Discovery of the first A_1 adenosine receptor ligand based on chromone scaffold

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Supplementary information

A-Chemistry

General

Chromone carboxylic acids, aniline derivatives, phosphorus (V) oxychloride, benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), *N*,*N*-diisopropylethylamine (DIPEA), dimethylformamide (DMF) and its derivatives were purchased from Sigma-Aldrich Química S.A. (Sintra, Portugal). All other reagents and solvents were *pro analysis* grade and

were acquired from LABCHEM (Lisbon, Portugal), Sigma-Aldrich Química S.A. (Sintra, Portugal) and used without additional purification.

Thin-layer chromatography (TLC) was carried out on pre-coated silica gel 60 F254 (Merck) with layer thickness of 0.2 mm. For analytical control the following systems were used: ethyl acetate/petroleum ether, ethyl acetate/methanol, chloroform/methanol in several proportions. The spots were visualized under UV detection (254 and 366 nm). Flash column chromatography was performed using silica gel 60 (0.040-0.063 mm) (Carlo Erba Reactifs – SDS, France). Following the workup and after extraction, the organic phases were dried over anhydrous sodium sulfate and solvents evaporated under reduced pressure in a Buchi Rotavapor.

Microwave-assisted synthesis was performed in a Biotage® Initiator Microwave Synthesizer.

^{1}H and ^{13}C spectra NMR spectra were acquired at room temperature and recorded on a Bruker Avance III operating at 400 and 100 MHz, respectively. Chemical shifts were expressed in δ (ppm) values relative to tetramethylsilane (TMS) as internal reference; coupling constants (J) were given in Hz. Assignments were also made from DEPT (distortionless enhancement by polarization transfer) (underlined values).

Synthetic procedures for obtaining chromone phenylcarboxamides

Method A: To a solution of chromone-2-carboxylic acid (1 g, 5.26 mmol) in DMF (12 mL) at 4ºC was added *N*,*N*-diisopropylethylamine (0.92 ml, 5.26 mmol) and a solution of PyBOP (2.73 mg, 5.26 mmol) in CH_2Cl_2 (12 mL). The mixture was kept in an ice bath and stirred for half hour. After this period, the corresponding aniline (5.26 mmol) was added and the mixture was allowed to warm up to room temperature. The reaction was kept with stirring for 4 hours. Upon completion, the mixture was diluted with dichloromethane (20 mL), washed with H_2O (2X10 mL) and with saturated $NaHCO_3$ solution (2X10 mL). The organic phase was dried with Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography or recrystallization.

***N*-(4-nitrophenyl)-4-oxo-4*H*-chromene-2-carboxamide (2)** the synthetic procedure and structural elucidation data was described elsewhere [1]

***N*-(4-bromophenyl)-4-oxo-4*H*-chromene-2-carboxamide (5)** the synthetic procedure and structural elucidation data was described elsewhere [2]

***N*-(*p*-tolyl)-4-oxo-4*H*-chromene-2-carboxamide (8)** **T**he synthetic procedure and structural elucidation data was described elsewhere [3]

***N*-(*m*-tolyl)-4-oxo-4*H*-chromene-2-carboxamide (9)**

Yield: 76%. ^{1}H-NMR (400 MHz, DMSO) δ = 2.34 (3H, *s*, CH_3), 6.97 (1H, *s*, H3), 7.03 (1H, *d*, J = 7.8, H4'), 7.31 (1H, *dd*, J = 8.0, 7.8, H5'), 7.57 (1H, *ddd*, J = 8.0, 7.1, 1.1, H6), 7.61-7.64 (2H, *m*, H2', H6'), 7.85 (1H, *d*, J = 8.5, H8), 7.94 (1H, *ddd*, J = 8.6, 7.0, 1.6, H7), 8.09 (1H, *dd*, J = 8.0, 1.4, H5), 10.68 (1H, *s*, CONH). ^{13}C-NMR (100 MHz, DMSO) δ = 21.1 (CH_3), 111.0 (C3), 118.2 (C6'), 119.0 (C8), 121.6 (C4'), 123.7 (C4a), 124.9 (C6), 125.6 (C2'), 126.1 (C5), 128.6 (C5'), 135.0 (C7), 137.4 (C3'), 138.1 (C1'), 155.1 (C8a), 155.7 (C2), 157.6 (CONH), 177.3 C(4). EI/ME m/z (%): 280 ($M^{+\bullet}$+1, 4), 279 ($M^{+\bullet}$, 31), 278 (100), 250 (25), 223 (2), (130 (4), 106 (2), 89 (3).

***N*-(*o*-tolyl)- 4-oxo-4*H*-chromene-2-carboxamide (10)**

Yield: 38%. ^{1}H-NMR (400 MHz, DMSO) δ = 2.28 (3H, *s*, CH_3), 6.97 (1H, *s*, H3), 7.41-7.23 (4H, *m*, H3', H4', H5', H6'), 7.57 (1H, *ddd*, J = 8.0, 7.1, 1.0, H6), 7.86 (1H, *d*, J = 8.0, H8), 7,93 (1H, *ddd*, J = 8.6. 7.1, 1.6, H7), 8.10 (1H, *dd*, J = 8.0, 1.6, H5), 10.53 (1H, *s*, CONH). ^{13}C-NMR (100 MHz, DMSO) δ = 19.2 (CH_3), 112.4 (C3), 120.4 (C8), 125.1 (C4a), 126.3 (C6), 127.5 (C6'), 127.6 (C5), 127.9 (C5'), 128.3 (C4'), 131.9 (C3'), 135.3 (C2'), 136.2 (C1'), 136.5 (C7), 156.5 (C8a), 157.0 (C2), 159.3 (CONH), 178.7 (C4). EI/ME m/z (%): 280 ($M^{+\bullet}$+1, 22), 279 ($M^{+\bullet}$, 100), 278 (88), 278 (19), 262 (16), 250 (23), 159 (14), 146 (11), 145 (11), 134 (15), 131 (12), 130 (16), 121 (30), 107 (15), 106 (73), 89 (53), 77 (20).

