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Derivatives of sterically hindered phenols and pyridinecarboxylic acids as prospective radioprotectors

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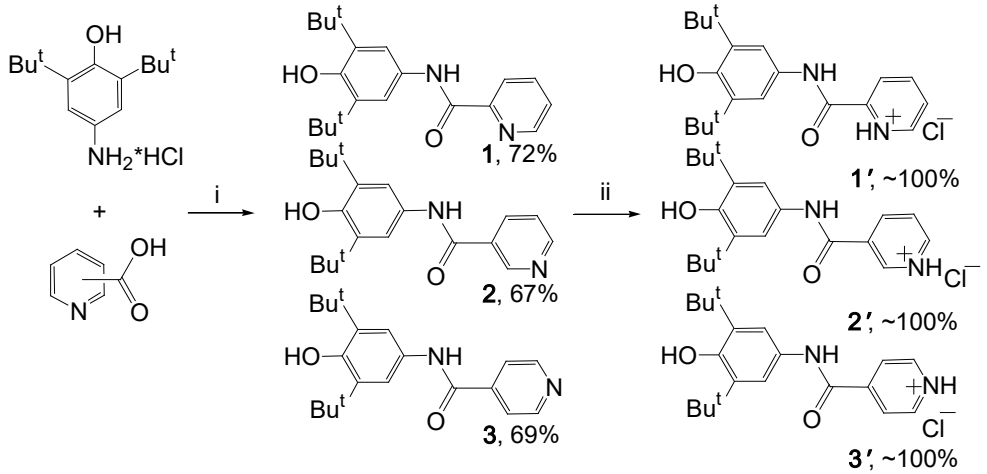
2-Picolinic acid (99%), nicotinic acid (≥98%), isonicotinic acid (99%), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC HCl, ≥98%), 4-(dimethylamino)pyridine (DMAP, ≥98%), neocuproine (2,9-dimethyl-1,10-phenanthroline, 99%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), linoleic acid (99%), soybean lipoxygenase type 1-B (158000 units/mg), xanthine, ethylenediaminetetraacetic acid (EDTA), and were purchased from Sigma-Aldrich (USA). Trolox (97%) was purchased from Acros Organics (Belgium). The solvents (EtOH (95%), MeOH, petroleum ether (40-70°C), DMSO, and CH_2Cl_2) were used as supplied. 2,6-Di-*tert*-butyl-4-aminophenol was obtained according to the known procedure [A. Rieker, K. Scheffler, R. Mayer, B. Narr and E. Müller, *Justus Liebigs Ann. Chem.,* 1966, **693**, 10].

Infrared spectra in the region of 4000–370 cm^{-1} were obtained using IR200 Nicolet spectrometer (KBr; Thermo Fisher Scientific, USA). The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ and DMSO-d_6 using Avance-400 spectrometer (Bruker, Germany) operating at 400.1 MHz (^1H) and 100.6 MHz (^{13}C). Chemical shifts are given in ppm using ^1H-TMS as an internal reference. Elemental analysis was performed on 2400 Series II elemental analyzer (PerkinElmer, USA).



Scheme S1 *Reagents and conditions:* i, EDC HCl, DMAP, CH_2Cl_2, 0°C; ii, HCl, MeOH, 70 °C, 15 min.

General synthesis method of the compounds 1–3 and their water-soluble hydrochlorides 1′–3′
Synthesis of compounds **1-3** was carried out as follows. To a mixture of 0.1 mmol of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC*HCl) and 0.01 4-(dimethylamino)pyridine (DMAP) in 30 ml of ice-cold dry CH_2Cl_2, 0.1 mmol of the corresponding pyridinecarboxylic acid was added and vigorously stirred. After that a mixture of 0.1 mmol 4-amino-2,6-di-*tert*-butylphenol and 0.1 mmol of triethylamine in 5 ml of dry CH_2Cl_2 was added to the initiate the reaction, and the mixture was stirred overnight at room temperature. The solvent was removed *in vacuo*, and the residue was rinsed with distilled water and dried in desiccator.

N-(3,5-Di-tert-butyl-4-hydroxyphenyl)pyridine-2-carboxamide **1**: red solid, mp 168-170°C, yield was 72%, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.48 (s, 18H, Bu^t), 5.12 (s, 1H, OH) 7.47(dd, 1H, CH-Py, ^{3}J 13.1 Hz, ^{3}J 0.7 Hz), 7.62(s, 2H, CH-Ar), 7.90 (td, 1H, CH-Py, ^{3}J 17.0 Hz, ^{3}J 1.6 Hz), 8.31 (d, 1H, CH-Py, ^{3}J 7.9 Hz), 8.63 (d, 1H, CH-Py, ^{3}J 4.7 Hz), 9.86 (br.s, 1H, NH(C=O)). ^{13}C NMR (100 MHz, $CDCl_3$, 25°C) δ: 29.82 (C(<u>C</u>H$_3$)$_3$), 34.12 (<u>C</u>(CH$_3$)$_3$), 116.84 (C2-Ar), 121.80 (CH-Py), 125.77 (CH-Py), 129.43 (C1-Ar), 136.15 (CH-Py), 137.24 (CH-Py), 147.48 (CH-Py), 149.77 (C4-Ar), 150.25 (C3-Ar), 161.27 (NH(<u>C</u>=O). IR (KBr, ν/cm^{-1}): ν(OH) 3528.6; ν(N-H) 3330.5; ν(C-H) 2958.3-2873.4; ν(C=O) 1668.1; ν(C-C, Py, Ph) 1605.0, 1587.1; ν(N-C=O) 1540.5; 1433.8; 1234.2; 1115.6; 1090.1; 997.5. Found (%): C, 73.65; H, 8.16; N, 8.44. Calc. for $C_{20}H_{26}N_2O_2$(%): C, 73.58; H, 8.04; N, 8.58.

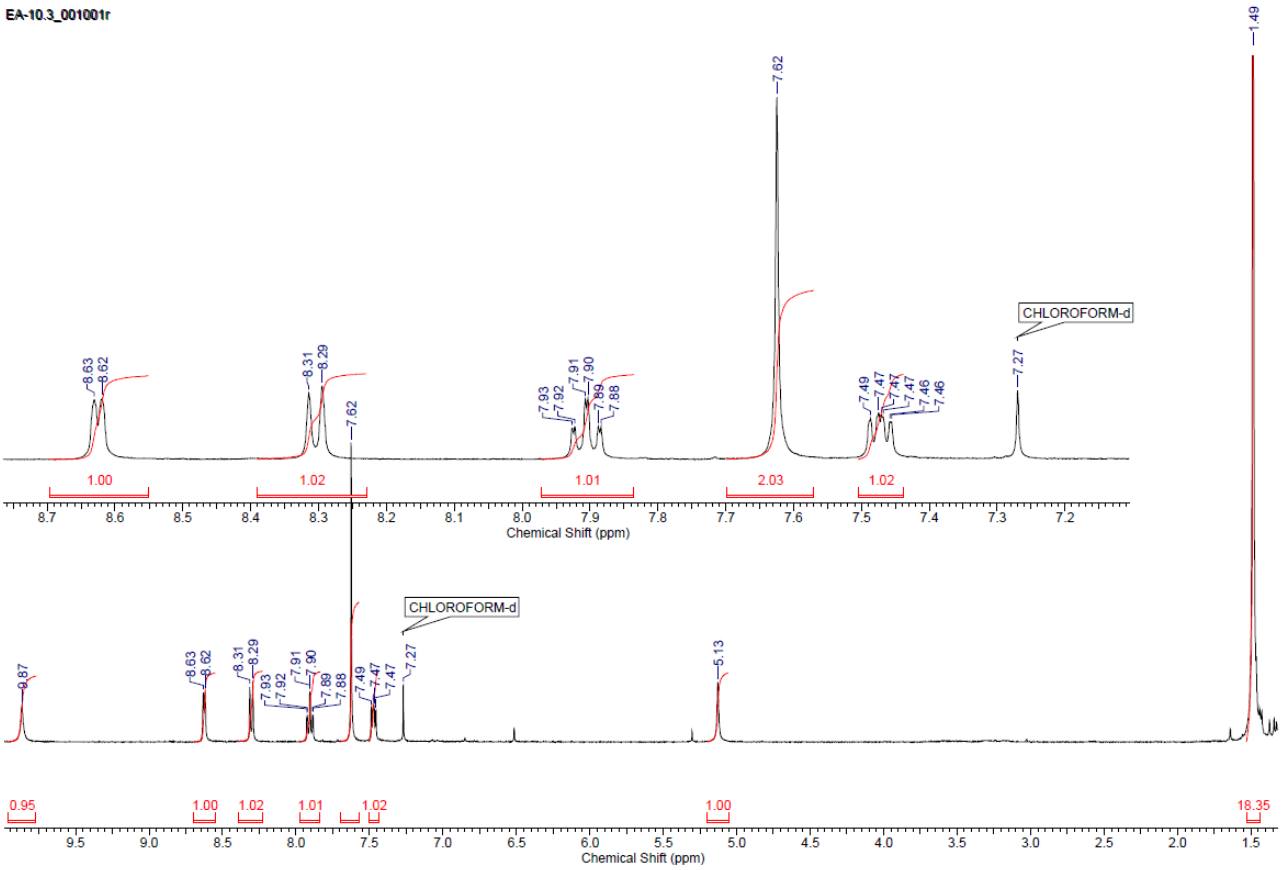


Figure S1. The ^{1}H NMR spectrum of the compound **1**.

