

Phenazine *N,N'*-dioxide scaffold as selective hypoxic cytotoxin pharmacophore. Structural modifications looking for further DNA topoisomerase II-inhibition activity

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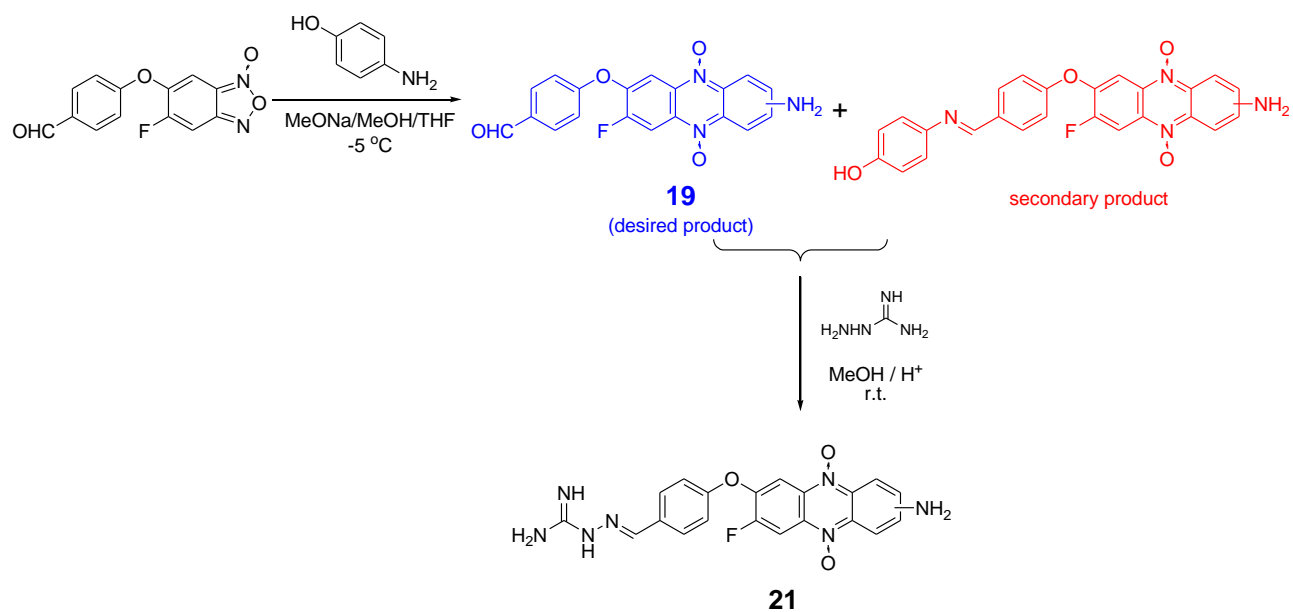


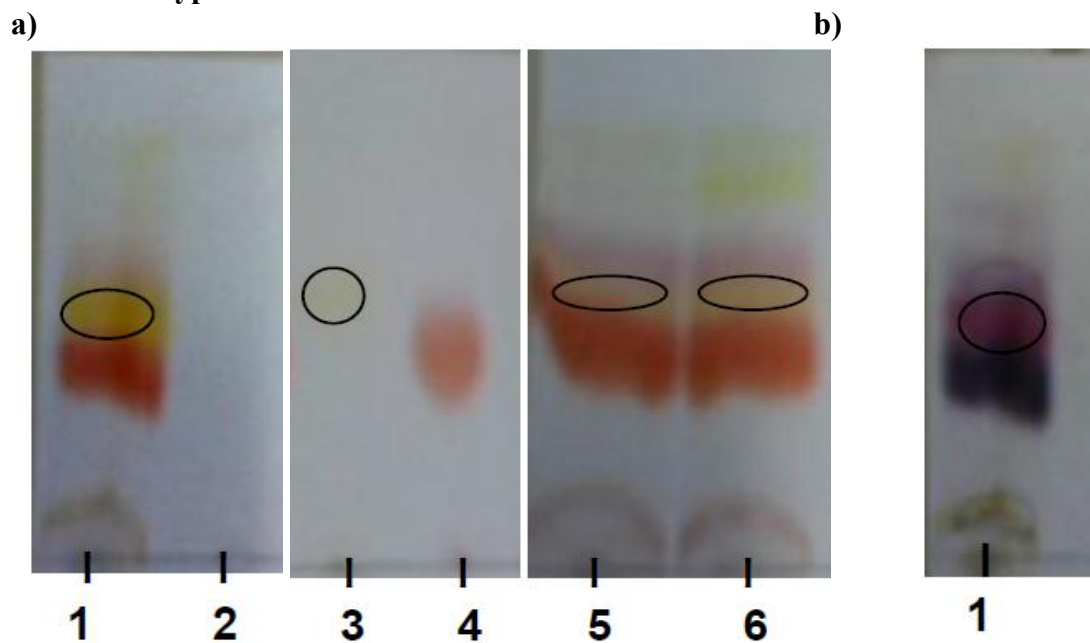
Figure S1. Synthetic scheme and results in the preparation of PDO **19**. This compound was obtained mixed with the corresponding imine (secondary product). This mixture treated with aminoguanidine yield PDO **21**.

Table S1. Proportions of 7- and 8-Isomers of Studied Compounds.

| compound | 7:8 isomers ratio^a |
|-----------------|--------------------------------------|
| 6 | 50 : 50 |
| 7 | 55 : 45 |
| 8 | 50 : 50 |
| 9 | 53 : 47 |
| 10 | 51 : 49 |
| 11 | 44 : 56 |
| 12 | 52 : 48 |
| 13 | 65 : 35 |
| 14 | 59 : 41 |
| 15 | 56 : 44 |
| 16 | 55 : 45 |
| 17 | 58 : 42 |
| 18 | 60 : 40 |
| 19 | 60 : 40 ^b |
| 20 | 65 : 35 ^b |
| 21 | 58 : 42 ^b |
| 22 | 60 : 40 ^b |
| 23 | 56 : 44 |
| 25 | 50 : 50 ^b |

^a Determined by ¹H-NMR from the isolated products. ^b 7-fluoro- and 8-fluoro-isomers.

Simulated hypoxia



Simulated normoxia

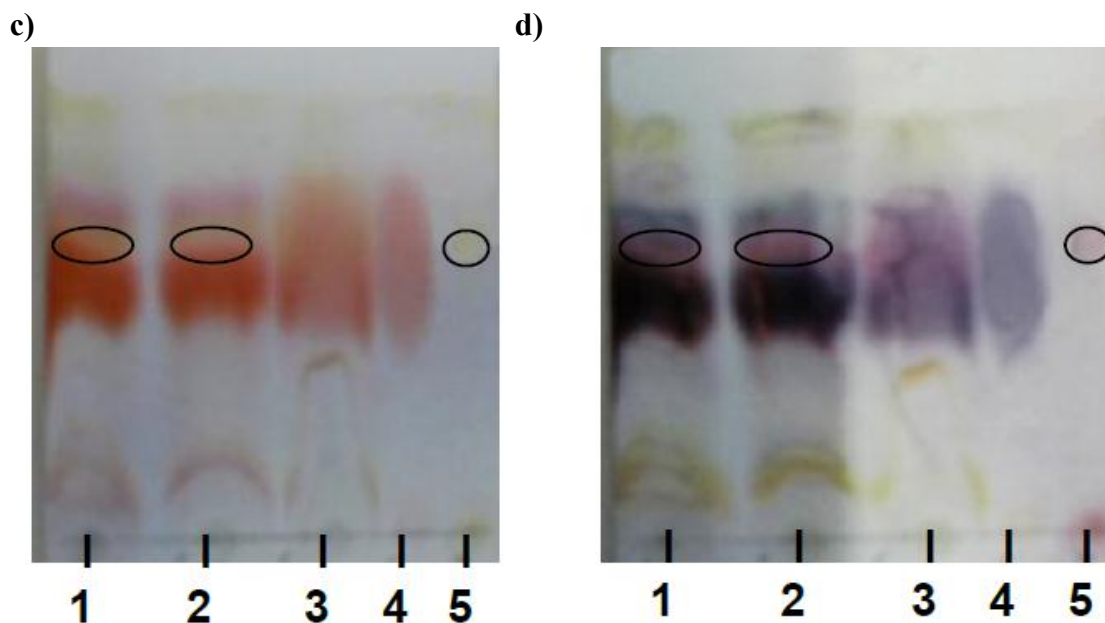


Figure S2. TLC chromatograms (see Material and methods for experimental conditions) taken after 30 min of incubation of PDO 12 with different protein fractions and in different gasification conditions. **Simulated hypoxia:** **a)** Spots without revealed (PDO, orange; phenazine monoxides, yellow); **b)** Run 1 spots visualised by spraying with a solution of *p*-anisaldehyde:H₂SO₄(c):EtOH (95:4:1) followed by heating. Runs: **1.** Incubation with S9 fraction; **2.** Control of enzymatic fractions; **3.** PDO 23; **4.** PDO 12; **5.** Incubation with cytosolic fraction; **6.** Incubation with microsomal fraction. **Simulated normoxia:** **c)** Spots without revealed (PDO, orange; phenazine monoxides, yellow); **d)** Spots visualised by spraying with a solution of *p*-anisaldehyde:H₂SO₄(c):EtOH (95:4:1) followed by heating. Runs: **1-3.** Incubations with cytosolic, microsomal, and S9 fractions; **4.** PDO 12; **5.** PDO 23.

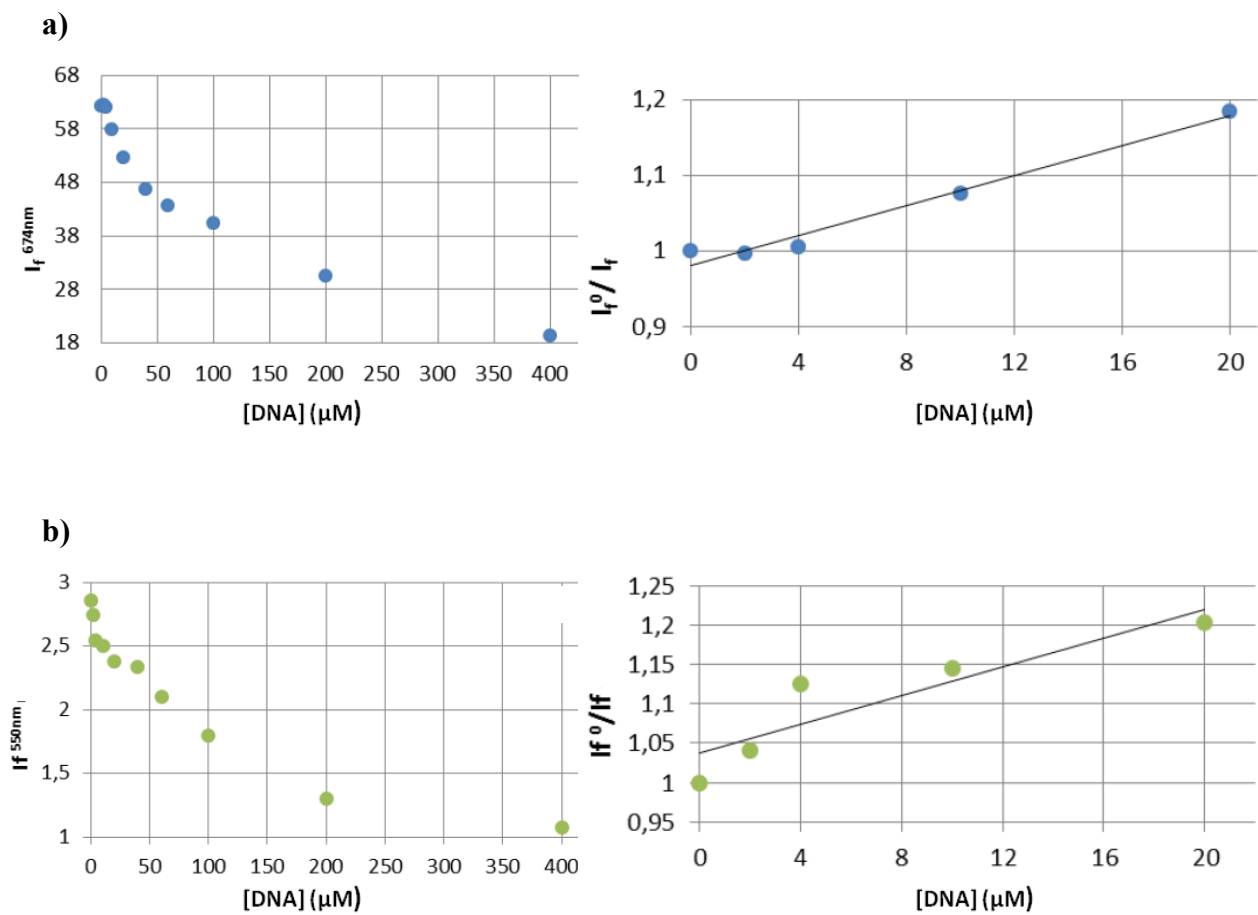


Figure S3. Stern–Volmer quenching plot (right) from the fluorescence data with increasing concentrations of DNA in PBS (left). **a)** For toluidine blue (reference compound). **b)** For PDO 7.

