Molecular Docking and *in vitro* Antileishmanial Evaluation of Chromene-2-thione Analogues

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Supplementary Information

Contents

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Material and general methods

1. General

All the reagents were purchased from Merck, Aldrich and Fluka, and they were used as received. All ^{1}H and ^{13}C NMR spectra were recorded on JEOL AL 300 FT-NMR spectrometer. Chemical shifts are given as δ value with reference to tetramethylsilane (TMS) as the internal standard. The IR spectra were recorded on Varian 3100 FT-IR spectrophotometer. Mass spectra were recorded at 70 eV ionizing voltage on a JEOL-D300 MS instrument. The C, H, and N analyses were performed from microanalytical laboratory with an Exeter Analytical Inc. "Model CE-400 CHN Analyzer". All the reactions were monitored by TLC using precoated sheets of silica gel G/UV-254 of 0.25 mm thickness (Merck $60F_{254}$) using UV light (254 nm/365 nm) for visualization. Melting points were determined with Büchi B-540 melting point apparatus and are uncorrected.

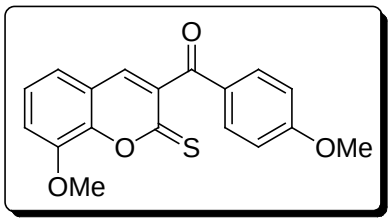
2. Experimental procedure for the synthesis of chromene-2-thione and benzo[*f*]chromene-2-thione

A mixture of β-oxodithioester (1.0 mmol), salicylaldehyde (or 2-hydroxy-1-naphthaldehyde) (1.2 mmol) and urea (1.2 mmol) were treated under solvent-free conditions in the presence of $InCl_3$ (20 mol%, 0.2 mmol, 0.044 g) with constant stirring at 100 ^{o}C. The heating was continued until the completion of the reaction (2-3 hours, monitored by TLC). The reaction mixture was treated with water (20 ml) and extracted with ethyl acetate (2 × 20 ml). The combined organic layers were washed with brine (1 × 20 ml) and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum to yield the product, which was then purified either by recrystallization

from ethyl acetate or by column chromatography over silica gel using increasing amount of ethyl acetate in *n*-hexane.

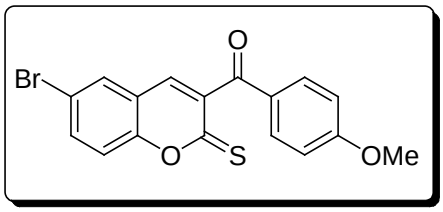
3. Spectral and analytical data

3-(4-Methoxybenzoyl)-8-methoxy-2*H*-chromene-2-thione (3a):



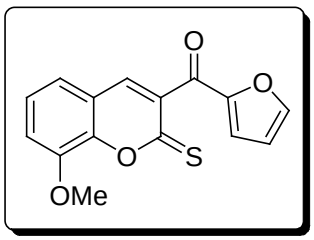
Yellow solid: mp 141-143 ^{o}C. IR (KBr): 3064, 2925, 1648, 1597, 1547, 1267, 1172 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.90 (d, J = 8.7 Hz, 2H, ArH), 7.54 (s, 1H, ArH), 7.27 (d, J = 8.4 Hz, 1H, ArH), 7.17-7.10 (m, 2H, ArH), 6.92 (d, J = 8.7 Hz, 2H, ArH), 4.01 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.9, 190.8, 164.2, 146.9, 146.7, 139.7, 133.2, 132.1, 128.5, 125.7, 120.7, 119.5, 114.4, 114.0, 56.2, 55.5. FAB MS (*m/z*): 327 (M^++1); Anal. Calcd. for $C_{18}H_{14}O_4S$: C, 66.24%; H, 4.32%. Found: C, 66.37%; H, 4.21%.

6-Bromo-3-(4-methoxybenzoyl)-2*H*-chromene-2-thione (3b):



Yellow solid: mp 207-208 ^{o}C. IR (KBr): 3050, 2921, 1659, 1601, 1553, 1261, 1234, 1174 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.89 (d, J = 9.0 Hz, 2H, ArH), 7.74-7.70 (m, 2H, ArH), 7.46 (s, 1H, ArH), 7.40 (d, J = 8.7 Hz, 1H, ArH), 6.94 (d, J = 9.0 Hz, 2H, ArH), 3.88 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 193.0, 190.3, 164.5, 155.7, 140.4, 135.7, 132.2, 131.3, 130.5, 128.3, 121.5, 118.5, 118.3, 114.2, 55.6. FAB MS (*m/z*): 377 (M^++1); Anal. Calcd. for $C_{17}H_{11}BrO_3S$: C, 54.41%; H, 2.95%. Found: C, 54.53%; H, 3.09%.

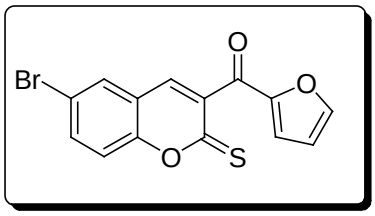
3-(Furan-2-oyl)-8-methoxy-2*H*-chromene-2-thione (3c):



Orange crystals: mp 169-170 ^{o}C. IR (KBr): 2928, 1657, 1600, 1559, 1257, 1170 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.61 (d, J = 6.3 Hz,

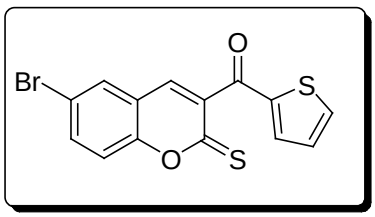
1H, ArH), 7.59 (s, 1H, ArH), 7.29-7.11 (m, 4H, ArH), 6.57 (dd, *J* = 1.5 Hz, 1.8 Hz, 1H, ArH), 4.01 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.5, 179.6, 151.7, 147.5, 146.7, 138.3, 133.7, 125.7, 120.4, 120.1, 119.6, 114.7, 112.8, 56.3. FAB MS (*m/z*): 287 (M^++1); Anal. Calcd. for $C_{15}H_{10}O_4S$: C, 62.93%; H, 3.52%. Found: C, 62.69%; H, 3.31%.

3-(Furan-2-oyl)-6-bromo-2*H*-chromene-2-thione (3d):



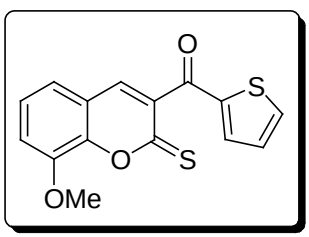
Yellow crystals: mp 198-199 ^oC. IR (KBr): 3119, 1639, 1552, 1460, 1363, 1240, 1174 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.75-7.70 (m, 2H, ArH), 7.63 (s, 1H, ArH), 7.51 (s, 1H, ArH), 7.38 (d, *J* = 8.7 Hz, 1H, ArH), 7.28 (d, *J* = 3.3 Hz, 1H, ArH), 6.60–6.59 (m, 1H, ArH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.4, 179.0, 155.7, 151.5, 147.6, 138.8, 135.9, 131.7, 130.6, 121.2, 120.2, 118.4, 118.2, 112.9. FAB MS (*m/z*): 337 (M^++1); HRMS (ESI^+) Calcd for $C_{14}H_7BrO_3S$: 333.9299. Found 333.9210.

3-(Thien-2-oyl)-6-bromo-2*H*-chromene-2-thione (3e):



Yellow crystal: mp 202-203 ^oC. IR (KBr): 3054, 2923, 1639, 1601, 1549, 1236, 1162 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.77-7.69 (m, 3H, ArH), 7.64 (d, *J* = 3.3 Hz, 1H, ArH), 7.50 (s, 1H, ArH), 7.39 (d, *J* = 8.7 Hz, 1H, ArH), 7.13 (t, 1H, ArH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.4, 183.7, 155.7, 142.5, 139.6, 135.9, 135.1, 131.2, 131.1, 130.6, 128.4, 121.2, 118.5, 118.2. FAB MS (*m/z*): 353 (M^++1). Anal. Calcd. for $C_{14}H_7BrO_2S_2$: C, 47.87%; H, 2.01%. Found: C, 47.63%; H, 2.32%.

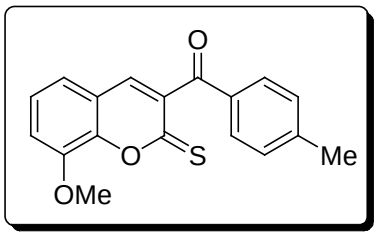
3-(Thien-2-oyl)-8-methoxy-2*H*-chromene-2-thione (3f):



Yellow solid: mp 167-168 ^oC. IR (KBr): 3025, 1632, 1602, 1549, 1405, 1234, 1158 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.74 (d, *J* = 4.5 Hz,

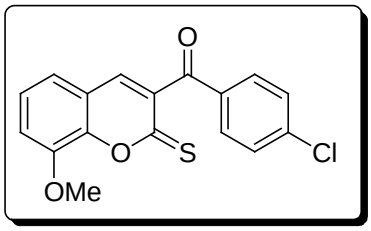
1H, ArH), 7.65 (d, *J* = 3.3 Hz, 1H, ArH), 7.57 (s, 1H, ArH), 7.31-7.25 (m, 1H, ArH), 7.18-7.10 (m, 3H, ArH), 4.01 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.6, 184.9, 146.2, 144.2, 143.1, 138.5, 135.6, 134.9, 127.9, 125.0, 121.2, 120.1, 119.7, 111.5, 56.1. FAB MS (*m/z*): 303 (M^++1); HRMS (ESI^+) Calcd. for $C_{15}H_{10}O_3S_2$: 302.0071. Found 302.0054.

3-(4-Methylbenzoyl)-8-methoxy-2*H*-chromene-2-thione (3g):



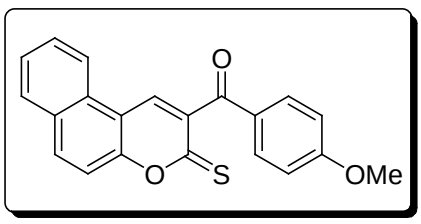
Yellow solid: mp 164-165 ^oC. IR (KBr): 3054, 2961, 2922, 1658, 1604, 1564, 1241, 1158 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.82 (d, *J* = 8.1 Hz, 2H, ArH), 7.55 (s, 1H, ArH), 7.18-7.11 (m, 5H, ArH), 4.01 (s, 3H, OCH_3), 2.41 (s, 3H, $ArCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 193.0, 192.1, 146.9, 145.0, 139.9, 133.5, 133.1, 129.8, 129.6, 126.0, 120.9, 119.3, 114.5, 56.4, 21.9. FAB MS (*m/z*): 311 (M^++1); Anal. Calcd. for $C_{18}H_{14}O_3S$: C, 69.66%; H, 4.55%. Found: C, 69.81%; H, 4.25%.

3-(4-Chlorobenzoyl)-8-methoxy-2*H*-chromene-2-thione (3h):



Yellow solid: mp 176-177 ^oC. IR (KBr): 2966, 2932, 1674, 1568, 1468, 1376, 1236, 1169 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.85 (d, *J* = 8.1 Hz, 2H, ArH), 7.59 (s, 1H, ArH), 7.42 (d, *J* = 8.1 Hz, 2H, ArH), 7.30-7.25 (m, 1H, ArH), 7.20-7.15 (m, 2H, ArH), 4.02 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.7, 191.3, 147.1, 146.5, 140.3, 138.9, 134.2, 134.1, 131.0, 129.2, 126.0, 120.7, 119.9, 114.5, 56.3. FAB MS (*m/z*): 331 (M^++1); Anal. Calcd. for $C_{17}H_{11}ClO_3S$: C, 61.73%; H, 3.35%. Found: C, 61.61%; H, 3.39%.

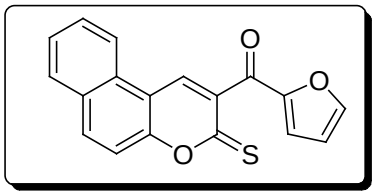
3-(4-Methoxybenzoyl)-benzo[*f*]2*H*-chromene-2-thione (3i):



Yellow solid: mp 246-247 ^oC. IR (KBr): 3067, 1662, 1596, 1547, 1259, 1203 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 8.34 (s,

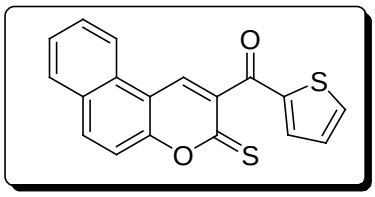
1H, ArH), 8.23 (d, *J* = 8.1 Hz, 1H, ArH), 8.11 (d, *J* = 9.0 Hz, 1H, ArH), 7.96 (d, *J* = 8.4 Hz, 3H, ArH), 7.74-7.63 (m, 3H, ArH), 6.95 (d, *J* = 8.7 Hz, 2H, ArH), 3.88 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.6, 191.2, 164.3, 157.6, 141.0, 139.1, 135.0, 134.5, 132.2, 130.6, 129.5, 129.2, 128.9, 128.6, 127.0, 121.6, 116.4, 115.2, 114.0, 113.8, 55.5. FAB MS (*m/z*): 347 (M^++1); Anal. Calcd. for $C_{21}H_{14}O_3S$: C, 72.81%; H, 4.07%. Found: C, 72.59%; H, 3.92%.

