Laura Friggeri, Luigi Scipione, Roberta Costi, Marcel Kaiser, Francesca Moraca, Claudio Zamperini, Bruno Botta, Roberto Di Santo, Daniela De Vita, Reto Brun and Silvano Tortorella

New promising compounds with *in vitro* nanomolar activity against *Trypanosoma cruzi*

SUPPORTING INFORMATION

Content

Chemical synthesis

All reagents and solvents were of high analytical grade and were purchased from Sigma-Aldrich (Milano, Italy). 2-(1*H*-imidazol-1-yl)-1-phenylethanol derivatives **1-5** were prepared according to literature procedure.[14] Melting points were determined on Tottoli apparatus (Buchi) and are uncorrected. Infrared spectra were recorded on a Spectrum One ATR Perkin Elmer FT-IR spectrometer. ^{1}H NMR spectra were acquired on a Bruker AVANCE-400 spectrometer at 9.4 Tesla, in DMSO, CD_3OD or $CDCl_3$ at 27 °C; chemical shift values are given in δ (ppm) relatively to TMS as internal reference. Coupling constants are given in Hz.

Mass spectrometric experiments were carried out with a 2000 Q TRAP instrument (Applied Biosystems); a commercial hybrid triple-quadrupole linear ion-trap mass spectrometer (Q1q2QLIT), equipped with an ESI source and a syringe pump have been used.

The examined compounds were previously dissolved in methanol (10^{-5} M) and aqueous HCl was added just before the injection. The molecular peaks (m/z) have been observed as $[M+H]^+$.

Elemental analyses were obtained by a PE 2400 (Perkin-Elmer) analyzer and the analytical results were within ± 0.4 % of the theoretical values for all compounds.

Synthesis of 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl (3-bromopyridin-4-yl)carbamate (6)

3-Bromo-4-aminopyridine (170 mg, 0.98 mmol) and triethylamine 0.64 mL (4.59 mmol, d = 0.727 g mL^{-1}) were dissolved in 12 mL of anhydrous benzene and thereafter 5 mL of anhydrous benzene containing triphosgene (235 mg, 0.79 mmol) were dropwise added. The obtained pale yellow suspension was refluxed for 5 h, then it was cooled at r.t, added of 2 mL of anhydrous acetonitrile containing metronidazole (170 mg, 0.99 mmol) and stirred for 12 h at r.t.

After this time the suspension was added of 15 mL of saturated aqueous Na_2CO_3 and extracted with CH_2Cl_2 (3 x 10 mL). The combined organic fractions were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give a crude residue. Pure 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethyl (3-bromopyridin-4-yl)carbamate (**6**) was obtained by silica gel column chromatography, using ethyl acetate as eluent. **6** was crystallized by methanol/diethyl ether to give 250 mg of pure pale yellow crystalline solid (68 % yield). Mp = 190-192°C. I.R (neat) 1736, 1266, 1235, 1188, 1151, 1069, 1028 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.64 (s, 1H, H2 py), 8.44 (d, 1H, J = 5.6 Hz, H6 py), 8.07 (d, 1H, J = 5.6 Hz, H5 py), 8.00 (s, 1H, imidazole), 7.31 (bs, 1H, NH), 4.69 (t, 2H, J = 5.1 Hz, CH_2-O), 4.60 (t, 2H, J = 5.1 Hz, CH_2-N), 2.55 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$): δ 151.5, 151.3, 150.6, 149.3, 142.4, 138.6, 133.2, 113.4, 110.1, 63.9, 45.1, 14.3. Anal. Calcd. for C12H12BrN5O4: C, 38.94; H, 3.27; N, 18.92. Found: C, 39.01; H, 3.27; N, 18.99. MS-ESI$^+$ found 371.2 $[M + H]^+$.

Synthesis of 2-(morpholin-4-yl)ethyl (2,6-dichloropyridin-4-yl)carbamate (7)

2,6-Dichloro-4-aminopyridine (278 mg, 1.71 mmol) and triethylamine (0.86 mL, d = 0.727 g mL^{-1}) were suspended in 15 mL of anhydrous benzene and then triphosgene (310 mg, 1.04 mmol) dissolved in 5 mL of anhydrous benzene was dropwise added. The obtained yellow suspension was refluxed for 5 h, then it was cooled at r.t, added of 0.25 mL of 2-(morpholin-4-yl)ethanol (2.06 mmol, d = 1.083 g mL^{-1}) and overnight stirred at r.t. The obtained suspension was added of 20 mL of saturated aqueous Na_2CO_3 and extracted with CH_2Cl_2 (5 x 10 mL). The combined organic fractions were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give a crude residue crystallized from CH_3CN to obtain pure 2-(morpholin-4-yl)ethyl (2,6-dichloropyridin-4-yl)carbamate (**7**) in 70 % yield (383 mg). Mp = 121-122°C. I.R (neat) 1736, 1375, 1248, 1216, 1106, 1069 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.62 (bs, 1H, NH), 7.38 (s, 2H, H3 and H5 py), 4.33 (t, 2H, J = 5.5 Hz, CH_2-O), 3.75 m, 4H, CH_2-O morpholine), 2.68 (t, 2H, J = 5.5 Hz, CH_2-N), 2.53 (t, 4H, J = 4.5 Hz, CH_2-N morpholine). ^{13}C NMR

($CDCl_3$):δ 152.3, 151.2, 149.1, 111.1, 66.8, 62.6, 57.2, 53.7. Anal. Calcd. for C12H15Cl2N3O3: C, 45.02; H,4.72; N,13.12. Found: C, 44.95; H, 4.73; N, 13.10. MS-ESI$^+$ found 321.2 $[M + H]^+$.

Synthesis of 2-(morpholin-4-yl)ethyl (2-chloropyridin-4-yl)carbamate (8)

8 was prepared as described for **7**, using 2-chloro-4-aminopyridine (220 mg, 1.71 mmol), triethylamine (0.86 mL, d = 0.727 g mL^{-1}), triphosgene (310 mg, 1.04 mmol) and 0.25 mL of 2-(morpholin-4-yl)ethanol (2.06 mmol, d = 1.083 g mL^{-1}).

The crude residue was purified by silica gel column chromatography, using methanol / ethylacetate (1:1) as eluent obtaining 293 mg (yield 60 %) of 2-(morpholin-4-yl)ethyl (2-chloropyridin-4-yl)carbamate (**8**). Mp = 110-111°C. I.R (neat) 1733, 1273, 1245, 1212, 1110, 1065 cm^{-1}. ^1H NMR($CDCl_3$): δ 8.23 (d, 1H, J = 5.6 Hz, H6 py), 7.51 (d, 1H, J = 1.9 Hz, H3 py), 7.50 (bs, 1H, NH), 7.24 (dd, 1H, J = 5.6 and J = 1.9 Hz, H5 py), 4.34 (t, 2H, J = 5.5 Hz, CH_2-O), 3.75 (m, 4H, CH_2-O morpholine), 2.70 (t, 2H, J = 5.5 Hz, CH_2-N), 2.54 (m, 4H, CH_2-N morpholine). ^{13}C NMR ($CDCl_3$):δ 152.9, 152.5, 150.1, 147.4, 112.3, 111.5, 66.7, 62.3, 57.2, 53.7. Anal. Calcd. for C12H16ClN3O3: C, 50.44; H, 5.64; N, 14.71; Found: C, 50.62; H, 5.65; N, 14.73. MS-ESI$^+$ found 286.7 $[M + H]^+$.

General procedure for the enantioselective synthesis of 2-(1H-imidazol-1-yl)-1-phenylethanols

Example: Synthesis of (*S*)-2-(1H-imidazol-1-yl)-1-phenylethanol is reported. A Schlenk tube was charged with dichloromethane (5 mL), triethylamine (0.3 mL, 2.4mmol), 1-(phenyl)-2-imidazol-1-yl-ethanone (250 mg, 1.22 mmol) and [(*R*,*R*)-TsDPEN Ru-(Cymene)Cl (3 mg, 5 μmol). A nitrogen atmosphere was established and then formic acid (110 mg, 2.4 mmol) was added over a period of an hour *via* GC-syringe. During the addition, the temperature rose slowly to 30°C. The mixture was heated at 40°C for 26 h .The suspension obtained was centrifugated and the white solid was collected by filtration and washed with dichloromethane obtainig the pure (*S*)-2-(1H-imidazol-1-yl)-1-

phenylethanol. (*R*)-2-(1H-imidazol-1-yl)-1-phenylethanol was prepared with the above described procedure, using [(*S*,*S*)-TsDPEN Ru-(Cymene)Cl as catalyst. Spectroscopic data of the alcohols are in agreement with those of the racemic alcohols described in literature. From obtained enantiopure alcohols (*R*)-**1,** (*S*)-**1,** (*R*)-**3,** (*S*)-**3,** (*R*)-**5,** (*S*)-**5** were prepared using the procedure previously reported for racemic compounds.[14] The enantiomeric purity of these compounds was verified by chiral HPLC analysis on Chiralpak IC column (250 x 4.6 mm i.d), temperature 25 °C