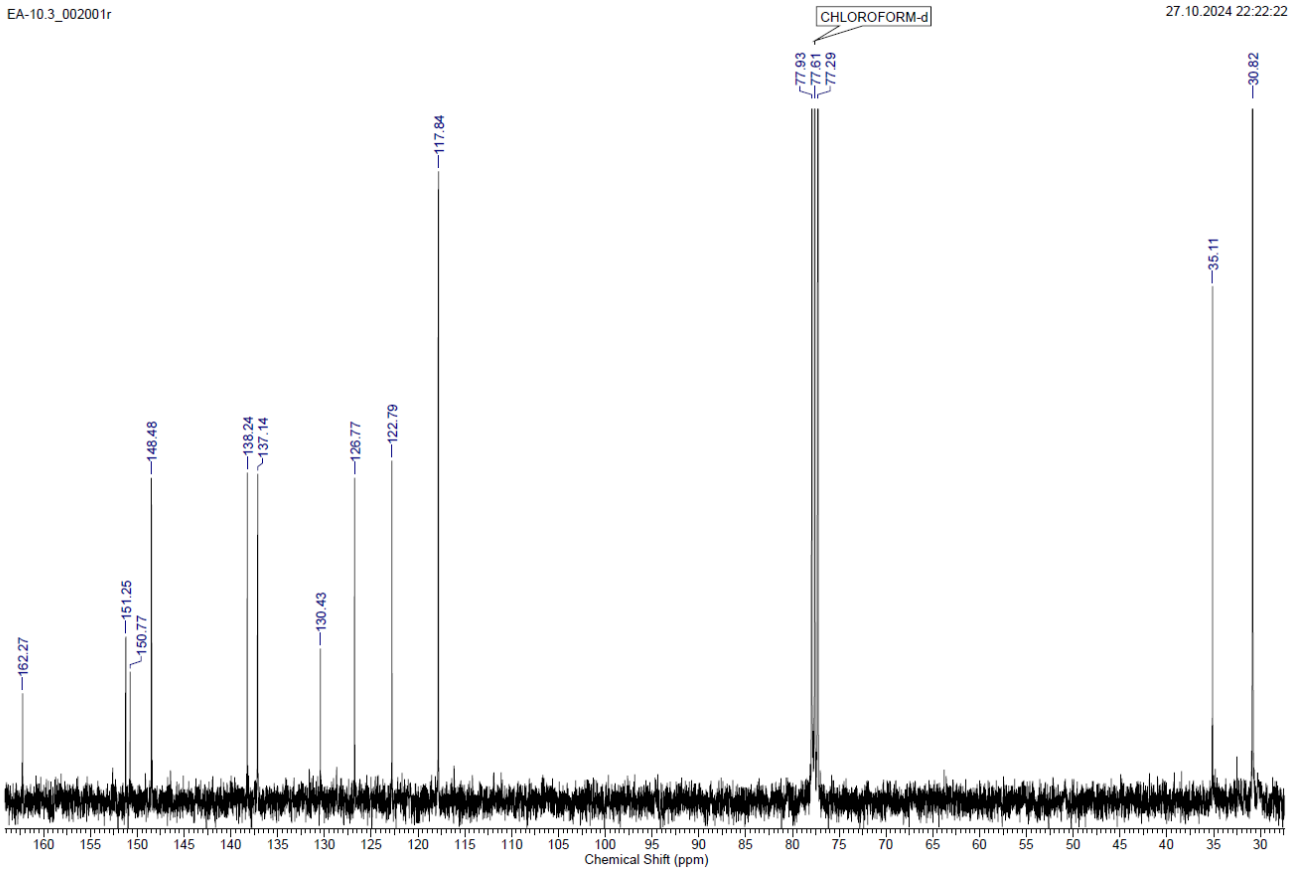


Figure S2. The ^{13}C NMR spectrum of the compound **1**.

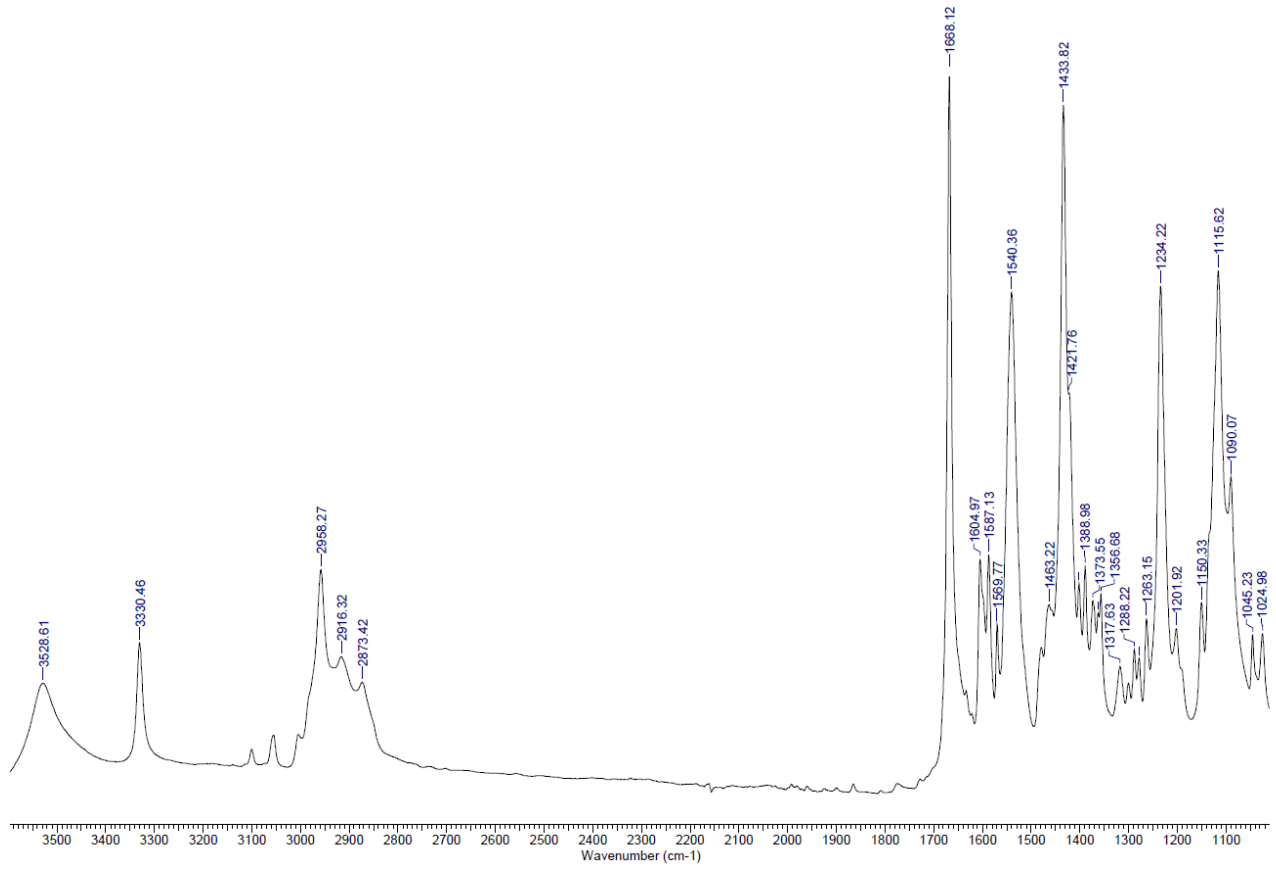


Figure S3. The IR spectrum of the compound **1**.

N-(3,5-Di-tert-butyl-4-hydroxyphenyl)pyridine-3-carboxamide **2**: red solid, mp 227-230 °C, yield was 67%, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.44 (s, 18H, Bu^t), 5.19 (s, 1H, OH) 7.45(m, 2H, CH-Py, CH-Ar), 8.06 (br.s, 1H, CH-Py), 8.23 (dt, 1H, CH-Py, ^{3}J 11.4 Hz, ^{3}J 3.6 Hz), 8.75 (dd, 1H, CH-Py, ^{3}J 6.2 Hz, ^{3}J 1.5 Hz), 8.31 (d, 1H, CH-Py, ^{3}J 7.9 Hz), 8.63 (d, 1H, CH-Py, ^{3}J 4.7 Hz), 9.10 (br.s, 1H, NH(C=O)). ^{13}C NMR (100 MHz, $CDCl_3$, 25°C) δ: 29.75 (C(<u>C</u>H$_3$)$_3$), 34.09 (<u>C</u>(CH$_3$)$_3$), 117.90 (C2-Ar), 128.93 (CH-Py), 130.69 (C1-Ar), 135.01 (CH-Py), 136.23 (C3-Ar), 147.38 (CH-Py), 150.82 (C4-Ar), 151.79 (CH-Py), 163.27 (NH(<u>C</u>=O). IR (KBr, ν/cm^{-1}): ν(OH) 3637.6; ν(N-H) 3310.2; ν(C-H) 2957.3-2869.1; ν(C=O) 1641.6; ν(C-C, Py, Ph) 1602.6, 1539.9; ν(N-C=O) 1433.3; 1419.8; 1360.6; 1311.4; 1231.3; 1152.7; 1116.1; 1024.1. Found (%): C, 73.69; H, 8.09; N, 8.45. Calc. for $C_{20}H_{26}N_2O_2$(%): C, 73.58; H, 8.04; N, 8.58.

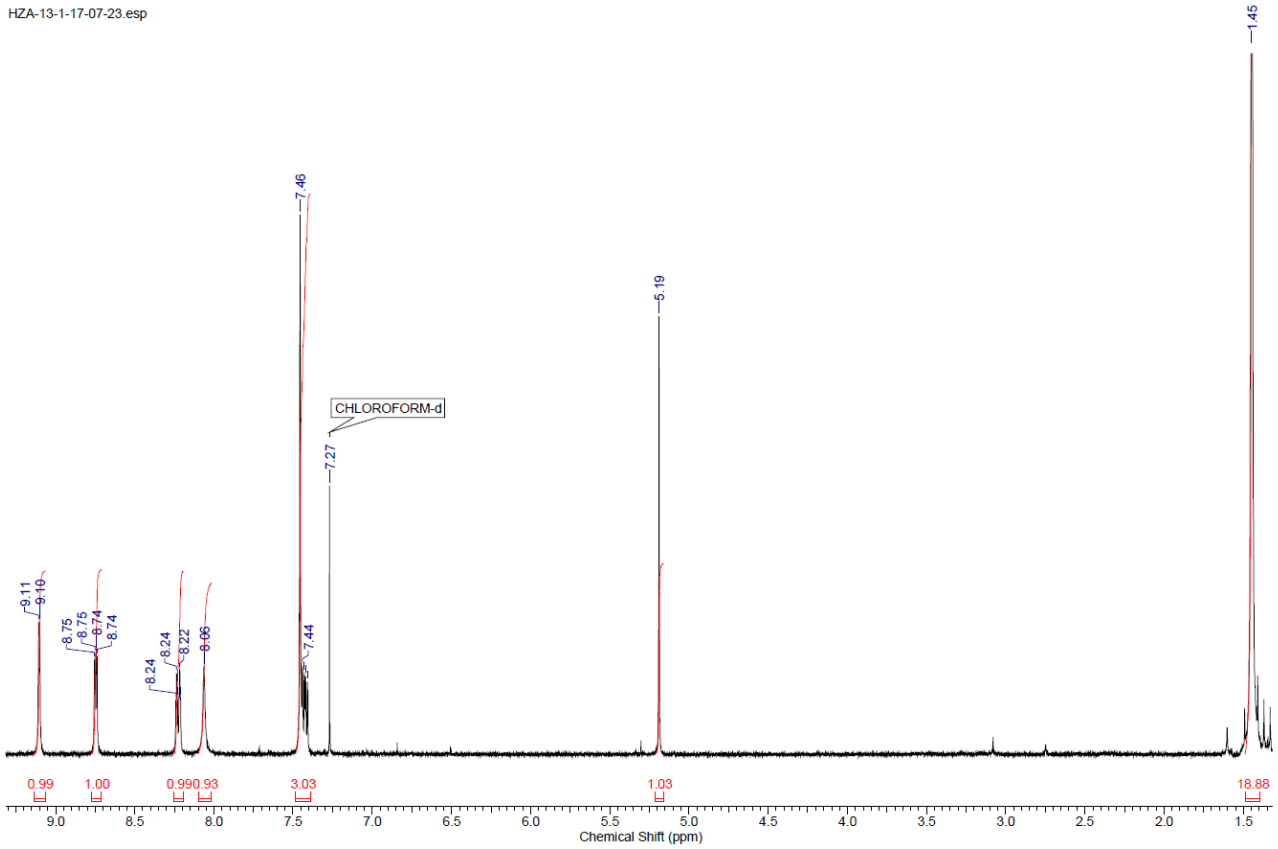


Figure S4. The ^{1}H NMR spectrum of the compound **2**.

