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DESIGN, SYNTHESIS, CHARACTERIZATION AND EVALUATION OF THE ANTICANCER PROPERTIES OF OXA-TRIAZA-CYCLOPENTA [B] PHENANTHRENE DICARBOXYLIC ESTER DERIVATIVES

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ABSTRACT

The wide range of biologic activities of pyrazole makes it one of the most active classes of heterocyclic compounds. Pyrazole derivatives are used extensively to synthesize fused heterocyclic systems. Of these, pyrazalo pyrimidines have high chemical and pharmacologic importance as they structurally mimic biogenic purines. Here we describe the synthesis of quadracyclic regioisomers obtained from the cyclocondensation of ethyl 5-amino-1H-pyrazole-4-carboxylate and (7-chloro-4-oxo-chroman-3-yl)-oxo-acetic acid ethyl ester. Further studies demonstrated the requirement of orthogonal esters such that one of the ethyl groups must be replaced with a methyl group. We synthesized the corresponding quadracyclic regioisomers from methyl 5-amino-1H-pyrazole-4-carboxylate and (7-chloro-4-oxo-chroman-3-yl)-oxo-acetic acid ethyl ester. The present study aimed to characterize the diethyl and two positional ester isomers using HPLC, LCMS, NMR and XRD (crystallization) techniques. The compounds were also screened for their in vitro antiproliferative activity by MTT assay. The anti-proliferative activities of two of the derivative compounds, **6** and **9a**, were superior to the currently used anticancer agent paclitaxel.

KEYWORDS: Synthesis, Pyrazole, Dicarboxylic ester, Cyclopenta[b]phenanthrene, Anticancer activity.

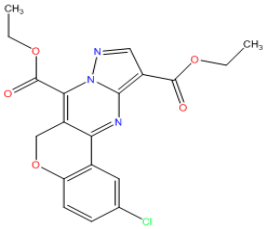
ABBREVIATIONS: HPLC, high performance liquid chromatography; LCMS, liquid chromatography mass spectroscopy; NMR, nuclear magnetic resonance; XRD, X-ray diffraction.

INTRODUCTION

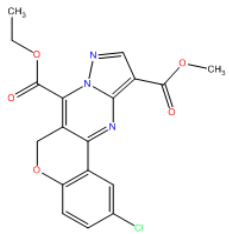
Pyrazole represents one of the most active classes of heterocyclic compounds, as many of them possess a wide range of biologic activities, including potential inhibition of human immunodeficiency virus type 1,[1] analgesia,[2,3] antihypertensive actions[4] and anticancer activity.[5,6] Also, compounds containing substituted pyrimidine derivatives are of significant biologic importance and may be used as antibacterial,[7] antifungal,[8] antitumor,[9] antiviral,[10] anti-inflammatory,[11] and antihypertensive[12] agents.

In general, pyrazole derivatives are utilized for the synthesis of other fused heterocyclic systems. Among these, pyrazolo pyrimidines, which structurally mimic biogenic purines[13,14] have high chemical and pharmacologic importance and are bioisosteres to triazolo-thieno pyrimidines,[15] imidazoquinazolines[16] and pyrimidoquinazolines.[17] Many analogues of pyrazolo[1,5-a]pyrimidine are associated with diverse pharmacologic activities,[18-22] including tuberculostatic,[23] antimicrobial,[24] neuroleptic,[25] central nervous system depressant[26] and antihypertensive[27] activities. Moreover, pyrazolo-pyrimidines have useful properties as antimetabolites in purine biochemical reactions.

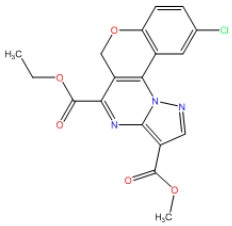
In the present investigation, we designed and synthesized novel quadracyclic diester derivatives incorporating pyrazolo-pyrimidine as a counterpart. We also synthesized the corresponding orthogonal diester derivatives **6, 9a** and **9b,** as shown in **Fig. 1**, which inhibited the growth of a HeLa cell line.



3-Chloro-6H-5-oxa-7a,8,11-triaza-cyclopenta[b]phenanthrene-7,10-dicarboxylic acid diethyl ester **(6)**



3-Chloro-6H-5-oxa-7a,8,11-triaza-cyclopenta[b]phenanthene-7,10-dicarboxylic acid 7-ethyl ester 10-methyl ester **(9a)**



9- Chloro-6H-7-oxa-1,4,11c-triaza-cyclopenta[c]phenanthene-3,5-dicarboxylic acid 5-ethyl ester 3-methyl ester (**9b)**

Fig.1. Orthogonal diester derivatives of 6,9a and 9b

MATERIALS AND METHODS

Samples, chemicals and reagents

All chemicals for synthesis was purchased from Sigma-Aldrich Chemicals Pvt. Ltd., (Bangalore, India). HPLC grade acetonitrile, sodium bicarbonate AR grade and dichloromethane LR grade was purchased from Rankem (Bangalore, India) and trifluoroacetic acid (for IR and NMR Spectroscopy) was purchased from Spectrochem (Bangalore, India). Formic acid (puriss AR) was purchased from Spectrochem. Water used for the preparation of the mobile phase was purified using Merck Millipore Milli-Q® Ultrapure Water (Merck KGaA, Darmstadt, Germany). Solvents for NMR, DMSO-d6, (99.80%D, with 0.03% TMS, v/v) was purchased from Eurisotop (St-Aubin Cedex, France).