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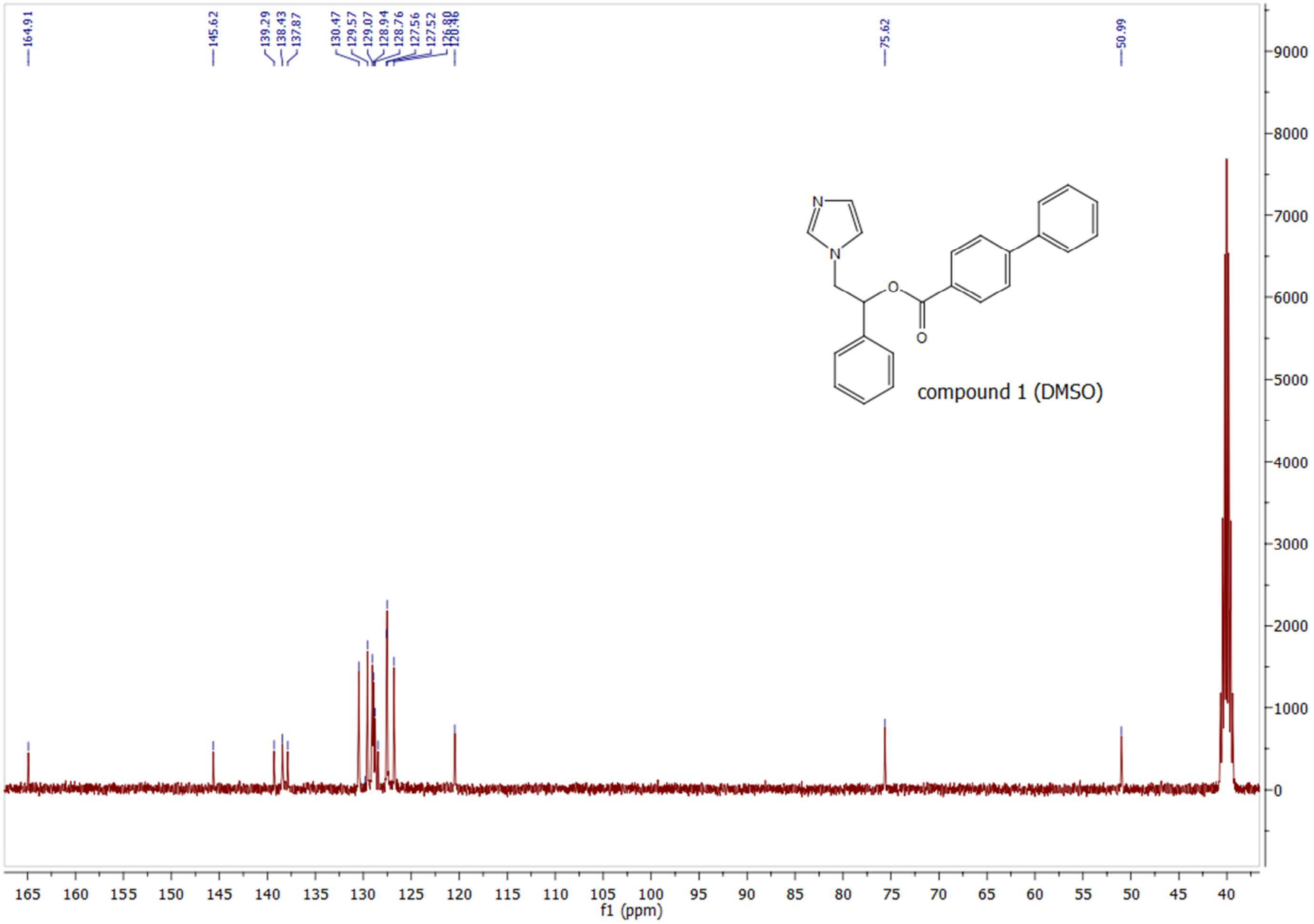
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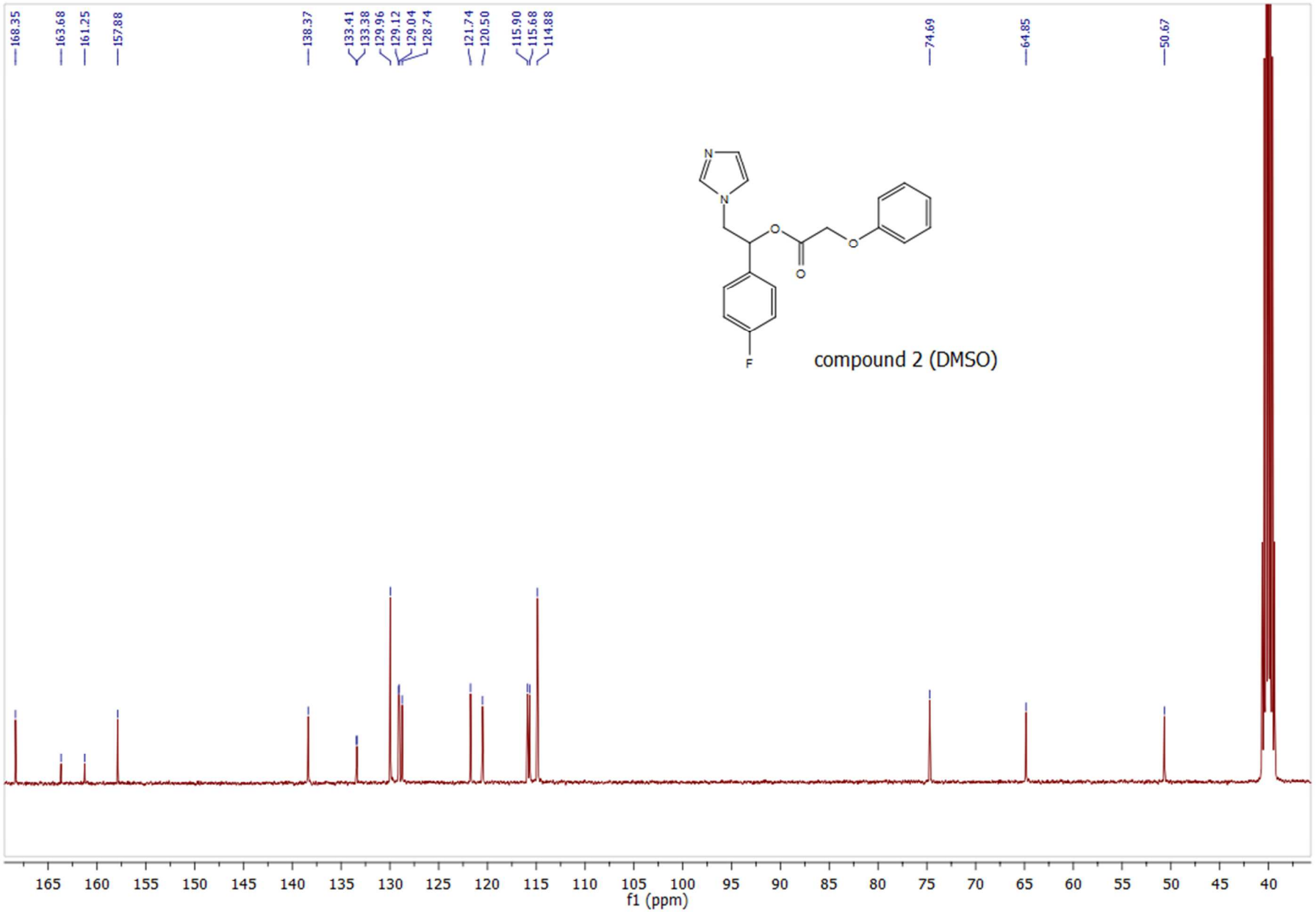
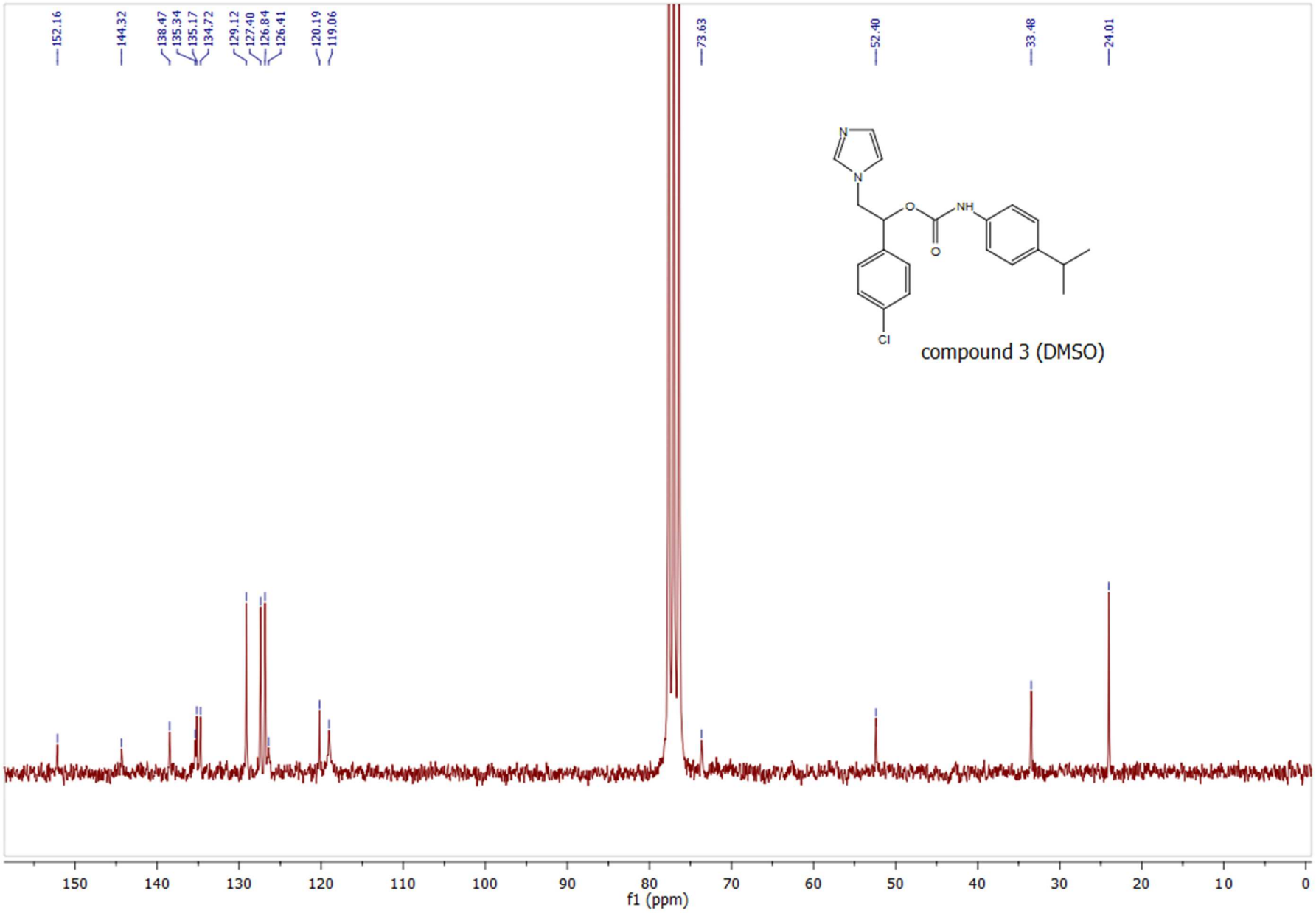
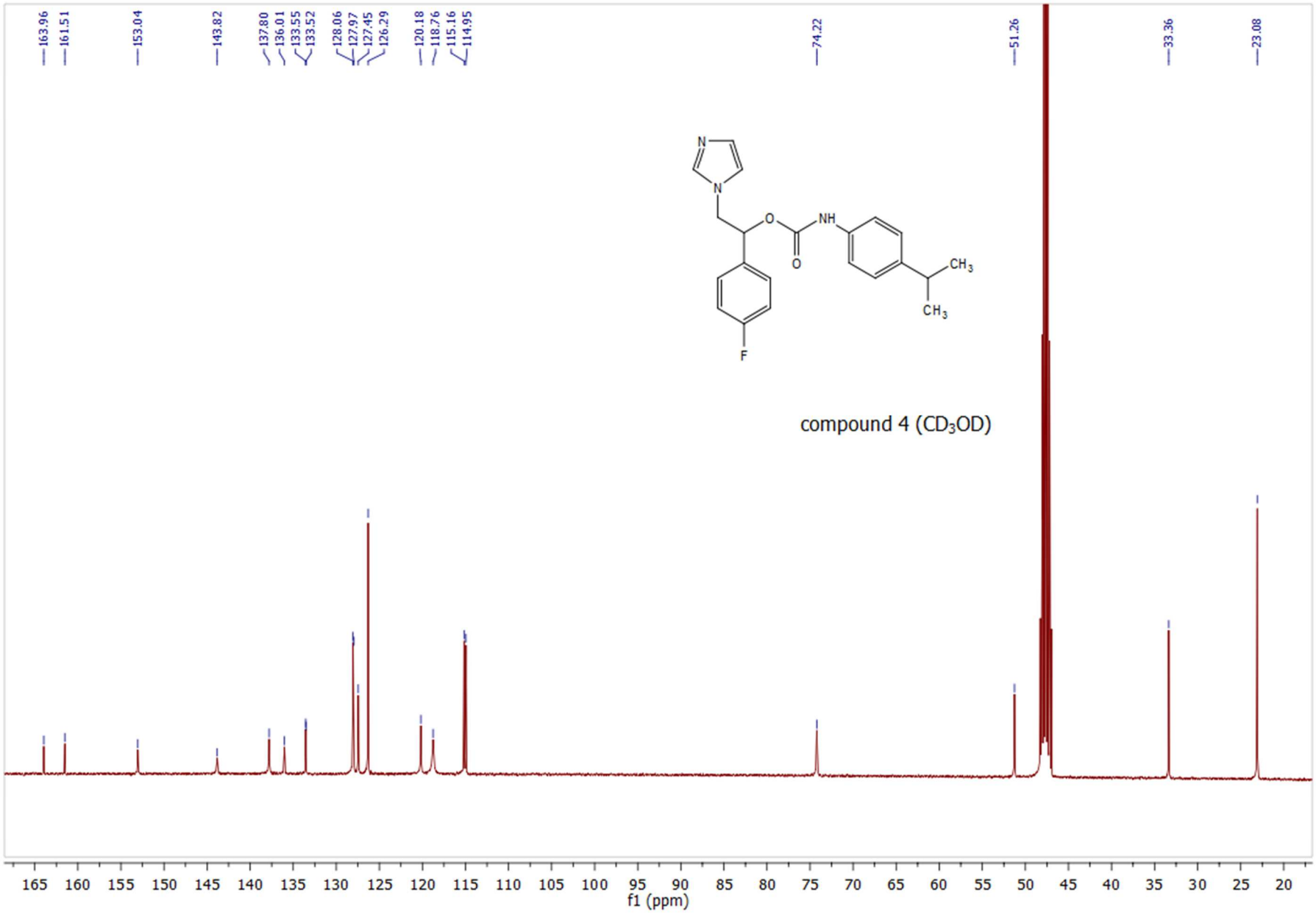
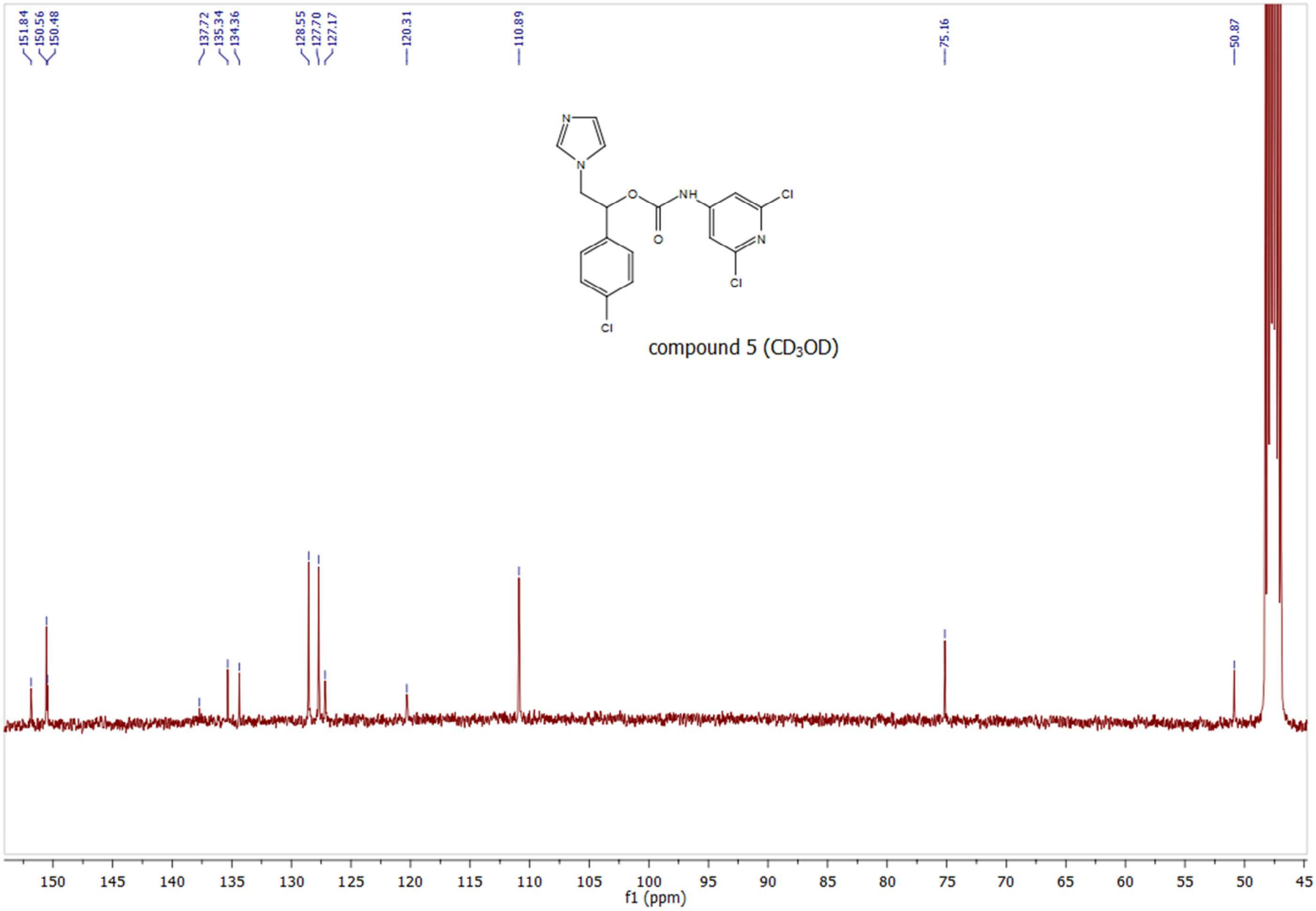
145.62
139.29
138.43
137.87
130.47
129.57
129.07
128.94
128.76
127.56
127.52
126.80
120.46
75.62
50.99
N
N
O
O
compound 1 (DMSO)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
165 160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40
f1 (ppm)


