Supplementary data
(For online publication)

Discovery of 2-aminoimidazole and 2-aminoimidazolyl-thiazoles as non-xanthine human adenosine A₃ receptor antagonists: Structure-Activity Relationships and Molecular Modeling studies

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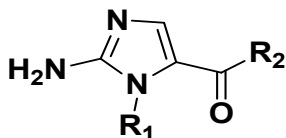
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Experimental section

Table-S1: Binding affinities of 2-aminoimidazole derivatives for human adenosine receptors

Compound	R_1	R_2	6a-6l Binding experiments K_i (µM)			Adenylyl cyclase activity K_i (µM)
			$hA_1{}^a$	$hA_{2A}{}^b$	$hA_3{}^c$	$hA_{2B}{}^d$
6a	$4\text{-}FC_6H_4$	$4\text{-}MeC_6H_4$	> 100	> 100	> 100	> 30
6b	$4\text{-}FC_6H_4$	$4\text{-}OMeC_6H_4$	> 100	> 100	> 100	> 30
6c	$4\text{-}FC_6H_4$	$4\text{-}ClC_6H_4$	> 100	> 100	> 100	> 30
6d	$4\text{-}ClC_6H_4$	$4\text{-}MeC_6H_4$	> 100	> 100	> 100	> 30
6e	$4\text{-}ClC_6H_4$	$4\text{-}C_5H_4N$	> 100	> 100	32.6 (25.8 – 41.1)	> 30
6f	C_6H_5	$4\text{-}SO_2MeC_6H_4$	> 100	> 100	> 100	> 30
6g	$4\text{-}CF_3C_6H_4$	$4\text{-}ClC_6H_4$	> 100	39.0 (27.5 – 55.4)	15.2 (12.5 – 18.4)	> 30
6h	$2\text{-}ClC_6H_4$	$4\text{-}MeC_6H_4$	> 100	> 100	22.7 (21.6– 23.7)	> 30
6i	$3\text{-}ClC_6H_4$	$4\text{-}MeC_6H_4$	> 100	> 100	34.8 (31.7- 38,1)	> 30
6j	$3\text{-}Cl\ 4\text{-}FC_6H_4$	$4\text{-}ClC_6H_4$	> 100	> 100	27.8 (22.1 – 34.9)	> 30
6k	$4\text{-}CF_3C_6H_4$	$4\text{-}C_5H_4N$	> 100	> 100	> 100	> 30
6l	$4\text{-}FC_6H_4$	$4\text{-}C_5H_4N$	> 100	> 100	> 100	> 30

[a] Displacement of specific [^{3}H]CCPA binding at human A_1 receptors expressed in CHO cells.

[b] Displacement of specific [^{3}H]NECA binding at human A_{2A} receptors expressed in CHO cells.

[c] Displacement of specific [^{3}H]HEMADO binding at human A_3 receptors expressed in CHO cells.

[d] Inhibition of NECA-stimulated adenylyl cyclase activity at human A_{2B} receptors expressed in CHO cells.

Data are expressed as geometric means, with 95% confidence limits in parentheses.

Biology

Receptor-radioligand binding studies were performed as previously described by Klotz et al [1]. The membranes for radioligand binding were prepared from CHO cells stably transfected with human adenosine receptor subtypes in a two-step procedure. In a first low-speed step (1,000x g) cell fragments and nuclei were removed. The crude membrane fraction was sedimented from the supernatant at 100,000x g. The membrane pellet was re-suspended in the buffer used for the respective binding experiments, frozen in liquid nitrogen and stored at -80°C. For the measurement of adenylyl cyclase activity only one high-speed centrifugation of the homogenate was used. The resulting crude membrane pellet was re-suspended in 50mM Tris/HCl, pH 7.4 and immediately used for the cyclase assay.

For agonist radioligand binding at A_1 adenosine receptors 1 nM [^{3}H]CCPA was used, whereas 10 nM [^{3}H]NECA were used for A_{2A} receptors. For A_3 adenosine receptors the newly developed high-affinity agonist [^{3}H]HEMADO[2] at a concentration of 1 nM was used.

Non-specific binding of [^{3}H]CCPA was determined in the presence of 1 mM theophylline, in the case of [^{3}H]NECA and [^{3}H]HEMADO 100 μM R-PIA was used. K_i-values from competition experiments were calculated with the program SCTFIT. [3]

Radioligand binding at A_{2B} adenosine receptors is problematic as no high-affinity ligand is available for this subtype. Therefore, inhibition of NECA-stimulated adenylyl cyclase activity was determined as a measurement of affinity of compounds. No detectable interaction of the compounds under investigation with the A_{2B} AR was observed.

References:

1. Klotz, K.-N. et al *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1998,***357,* 1.
2. Klotz, K.-N.; Falgner, N.; Kachler, S.; Lambertucci, C.; Vittori, S.; Volpini, R.; Cristalli, G. *Eur. J. Pharmacol.***2007,** *556,*14.
3. De Lean, A. et al. *Mol. Pharmacol.***1982,** *21*, 5.

Molecular modeling studies

Software overview

General molecular modeling operations, such as drawing of ligand structures, partial charges computation, energetic analysis and visual inspection of docking poses, were performed using the MOE suite (Molecular Operating Environment, version 2015.1001) [1]. Shape similarity studies were conducted with the vROCS application of the OpenEye Scientific Software [7]. GOLD (Genetic Optimization for Ligand Docking, version 5.2) suite [2] was used for docking simulations. Calculation of ligand partial charges was performed with the software MOPAC [3] as implemented in the MOE suite. CHIMERA [4] was used for image production and GNUPLOT 4.6 [5] to construct plots. Video assembly was carried out using MEncoder [6]. Molecular modeling studies have been performed on a 8 CPU (Intel® Xeon® CPU E5-1620 3.70 GHz) linux workstation.

Three-dimensional structures of Adenosine Receptors

A shape similarity comparison was conducted among compound **12b**, chosen as representative of the series under examination, and all the ligands retrieved from A_{2A} AR PDB structures available to date. Consequently, 3UZC [8] crystallographic structure was chosen because its co-crystallized ligand, 4-(3-amino-5-phenyl-1,2,4-triazin-6-yl)-2-chlorophenol, showed highest ROCS Tanimoto Combo value for compound **12b**. Homology models of A_3 and A_1 ARs, constructed using 3UZC structure as a template, were retrieved from the *Adenosiland* web-platform [9,10]. The protein residues have been named according the generic Ballesteros Weinstein numbering system [11] throughout this work.

Molecular docking

MOE-Builder tool was employed to model three-dimensional structures of ligands and the MOE-Protonate-3D [12] tool was used to predict ionization states of ligands and proteins. Newly added protein hydrogens were minimized using Amber12EHT force field, while ligand structures were subjected to MMFF94x energy minimization, fixing the root mean square (rms) gradient cutoff at 0.1 kcal mol^{-1} A^{-2}. Taking advantage of a docking benchmark previously performed in our laboratory [13,14], GOLD docking tool [2] was selected as conformational search program and GoldScore as scoring function. For each compound, 20 docking runs were performed on each

receptor subtype, searching in a sphere of 20 Å radius centered on Asn6.55 residue. After computing Amber12EHT partial charges for receptor atoms and PM3/ESP atomic partial charges for ligands poses, electrostatic and van der Waals interactions were calculated with MOE.