3-(Furan-2-oyl)-benzo[*f*]2*H*-chromene-2-thione (3j):



Yellow solid: mp 252-253 ^oC. IR (KBr): 3030, 1641, 1558, 1462, 1284, 1170 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 8.39 (s, 1H, ArH), 8.25 (d, *J* = 8.1 Hz, 1H, ArH), 8.11 (d, *J* = 9.0 Hz, 1H, ArH), 7.96 (d, *J* = 7.8 Hz, 1H, ArH), 7.76-7.62 (m, 4H, ArH), 7.33 (d, *J* = 3.6 Hz, 1H, ArH), 6.61-6.59 (m, 1H, ArH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.3, 179.9, 157.7, 151.8, 147.5, 137.7, 134.9, 130.6, 130.0, 129.2, 129.0, 128.8, 127.0, 121.6, 120.1, 116.4, 115.0, 112.8. FAB MS (*m/z*): 307 (M^++1); Anal. Calcd. for $C_{18}H_{10}O_3S$: C, 70.57%; H, 3.29%. Found: C, 70.69%; H, 3.41%.

3-(Thien-2-oyl)-benzo[*f*]2*H*-chromene-2-thione (3k):



Yellow solid: mp 267-268 ^oC. IR (KBr): 3058, 1634, 1554, 1407, 1352, 1198 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 8.37 (s, 1H, ArH), 8.23 (d, *J* = 8.4 Hz, 1H, ArH), 8.12 (d, *J* = 9.0 Hz, 1H, ArH), 7.96 (d, *J* = 7.5 Hz, 1H, ArH), 7.78-7.63 (m, 5H, ArH), 7.15-7.12 (m, 1H, ArH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.2, 184.6, 176.7, 157.7, 151.3, 147.4, 135.6, 135.1, 134.9, 129.3, 129.2, 129.0, 128.4, 127.0, 121.6, 116.5, 112.8. FAB MS (*m/z*): 323 (M^++1); Anal. Calcd. for $C_{18}H_{10}O_2S_2$: C, 67.06%; H, 3.13%. Found: C, 66.91%; H, 3.26%.

4. Computational studies

AutoDock 4.2 was used to perform molecular docking of small molecules on macromolecular protein by treating the ligand as conformationally flexible. Autodock 4.2 also has a free-energy scoring function, which uses AMBER force field to estimate the free-energy of binding of a ligand to its target. Docking simulations were performed using AutoDock4.2, implementing Lamarckian Genetic Algorithm (LGA).[1] Gridmaps for each atom type in the ligand were generated as well as electron density maps and desolvation maps were formed using the Autogrid4 utility in AutoDock. The following specifications were used for the grids. Grid size was 120 Å for the three coordinates (X,Y,Z) with grid spacing set to 0.375 Å , the X, Y, and Z coordinates were fixed at 21.0, 30.0, and 5.0, respectively. Nonflexible docking was carried out and the active site of receptor was kept rigid. Docking was carried out with 20 LGA runs (ga_run, 20), initial population size of 300 (ga_pop_size, 300) and 250000 energy evaluations (ga_num_evals, 250000). Other docking parameters were set to default. To identify the possible targets, a series of compounds have been docked against several target enzymes of the *Leishmania* parasite (like trypanothione synthetase, spermidine synthase and trypanothione reductase). We found acceptable docking statistics with the enzyme trypanothione reductase. Thus, the docking results with trypanothione reductase (2JK6) were further analysed.

Free energy of binding values for each ligand is given in **Table 1**, along with description of interacting residues.

Table 1 Docking statistics of the compounds with Trypanothione reductase and interaction of these compounds with the protein

Entry	Binding energy	H-Bond	Possible Hydrophobic Interactions
3a	-7.78	2JK6:Met-400(A):NH	Pro-398, Phe-396, Glu-467, Glu-466, Thr-463 from A chain. Leu-62, Lys-61 from B chain
3b	-8.09		Val-58, Lys-61, Leu-62, Thr-65 from B chain. Phe-396, Thr-397, Pro-398, Leu-399, Met-400, His-461 and Pro-462 from A chain
3c	-6.82		Val-58, Lys-61, Leu-62, Thr-65 from B chain. Thr-463, Pro-398, Phe-396, Leu-399, Met-400 and Pro-462 from A chain
3d	-7.63	2JK6:Lys-61(B):HZ2	Val-58, Lys-61, Leu-62 from B chain. Phe-396, Pro-398, Leu-399, Thr-463 and Ser-464 from A chain
3e	-7.54		Val-58, Lys-61, Leu-62, Thr-65 from B chain. Phe-396, Pro-398, Met-400, His-461 and Pro-462 from A chain
3f	-7.35		Lys-61, Leu-62, Thr-65, Phe-396, Pro-398, Met-400 and Pro-462
3g	-8.22	2JK6:Lys-61(B):HZ2	Leu-62, Thr-65 from B chain. Phe-396, Thr-397, Pro-398, Met-400, Pro-462 and Thr-463 from A chain.
3h	-8.11		Val-58, Lys-61, Leu-62, Thr-65 from B chain. Phe-396, Pro-398, Met-400, Pro-462 and Thr-463 from A chain.
3i	-9.20		Val-58, Lys-61, Leu-62, Thr-65 from B chain. Phe-396, Leu-399, Met-400, Thr-463, Glu-466 and Glu-467 from A chain.
3j	-8.17	2JK6:Lys-61(B):HZ2 2JK6:Leu-399(A):HN	Phe-396, Pro-462, Thr-463 and Ser-464 from A chain.
3k	-8.61	2JK6:Ser-464(A):SH and OH	Val-58, Lys-61, Leu-62 from B chain. Phe-396, Pro-398, Leu-399 and Met-400 from A chain.

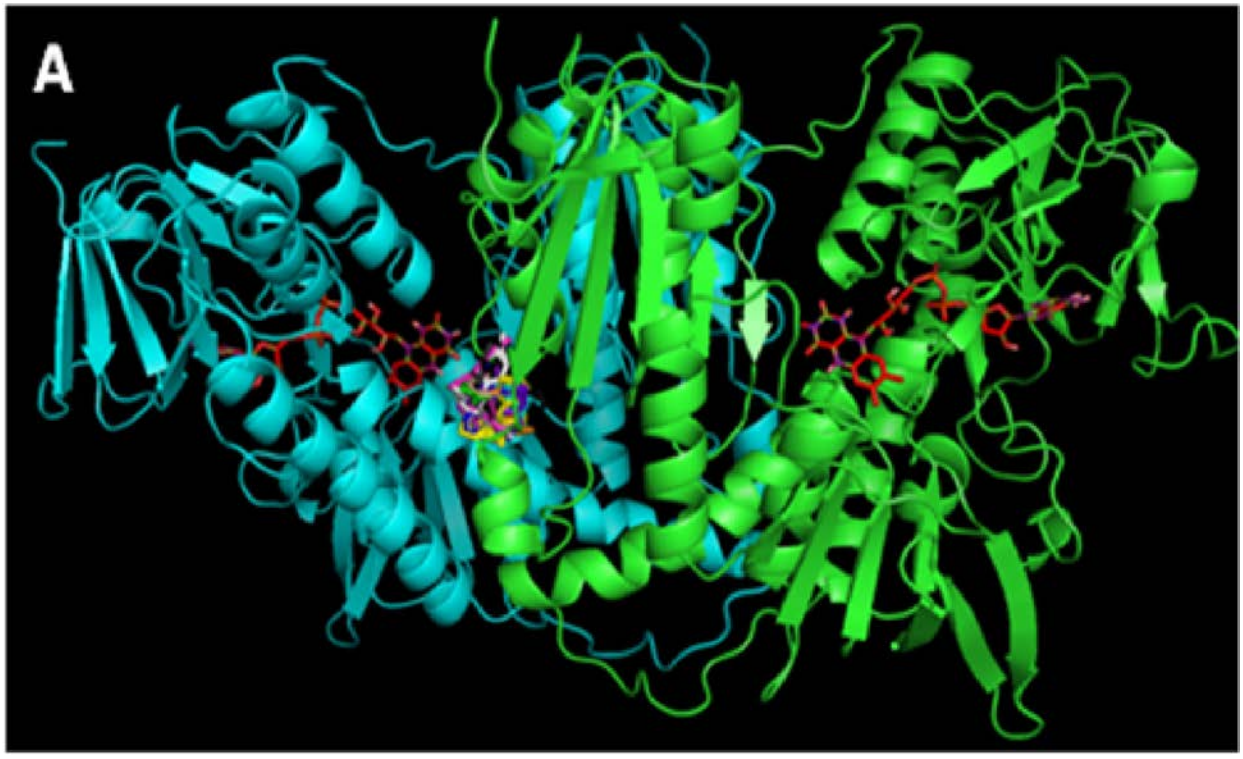


Figure 1 Orientation of all eleven ligands (shown in multicolour) within the interface of chain A (green colour) and chain B (blue colour) of 2JK6 is depicted. FAD molecules (red colour) within 2JK6 are shown, one within the A chain and another within the B chain.

All of the eleven compounds dock at a particular position, near the active site of TryR (Figure 1) where co-factor FAD (shown in red colour) is bound. The conserved residues near the active site which are involved in bonding and non-bonding interactions with the ligands are as follows: Val-58, Lys-61, Leu-62, Thr-65 from B chain and Phe-396, Thr-397, Pro-398, Leu-399, Met-400, His-461, Pro-462, Thr-463 and Ser-464 from A chain. Lys-61, Leu-399, Met-400 and Ser-464 mainly form hydrogen bonds with the ligands. The ligand **3i** shows the least free energy of binding followed by **3k**, **3g**, **3j**, **3h**, **3b**, **3a**, **3d**, **3e**, **3f** and **3c**. The detailed interaction profiles have been shown in Figure 2-6.

4.1 Representative interaction profiles

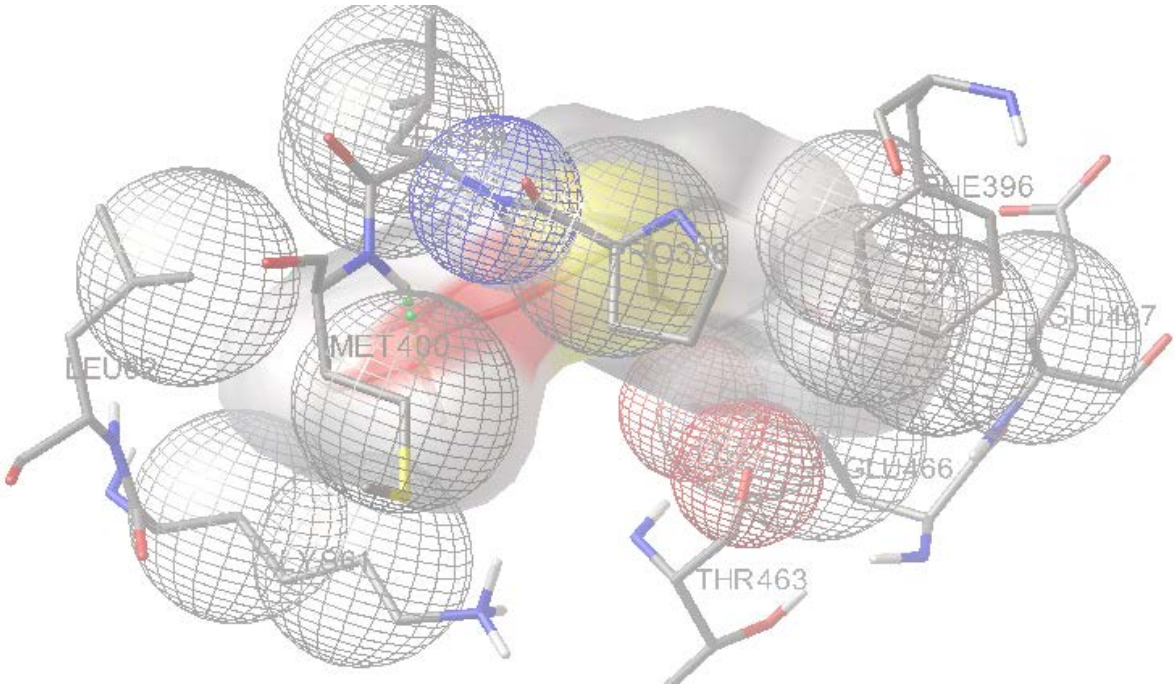


Figure 2 This figure is a representative of compounds making one hydrogen bond with Met-400(A) of Trypanothione reductase. Here it shows compound **3a**.

