Supporting Information

Synthesis of novel tricyclic pyrazolo(1,4)oxathiinopyrazines and evaluation of their competency towards the inhibition of lactate dehydrogenase activity

Authors

Prasun Mukherjee[1], Asish R. Das*[1], Raghwendra Mishra[2], Shovonlal Bhowmick[3], Achintya Saha[3]

Affiliations

1 Department of Chemistry, University of Calcutta, Kolkata-700009, India

2 Department of Physiology, University of Calcutta, Kolkata-700009, India

3 Department of Chemical Technology, University of Calcutta, Kolkata-700009, India

Correspondence

Prof. Asish R. Das

Department of Chemistry

University of Calcutta

92-A.P.C. road

700009 Kolkata

India

Tel.: +913323501014, +919433120265; fax: +913323519754

Email: ardchem@caluniv.ac.in , ardas66@rediffmail.com

I. Materials and method:

^{1}H-NMR and ^{13}C-NMR spectral analysis were carried out on 300 MHz, 75 MHz instruments where tetramethylsilane (TMS) was used as internal standard. Infrared spectra were recorded in KBr pallets in reflection mode on a FTIR spectrophotometer. High Resolution Mass Spectra were obtained using a mass spectrometer. Suitable single crystals of compound **3c** was mounted on an X-ray diffractometer equipped with a graphite monochromator. All the reactions were monitored by thin layer chromatography carried out on aluminum-blocked silica gel plates coated with silica gel G under UV light and also by exposure to iodine vapor for detection. Melting points were recorded on a Köfler Block apparatus and are uncorrected. Synthetic grade chemicals from available companies were used for carrying out the organic reactions. For column chromatography 100-200 mesh silica gel was used. All the organic solvents, used in the reaction, were appropriately dried and distilled prior to use.

Experimental Section

General Procedure for the Synthesis of Pyrazolone Derivatives:

Sodium acetate (328 mg, 4.0 mmol) was added in a suspension of aromatic hydrazine hydrochloride derivatives (4.0 mmol) in 5 ml of EtOH and 1 ml of water, and the mixture was stirred at rt for 5 min. Then, to the mixture ethyl acetoacetate (521 mg, 4.0 mmol) was added, and the resultant mixture was heated to reflux for 3 h. After that, the mixture was poured dropwise onto crushed ice (50 g) with vigorous stirring, and the resulting precipitate was then filtered off and crystallized from EtOH. These pyrazolone derivatives were then employed for the synthesis of pyrazolo[4',3':5,6][1,4]oxathiino[2,3-b]pyrazine derivatives without further purification.

General Procedure for the Synthesis of pyrazolo[4',3':5,6][1,4]oxathiino[2,3-b]pyrazines (3a-c) from 2,3-dichloropyrazine:

In a 25 ml RB flask fitted with a condenser, were added pyrazolone derivatives (1.5 mmol), elemental sulfur (1.3 equiv, 62 mg), triethylamine (1 drop) and 2 ml ethanol. The resulting mixture was then heated to reflux for 40 min. After that the volatiles were removed under vacuo followed by the addition of 2,3-dichloropyrazine (1.0 mmol, 149 mg), Cs_2CO_3 (2.2 equiv) and DMSO (2 ml). The resulting mixture was then stirred at 80 ^oC for 1 h. After completion of the reaction (monitored by TLC) the mixture was cooled to rt and quenched by addition of H_2O followed by the extraction with EtOAc (3 × 10 ml). The combined organic layers were washed with brine and dried over anhydrous Na_2SO_4. The solvent was then removed in vacuo and the residue was purified by column chromatography over silica gel (100-200 mesh) with ethyl acetate and petroleum ether (1:91:4, v/v) as the eluent to afford the pyrazolo[4',3':5,6][1,4]oxathiino[2,3-b]pyrazines (**3a-c**).

Determination of LDH inhibition and calculation of IC_{50} values

Preparation of LDH rich plasma

Peripheral blood was drawn from healthy volunteers (n=3, all male, age range of 22-26 years) from antecubital vein and collected in heparinized vials. The heparinized blood was then treated with sub-hemolytic dose of *Naja kaouthia* venom (2µg/ml) for 1 hour at room temperature as per standard protocol of our laboratory [1]. This dose-time incubation pair is characterized by morphological changes of erythrocyte accompanied with increased LDH leakage without any significant hemolysis. After the completion of incubation period plasma (rich in LDH activity) was separated from the treated blood by centrifugation (5000 rpm for 5 minutes at 4^oC).