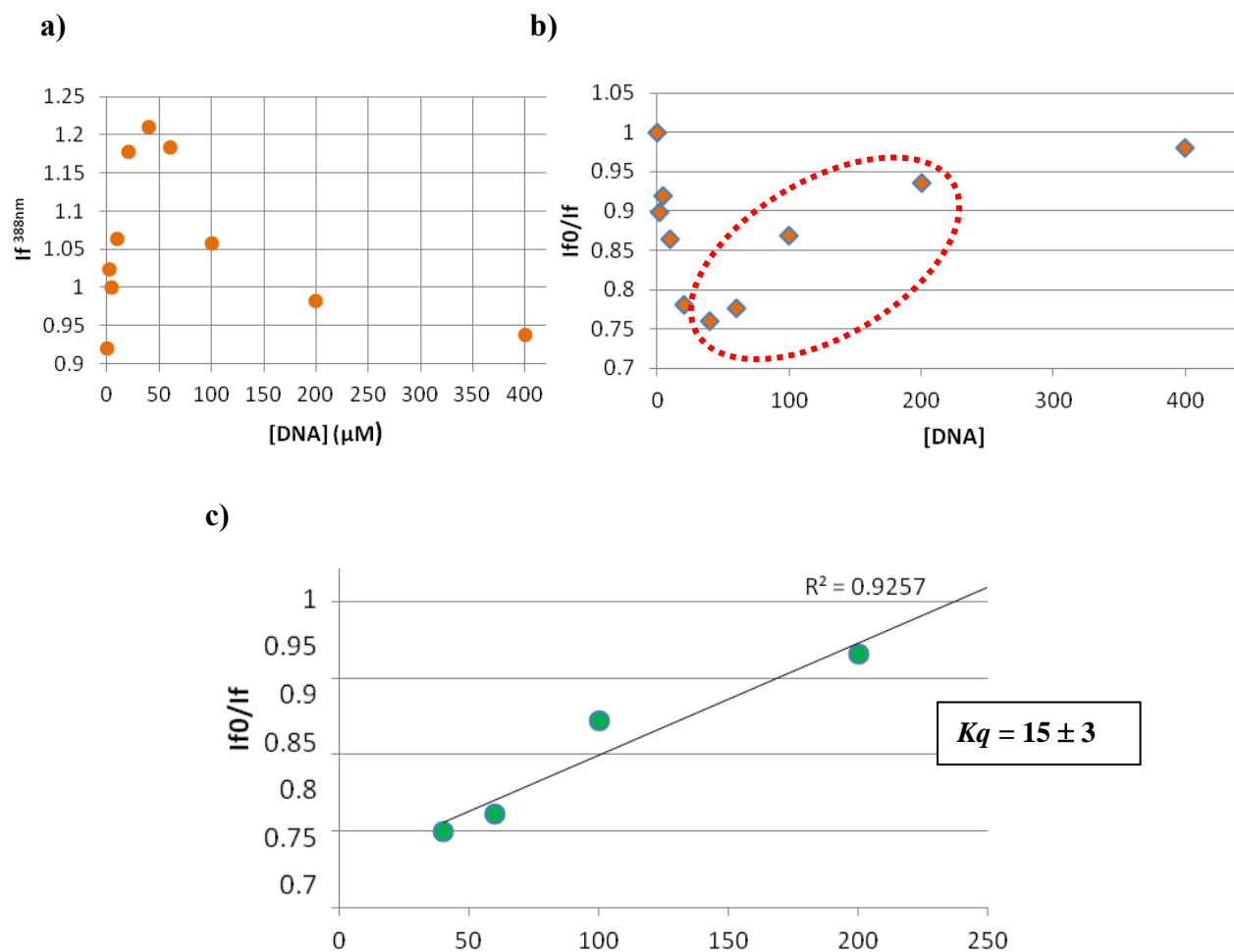
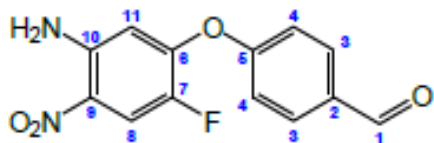


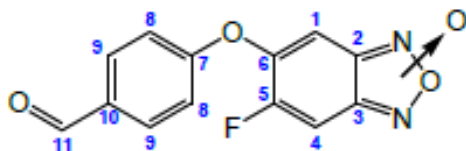
Figure S4. a) Variation of fluorescence of PDO 6 with increasing concentrations of DNA. b) Stern–Volmer quenching plot from the fluorescence data with increasing concentrations of DNA in PBS for PDO 6. The red circle point to the region used to determine the K_p (c). c) K_p determination for PDO 6 in the DNA concentrations range 40-200 μM .

Detailed experimental procedures and spectroscopic characterization of benzofuroxan (IV)



Synthesis of 4-(5-amino-2-fluoro-4-nitrophenoxy)benzaldehyde.

Dried molecular sieves (3 Å) were loaded into the main chamber of a Soxhlet extractor equipment. Then the extractor was placed onto a flask containing a mixture of 4,5-difluoro-2-nitroaniline (4.5 mmol), *p*-hydroxybenzaldehyde (4.1 mmol), anhydrous potassium carbonate (4.1 mmol), 18-crown-6 (4.1 mmol) and dried toluene (70 mL). The mixture was heated at reflux during 2.5 h. After that, the toluene was evaporated *in vacuo* and the residue was dissolved in EtOAc (50 mL) and washed with an aqueous solution of sodium hydroxide (10 %) (3 × 20 mL). The organic phase was dried over anhydrous Na₂SO₄ and evaporated *in vacuo*. The formed solid corresponded to the desired product. Green solid (91 %). ¹H-NMR (CDCl₃+D₂O, 400 MHz) δ (ppm): 10.01 (1H, s, H₁), 8.04 (1H, d, J= 10.8Hz, H₈), 7.94 (2H, d, J= 8.6 Hz, H₃), 7.21 (2H, d, J= 8.6 Hz, H₄), 6.41 (1H, d, J= 6.8 Hz, H₁₁). ¹³C-NMR (CDCl₃, 100 MHz) δ (ppm): 191.8, 160.7, 151.0, 146.5, 137.1, 132.6, 130.1, 127.5, 119.1, 114.5, 108.8. MS, *m/z* (%): 276 (M⁺, 100), 260 (M⁺ - 16, 2), 246 (M⁺ - 30, 10), 230 (M⁺ - [NO₂], 10).



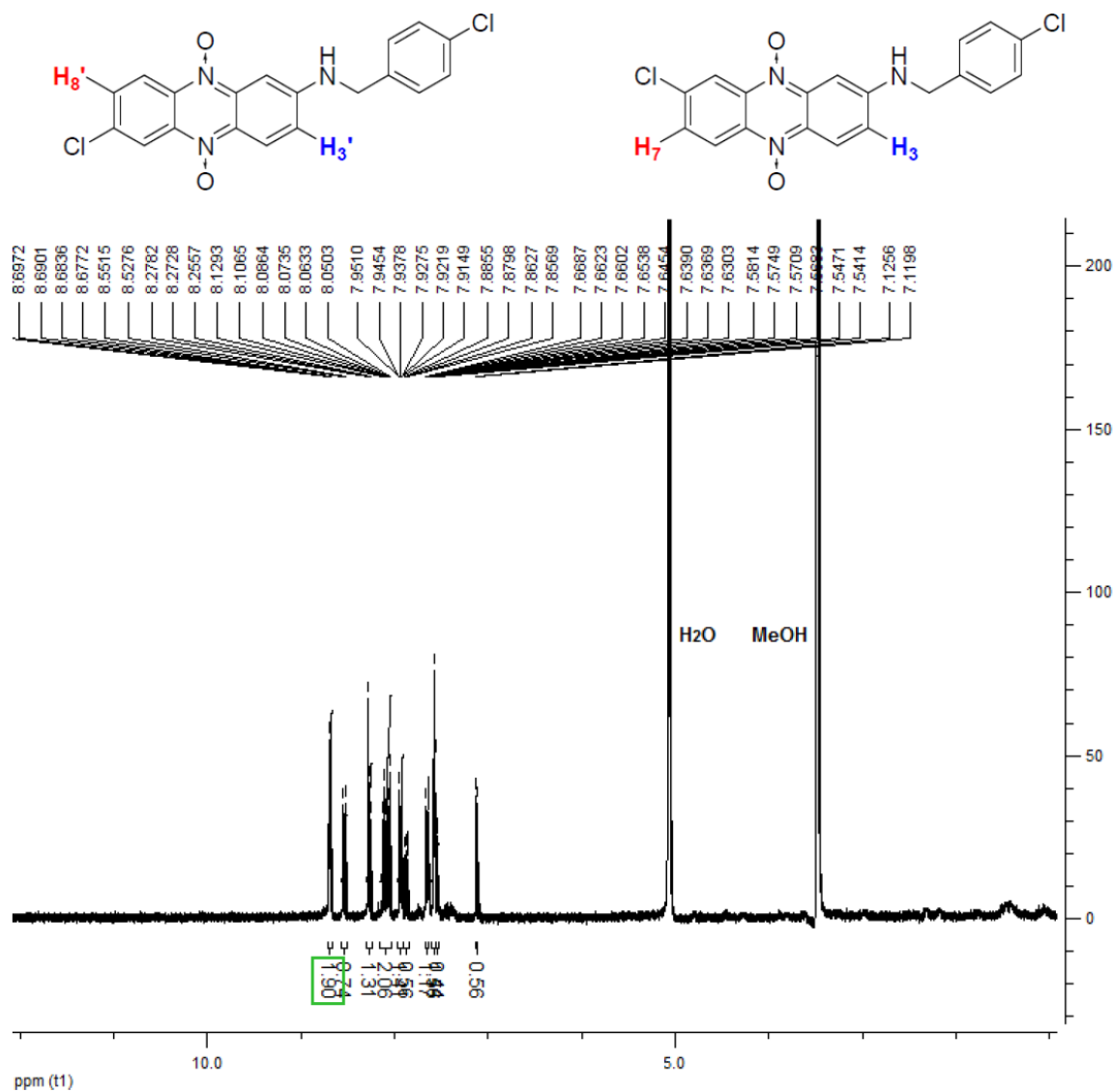
Synthesis of 5-fluoro-6-(4-formylphenoxy)benzo[1,2-c][1,2,5]oxadiazole (IV).