168.35
163.68
161.25
157.88
138.37
133.41
133.38
129.96
129.12
129.04
128.74
121.74
120.50
115.90
115.68
114.88
74.69
64.85
50.67
N
N
O
O
O
F
compound 2 (DMSO)
165 160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40
f1 (ppm)


152.16
144.32
138.47
135.34
135.17
134.72
129.12
127.40
126.84
126.41
120.19
119.06
73.63
52.40
33.48
24.01
N
N
O
NH
O
Cl
compound 3 (DMSO)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


163.96
161.51
153.04
143.82
137.80
136.01
133.55
133.52
128.06
127.97
127.45
126.29
120.18
118.76
115.16
114.95
74.22
51.26
33.36
23.08
N
N
O
O
NH
CH3
CH3
F
compound 4 (CD3OD)
165 160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20
f1 (ppm)


151.84
150.56
150.48
137.72
135.34
134.36
128.55
127.70
127.17
120.31
110.89
75.16
50.87
N
N
O
NH
Cl
O
N
Cl
Cl
compound 5 (CD3OD)
150
145
140
135
130
125
120
115
110
105
100
95
90
85
80
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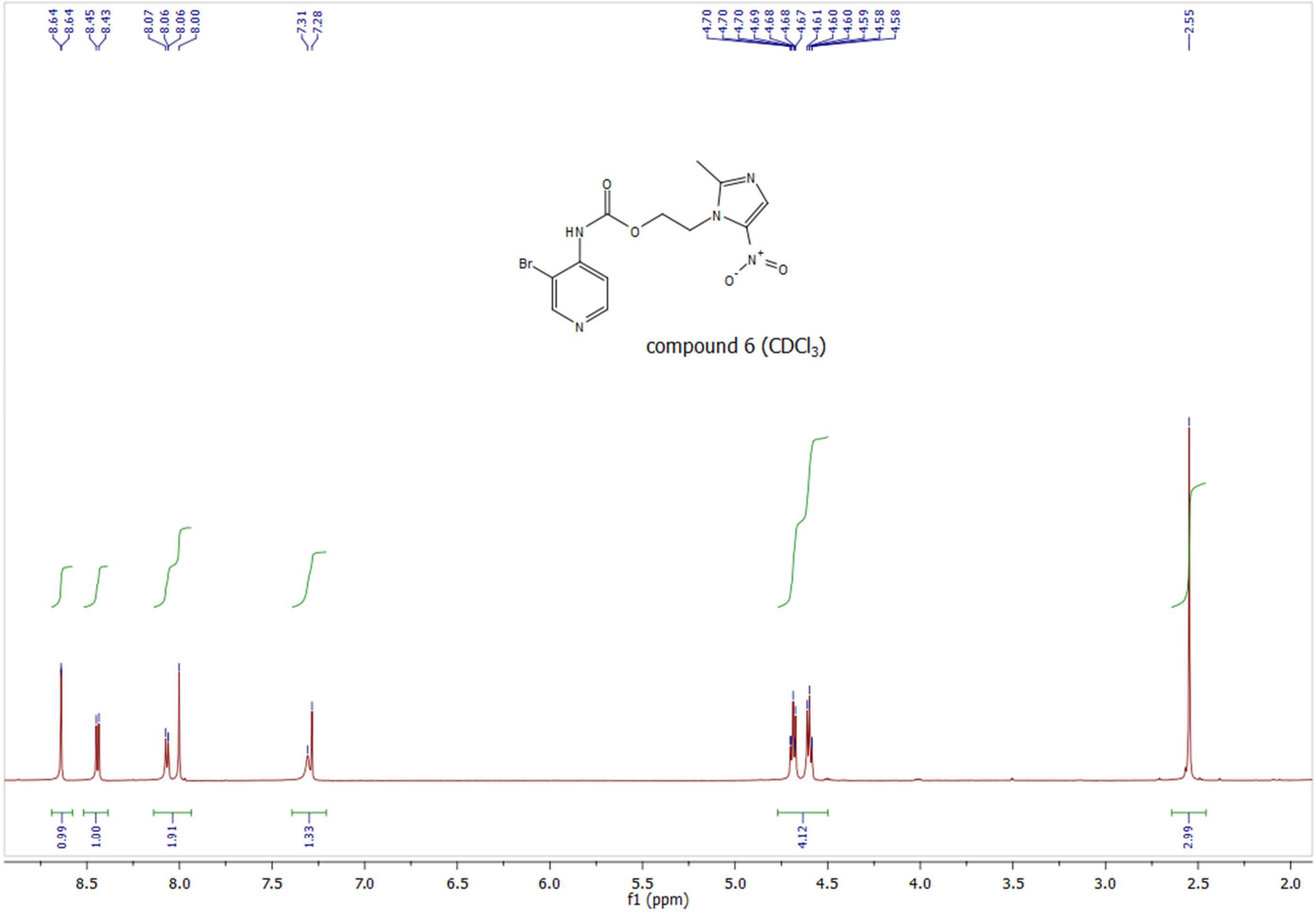
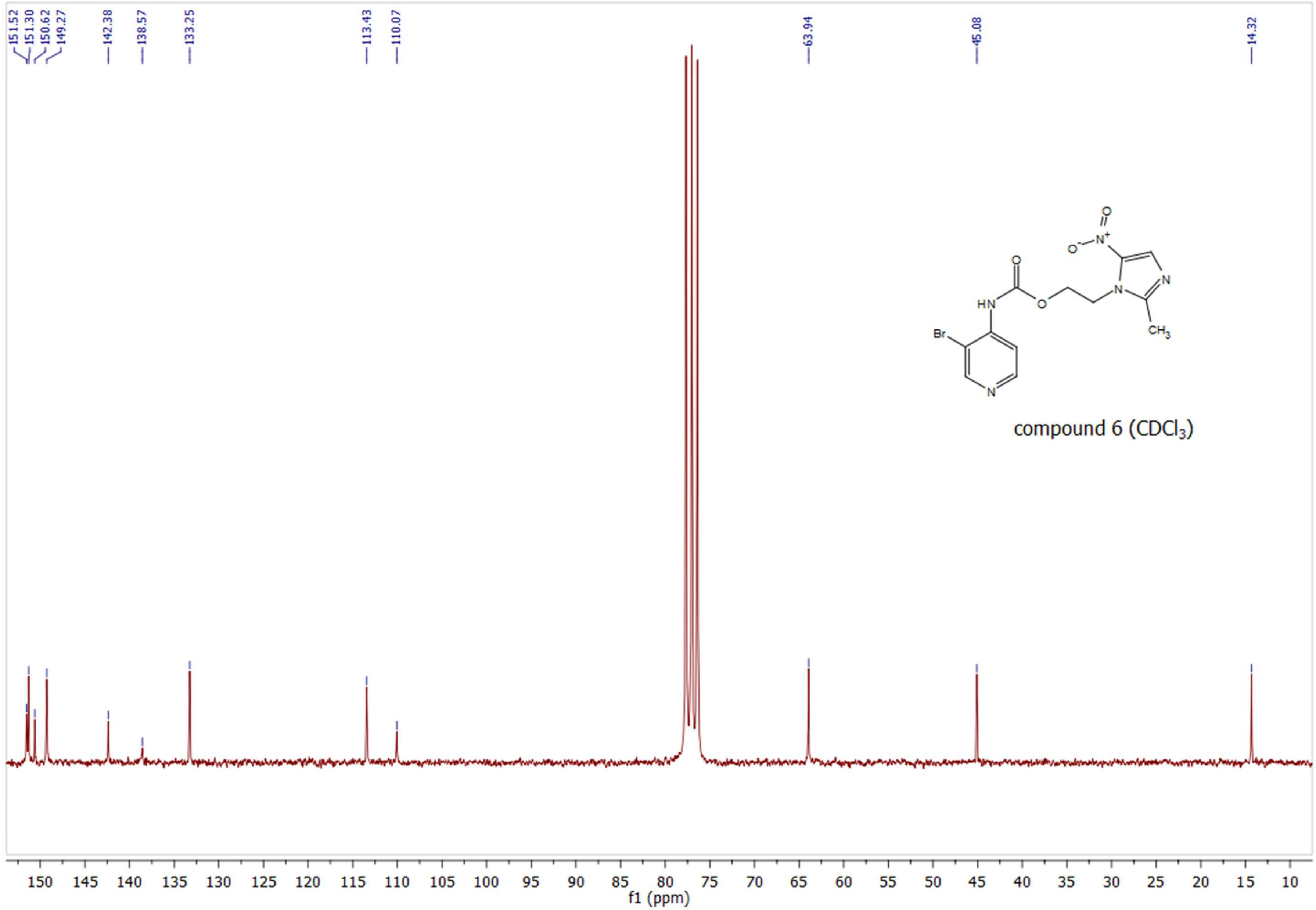
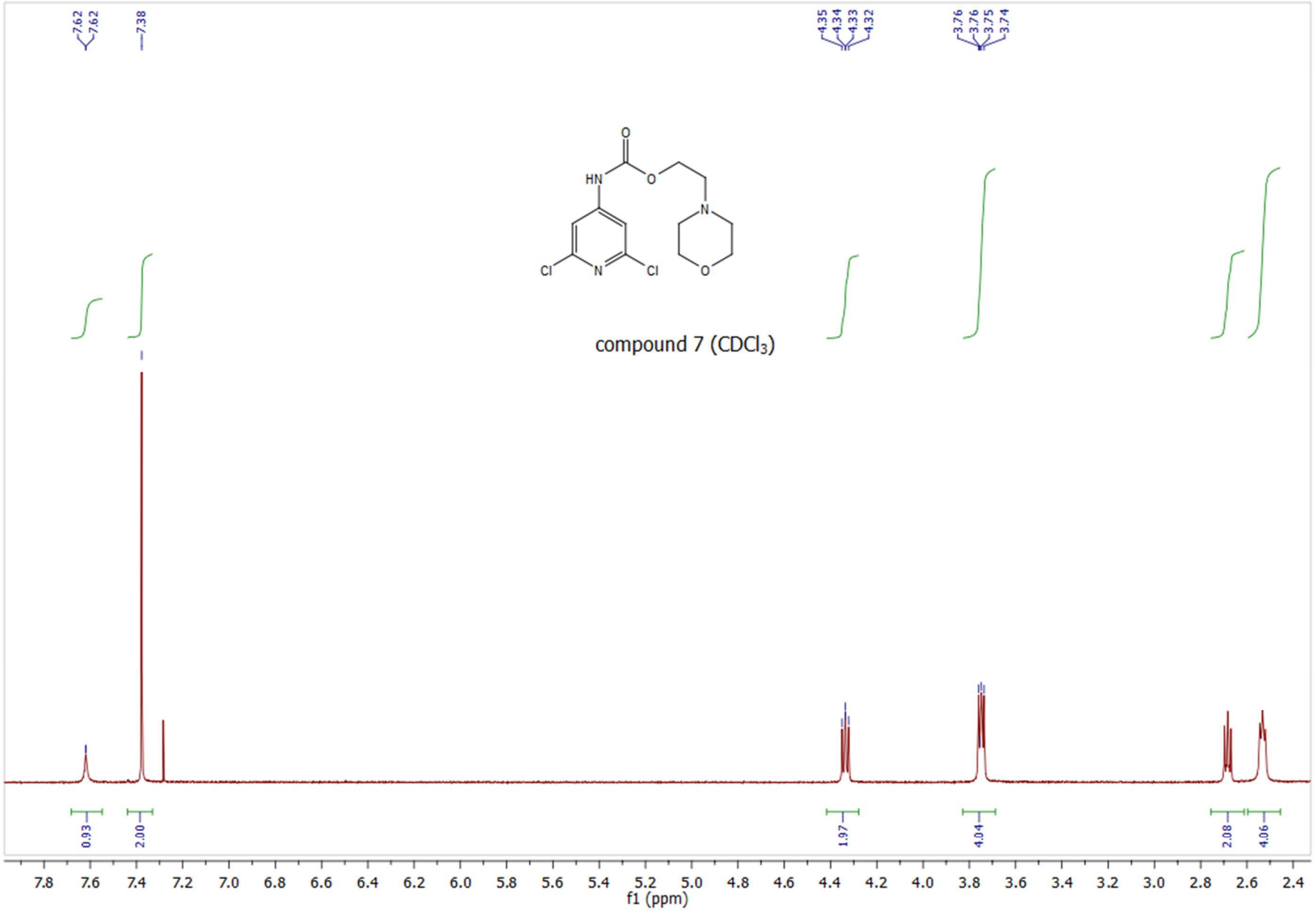
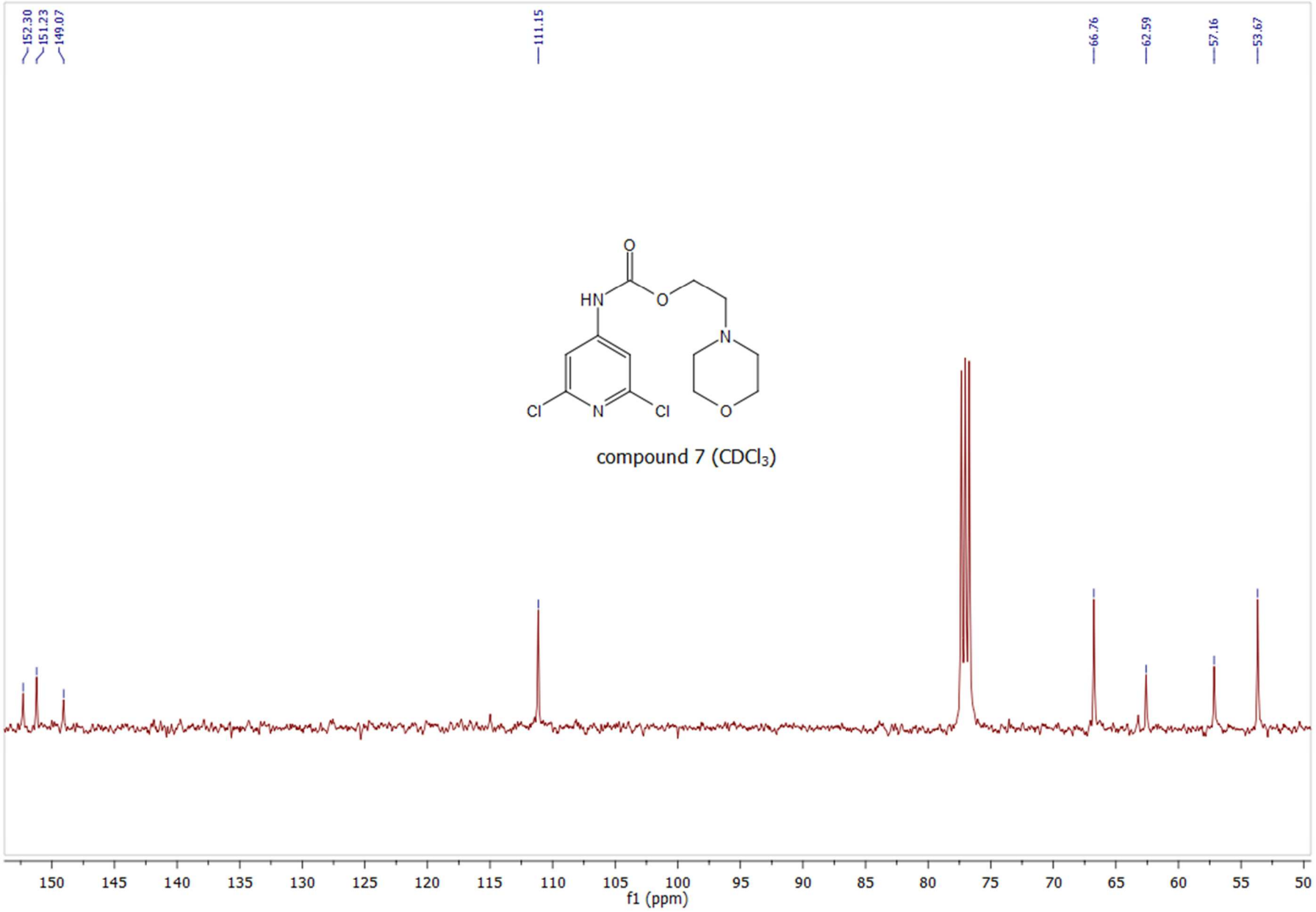
70
65
60
55
50
45
f1 (ppm)