Interaction Energy Fingerprints (IEFs)

Protein residues within 4.5 Å of the selected pose of compound **12b** were identified as defining the binding pocket of the receptor. Per residue electrostatic and hydrophobic interactions (named IEele and IEhyd, respectively) were calculated between each binding site residue and the ligand, using MOE [13,15]. In particular, IEele, measured in kcal/mol, were computed with Amber12EHT force field. Instead, IEhyd, expressed by an adimensional score (the higher the better), were computed as contact hydrophobic surfaces. The data were plotted on a heat map, called Interaction Energy Fingerprints (IEFs), in which residues are reported on the x-axis and the energetic values are rendered by a colorimetric scale. As regards IEele, colors from blue to red depict energy values ranging from negative to positive ones; for IEhyd, colors from white to dark green represent scores going from 0 to positive values.

MMsDocking video maker

A in-house tool, named MMsDocking video maker, was used to produce videos with the aim to facilitate the visualization and analysis of data obtained from the docking simulations. The videos show for each ligand the selected docking pose, scoring values, experimental binding data and per residue IEhyd and IEele of most relevant residues. IEhyd and IEele were computed as previously described and plotted with GNUPLOT [5], molecular representation of docked compounds were obtained using CHIMERA [4] software, 2D depictions of the compounds were drawn with RDKit toolkit [16]. Images were assembled into videos using MEncoder[6].

SUPPLEMENTARY MATERIAL

Videos SM1-SM2-SM3: Videos reporting the selected docking poses on A_3, A_{2A} and A_1 adenosine receptors binding sites, respectively. Ligands are rendered by light-blue sticks, and protein residues by tan sticks. Protein residues mainly involved in binding are shown. For A_{2A} and A_1 receptors, in addition to highly interacting residues, residues at equivalent positions to those selected for A_3 receptor are reported, to enable inter-receptors comparison. The heat maps depicted in the

background report the electrostatic and hydrophobic contributions of the selected residues ("ele" and "hyd" labels identify the major contribution type of the residue) by a colorimetric scale going from blue to green for negative to positive values. Hydrogen bonds are represented by yellow lines.

REFERENCES

1. Chemical Computing Group Inc. Molecular Operating Environment (MOE) [Internet]. Available from: http://www.chemcomp.com

2. Cambridge Crystallographic Data Centre: 12 Union Road, Cambridge CB2 1EZ, UK. GOLD suite, version 5.2 [Internet]; Available from: http://www.ccdc.cam.ac.uk

3. Stewart JJ. Optimization of parameters for semiempirical methods V: modification of NDDO approximations and application to 70 elements. J Mol Model 2007;13:1173–1213

4. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera: a visualization system for exploratory research and analysis. J Comput Chem 2004;25:1605–12.

5. Gnuplot [Internet]. Available from: http://www.gnuplot.info/index.html

6. MEncoder [Internet]. Available from: http://www.mplayerhq.hu/design7/projects.html

7. Hawkins PCD, Skillman AG, Nicholls A. Comparison of Shape-Matching and Docking as Virtual Screening Tools. J Med Chem. 2007 Jan 1;50(1):74–82.

8. Congreve M, Andrews SP, Doré AS, Hollenstein K, Hurrell E, Langmead CJ, et al. Discovery of 1,2,4-triazine derivatives as adenosine A(2A) antagonists using structure based drug design. J Med Chem. 2012 Mar 8;55(5):1898–903.

9. Floris M, Sabbadin D, Medda R, Bulfone A, Moro S. Adenosiland: Walking through adenosine receptors landscape. Eu J Med Chem 2012;58:248–57.

10. Floris M, Sabbadin D, Ciancetta A, Medda R, Cuzzolin A, Moro S. Implementing the "Best Template Searching" tool into Adenosiland platform. In Silico Pharmacol 2013;20;1-25.

11. Ballesteros JA, Weinstein H. Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors, Methods in Neurosciences 1995; 25:366–428.

12. Labute P. Protonate3D: assignment of ionization states and hydrogen coordinates to macromolecular structures. Proteins. 2009;75:187–205.

13. Ciancetta A, Cuzzolin A, Moro S. "Alternative Quality Assessment Strategy to Compare Performances of GPCR-Ligand Docking Protocols: The Human Adenosine A2A Receptor as a Case Study." J Chem Inf Model 2014;54:2243-2254.

14. Cuzzolin A, Sturlese M, Malvacio I, Ciancetta A, Moro S. DockBench: An Integrated Informatic Platform Bridging the Gap between the Robust Validation of Docking Protocols and Virtual Screening Simulations. Molecules 2015;20:9977–9993.

15. Ciancetta A, Sabbadin D, Federico S, Spalluto G, Moro S. "Advances in Computational Techniques to Study GPCR-Ligand Recognition." Trends Pharmacol Sci. 2015;36:878-890.

16. RDKit: Cheminformatics and Machine Learning Software [Internet]. Available from: http://www.rdkit.org

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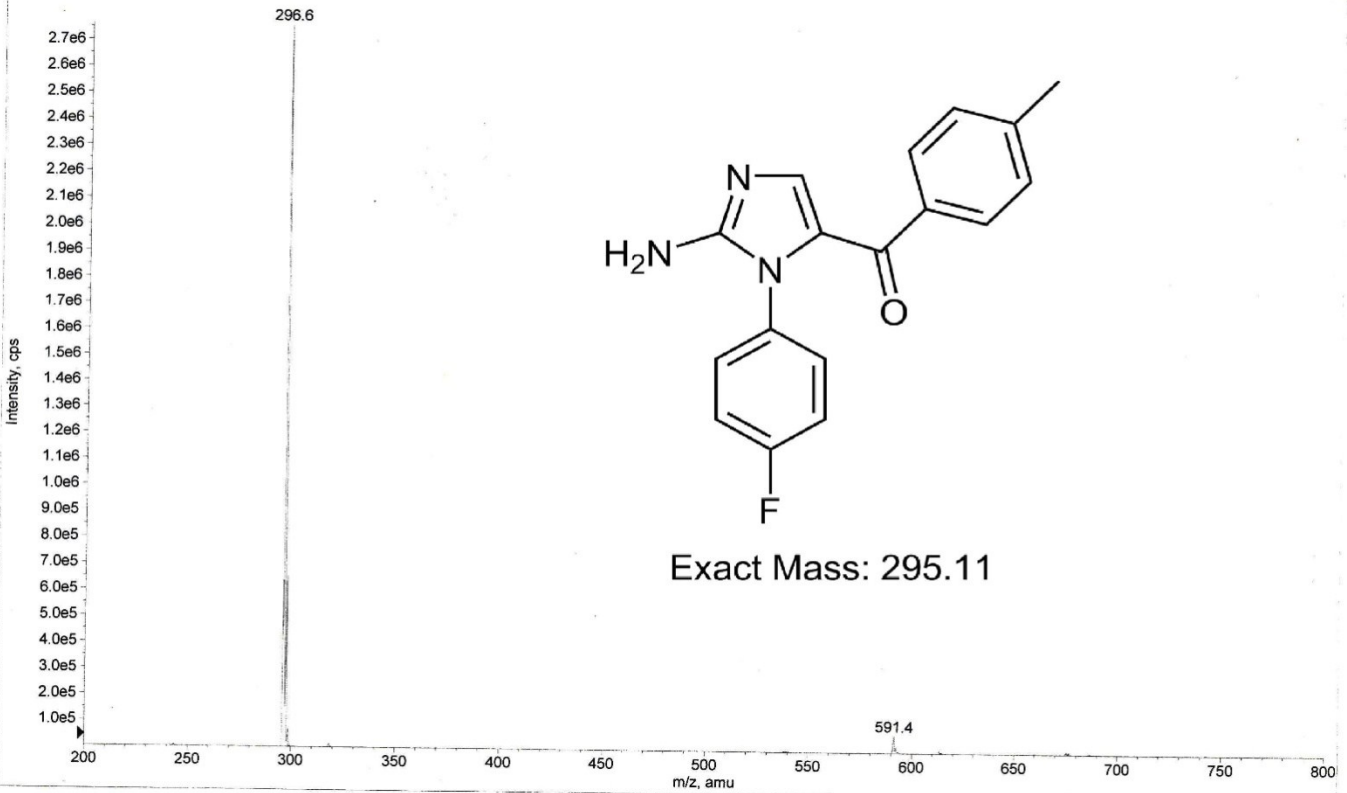
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116.791
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77.003
76.579
21.468