A solution of 4-(5-Amino-2-fluoro-4-nitrophenoxy)benzaldehyde (4.3 mmol) in acetone (19 mL) and glacial acetic acid (12 mL) was cooled at 0 °C and a solution of sodium nitrite (4.3 mmol) in concentrated hydrochloric acid (1.2 mL) and water (3.3 mL) was added dropwise. Then the reaction mixture was stirred during 30 min at 0 °C. After that, a solution of sodium azide (4.3 mmol) and sodium acetate (4.3 mmol) in water (1.1 mL) was added dropwise and the reaction mixture was raised to room temperature and stirred for 2 h. The acetone was evaporated *in vacuo* and the residue was dissolved in EtOAc (50 mL) and washed with an aqueous solution of sodium hydroxide (10 %) (3 × 20 mL). The organic phase was dried over anhydrous Na₂SO₄ and evaporated *in vacuo*. The residue was dissolved in toluene (75 mL) and the solution was heated at reflux for 2 h. The toluene was evaporated *in vacuo*. The residue was purified by chromatography (SiO₂, petroleum ether:EtOAc, 8:2) yielding the desired product as a yellow solid (71 %). ¹H-NMR (CDCl₃, 400 MHz) δ (ppm): 10.05 (1H, s, H₁₁), 8.01 (2H, d, J= 9.1 Hz, H₉), 7.30 (2H, d, J= 9.0 Hz, H₈), 7.45-7.20 (1H, bs, H₁), 7.25-7.05 (1H, bs, H₄). ¹³C-NMR (CDCl₃, 100 MHz) δ (ppm): 190.7, 159.4, 159.3, 149.0, 134.3, 132.7, 118.9, 118.4, 113.1. MS, *m/z* (%): 274 (M⁺, 100), 258 (M⁺ - [O], 15), 228 (M⁺ - [NO₂], 2), 213 (M⁺ - [N₂O₂] - [H], 85).

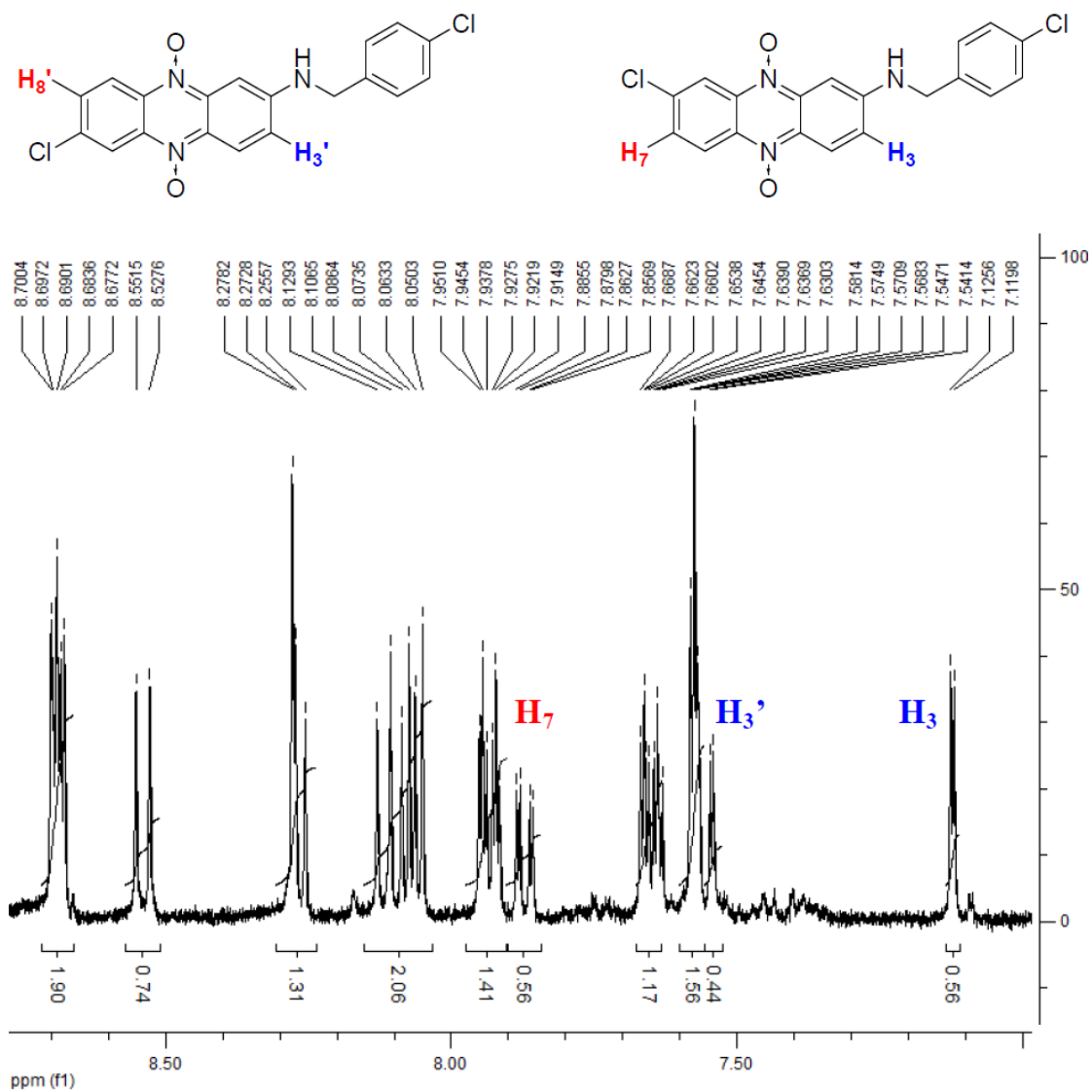
Selected NMR spectra

7(8)-Chloro-2-(4-chlorobenzylamino)phenazine 5,10-dioxide (11)

(7:8 isomers ratio, 44:56)



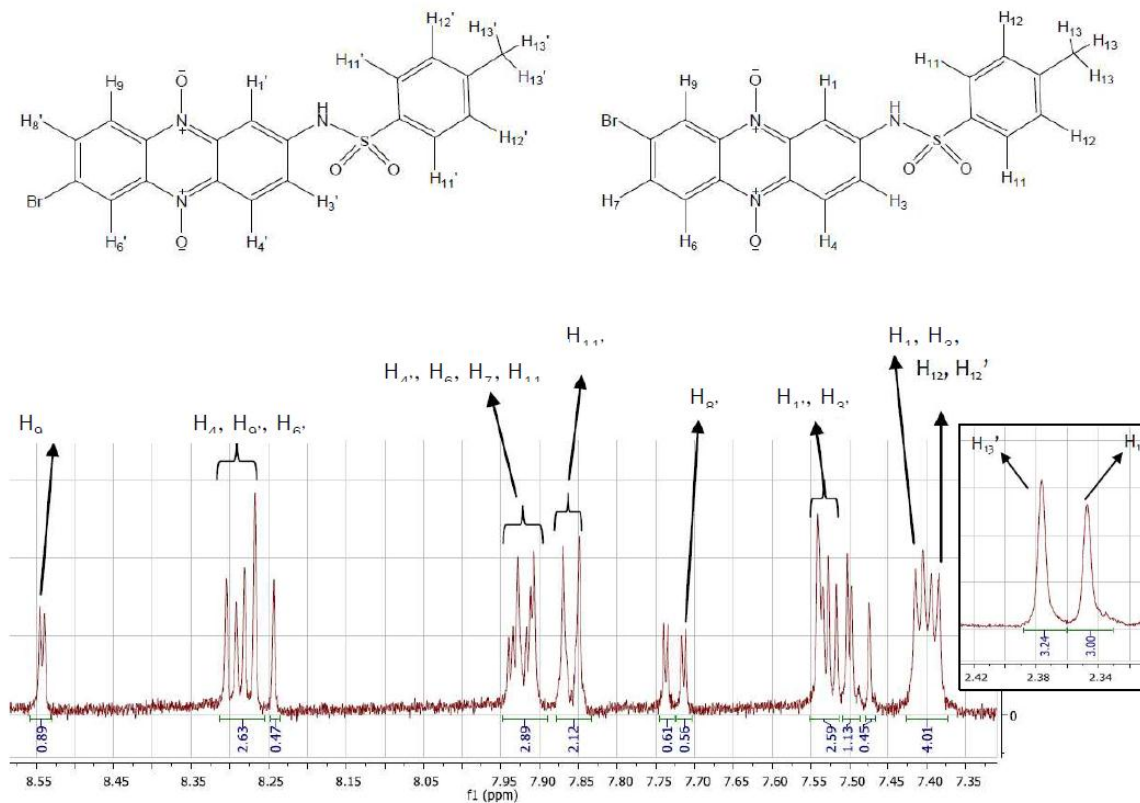
¹H NMR spectrum recorded on a Bruker DPX-400 spectrometer at 298 K and using CD₃OD:D₂O (9:1) as solvent.



Selected region, aromatics, of the proton NMR spectrum recorded on a Bruker DPX-400 spectrometer at 298 K and using $CD_3OD:D_2O$ (9:1) as solvent.

7(8)-Bromo-2-(4-methylphenylsulfonylamino)phenazine 5,10-dioxide (12)

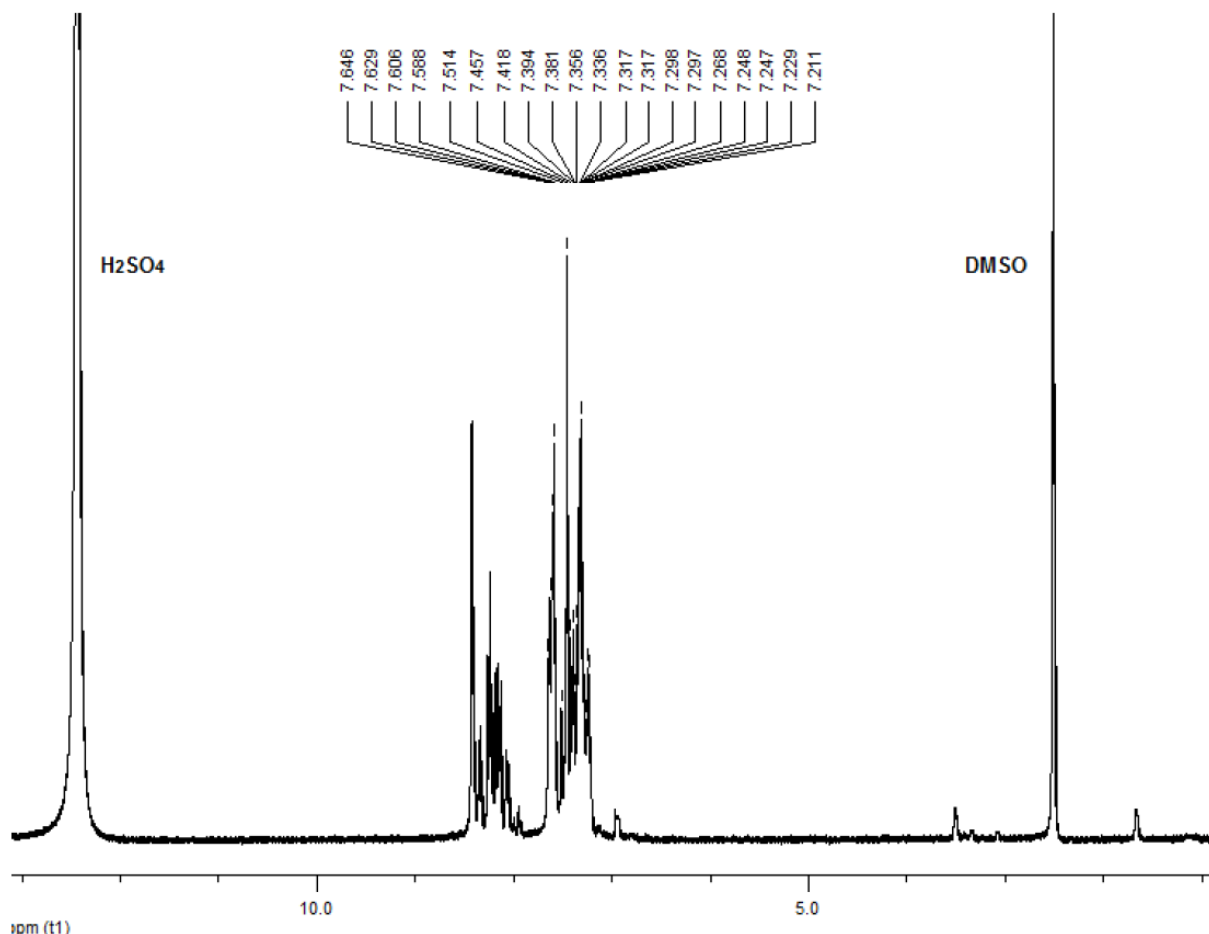
(7:8 isomers ratio, 52:48)