Synthesis

Procedure for the synthesis of ethyl 5-amino-1H-pyrazole-4-carboxylate (2)

Dimethyl formamide dimethylacetal (47.40 g, 397.8 mmol) was added dropwise to a solution of ethylcyanoacetate **1** (25g, 221.0 mmol) in dimethylformamide (25 mL) and acetic acid (37.5 mL) at room temperature under nitrogen over a period of 30 min and the mixture was stirred at room temperature for 2 h. The reaction mixture was cooled to 0°C and hydrazine hydrate (80%, 25 mL) was added dropwise to the mixture over a period of 45 min and the

mixture was then heated to 50 °C for 2 h. Completion of the reaction was monitored by TLC. After the reaction was complete, the reaction mixture was diluted with ice-cold water (1 L) and then extracted with ethyl acetate (2 × 250 mL). The combined ethyl acetate layer was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The crude material obtained was purified by column chromatography over silica gel and eluted with 2%-5% methanol in chloroform to obtain the product **4** as an off-white solid (24 g, 70%). MP: 106.7 – 108.9°C; IR (ATR, cm -1) υ: 1125.41 (C - O), 1337.61 (C - N), 1496.40 (C - C), 1615.86 (N-H bend), 1662.37 (ester C = O), 2972.92 (C - H), 3193.79 (N-H stretch); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 1.23 (3H, t, J =7.2 Hz, ester- CH_3), 4.16 (2H, q, J = 6.9 Hz, ester– CH_2), 5.99 (2H, bs, pyrazole- NH_2), 7.47 (1H, bs, pyrazole – CH), 11.84 (1H, bs, pyrazole - NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 14.92 (ester– CH_3), 59.10 (ester - CH_2), 94.00 (pyrazole - C-3), 139.93 (pyrazole - C-4), 151.86 (pyrazole - C-5), 164.27 (carbonyl from ester); LC - MS (ESI, m/z): 156.7 (M +H).

Step-02: Procedure for the synthesis of (7-chloro-4-oxo-chroman-3-yl)-oxo-acetic acid ethyl ester (**5)**

Lithium hexamethyldisilazide (219 mL, 219 mmol, 1M in THF) was added to a solution of 7-chloro- 2, 3-dihydrochromen- 4-one, **3** (25g, 136.9 mmol**)** in THF (250 mL) at -78°C under nitrogen. After stirring for 1 h, diethyl oxalate, **4** (42.01g, 287.4 mmol) was added dropwise at -78°C and stirred at -78°C for 1 h. The reaction was quenched at -78°C with 1 N HCl (250 mL), slowly warmed to 0°C and the pH was adjusted to 3. The reaction mixture was allowed to come to room temperature and the layers were separated. The aqueous layer was extracted with ethyl acetate (2 × 250 mL) and then the combined organic layer was washed with water (1 × 200 mL) followed by brine (1 × 200 mL) and dried over sodium sulfate. The organic phase was concentrated under reduced pressure to obtain a dark green-colored semi-solid. The crude semi-solid was placed in heptane and stirred at 0°C to 5°C for 1 h and the solid was filtered and dried under vacuum at room temperature for 3 h to obtain **5** as a pale yellow solid (31.34 g, 81%). MP: 186.6 – 188.7°C; IR (ATR, cm -1) υ: 1720.8 (C = O), 1699.34 (C = O), 1662.37 (ester C = O); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 1.34 (3H, t, J =7.2 Hz, ester- CH_3), 4.19 (2H, q, J = 6.8 Hz, ester– CH_2), 5.78 (2H, s, O-CH_2), 7.47 (1H, d, J = 8.4 Hz), 7.51 (1H, s), 8.23 (1H, d, J = 8.4 Hz), 11.84 (1H, bs); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 14.92, 59.10, 60.84, 125.6, 128.4, 131.08, 138.67, 141.34, 144.32, 141.56, 158.37, 161.29, 164.3; LC - MS (ESI, m/z): 283.71 (M +H).

Step-03: 3-Chloro-6H-5-oxa-7a,8,11-triaza-cyclopenta[b]phenanthrene-7,10-dicarboxylic acid diethyl ester **(6)**

Ethyl 5-amino-1H-pyrazole-4-carboxylate **2** (5 g, 32.1 mmol) and (7-chloro-4-oxo-chroman-3-yl)-oxo-acetic acid ethyl ester **5** (9.08 g, 32.1 mmol) were suspended in acetic acid (40 mL) and heated to reflux for 5 h under inert atmosphere. The reaction mixture became a clear solution during heating. The resultant reaction mixture was quenched with water (800 mL) and the precipitated solid was filtered, washed with water and dried under vacuum to obtain the crude diester as a pale yellow solid (10.58 g, 82%). The solid was purified by preparative HPLC to attain the diethyl ester derivative.

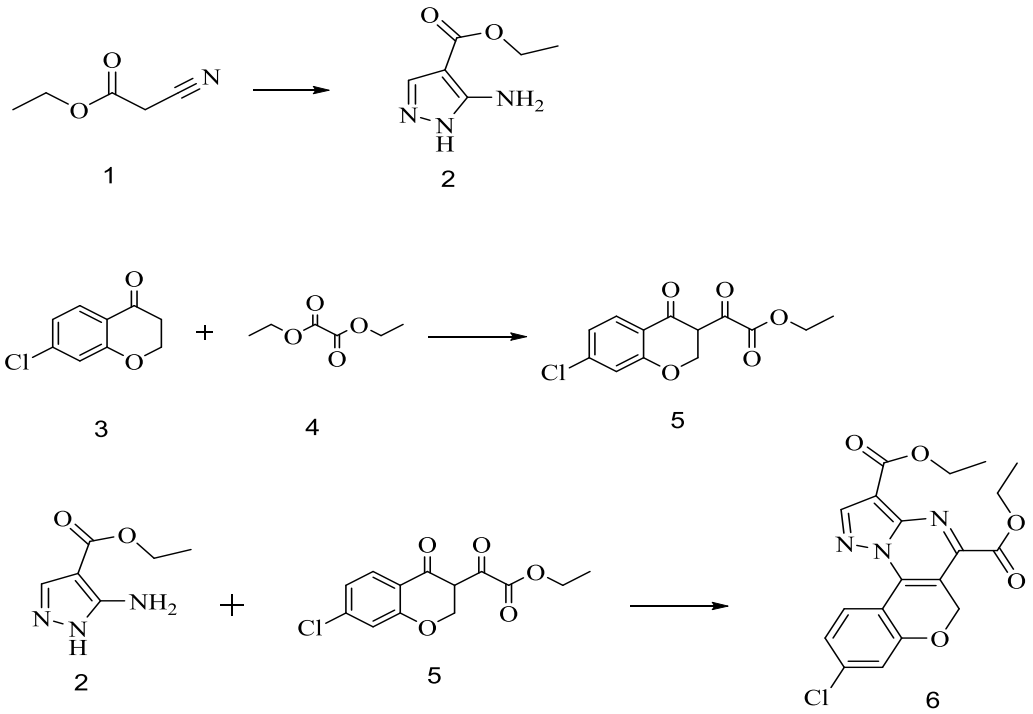
Procedure for the synthesis of methyl 5-amino- 1H-pyrazole- 4-carboxylate (8)