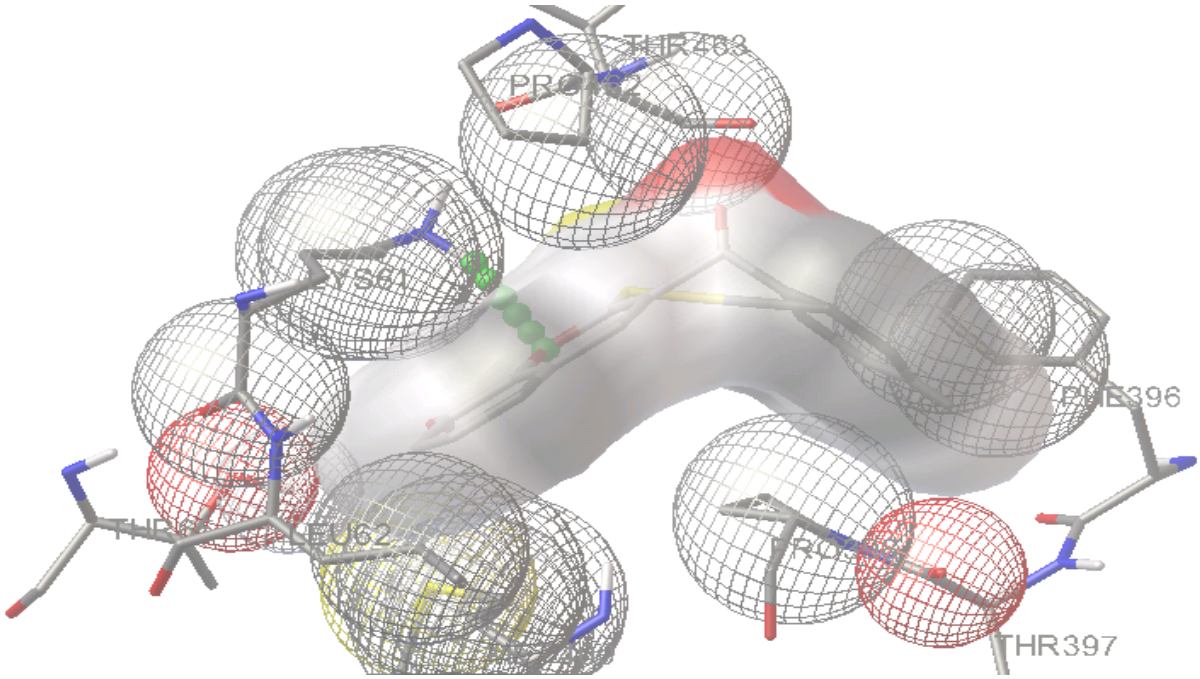


Figure 3 This figure is a representative of compounds making one hydrogen bond with Lys-61(B) of Trypanothione reductase. Here it shows compound **3g**.

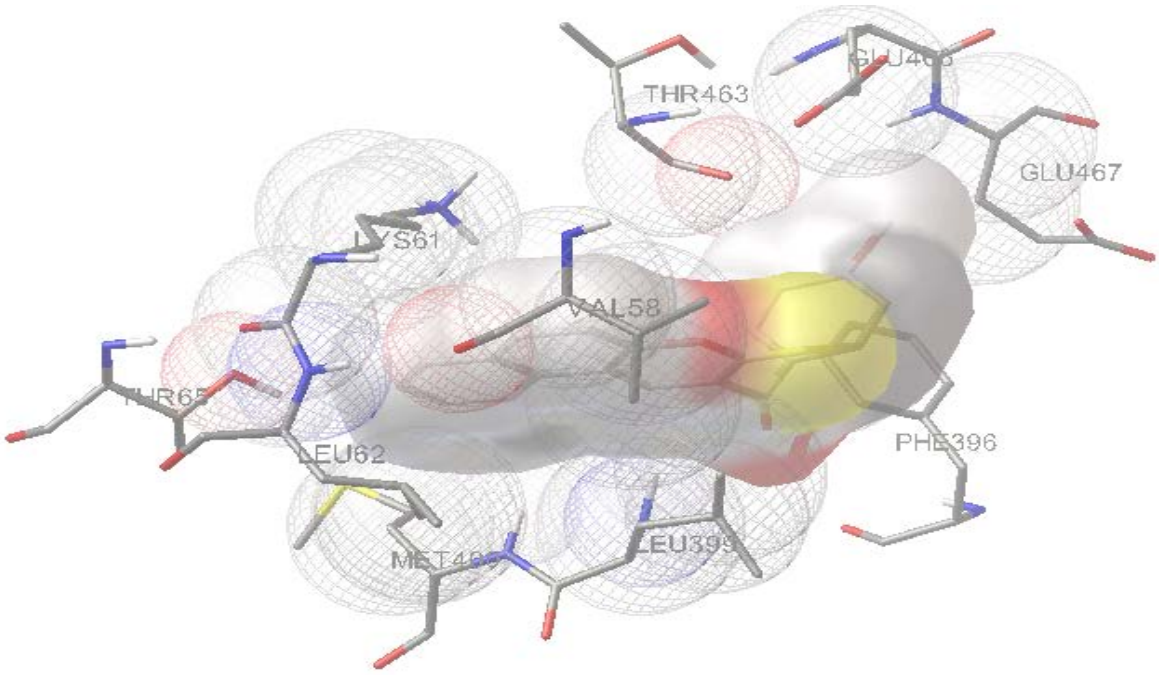


Figure 4 This figure is a representative of compounds making no hydrogen bonds with Trypanothione reductase. Only possible hydrophobic interactions are shown. Here it shows compound **3i**, which shows the highest binding affinity and lowest binding energy with Trypanothione reductase.

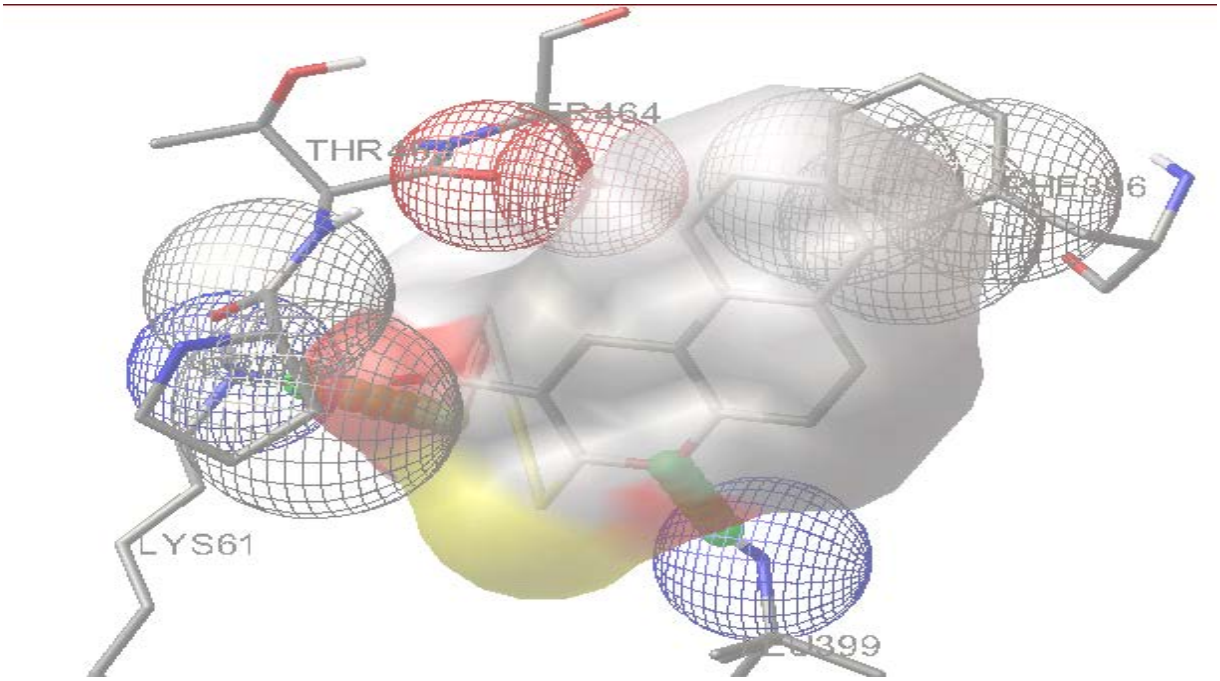


Figure 5 This figure is a representative of compounds making two hydrogen bonds, with Lys-61(B) and Leu-399(A) of Trypanothione reductase. Here it shows compound **3j**.

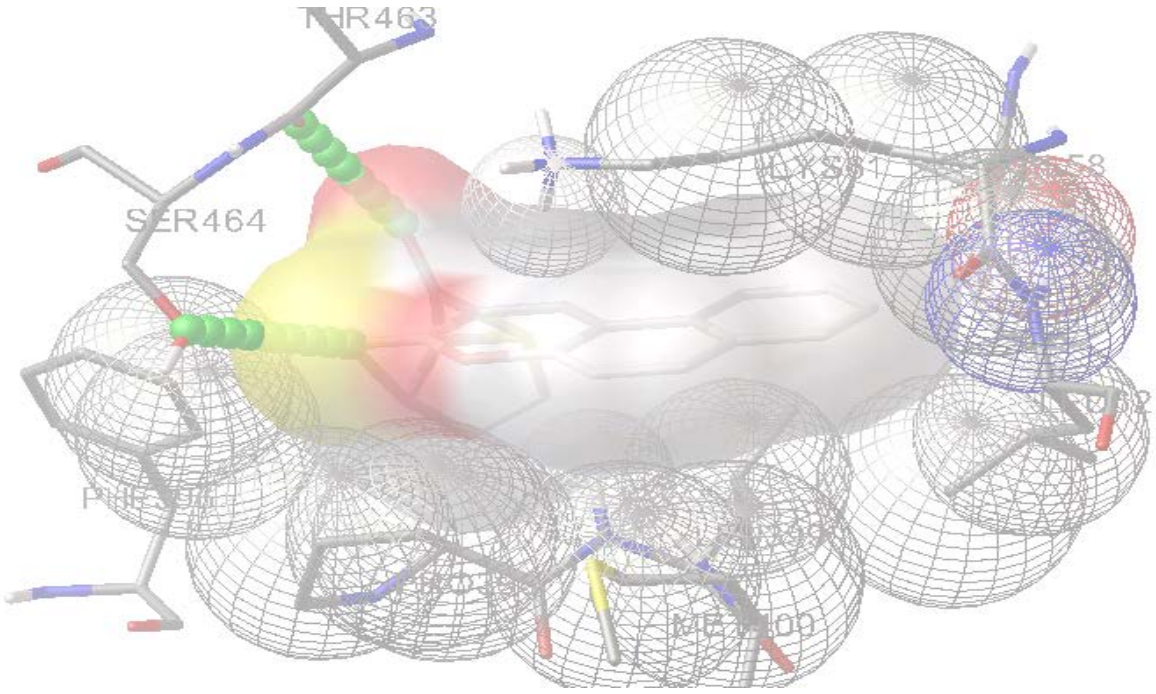


Figure 6 This figure is a representative of compounds making two hydrogen bonds with Ser-464(A) of Trypanothione reductase. Here it shows compound **8k**.

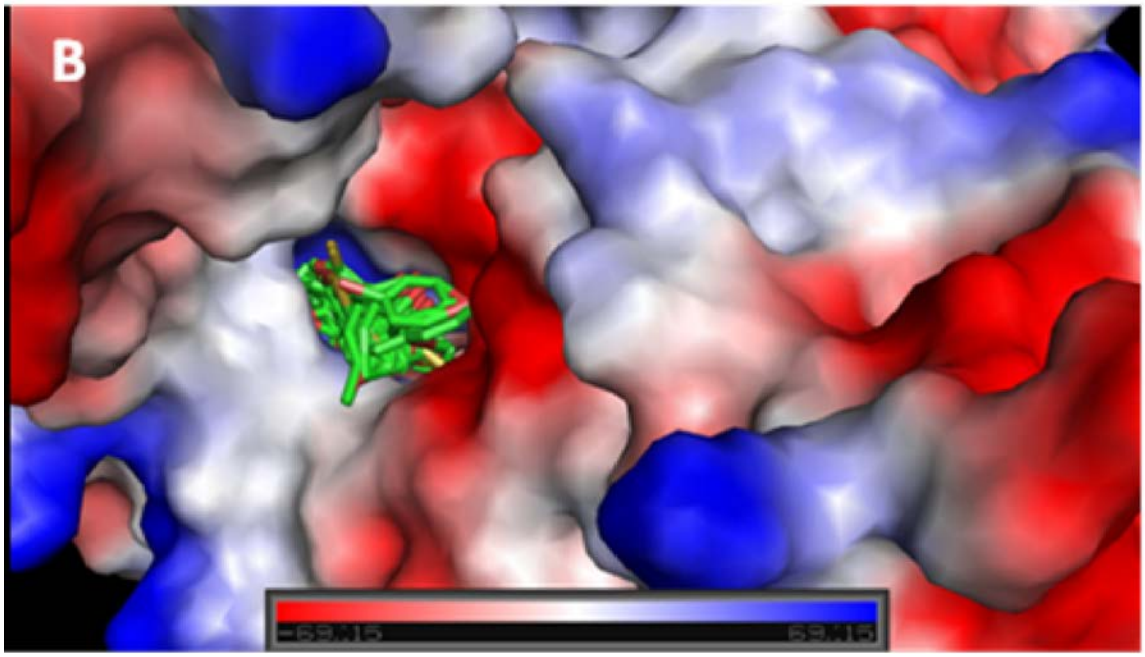


Figure 7 Surface electrostatic potential of Trypanothione reductase (2JK6) is shown, where red depicts negative charge and blue depicts positive charge on the surface of the molecule. This surface depiction shows that all the ligands are positioned inside a cavity formed at the dimeric interface of the protein. The orientation of each ligand is different from the other.

