

Novel Isatin-Derived Thiosemicarbazones: Synthesis, Characterization, and Assessment of Antioxidant Activity

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ABSTRACT

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A series of new of isatin-derived thiosemicarbazones (1–8) were synthesized using different isatin derivatives and *N*-(4-ethylphenyl)hydrazinecarbothioamide. The intermediated thiosemicarbazide was obtained by the reaction of 1-ethyl-4-isothiocyanatobenzene with hydrazine monohydrate. The identities and purities of all newly compounds were confirmed through standard spectroscopic techniques, including proton and carbon nuclear magnetic resonance (^1H and ^{13}C NMR), Fourier-transform infrared (FT-IR) spectroscopy, and elemental analysis. In this study, the antioxidant potential of the newly synthesized compounds was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH $^{\cdot}$) free radical scavenging assay. Compound 7 showed higher activity than the standard Trolox and the other compounds. The remaining compounds exhibited lower activity than the standard. The IC_{50} values of the compounds were determined to range from 15.88 ± 1.21 to 145.34 ± 2.68 μM . The antioxidant activity of the compounds decreased in the following order: $7 > 2 > 3 > 8 > 6 > 5 > 4 > 1$. Particular attention was given to the relationship between structural modifications and antioxidant activity, with a focus on how different functional groups influence the compounds' ability to neutralize free radicals.

1. Introduction

Thiosemicarbazones represent a significant class of compounds in synthetic organic chemistry, distinguished by their organosulfur core featuring the $-\text{NH}-\text{C}(=\text{S})\text{NH}-\text{N}=\text{}$ moiety. Due to their versatile structures and high reactivity, they are frequently used as key intermediates in the development of a wide range of biologically active substances. Extensive research has highlighted their diverse pharmacological and biological properties, including urease inhibitor [1-3], anticonvulsant [4], anticancer [5, 6], antitubercular [7], cytotoxic [8], antiviral [9], antioxidant [10, 11], antibacterial [12], and antimicrobial [13] properties.

Isatin and its derivatives (1*H*-indole-2,3-dione) represent a significant class of aromatic heterocyclic compounds. The isatin core serves as a valuable scaffold in the design and

development of biologically active molecules and potential drug candidates. Structural modifications of the isatin framework have led to a wide range of pharmacological activities, including anti-fungal [14-16], anti-oxidant [17-19], anti-bacterial [14, 20-22], enzyme inhibitory [14, 23-25], anti-cancer [26, 27], antiviral [28, 29], anti-convulsant [30], anti-tubercular [31], and analgesic [32].

Reactive oxygen species (ROS) and free radicals are widely recognized as key contributors to the onset and progression of various pathological conditions, such as metabolic disorders, ischemia-reperfusion injury, chronic inflammation, age-related cellular damage, and multiple types of cancer [33-35]. The excessive accumulation of these reactive molecules disrupts redox balance within

biological systems, thereby playing a pivotal role in disease pathogenesis. As a result, antioxidants have attracted significant scientific interest for their potential to mitigate oxidative stress and lower the risk of serious health conditions [34]. By neutralizing free radicals, antioxidants serve a critical function in protecting the human body from a wide range of diseases [36, 37]. This has led to ongoing efforts in the design and synthesis of novel antioxidant agents, which remain a major area of research focus.

In this paper, new isatin-derived thiosemicarbazones were synthesized and comprehensively characterized through spectroscopic techniques, including FT-IR, ^1H -NMR, and ^{13}C -NMR, as well as elemental analysis. Given the critical role of antioxidants in neutralizing free radicals and reducing their detrimental effects on human health, the antioxidant activity of the synthesized compounds was evaluated *in vitro* using the DPPH $^{\bullet}$ free radical scavenging assay. The potency of each compound was quantified by determining its IC_{50} value. Furthermore, the study explored the relationship between molecular structure and antioxidant efficacy, with particular attention to how various substituent groups influence radical scavenging performance.

2. General Methods

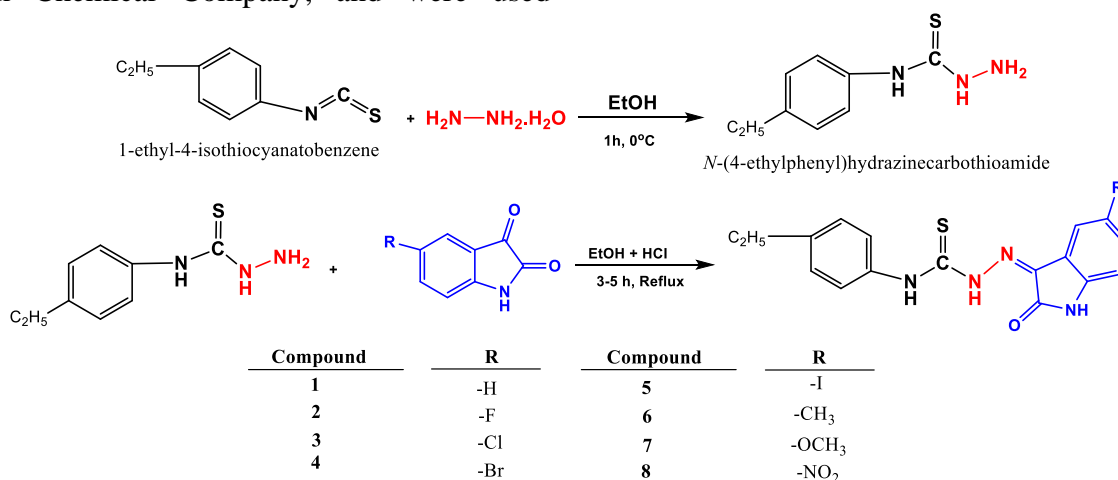
2.1. Materials and instruments

All chemical materials were purchased from Sigma-Aldrich, Acros Organics, Isolab, or Merck Chemical Company, and were used

without further purification. The solvents were of spectroscopic grade. A Stuart SMP 30 melting point apparatus was used to determine the melting points ($^{\circ}\text{C}$). The elemental analysis was performed on a Eurovector EA3000-Single. A Bruker Alpha Fourier transform IR (FT-IR) spectrometer was used to record for infrared spectra. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX-400 spectrometer (400 and 101 MHz) in $\text{DMSO}-d_6$. Antioxidant spectrophotometric measurements were performed using Biotek Epoch 2 Microplate Reader.

2.2. Synthesis of new isatin-derived thiosemicarbazones

A mixture of 1-ethyl-4-isothiocyanatobenzene (6.00 mmol) and hydrazine monohydrate (6.00 mmol) was added dropwise to 20 mL of ethanol under vigorous stirring while maintaining the temperature in an ice bath. Refrigeration of the reaction mixture overnight resulted in the precipitation of the thiosemicarbazide product, which was isolated by filtration, washed twice with cooled ethanol (96%), and dried in air. Subsequently, a few drops of HCl were added to a solution of the intermediated thiosemicarbazide (4.00 mmol) and various isatins (4.00 mmol) in 20 mL of aqueous ethanol (96%), refluxed at 78°C for 3 to 5 hours. After completion, the solid product was isolated by filtration, washed, and air-dried. The successful synthesis of the target compounds, obtained in good yields (61–89%), is illustrated in Scheme 1. The procedure followed was adapted from previously reported methods with slight modifications [38].



Scheme 1. Synthesis route of new isatin-derived thiosemicarbazones

2.3. Antioxidant activity (DPPH[•] scavenging)

The free radical scavenging activity of the samples was determined using the DPPH[•] (1,1-diphenyl-2-picryl-hydrazil) method [39]. 150 μ L of different concentrations of samples or standards (Trolox), 50 μ L of 0.1 mM DPPH[•] have homogeneously mixed in a 96-well plate. The samples have waited in the dark at room temperature for 30 min. The absorbance values of each mixture were measured at 517 nm using the BioTek Epoch 2 microplate spectrophotometer and determined the results by calculating the IC₅₀ (μ g/mL) values [40].

2.4. Statistical analysis

Each variable in the biological activity was evaluated three times, yielding an average \pm standard deviation. The results were statistically examined using SPSS 20.0. The analytic mean would be regarded as homogenous across more than two groups if the data distribution were regular. Given that Tukey's HSD_{a,b} is a multiple comparison process, it was used. There were statistically significant differences ($p < 0.05$) between the values derived from each activity analysis and the samples and standards.

3. Results and Discussion

3.1. Physicochemical data

Tables 1 and 2 present the experimental findings related to physicochemical properties, yields, melting points, and elemental analyses.