Dimethyl formamide dimethylacetal (45.40 g, 375.7 mmol) was added dropwise to a solution of methylcyanoacetate **7** (25 g, 250.4 mmol) in dimethylformamide (25 mL) and acetic acid (37.5 mL) at room temperature under nitrogen over a period of 30 min and then stirred at room temperature for 2 h. The reaction mixture was cooled to 0°C and hydrazine hydrate (80%, 25 mL) was added dropwise over a period of 45 min and the mixture was heated to 50°C for 2 h. Completion of the reaction was monitored by TLC. After the reaction was completed, the reaction mixture was diluted with ice-cold water (1 L) and then extracted with ethyl acetate (2 × 250 mL). The combined ethyl acetate layer was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The crude material was purified by column chromatography over silica gel, eluted with 2%-5% methanol in chloroform to obtain product **4** as off-white solid (23 g, 72%). MP: 100.2 – 102.1°C; IR (ATR, cm -1) υ: 1124.31 (C - O), 1336.42 (C - N), 1494.37 (C - C), 1613.66 (N-H bend), 1661.16 (ester C = O), 2970.52 (C - H), 3190.87 (N-H stretch); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 3.69 (3H, s, ester- CH_3), 5.88 (2H, bs, pyrazole- NH_2), 7.45 (1H, bs, pyrazole – CH), 11.80 (1H, bs, pyrazole - NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 64.34 (ester–CH_3), 94.64 (pyrazole - C-3), 139.56 (pyrazole - C-4), 150.48 (pyrazole - C-5), 163.78 (carbonyl from ester); LC - MS (ESI, m/z): 142.8 (M +H).

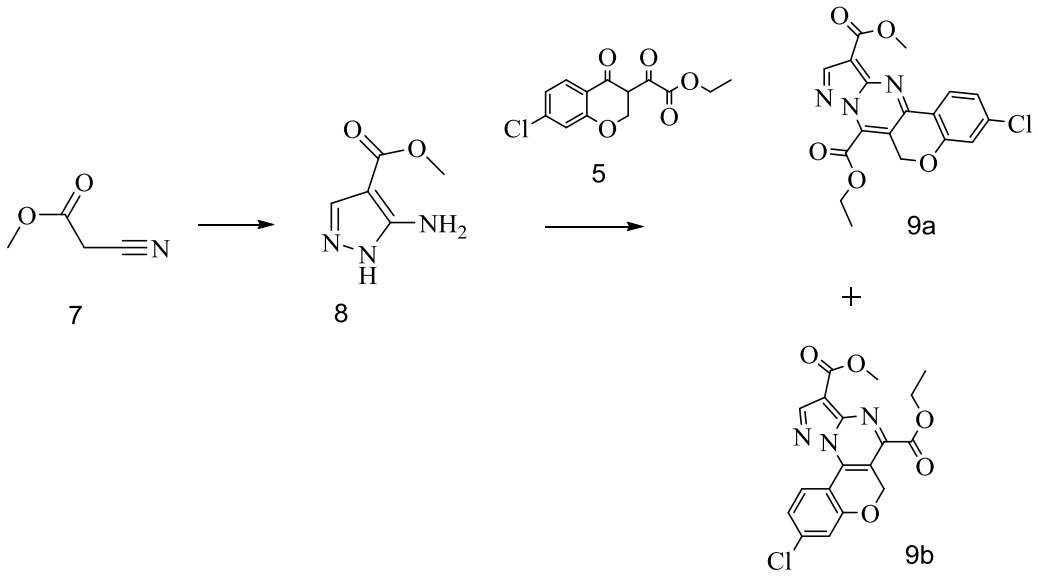
Procedure for the synthesis of 3-chloro-6H-5-oxa-7a,8,11-triazacyclopenta[b] phenanthrene-7,10-dicarboxylic acid 7-ethyl ester 10-methyl ester (9a) and 9- chloro-6H-7-oxa-1,4,11c-triaza-cyclopenta[c]phenanthene-3,5-dicarboxylic acid 5-ethyl ester 3-methyl ester (9b)

Methyl 5-amino-1H-pyrazole-4-carboxylate **8** (5 g, 35.3 mmol) and (7-chloro-4-oxo-chroman-3-yl)-oxo-acetic acid ethyl ester **5** (35.3 g, 35.3 mmol) were suspended in acetic acid (40 mL) and heated to reflux for 5 h under inert atmosphere. The reaction mixture became a clear solution during heating. The resultant reaction mixture was quenched with water (800 mL) and the precipitated solid was filtered, washed with water and dried under vacuum to obtain the crude diester as pale yellow solid (11 g, 80% crude). The solid material was purified by preparative HPLC to obtain the regioisomers.