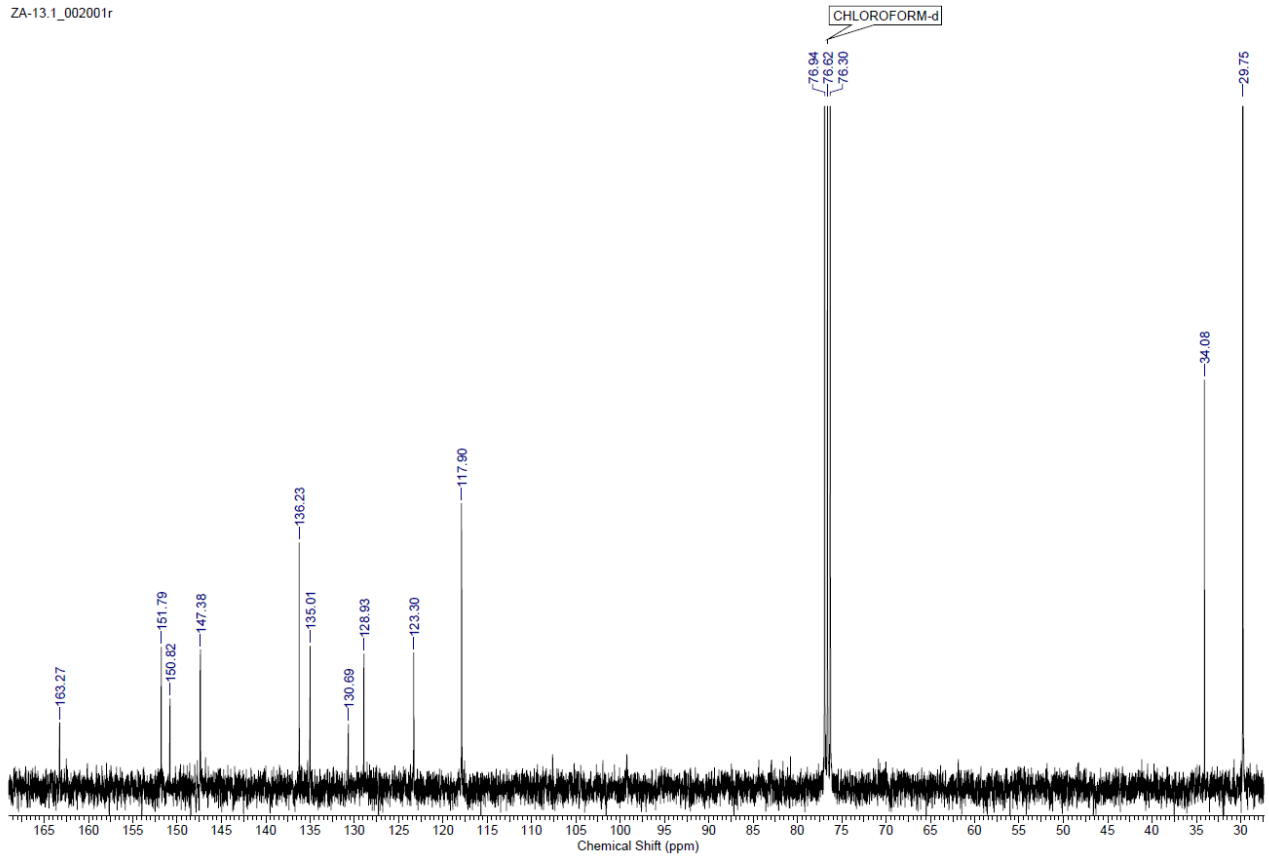


Figure S5. The ^{13}C NMR spectrum of the compound **2**.

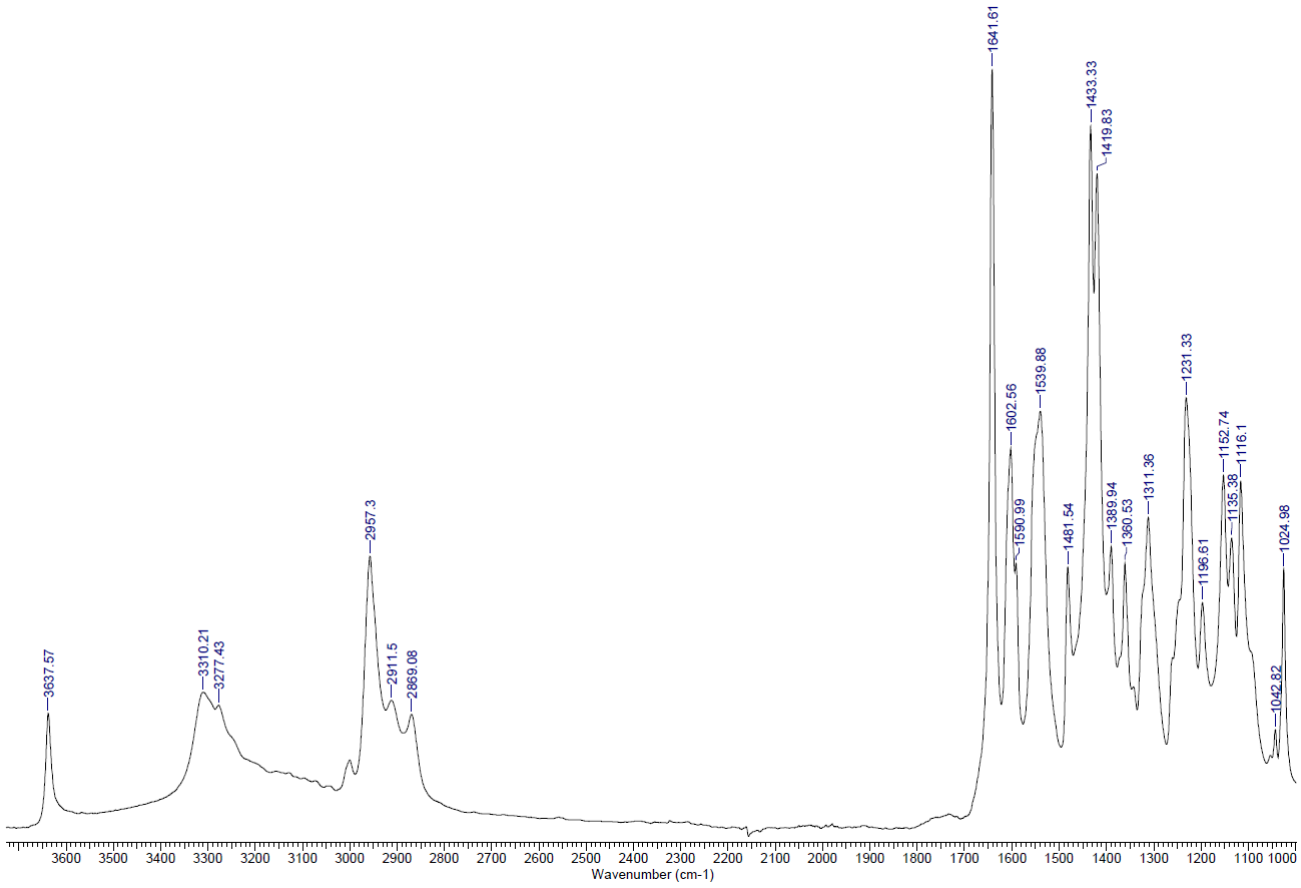


Figure S6. The IR spectrum of the compound **2**.

N-(3,5-Di-tert-butyl-4-hydroxyphenyl)pyridine-4-carboxamide **3**: red solid, mp 228-231 °C, yield was 69%, ^{1}H NMR (400 MHz, DMSO-d^{6}) δ: 1.38 (s, 18H, Bu^{t}), 6.87 (s, 1H, OH) 7.58 (s, 2H, CH-Ar), 7.84 (d, 2H, CH-Py, ^{3}J 6.1 Hz), 8.76 (d, 2H, CH-Py, ^{3}J 6.1 Hz), 10.19 (s, 1H, NH(C=O)). ^{13}C NMR (100 MHz, DMSO-d^{6}, 25°C) δ: 30.37 (C(<u>C</u>H$_3$)$_3$), 34.79 (<u>C</u>(CH$_3$)$_3$), 117.54 (C2-Ar), 121.52 (CH-Py), 131.17 (C1-Ar), 135.01 (CH-Py), 139.77 (C3-Ar), 142.30 (CH-Py), 150.20 (CH-Py), 150.46 (C4-Ar), 163.27 (NH(<u>C</u>=O)). IR (KBr, ν/cm^{-1}): ν(OH) 3628.9; ν(N-H) 3309.3; ν(C-H) 2956.3-2870.1; ν(C=O) 1642.1; ν(C-C, Py, Ph) 1599.2; ν(N-C=O) 1540.4; 1433.8; 1360.5; 1233.7; 1107.4; 1065.0. Found (%): C, 73.47; H, 8.14; N, 8.53. Calc. for $C_{20}H_{26}N_2O_2$(%): C, 73.58; H, 8.04; N, 8.58.