Preparation of drug

The stock solutions of the drugs (**3a**, **3b** and **3c**) were made by dissolving 2mg of drug/ ml in absolute ethanol. The stock solution was then diluted 1:1 with distilled water to obtain a working concentration of 1mg/ml (1µg drug/µl).

LDH Assay

In order to evaluate the LDH inhibition potential of the newly synthesized drugs, 100 µl aliquots of the LDH rich plasma were incubated with the graded doses of the compounds (0, 2,4,8 and 16 µg) for 1 hour at room temperature. LDH activity of the different aliquots were then assessed by monitoring NADH absorbance at 340nm according to manufacturer's guideline using a Shimadzu spectrophotometer (Model UV-1800) with commercially available LDH (P-L) kit (Crest Biosystems, Coral Clinical Systems, India). All tests were blanked with equal volume of 1:1 ethanol:water solution to eliminate the effect of ethanol, if any.

Calculation of IC_{50} values

The LDH activity at different doses were expressed as the percent of control (0 µg dose) and plotted against respective doses. The linear fit equation thus obtained was used to determine IC_{50} value for each drug. Statistical analysis was performed using statistical program packages OriginPro 8 (OriginLab, Northampton, MA, US).

ADMET analysis

Using the QikProp tool of Schrödinger suite,[12] ADME properties of the three synthesized novel derivatives were predicted at the 95% level of significance. The QikProp computes over twenty bio-physical descriptors including evaluation of the acceptability of Lipinski's rule of five [2]. Prediction is based on comparing a molecule's properties with those of 95% of known drugs. All the compounds were further analyzed for toxicity studies. The

hepatotoxicity probability was predicted with the ADMET descriptors in Discovery Studio 2.5 (DS) [3]. The developmental toxicity potential was predicted using Toxicity Estimation Software Tool (TEST) (version 4.2) [4]. Before performing the ADMET prediction, the compound is subjected to reach its neutralized state for used in QikProp, DS and TEST.

Molecular docking

From the Protein Data Bank (PDB) accessible at www.rcsb.org [5], which is a repository for 3-dimensional (3D) structural data of large biological molecules, a high resolution X-ray crystallographic structure of LDH (PDB ID: 4ZVV) [6] was downloaded. Using protein preparation wizard in Schrödinger suite, the LDH protein was preprocessed before docking to determine its automatically optimal protonation state by addition of hydrogen atoms, removal of non-essential waters, creation of disulfide bonds, and filling of missing side chains and loops. Using OPLS2005 force field in Impact Refinement module (Impref) [7], a restrained minimization was performed to get the energy minimized structure. In order to get the correct Lewis structures, and also to eliminate and reduce downstream computational errors, LigPrep module [7] was used to generate accurate energy minimized 3D molecular structures of the synthesized novel derivatives. Molecular docking studies have been employed for the synthesized derivatives with target protein LDH, which was performed in Glide program using Maestro graphical user interface of Schrödinger suite [7]. The grid was generated using receptor grid generation panel. To specify the grid box, already attached ligand was picked, and grid centre was defined for the active site, and box sizes were also set by extending it to a combination of side chain and backbone amino acid residues. After successful grid generation, a standard precision (SP) docking method was executed in Glide module of Schrödinger suite [7].

Molecular dynamics

Using the Desmond module in Schrödinger (Desmond Molecular Dynamics System, and Maestro-Desmond Interoperability Tools, Schrödinger) [7], molecular dynamics (MD) simulation study was performed on the three interacting modeled of docked complexes. Using Desmond system builder program, the molecular system was solvated by predefined TIP3P (transferable intermolecular potential 3P) [8] solvent model of orthorhombic water box shape to ensure that the complete surface of each complex was properly enclosed by the solvent. The shape and size of the orthorhombic periodic boundary conditions unit buffered at 10 Å distances were specified. In order to maintain electric neutrality and equilibrated state of the system, essentials counter ions were added. The systems were minimized and pre-equilibrated before production of MD simulations, keeping the default relaxation program in Desmond. Minimization state of the system was reached with maximum 2000 iterations, employing steepest descent and LBFGS (limited-memory Broyden–Flecher–Goldfarb–Shanno) algorithm. The MD simulation run was performed at 40 ns for compound **1** bounded docked complex, and at 100 ns for other two studied docked complexes using a Nose-Hoover thermostat temperature of 300 K, and in 1.2 ps of Martyna-Tobias-Klein barostat bar pressure under the NPT (N = number of particle, P = system pressure, T = temperature) ensemble.