Scheme 1



Scheme 2



Analytical and preparative high-performance liquid chromatography

An Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA.) equipped with a binary pump, autosampler and photodiode array detector was used. The analysis was carried out on an Agilent Zorbax Eclipse XDB C18, 150 mm × 4.6 mm, 5-µm particle size column (Agilent Technologies) using 0.01% trifluoroacetic acid in water and acetonitrile:methanol (1:1) as a mobile phase with a gradient elution at a flow rate of 1 ml/min at a temperature of 40°C. UV detection was carried out at 210 nm. Data acquisition time was 12 min. The data were recorded using Chemstation software (Agilent Technologies). The major component **(6)** was observed at a retention time of 6.7 min. The chromatogram showed a percent composition of 65%.

To elucidate the formation of the compound, we introduced the methyl and ethyl ester orthogonally. The reaction was continued and the product **(9a and 9b)** formed was submitted for HPLC purity after workup. Two peaks were eluted at retention times of 5.95 and 6.26 min. The HPLC conditions were the same as used for derivative **6**.

Purification of the crude **6, 9a** and **9b** was performed on an Agilent 1200 series Preparative HPLC (Agilent Technologies) equipped with a binary pump, autosampler and photodiode array detector using acetonitrile and 0.1% trifluoroacetic acid in water as the mobile phase with a Phenomenex GEMINI-NX C18, 150 mm × 21.2 mm and 5-µm particle size column. The major fraction of Compd-1 was collected and injected into HPLC and LCMS. The purity was 99% and the mass was confirmed.The two major peaks of **9a** and **9b** were also collected separately. The preparative effluents were then concentrated on a Buchi Rotavapor®. The compounds were then treated with a saturated sodium bicarbonate solution, extracted using dichloromethane and concentrated. The resultant free bases had a purity of 99% with a yield of 150 mg for **6** and 98.5% and 98.6% purity with a yield of 100 mg and 80 mg, respectively, for **9a** and **9b**.

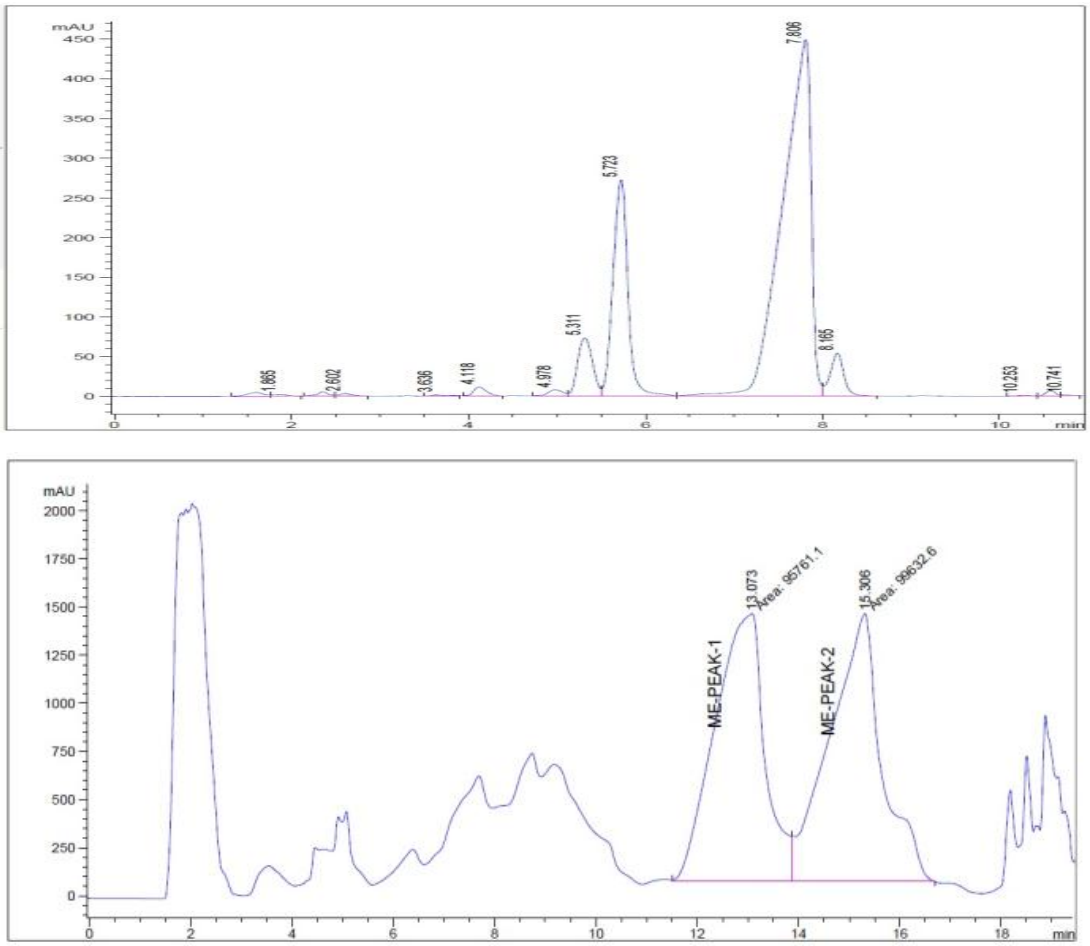


Fig. 2: Preparative Chromatograms of (6) and (9a and 9b)

Mass Spectroscopy

Mass spectral analysis was performed on a single quadruple mass spectrometer (Shimadzu Corporation, Kyoto, Japan) and triple quadrupole mass spectrometer MDS Sciex model API2000 (AB Sciex Pte. Ltd., Foster City, CA, USA). Formic acid (0.1%) and acetonitrile was used as the mobile phase and Synergi 2.5 µm MAX-RP 100 A Mercury (Phenomenex Inc.) as the stationary phase. Detection of ions was performed in electrospray ionization, positive ion mode. The positive masses were confirmed as 402.0 for **6** and 388.0 for **9a** and **9b**.