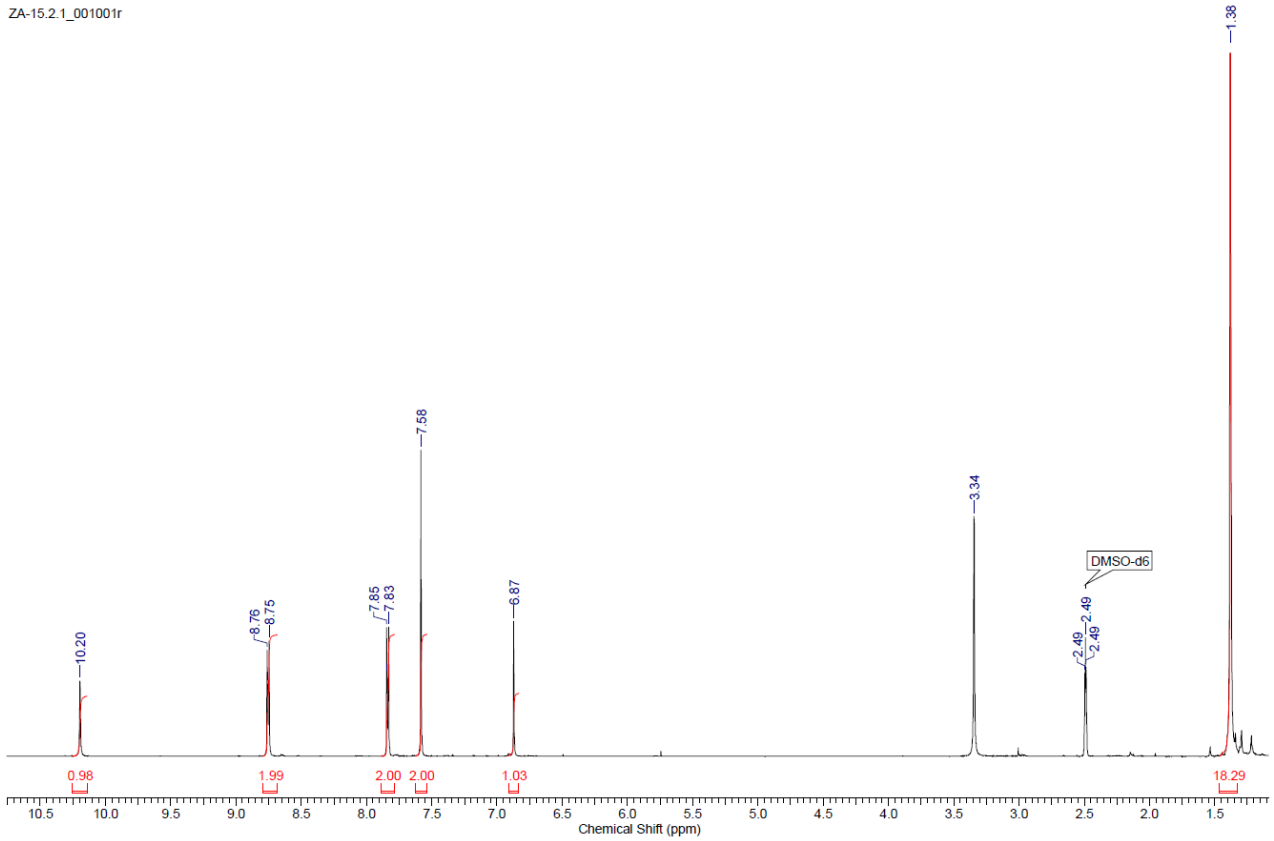


Figure S7. The ^{1}H NMR spectrum of the compound **3**.

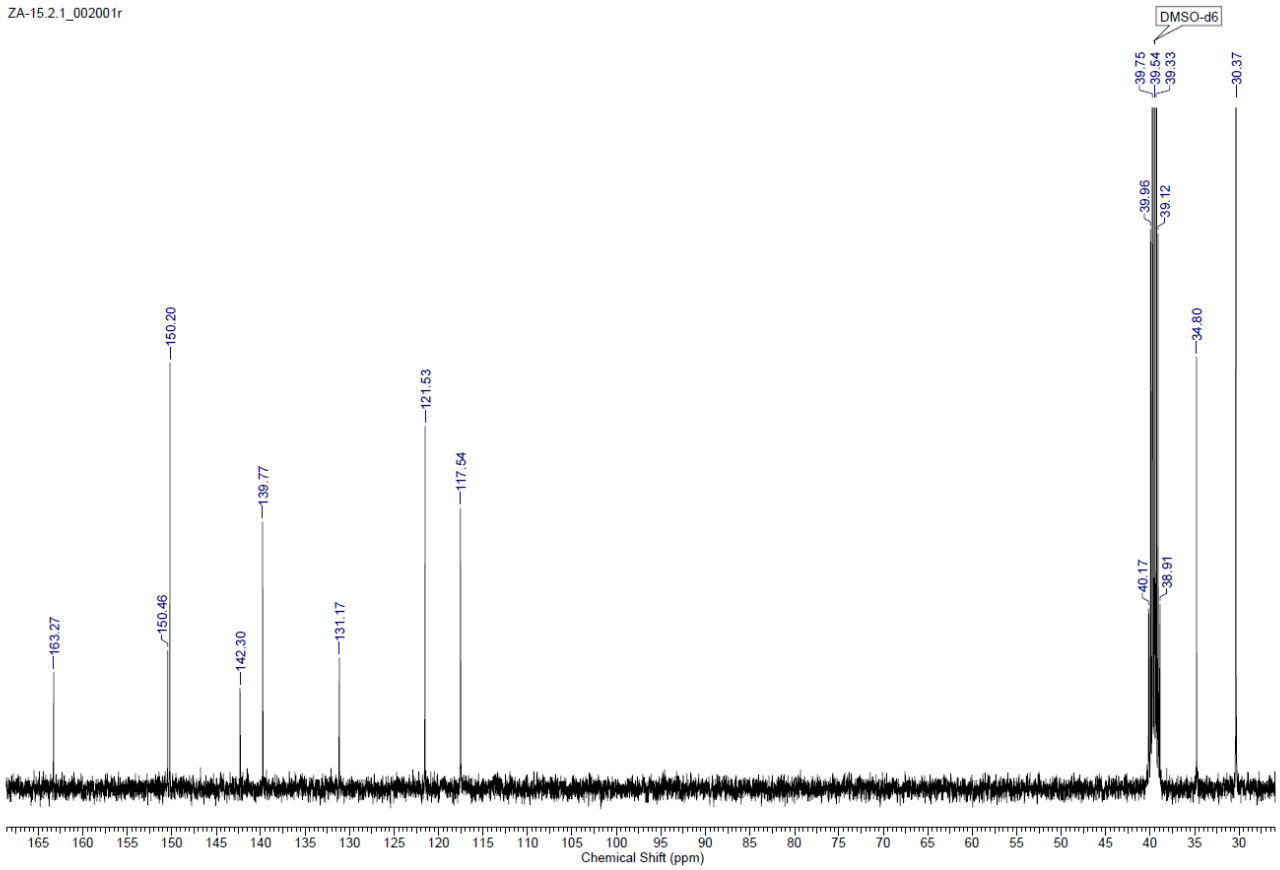


Figure S8. The ^{13}C NMR spectrum of the compound **3**.

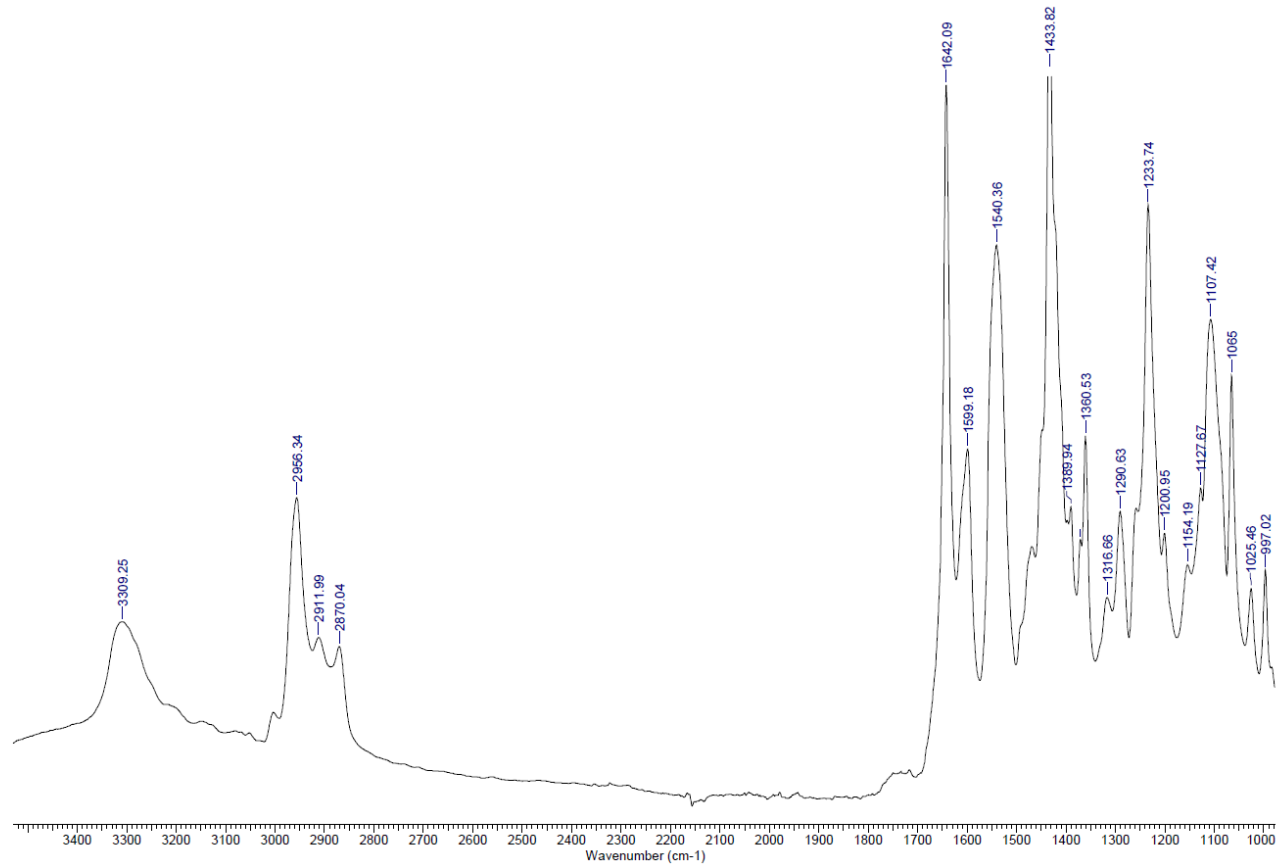


Figure S9. The IR spectrum of the compound **3**.

Hydrochlorides **1′-3′** were prepared by the following procedure. A solution of 0.1 mmol of the corresponding pyridine amide in MeOH was prepared and 0.33 mmol of concentrated HCl was then added to the solution. The mixture was stirred and heated at 70°C for 15 minutes, after which it was cooled to room temperature. The solvent was removed under vacuum. The residue was left in a desiccator for several days to allow any remaining solvent to evaporate.

2-[N-(3,5-Di-tert-butyl-4-hydroxyphenyl)carbamoyl]pyridinium chloride **1′**: red solid, mp 172-175°C, yield was quantitative, ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.48 (s, 18H, Bu^t), 5.18 (s, 1H, OH), 7.74 (s, 2H, CH-Ar), 7.79 (br s, 1H, CH-Py), 8.59 (br t, 1H, CH-Py), 8.59 (br d, 1H, CH-Py, ^{3}J 6.7 Hz), 8.74 (br s, 1H, CH-Py), 10.69 (br.s, 1H, NH(C=O)). ^{13}C NMR (100 MHz, $CDCl_3$, 25°C) δ: 29.83 (C($\underline{C}H_3)_3$), 34.13 ($\underline{C}(CH_3)_3$), 117.23 (C2-Ar), 123.55 (CH-Py), 126.31 (CH-Py), 129.29 (C1-Ar), 136.14 (C3-Ar), 139.46 (CH-Py), 140.58 (CH-Py), 145.68 (C4-Ar), 150.55 (CH-Py), 159.43 (NH($\underline{C}$=O). IR (KBr, ν/cm^{-1}): ν(OH) 3631.3; ν(N-H) 3330.1; ν(C-H) 2957.8-2857.8; ν(C=O) 1669.1; ν(C-C, Py, Ph) 1599.2, 1519.6; ν(N-C=O) 1543.7; 1434.8; 1358.6; 1233.3; 1148.4; 1118.0; 1099.7. Found (%): C, 66.24; H, 7.46; N, 7.77. Calc. for $C_{20}H_{27}N_2O_2Cl$(%): C, 66.19; H, 7.51; N, 7.72.