Table 1. The physicochemical data of the products

Comp.	M.P. (°C)	Yields (%)	Colour	Mol. Formula
1	223-224	76	Yellow	C ₁₇ H ₁₆ N ₄ OS
2	248-249	74	Dark Yellow	C ₁₇ H ₁₅ FN ₄ OS
3	226-227	61	Orange	C ₁₇ H ₁₅ ClN ₄ OS
4	230-231	72	Orange	C ₁₇ H ₁₅ BrN ₄ OS
5	253-254	65	Dark Yellow	C ₁₇ H ₁₅ IN ₄ OS
6	208-209	89	Dark Orange	C ₁₈ H ₁₈ N ₄ OS
7	227-228	77	Brick Red	C ₁₈ H ₁₈ N ₄ O ₂ S
8	225-226	63	Light Orange	C ₁₇ H ₁₅ N ₅ O ₃ S

Table 2. Elemental analysis results of the products

Comp.	Calculated			Experimental		
	C%	H%	N%	C%	H%	N%
1	62.94	4.97	17.27	63.01	4.98	17.24
2	59.63	4.42	16.36	59.59	4.43	16.38
3	56.90	4.21	15.61	56.95	4.19	15.59
4	50.63	3.75	13.89	50.65	3.76	13.86
5	45.34	3.36	12.44	45.31	3.36	12.43
6	63.88	5.36	16.56	63.80	5.34	16.58
7	61.00	5.12	15.81	59.95	5.15	15.83
8	55.27	4.09	18.96	55.30	4.08	18.94

3.2. Interpretation of vibrational frequencies

For all compounds 1–8, amine group (–NH) vibration signals of isatin ring and thiosemicarbazone moiety were detected at 3326 – 3288 cm^{-1} and 3244 – 3142 cm^{-1} , respectively. Aromatic proton vibrations were observed in the 3089 – 3019 cm^{-1} range, while aliphatic proton vibrations were detected between 2927 and 2817 cm^{-1} . The –C=N stretching vibrations of the azomethine (imine) group were observed at 1668 – 1589 cm^{-1} , the –C=C stretching vibrations were observed at 1605 – 1525 cm^{-1} . The –C=O signals of the isatin ring were observed at 1706 – 1671 cm^{-1} , the characteristic –C=S stretching bands of the thiosemicarbazone moiety were found at 1414 – 1304 cm^{-1} , and the –C–N stretching vibrations were observed in the range of 1250 – 1129 cm^{-1} .

Table 3. FT-IR frequencies of the compounds (cm^{-1})

Comp.	ν_{NH}	$\nu_{\text{C=O}}$	$\nu_{\text{C=N}}$	$\nu_{\text{C=C}}$	$\nu_{\text{C=S}}$	$\nu_{\text{C-N}}$
1	3288, 3142	1684	1608	1590	1340	1199, 1137
2	3314, 3240	1686	1631	1587	1372	1250, 1134
3	3317, 3240	1689	1606	1586	1306	1197, 1151
4	3292, 3244	1706	1668	1605	1298	1197, 1129
5	3321, 3169	1684	1605	1525	1304	1205, 1149
6	3304, 3189	1675	1612	1534	1398	1200, 1131
7	3326, 3156	1671	1589	1529	1306	1195, 1143
8	3310, 3181	1699	1614	1531	1414	1220, 1148

For compounds 2–5, the –C–F, –C–Cl, –C–Br, and –C–I stretching vibrations were observed at 940, 882, 767, and 636 cm^{-1} , respectively. For compound 7, the –C–O stretching vibration was observed at 1143 cm^{-1} . The asymmetric and symmetric stretching peaks of the nitro group (–

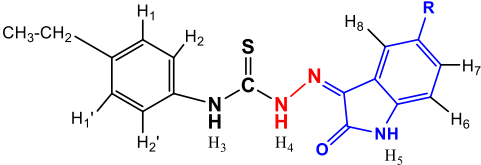
NO₂) were observed at 1531 and 1335 cm⁻¹ for compound 8. The FT-IR peaks of all compounds are presented in Table 3. The frequency data for all of the compounds were consistent with those reported for similar compounds in the literature [17, 18, 41-43].

3.3. ¹H NMR analysis

The ¹H NMR spectra of all compounds were attained in DMSO-*d*₆ solution, and the chemical shifts are summarized in Table 4. In all spectra, the DMSO-*d*₆ were seen at around 2.00 and 2.55 ppm (quintet) and 3.40 ppm (variable, depending on the solvent and concentration), respectively [44]. For compounds 1–8, the –NH proton signals (H5) of the isatin ring were detected as singlets in the ranges 11.04 – 10.73 ppm. The –N³H and –N⁴H proton signals

of the thiosemicarbazone moiety were detected as singlets in the ranges of 11.86 – 11.06 and 12.78 – 12.54 ppm, respectively. The aromatic protons (H1-H2) of the 4-ethylphenyl ring were observed as a doublet at 7.53 – 7.25 ppm. The isatin aromatic protons (H6-H8) were detected at between 8.70 and 6.80 ppm for all compounds (all ¹H NMR spectra are given in Figures 1–8). For all compounds, the proton signal of the methylene group (–CH₂) was observed as a quartet at 2.68 – 2.61 ppm (2H, q); the –CH₃ proton signal was detected as a triplet at 1.24 – 1.19 ppm (3H, t). For compounds 6 and 7, the proton signal of the methyl (–CH₃) and the methoxy group (–OCH₃) was detected as a singlet at 2.31 and 3.77 ppm, respectively. These results are consistent with values reported for similar compounds in the literature [17, 43, 45].

Table 4. ¹H NMR data of the compounds, (δ/ppm)



Comp.	Ar-NH H3	NH-N H4	isatin NH H5	isatin H H6-H8	ArH H1-H2	CH ₃ -CH ₂
1	11.26 (s, 1H)	12.78 (s, 1H)	10.76 (s, 1H)	7.79-7.77 (d), 7.40-7.38 (t), 7.13-7.10 (t), 6.96-6.94 (d)	7.52-7.50 (d), 7.27-7.25 (d)	2.66-2.61 (q), 1.23-1.19 (t)
2	11.25 (s, 1H)	12.65 (s, 1H)	10.78 (s, 1H)	7.65-7.63 (d), 7.20-7.18 (t), 6.95-6.93 (d)	7.52-7.50 (d), 7.28-7.26 (d)	2.67-2.61 (q), 1.23-1.19 (t)
3	11.34 (s, 1H)	12.59 (s, 1H)	10.83 (s, 1H)	7.89 (s, 1H), 7.40-7.38 (d), 6.96-6.94 (d)	7.51-7.49 (d), 7.28-7.26 (d)	2.67-2.61 (q), 1.23-1.19 (t)
4	11.35 (s, 1H)	12.59 (s, 1H)	10.84 (s, 1H)	8.01 (s, 1H), 7.51-7.49 (d), 6.91-6.89 (d)	7.51-7.49 (d), 7.28-7.26 (d)	2.67-2.61 (q), 1.23-1.19 (t)
5	11.41 (s, 1H)	12.61 (s, 1H)	10.85 (s, 1H)	8.16 (s, 1H), 7.69-7.67 (d), 6.82-6.80 (d)	7.50-7.48 (d), 7.28-7.26 (d)	2.67-2.61 (q), 1.23-1.19 (t)
6	11.14 (s, 1H)	12.76 (s, 1H)	10.73 (s, 1H)	7.61 (s, 1H), 7.18-7.16 (d), 6.84-6.82 (d)	7.53-7.51 (d), 7.27-7.25 (d)	2.66-2.61 (q), 1.23-1.19 (t), 2.31 (s)
7	11.06 (s, 1H)	12.77 (s, 1H)	10.74 (s, 1H)	7.42 (d), 6.97-6.95 (dd), 6.87-6.85 (d)	7.51-7.48 (d), 7.28-7.26 (d)	2.67-2.61 (q), 1.23-1.19 (t), 3.77 (s)
8	11.86 (s, 1H)	12.54 (s, 1H)	11.04 (s, 1H)	8.70 (s, 1H), 8.30-8.28 (d), 7.15-7.13 (d)	7.50-7.48 (d), 7.30-7.28 (d)	2.68-2.62 (q), 1.24-1.20 (t)

s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), and m (multiplet).