***N*-(4-Methoxyphenyl)-4-oxo-4*H*-benzopyran-2-carboxamide (11)** the synthetic procedure and structural elucidation data was described elsewhere [2]

Method B: To a solution of chromone-2-carboxylic acid (0.5 g, 2.63 mmol) in DMF (4 mL), $POCl_3$ (241 μl, 2.6 mmol) was added. The mixture was stirred at room temperature for 30 min, with *in situ* formation of the corresponding acyl chloride. Then the corresponding aniline (2.63 mmol) was added. The system was heated at 120 °C for 5 min in a microwave apparatus. Upon

completion, the mixture was diluted with dichloromethane (20 mL), washed with H_2O (2X10 mL) and with saturated $NaHCO_3$ solution (2X10 mL). The organic phase was dried with Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography or recrystallization.

***N*-(3-nitrophenyl)-4-oxo-4*H*-chromene-2-carboxamide (3)**

Yield: 38%. ^{1}H-NMR (400 MHz, DMSO) δ = 7.03 (1H, *s*, H3), 7.58 (1H, *ddd*, J = 8.1, 7.2, 1.1 Hz, H6), 7.73 (1H, dd, J = 8.2, 8.2 Hz, H5'), 7.85 (1H, dd, J = 8.5, 0.7 Hz, H8), 7.95 (1H, *ddd*, J = 8.7, 7.1, 1.7 Hz, H7), 8.06 (1H, *ddd*, J = 8.2, 2.3, 0.9 Hz, H4'), 8.09 (1H, *dd*, J = 8.0, 1.5 Hz, H6'), 8.26 (1H, *dd*, J = 8.0, 1.6 Hz, H5), 8.78 (1H, *dd*, J = 2.2 Hz, H2'), 11.16 (1H, *s*,NH). ^{13}C-NMR (100 MHz, DMSO) δ = 111.5 (C2'), 115.3 (C3), 119.0 (C8), 119.5 (C6'), 123.8 (C4a), 125.1 (C6), 126.3 (C5), 127.0 (C4'), 130.4 (C5'), 135.3 (C7), 138.8 (C1'), 148.0 (C3'), 155.1 (C8a), 155.2 (C2), 158.4 (CONH), 177.4 (C4). EI/ME m/z (%): 310 ($M^{+\bullet}$,63), 309 (61), 263 (32), 173 (51), 145 (56), 89 (100).

***N*-(2-nitrophenyl)-4-oxo-4*H*-chromene-2-carboxamide (4)**

Yield: 60%. ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.29 (1H, *s*, H3), 7.34 (1H, *ddd*, J = 8.5, 7.3, 1.3 Hz, H6), 7.51 (1H, *ddd*, J = 8.1, 7.1, 1.1 Hz, H4'), 7.72 (1H, dd, J = 8.5, 0.7 Hz, H8), 7.86 – 7.75 (2H, *m*, H7, H5'), 8.25 (1H, *dd*, J = 8.0, 1.4 Hz, H5), 8.35 (1H, *dd*, J = 8.5, 1.5 Hz, H3'), 8.99 (1H, *dd*, J = 8.5, 1.3 Hz, H6'), 12.17 (1H, *s*, NH). ^{13}C-NMR (100 MHz, DMSO) δ = 113.2 (C3), 118.6 (C8), 122.2 (C6'), 124.4 (C4a), 124.8 (C4'), 126.2 (C6), 126.3 (C3'), 126.5 (C5'), 133.8 (C1'), 135.1 (C7), 136.6 (C5'), 136.9 (C2'), 153.9 (C8a), 155.3 (C2), 157.9 (CONH), 178.1 (C4). EI/ME m/z (%): 310 ($M^{+\bullet}$,52), 309 (26), 265 (23), 264 (100), 145 (22), 89 (57).

***N*-(3-bromophenyl)-4-oxo-4*H*-chromene-2-carboxamide (6)** the synthetic procedure and structural elucidation data was described elsewhere [2]

***N*-(2-bromophenyl)-4-oxo-4*H*-chromene-2-carboxamide (7)** the synthetic procedure and structural elucidation data was described elsewhere [2]

***N*-(3-methoxyphenyl)-4-oxo-4*H*-chromene-2-carboxamide (12)** the synthetic procedure and structural elucidation data was described elsewhere [2]

***N*-(2-methoxyphenyl)-4-oxo-4*H*-chromene-2-carboxamide (13)** the synthetic procedure and structural elucidation data was described elsewhere [2]

***N*-(2-nitrophenyl)-4-oxo-4*H*-chromene-3-carboxamide (14)** This compound was obtained with a slight variation of method B: To a solution of chromone-2-carboxylic acid (0.5 g, 2.63 mmol) in DMF (4 mL), $POCl_3$ (241 μl, 2.6 mmol) was added. The mixture was stirred at room temperature for 30 min, with *in situ* formation of the corresponding acyl chloride. Then the 2-nitroaniline (0.4 g, 2.63 mmol) was added and the system was stirred for 5 hours. Upon completion, the mixture was diluted with dichloromethane (40 mL), washed with H_2O (2X10 mL) and with saturated $NaHCO_3$ solution (2X10 mL). The organic phase was dried with Na_2SO_4, filtered, concentrated under reduced pressure and the residue was washed with Et_2O and MeOH. Yield: 60%. ^{1}H-NMR (400 MHz, DMSO) δ = 7.39 (1H, *ddd*, J = 8.5, 7.3, 1.3 Hz, H4'). 7.66 (1H, *ddd*, J = 8.0, 7.2, 1.1 Hz, H6), 7.85 – 7.77 (2H, m, H8, H7), 7.95 (1H, ddd, *J* = 8.7, 7.3, 1.7 Hz, H5'), 8.17 (1H, *dd*, J = 8.5, 1.5 Hz, H3'), 8.28 (1H, dd, *J* = 8.0, 1.4 Hz, H5), 8.55 (1H, *dd*, J = 8.7, 1.3 Hz, H6'), 9.24 (1H, *s*, H2), 12.49 (1H, s, NH). ^{13}C-NMR (100 MHz, DMSO) δ = 115.1 (C3), 118.8 (C8), 123.5 (C4a), 124.3 (C6'), 124.9 (C6), 125.6 (C4'), 125.7 (C3'), 127.0 (C5), 132.3 (C1'), 135.0 (C5'), 135.6 (C7), 139.4 (C2'), 155.7 (C8a), 161.5 (CONH), 164.6 (C2), 176.4 (C4). EI/ME m/z (%): 310 ($M^{+\bullet}$,33), 265 (38), 264 (100), 174 (41), 173 (92), 121 (81).