Pharmacophore model generation

The pharmacophore model was derived using HipHop algorithm in DS 2.5[3] to explore the common features among the biologically active synthesized compounds. HipHop, also recognized as a common-feature hypothesis holds up-to 11 different features that include hydrogen bond donor, hydrogen bond acceptor, ring aromatic, hydrogen bond acceptor lipid, hydrophobic, hydrophobic aromatic, hydrophobic aliphatic, negative ionizable, positive ionizable, negative charge, and positive charge for hypothesis generation within a three-dimensional (3D) space, which are essential for implying biological actions of compounds.

For building the pharmacophore model for the present work, 10 features were selected, except hydrogen bond acceptor lipid feature.

References

[1] Mishra R, Sarkar D, Bhattacharya S, Mallick S, Chakraborty M, Mukherjee D, Kar M, Mishra R. Quantifying morphological alteration of RBC population from light scattering data. Clinical Hemorheology and Microcirculation 2015; 59: 287-300

[2] Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Advanced drug delivery reviews 2001; 46: 3-26

[3] Discovery Studio 2.5, (2009), Accelrys Software Inc., San Diego.

[4] USEPA, T.E.S.T. (Toxicity Estimation Software Tool), (2016), http://www.epa.gov/ORD/NRMRL/std/cppb/qsar/index.html.

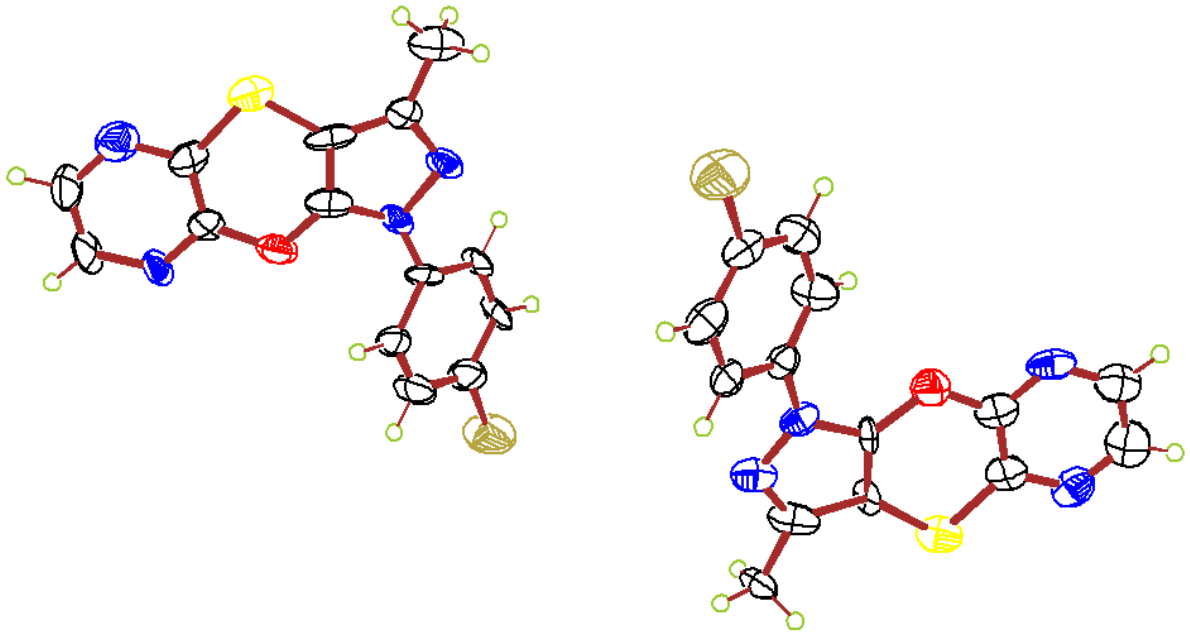
[5] Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE, The Protein Data Bank. Nucleic acids research, 2000; 28: 235-242