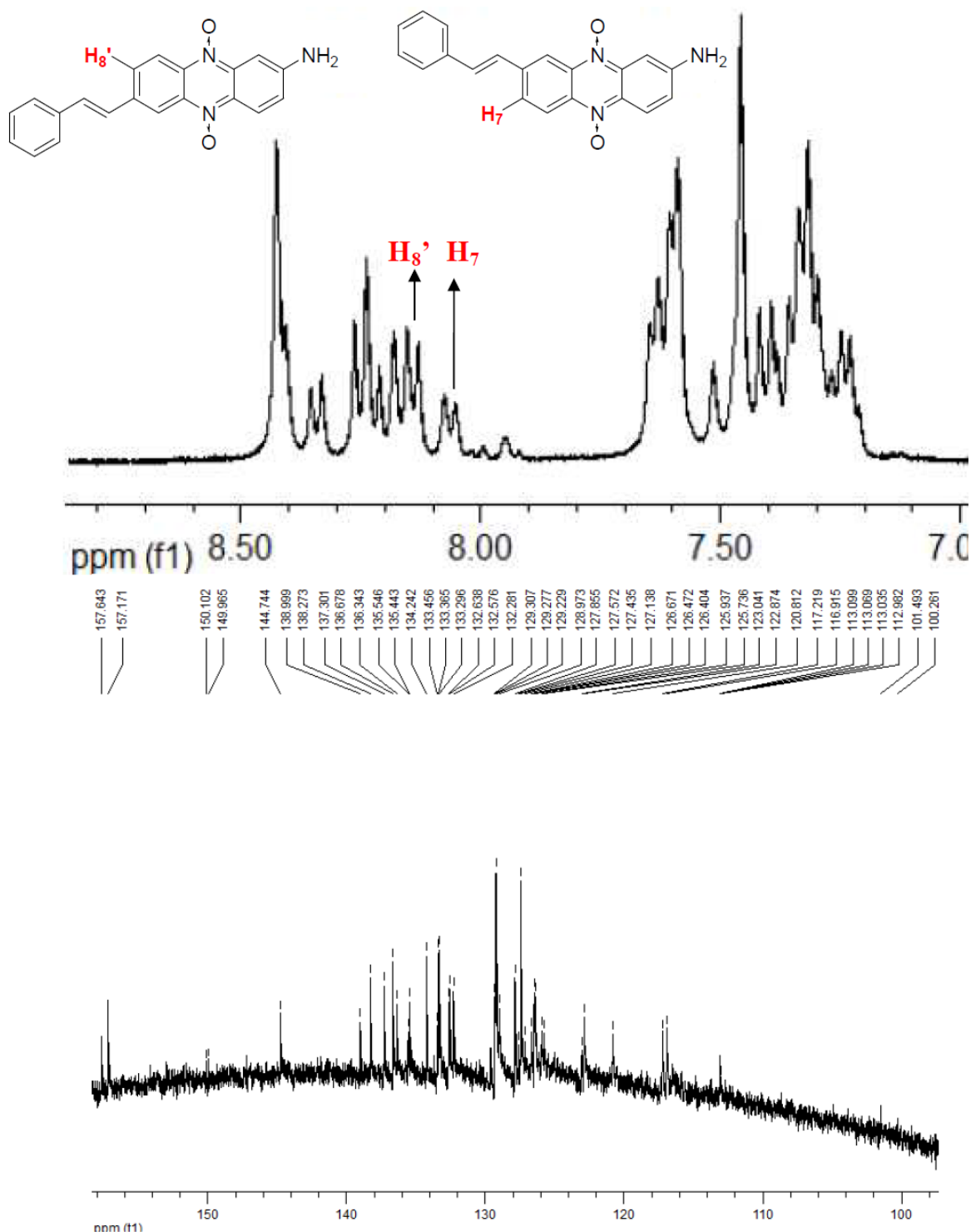
Selected region, aromatics, of the proton NMR spectrum recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO-*d*₆:D₂O (1:1) as solvent. Inset: region of the methyl-protons.

2-Amino-7(8)-(E-2-phenylethynyl)phenazine 5,10-dioxide (13)

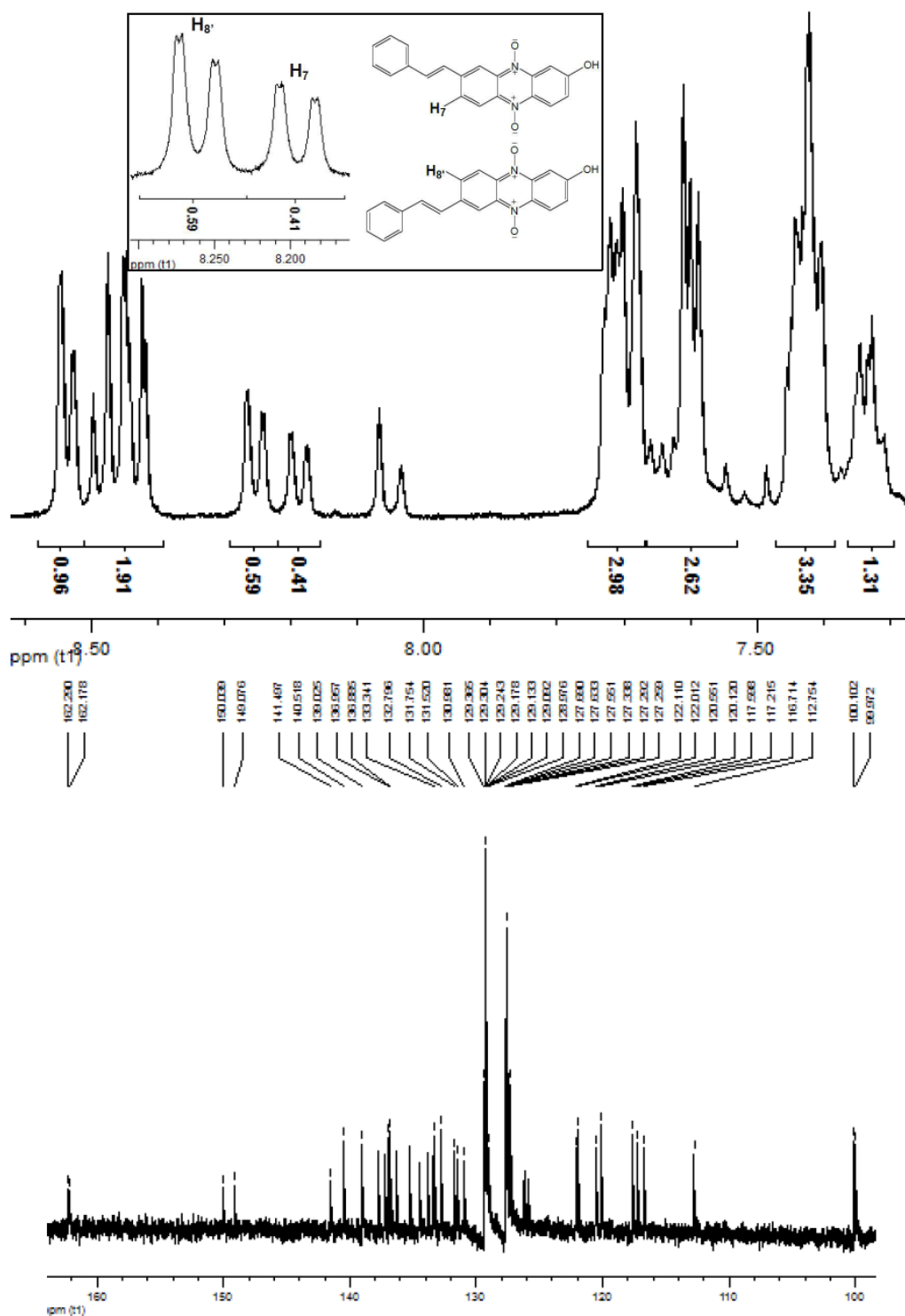
(7:8 isomers ratio, 65:35)



¹H NMR spectrum recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO-*d*₆:D₂SO₄ (9.5:0.5) as solvent.



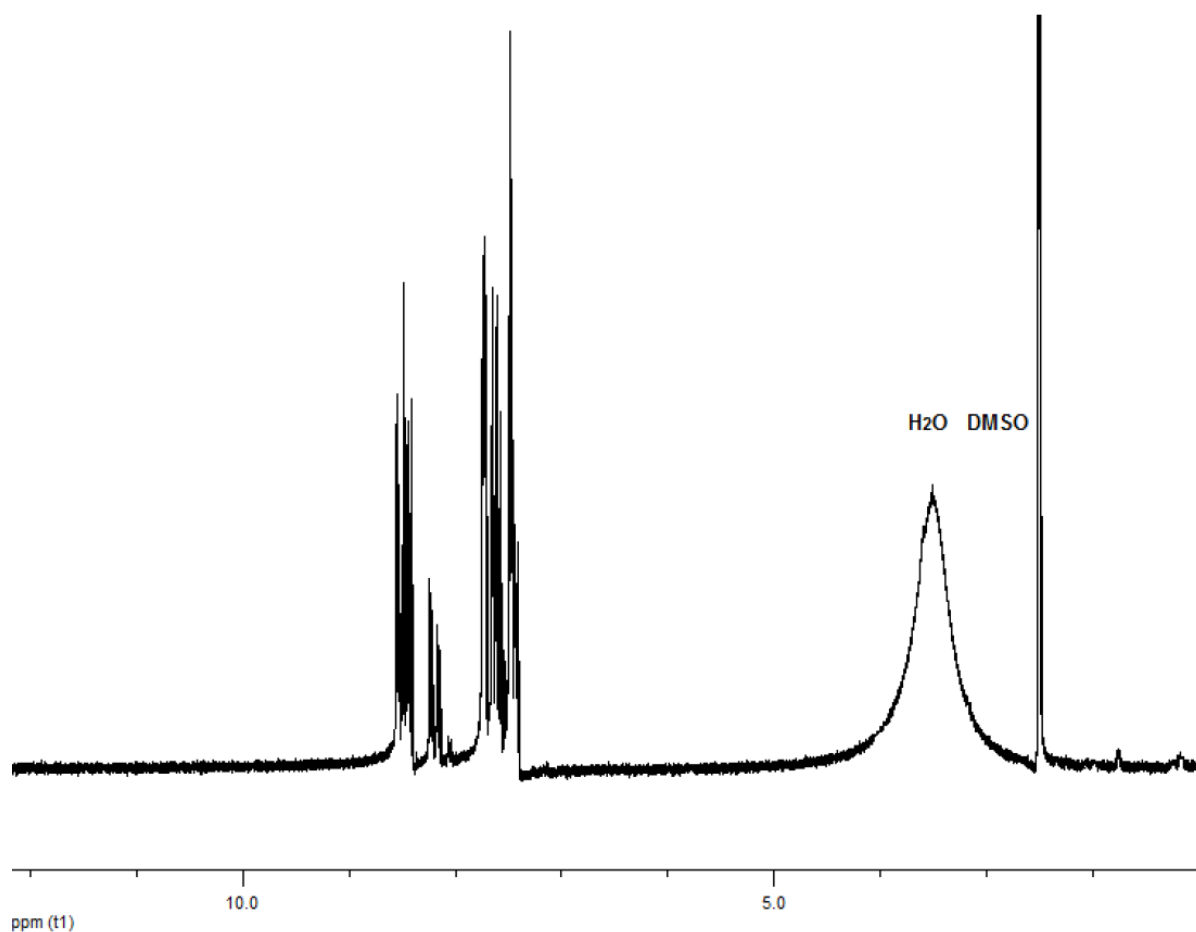
Selected regions, aromatics, of the proton and carbon NMR spectra recorded on a Bruker DPX-400 spectrometer at 298 K and using $DMSO-d_6:D_2SO_4$ (9.5:0.5) as solvent.