B-Pharmacology

Radioligand binding Assays

CHO Membrane Preparation. All methods for membrane preparation for radioligand binding experiments followed the procedures described earlier[4]. Membranes for radioligand binding were prepared from cells stably transfected with the human adenosine receptor subtypes (A_1, A_{2A}, and A_3 expressed on CHO cells) in a two-step procedure. In the first low-speed step (1,000g for 4 min), the cell fragments and nuclei were removed. The crude membrane fraction was sedimented from the supernatant at 100,000g for 30 min. The membrane pellet was then resuspended in the specific buffer used for the respective binding experiments, frozen in liquid nitrogen, and stored at -80 ºC. For the measurement of the adenylyl cyclase activity stimulated by activation of A_{2B} receptors expressed in CHO cells, only one step of centrifugation was used in which the

homogenate was sedimented for 30 min at 54,000g. The resulting crude membrane pellet was resuspended in 50 mM Tris/HCl, pH 7.4 and immediately used for the adenylyl cyclase assay.

Human Cloned A_1, A_{2A}, A_3 Adenosine Receptor Binding Assay. Binding of [^{3}H]CCPA to CHO cells transfected with the human recombinant A_1 adenosine receptor was performed as previously described[4]. Competition experiments were performed for 3h at 25˚C in 200 µL of buffer containing 1 nM [^{3}H] CCPA, 0.2 U/mL adenosine deaminase, 20 µg of membrane protein in 50 mM Tris/HCl, pH 7.4 and tested compound in different concentrations. Nonspecific binding was determined in the presence of 1 mM theophylline. Binding of [^{3}H]NECA to CHO cells transfected with the human recombinant A_{2A} adenosine receptor was performed following the conditions as described for the A_1 receptor binding[4]. In the competition experiments, samples containing a protein concentration of 50 µg, 10 nM [^{3}H]NECA and tested compound in different concentrations were incubated for 3h at 25°C. Nonspecific binding was determined in the presence of 100 µM R-PIA (R-*N*^{6}-phenylisopropyladenosine). Binding of [^{3}H]HEMADO to CHO cells transfected with the human recombinant A_3 adenosine receptors was carried out as previously described[5]. The competition experiments were performed for 3h at 25 °C in buffer solution containing 1 nM [^{3}H]HEMADO, 20 µg membrane protein in 50mM Tris-HCl, 1 mM EDTA (ethylenediaminotetraacetate), 10 mM $MgCl_2$, pH 8.25 and tested compound in different concentrations. Nonspecific binding was determined in the presence of 100 µM R-PIA.

Adenylyl Cyclase Activity. Because of the lack of a suitable radioligand for a *h*A_{2B} receptor binding assay, the potency of antagonists at A_{2B} receptor (expressed on CHO cells) was determined in adenylyl cyclase experiments instead. The procedure was carried out as described previously with minor modifications[6]. Membranes were incubated with about 150000 cpm of [α-^{32}P]ATP for 20 min in the incubation mixture as described,[4] without EGTA and NaCl. IC_{50} values for concentration-dependent inhibition of NECA-stimulated adenylyl cyclase caused by antagonists were calculated with the Hill equation. Hill coefficients in all experiments were near unity. Dissociation constants (K_i) for antagonist were then calculated with the Cheng and Prusoff equation[7]

C. X-Ray structural analysis

Although *N*-(2-nitrophenyl)-4-oxo-4*H*-chromene-2-carboxamide, **4,** CCDC-1413102, Cambridge Structural Database[7] was previously published[8] a new structural determination using a crystal

from a different batch was carried out. In this new determination CuKα radiation was used as opposed to MoKα radiation which was used in the original determination[8]. The structure is the same although the accuracy of the bond lengths and angles has been improved.

The main feature of the molecular structure of this compound, as reported in the above paper, is the formation of three intramolecular hydrogen bonds: 1) between the amino H and the O of the pyran ring, 2) between the amino H and one of the *ortho* nitro group O atoms and 3) between the *ortho* C-H group and the amide carbonyl O atom forming pseudo 6-membered, 5-membered and 6-membered rings respectively. In the present structure the angle between the 10 atoms of the chromone ring and the exocyclic benzene ring is 6.11(6)º and the nitro group is twisted out of the plane of the exocyclic benzene ring by 15.05(16)ºThe structure of *N*-(2-nitrophenyl)-4-oxo-4*H*-chromene-3-carboxamide, **14,** is discussed by Gomes *et al.*[9]

D-Molecular Modeling

Homology models of *h*A1

Construction of homogoly models was previously published by our research group[10]. The models of hA1 were developed with the help of MOE software[11]. As described previously, we used the crystallized structure of the hA2A adenosine receptor (PDB code: 3EML)[12] as a template to construct our homology modeling. The alignment between our template (3EML) and the hA1 protein was performed the same manner as Katritch *et al.*[13] in a previous publication with good results in modeling construction, and taking into account highly conserved residues in the transmembrane helices (TMs). In the models generation, if the residue is identical, all heavy atom coordinates are copied from the template to the new target; if the residue is different, only the backbone is considered. Residues in loops with no assigned coordinates were constructed with the loop construction method implemented in MOE considering compatible high resolution fragments extracted from the Protein Data Bank. Loops selection is made according to a Boltzmann-weighted function and side chains are assembled using a rotamer library from the PDB. Best model scored with the Generalized Born/Volume Integral (GB/VI) method was selected. Posteriorly, we evaluated the model geometry quality assessing Ramachandran plots, bond lengths, bond angles, dihedrals, sidechain rotamers and non-bonded contacts though the Protein Geometry module. Some disulfide bridges conserved between receptors, such as the disulfide bridge between Cys169 and Cys80 in the hA1, were manually assessed. The protein

pocket in hA_1 was optimized through the Induce Fit Docking protocol[14]. Though this procedure we docked with Glide SP (Standard Precision) a high affinity ligand to the sub-type model and optimized the pocket side chains with Prime[14]. Different protein models for hA_1 were extracted from the Induce Fit Docking. For the final selection of the homology modeling, we assessed the models in a similar manner as Katritch *et al.*[13], measuring: 1) the ability to differentiate between decoys and true ligands and 2) the ability to discriminate between sub-type high affinity selective ligands. The best model for hA1 yielded in both tests areas under the receiver operating characteristic (ROC) curves greater than 0.80. This model was selected as starting point for docking simulations.