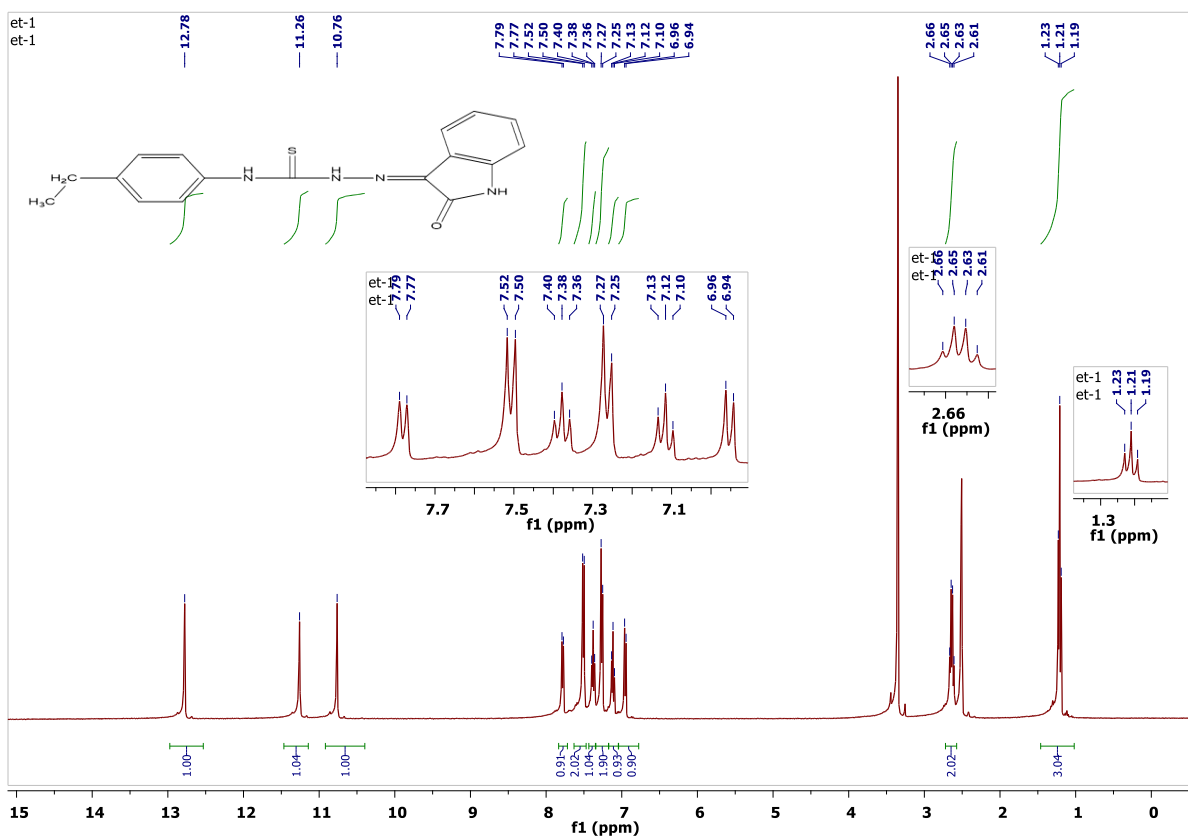


Figure 1. ¹H NMR spectrum of compound 1

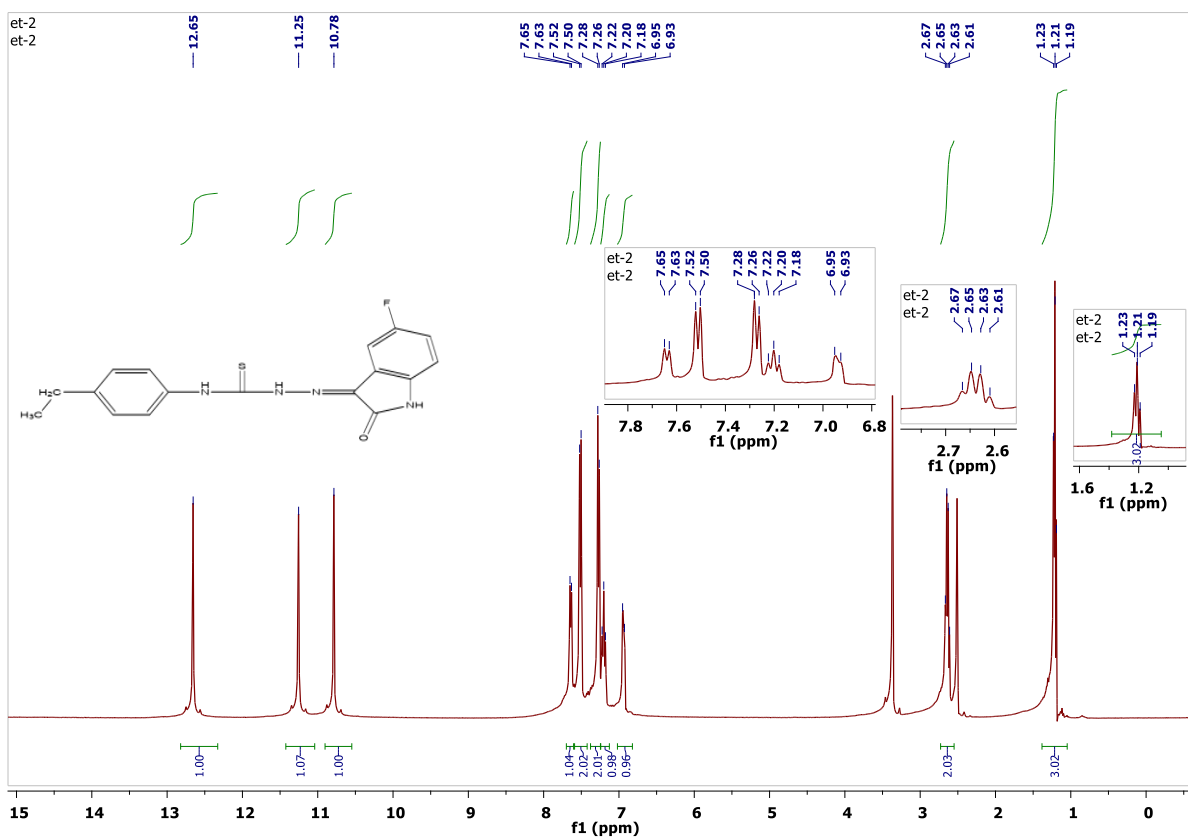


Figure 2. ¹H NMR spectrum of compound 2