[6] Boudreau A, Purkey HE, Hitz A, Robarge K, Peterson D, Labadie S, Kwong M, Hong R, Gao M, Del Nagro C, Pusapati R, Ma S, Salphati L, Pang J, Zhou A, Lai T, Li Y, Chen Z, Wei B, Yen I, Sideris S, McCleland M, Firestein R, Corson L, Vanderbilt A, Williams S, Daemen A, Belvin M, Eigenbrot C, Jackson PK, Malek S, Hatzivassiliou G, Sampath D, Evangelista M, O'Brien T. Metabolic plasticity underpins innate and acquired resistance to LDHA inhibition, Nature chemical biology 2016; 12: 779-786

[7] Schrödinger, L. (2014) New York, USA. www.schrodinger.com.

[8] Mark P, Nilsson L. Structure and Dynamics of the TIP3P, SPC, and SPC/E Water Models at 298 K. The Journal of Physical Chemistry A, 2001; 105: 9954-9960

II. X-ray Crystallography Data of Compound 3c (CCDC 1828414):



The X-ray structure of **3c**. The ellipsoid contour percent probability level is 50%.

Single crystal X-ray data for compound 3c (CCDC 1828414):

Single crystals suitable for X-ray diffraction of **3c** were grown from ethyl acetate. The crystals were carefully chosen using a stereo zoom microscope supported by a rotatable polarizing stage. The data was collected at 296(2) K on a CCD diffractometer with graphite monochromated Mo-Kα radiation (0.71073Å). The data was processed using the package SAINT [1]. Structure was solved by direct and Fourier methods and refined by fullmatrix least squares based on F2 using SHELXTL [2] and SHELXL-97 [3] packages.

Table 1. Crystallographic data for the compound 3c

Compounds	**3c**
empirical formula	C_{14} H_9 Cl N_4 O S
fw	316.76
crystal system	Monoclinic

space group	*P 21/n*
a (Å)	6.877(3)
b(Å)	17.060(7)
c (Å)	23.960(10)
α (°)	90.00
β (°)	97.017(9)
γ (°)	90.00
V (Å^3)	2790(2)
Z	8
T, K	296(2)
Wavelength (Å)	0.71073
2θ (°)	2.94-50.62
μ (mm^{-1})	0.427
ρ_{calcd} (g cm^{-3})	1.508
F(000)	1296
absorption correction	multi-Scan
index ranges	$-8 \le h \le 8$
	$-18 \le k \le 20$
	$-28 \le l \le 27$
reflections collected	18393
independent reflections (R_{int})	4949 (0.1321)
Goodness-of-fit on F^2	1.073
$R_1{}^a$ /w$R_2{}^b$ ($I > 2\sigma(I)$)	0.1394 / 0.3295
$R_1{}^a$/w$R_2{}^b$ (for all data)	0.2219/ 0.3645
Largest diff. peak/hole / e Å^{-3}	0.629/ -0.472

$^a R_1 = [\sum||F_o| - |F_c||/\sum|F_o|]$. $^b wR_2 = [\sum w(F_o^2 - F_c^2)^2/\sum wF_o^4]^{1/2}$

References:

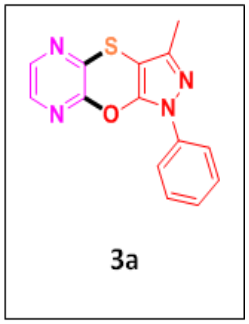
[1]. *APEX-II, SAINT-Plus,* and *TWINABS;* Bruker-Nonius AXS Inc.: Madison, WI, 2004.

[2] *SHELXTL,* version 6.10; Bruker AXS Inc.: Madison, WI, 2002.

[3] Sheldrick, G. M. *SHELXL-97, Crystal Structure Refinement Program;* University of Gö ttingen Gö ttingen Germany, 1997.