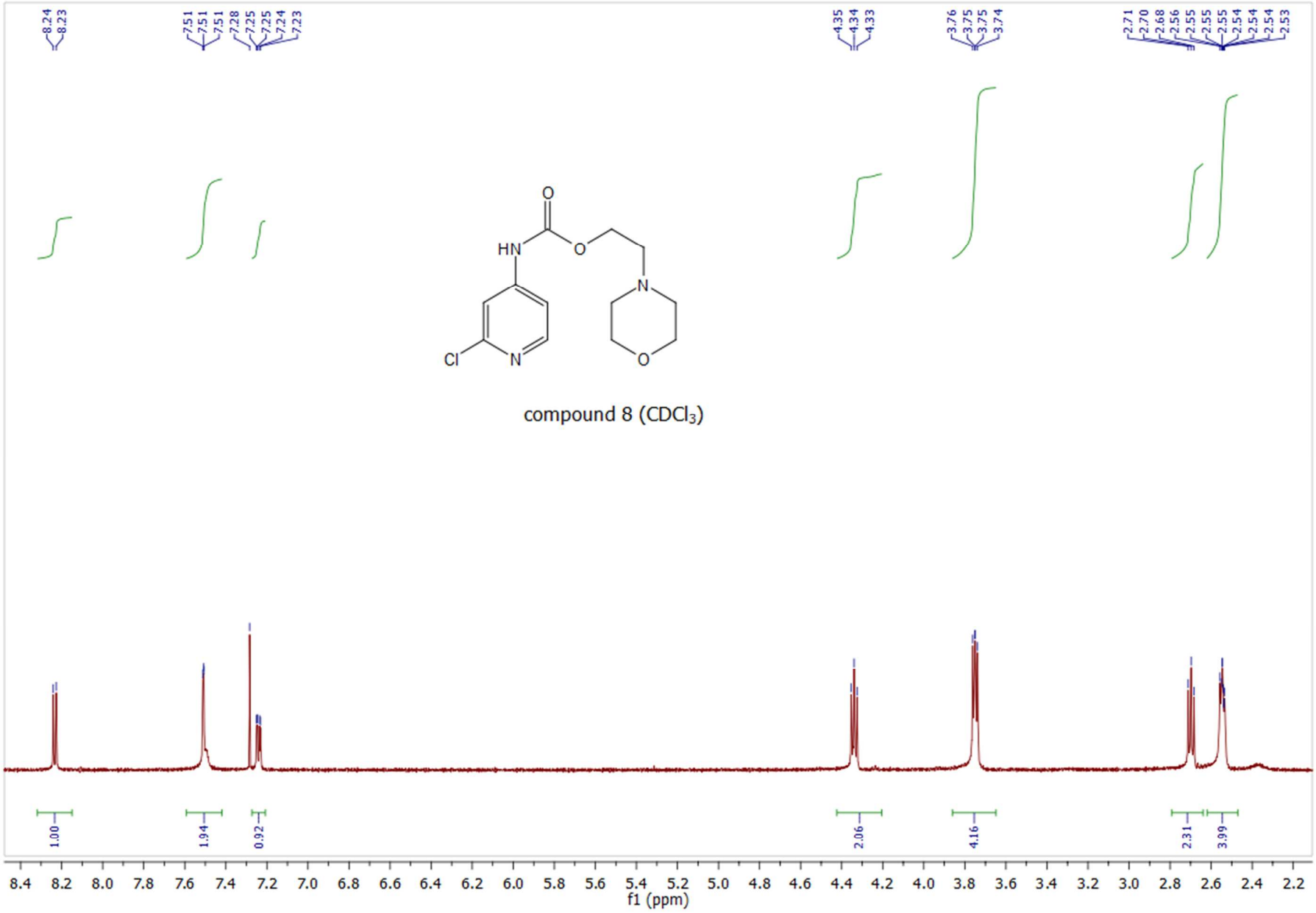


compound 6 ($CDCl_3$)

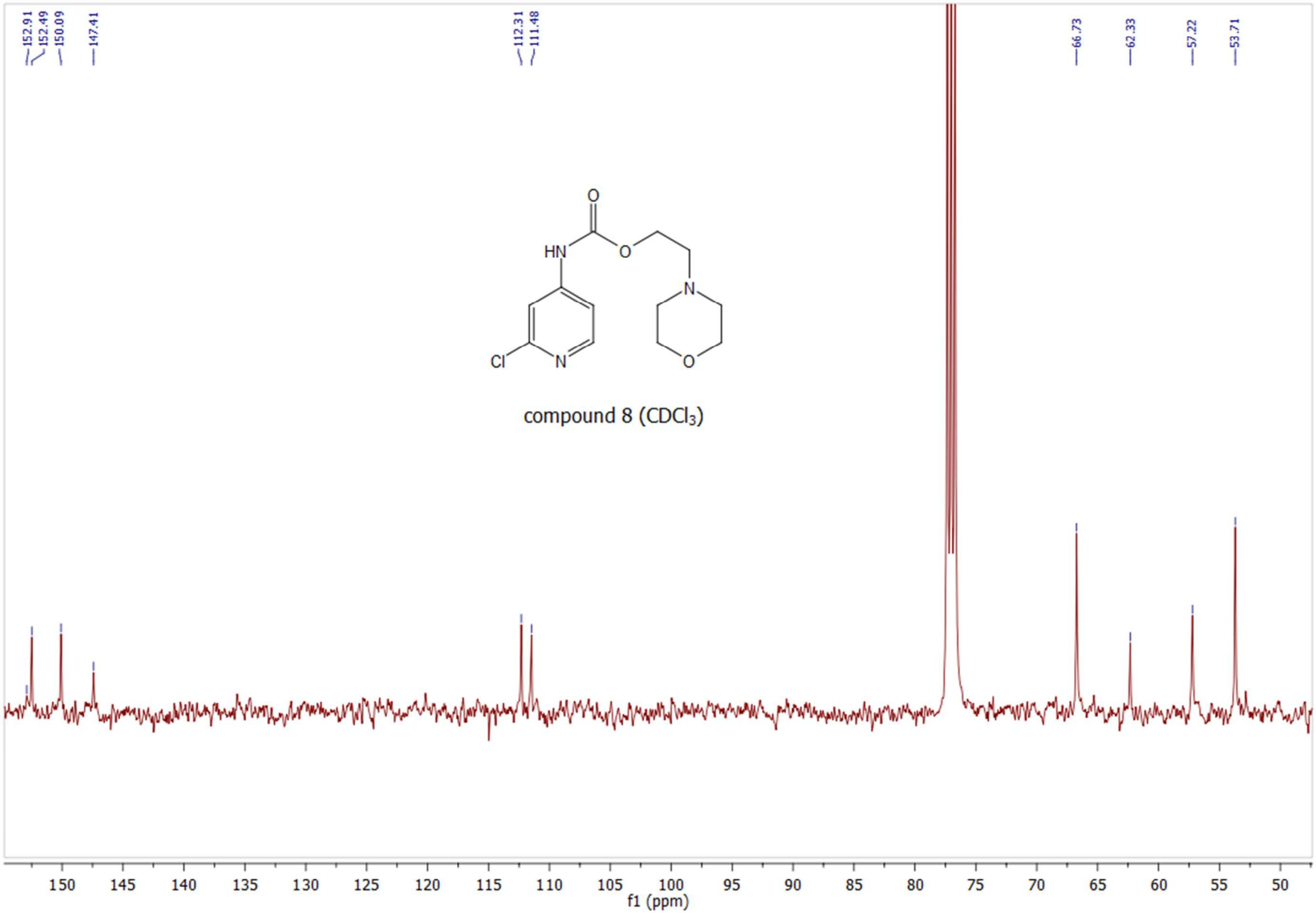

151.52
151.30
150.62
149.27
142.38
138.57
133.25
113.43
110.07
63.94
45.08
14.32
HN
O
Br
N
N
N
CH3
compound 6 ($CDCl_3$)
150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10
f1 (ppm)


7.62
7.62
7.38
4.35
4.34
4.33
4.32
3.76
3.76
3.75
3.74
HN
O
O
N
Cl
N
Cl
O
compound 7 (CDCl3)
0.93
2.00
1.97
4.04
2.08
4.06
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
f1 (ppm)


152.30
151.23
149.07
111.15
66.76
62.59
57.16
53.67
O
HN
O
N
Cl
N
Cl
O
compound 7 ($CDCl_3$)
150
145
140
135
130
125
120
115
110
105
100
95
90
85
80
75
70
65
60
55
50
f1 (ppm)



compound 8 ($CDCl_3$)


152.91
152.49
150.09
147.41
112.31
111.48
66.73
62.33
57.22
53.71
O
HN
O
N
Cl
N
O
compound 8 ($CDCl_3$)
150
145
140
135
130
125
120
115
110
105
100
95
90
85
80
75
70
65
60
55
50
f1 (ppm)

Elemental analysis of compounds 1-5

Compound 1. Anal. Calcd. for C24H20N2O2: C, 78.24; H, 5.47; N, 7.60. Found: C, 78.01; H, 5.48; N, 7.59.