Protein contact potential was generated for all the docked conformations (Figure 7). This depicted the whole surface of the protein. Red and blue colors show negative and positive charges respectively. In this depiction, all the ligands were seen to be bound inside a cavity formed at the interface of chain A and chain B i.e. dimeric interface of the enzyme.

4.2 Lipinsky rule of five: Lipinsky rule of five states that a molecule likely to be developed as an orally active drug candidate should show no more than one violation of the following four criteria – it should not have more than 5 hydrogen bond donors; it should not have more than 10 hydrogen bond acceptors; it should not have molecular weight greater than 500 Da and it should not have octanol-water partition coefficient greater than 5. Molecular properties of all ligands were calculated by molinspiration and the values of each property are tabulated in Table 2. All these compounds do not show any violation of Lipinski's Rule and shows best drug like properties.

Table 2 "Drug like" properties according to Lipinski's Rule of the top ten hits of docking experiments

Compound	**nViol**	**natoms**	**miLogP**	**MW**	**nON**	**nOHNH**	**nrotb**
Acceptable range→			<5	<500	<10	<5	
3a	0	23	3.805	326.37	4	0	4
3b	0	22	3.87	379.27	3	0	3
3c	0	20	2.627	290.34	4	0	3
3d	0	19	3.071	339.21	3	0	2
3e	0	19	3.713	355.27	2	0	2
3f	0	20	3.269	306.40	3	0	3
3g	0	22	3.818	314.406	3	0	3
3h	0	22	4.048	334.82	3	0	3
3i	0	25	4.956	346.40	3	0	3
3j	0	22	4.156	306.34	3	0	2
3k	0	22	4.798	322.41	2	0	2

The molecular properties of these top ten compounds were predicted in molinspiration using the SMILES format (http://www.molinspiration.com/cgi-bin/properties?textMode=1). **nViol**, No. of violations; **miLogP**, molinspiration predicted LogP; **natoms**, no. of atoms; **MW**, molecular weight; **nON**, number of hydrogen bond acceptors; **nOHNH**, number of hydrogen bond donors; **nrotb**, Number of rotatable bond.

5. *In vitro* Antileishmnial evaluation

5.1 *Leishmania donovani* parasite culture

L. donovani parasites LEM-138 (MHOM/ IN/00/DEVI) were cultured in RPMI–1640 medium (HyClone) supplemented with 0.2% $NaHCO_3$, 2.05 mM L-Glutamine, 12 mM HEPES buffer (HiMedia, India), 15% (v/v) heat inactivated FBS (Gibco, Germany) and 50 mg/L gentamicin at 25 ^{o}C.[2] These reference *L. donovani* parasites were used for *in vitro* experiments.

5.2 *In vitro* assay on *L. donovani* promastigotes

Antileishmanial activities of the compounds were tested *in vitro* using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay against a culture of *L. donovani* promastigotes in dimethyl sulfoxides (DMSO). Working solutions of all synthesized compounds, at starting concentrations of 800 µg/mL were prepared in RPMI media and experiments were performed as described previously.[3]

5.3 *In vitro* assay on *Leishmania donovani* axenic amastigotes

L. donovani axenic culture of amastigotes was passaged from promastigotes by changing the pH to 5.5 and gradually increasing the temperature (from 26 to 37 ^{o}C). Drug dilutions were prepared in DMSO and an appropriate concentration of each drug was used in triplicate (10-160 µg/mL). The MTT assay described as above was used for evaluation of drug activity against *L. donovani* amastigotes. The mean percentage of post treatment viable amastigotes was calculated relative to control and results were expressed as concentration inhibiting the parasitic growth.

5.4 *In vitro* susceptibility assay on intramacrophage amastigotes

J774A.1 macrophages in 8 well Labtek culture slides in serum free RPMI media were infected with *L. donovani* parasites at a ratio of 10:1 (leishmania: macrophage). Cells were incubated for 24 hours at 37°C in 5% CO_2. Non internalized promastigotes were removed and infected cells were treated with different concentrations in duplicates (5-20 µg/mL) of selected chemical compounds **3b** [6-Bromo-3-(4-methoxybenzoyl)-2*H*-chromene-2-thione], **3c** [3-(Furan-2-oyl)-8-methoxy-2*H*-chromene-2-thione], **3h** [3-(4-Chlorobenzoyl)-8-methoxy-2*H*-chromene-2-thione] and **3k** [3-(Thien-2-oyl)-benzo[*f*]2*H*-chromene-2-thione] chosen because they had shown good antileishmanial activity in promastigote and axenic amastigote assays. The cultures were incubated for 72 h at 37°C in 5% CO_2, and the slides were fixed in absolute methanol for 5 min and stained with Giemsa dye. At least 100 cells per well were counted to determine the percentage of macrophages infected and the number of parasites per infected cell. The percentage inhibition relative to untreated macrophages was calculated on the basis of the comparison of total amastigotes per 100 macrophage.[4] The IC_{50} was determined for each chemical compounds.

5.5 Cytotoxicity assay on human PBMCs

In vitro cytotoxicity was determined against human PBMC (peripheral blood mononuclear cells) separated from the heparinized blood of a normal healthy individual by Ficoll-Hypaque (Sigma,USA) density gradient centrifugation and then incubated in a 96 well plate in 200 µl per well at a concentration of 5×10^4 cells. The plates were incubated overnight in a CO_2 incubator with a supply of 5% CO_2 at 37 ^oC. The cells were then incubated for 48 hours with the chemically synthesized compounds and reference drug amphotericin B, in triplicate at both IC_{50} and $2 \times IC_{50}$ concentrations of the promastigote susceptibility assay. Three wells were left as

control wells. MTT assay was then performed to assess the cell proliferation or viability. The result was expressed as percent reduction in cell viability compared to untreated control wells. The percentage of cells killed at IC_{50} and 2 × IC_{50} were obtained from the optical density (OD) against drug concentration taking the OD of the control well as 100% growth.[3, 5]

5.6 Statistical analysis

GraphPad Prism5 version was used to calculate the CC_{50} and IC_{50} values. An unpaired t-test (two-tailed) was applied to determine the significance of the differences between the cytotoxicity and antileishmanial activity of the chemical compounds. The results of various *In vitro* analyses is summarised in Table 3.

Table 3 Antilieshmanial activity and cytotoxicity of chromene-2-thione conjugates

Compound	IC_{50} (µM)			Cytotoxicity (%)	
	Axenic amastigotes	Promastigotes	Intracellular amastigotes	At IC_{50}	At 2 × IC_{50}
3a	502	1398	-	16.63	14.82
3b	198	450	17	32.91	40.51
3c	65	1224	26	12.31	25.96
3d	524	1030	-	10.72	17.57
3e	83	4634	-	10.95	6.66
3f	927	4300	-	11.08	19.52
3g	446	744	-	9.40	33.14
3h	73	48	24	0.46	20.22
3i	221	335	-	15.11	26.96
3j	37	2774	-	12.03	27.79
3k	36	97	22	28.84	24.63
Amphotericin B	0.061	0.465	-	16.02	24.40

A comparison of IC_{50} values for amastogotes *Vs* promastogotes have been shown through a graph in Figure 8.

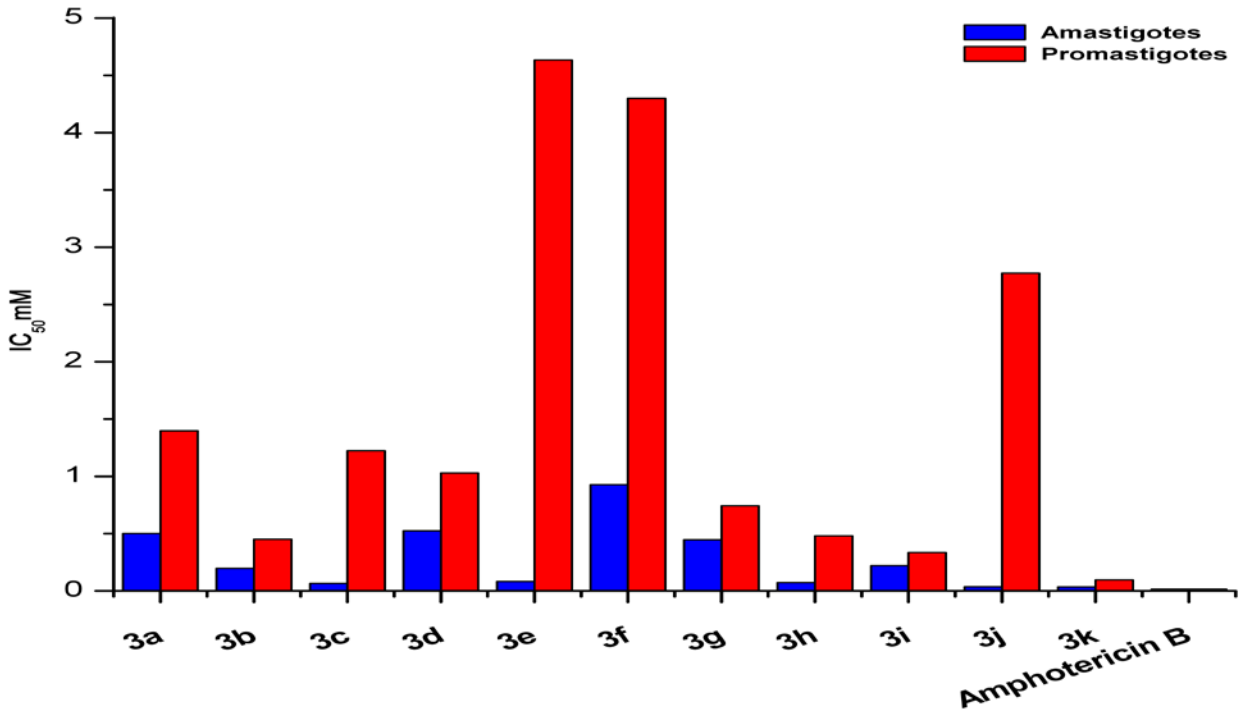
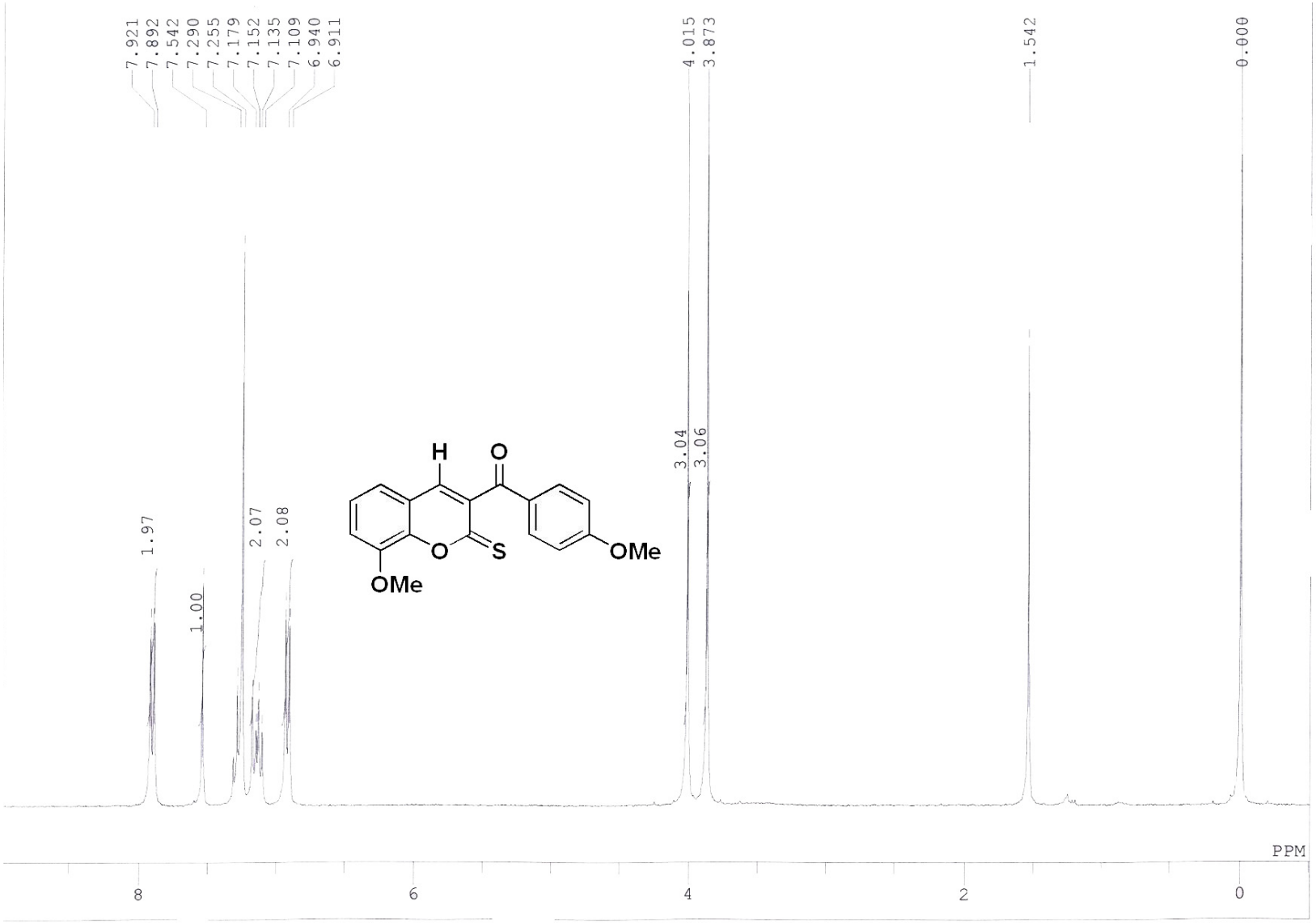