Exact Mass: 311.11

1H-NMR   / CDCl3

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7.802
7.777
7.399
7.377
7.361
7.354
7.348
7.332
7.266
7.233
7.226
7.206
7.184
7.177
6.957
6.928
4.947
4.865
3.964
3.871
1.844
1.254
0.000

2.009
3.064
2.088
2.044

2.009
3.064
2.088
2.044
1.969
6.112
2.999

13C-NMR / CDCl3

181.385
164.122
162.807
160.819
153.161
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128.131
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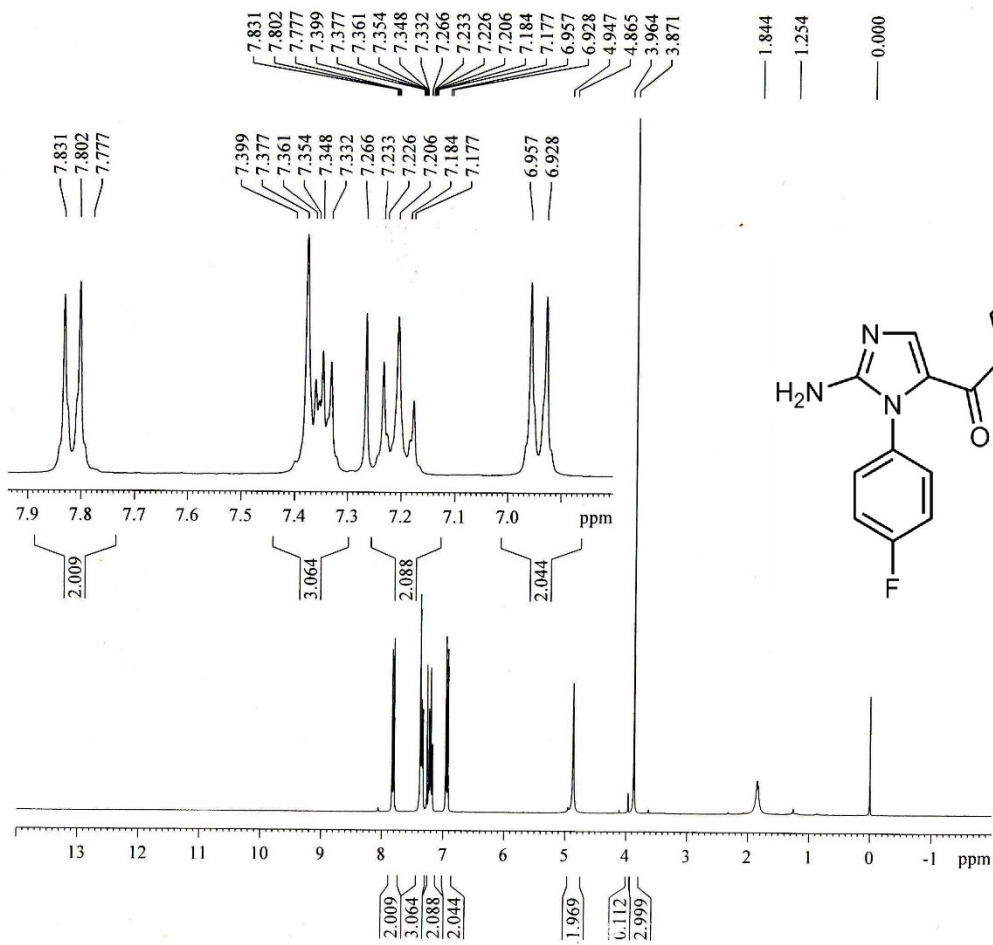
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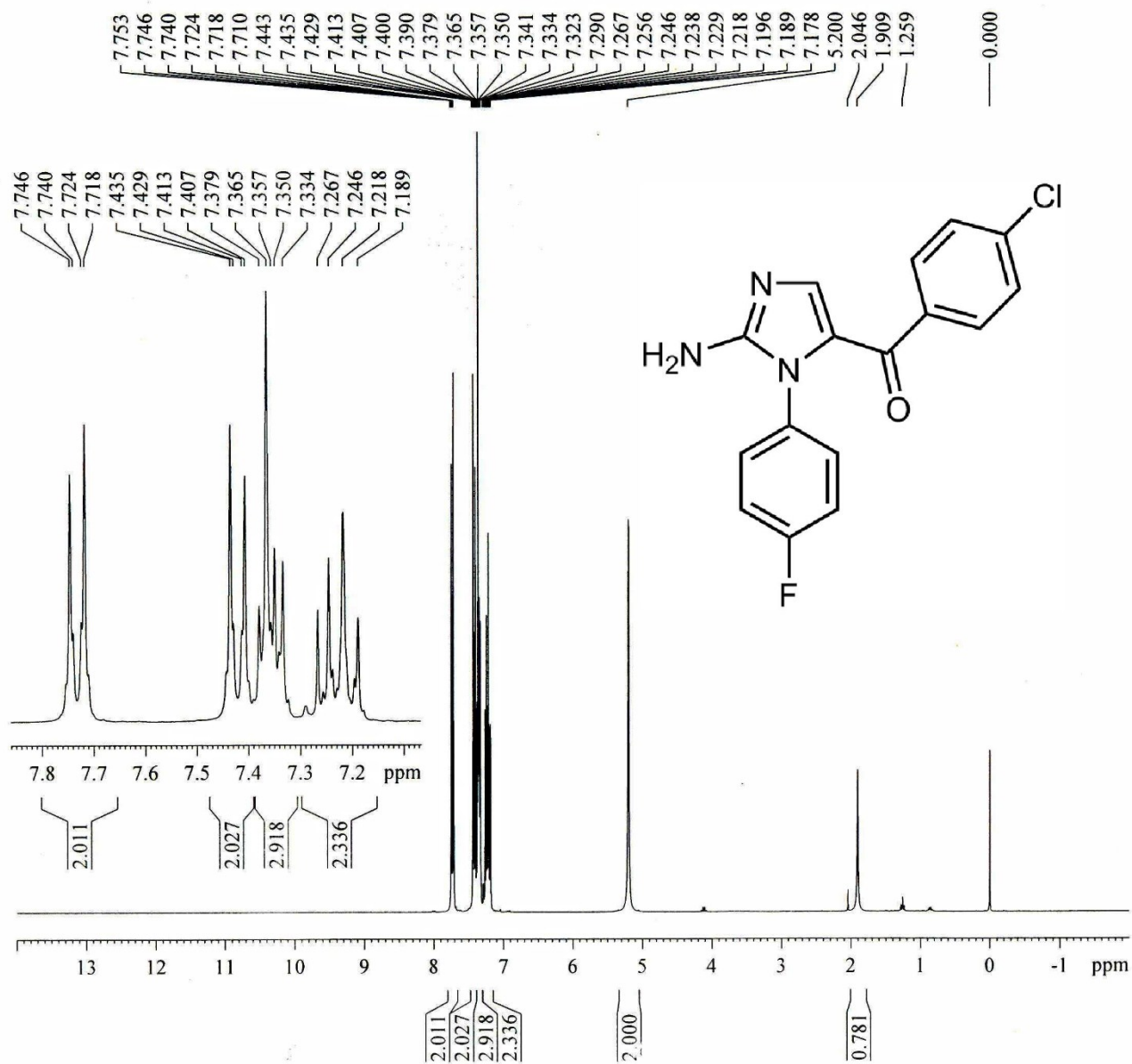
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0.000