Molecular docking of adenosine receptors

Molecular docking simulations were carried out with Schrödinger package[14]. Ligands database was prepared with the help of LigPrep module that generates tautomers, different protonation states (pH=7.0 ± 2.0) and initially optimizes the geometry of the molecular structures. Docking simulations were carried out using the hA1 homology model. A receptor grid was calculated and centered in the protein pocket with a van der Waals radius scaling factor of 1.0 and a partial atomic charge cut-off of 0.25. The compounds were docked to the protein through Glide SP mode (standard precision)[14]. We collected ten poses for each ligand. SP docking score was used to select the pose representative of the calculations. Final ligand binding modes along with the protein pocket (5 Å from the ligand) were optimized using MM-GBSA in Prime[14].

RMSD between conformational analysis, X-ray and docking conformations

The most stable conformations for compounds **2-4** in vacuum were calculated through conformational analysis using Macromodel[14]. The force field used in the calculations was OPLS_2005. Non-bonded cut-off distances were 4.0, 7.0 and 12.0 Å for H-bond, *van der Waals* and electrostatic interactions respectively. The minimization method was Polak-Ribiere Conjugate Gradient with a maximum of 2,500 iterations and a gradient convergence threshold of 0.05. The engine search methodology was Monte Carlo Multiple Minimum (MCMM). The global minimum potential energy structures were retained as representative of the calculation.
The RMSD (root mean square deviation) between the poses extracted from the hA_1 docking and the minimum energy conformations determined through MCMM are 0.12, 2.05 and 1.27 Å for

compounds **4**, **3** and **2**, respectively. On the other hand, the RMSD between the XRay conformation for compound **4** and the MCMM and hA_1 docking conformations were 0.67 and 0.53 Å respectively.

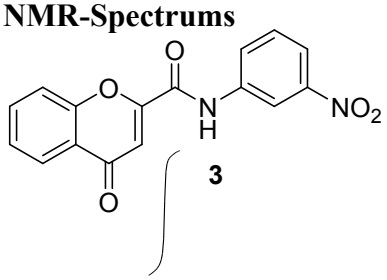
E- Evaluation of drug-like properties

Calculation drug physicochemical properties were performed using the Molinspiration Cheminformatics® [http://www.molinspiration.com].

F- Evaluation of compound´s purity

The purity of the final products (>97% purity) was verified by high-performance liquid chromatography (HPLC) equipped with a UV detector. Chromatograms were obtained in an HPLC/DAD system, a Jasco instrument (pumps model 880-PU and solvent mixing model 880-30, Tokyo, Japan), equipped with a commercially prepacked Nucleosil RP-18 analytical column (250 mm x 4.6 mm, 5 μm, Macherey-Nagel, Duren, Germany), and UV detection (Jasco model 875-UV) at the maximum wavelength of 254 nm. The mobile phase consisted of a methanol/water or acetonitrile/water (gradient mode, room temperature) at a flow rate of 1 mL/min. The chromatographic data was processed in a Compaq computer, fitted with CSW 1.7 software (DataApex, Czech Republic).

NMR-Spectrums



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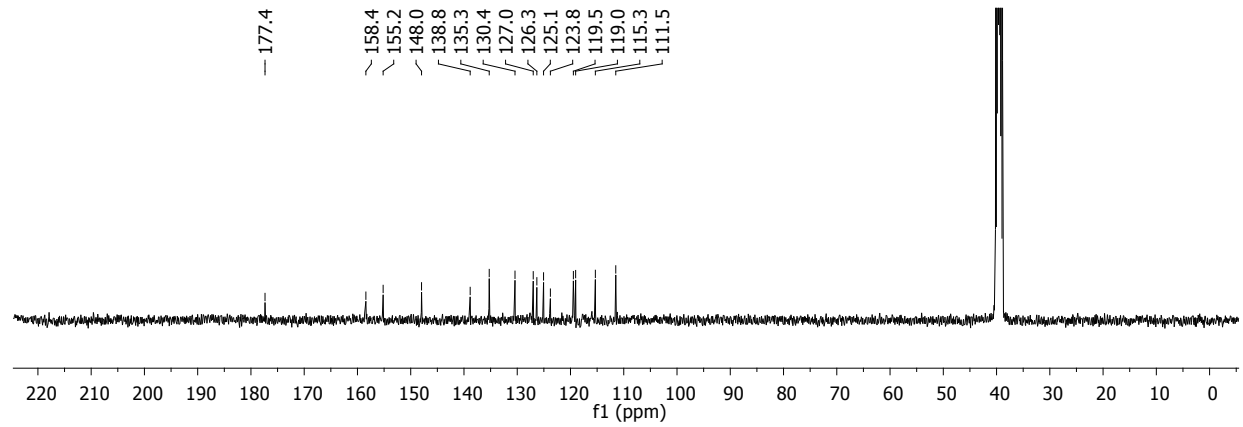