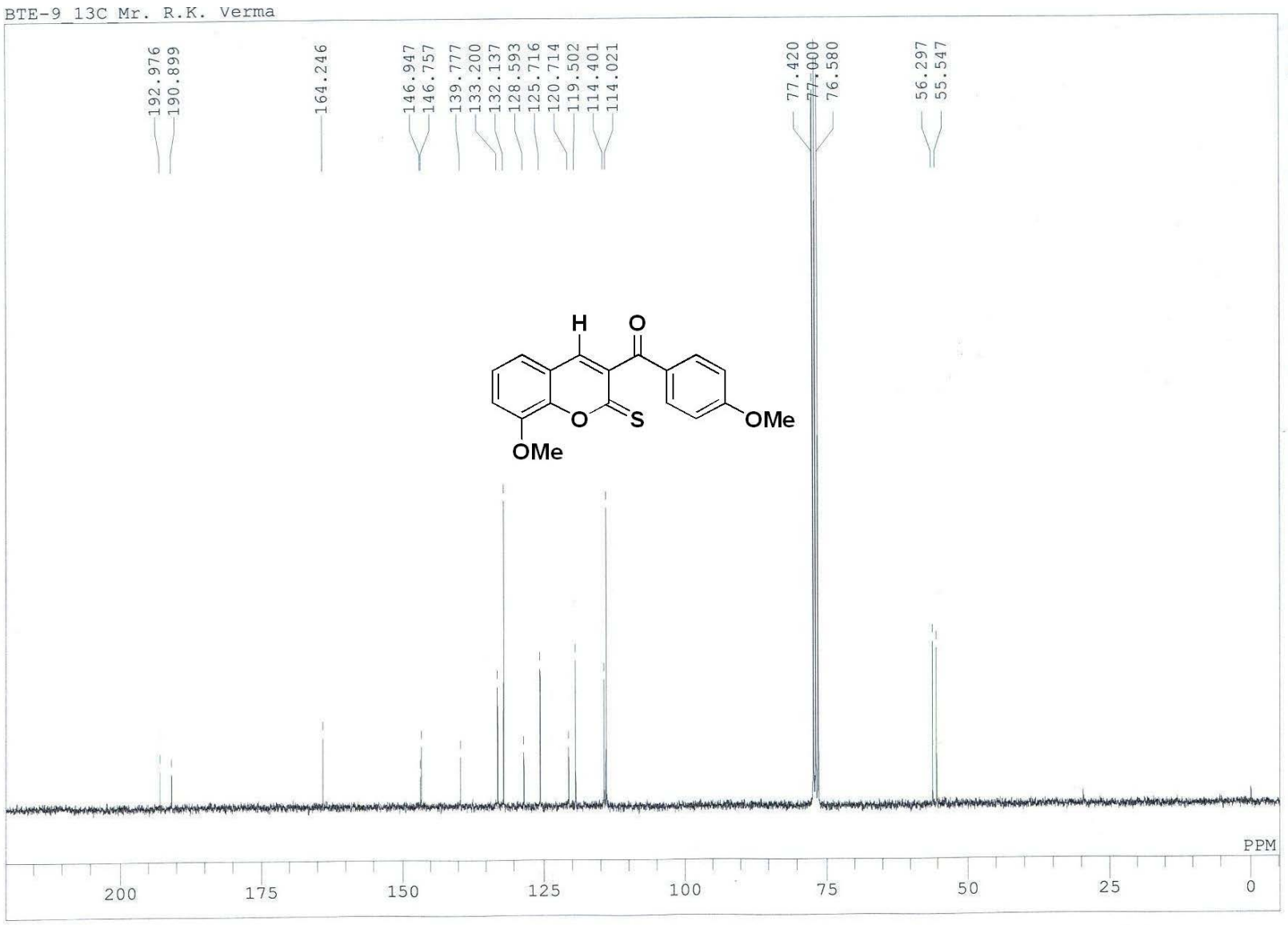
Figure 8 Comparison of IC_{50} values of compounds against amastigotes and promastigotes.

6. ^{1}H and ^{13}C NMR of some selected derivatives

^{1}H NMR of 3a



^{13}C NMR of 3a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119.502
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77.000
76.580
56.297
55.547
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OMe
OMe
PPM
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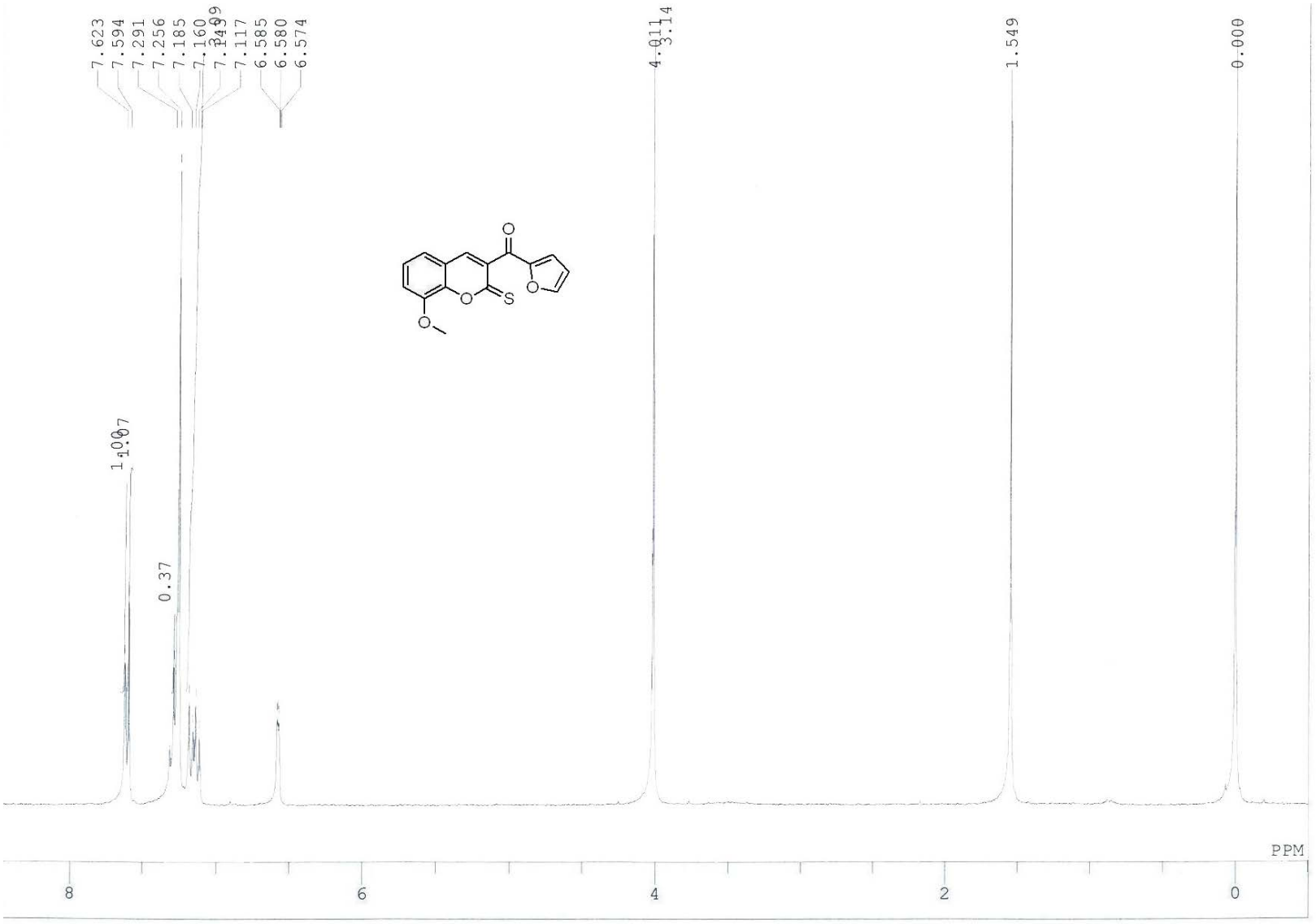
175
150
125
100
75
50
25
0