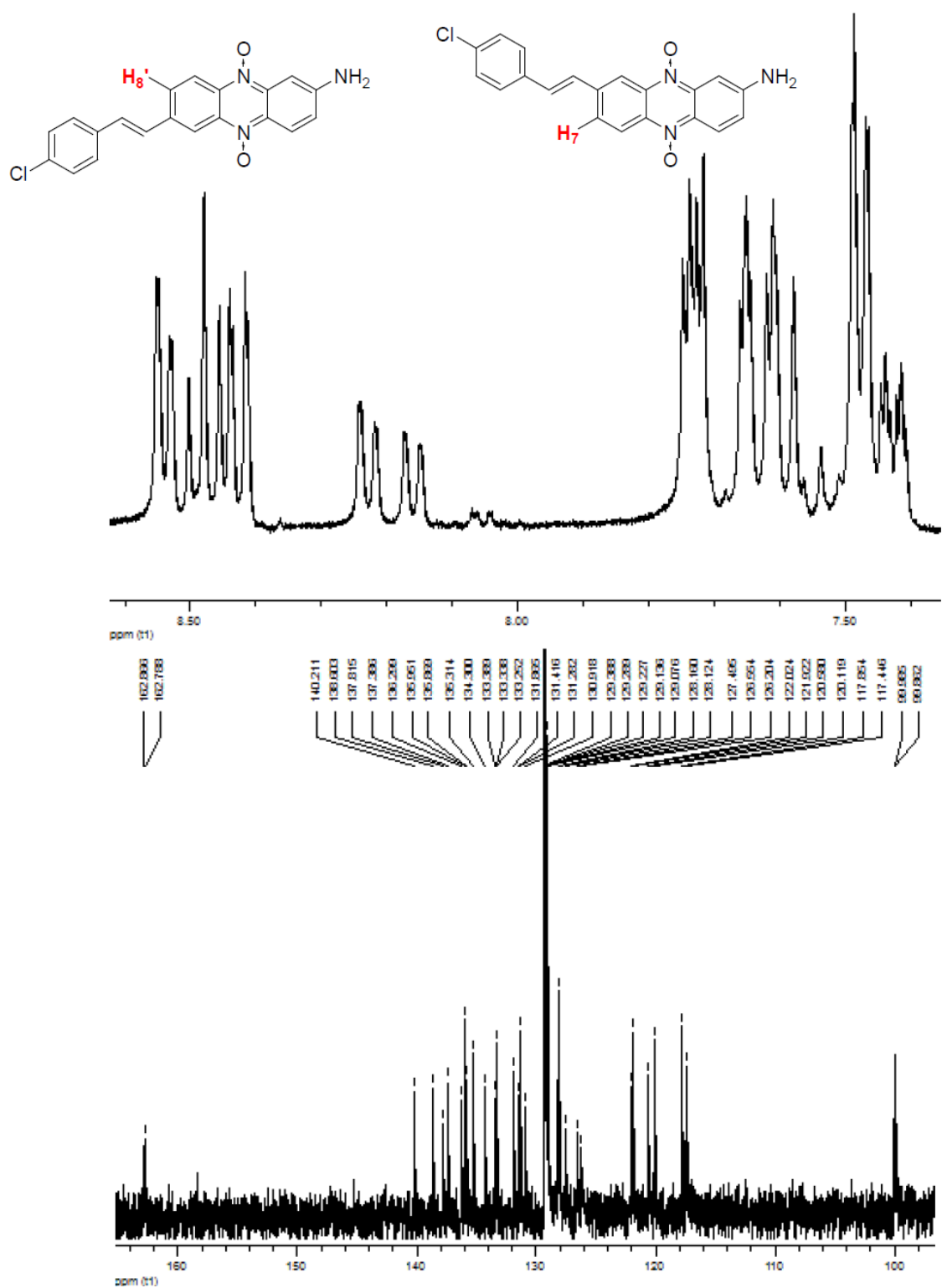
Selected regions, aromatics, of the proton and carbon NMR spectra recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO-*d*₆:D₂O (9.5:0.5) as solvent.. Inset: protons that allowed to determine the ratio of isomers.

2-Amino-7(8)-[E-2-(4-chlorophenyl)ethenyl]phenazine 5,10-dioxide (15)

(7:8 isomers ratio, 56:44)



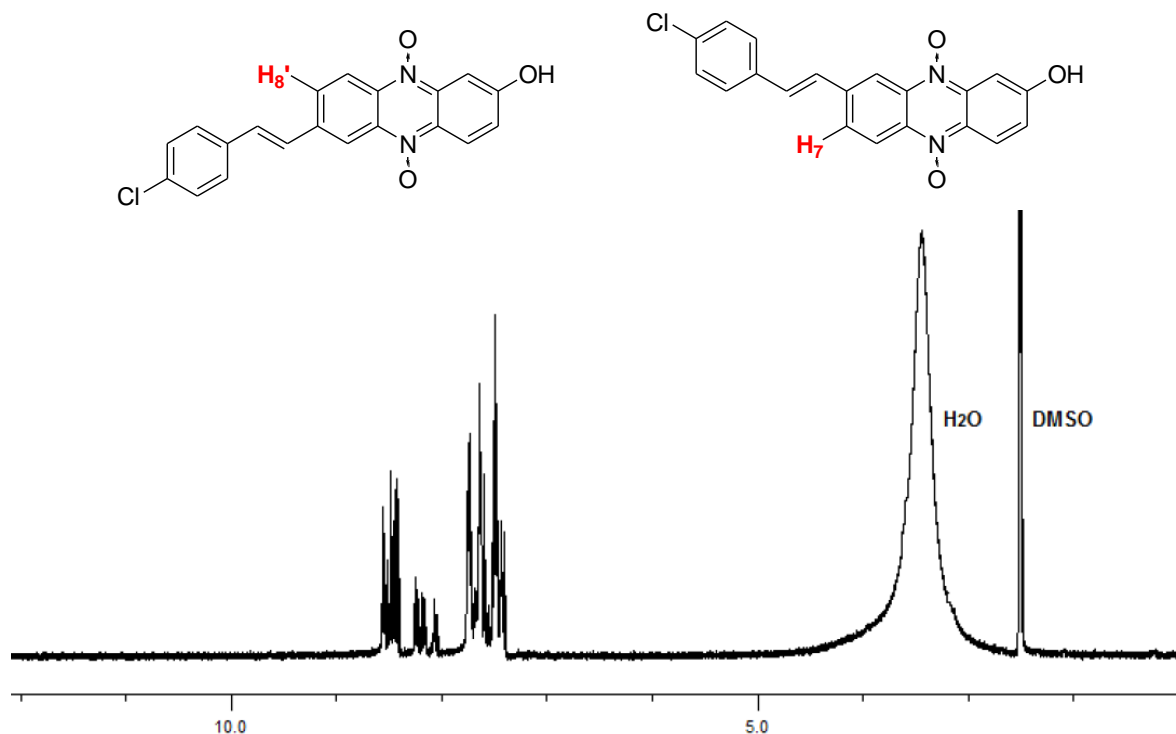
¹H NMR spectrum recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO-*d*₆:D₂O (9.5:0.5) as solvent.



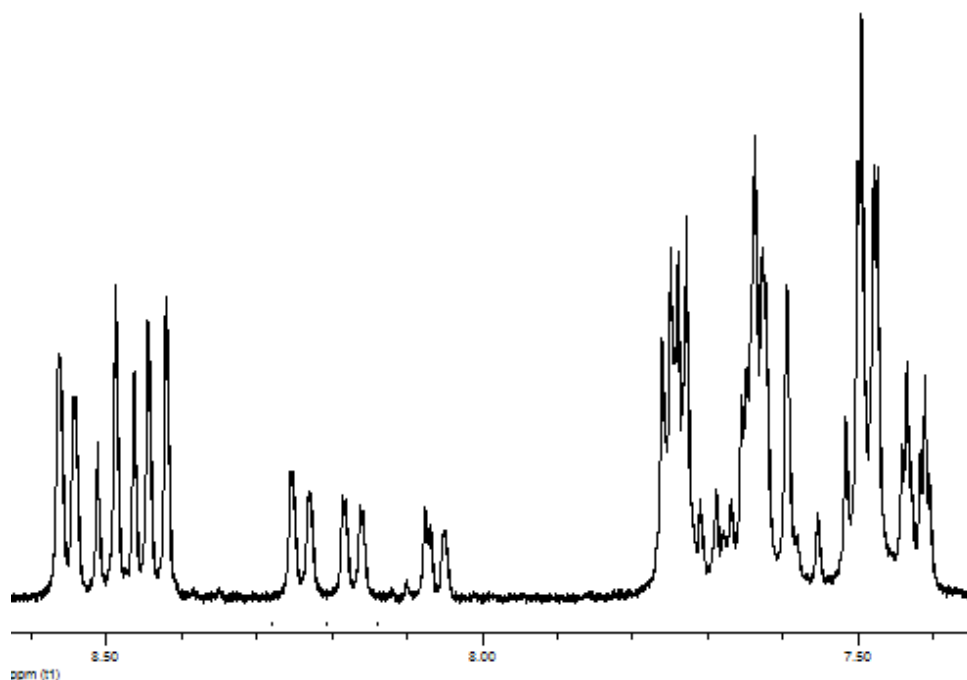
Selected regions, aromatics, of the proton and carbon NMR spectra recorded on a Bruker DPX-400 spectrometer at 298 K and using $DMSO-d_6:D_2O$ (9.5:0.5) as solvent.