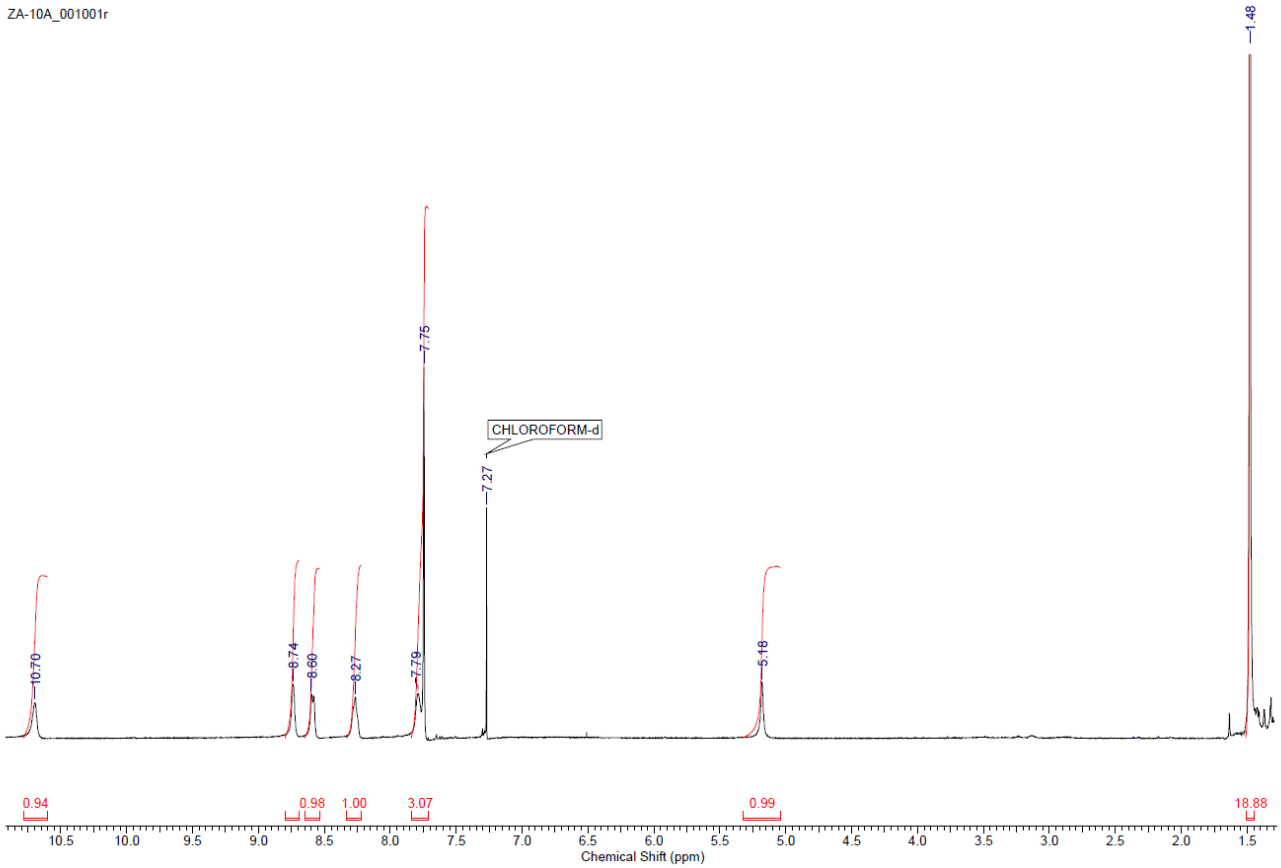


Figure S10. The ^{1}H NMR spectrum of the compound **1′**.

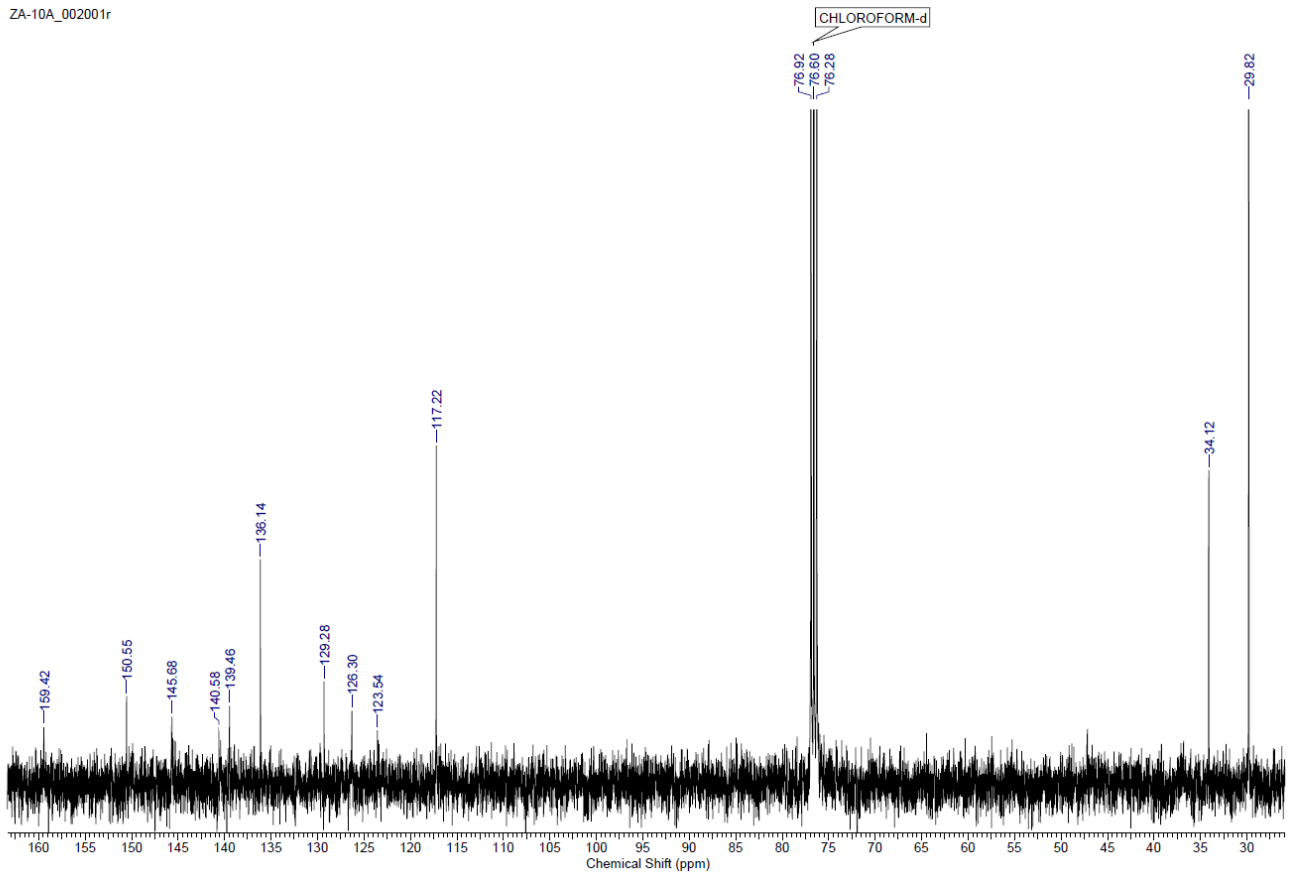


Figure S11. The ^{13}C NMR spectrum of the compound **1′**.

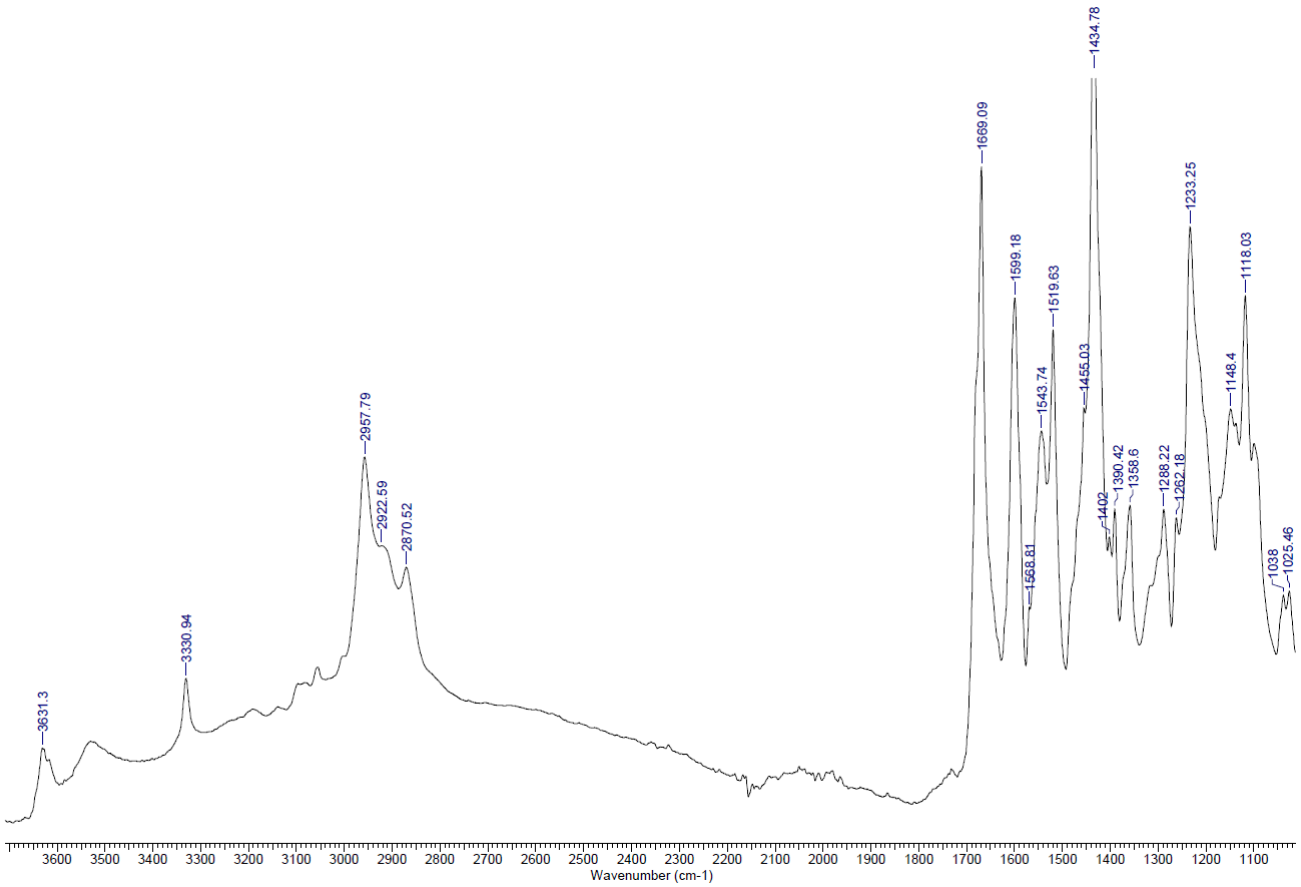


Figure S12. The IR spectrum of the compound **1′**.