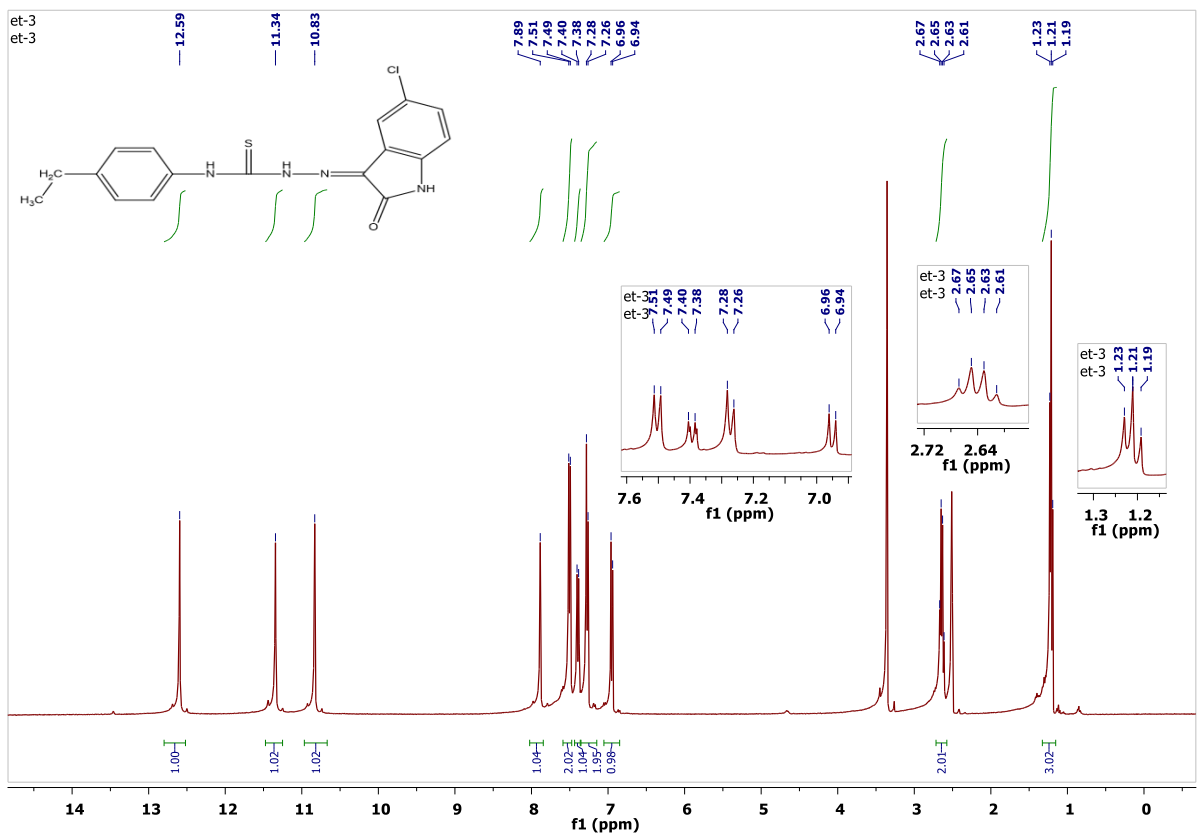


Figure 3. ¹H NMR spectrum of compound 3

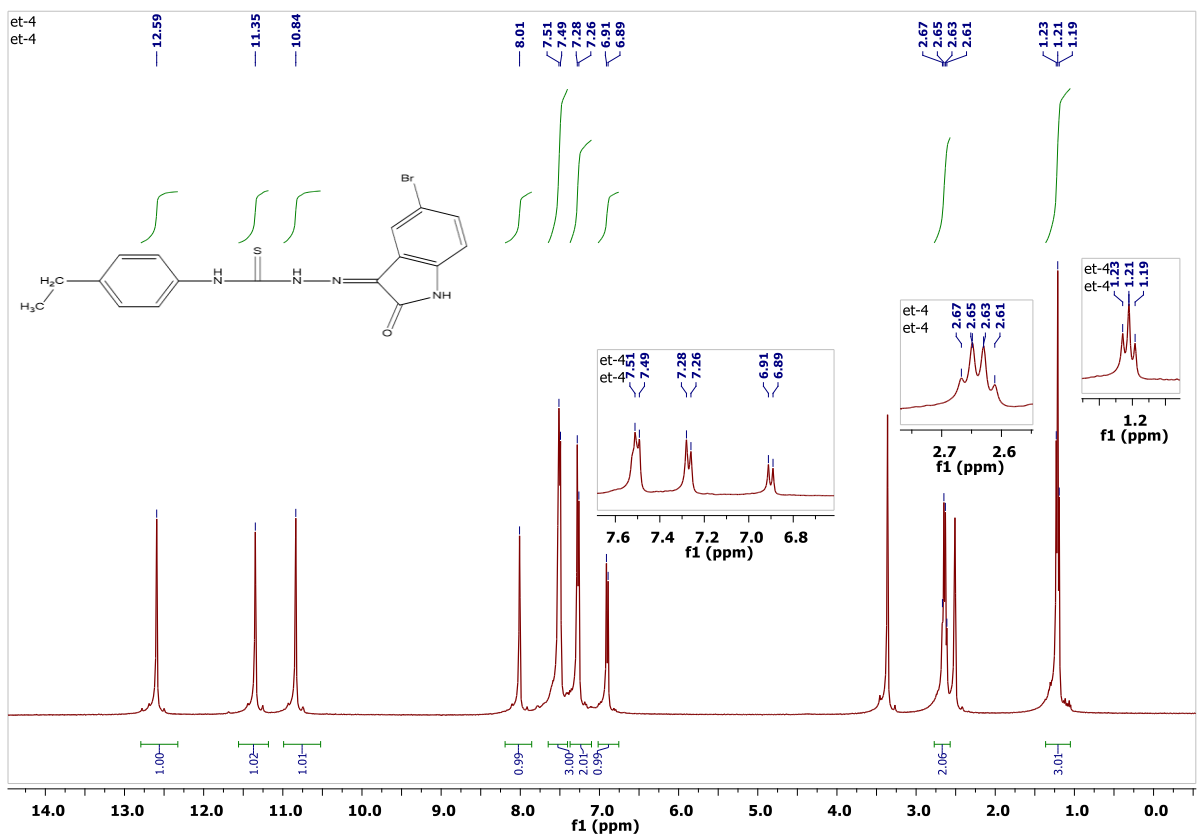
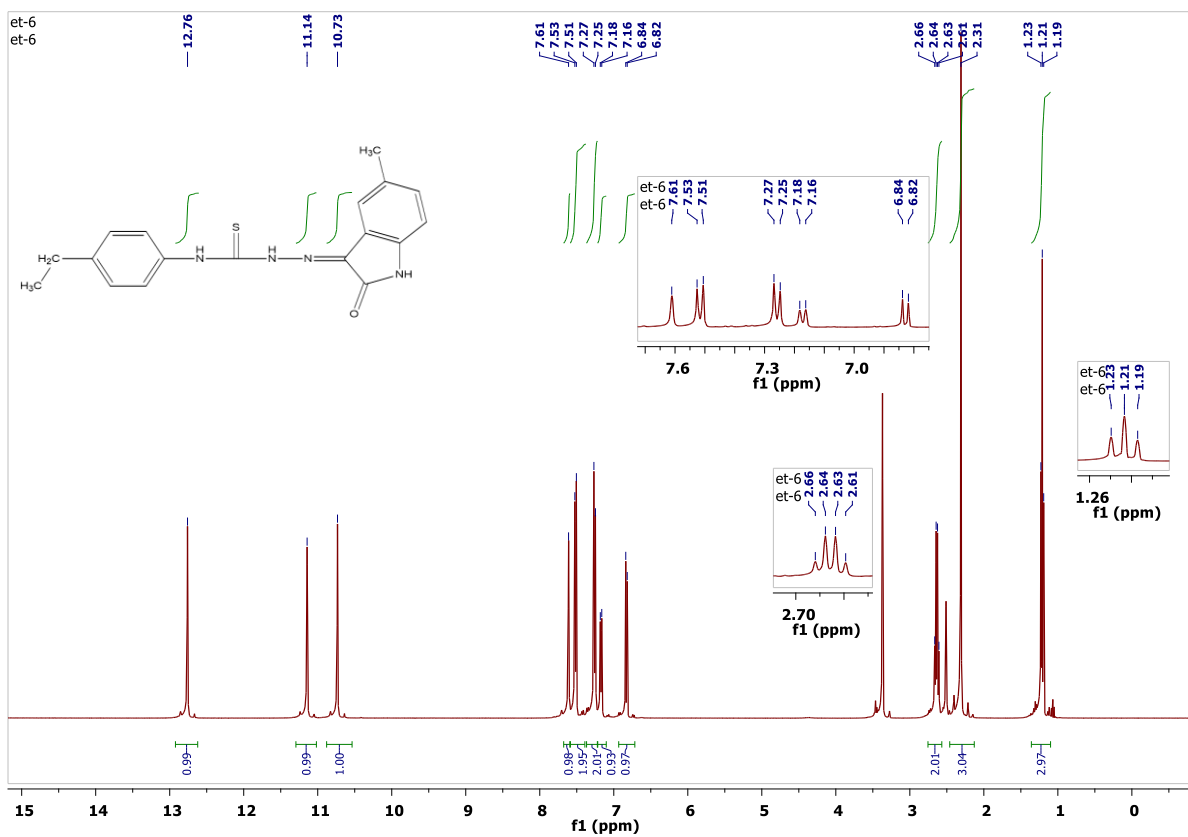
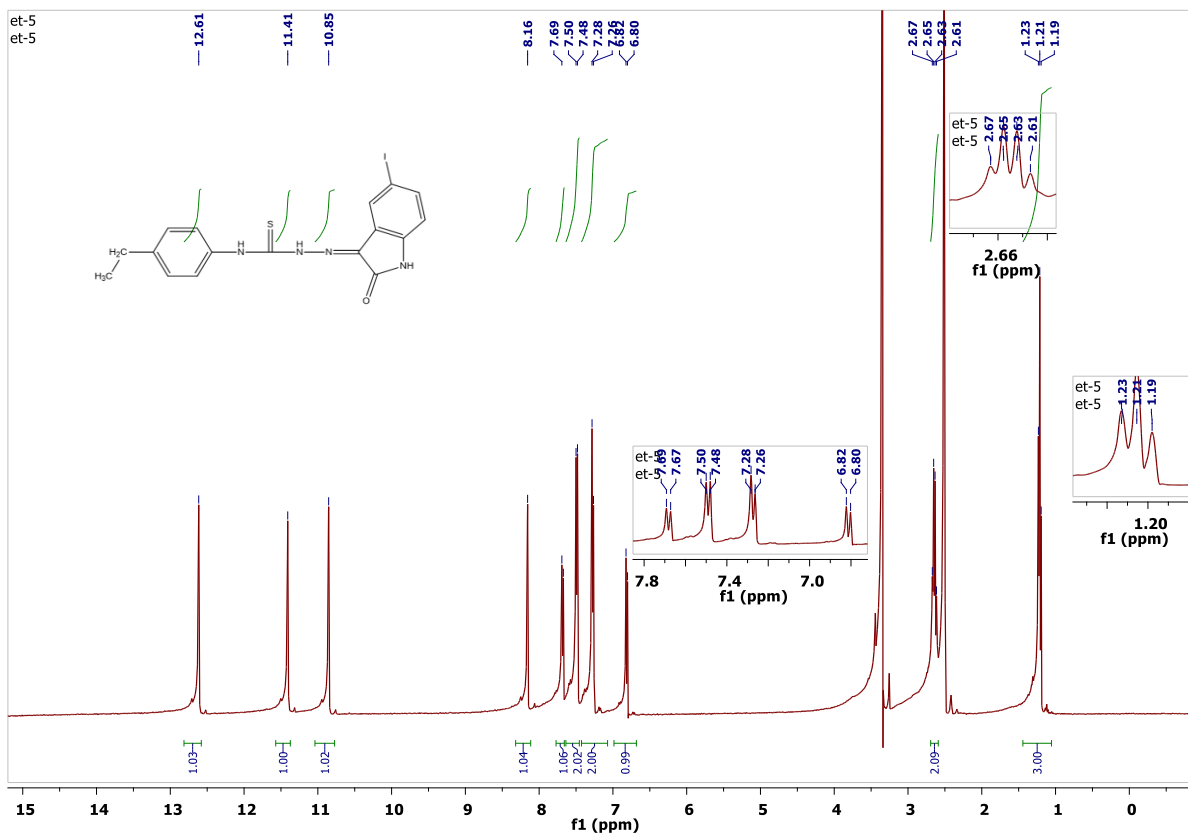