7(8)-[E-2-(4-Chlorophenyl)ethenyl]-2-hydroxyphenazine 5,10-dioxide (16)

(7:8 isomers ratio, 55:45)



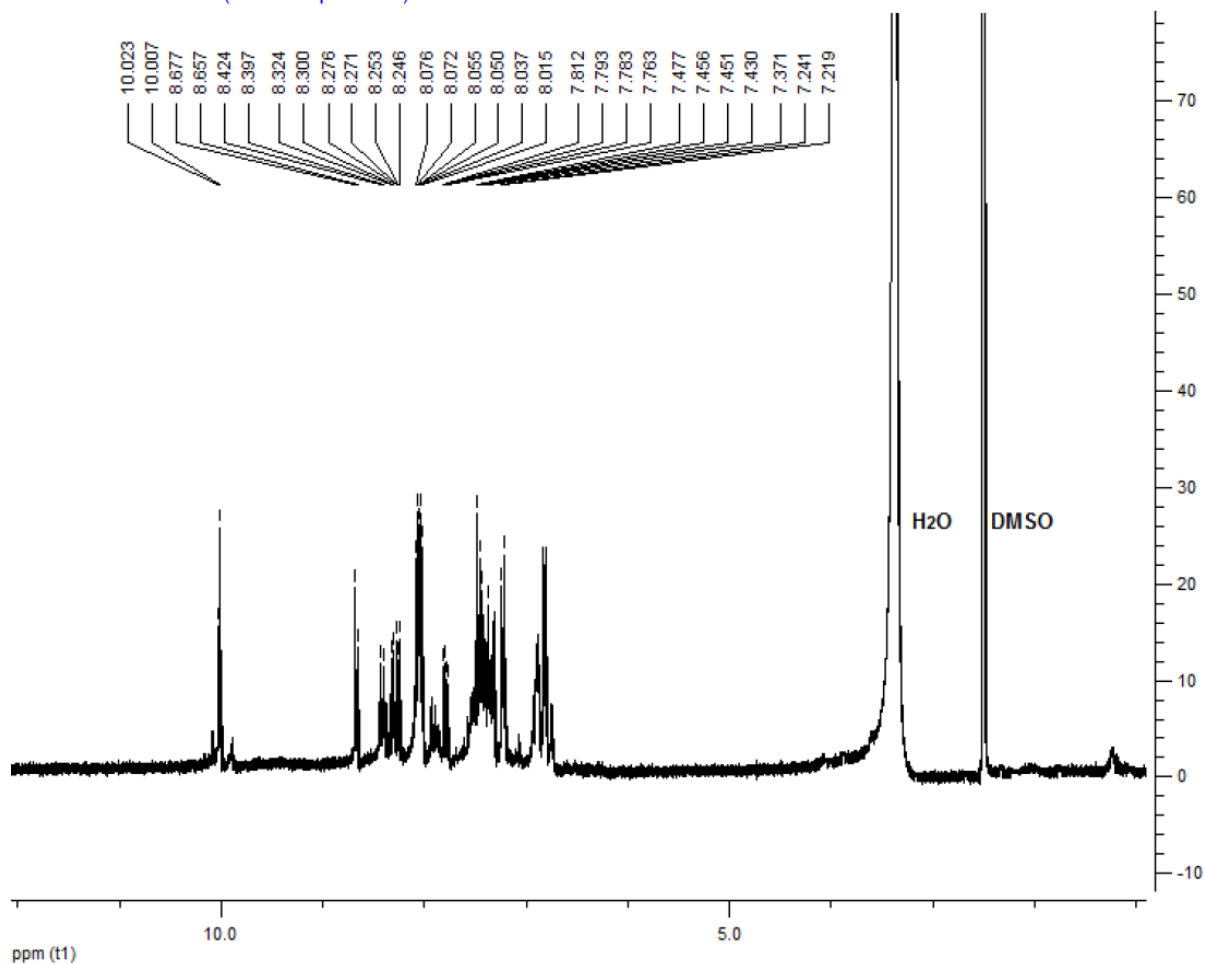
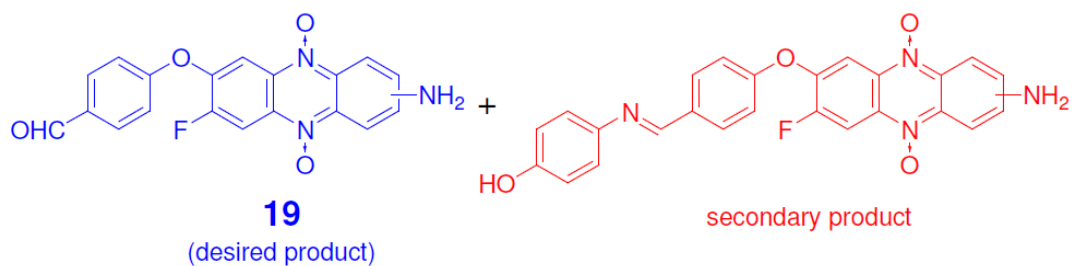
1H NMR spectrum recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO- d_6 :D $_2$ O (9.5:0.5) as solvent.



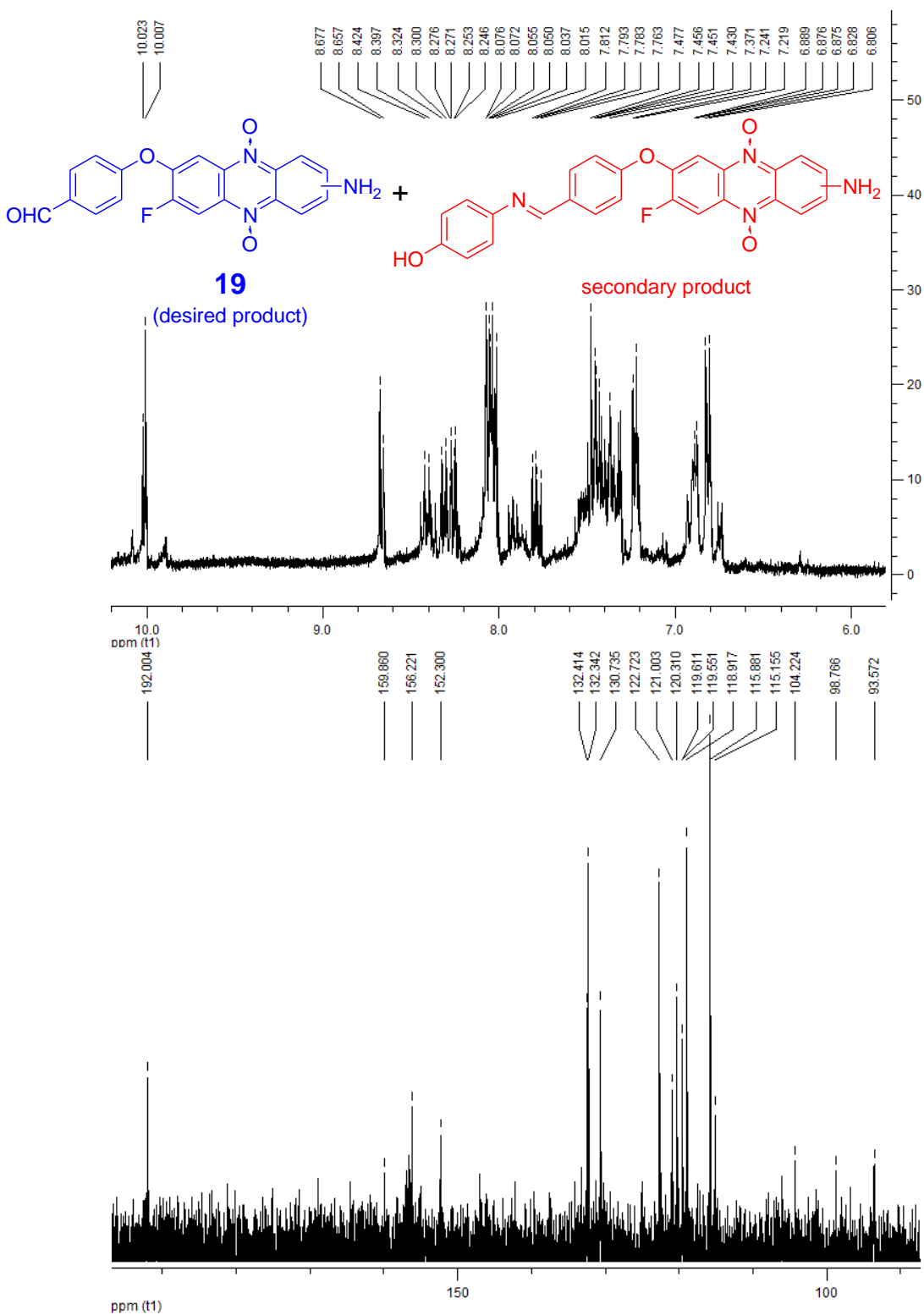
Selected regions, aromatics, of the proton NMR spectrum recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO- d_6 :D $_2$ O (9.5:0.5) as solvent.

2-Amino-7(8)-fluoro-8(7)-(4-formylphenoxy)phenazine 5,10-dioxide (19)

(as mixture of aldehyde and the corresponding imine)



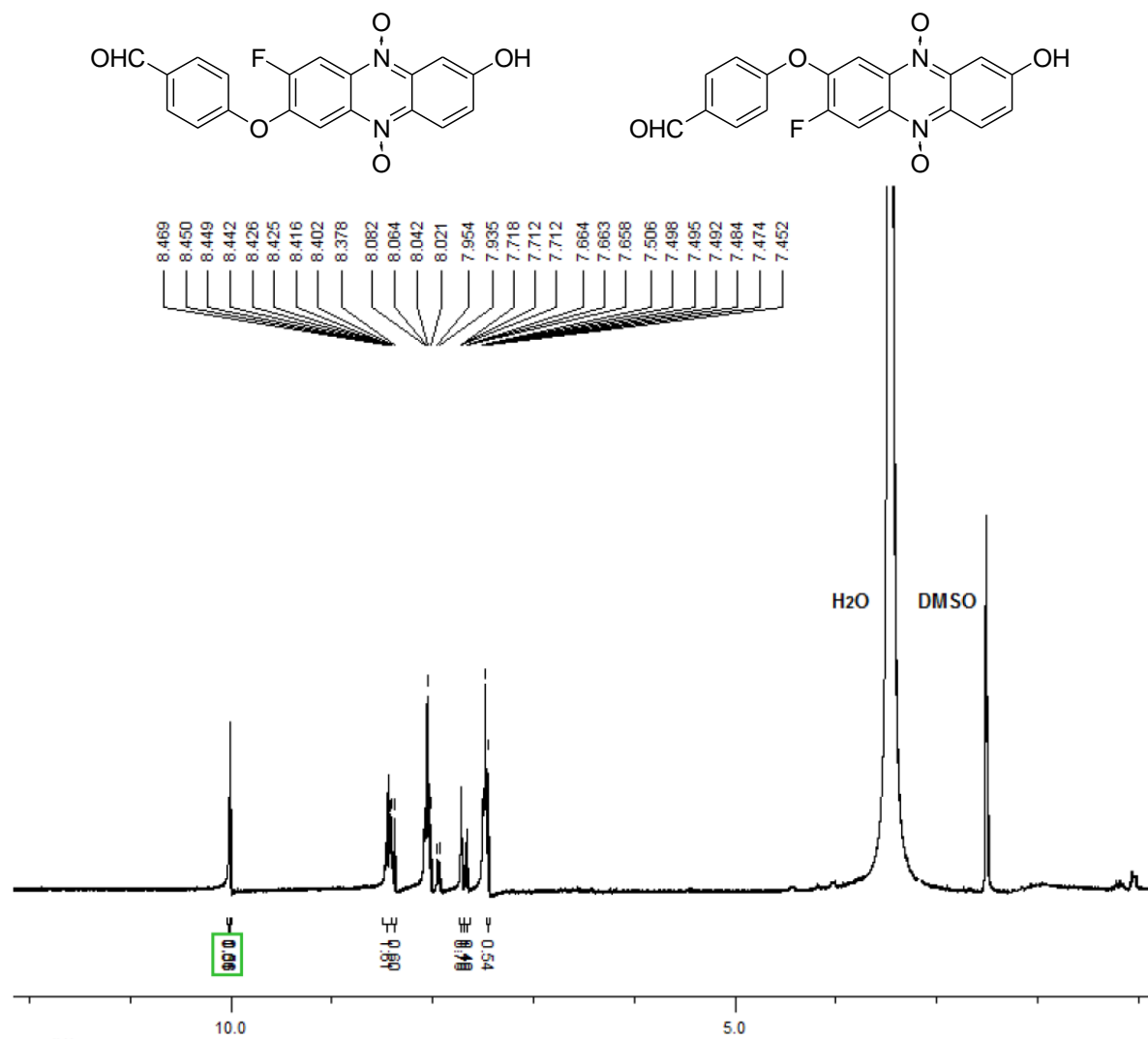
¹H NMR spectrum recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO-*d*₆:D₂O (9.5:0.5) as solvent.