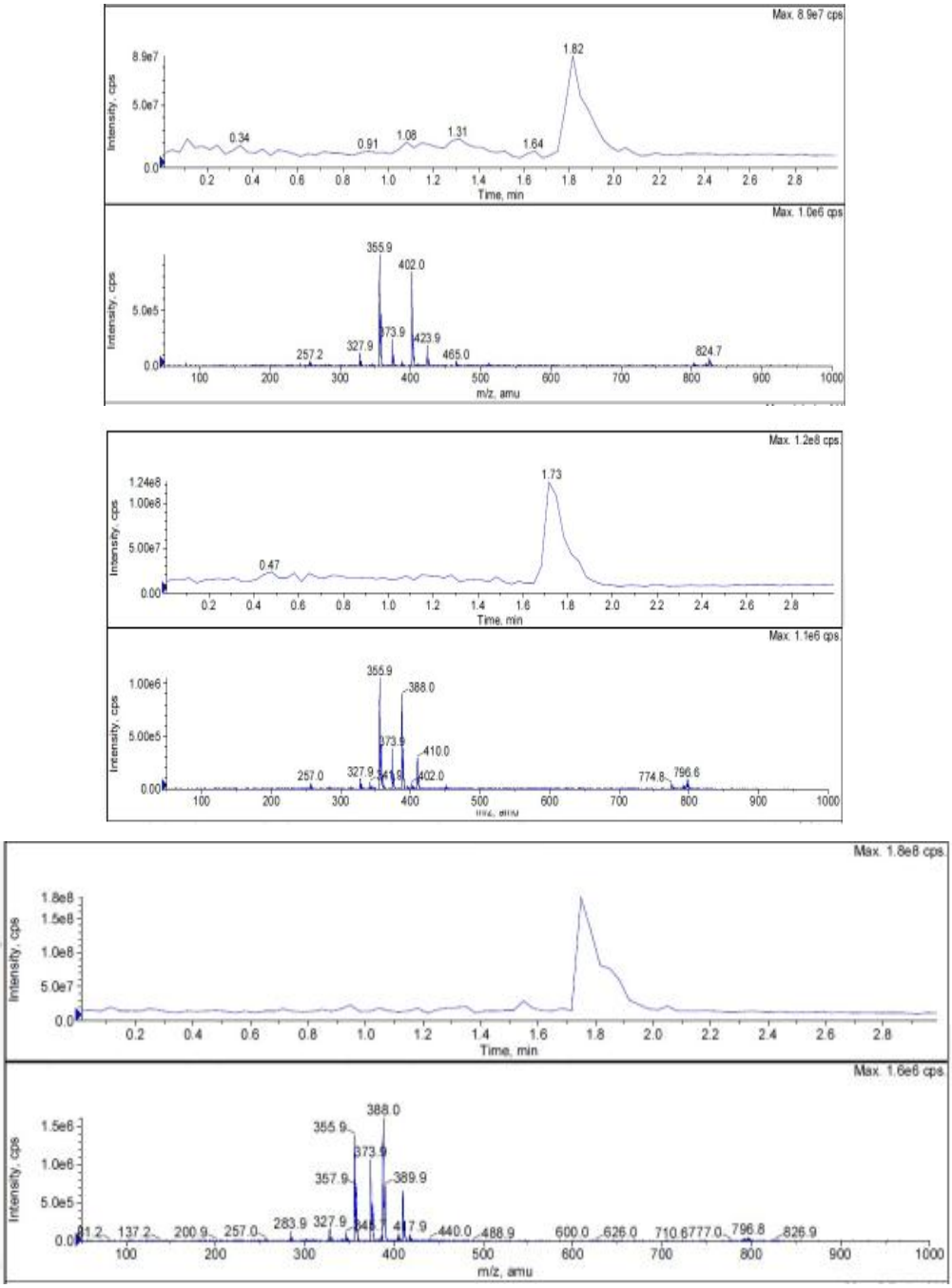


Fig. 3: Mass chromatograms for 6, 9a, and 9b

NMR spectroscopy

The ^{1}H, ^{13}C and two dimensional (2D) NMR experiments such as Heteronuclear Single Quantum Coherence Spectroscopy (HSQC) and Heteronuclear Multibond Coherence Spectroscopy (HMBC) were performed on a Varian Mercury plus 400 MHz NMR instrument (Varian Medical Systems Inc., Palo Alto, CA, USA) at 25°C in DMSO-d6. The ^{1}H chemical shift values were reported on the ι scale in ppm, relative to TMS (ι = 0.00 ppm) and in the ^{13}C NMR the chemical shift values were reported relative to DMSO-d6 (ι = 39.50 ppm).

Table 1: Comparative ^{1}H NMR assignments for 6, 9a and 9b

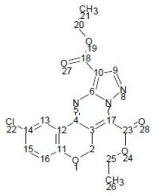
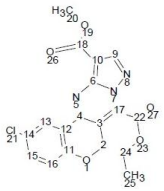
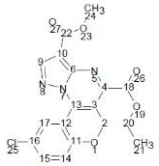
6	9a	9b
		
(Atom #), δppm, multiplicity, j(Hz)	**(Atom #), δppm, multiplicity, j(Hz)**	**(Atom #), δppm, multiplicity, j(Hz)**
(21,26)1.38, q, 7.25	(25)1.51, t, 7.12	(21)1.39, t, 7.12
(20)4.33, q, 6.98	(20)4.00, s	(24)3.86, s
(25)4.57, q, 6.98	(24)4.65, q, 7.25	(20)4.46, q, 6.98
(2)5.45, s	(2)5.33, s	(2)5.59, s
(16)7.15, d, 8.87	(14)6.98, d, 8.60	(14)7.22, d, 8.86
(15)7.57, dd,8.87 &2.69	(15)7.40, dd, 8.73&2.55	(15)7.67, dd, 8.87&2.69
(13)8.11, d, 2.69	(13)8.40, d, 2.42	(9)8.94, s
(9)8.65, s	(9)8.58, s	(17)9.21, d, 2.42