7.746, 7.740, 7.724, 7.718, 7.435, 7.429, 7.413, 7.407, 7.379, 7.365, 7.357, 7.350, 7.334, 7.267, 7.246, 7.218, 7.189

2.011   2.027   2.918   2.336

2.011, 2.027, 2.918, 2.336   2.000   0.781

13C-NMR / CDCl3

180.886
164.217
160.906
153.883
141.195
138.140
137.133
131.504
130.102
129.023
128.907
128.618
127.734
116.986
116.681
77.430
77.210
77.006
76.583

190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

Exact Mass: 311.08

1H-NMR / CDCl3

13C-NMR **/ CDCl3**

182.046
153.431
142.561
140.640
135.965
134.810
134.353
129.924
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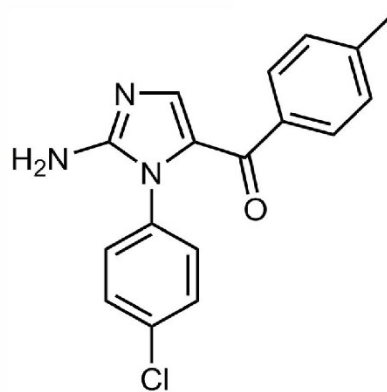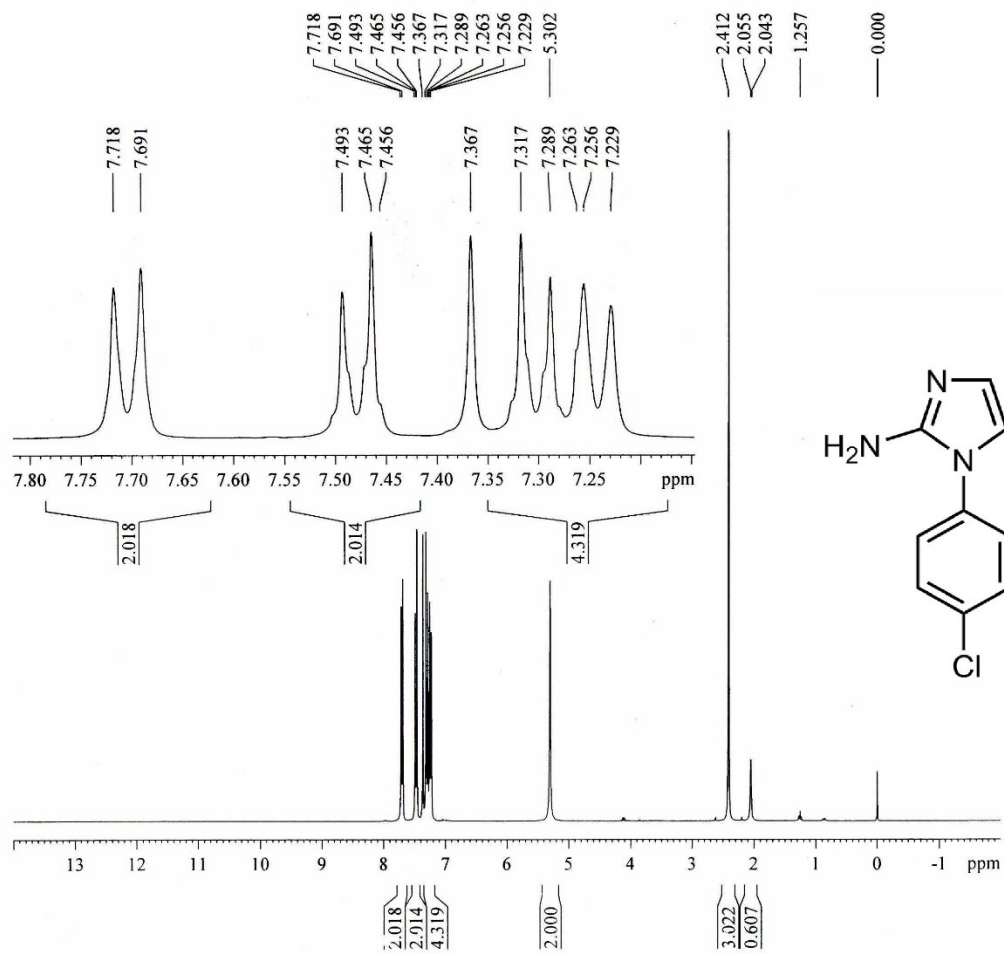
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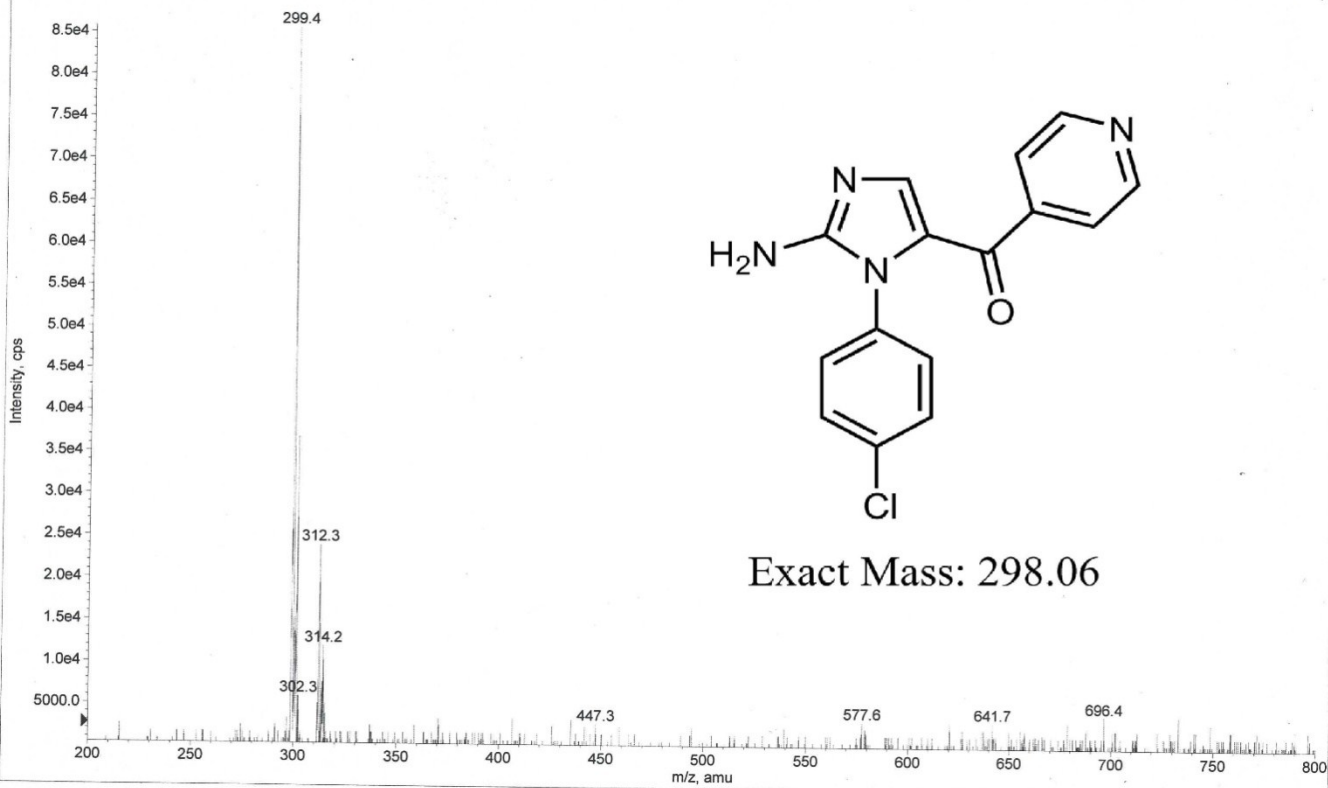
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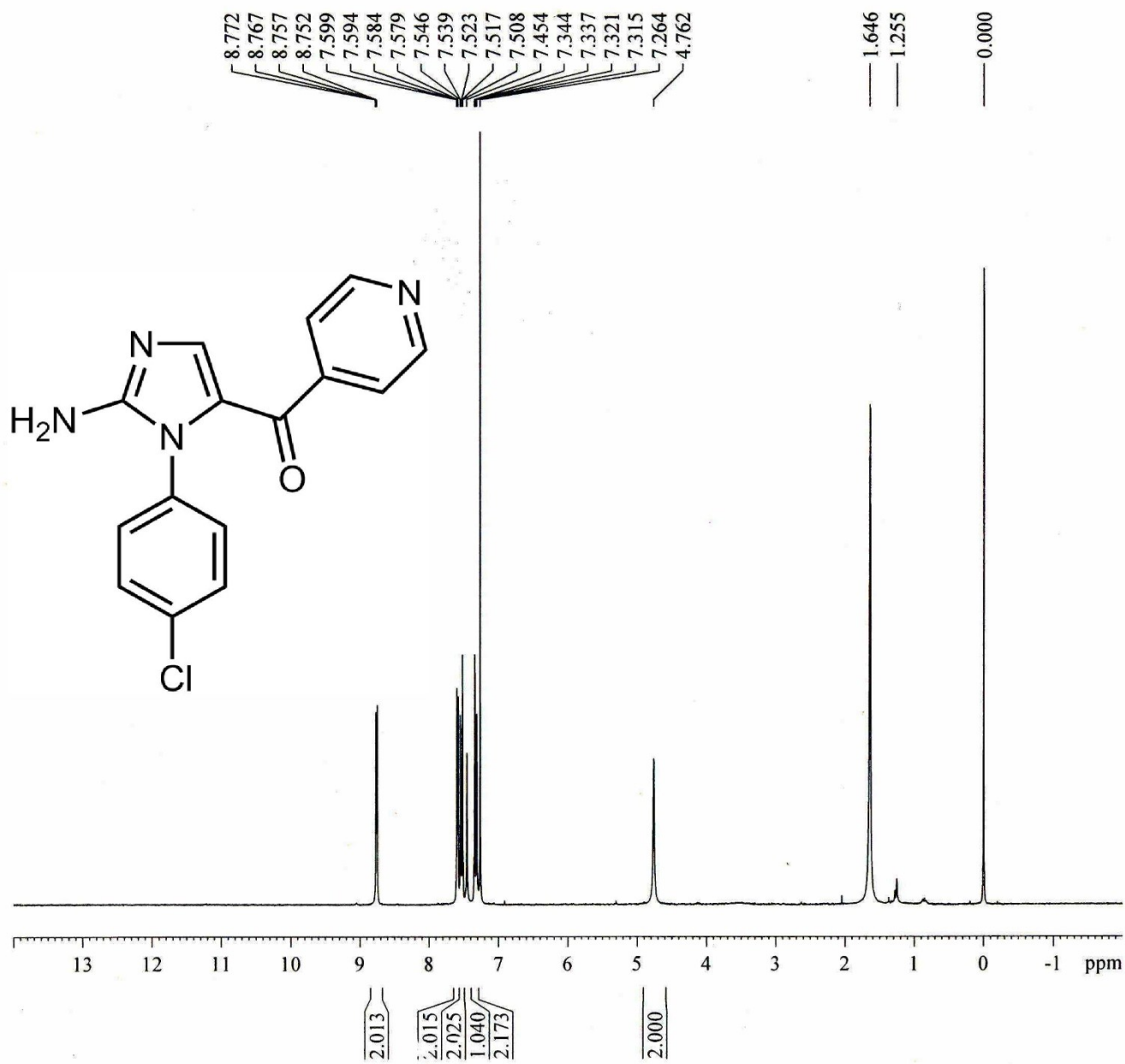
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7.454
7.344
7.337
7.321
7.315
7.264
4.762