^{1}H NMR of 3c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6
4
2
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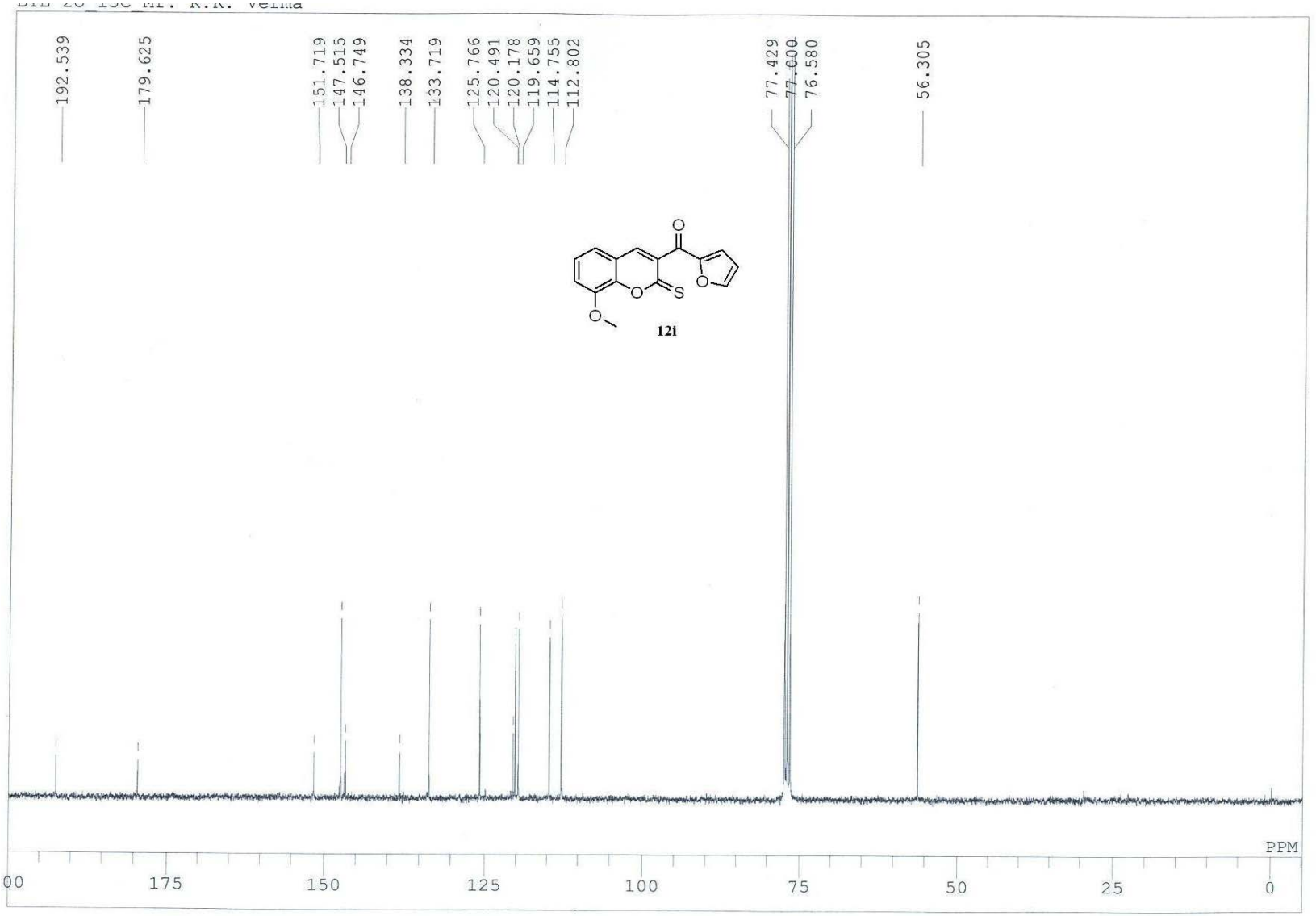
^{13}C NMR of 3c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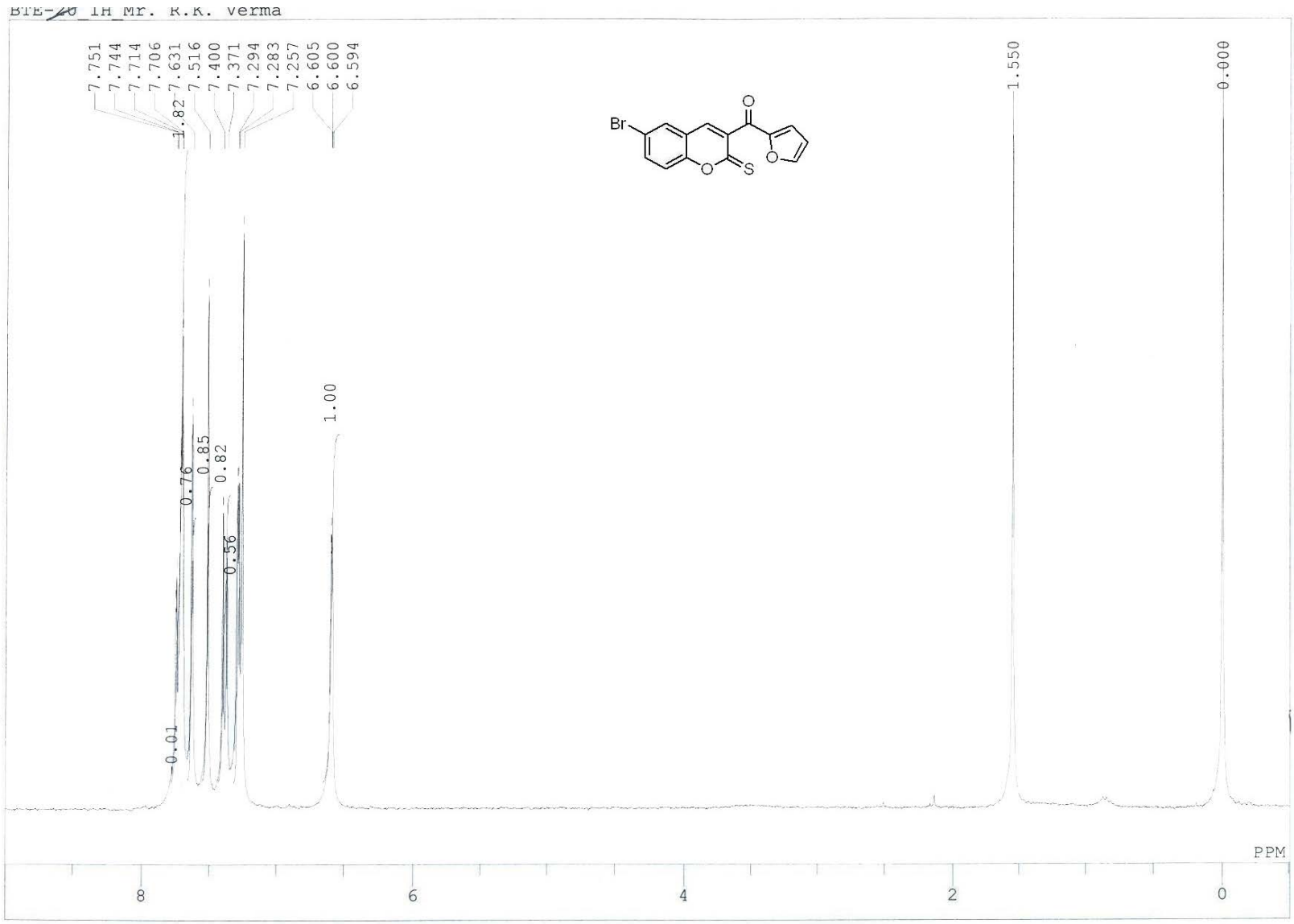
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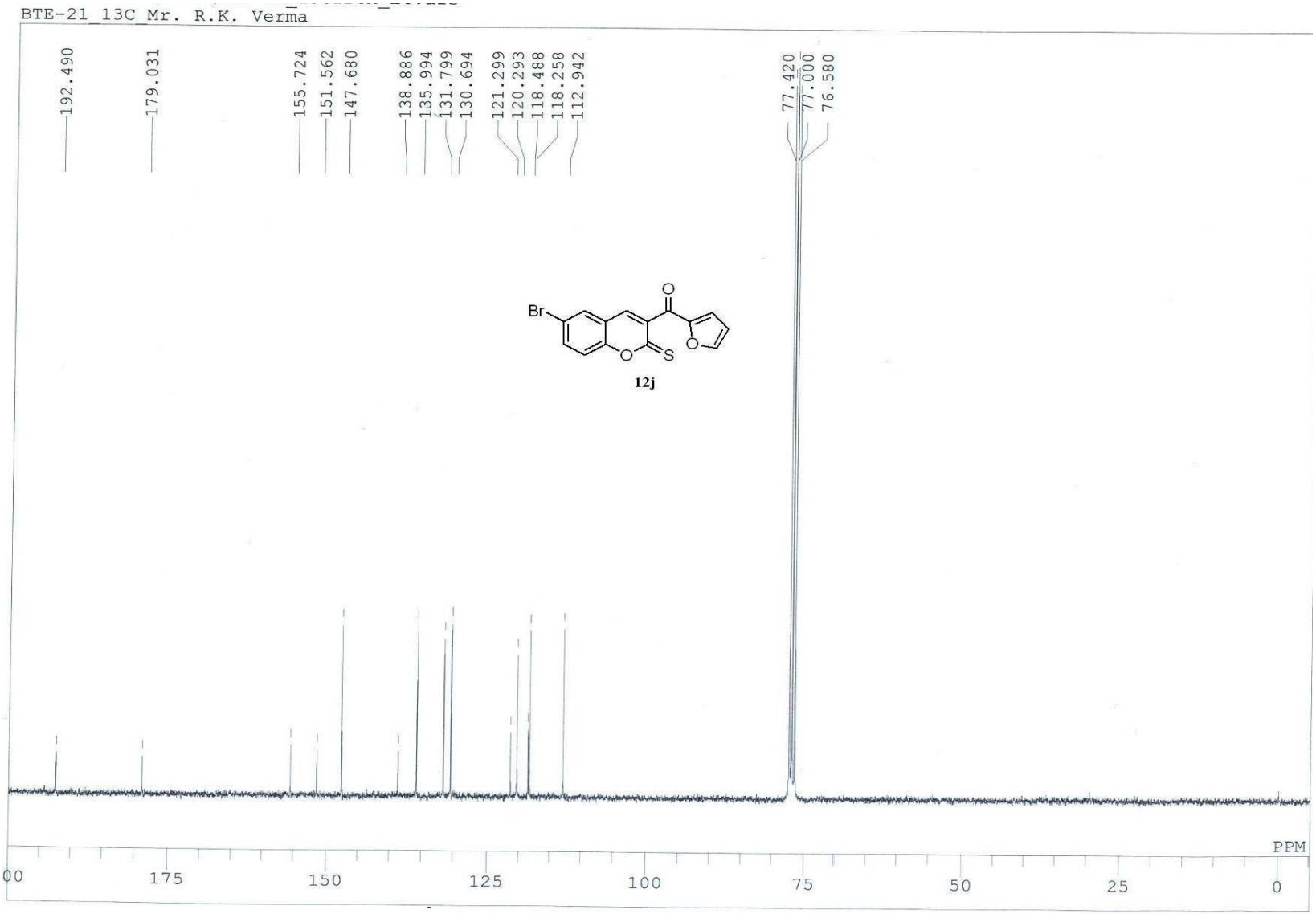
^{13}C NMR of 3d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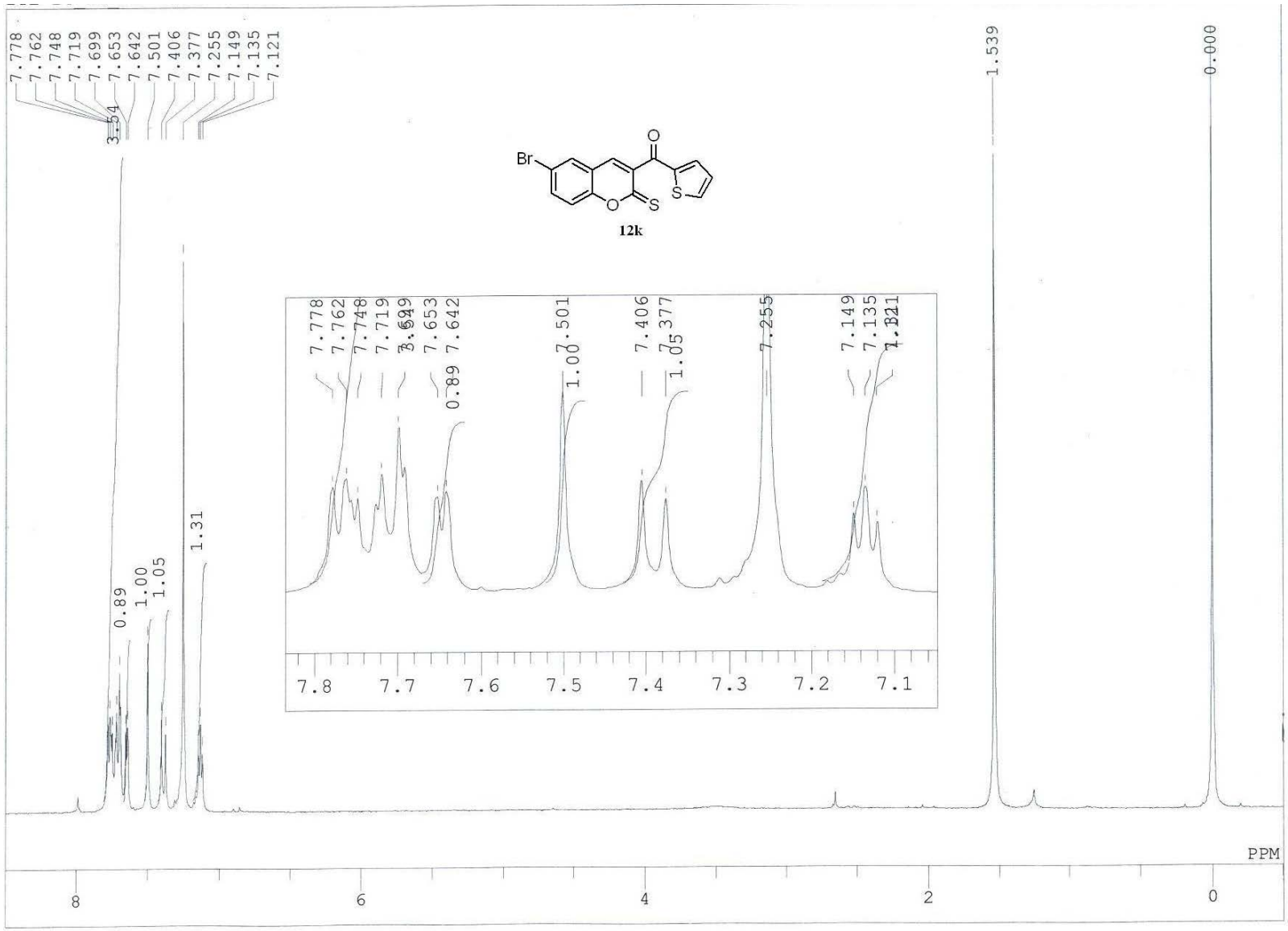
^{1}H NMR of 3e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7.121
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1.539
0.000
0.89
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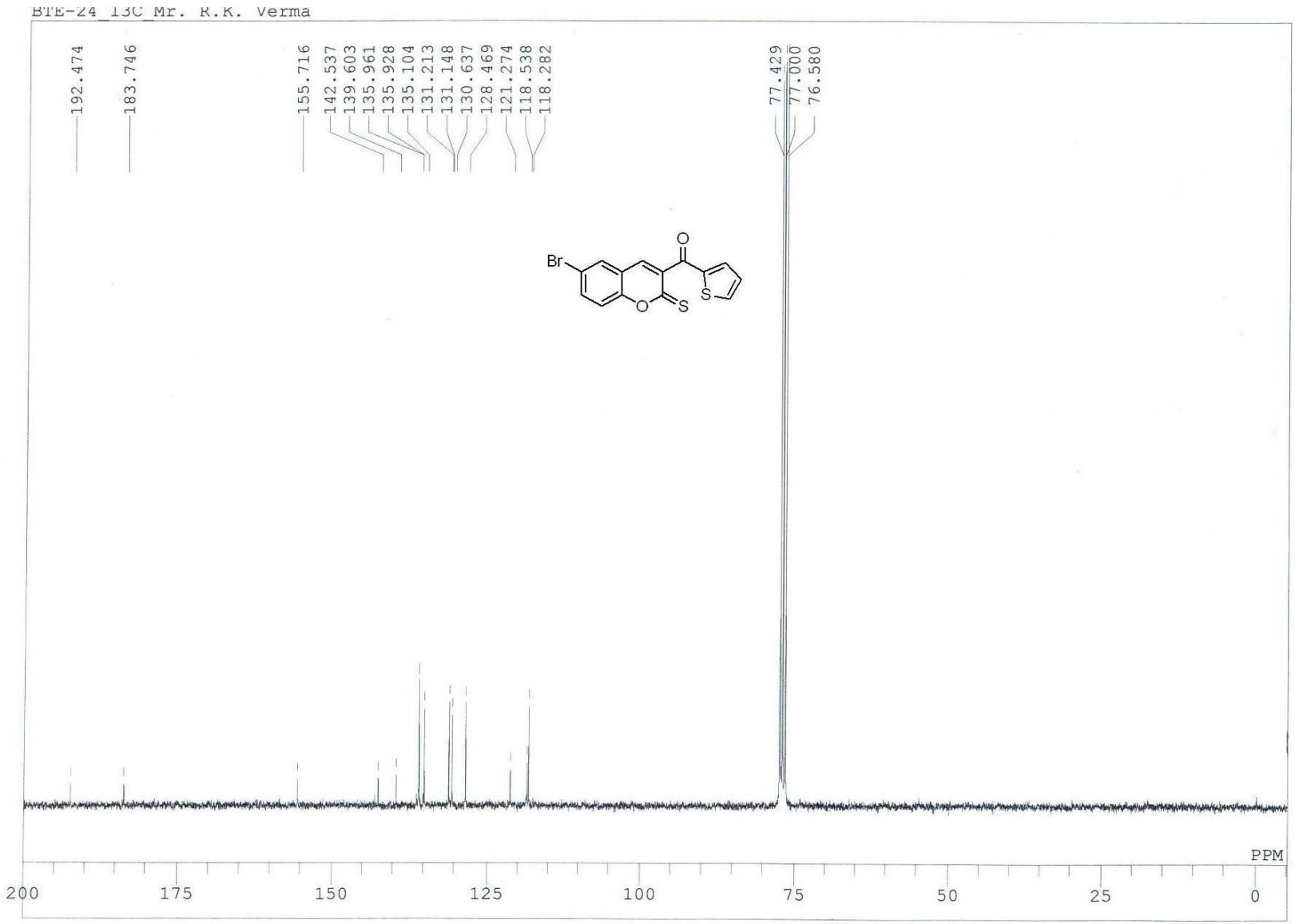