Table 2: Comparative ^{13}C NMR assignments for 6, 9a and 9b

6	9a	9b
Atom #),δppm	**(Atom #),δppm**	**(Atom #),δppm**
(2) 64.002	(2) 64.428	(2) 63.995
(3) 112.468	(3) 112.552	(3) 113.330
(4) 149.993	(4) 150.690	(4) 146.543
(6)146.973	(6) 147.630	(6) 146.422
(9) 147.581	(9) 148.235	(9) 148.464
(10) 102.557	(10) 103.365	(10) 103.126
(11) 156.219	(11) 156.163	(11) 156.295
(12) 121.737	(12) 121.747	(12) 116.164
(13) 124.378	(13) 125.865	(13) 136.815
(14) 126.720	(14) 128.537	(14) 119.336
(15) 133.840	(15) 134.099	(15) 134.159
(16) 119.902	(16) 119.132	(16) 125.698
(17) 132.938	(17) 133.276	(17) 128.855
(18) 161.380	(18) 162.662	(18) 163.521
(20) 59.860	(20) 51.777	(20) 62.493
(21) 14.286	(22) 159.158	(21) 13.941
(23) 158.615	(24) 64.048	(22) 161.632
(25) 63.820	(25) 14.140	(24) 51.441
(26) 13.841		

All protonated carbons of **6** were assigned by analysis of the HSQC and DEPT spectra. The DEPT spectrum confirmed the presence of four aromatic –CH carbons, three aliphatic –CH2 carbons and two aliphatic –CH3 carbons. The –CH2 carbons of C2, C20 and C25 were observed at δC 63.75, 59.60 and 63.57, respectively. The correlation between CH2 (H20,25

δH =4.33,4.57) of the ester groups with the carbonyl carbons (C18,C23 δC 161.380,158.615) were established from the HMBC data. The HMBC correlation of H9 with C10 and C6 confirms the quarternary carbons of the pyrazole ring. The –CH2 (H2, δH =5.45) of the chromane ring correlated with C3, C4, C11, C17 and C23. Correlations were also observed between H13 to C14, C15, C11, and C4; H15 to C14 and C11; H16 to C11, C12, C14 and C4.

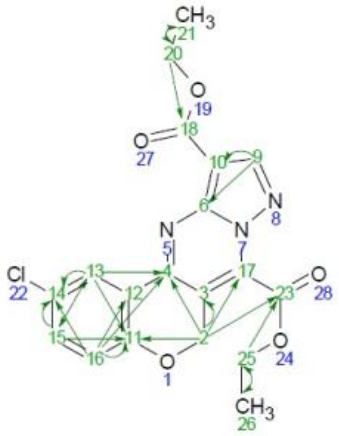


Fig.4. ^{1}H-^{13}C HMBC correlations of 6

Table 3: ^{1}H-^{13}C HMBC assignment for 6

#	Atom#	C Shift	XHn	H Shift	H HMBC	C HMBC
1	2		CH2	5.447		158.55, 149.97, 156.22, 132.93, 112.47
2	3	112.465	C		5.45	
3	4	149.966	C		5.45, 7.15, 8.12	
4	6	146.946	C		8.65	
5	9		CH	8.647		102.56, 146.95
6	10	102.562	C		8.65	
7	11	156.218	C		5.45, 7.15, 7.58, 8.12	
8	12	121.728	C		7.15	
9	13	124.377	CH		7.58	
10	13		CH	8.115		156.22, 133.84, 126.74, 149.97
11	14	126.736	C		7.15, 7.58, 8.12	
12	15		CH	7.575		156.22, 124.38, 126.74
13	15	133.844	CH		8.12	
14	16		CH	7.148		149.97, 156.22, 121.73, 126.74
15	17	132.933	C		5.45	
16	18	161.293	C		4.33	
17	20		CH2	4.332		161.29, 14.28
18	20	59.831	CH2		1.37	
19	21	14.285	CH3		4.33	
20	21		CH3	1.37		59.83
21	23	158.552	C		4.57, 5.45	

22	25	63.785	CH2		1.39	
23	25		CH2	4.571		158.55, 13.73
24	26	13.732	CH3		4.57	
25	26		CH3	1.393		63.79

9a

All protonated carbons of **9a** were assigned by HSQC and DEPT spectra. The analyses confirmed the presence of four aromatic –CH carbons, two aliphatic –CH2 carbons and two aliphatic –CH3 carbons. The –CH2 carbons of C2 and C24 were observed at δC 64.428 and 64.048, respectively. Correlations between -CH2 (H24 δH =4.65) of the ethyl ester group with the carbonyl carbon (C22 δC 159.158) and between –CH3 (H20 δH =4.00) of the methyl ester group with the carbonyl carbon (C18 δC 162.662) were established from the HMBC data. The HMBC correlation of H9 with C10, C6, and 17 confirmed the presence of the quarternary carbons of the pyrazole ring. The –CH2 (H2, δH =5.45) of the chromane ring correlated with C3, C4, C11 and C17. Correlations were also observed between H13 to C14, C15, C11 and C4; H15 to C13, C14 and C11; H16 to C11, C12, C14 and C4.