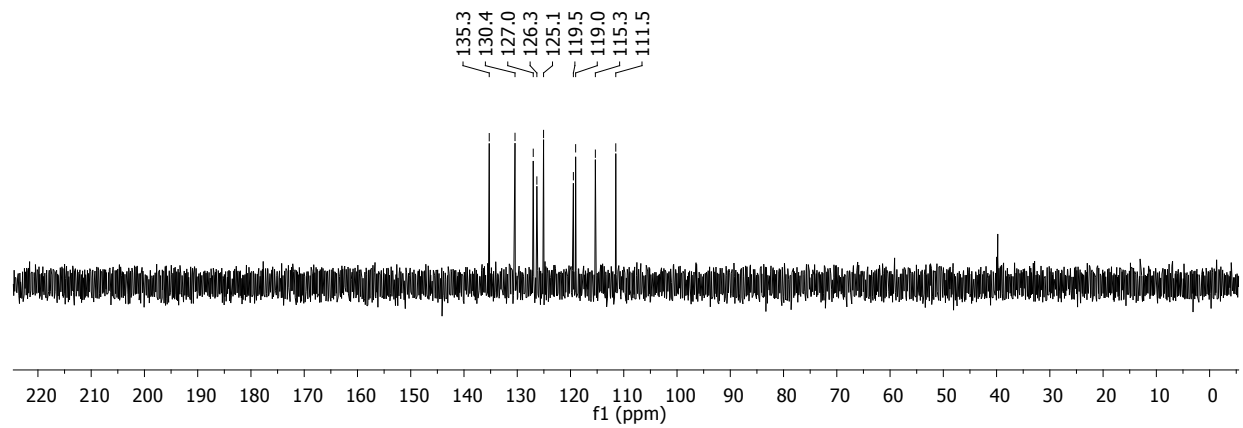

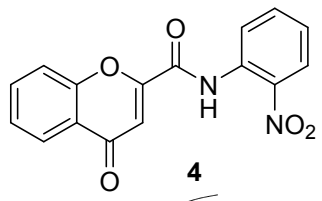
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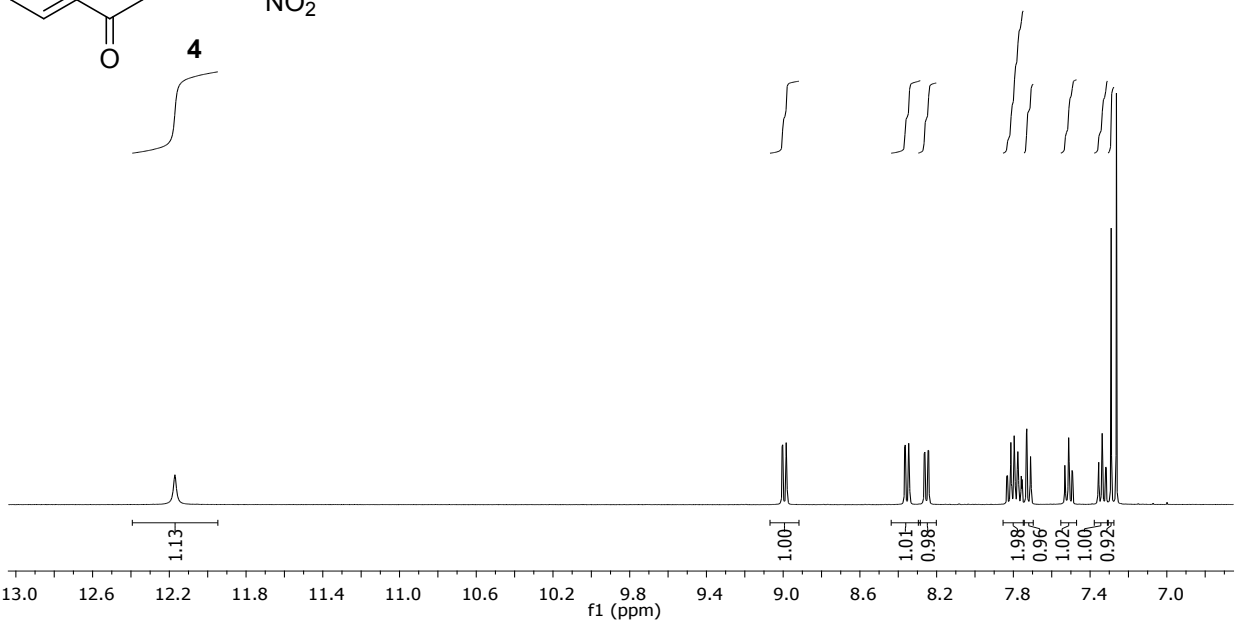
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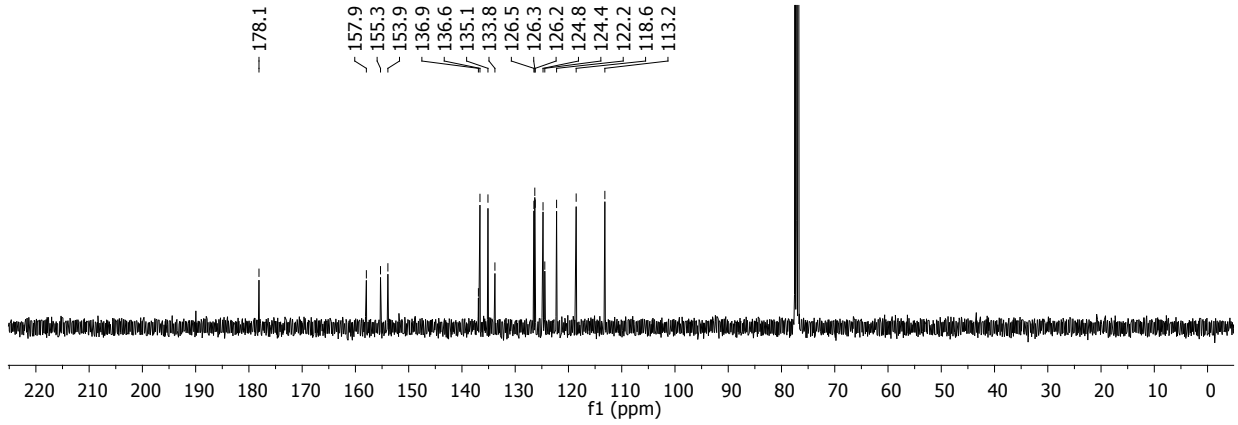