1.646
1.255

0.000

13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 ppm

2.013

2.015
2.025
1.040
2.173

2.000

13C-NMR / CDCl3

180.166

153.858
150.364
145.330
142.633
135.477
133.762
130.195
128.406
127.484
122.204

77.427
77.209
77.004
76.581

-0.018

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Max. 7.4e4 cps

Exact Mass: 341.08

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8.000
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7.859
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7.466
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7.441
7.429
7.424
7.415
7.406
7.393
7.386
7.382
7.366
7.324
7.300
7.265
7.240
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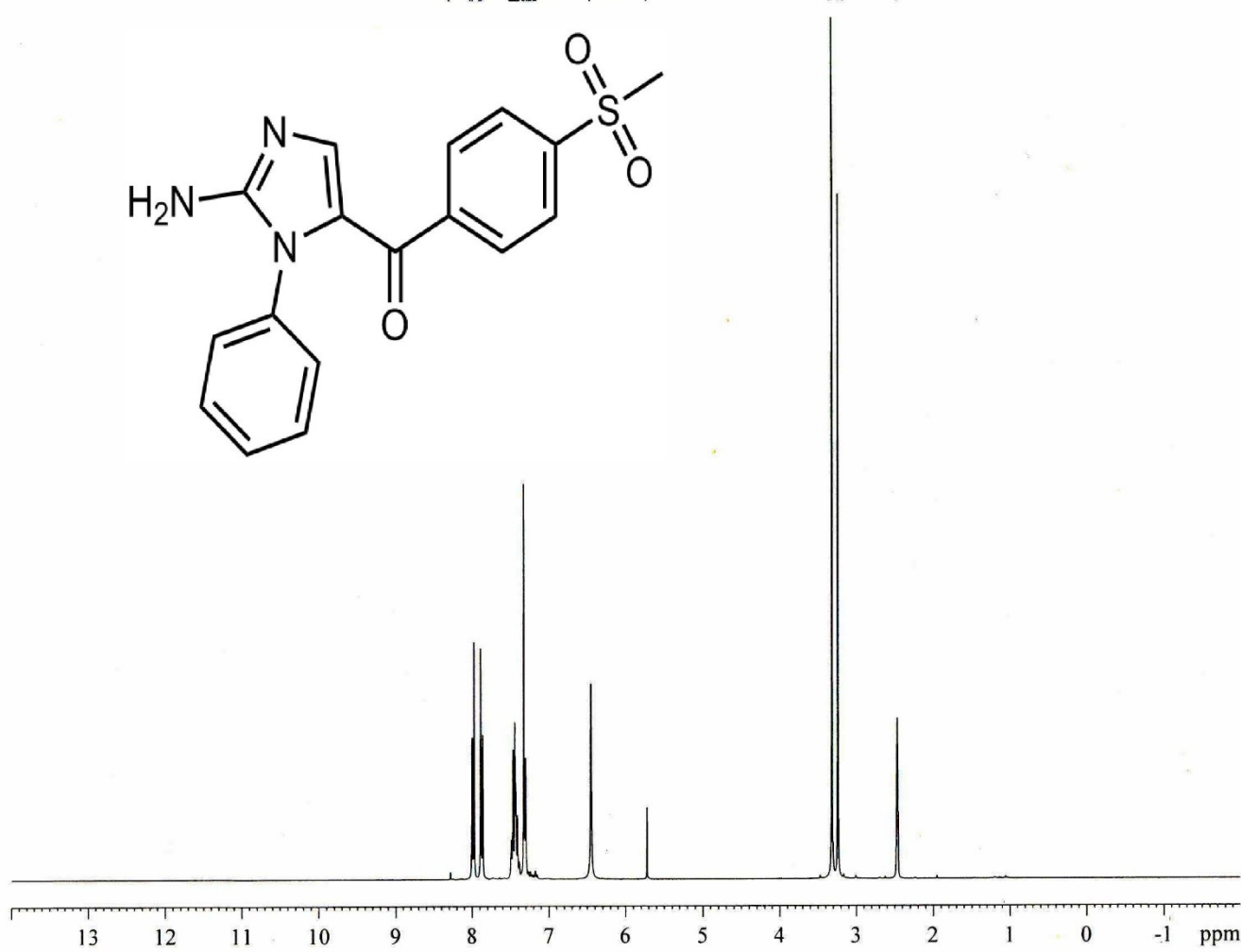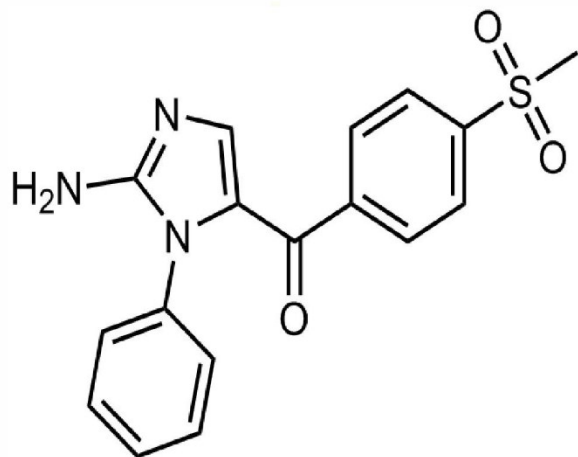
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0.236
2.022
0.235
3.019

13C-NMR / DMSO-d6

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143.513
142.616
135.947
129.219
129.124
128.097
127.220
127.024
126.842

54.902
43.356
40.337
40.059
39.782
39.503
39.225
38.947
38.669

190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

Exact Mass: 365.05

Exact Mass: 311.08

Exact Mass: 311.08

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406.5

1H-NMR/ IT-1 / DMSO-d6
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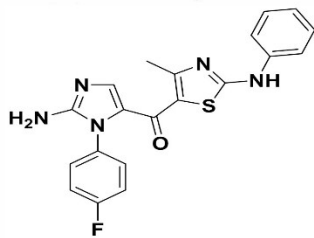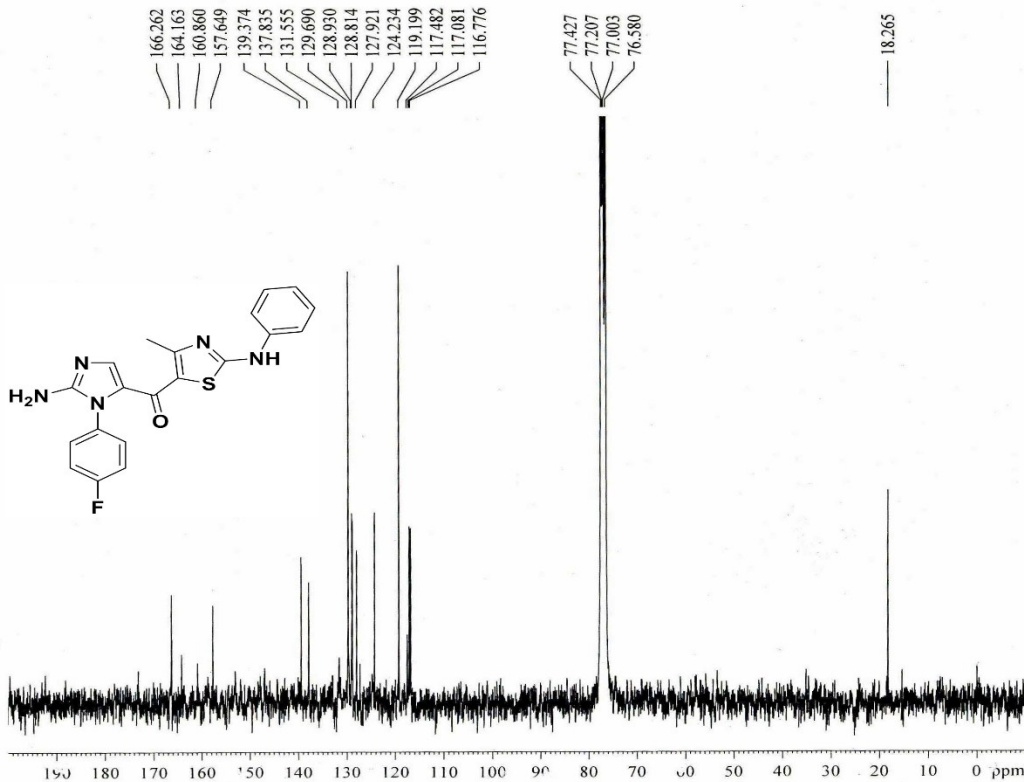F2 - Acquisition Parameters
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2.000

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0.088

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CADILA
PHARMACEUTICALS
LIMITED