Compound 2. Anal. Calcd. for C19H17FN2O3: C, 67.05; H, 5.03; N, 8.23. Found: C, 67.28; H, 5.02; N, 8.22.

Compound 3. Anal. Calcd. for C21H22ClN3O2: C, 65.71; H, 5.78; N, 10.95. Found: C, 65.48; H, 5.78; N, 10.93.

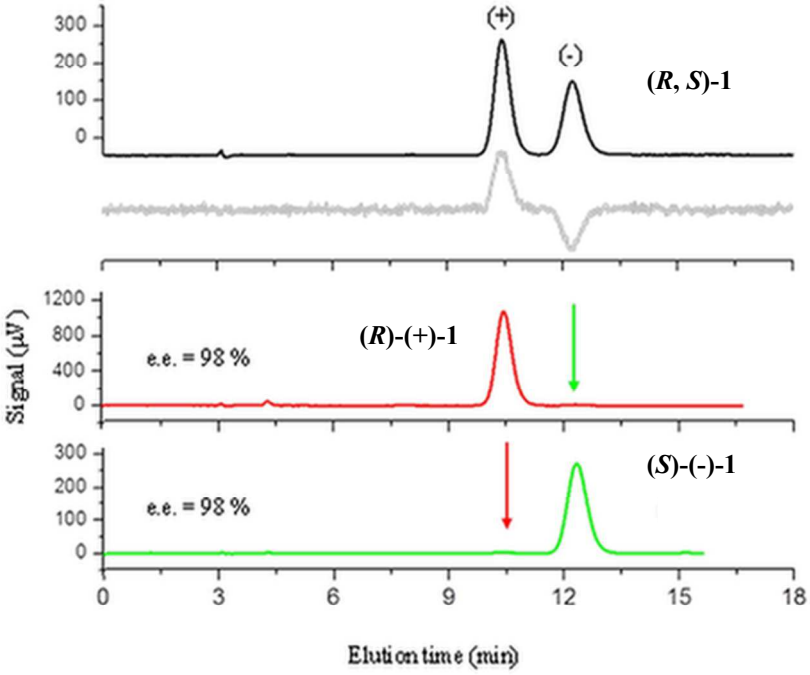
Compound 4. Anal. Calcd. for C21H22FN3O2: C, 68.65; H, 6.04; N, 11.44. Found: C, 68.39; H, 6.05; N, 11.42.

Compound 5. Anal. Calcd. for C17H13Cl3N4O2: C, 49.60; H, 3.18; N, 13.61. Found: C, 49.45; H, 3.19; N, 13.59.

Enantioselective HPLC

The analytical HPLC apparatus consisted of a PerkinElmer (Norwalk, CT, USA) 200 LC pump equipped with a Rheodyne (Cotati, CA, USA) injector, a 20 mL sample loop, an HPLC Dionex CC-100 oven (Sunnyvale, CA, USA) and a Jasco (Jasco, Tokyo, Japan)Model CD 2095 Plus UV/CD detector. HPLC analysis were performed using the commercially available 250 mm-4.6 mm I.D. Chiralcel OD column(Chiral Technologies Europe, Illkirch, France).

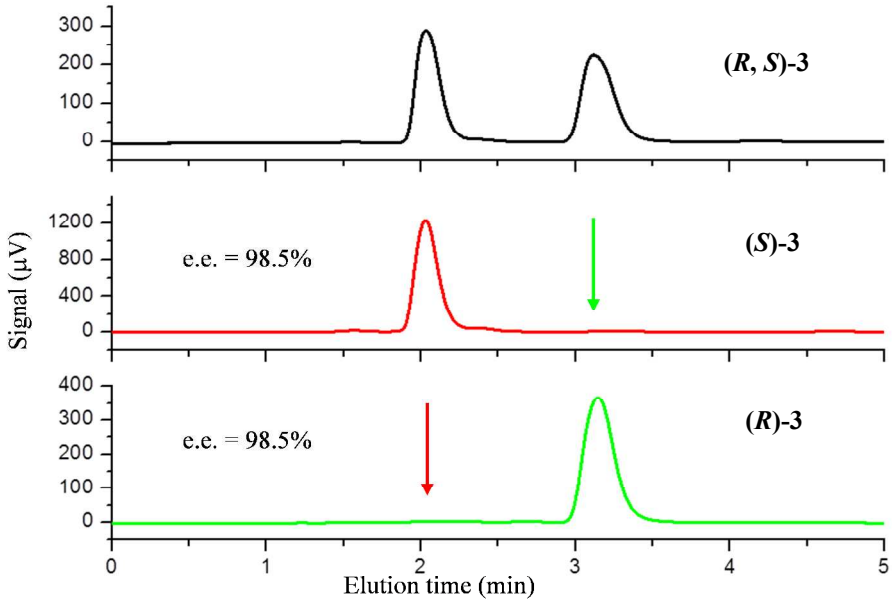
Enantiomeric purity of compounds (*R*)-**1**, (*S*)-**1**, (*R*)-**3**, (*S*)-**3**, (*R*)-**5** and (*S*)-**5**



Column: Chiralcel OD (250 mm x 4.6 mm i.d.); eluent: n-hexane/ethanol 50:50 (v/v);

flow rate: 1 mL/min;

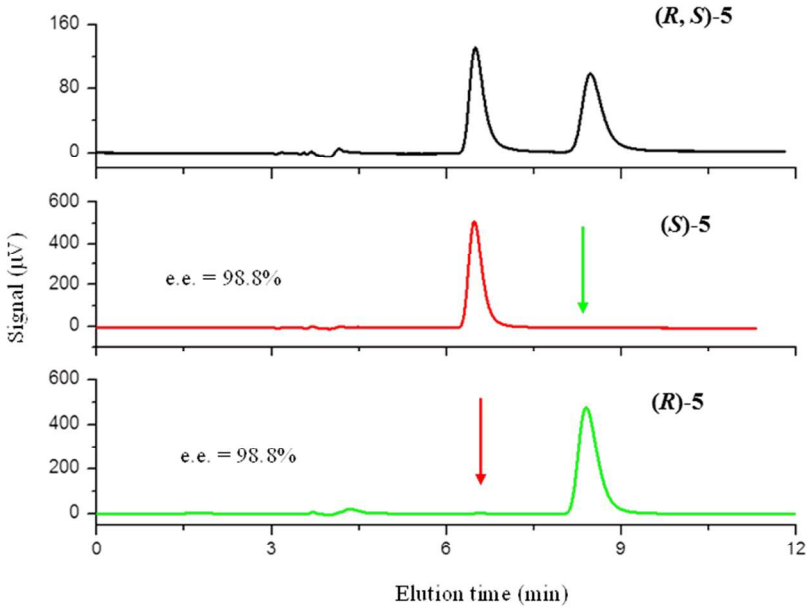
detector: UV and CD (grey line) at 275 nm; temperature: 25 °C.



Column: Chiralpak IA-3 (100 mm x 4.6 mm i.d.); eluent: n-hexane/ethanol 40:60 (v/v);

flow rate: 1 mL/min;

detector: UV at 254 nm; temperature: 25 °C.



Column: Chiralpak IC (250 mm x 4.6 mm i.d.); eluent: n-hexane/2-propanol/DEA 60:40:0.1 (v/v/v);

flow rate: 1 mL/min;

detector: UV at 254 nm; temperature: 25 °C.

Molecular Modelling

X-ray crystallographic structures of the sterol 14α-demethylase of *T.cruzi* ($CYP51_{Tc}$, PDB ID: 2WX2), *L. infantum* ($CYP51_{Li}$, PDB ID: 3L4D) and *T. brucei* ($CYP51_{Tb}$, PDB ID: 3GW9) were downloaded from the Protein Data Bank (PDB). The first two structures were co-crystallized with fluconazole and the last, 3GW9, with the VNI (N-[(1R)-1-(2,4-dichlorophenyl)-2-(1H-imidazol- 1-yl)ethyl]-4-(5-phenyl-1,3,4-oxadiazol-2- yl)benzamide). The three proteins were chosen according to the best resolution among all structures of $CYP51_{Tc}$, $CYP51_{Tb}$ and $CYP51_{Li}$ available in the PDB.

Proteins pre-tratment was performed by means of the *Protein Preparation Wizard* tool of Maestro9.2 suite by adding hydrogens, filling missing side chains using *Prime* and removing water molecules. For each structure a 5000 step energy minimization in explicit water, using OPLS2005 as force field was performed by means of Macromodel.

Among the scoring functions available within GOLD5.1 (ASP score, PLP score, ChemScore and GoldScore), the pose of the co-cristallized ligand in all the three proteins was only reproduced by the ASP score with the following parameters: 200% of efficiency, 50 runs for each ligand, early termination turned off and the coordination to the heme-iron to be consider as a constraint. Hence, those settings were used for the subsequent docking calculation of compounds **1-5** to check their orientation in the active site of $CYP51_{Tc}$, $CYP51_{Tb}$ and $CYP51_{Li}$.

Docking poses. $CYP51_{Tc}$, PDB ID: 2WX2 Resolution: 2.27Å.

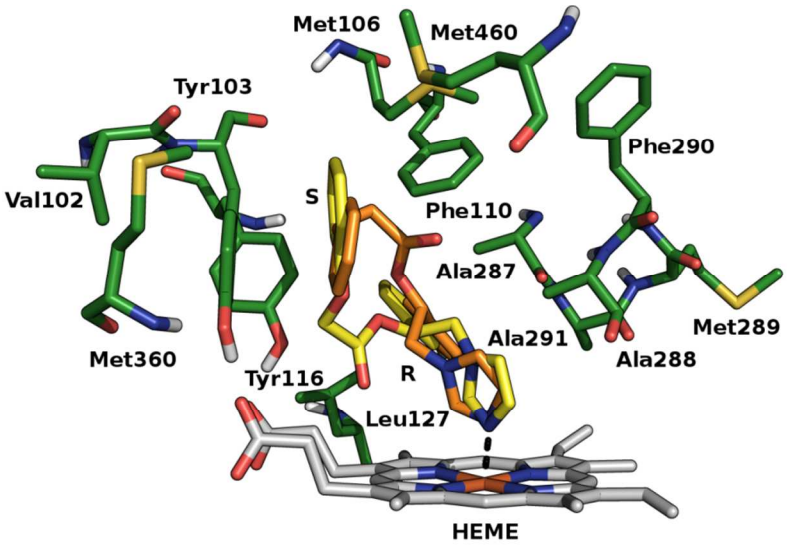


Figure S1. Docking pose of (*R*)- and (*S*)-enantiomers of compounds **2**

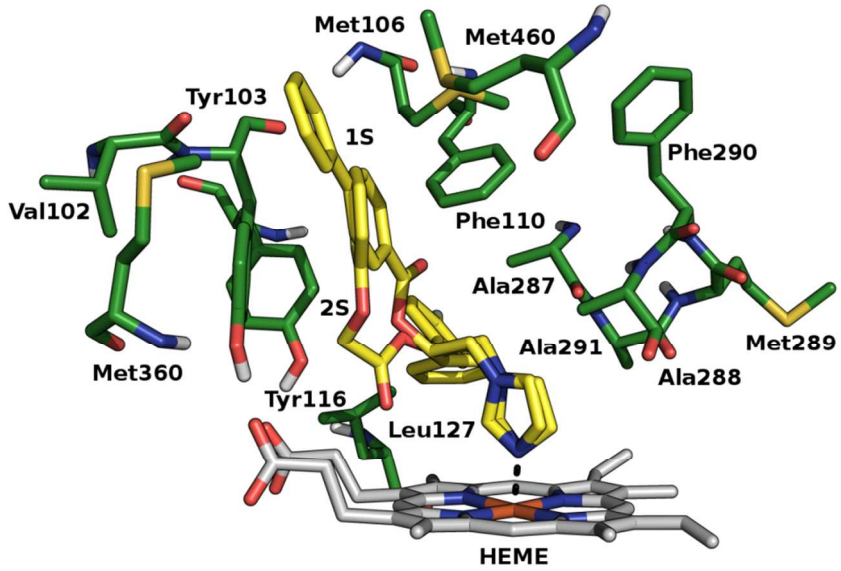


Figure S2. Comparison of docking pose of (*S*)-enantiomers of compounds **1** and **2**