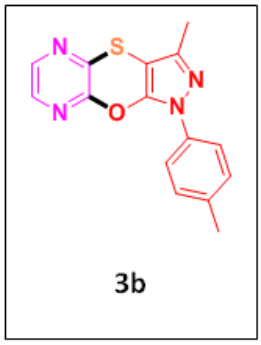
III. Characterization data for the products of 3a-c

3-methyl-1-phenyl-1H-pyrazolo[4',3':5,6][1,4]oxathiino[2,3-b]pyrazine 3a



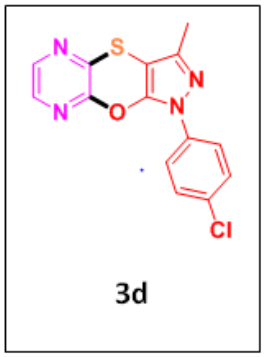
Off-white solid (232 mg, 82%); Mp: 150-152 ^{o}C; ^{1}H NMR (300 MHz; $CDCl_3$; Me_4Si): δ 2.12 (s, 3H), 7.23 (t, *J*=7.2 Hz, 1H), 7.38 (t, *J*=7.7 Hz, 2H), 7.62 (d, *J*=7.8 Hz, 1H), 7.78 (s, 1H), 8.03 (d, *J*=2.1 Hz, 1H), ^{13}C{^{1}H}NMR (75 MHz; $CDCl_3$; Me_4Si): δ 12.6, 87.2, 121.2, 121.3, 121.32, 121.4, 126.9, 129.2, 137.2, 138.1, 140.9, 142.6, 144.2, 152.2; HRMS (ESI-TOF) m/z Calcd for $[C_{14}H_{10}N_4OS +H]^+$: 283.0648, found: 283.0652.

3-methyl-1-(p-tolyl)-1H-pyrazolo[4',3':5,6][1,4]oxathiino[2,3-b]pyrazine 3b



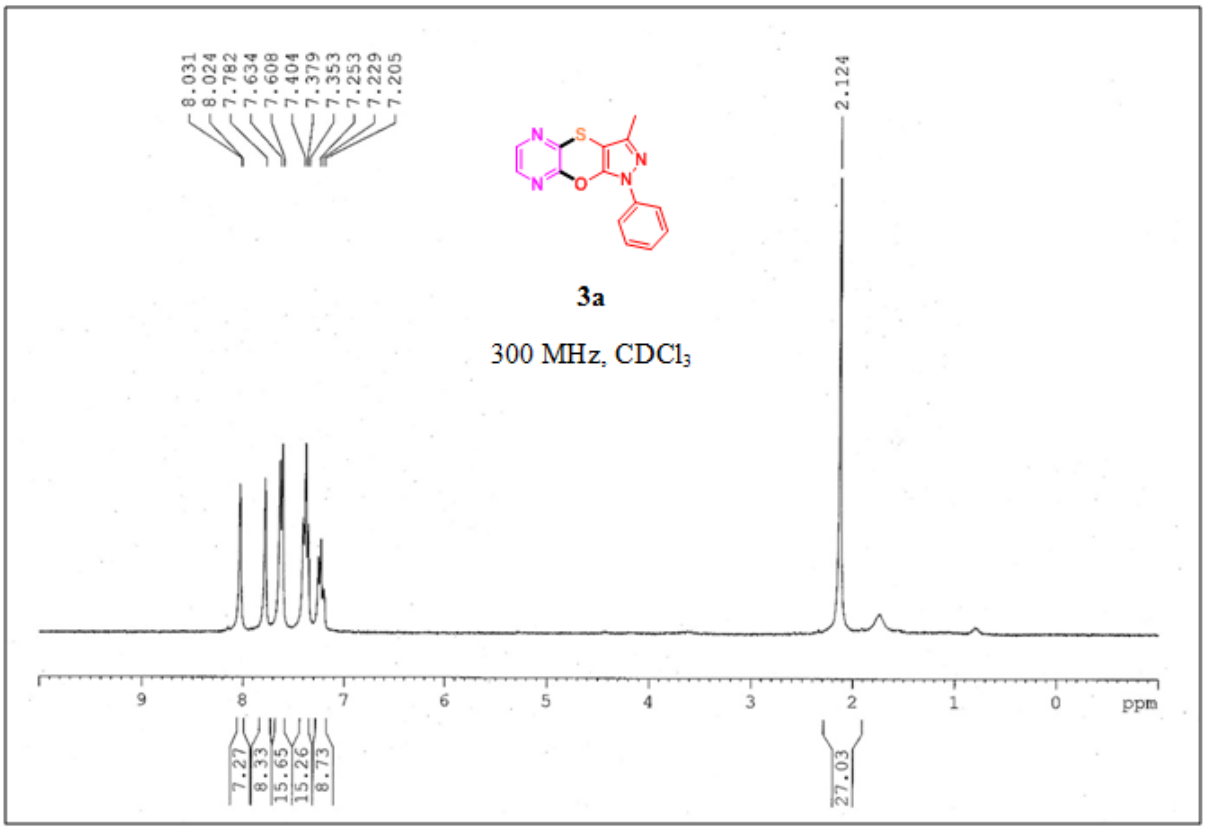
Yellow solid (261 mg, 88%); Mp: 146-148 ^{o}C; ^{1}H NMR (300 MHz; $CDCl_3$; Me_4Si): δ 2.10 (s, 3H), 2.29 (s, 3H), 7.16 (d, *J*=8.1 Hz, 2H), 7.48 (d, *J*=8.1 Hz, 2H), 7.75 (d, *J*=2.1 Hz, 1H); 7.99 (d, *J*=2.1 Hz, 1H), ^{13}C{^{1}H}NMR (75 MHz; $CDCl_3$; Me_4Si): δ 12.7, 21.0, 86.91, 121.2, 121.3, 121.4, 129.8, 134.9, 136.9, 138.2, 140.9, 141.0, 142.6, 144.0, 152.3; HRMS (ESI-TOF) m/z Calcd for $[C_{15}H_{12}N_4OS +H]^+$: 297.0805, found: 297.0827.