3-[N-(3,5-Di-tert-butyl-4-hydroxyphenyl)carbamoyl]pyridinium chloride **2′**: red solid, mp 221-224°C, yield was quantitative, ^{1}H NMR (400 MHz, DMSO-d^6) δ: 1.38 (s, 18H, Bu^t), 6.89 (br s, 1H, OH), 7.61 (s, 2H, CH-Ar), 8.01 (dd, 1Y, CH-Py, ^{3}J 13.5 Hz, ^{3}J 2.5 Hz), 8.85 (d, 1H, ^{3}J 8.1 Hz), 8.97 (br d, 1H, ^{3}J 4.8 Hz), 9.38 (s, 1H, CH-Py), 10.57(s, 1H, NH(C=O)). ^{13}C NMR (100 MHz, DMSO-d^6, 25°C) δ: 30.39 (C(<u>C</u>H$_3$)$_3$), 34.81 (<u>C</u>(CH$_3$)$_3$), 117.53 (C2-Ar), 125.99 (CH-Py), 131.05 (C1-Ar), 133.02 (CH-Py), 139.85 (C3-Ar), 141.83 (CH-Py), 143.57 (CH-Py), 146.05 (CH-Py), 150.60 (C4-Ar), 161.04 (NH(<u>C</u>=O)). IR (KBr, ν/cm^{-1}): ν(OH) 3627.5; ν(N-H) 3241.3; ν(C-H) 2953.5-2870.5; ν(C=O) 1666.7; ν(C-C, Py, Ph) 1630.0, 1602.6; ν(N-C=O) 1538.0; 1434.3; 1358.6; 1291.1; 1231.8; 1154.7; 1135.9; 1117.6. Found (%): C, 66.18; H, 7.53; N, 7.73. Calc. for $C_{20}H_{27}N_2O_2Cl$(%): C, 66.19; H, 7.51; N, 7.72.

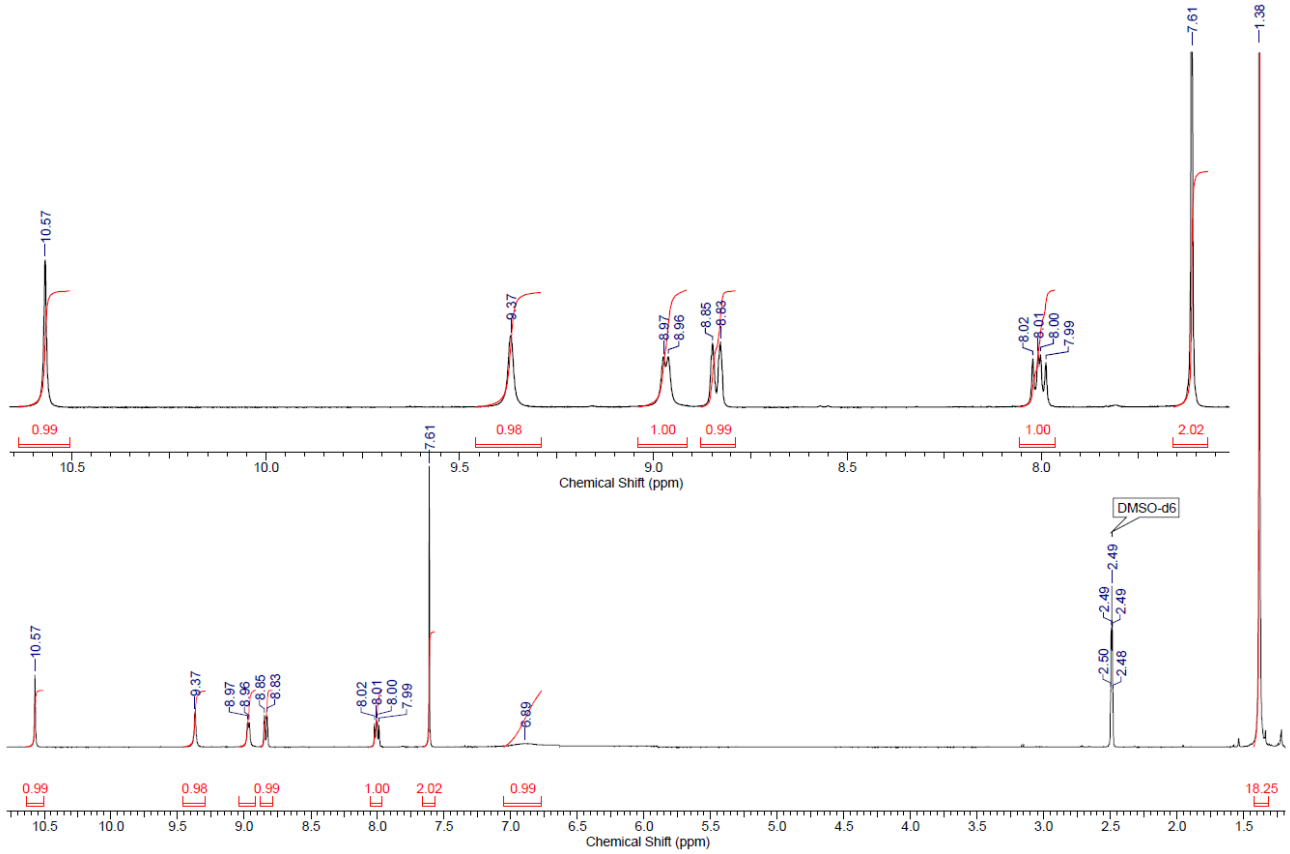


Figure S13. The ^{1}H NMR spectrum of the compound **2′**.

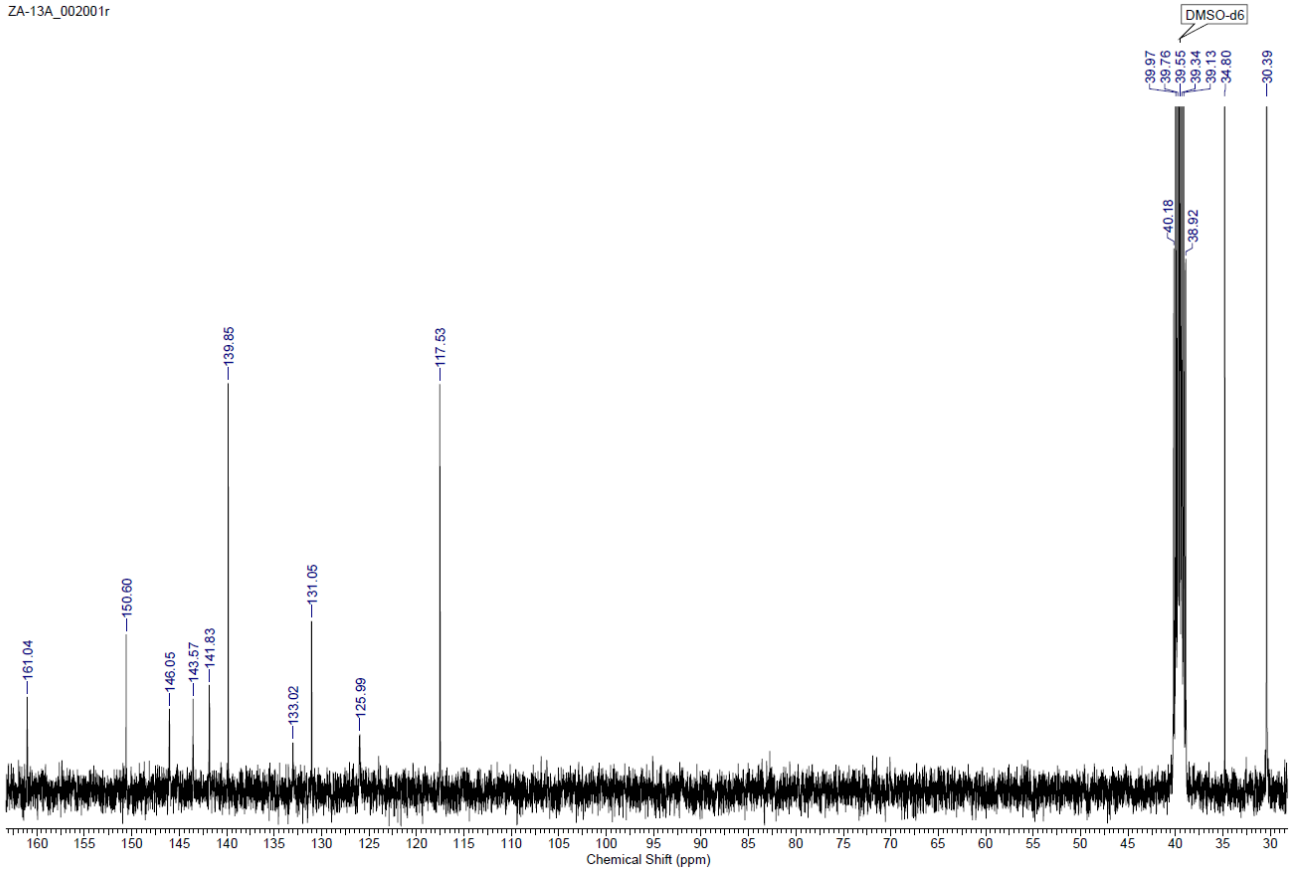


Figure S14. The ^{13}C NMR spectrum of the compound **2′**.