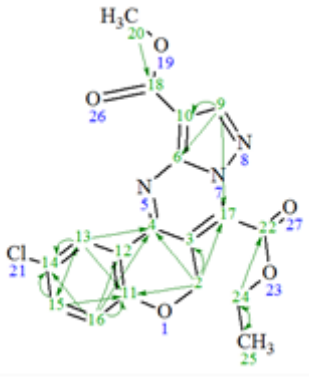


Fig.5. ^{1}H-^{13}C HMBC correlations of 9a

Table 4: ^{1}H-^{13}C HMBC assignment for 9a

#	Atom#	C Shift	XHn	H Shift	H HMBC	C HMBC
1	2		CH2	5.328		133.25, 150.68, 156.15, 112.53
2	3	112.53	C		5.33	
3	4	150.684	C		5.33, 6.98, 8.40	
4	6	147.614	C		8.58	
5	9		CH	8.58		103.35, 133.25, 147.61
6	10	103.353	C		8.58	
7	11	156.149	C		5.33, 6.98, 7.40, 8.40	
8	12	121.742	C		6.98	
9	13	125.855	CH		7.4	
10	13		CH	8.403		156.15, 134.08, 128.53, 150.68

11	14	128.528	C		6.98, 7.40, 8.40	
12	15	134.078	CH		8.4	
13	15		CH	7.401		125.86, 156.15, 128.53
14	16		CH	6.984		150.68, 156.15, 121.74, 128.53
15	17, 9	133.249	C, CH		5.33, 8.58	
16	18	162.629	C		4	
17	20		CH3	3.998		162.63
18	22	159.147	C		4.65	
19	24		CH2	4.653		159.15, 14.13
20	24	64.025	CH2		1.51	
21	25		CH3	1.512		64.03
22	25	14.129	CH3		4.65	

9b

All protonated carbons of **9b** were assigned by HSQC-DEPT spectra. The analyses confirmed the presence of four aromatic –CH carbons, two aliphatic –CH2 carbons and two aliphatic –CH3 carbons. The –CH2 carbons of C2 and C20 were observed at δC 63.995 and 62.493, respectively. Correlations between -CH2 (H20 δH =4.46) of the ethyl ester group with the carbonyl carbon (C18 δC 163.521) and between –CH3 (H24 δH =3.86) of the methyl ester group with the carbonyl carbon (C22 δC 161.632) were established from the HMBC data. The HMBC correlation of H9 with C10 andC6 confirmed the presence of the quarternary carbons of the pyrazole ring. The –CH2 (H2, δH =5.59) of the chromane ring showed correlations with C3, C4, C11 and C13. Correlations were also observed between H14 to C11, C12, C13 and C16; H15 to C11, C16 and C17; H17 to C11, C13, C15 and C16.

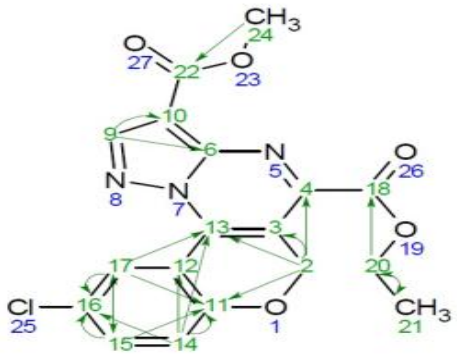


Fig.6. ^{1}H-^{13}C HMBC correlations of 9b

Table 5: ^{1}H-^{13}C HMBC assignment for 9b

#	Atom#	C Shift	XHn	H Shift	H HMBC	C HMBC
1	2		CH2	5.59		113.32,136.82,146.54,156.29
2	3	113.32	C		5.59	
3	4	146.54	C		5.59	
4	6	146.42	C		8.94	
5	9		CH	8.94		146.42,103.12
6	10	103.12	C		8.94	
7	11	156.29	C		5.59,7.23,7.67,9.21	
8	12	116.16	C		7.23	
9	13	136.82	C		5.59,7.23,9.21	
10	14		CH	7.23		156.29,116.16,136.82,125.70
11	15		CH	7.67		156.29,125.70,128.85
12	15	134.16	CH		9.21	
13	16	125.7	C		7.23,7.67,9.21	125.86, 156.15, 128.53
14	17		CH	9.21		156.29,136.82,134.16,125.70
15	17	128.85	CH		7.67	
16	18	163.51	C		4.46	
17	20		CH2	4.46		163.51,13.93
18	20	62.49	CH2		1.4	
19	21		CH3	1.4		62.49
20	21	13.93	CH3		4.46	
21	22	161.62	C		3.86	
22	24		CH3	3.86		161.62

FT-IR Spectroscopy

FT-IR spectra were recorded in the solid state using KBr dispersion on a PerkinElmer Spectrum Two FTIR Spectrometer (PerkinElmer, Waltham, MA, USA).