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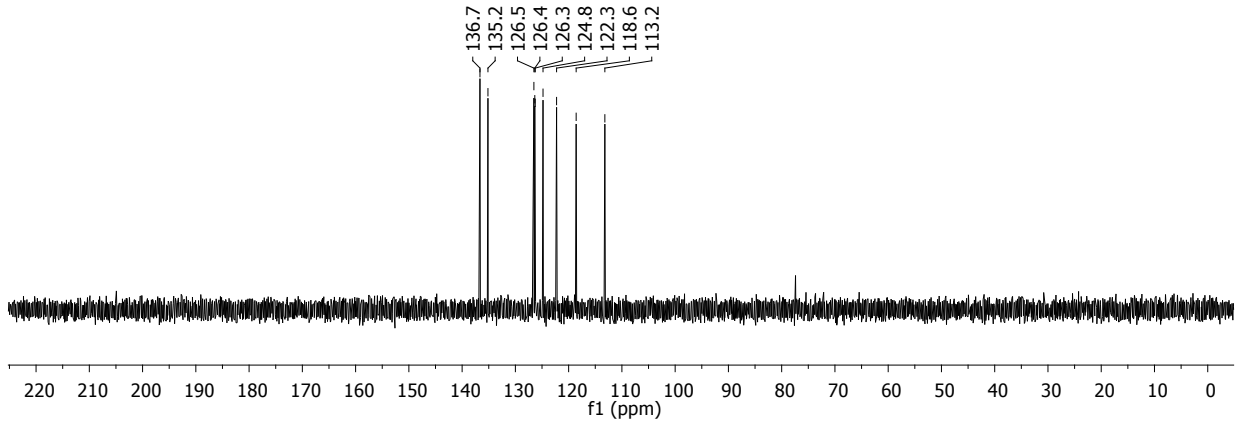
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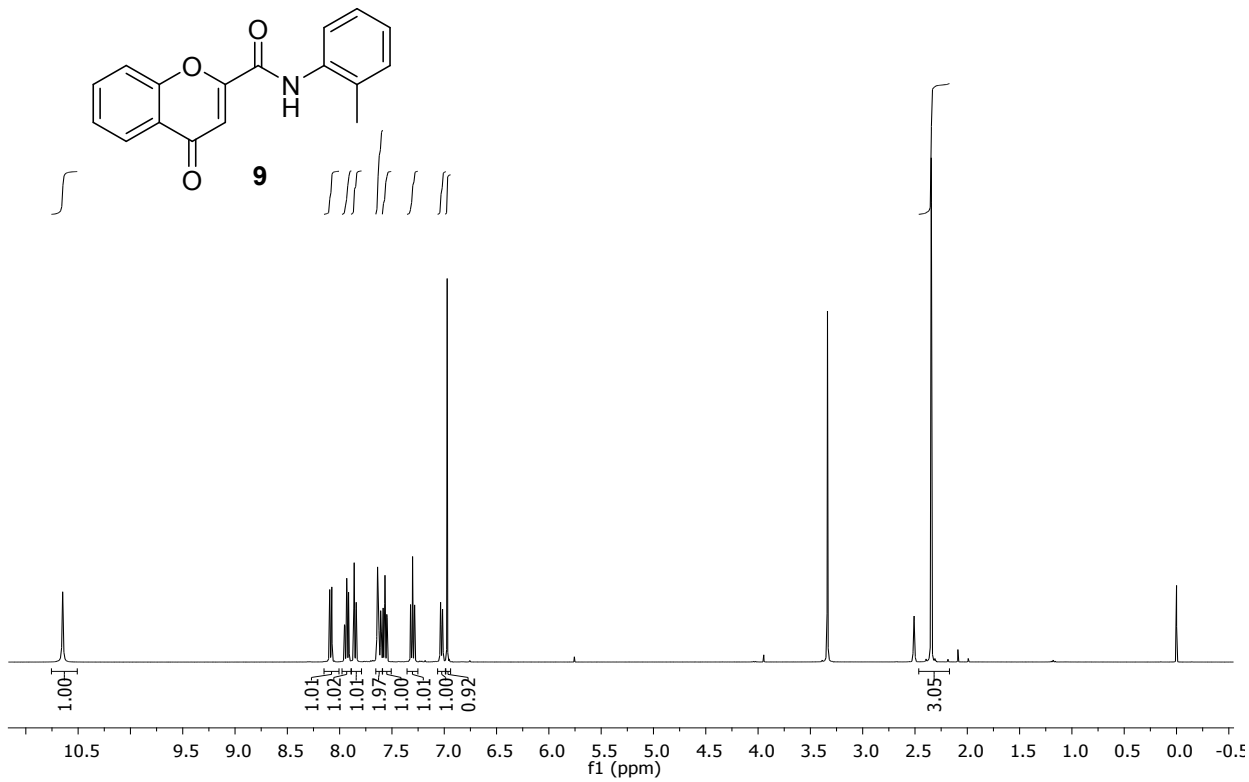
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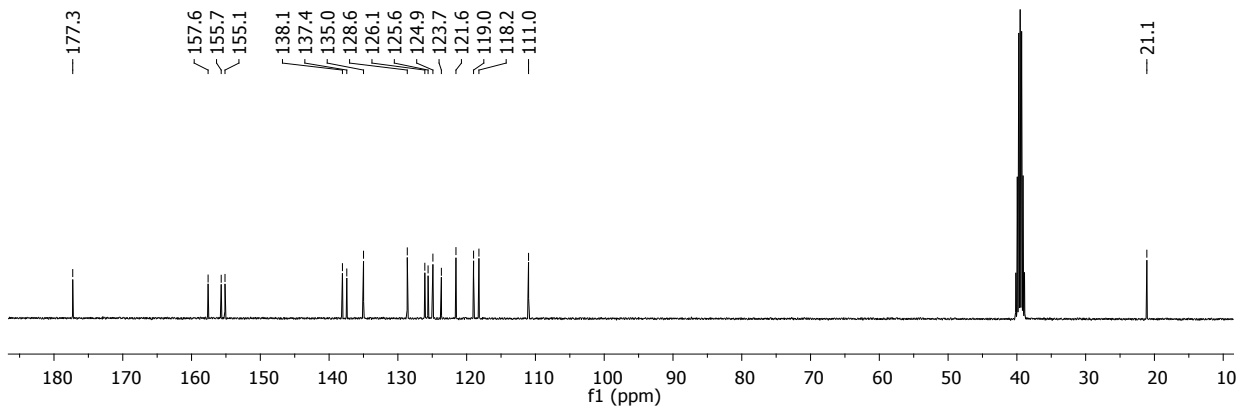