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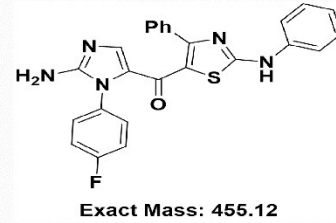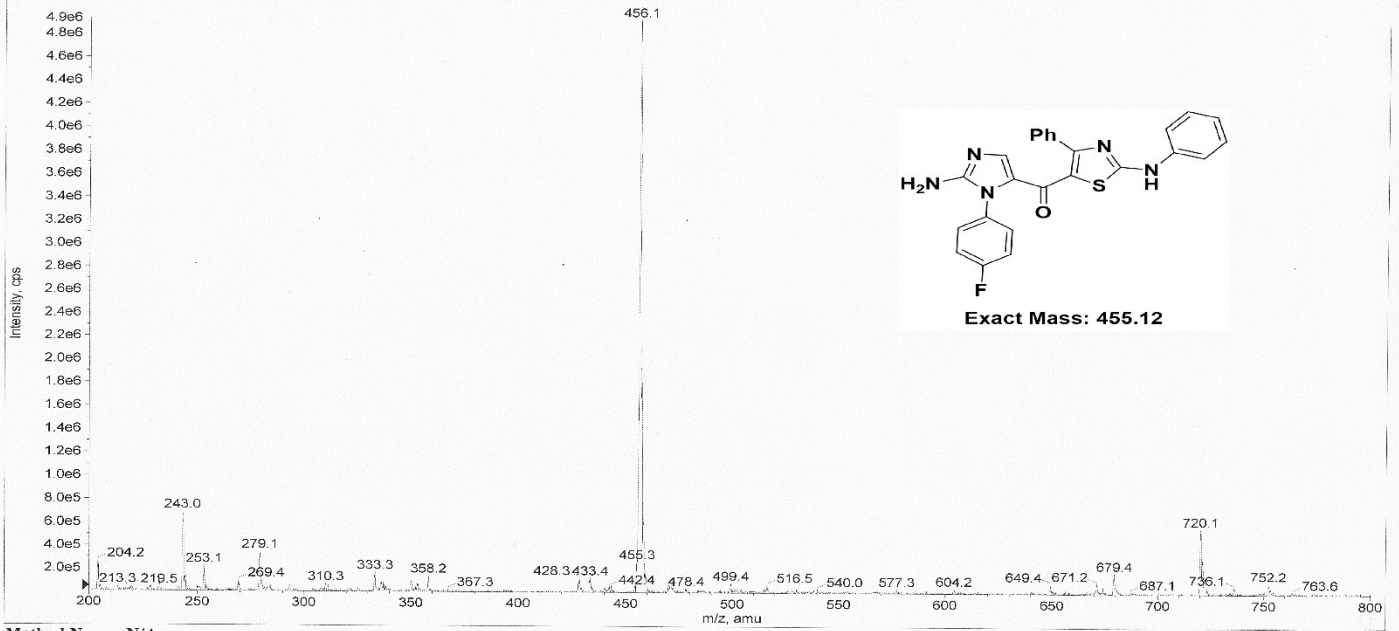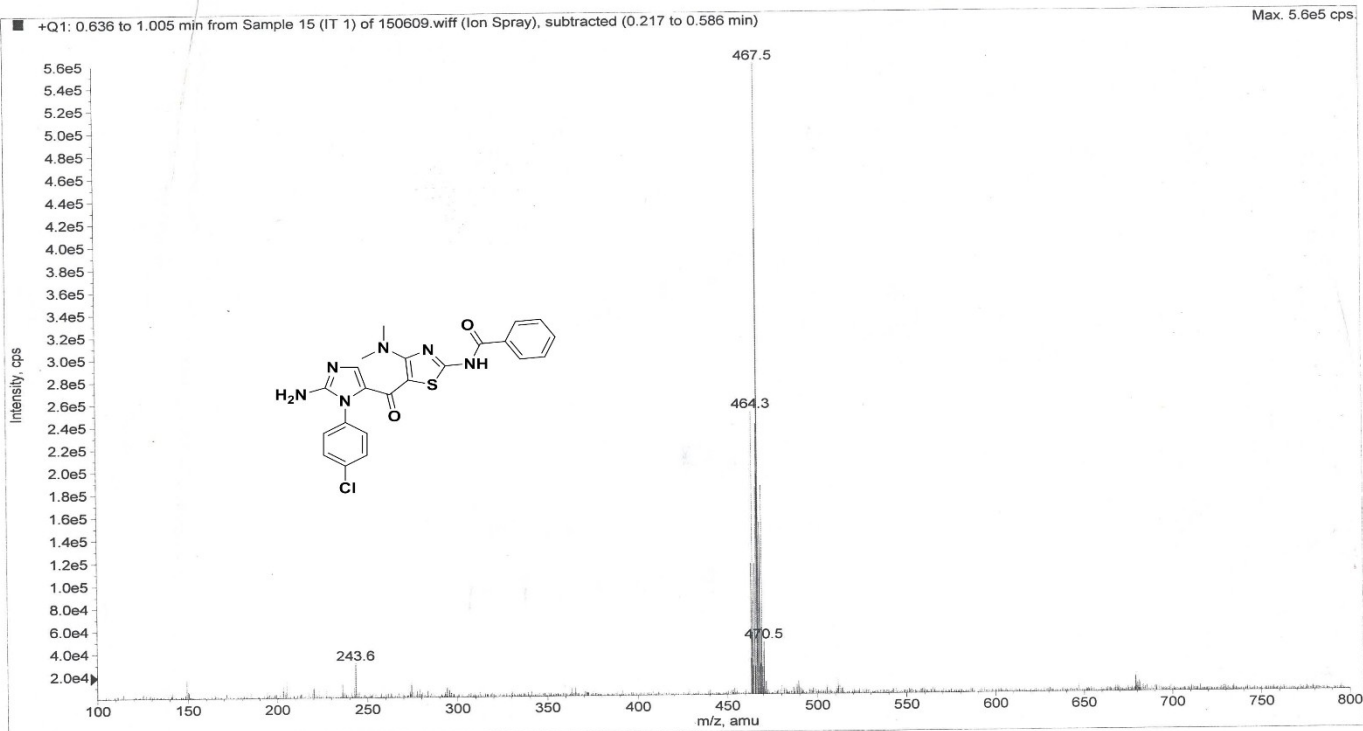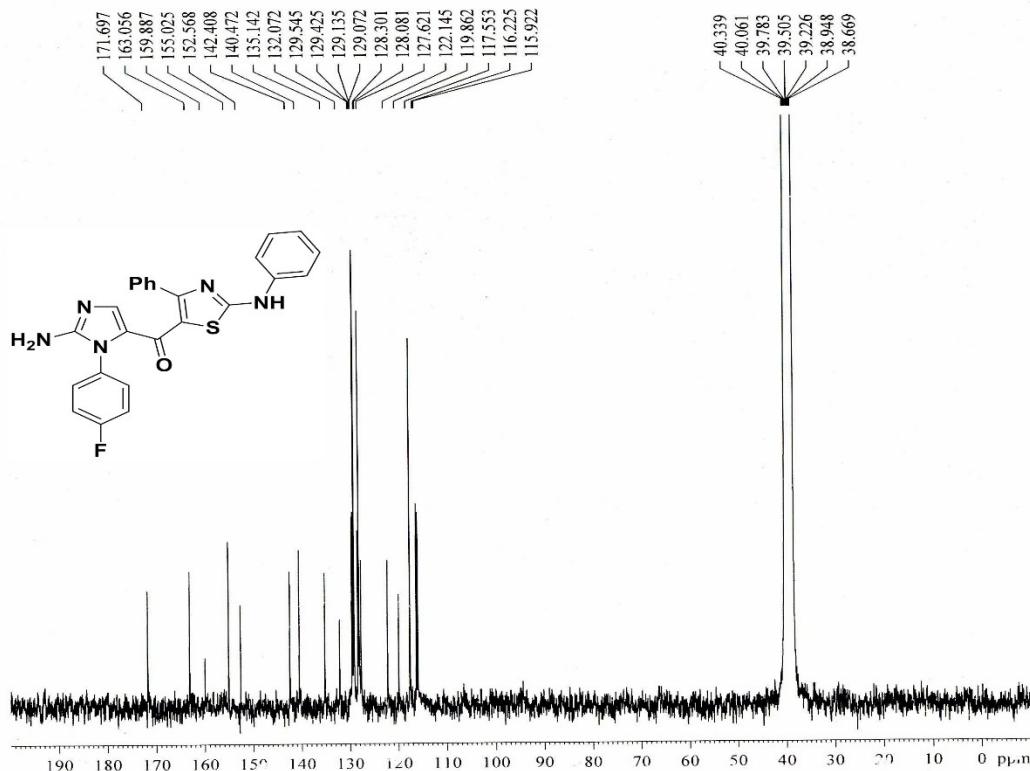LB   3.00 Hz
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■ +Q1: 0.251 to 0.452 min from Sample 15 (IT 2) of 061009.wiff (Ion Spray), subtracted (0.117 to 0.234 min)   Max. 1.0e6 cps.