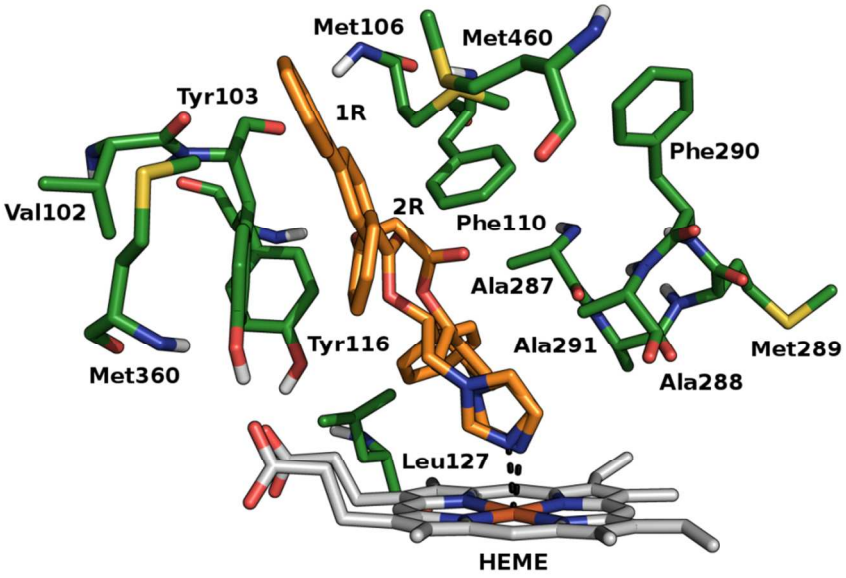


Figure S3. Comparison of docking pose of (*R*)-enantiomers of compounds **1** and **2**

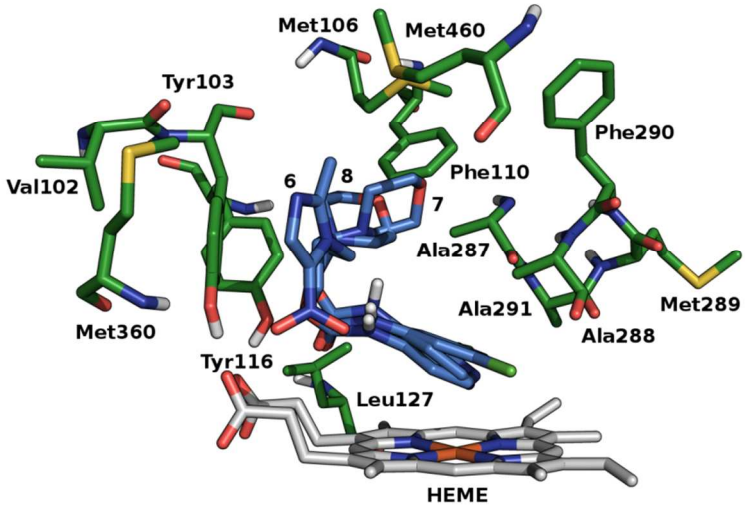


Figure S4. Docking pose of compounds **6-8**

Docking poses. $CYP51_{Tb}$, PDB ID: 3GW9 Resolution: 1.87Å.

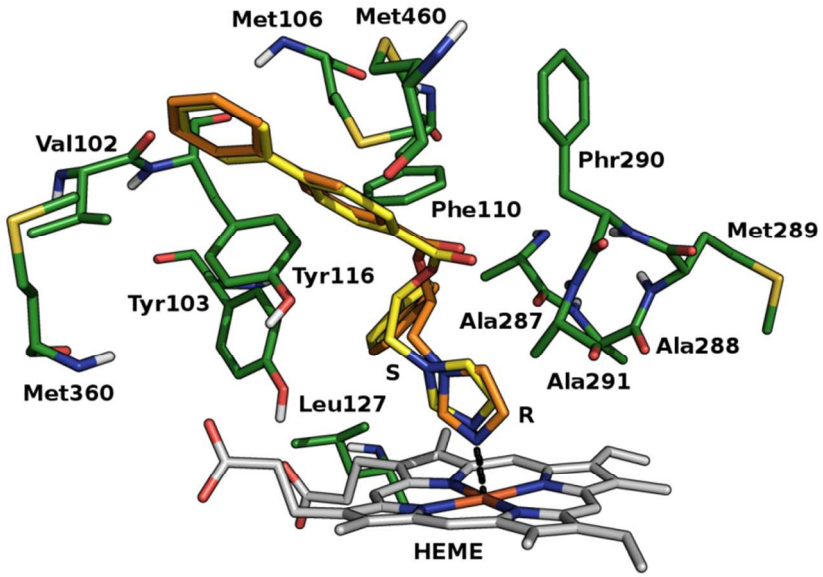


Figure S5. Docking pose of (*R*)- and (*S*)-enantiomers of compounds **1**

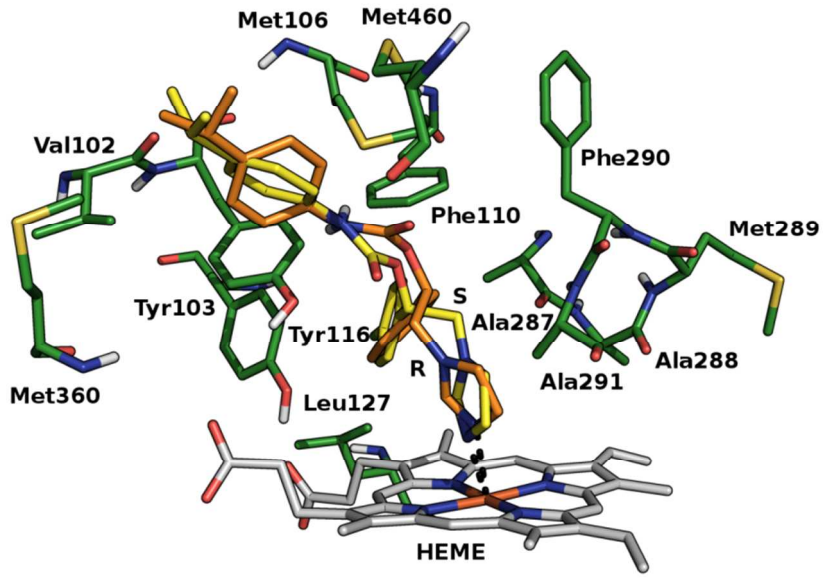


Figure S6. Docking pose of (*R*)- and (*S*)-enantiomers of compounds **3**

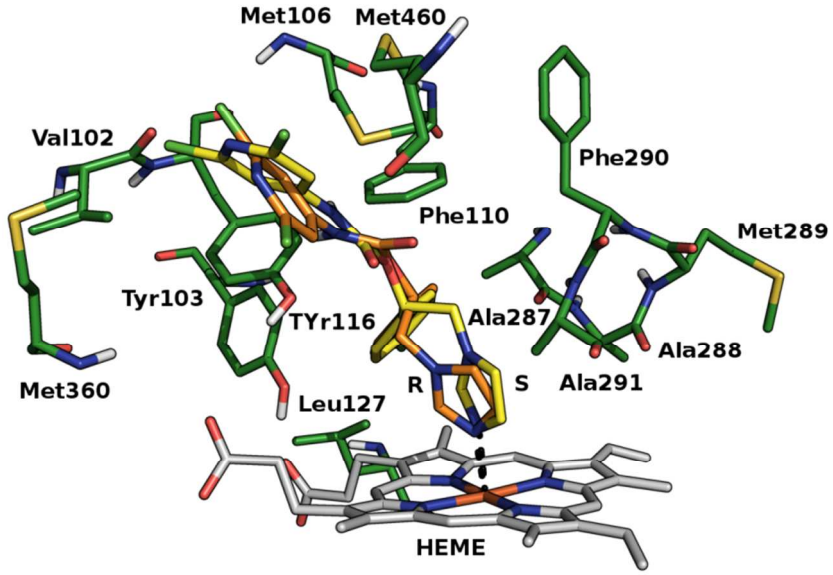


Figure S7. Docking pose of (*R*)- and (*S*)-enantiomers of compounds **5**

Docking poses. $CYP51_{Li}$, PDB ID: 3L4D Resolution: 2.75Å

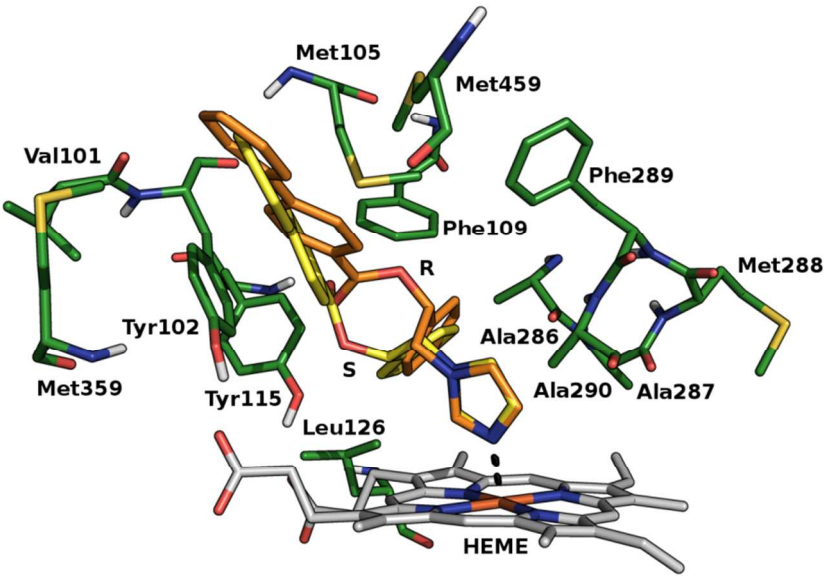


Figure S8. Docking pose of (*R*)- and (*S*)-enantiomers of compounds **1**

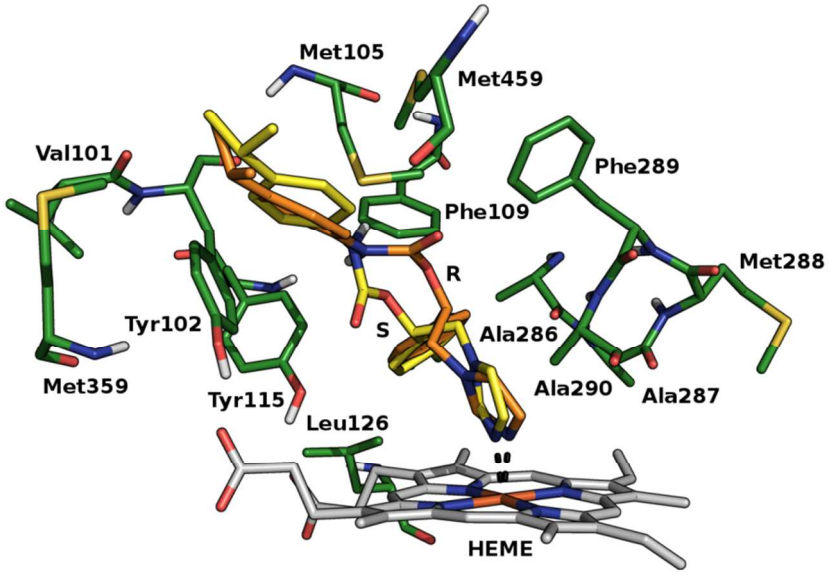


Figure S9. Docking pose of (*R*)- and (*S*)-enantiomers of compounds **3**

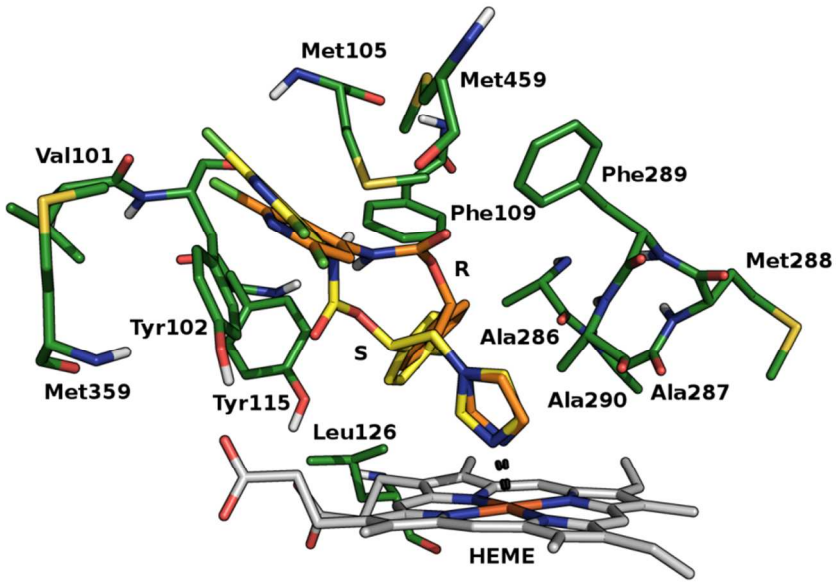


Figure S10. Docking pose of (*R*)- and (*S*)-enantiomers of compounds **5**

ADME assay

Chemicals. All solvents, reagents, were from Sigma-Aldrich Srl (Milan,Italy), Pooled Male Donors 20 mg/mL HLM (Human Liver Microsomes) were from BD Gentest-Biosciences (San Jose, California). Milli-Q quality water (Millipore, Milford, MA, USA) was used.

Microsomal Stability Assay. Each compound in DMSO solution was incubated at 37 °C for 60 min in 125mM phosphate buffer (pH 7.4), 5 μL of human liver microsomal protein (0.2 mg/mL), in the presence of a NADPH-generating system at a final volume of 0.5mL (compounds' final concentration, 50 μM); DMSO did not exceed 2% (final solution). The reaction was stopped by cooling in ice and adding 1.0 mL of acetonitrile. The reaction mixtures were then centrifuged for 15 min at 10000 rpm, and the parent drug and metabolites were subsequently determined by LC-UV-MS.