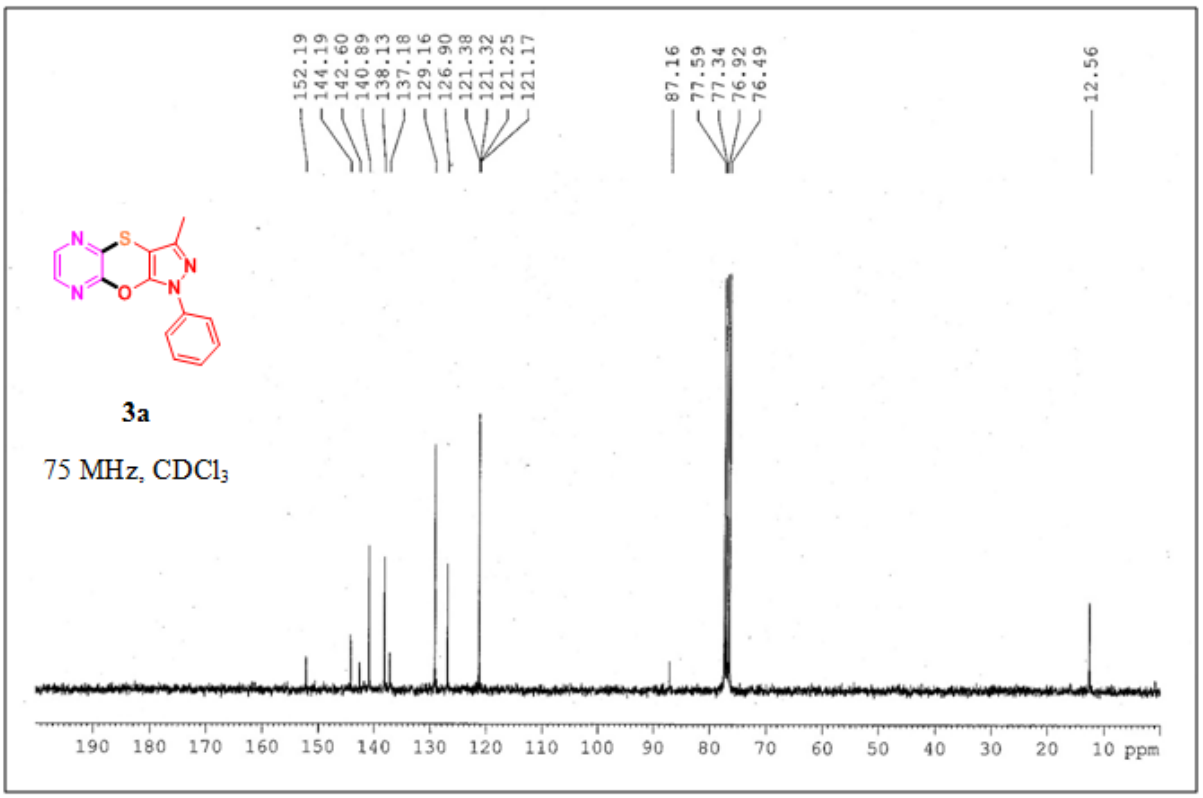
1-(4-chlorophenyl)-3-methyl-1H-pyrazolo[4',3':5,6][1,4]oxathiino[2,3-b]pyrazine 3c