Figure 4. ¹H NMR spectrum of compound 4



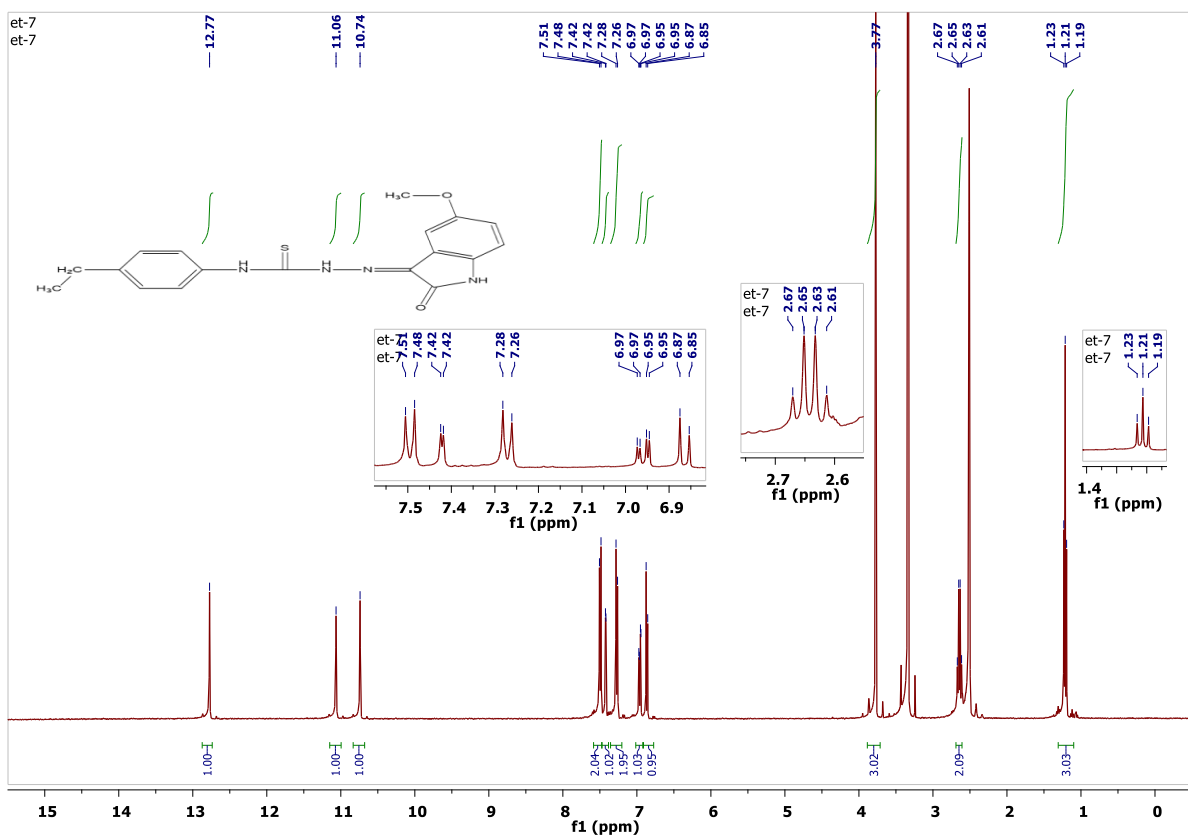


Figure 7. ¹H NMR spectrum of compound 7

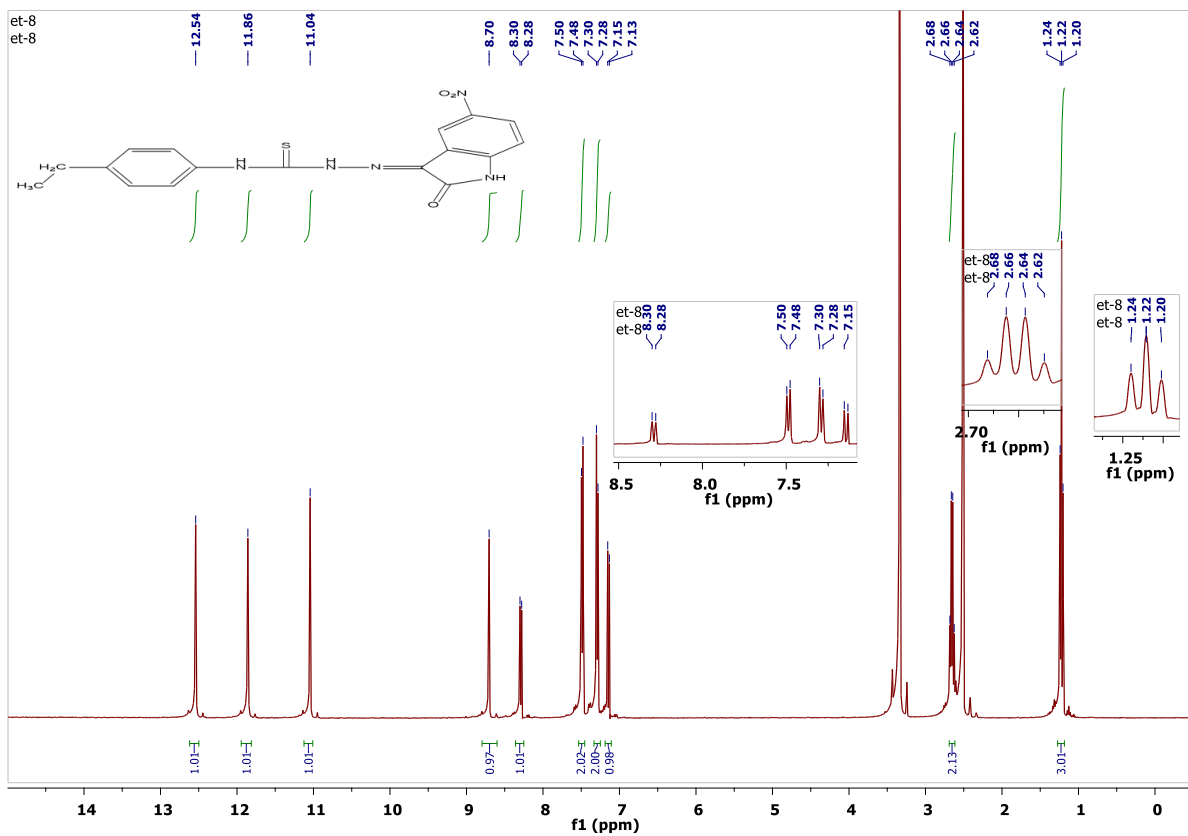
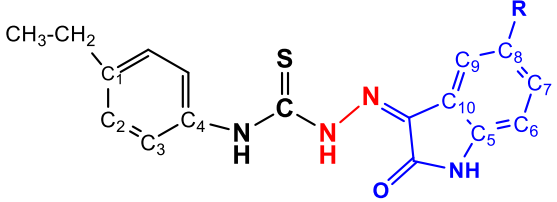


Figure 8. ¹H NMR spectrum of compound 8

3.4. ^{13}C NMR interpretations

The ^{13}C NMR chemical shifts of the synthesized compounds, measured in $\text{DMSO-}d_6$, are shown in Table 5.

Table 5. ^{13}C NMR data of the compounds, (δ/ppm)



Comp.	C1-C4	C=S	C=O	C=N	C5-C10	$\text{CH}_3/\text{CH}_2/\text{R}$
1	132.6	176.8	163.2	142.9	142.2, 120.4	16.1/28.2
	126.1				131.9, 121.9	
	122.8				128.1, 111.6	
	136.6					
2	136.4	176.7	163.3	142.3	132.0, 131.9	16.1/28.2
	128.2				112.7, 112.6	
	126.0				118.2, 117.9	
	139.2				159.9, 157.6	
					108.9, 108.6	
121.9, 121.8						
3	131.0	176.7	162.9	142.3	141.6, 121.5	16.1/28.2
	126.1				131.4, 128.2	
	122.3				127.0, 113.0	
	136.4					
4	131.2	176.7	162.8	142.3	141.9, 113.5	16.1/28.2
	126.1				133.8, 114.7	
	122.7				128.2, 124.2	
	136.4					
5	131.1	176.7	162.5	142.4	142.3, 128.2,	16.1/28.2
	126.2				139.6, 85.8	
	122.9				129.8, 113.9,	
	136.4					
6	131.8	176.7	163.2	142.1	140.7, 120.4	16.1/28.2 /21.1
	126.0				132.2, 132.7	
	122.2				128.1, 111.3	
	136.6					
7	132.9	176.8	163.3	142.3	136.5, 128.2	16.1/28.2 /56.1
	126.3				112.3, 155.8	
	121.2				107.1, 117.9	
	136.6					
8	130.6	176.9	163.5	143.2	147.9, 117.2,	16.1/28.2
	126.3				128.2, 142.5,	
	121.4				127.5, 111.7	
	136.4					

For compounds 1–8, the characteristic $-\text{C}=\text{S}$ peaks of the thiosemicarbazone moiety were detected at 176.9 – 176.7 ppm. The characteristic $-\text{C}=\text{O}$ peaks of the isatin ring were detected at 163.5 – 162.5 ppm. The other characteristic $-\text{C}=\text{N}$ peaks were observed in the ranges 143.2 – 142.1 ppm. The methyl ($-\text{CH}_3$) and methylene ($-\text{CH}_2$) carbon signals were observed at 16.1 and 28.2 ppm. The aromatic carbon atom (C1-C4) signals of phenyl region were detected in the ranges of 139.2 – 121.2 ppm. The aromatic carbon atom (C5-C10) signals of aldehyde region were observed in the ranges of 155.8 – 85.8 ppm (see ^{13}C NMR spectra Figures 9-16).

For compounds 6 and 7, the $-\text{CH}_3$ and $-\text{OCH}_3$ carbon atoms were observed at 21.1 and 56.1 ppm, respectively. For compound 5, the carbon C8 signal was observed at 85.8 ppm. In addition, the ^{13}C NMR spectrum of compound 2 revealed that the phenyl ring carbons (C5–C10) were split into doublets as a result of fluorine–carbon coupling. The presence of substituent groups ($-\text{F}$, $-\text{Cl}$, $-\text{Br}$, $-\text{I}$, $-\text{CH}_3$, $-\text{OCH}_3$, and $-\text{NO}_2$) in all compounds led to downfield shifts (155.8 – 129.8 ppm) of some carbon signals relative to the phenyl carbon signal observed at 128.5 ppm. These findings are in agreement with those reported for analogous compounds in earlier studies [17, 45, 46].