Chromatographic analysis were performed with an Agilent 1100 LC/MSD VL system (G1946C) (Agilent Technologies, Palo Alto, CA) constituted by a vacuum solvent degassing unit, a binary high-pressure gradient pump, an 1100 series UV detector, and an 1100 MSD model VL benchtop mass spectrometer.

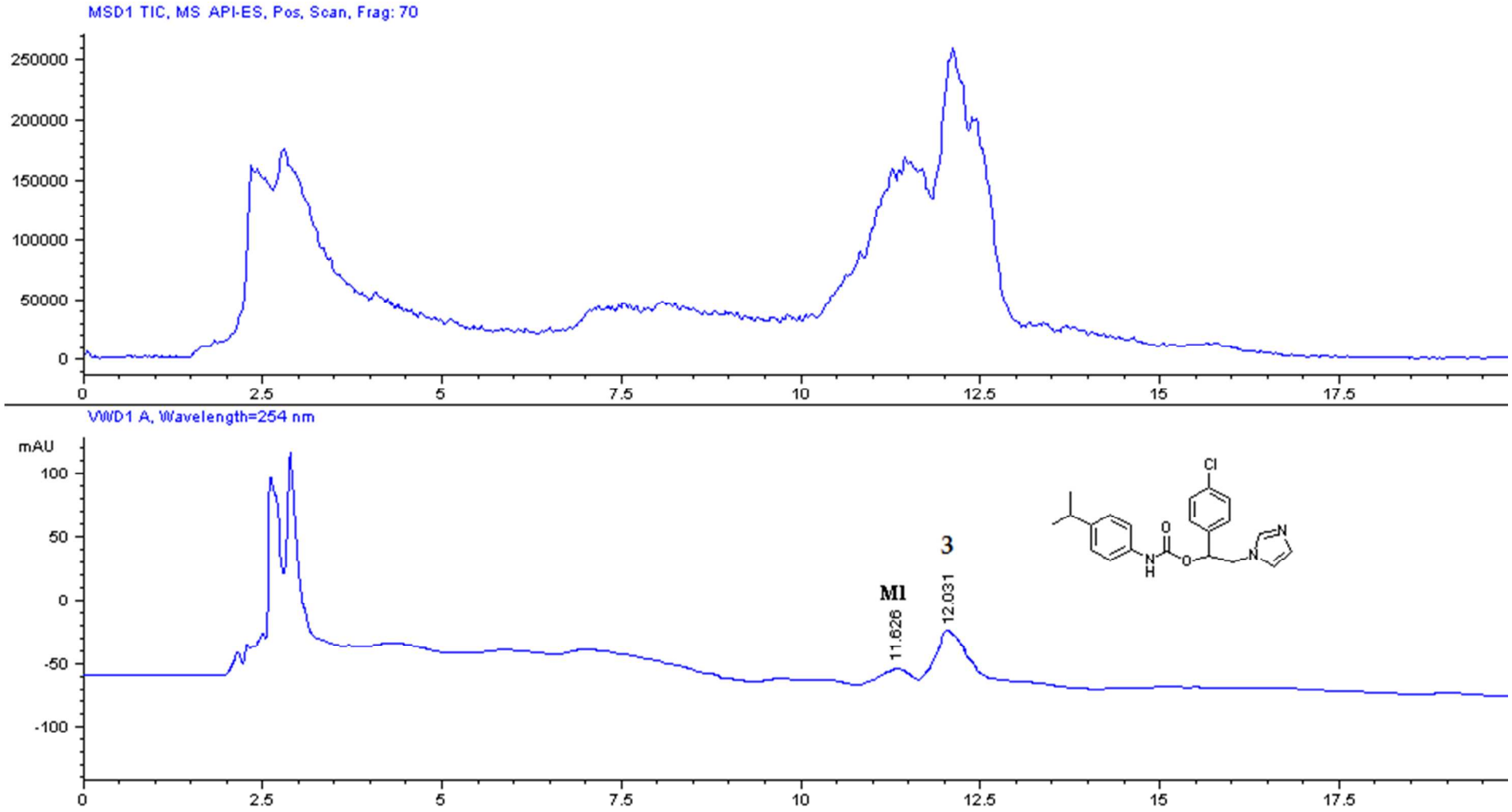
Chromatographic separation was obtained using a Varian Polaris 5 C18-A column (150 - 4.6 mm, 5 μm particle size) and gradient elution: eluent A being ACN and eluent B consisting of water. The analysis started with 2% of eluent A, which was rapidly increased up to 70% in 10 min, then slowly increased up to 98% in 20 min. The flow rate was 1.0 mL min-1 and injection volume was 20 μL.

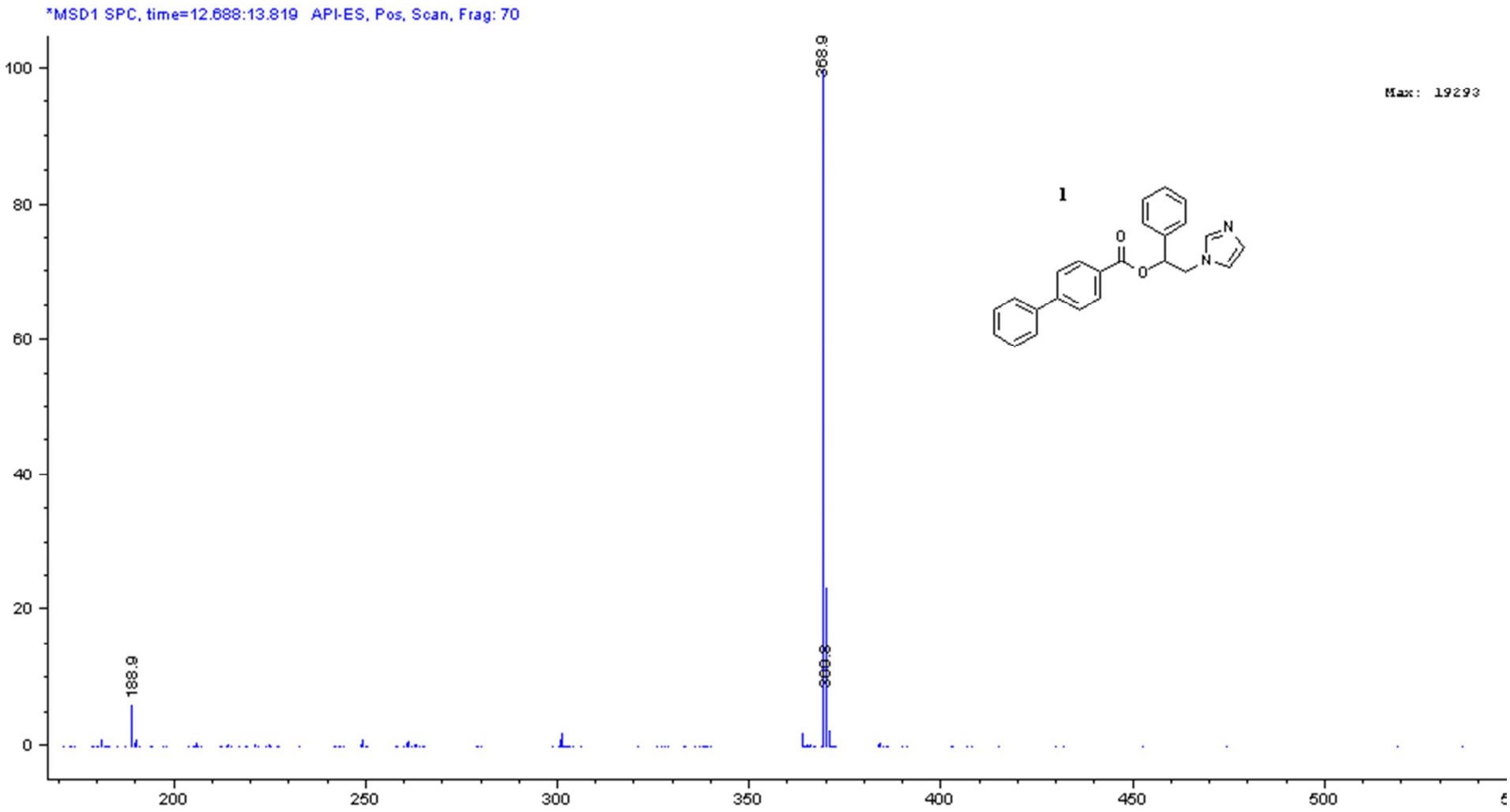
The Agilent 1100 series mass spectra detection (MSD) single-quadrupole instrument was equipped with the orthogonal spray API-ES (Agilent Technologies, Palo Alto, CA). Nitrogen was used as nebulizing and drying gas. The pressure of the nebulizing gas, the flow of the drying gas, the capillary voltage, the fragmentor voltage, and the vaporization temperature were set at 40 psi, 9 L/min-1, 3000 V, 70 V, and

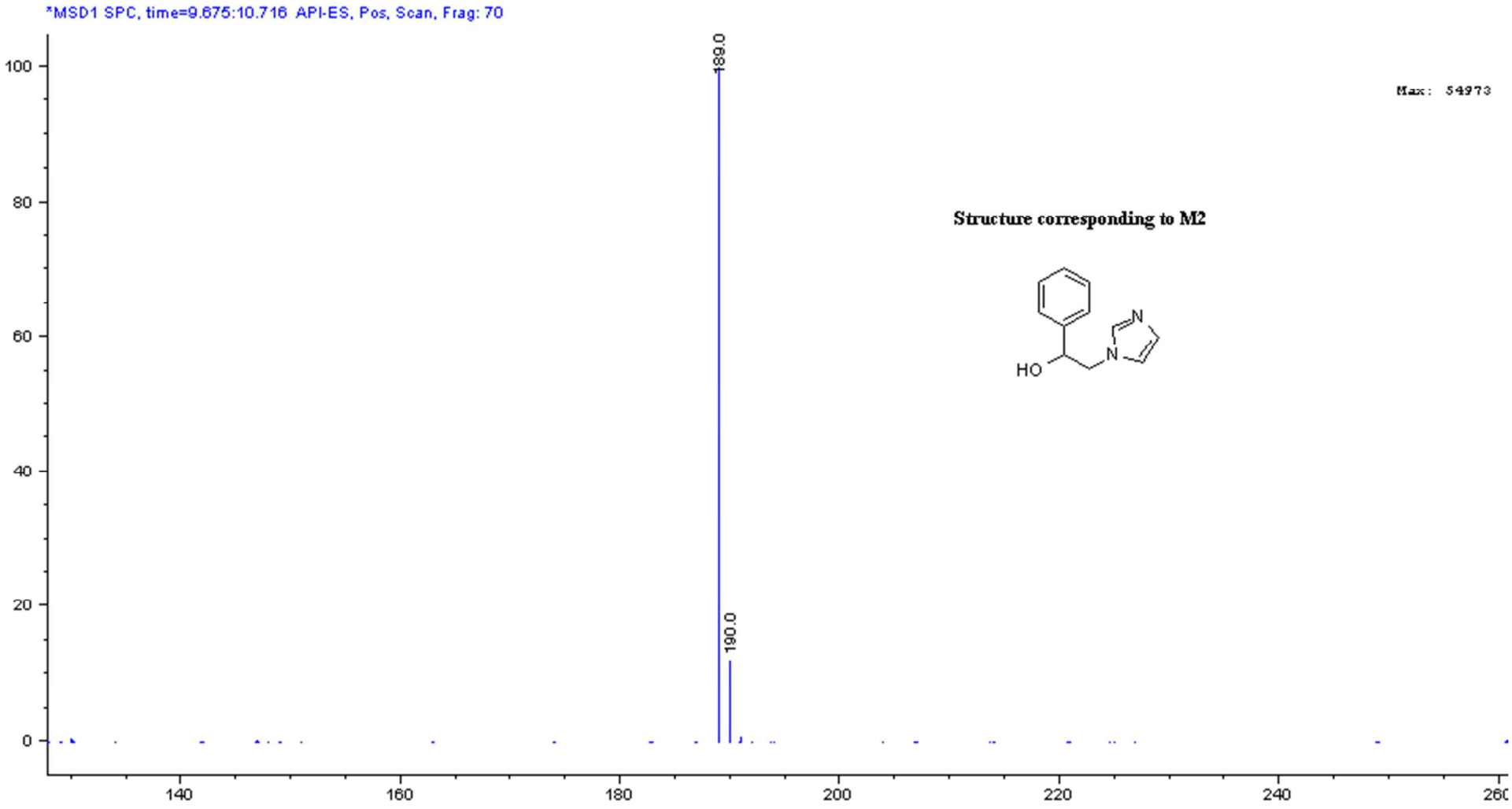
350°C, respectively. UV detection was monitored at 254 nm. The LC-ESI-MS determination was performed by operating the MSD in the positive ion mode. Spectra were acquired over the scan range m/z 100-1500 using a step size of 0.1 u.