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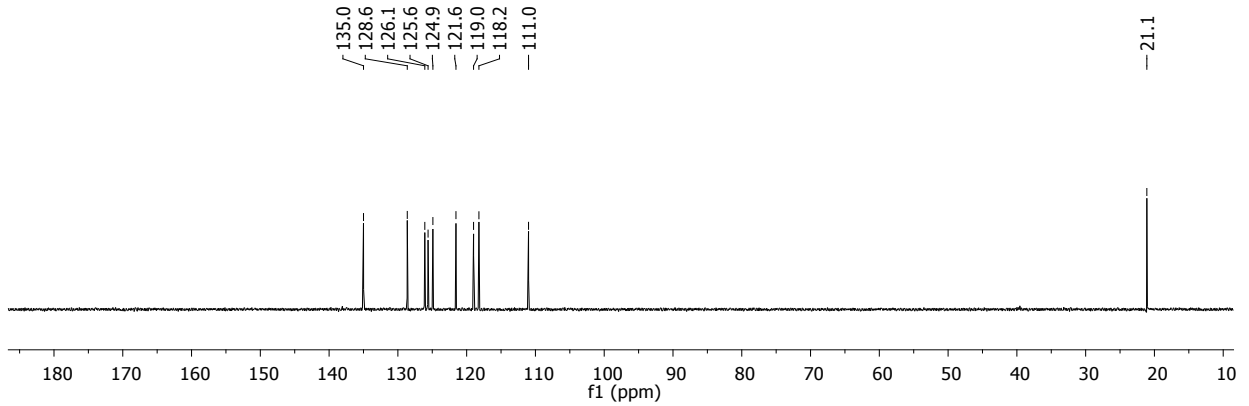
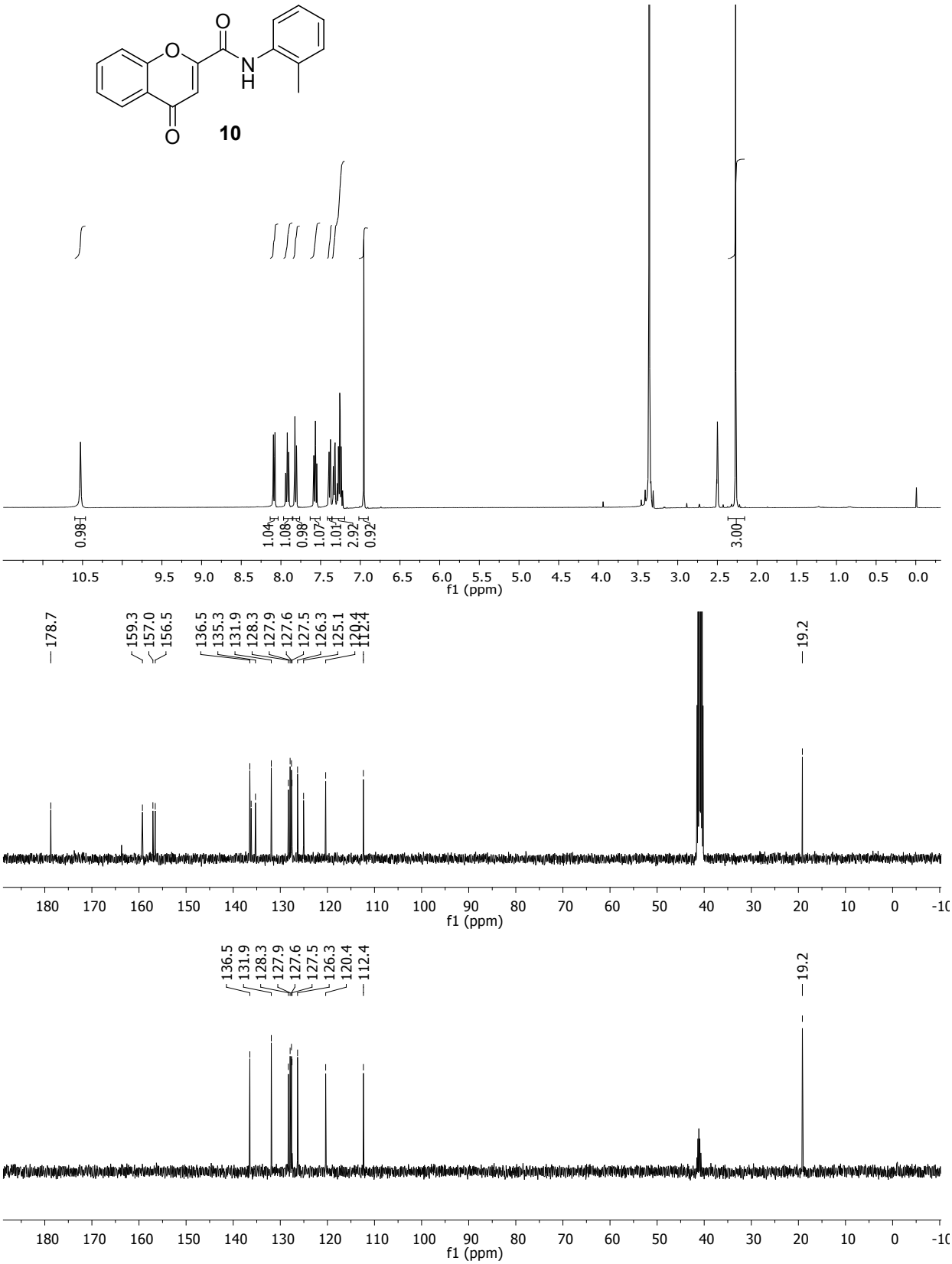
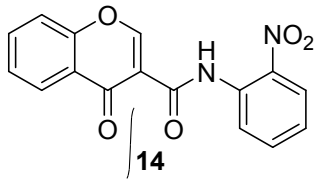
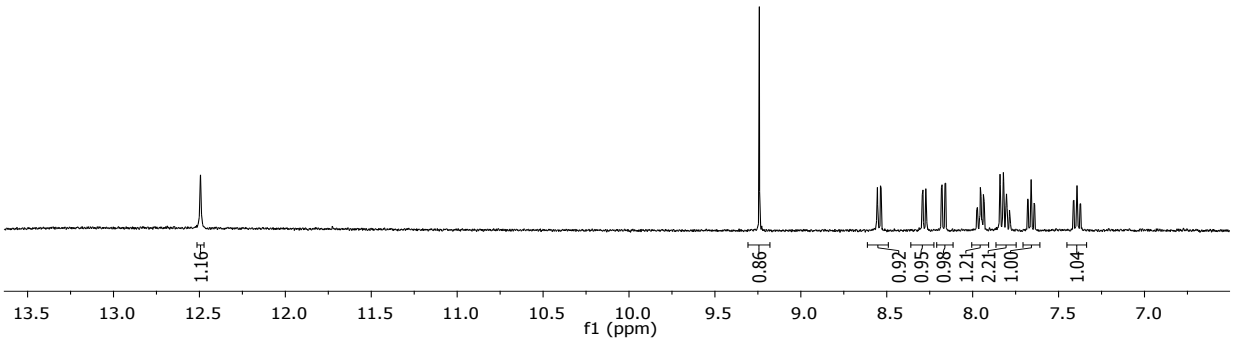
119.0
118.2
111.0
21.1
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