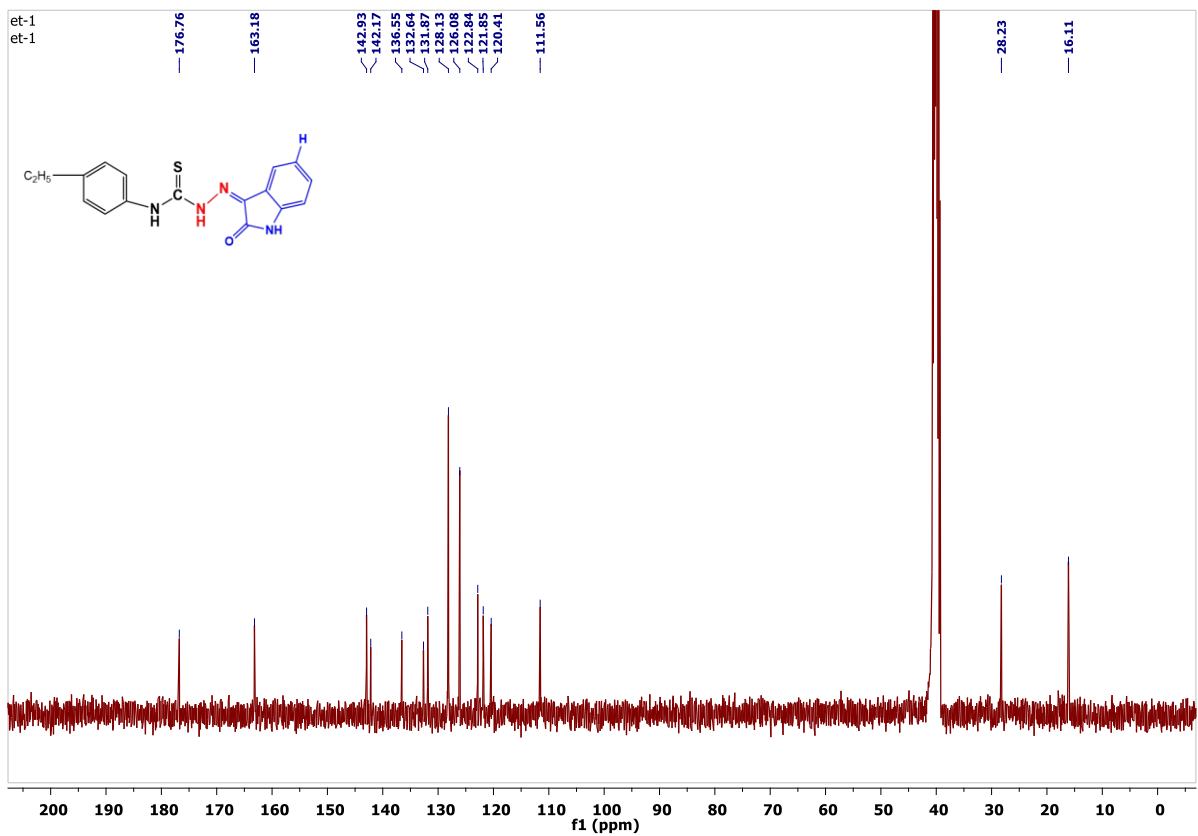


Figure 9. ¹³C NMR spectrum of compound 1

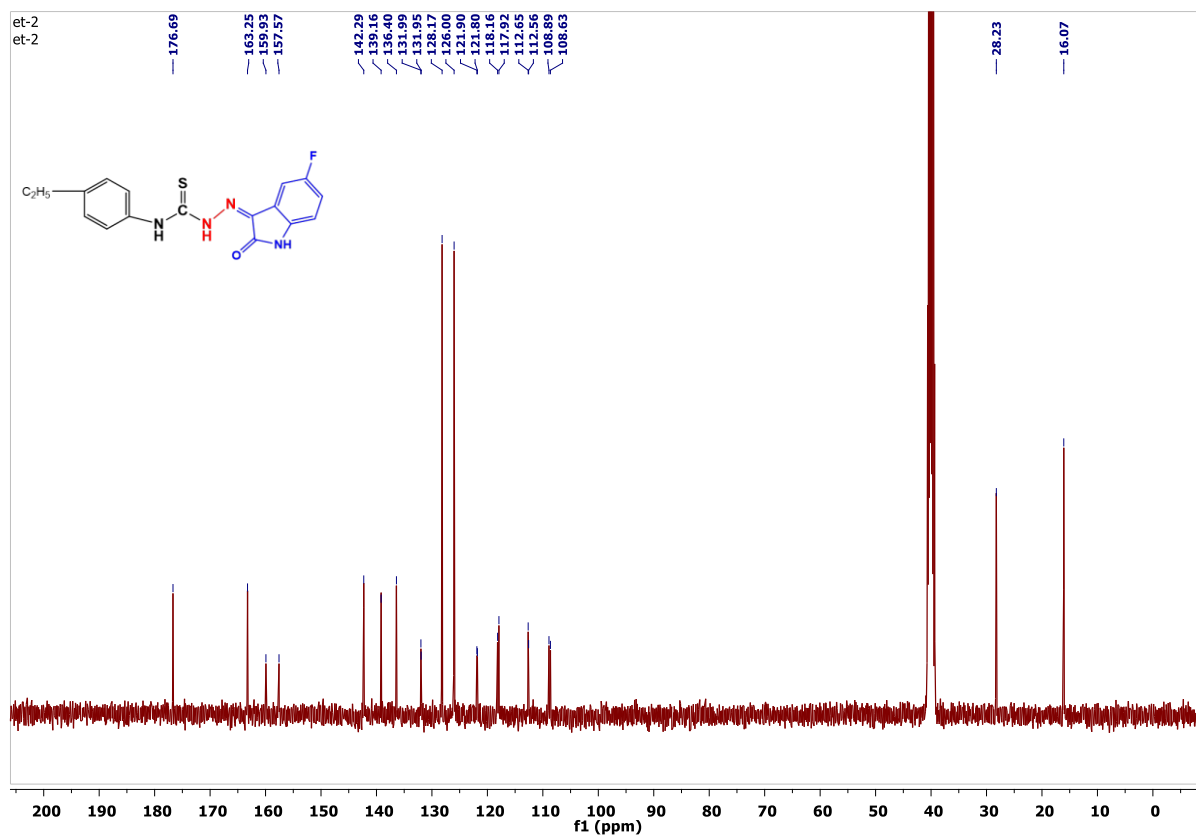


Figure 10. ¹³C NMR spectrum of compound 2

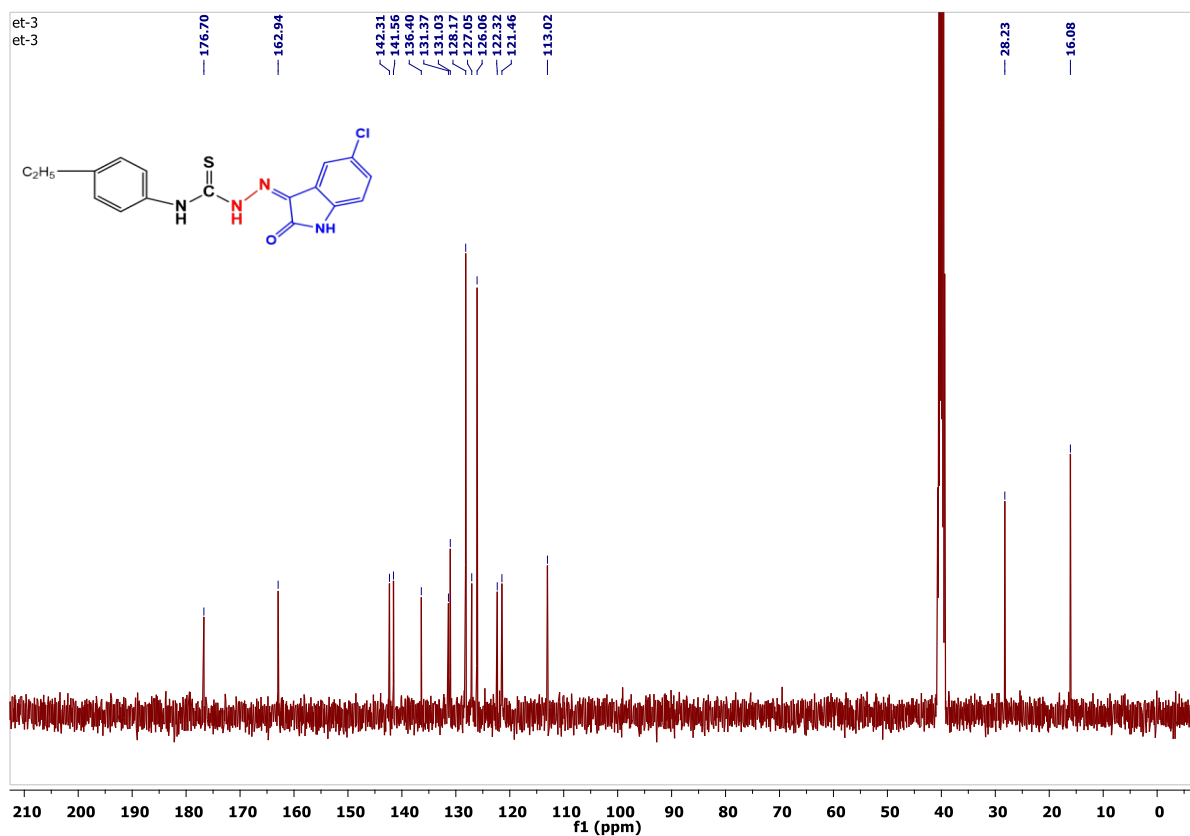


Figure 11. ¹³C NMR spectrum of compound 3

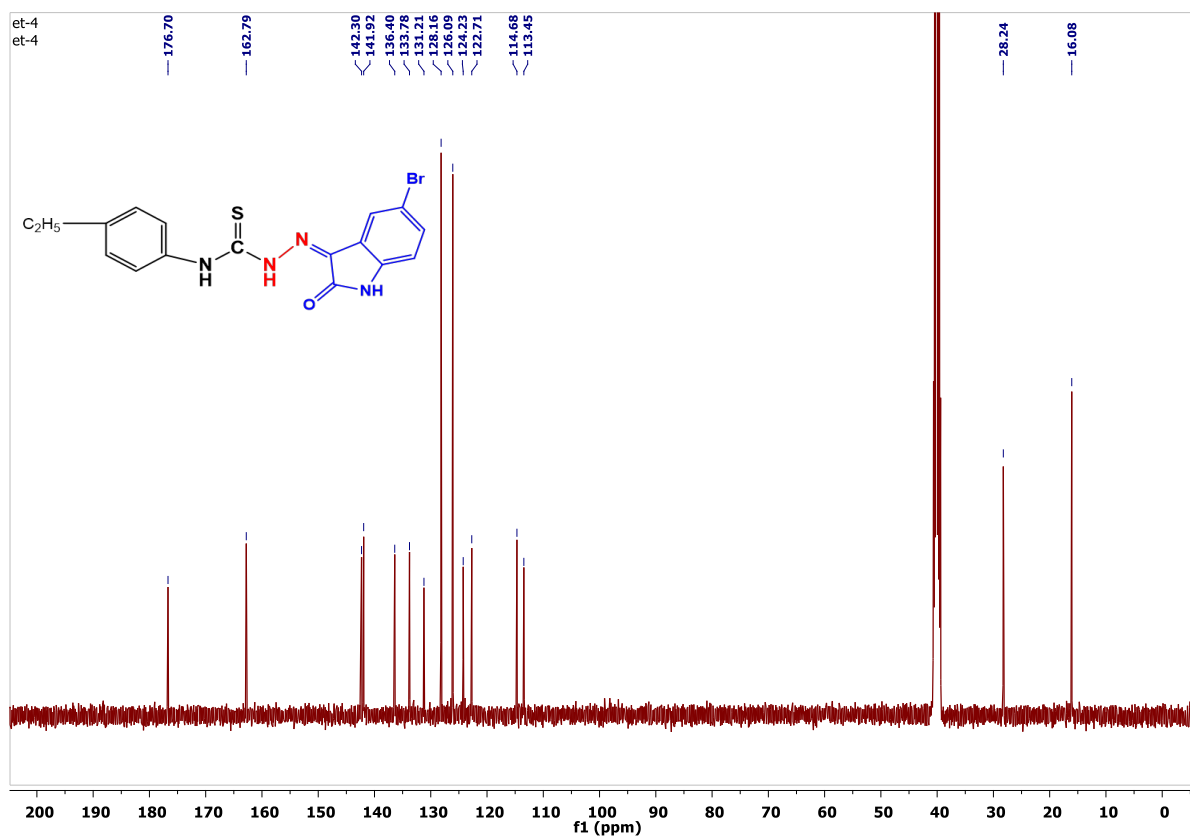


Figure 12. ¹³C NMR spectrum of compound 4

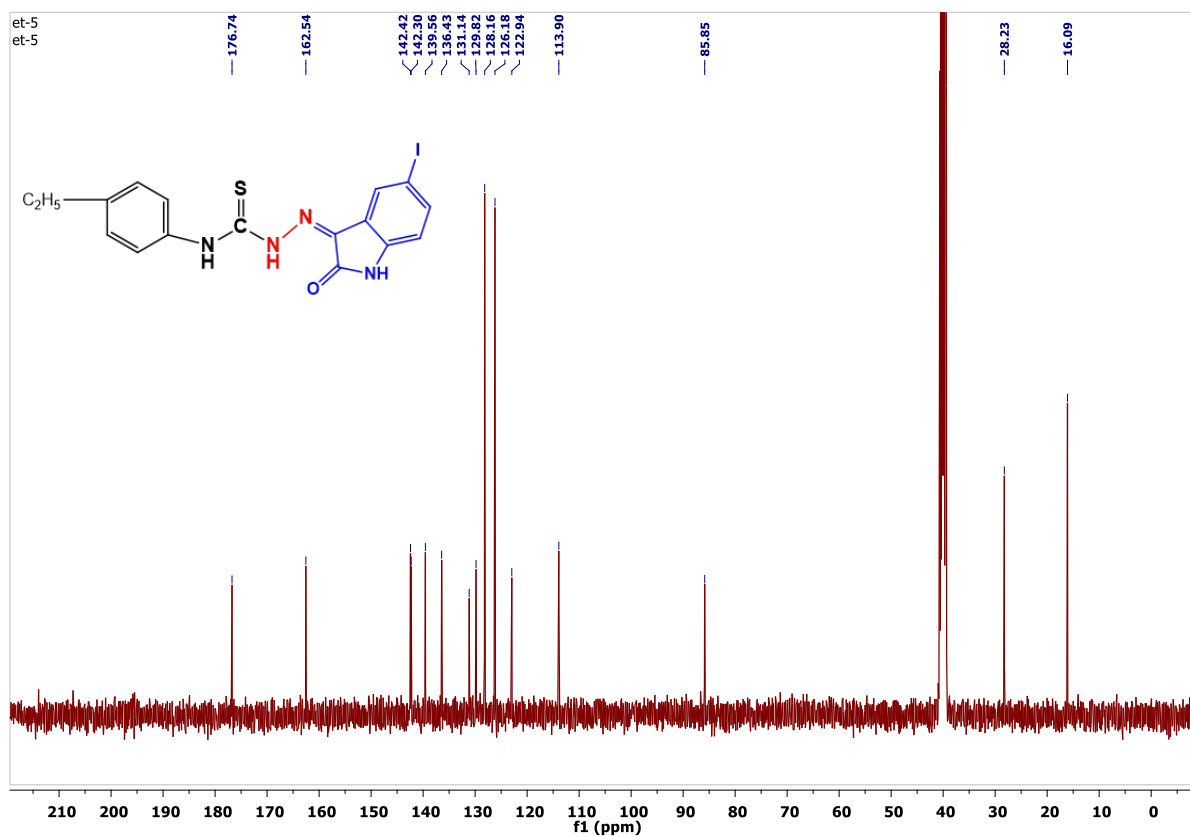


Figure 13. ¹³C NMR spectrum of compound 5

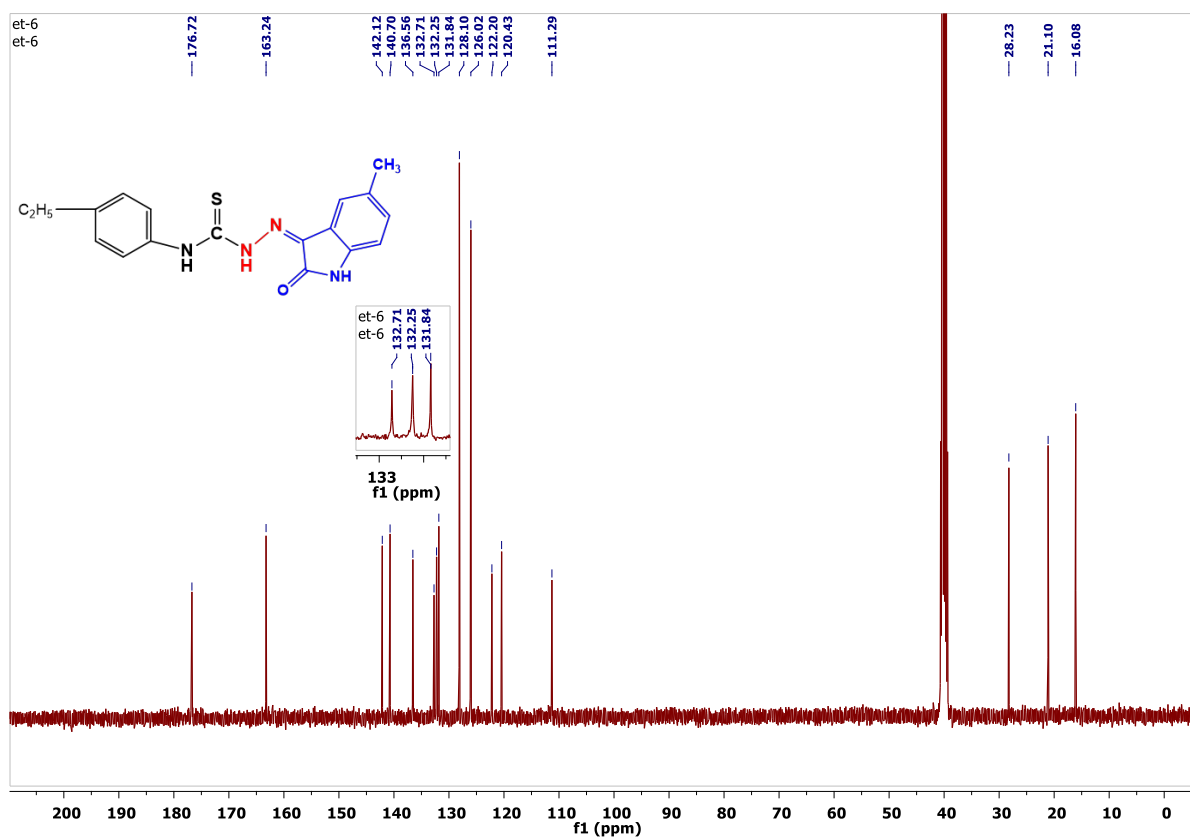


Figure 14. ¹³C NMR spectrum of compound 6

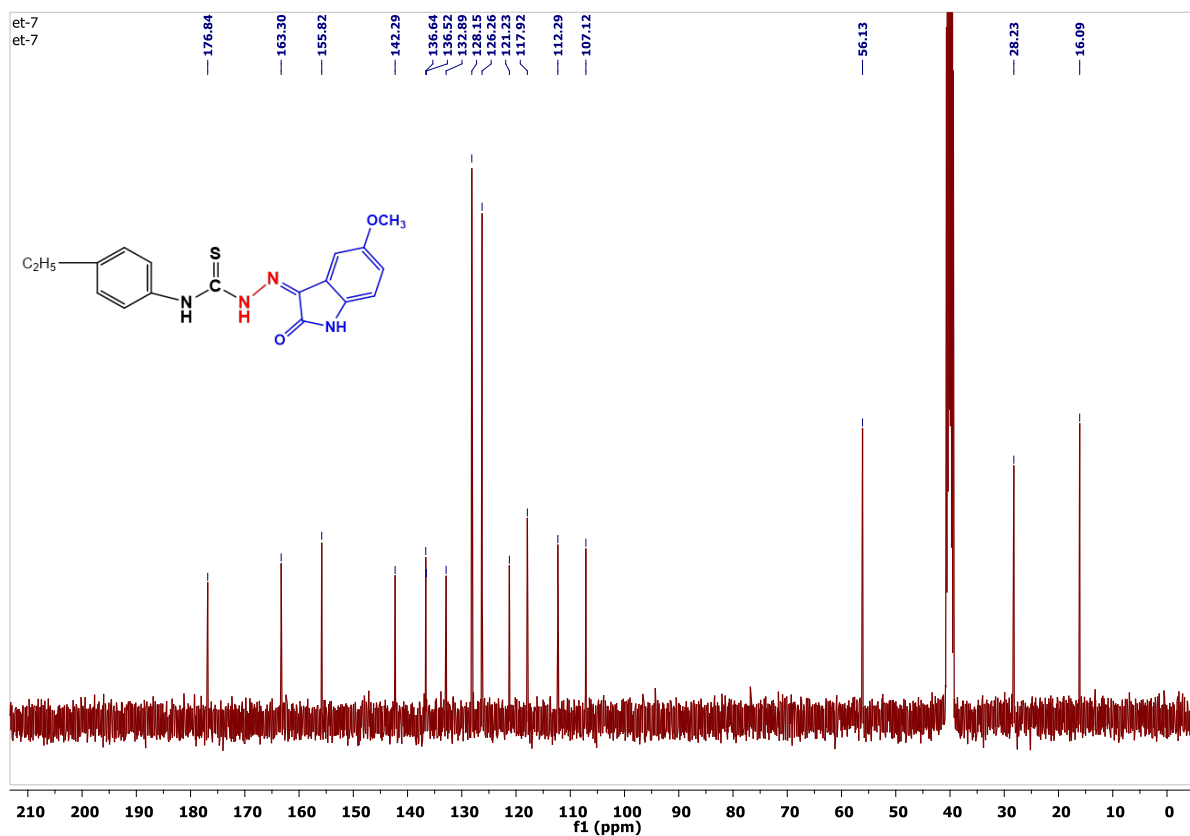


Figure 15. ^{13}C NMR spectrum of compound 7

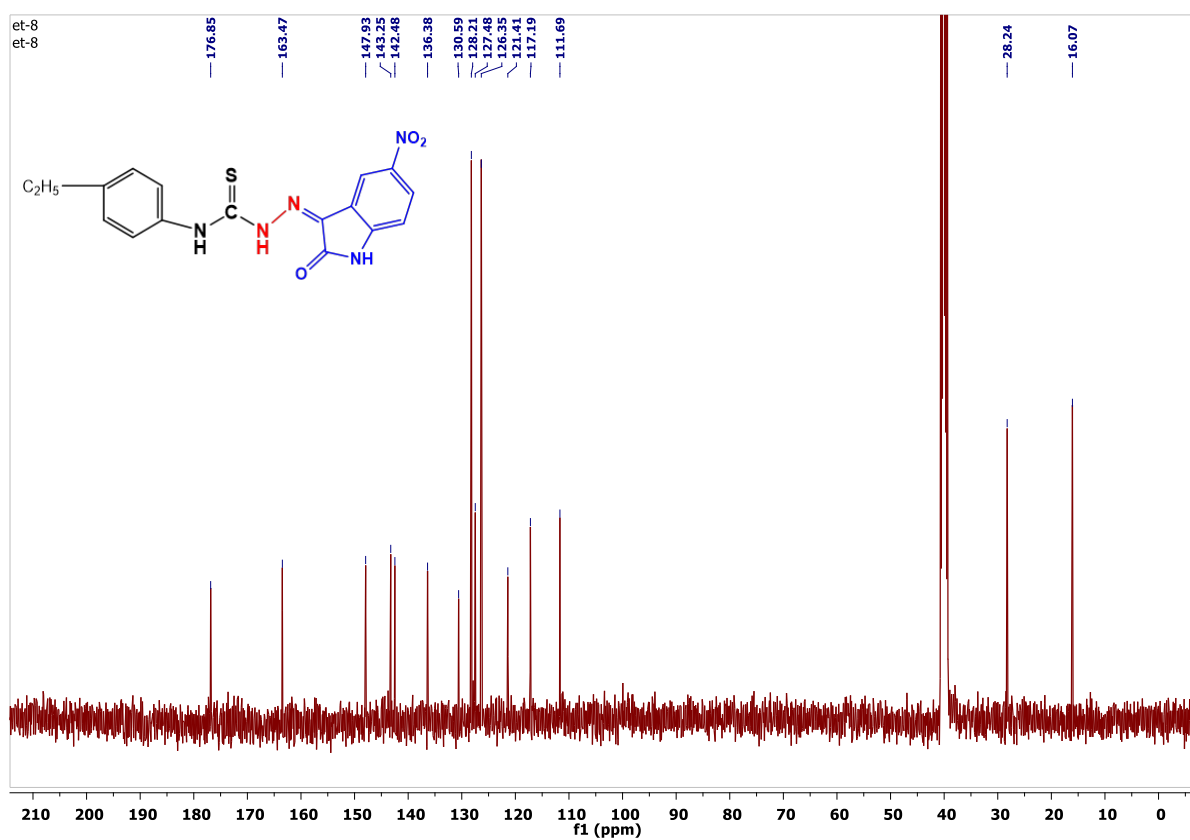


Figure 16. ^{13}C NMR spectrum of compound 8

3.5. Antioxidant activity outcomes

An antioxidant is defined as any substance that, even at low concentrations relative to an oxidizable substrate, delays or prevents the oxidation of that substrate [47]. It can be assumed that the antioxidant activity of the studied molecules is related to their ability to donate hydrogen atoms or to form structurally stable radicals after interacting with the DPPH free radical [48]. The antioxidant activity of all synthesized compounds was evaluated using the DPPH assay, and the results are expressed as IC₅₀ values, as presented in Table 6.

As seen in the Table 6, compound 7 showed higher activity than the standard trolox and other compounds. The other compounds showed lower activity than the standard. IC₅₀ values of the compounds were found between 15.88 ± 1.21 and 145.34 ± 2.68 μM. The order of decreasing antioxidant potential of the compounds is as follows: 7 > 2 > 3 > 8 > 6 > 5 > 4 > 1.

Table 6. IC₅₀ values for the synthesized compounds

Samples	R	DPPH [•] scavenging activity, (IC ₅₀ , μg/mL)