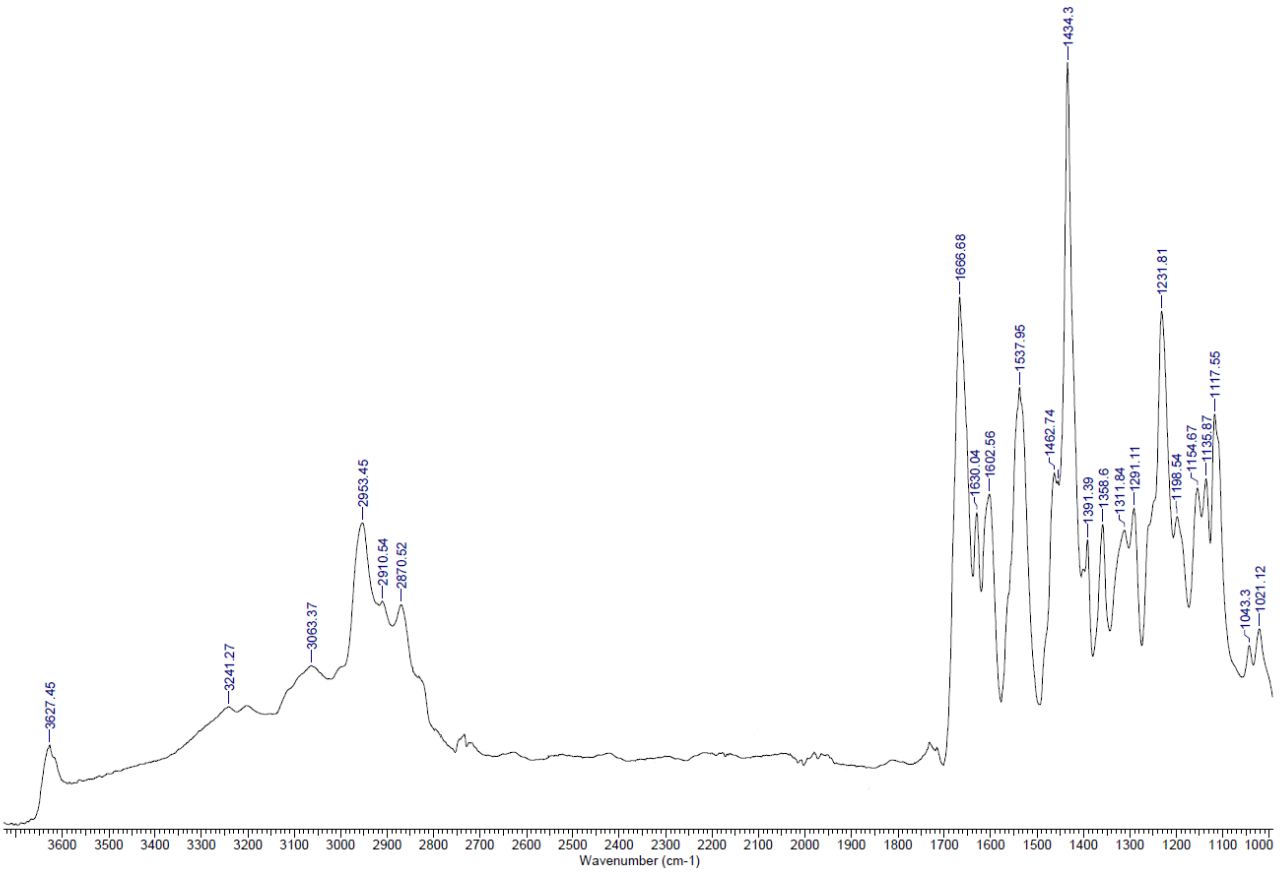


Figure S15. The IR spectrum of the compound **2′**.

4-[N-(3,5-Di-tert-butyl-4-hydroxyphenyl)carbamoyl]pyridinium chloride **3′**: red solid, mp 241-244°C, yield was quantitative, ^{1}H NMR (400 MHz, DMSO-d^6) δ: 1.38 (s, 18H, Bu^t), 6.96 (br s, 1H, OH), 7.62(s, 2H, CH-Ar), 8.30 (d, 2H, CH-Py, ^{3}J 6.4 Hz), 9.01 (d, 2H, CH-Py, ^{3}J 6.4 Hz), 10.60 (s, 1H, NH(C=O)). ^{13}C NMR (100 MHz, DMSO-d^6, 25°C) δ: 30.36 (C(<u>C</u>H$_3$)$_3$), 34.81 (<u>C</u>(CH$_3$)$_3$), 117.64 (C2-Ar), 124.11 (CH-Py), 130.86 (C1-Ar), 139.85 (C3-Ar), 145.12 (CH-Py), 149.88 (CH-Py), 150.80 (C4-Ar), 161.54 (NH(<u>C</u>=O)). IR (KBr, ν/cm^{-1}): ν(OH) 3627.5; ν(N-H) 3233.1; ν(C-H) 2953.0-2868.6; 2394.7; 2113.1; ν(C=O) 1663.3; ν(C-C, Py, Ph) 1608.9; ν(N-C=O) 1557.7; 1499.9; 1435.3; 1362.5; 1231.8; 1202.4; 1149.9; 1119.0. Found (%): C, 66.04; H, 7.48; N, 7.87. Calc. for $C_{20}H_{27}N_2O_2Cl$(%): C, 66.19; H, 7.51; N, 7.72.

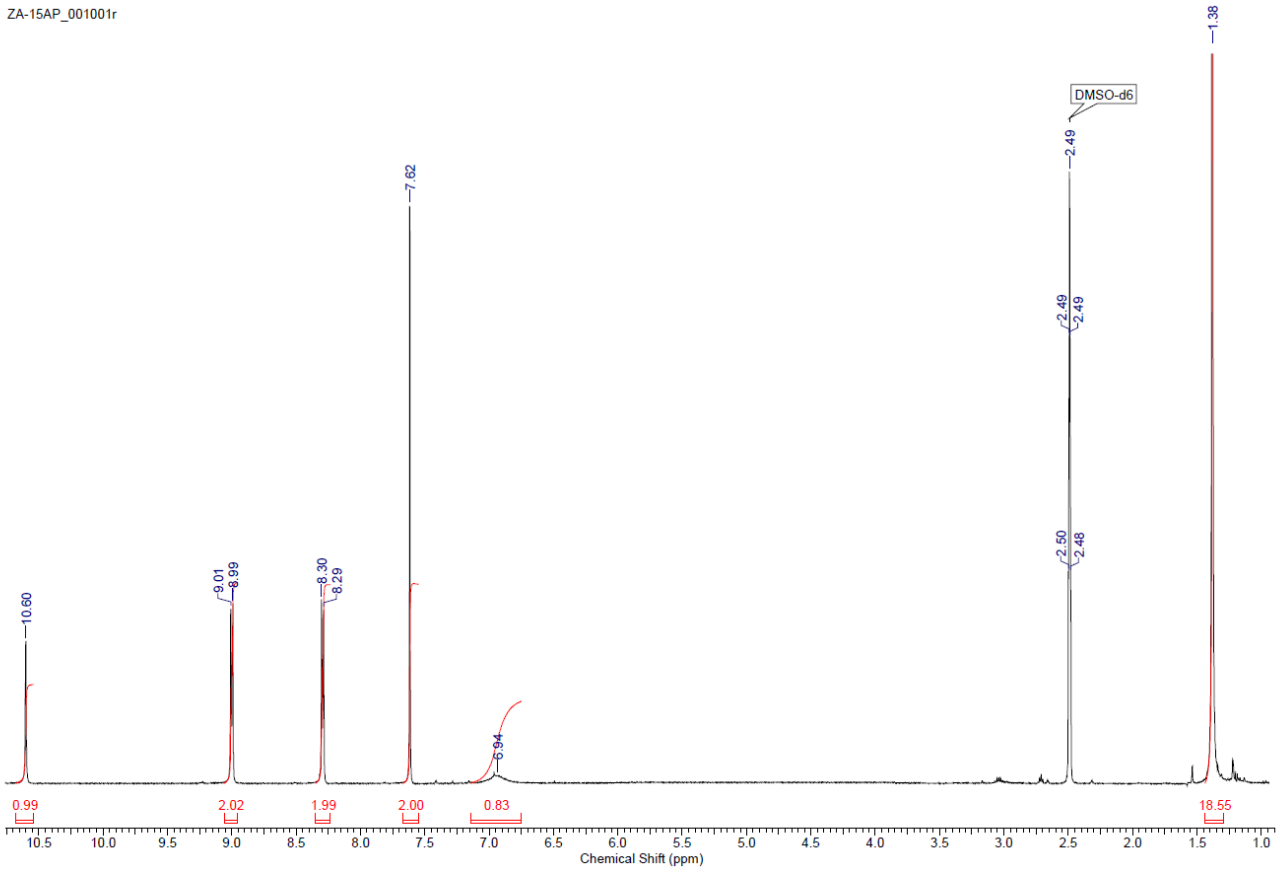


Figure S16. The ^{1}H NMR spectrum of the compound **3′**.

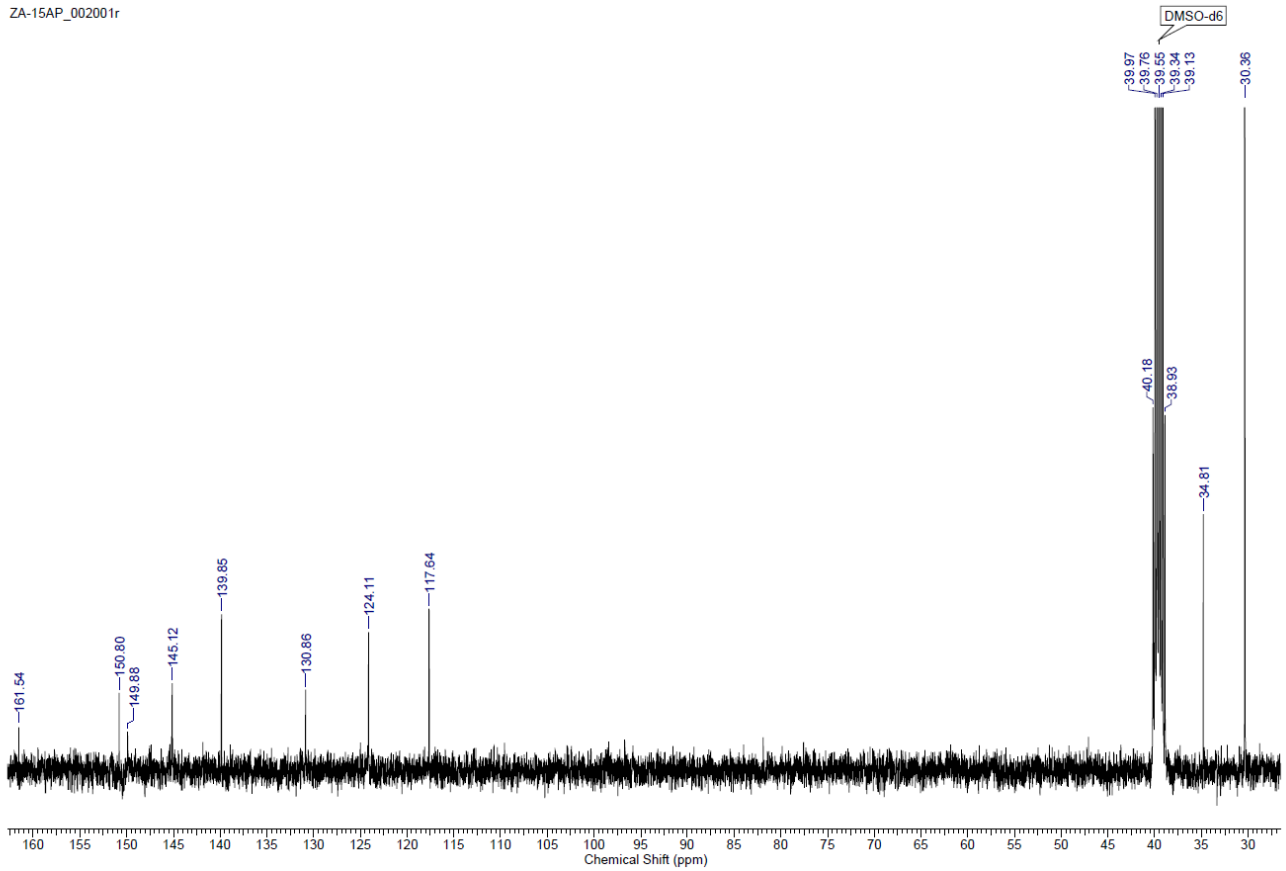


Figure S17. The ^{13}C NMR spectrum of the compound **3′**.