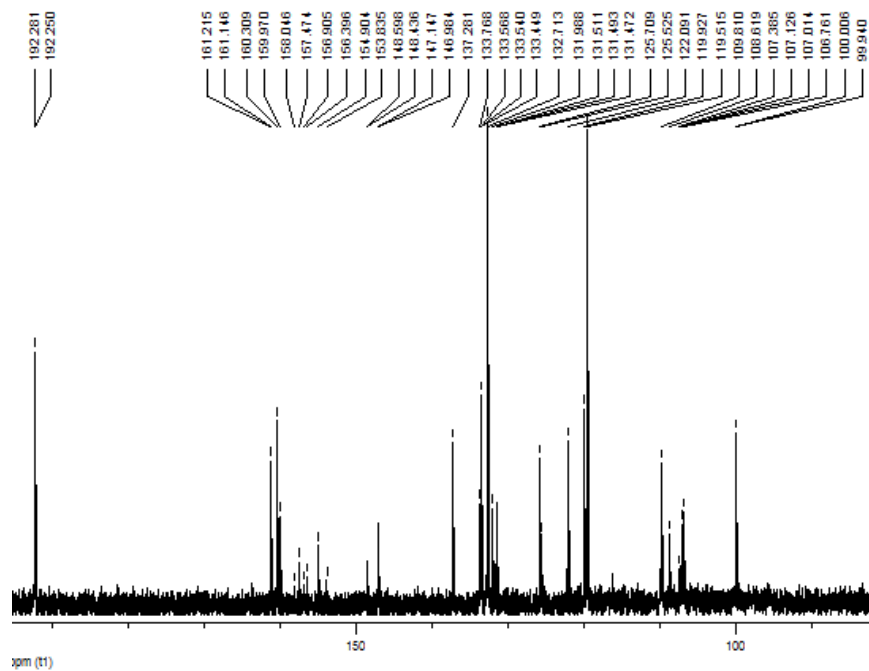
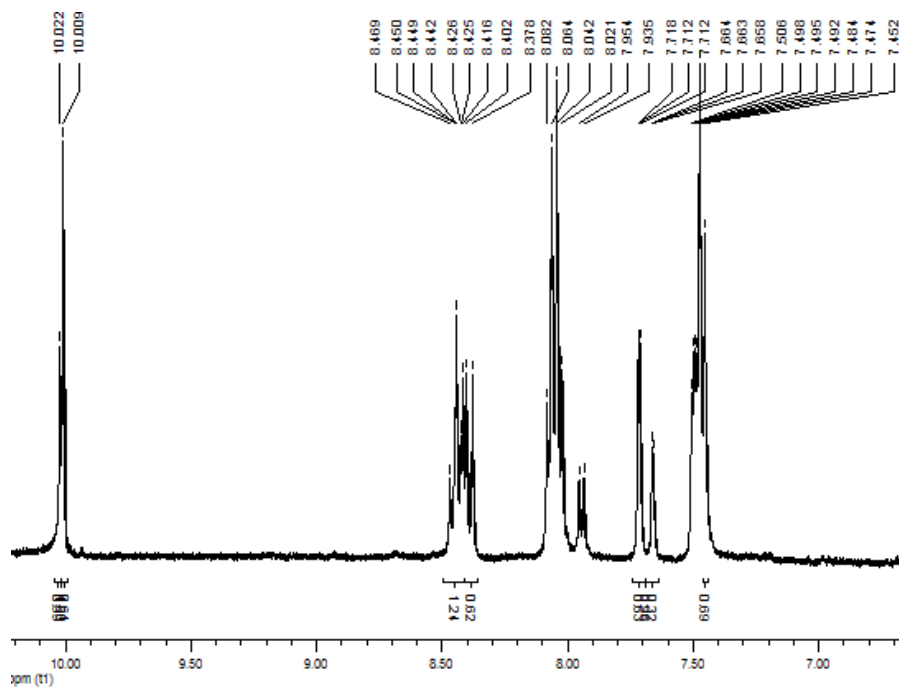
Selected regions, aromatics, of the proton and carbon NMR spectra recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO-*d*₆:D₂O (9.5:0.5) as solvent.

7(8)-Fluoro-8(7)-(4-formylphenoxy)-2-hydroxyphenazine 5,10-dioxide (20)

(7:8 isomers ratio, 65:35)



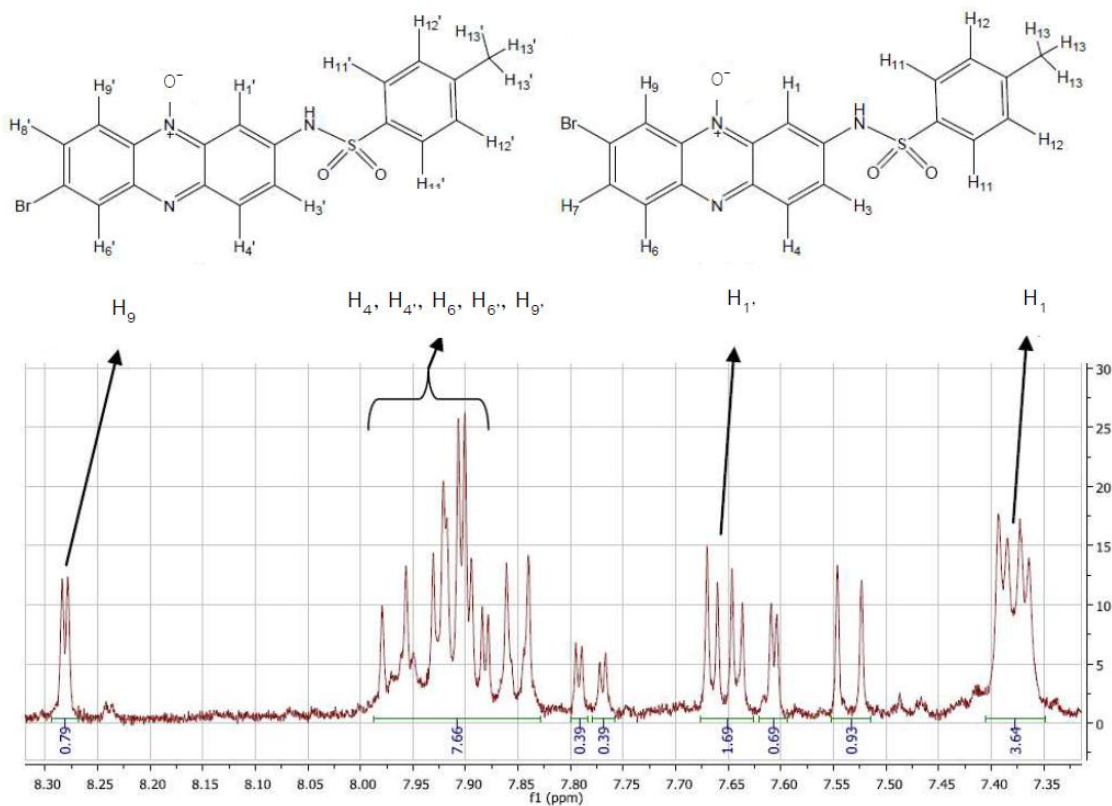
¹H NMR spectrum recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO-*d*₆:D₂O (9.5:0.5) as solvent.



Selected regions, aromatics, of the proton and carbon NMR spectra recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO- d_6 :D $_2$ O (9.5:0.5) as solvent.

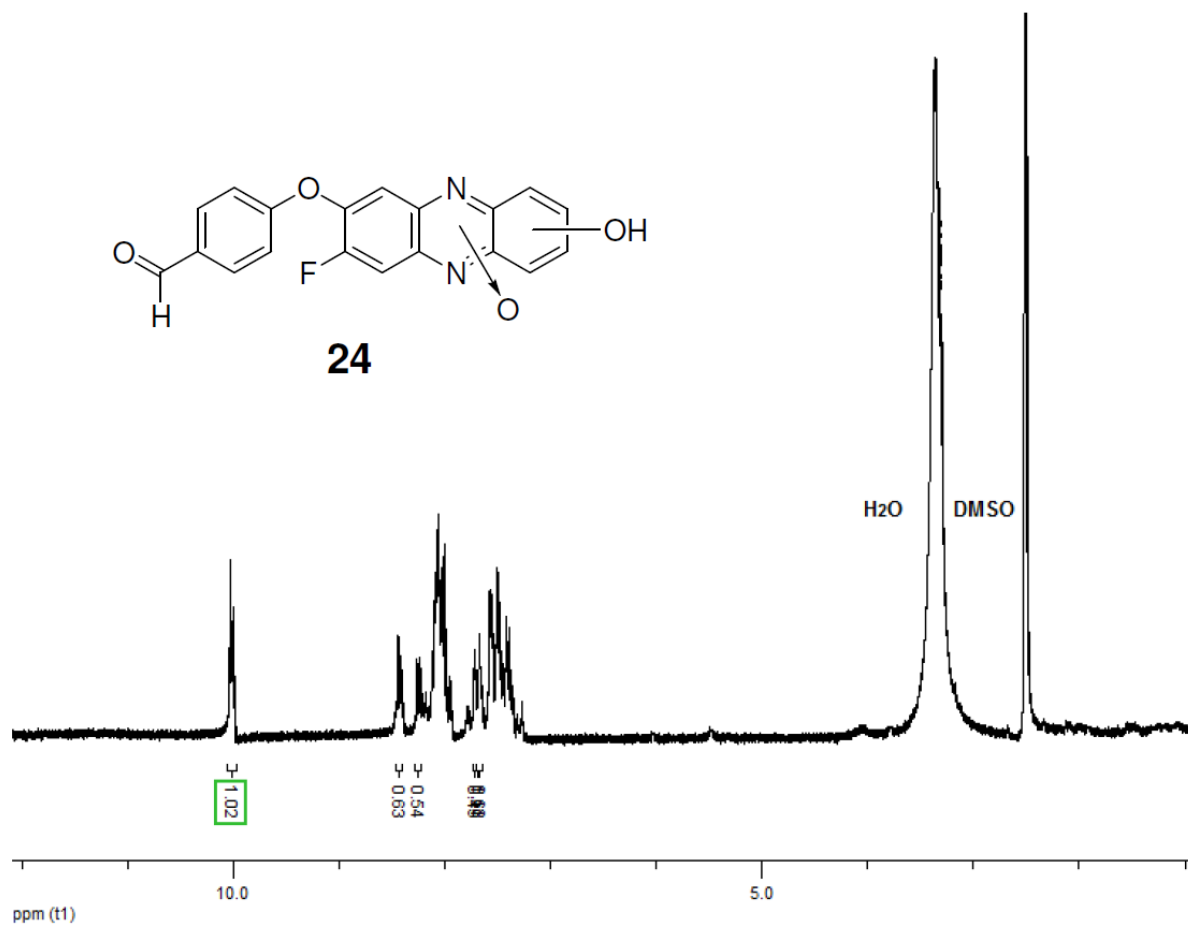
7(8)-Bromo-2-(4-methylphenylsulfonylamino)phenazine N^{10} -oxide (23)

(7:8 isomers ratio, 56:44)