^{13}C NMR of 3e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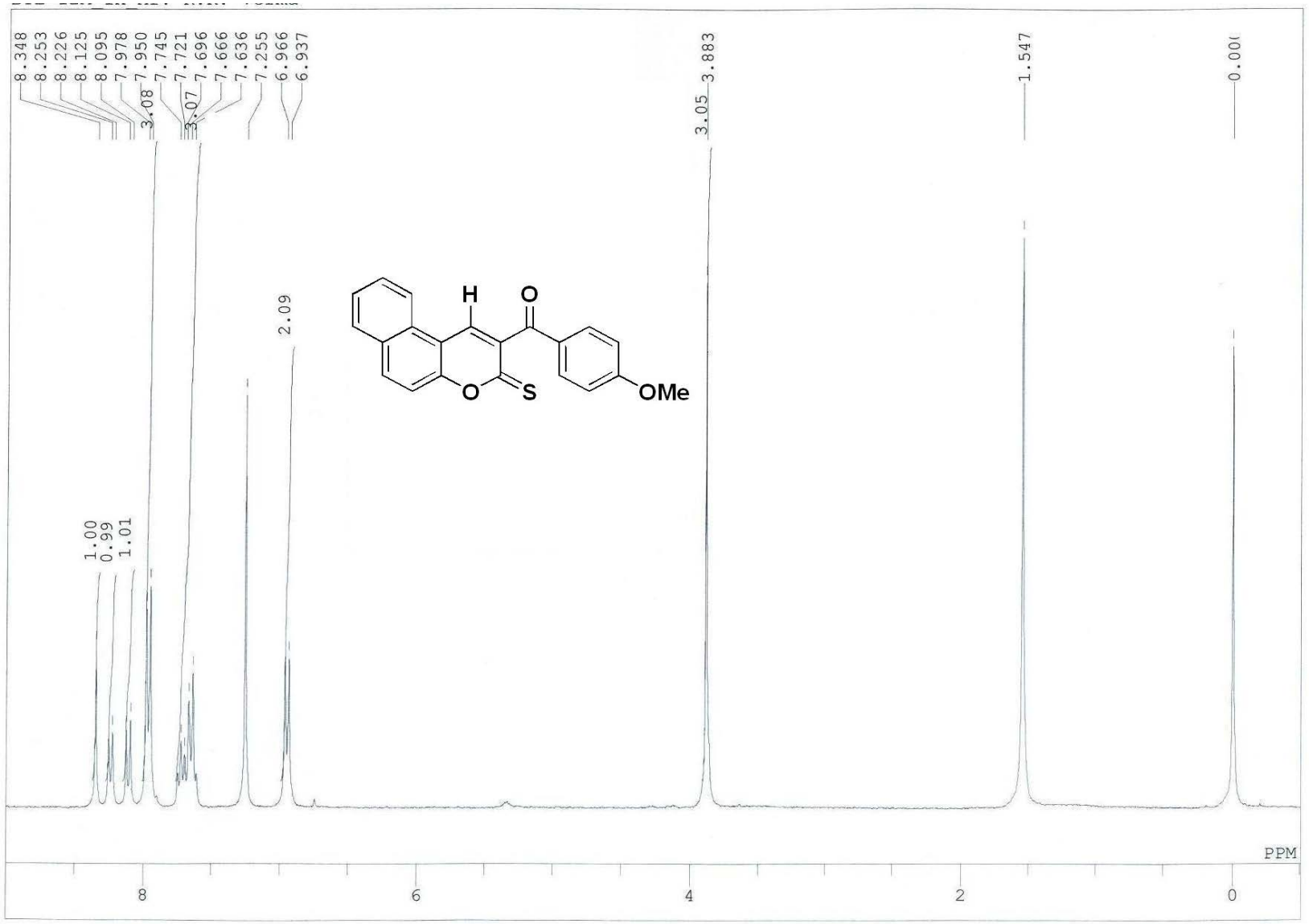
^{1}H NMR of 3i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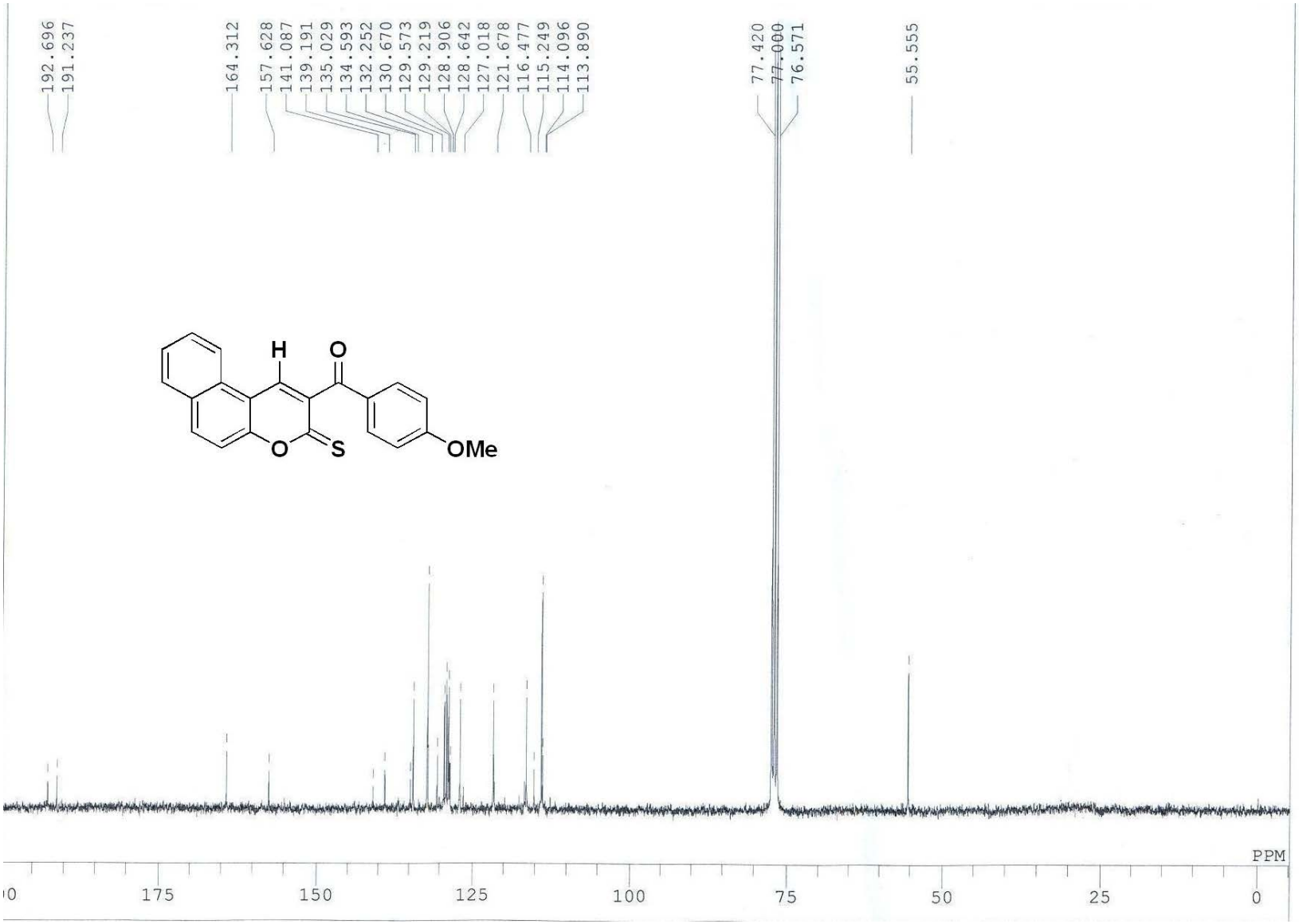
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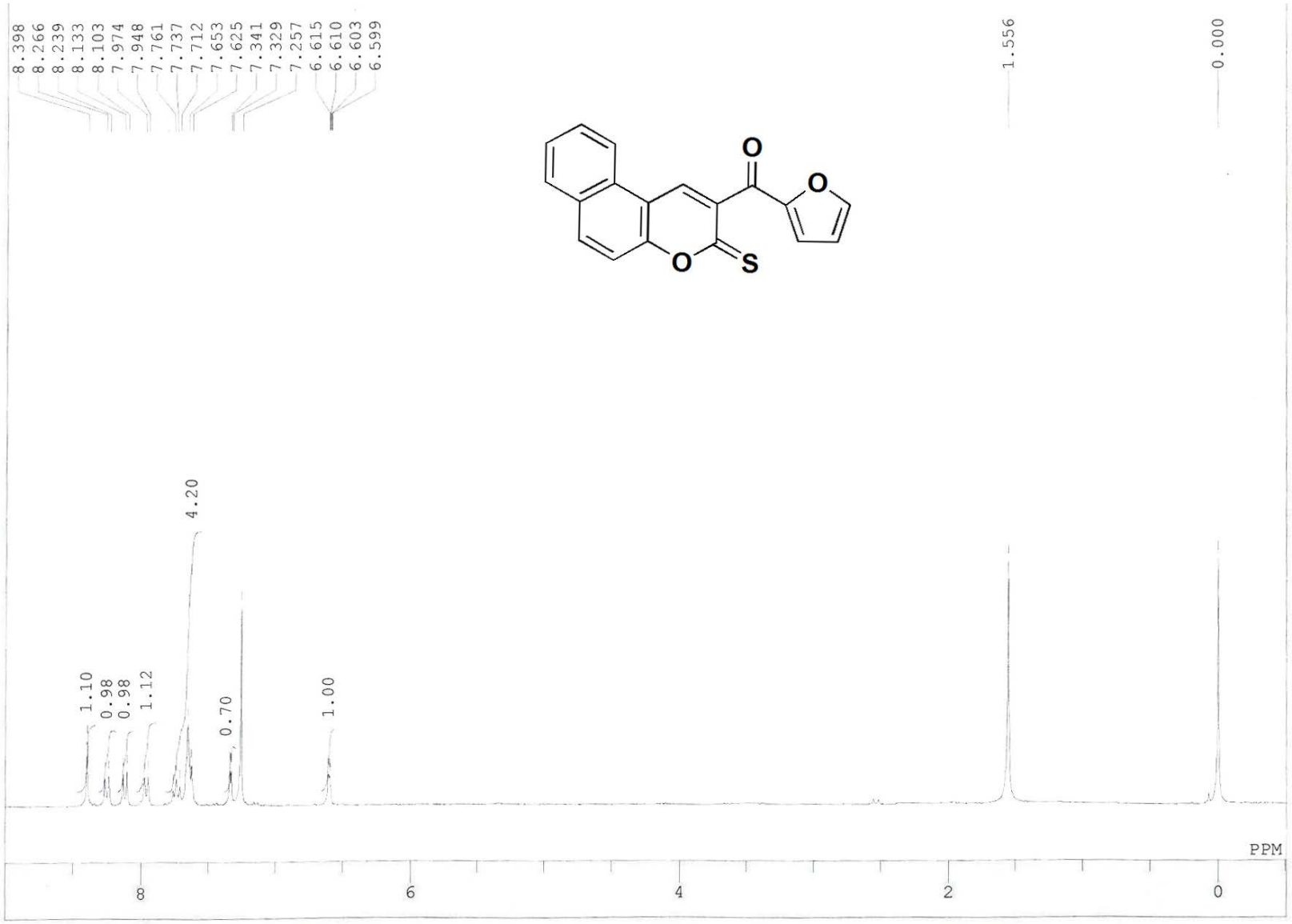
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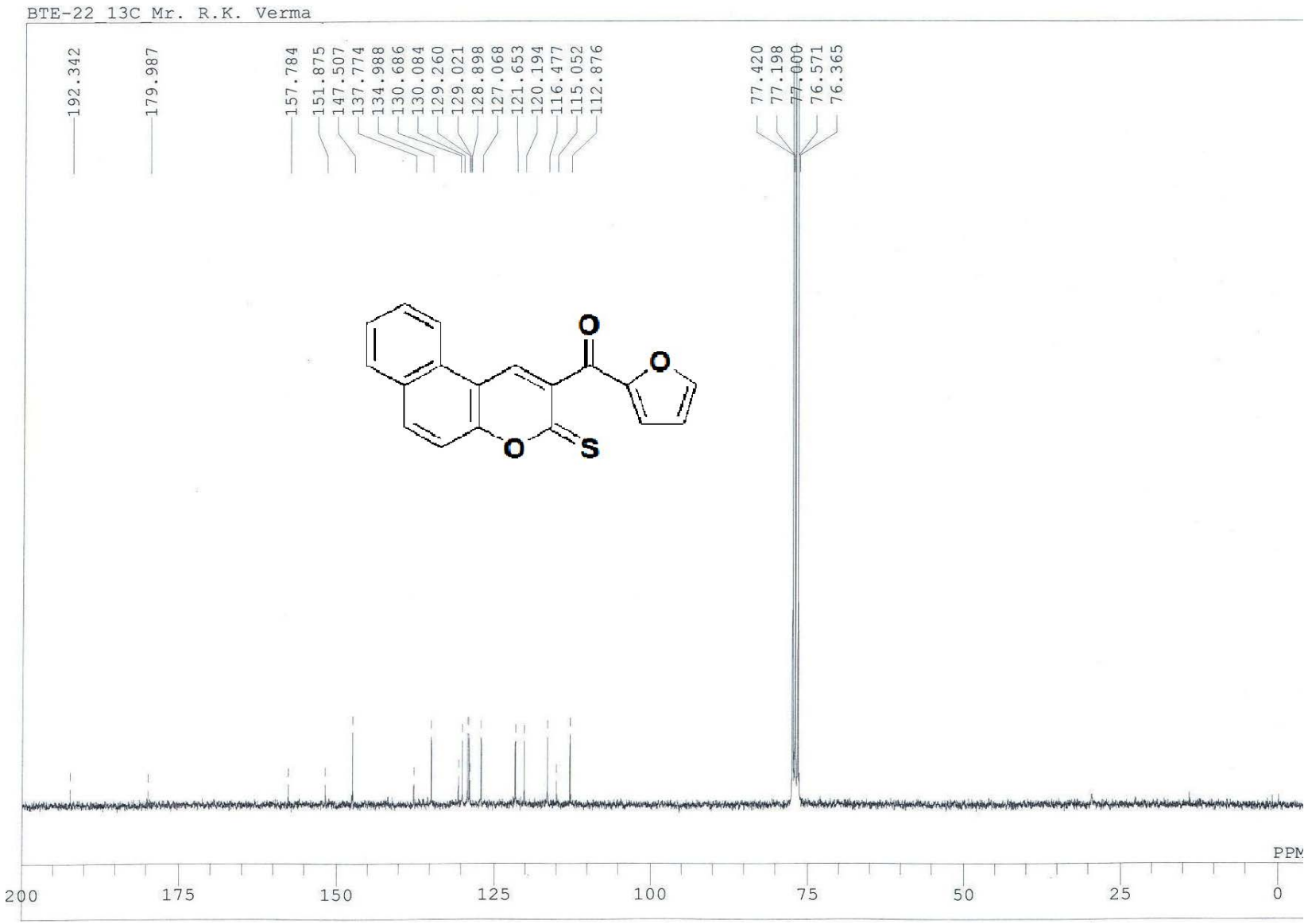
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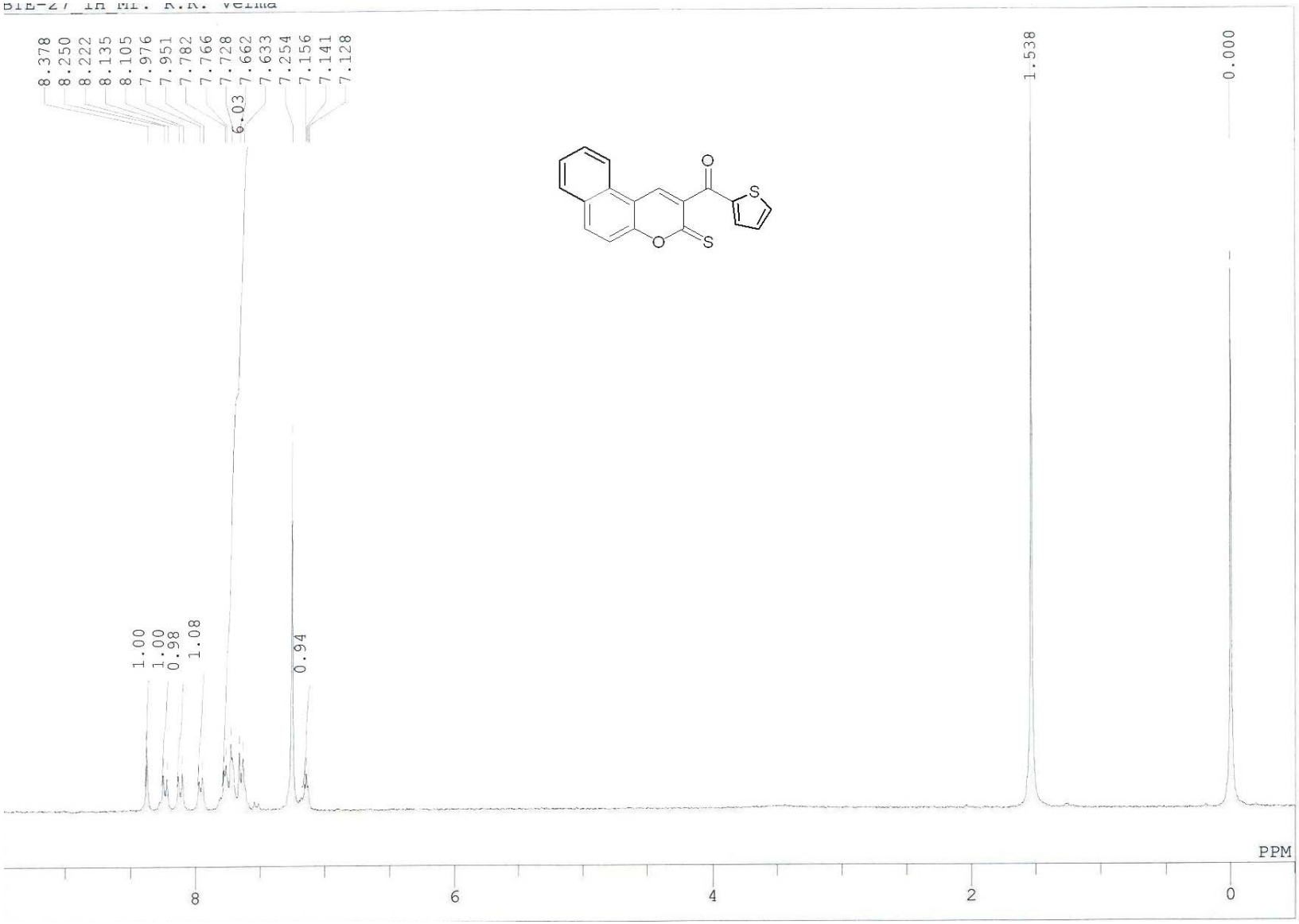
^{1}H NMR of 3k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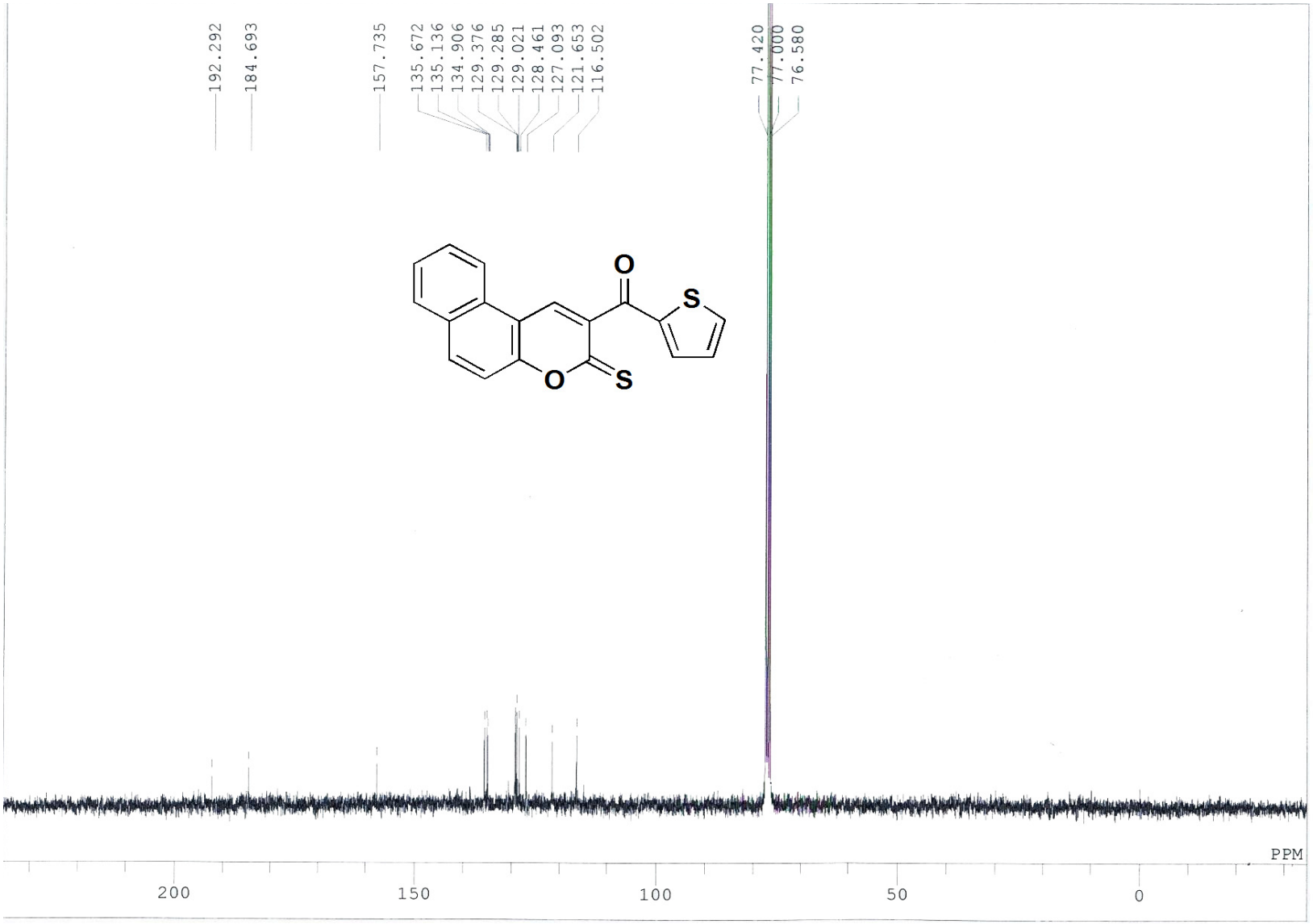
^{13}C NMR of 3k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7. Reference:

1. Venkatesan, S. K.; Shukla A. K.; Dubey, V. K. Molecular docking studies of selected tricyclic and quinone derivatives on trypanothione reductase of *Leishmania infantum*. *J. Comput. Chem.* **2010**, *31*, 2463-2472.
2. Maurya, R.; Mehrotra, S.; Prajapati, V. K.; Nylen, S.; Sacks, D.; Sundar, S. Evaluation of blood agar microtiter plates for culturing leishmania parasites to titrate parasite burden in spleen and peripheral blood of patients with visceral leishmaniasis. *J. Clin. Microbiol.* **2010**, *48*, 1932-1934.
3. Manandhar, K. D.; Yadav, T. P.; Prajapati, V. K.; Kumar, S.; Rai, M.; Dube, A.; Srivastava, O. N.; Sundar, S. Antileishmanial activity of nano-amphotericin B deoxycholate. *J. Antimicrob. Chemother.* **2008**, *62*, 376-380.
4. Kumar, D.; Kulshrestha, A.; Singh, R.; Salotra, P. In vitro susceptibility of field isolates of *Leishmania donovani* to Miltefosine and amphotericin B: correlation with sodium antimony gluconate susceptibility and implications for treatment in areas of endemicity. *Antimicrob. Agents Chemother.* **2009**, *53*, 835-838.
5. Prajapati, V. K.; Awasthi, K.; Gautam, S.; Yadav, T. P.; Rai, M.; Srivastava, O. N.; Sundar, S. Targeted killing of Leishmania donovani in vivo and in vitro with amphotericin B attached to functionalized carbon nanotubes. *J. Antimicrob. Chemother.* **2010**, *66*, 874-879.