394.3

416.4

Intensity, cps

100 150 200 250 300 350 400 450 500 550 600 650 700
m/z, amu

1H-NMR/ IT-2 / CDCl3
30.11.09

8.858 7.618 7.424 7.395 7.373 7.357 7.351 7.342 7.328 7.312 7.295 7.289 7.262 7.228 7.201 7.172 7.153 7.146 7.125 7.118 4.848 2.504 1.678 0.000

13 12 11 10 9 8 7 6 5 4 3 2 1 0 ppm

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13C-NMR/ IT-2 / CDCl3
30.11.09

166.262 164.163 160.860 157.649 139.374 137.835 131.555 129.690 128.930 128.814 127.921 124.234 119.199 117.482 117.081 116.776 77.427 77.207 77.003 76.580 18.265

190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

+Q1: 0.318 to 0.335 min from Sample 1 (PMC APIT3) of PMC APIT3.wiff (Turbo Spray)

Max. 4.9e6 cps.

Exact Mass: 455.12

1H-NMR/ IT-3 / DMSO-d6
30.11.09

Peaks: 10.595, 7.669, 7.643, 7.584, 7.579, 7.556, 7.402, 7.397, 7.391, 7.373, 7.347, 7.322, 7.300, 7.282, 7.270, 7.247, 7.155, 7.027, 7.002, 6.978, 6.318, 3.488, 3.480, 3.432, 3.414, 3.347, 3.288, 2.490, 1.383, 1.219, 0.930, 0.908, 0.843, 0.821

Integrals: 0.933, 2.000, 2.031, 9.202, 0.954, 1.094, 1.856, 0.115, 0.480, 0.178, 0.272, 0.039

13C peaks: 171.697, 163.056, 159.887, 155.025, 152.568, 142.408, 140.472, 135.142, 132.072, 129.545, 129.425, 129.135, 129.072, 128.301, 128.081, 127.621, 122.145, 119.862, 117.553, 116.225, 115.922

Solvent peaks: 40.339, 40.061, 39.783, 39.505, 39.226, 38.948, 38.669

CADILA
PHARMACEUTICALS
LIMITED

ppm axis: 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

+Q1: 0.636 to 1.005 min from Sample 15 (IT 1) of 150609.wiff (Ion Spray), subtracted (0.217 to 0.586 min)

Max. 5.6e5 cps

Intensity, cps axis: 5.6e5, 5.4e5, 5.2e5, 5.0e5, 4.8e5, 4.6e5, 4.4e5, 4.2e5, 4.0e5, 3.8e5, 3.6e5, 3.4e5, 3.2e5, 3.0e5, 2.8e5, 2.6e5, 2.4e5, 2.2e5, 2.0e5, 1.8e5, 1.6e5, 1.4e5, 1.2e5, 1.0e5, 8.0e4, 6.0e4, 4.0e4, 2.0e4

Peaks: 467.5, 464.3, 470.5, 243.6

m/z axis: 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800

m/z, amu

+Q1: 0.469 to 0.519 min from Sample 1 (PMC APIT7) of PMC APIT7.wiff (Turbo Spray)

Max. 1.3e7 cps.
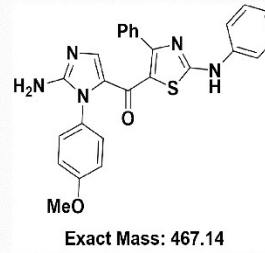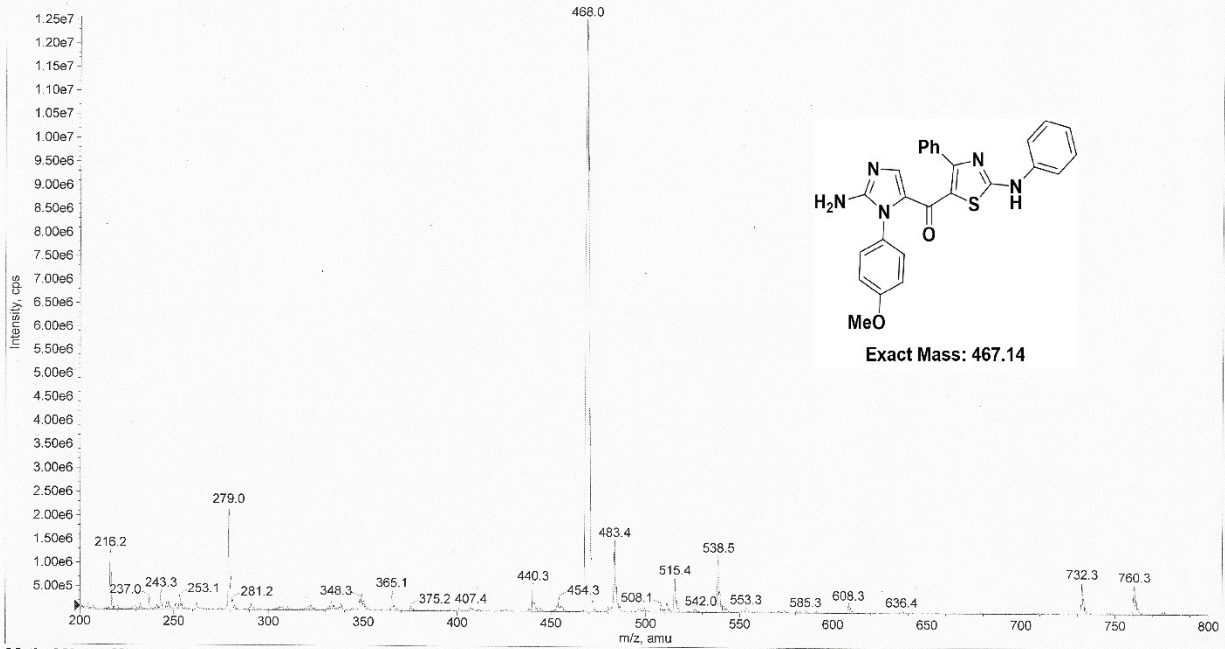
Exact Mass: 467.14

+Q1: 0.318 min from Sample 1 (PMC APIT8) of PMC APIT8.wiff (Turbo Spray)

Max. 6.8e6 cps.

Exact Mass: 461.15