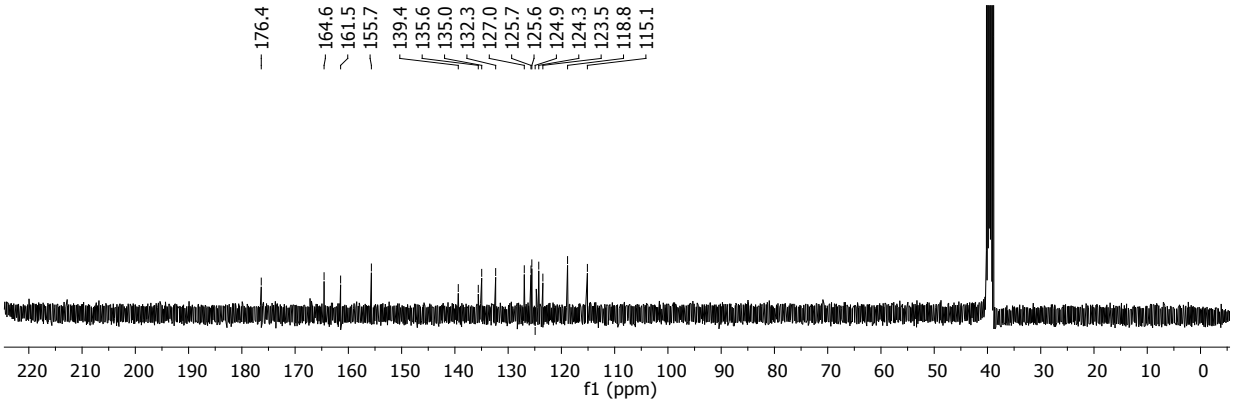

135.0
128.6
126.1
125.6
124.9
121.6
119.0
118.2
111.0
21.1
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

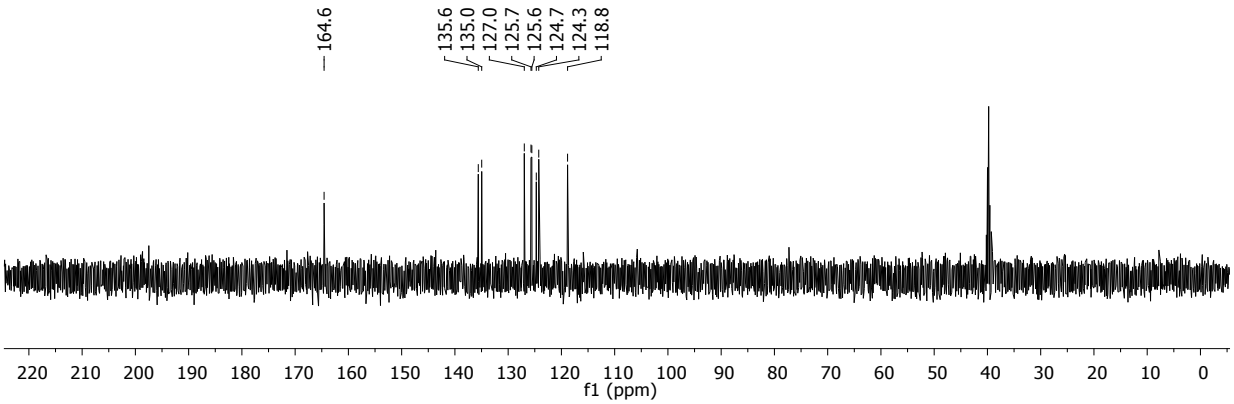

O
O
N
H
O
10
0.98
1.04
1.08
0.98
1.07
1.01
2.92
0.92
3.00
10.5 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)
178.7
159.3
157.0
156.5
136.5
135.3
131.9
128.3
127.9
127.6
127.5
126.3
125.1
120.4
112.4
19.2
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)
136.5
131.9
128.3
127.9
127.6
127.5
126.3
120.4
112.4
19.2
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


O
H
N
NO2
O
O

14


1.16
0.86
0.92
0.95
0.98
1.21
2.21
1.00
1.04
13.5 13.0 12.5 12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0
f1 (ppm)


176.4
164.6
161.5
155.7
139.4
135.6
135.0
132.3
127.0
125.7
125.6
124.9
124.3
123.5
118.8
115.1
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


164.6
135.6
135.0
127.0
125.7
125.6
124.7
124.3
118.8
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

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