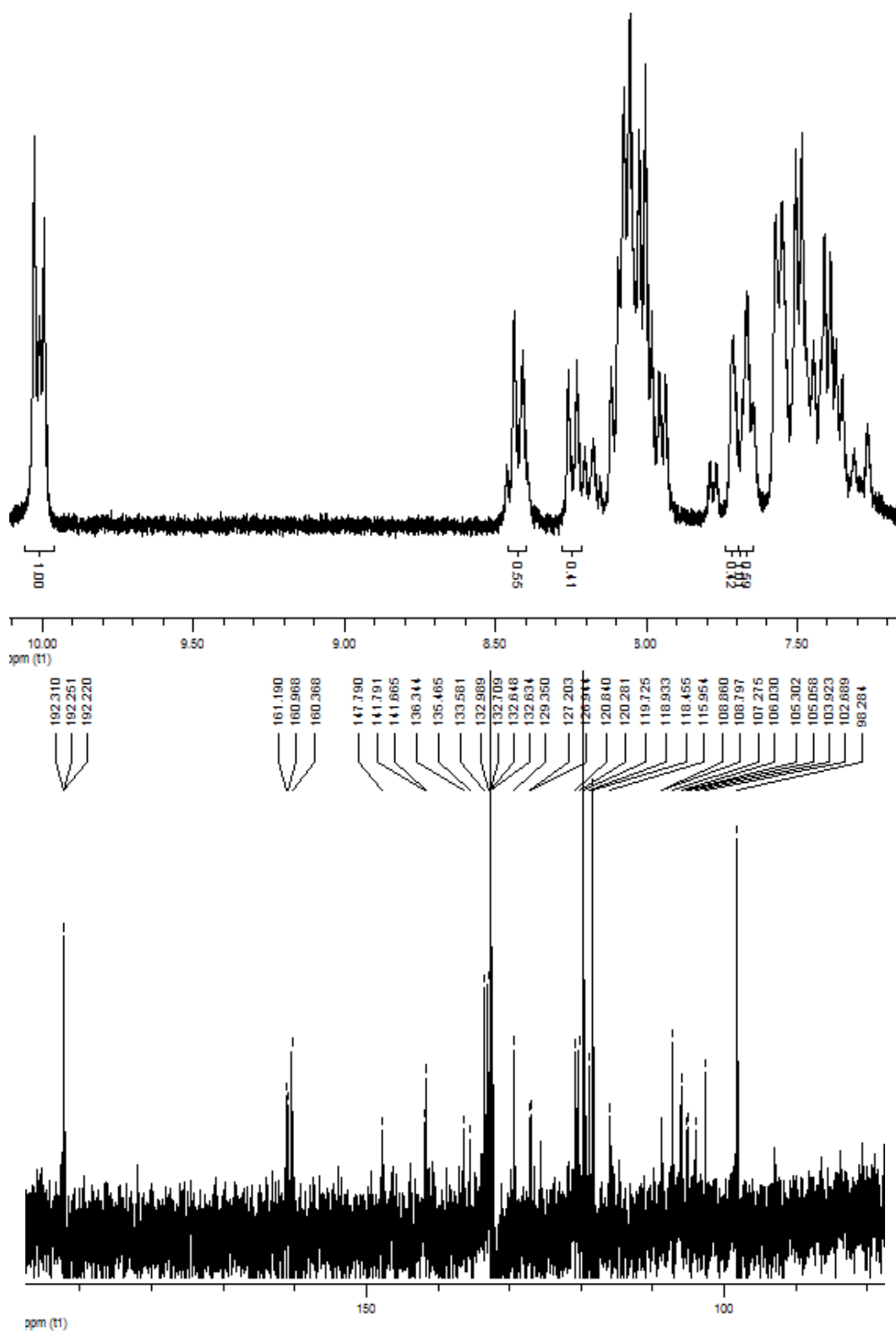


Selected regions, aromatics, of the proton NMR spectrum recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO- d_6 :D₂O (1:1) as solvent.

7(8)-Fluoro-8(7)-(4-formylphenoxy)-2-hydroxyphenazine N-oxide (24)



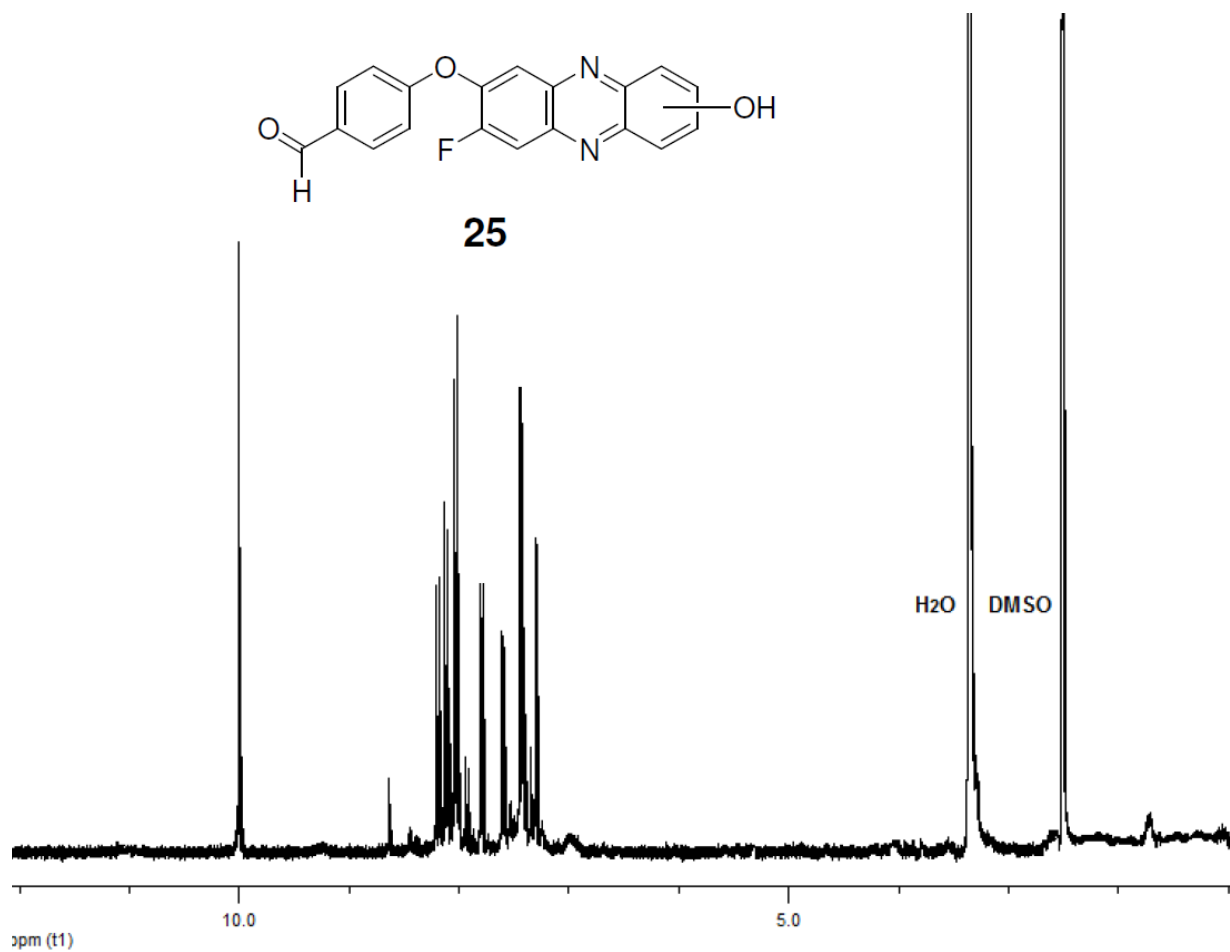
¹H NMR spectrum recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO-*d*₆:D₂O (9.5:0.5) as solvent.



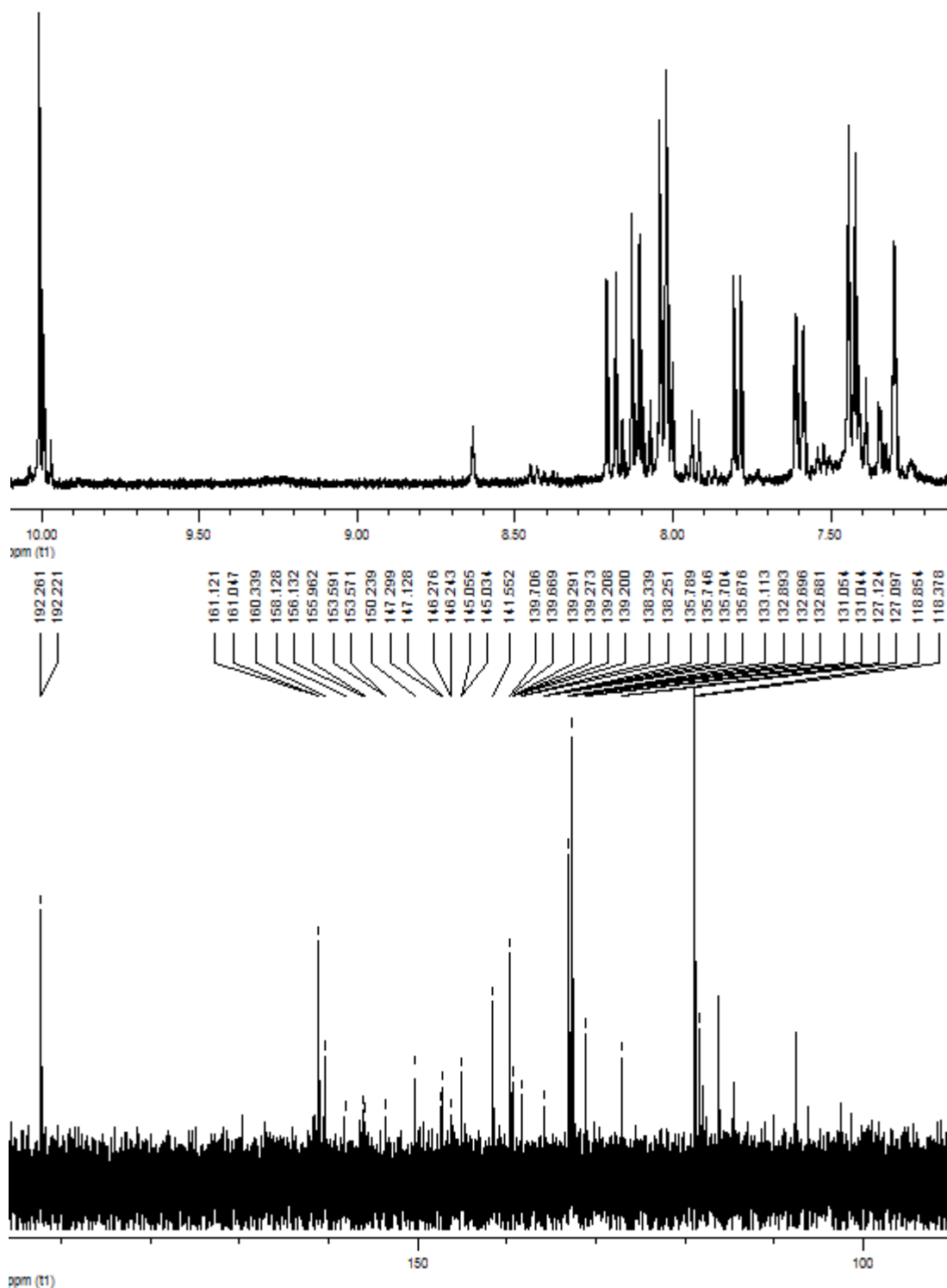
Selected regions, aromatics, of the proton and carbon NMR spectra recorded on a Bruker DPX-400 spectrometer at 298 K and using $\text{DMSO-}d_6\text{:D}_2\text{O}$ (9.5:0.5) as solvent.

7(8)-fluoro-8(7)-(4-formylphenoxy)-2-hydroxyphenazine (25)

(7:8 isomers ratio, 50:50)



¹H NMR spectrum recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO-*d*₆:D₂O (9.5:0.5) as solvent.



Selected regions, aromatics, of the proton and carbon NMR spectra recorded on a Bruker DPX-400 spectrometer at 298 K and using DMSO- d_6 :D₂O (9.5:0.5) as solvent.