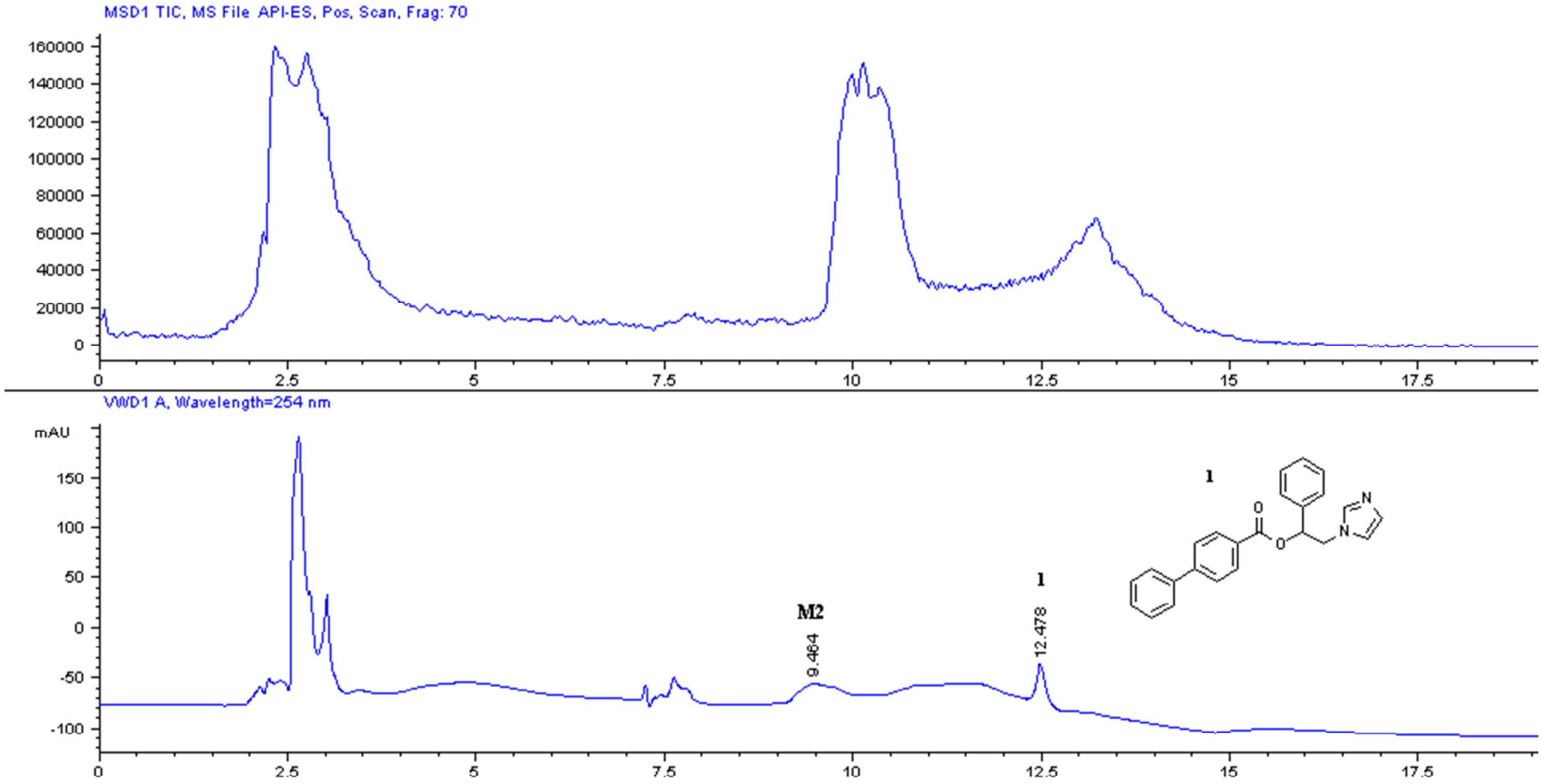
The percentage of not metabolized compound was calculated by comparison with reference solutions.

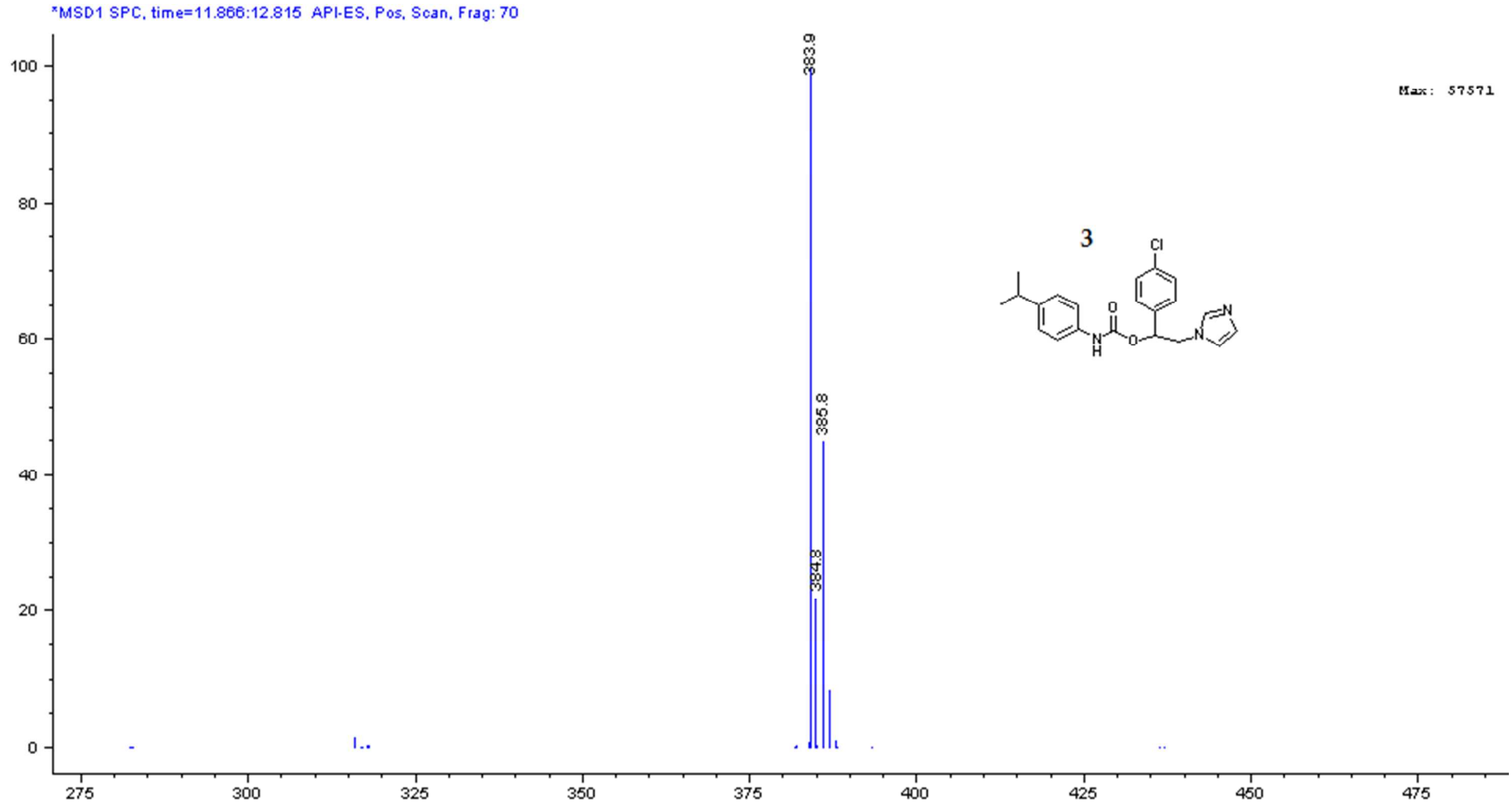
Cpd	metabolites formation (%)	Parent drug (%)
1	65.1	34.8
3	7.9	92.1

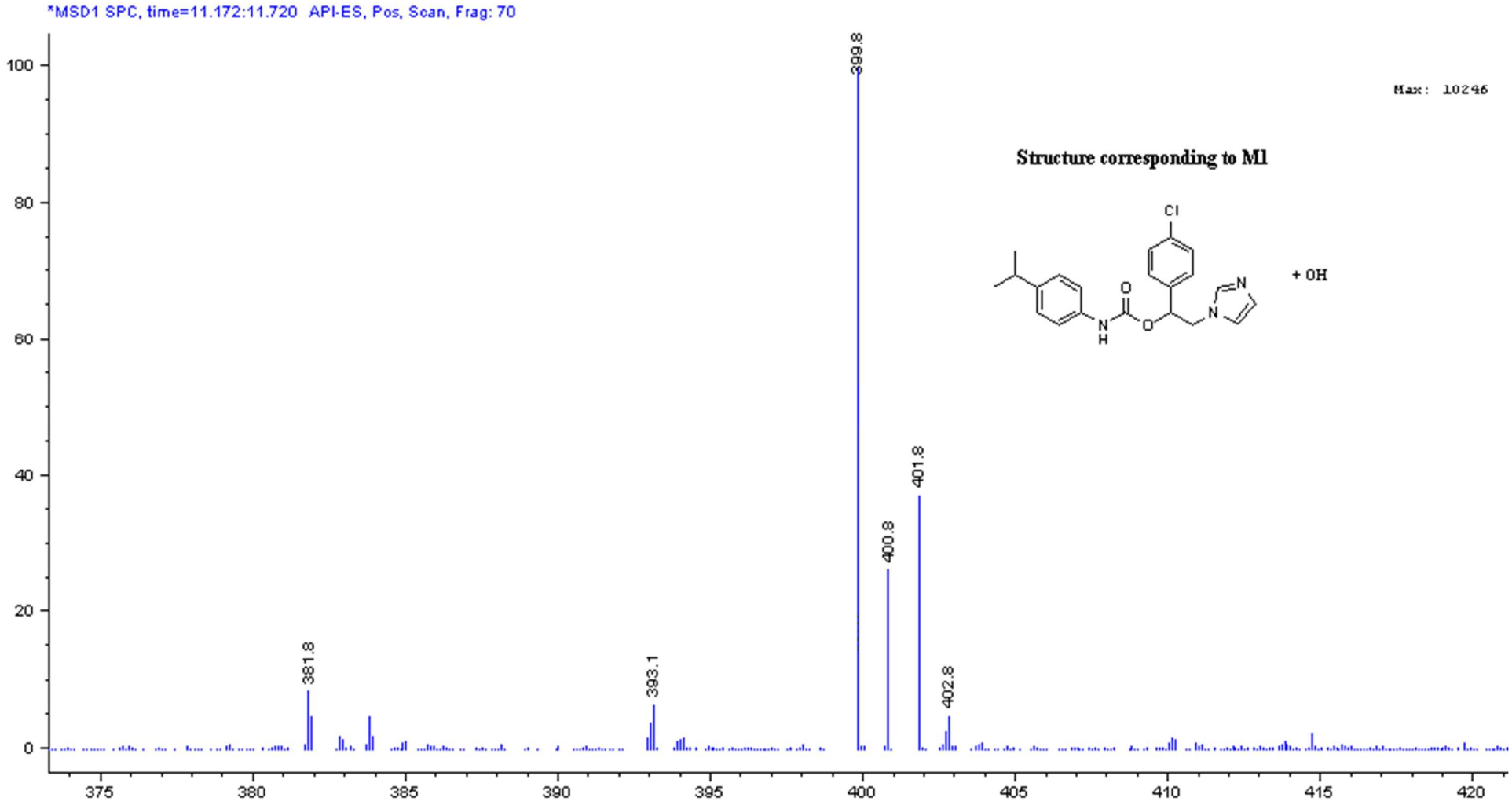

MSD1 TIC, MS API-ES, Pos, Scan, Frag: 70
250000
200000
150000
100000
50000
0
0
2.5
5
7.5
10
12.5
15
17.5
VWD1 A, Wavelength=254 nm
mAU
100
50
0
-50
-100
M1
11.626
3
12.031
Cl
N
O
N
H
O
N
0
2.5
5
7.5
10
12.5
15
17.5


*MSD1 SPC, time=12.688:13.819 API-ES, Pos, Scan, Frag: 70
Max: 19293
368.9
188.9
369.8
1
O
O
N
N
100
80
60
40
20
0
200
250
300
350
400
450
500


*MSD1 SPC, time=9.675:10.716 API-ES, Pos, Scan, Frag: 70
Max: 54973
189.0
190.0
Structure corresponding to M2
HO
N
N
100
80
60
40
20
0
140
160
180
200
220
240
260


MSD1 TIC, MS File API-ES, Pos, Scan, Frag: 70
160000
140000
120000
100000
80000
60000
40000
20000
0
0
2.5
5
7.5
10
12.5
15
17.5
VWD1 A, Wavelength=254 nm
mAU
150
100
50
0
-50
-100
M2
9.464
1
12.478
1
O
O
N
N


*MSD1 SPC, time=11.866:12.815 API-ES, Pos, Scan, Frag: 70
Max: 57571
383.9
385.8
384.8
3
Cl
O
N
H
O
N
100
80
60
40
20
0
275
300
325
350
375
400
425
450
475


*MSD1 SPC, time=11.172:11.720 API-ES, Pos, Scan, Frag: 70
Max: 10246
Structure corresponding to M1
+ OH
399.8
401.8
400.8
381.8
393.1
402.8
100
80
60
40
20
0
375
380
385
390
395
400
405
410
415
420