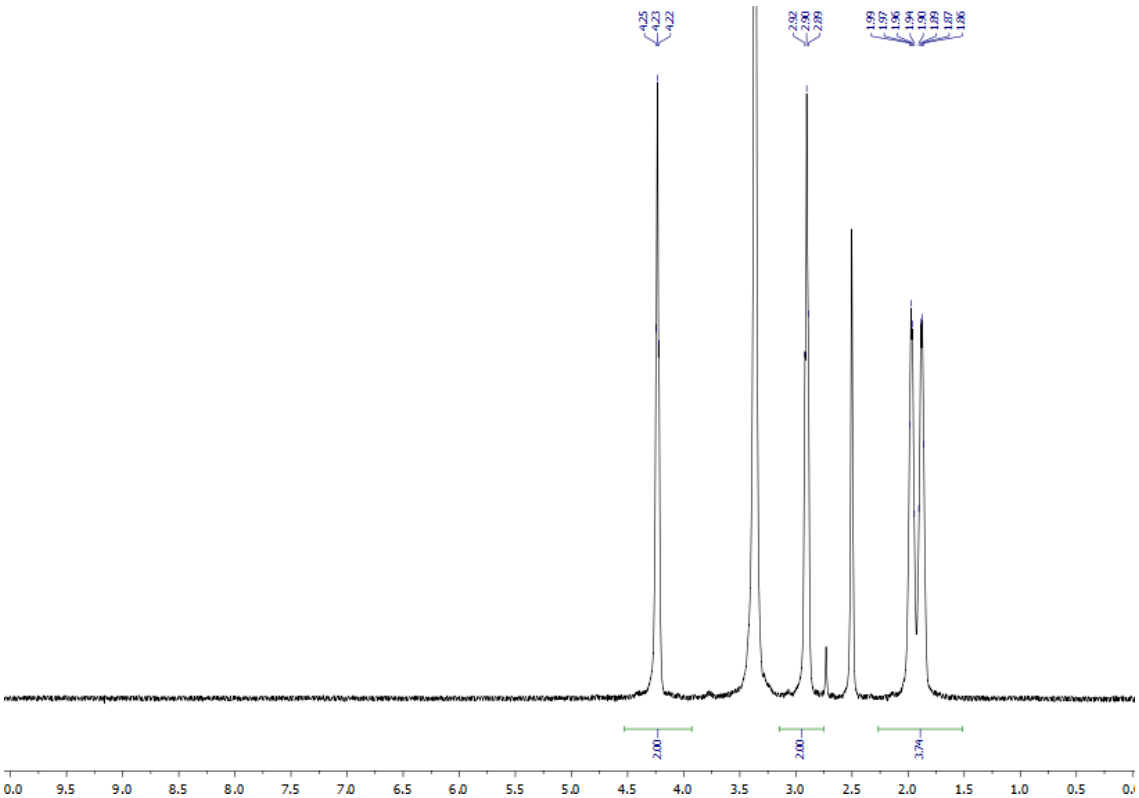

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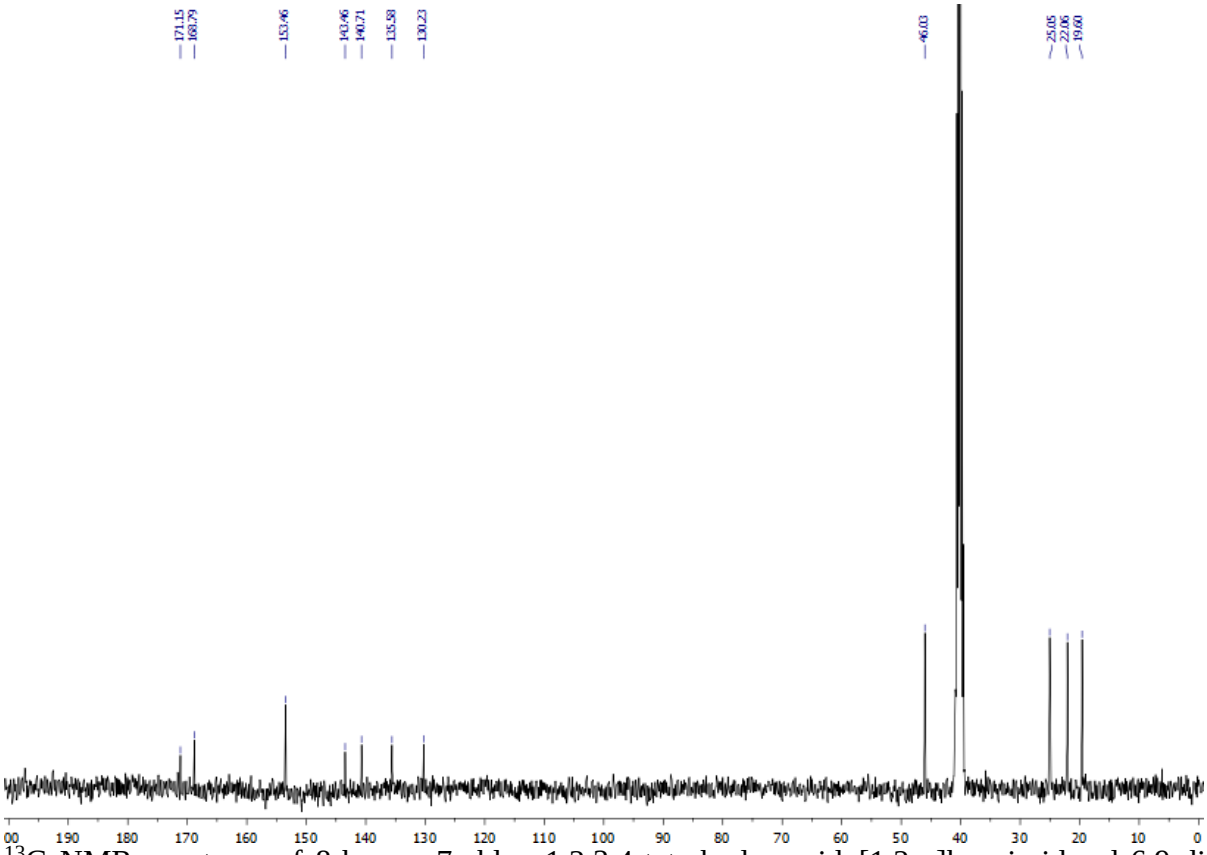

^{13}C NMR spectrum of 8-bromo-7-chloro1,2,3,4-tetrahydropyrido[1,2-*a*]benzimidazol-6,9-dione (**6b**) (DMSO-d_6)