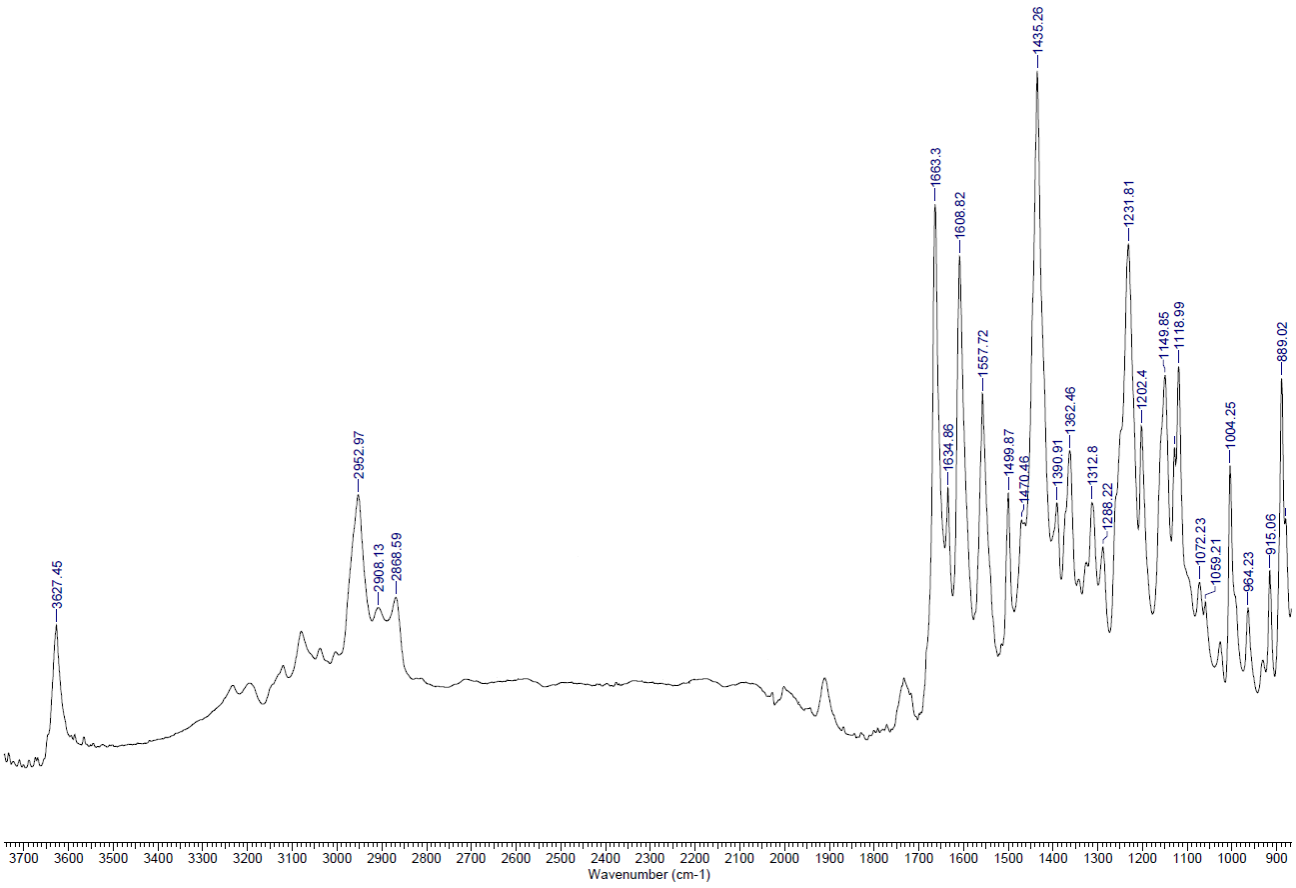


Figure S18. The IR spectrum of the compound **3′**.

Crystallographic data collection and structure determination

The X-ray diffraction analysis was carried out on D8 QUEST diffractometer (MoKα; Bruker, Germany). All calculations were carried using SHELXTL PLUS software. The structure was solved by dual methods and refined by least squares methods in the anisotropic approximation for non-hydrogen atoms.

Antioxidant assays

DPPH assay

The activity of the synthesized compounds as radical scavengers was estimated via colorimetric assay using stable radical 2,2-diphenyl-1-picrylhydrazyl. DPPH (0.75 mL, 0.2 mM) and solution of the test compound in EtOH (0.75 mL, its concentration was varied from 0.01 to 0.2 mM) were mixed in quartz cuvettes. After 30 min incubation at 20°C, the reduction in optical density at $\lambda = 517$ nm was measured using Multiscan GO plate spectrophotometer (Thermo Fisher Scientific, USA; this device was also used in the below tests). Based on the obtained data, the corresponding EC_{50} value (concentration required to obtain a 50% antioxidant effect) was calculated for each of the studied compounds. The experiment was repeated at least 3 times.

CUPRAC-test

To assess the reduction capability of the tested compounds, the known procedure was slightly modified: 50 μL of $CuCl_2$ solution in H_2O (0.01 M), 50 μL of neocuproine solution in MeOH (7.5 mM), 50 μL of ammonium acetate buffer (pH 7.0), along with 50 μL of the tested compound solution in MeOH (0.5 mM) were added in 96-well plate (NUNC, USA). Trolox (0.1, 0.125, 0.5, 1.0, 1.5 and 2.0 mM) was used as a reference compound. After 30 min incubation at 20°C, the optical density at $\lambda = 450$ nm was measured. Increase in the absorbance of the reaction mixture (A_1) in comparison with control (A_0) indicated the reduction capability of the test compound. The obtained data were expressed in Trolox equivalent antioxidant capacity (TEAC), which was calculated as TEAC = A_1/A_0. The experiment was repeated at least 3 times.

Lipoxygenase inhibition assay

The lipoxygenase activity was also evaluated spectrophotometrically: concentration of linoleic acid oxidation products was determined via optical density measured at $\lambda = 234$ nm ($\varepsilon_{234} = 25000\ M^{-1}\ cm^{-1}$). The analyzed solution contained 30 μL of borate buffer (pH 9.0), 100 μL of linoleic acid solution in borate buffer (0.45 mM), 3 μL of the studied compound solution in DMSO (1 mM). The reaction was initiated by the addition of 17 μL of soybean lipoxygenase solution in borate buffer (500 U). The inhibition rate I (%) of lipoxygenase was determined as $I = (v_0/v_0') \times 100\%$, where v_0 and v_0' are the initial rates of enzymatic reaction in the presence and absence (control) of the studied compound. In turn, it was calculated as $v_0 = \Delta C/\Delta t = \Delta A/(\Delta t \times \varepsilon) = tg\alpha/(\Delta t \times \varepsilon)$, where ΔA is the difference between A_1 and A_0 (absorbance of the reaction mixture in the presence of the tested compound 5 min after the beginning of the reaction and absorbance of the control solution, respectively). The experiment was repeated at least 3 times.

Lipid peroxidation of rat brain homogenate

The antioxidant properties of the synthesized compounds were evaluated by their ability to inhibit lipid peroxidation (LP) of the rat brain homogenate (1500 g). To assess Fe^{2+}-induced LP, the test compound or equal volume of DMSO, as well as 10 mM H_2O_2 or 0.5 mM $FeSO_4 \times 7H_2O$ were incubated with the rat brain homogenate for 1 h at 37°C. The degree of LP was assessed by the formation of trimetine complexes of secondary products with 2-thiobarbituric acid (TBA) according to the following procedure. After 30 (or 60) min incubation in a quartz cuvette, 1 mL of the studied suspension was mixed with 2 mL of the solution containing 2.6 mM TBA, 15% (w/v) of trichloroacetic acid, 0.25N HCl and 0.2% (w/v) of butylated hydroxytoluene. The samples were placed in the dark and stored at room temperature for 12–18 h. Then, they were centrifuged to remove protein precipitate and the differential absorbance (A_{532}–A_{630}) was measured in the supernatant.

Study of radioprotective activity

Commercial preparations of DNA isolated from salmon or sturgeon milt and depolymerized by ultrasound (Derinat®; (0.25–0.5) × 10^6 Da (≈400–800 base pairs); Technomedservice, Russia) and polyethylene glycol (PEG, 4000 Da; Paneko, Russia) were used. Their solutions and were prepared on the basis of aqueous saline buffer containing 10^{-2} M Na_2HPO_4 and 0.3 M NaCl (pH ≈7.4). Then the solutions of the nucleic acid of 1.5 mL volume each (its concentration in base pairs was 7.48 × 10^{-5} M – to calculate it, we used the value of extinction coefficient of ε_{260} = 13200 M^{-1} cm^{-1}) were irradiated with kilovoltage X-rays (LNK-268 machine (Diagnostika-M, Russia); 80 kVp, 250 Gy/min) within the dose range from 0 to 1000 Gy in the absence and presence of the compounds **1A**–**3A**. After that the cholesteric liquid-crystalline dispersions (CLCDs) were prepared on their basis: 800 μL of the irradiated solution were mixed with 400 μL of 60 wt.% solution of PEG so that the final concentration of the polymer in the system was 20 wt.%. The resulting mixtures were intensively stirred and kept at room temperature for at least 1.5 h.

The circular dichroism (CD) spectra were measured in quartz cuvettes of 1.0 cm × 1.0 cm (Hellma, Germany) using SKD-2 dichrograph (Institute of Spectroscopy of Russian Academy of Sciences, Russia). The CD spectra were presented as a dependence of the difference between the absorption of left- and right-hand polarized light $\Delta A = A_L - A_R$ on the wavelength λ.

Biological studies

Human colon carcinoma (HCT116) and non-tumor fibroblast immortalized with hTERT (WI38) cell lines were obtained from American Type Culture Collection (USA). The cells were cultivated at 37°C in humidified atmosphere with 5% CO_2. For this Dulbecco's modified Eagle's medium (DMEM; Paneko, Russia) supplemented with 10% fetal bovine serum (HyClone, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Paneko, Russia) was used.

The cytotoxicity of the compounds was assessed via classic formazan conversion assay (MTT-test). The cells were plated in 96-well plates (NUNC, USA; 5 × 10^3 cells in 190 μL of the culture medium per well). After 24 h of incubation at the abovementioned conditions, the cells were treated with the studied compounds up to their maximum concentration of 50 μM. Doxorubicin (Teva, Netherlands) was used as a control compound. After 72 h of incubation, 5 mg/ml of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, USA) was added to each well, then the plates were incubated for another 3 h under the same conditions. Then, the culture medium was removed, the precipitate with formazan crystals was dissolved in 100 μL DMSO, and the optical densities at λ = 570 nm were measured using Multiscan FC plate spectrophotometer (Thermo Fisher Scientific, USA). The percentage of the survived cells for each dose of the studied compounds was calculated as the ratio of the average optical density in the wells with this dose to the average optical density of the control wells (the latter values were taken as 100%). The experiment was repeated at least 3 times.