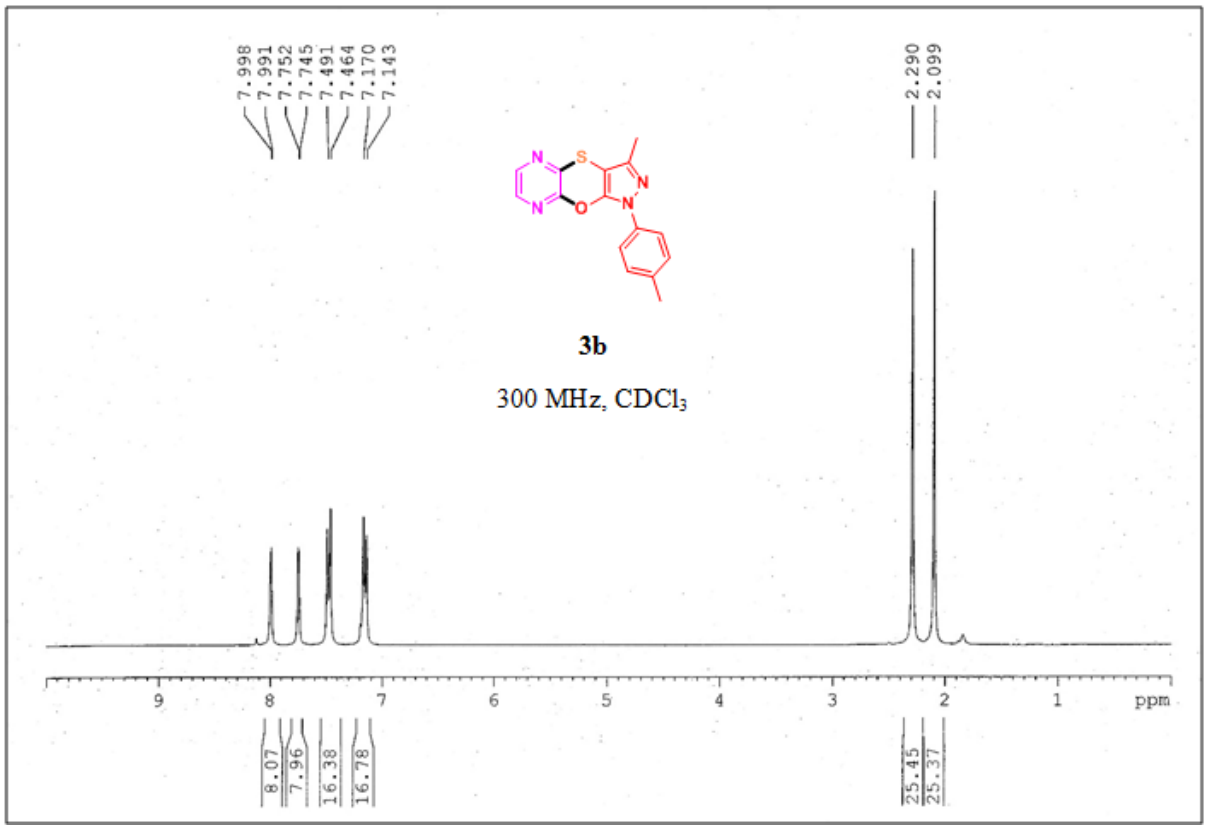


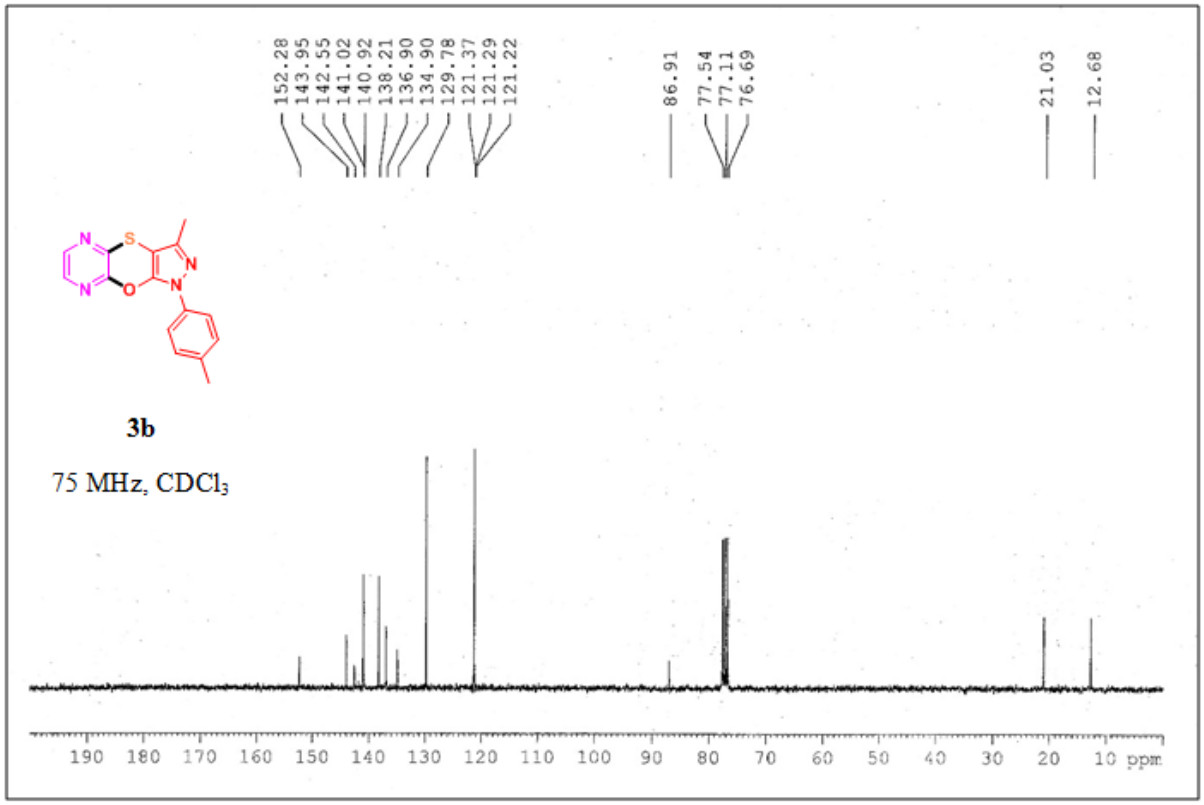
Off-white solid (266 mg, 84%); Mp: 156-158 ^{o}C; ^{1}H NMR (300 MHz; $CDCl_3$; Me_4Si): δ 2.11 (s, 3H), 7.34 (d, *J*=7.5 Hz, 2H), 7.59 (d, *J*=6.9 Hz, 2H), 7.79 (t, *J*=1.8 Hz, 1H); 8.04 (t, *J*=1.8 Hz, 1H), ^{13}C{^{1}H}NMR (75 MHz; $CDCl_3$; Me_4Si): δ 13.4, 88.4, 122.8, 122.9, 130.0, 130.1, 133.2, 136.6, 139.0, 141.5, 141.8, 143.4, 145.3, 152.8; HRMS (ESI-TOF) m/z Calcd for $[C_{14}H_9ClN_4OS +H]^+$: 317.0258, found: 317.0268.

IV. ^{1}H and ^{13}C NMR spectra for the products **3a-c**






7.998
7.991
7.752
7.745
7.491
7.464
7.170
7.143
2.290
2.099
3b
300 MHz, CDCl3
9
8
7
6
5
4
3
2
1
ppm
8.07
7.96
16.38
16.78
25.45
25.37


152.28
143.95
142.55
141.02
140.92
138.21
136.90
134.90
129.78
121.37
121.29
121.22
86.91
77.54
77.11
76.69
21.03
12.68
3b
75 MHz, CDCl3
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10 ppm