1	H	145.34±2.68
2	F	47.70±1.45
3	Cl	61.73±0.10
4	Br	114.88±1.31
5	I	92.81±2.33
6	CH ₃	83.70±3.05
7	OCH ₃	15.88±1.21
8	NO ₂	71.45±2.43
Trolox		26.27±0.42

The OCH₃ group (15.88 μg/mL), which had the lowest IC₅₀ value, exhibited the strongest antioxidant activity among all compounds in the study, with activity even higher than that of the reference standard Trolox (26.27 μg/mL). The OCH₃ group exerts a strong +M (mesomeric) electron-donating effect on the ring. This stabilizes the radical, increasing its electron density and demonstrating the strongest antioxidant activity. When halogen-containing derivatives were evaluated, the order of activity was determined as F > Cl > I > Br. The relatively low IC₅₀ value of fluorine compound 2 (47.70 μg/mL) may be attributed to the lack of steric hindrance caused by fluorine's small atomic radius. In contrast, larger volume halogens such as Br and I, due to their increased steric effects,

made interaction with the radical more difficult, reducing antioxidant activity and exhibiting higher IC₅₀ values. This is particularly supported by the fact that the bromine derivative (comp.4) had one of the weakest activities at 114.88 μg/mL.

The NO₂ substituent (IC₅₀ = 71.45 μg/mL), an electron-withdrawing group, showed moderate activity. The strong -M (mesomeric) and -I (inductive) effects of the nitro group reduced the electron density on the ring, hindering radical stabilization and reducing activity. Similarly, the methyl group (CH₃, IC₅₀= 83.70 μg/mL), although slightly electron-donating, was not as effective as OCH₃. This is due to the methyl group's lack of resonance contribution. In previous works, the compounds containing methoxy, fluoro, chloro, and nitro groups/atoms exhibited significantly higher antioxidant activity compared to various other substituted structures [49-51].

In conclusion, the antioxidant activity of the synthesized compounds is highly dependent on the electronic and steric properties of the substituents. The methoxy derivative demonstrated exceptional radical scavenging ability, even surpassing Trolox.

4. Conclusion

In this study, a series of new isatin-derived thiosemicarbazones (compounds 1–8) were synthesized with good yields ranging from 61% to 89%. The structures of all compounds were elucidated using spectroscopic techniques, including FT-IR, ¹H-NMR, ¹³C-NMR, and elemental analysis.

The *in vitro* antioxidant activity of the synthesized compounds was evaluated using the DPPH free radical scavenging assay. The IC₅₀ values of the newly obtained molecules ranged from 15.88 ± 1.21 to 145.34 ± 2.68 μM. The findings demonstrate that the electronic properties of substituents attached to the aromatic ring are decisive for antioxidant activity. Among them, compound 7 (-OCH₃) exhibited the most potent antioxidant activity against the DPPH radical.

Overall, structure-activity relationship (SAR) analysis confirms that electron-donating substituents significantly increase antioxidant capacity, while bulky groups tend to reduce activity. The insights obtained here can guide future structural optimization efforts aimed at developing potent antioxidant agents.

Article Information Form

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The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the author.

Artificial Intelligence Statement

No artificial intelligence tools were used while writing this article.

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