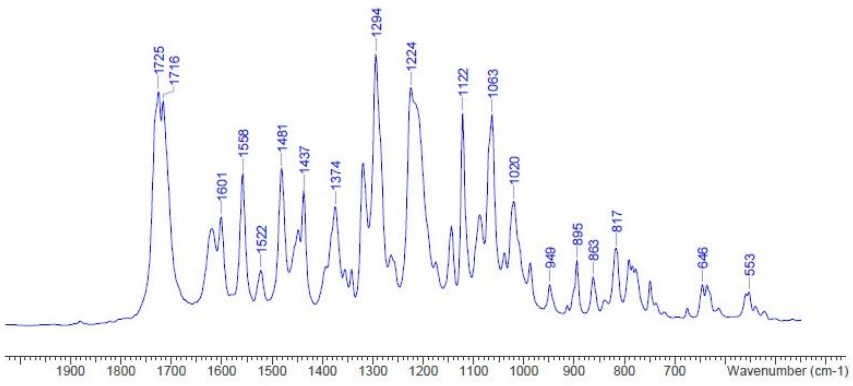


Fig.7A. FT-IR Spectra of 6

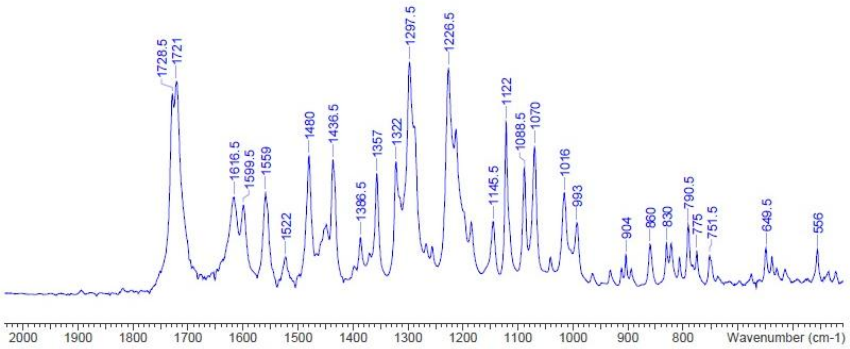


Fig.7B. FT-IR Spectra of 9a

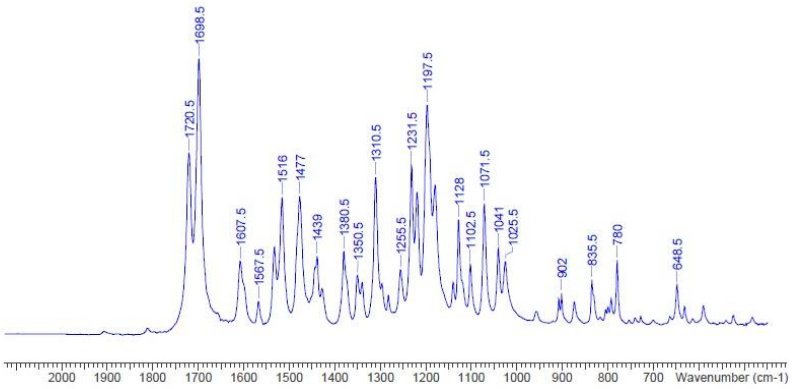
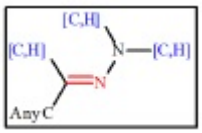
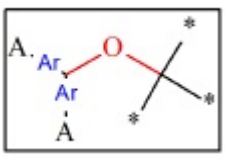
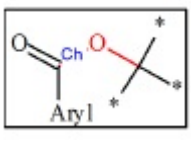
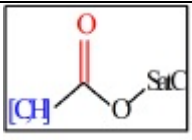


Fig.7C. FT-IR Spectra of 9b

Table 6: FT-IR spectral data for 6, 9a and 9b

Fragment	Description	Group	Wave No.			Mode of vibration
			6	9a	9b	
	Hydrazone	C=N	1601	1616.5	1607.5	C=N stretching
	Alkyl aryl ether	(C3)	1063	1070	1071.5	(al)C-O stretching
			1224	1226.5	1231.5	(ar)C-O stretching
	Aromatic acid alkyl ester	C-O-C	1294	1297.5	1310.5	C-O-C asym stretching
			1122	1122	1128	C-O-C sym stretching
	Conjugated or formic acid ester	C=O	1725 & 1716	1721 & 1728.5	1720.5 & 1698.5	C=O stretching

X-ray Diffraction data collection

Crystals of **6**, **9a** and **9b** were prepared with an acetonitrile:water mixture. A suitable single crystal of each compound was carefully selected under a polarizing microscope and the single crystal data were collected on a Bruker Kappa Apex2 CCD diffractometer at 293(2) K for **6** and 273(2) K for **9a** and **9b**. The X-ray generator was operated at 40 kV and 30 mA using Mo Kα(λ = 0.71073 A˚) radiation. Data were collected with a ω scan width of 0.5. The data reduction followed by empirical absorption corrections were applied with the various modules within the Apex2 software suite. The structures were solved by direct methods using the SHELXTL (Compound-1) and SHELXL-97 (Isomer-I and Isomer-II) packages and refined by full-matrix least-squares on F2. All non-hydrogen atoms were refined anisotropically and hydrogen atoms were refined using the riding model. The structures were drawn using Mercury 3.1 and Pymol.

Table 7: Crystallographic data of 6, 9a, 9b

Crystal data statistics	**6**	**9a**	**9b**
Empirical formula	C19 H16 Cl N3 O5	C18 H14 Cl N3 O5	C18 H14 Cl N3 O5
Molecular weight	401.80	387.78	387.78
Temperature/K	293	273	273
Crystal system	Triclinic	triclinic	Monoclinic
Space group	P-1	'P -1'	'P 21/c'
a/Å	7.412(4)	7.424(4)	7.346(6)
b/Å	11.592(5)	11.615(7)	27.60(2)
c/Å	13.087(5)	11.632(7)	8.631(7)
α/°	60.475(18)	113.543(7)	90
β/°	69.27(2)	101.164(6)	102.180(11)
γ/°	82.73(3)	99.915(4)	90
Volume/Å3	913.5(7)	866.8(9)	1710(2)
Z	2	2	4
Radiation	Mo Kα (λ = 0.71073)	Mo Kα (λ = 0.71073)	Mo Kα (λ = 0.71073)
Max 2θ	25.24	25.35	25.29
Reflections collected	3309	3038	3098
Independent reflections	2356	2200	2460
Data/restraints/parameters	2356 /0/255	2200/0/252	2460/0/244
F_{000}	424	394	764
Goodness-of-fit on F2	1.090	1.058	0.882
R_factor _gt	R1 = 0.0608 ,	R=0.0540	R=0.064
WR_Factor _gt	0.1184	WR=0.1687	WR=0.153

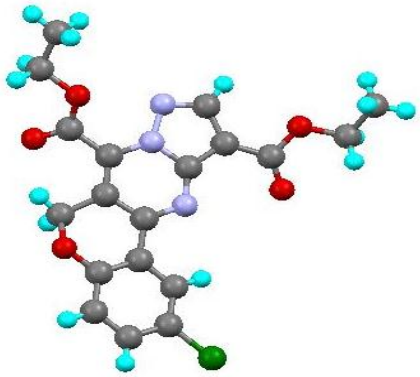


Fig. 8A. 3-Chloro-6H-5-oxa-7a,8,11-triaza-cyclopenta[b]phenanthrene-7,10-dicarboxylic acid diethyl ester (6)

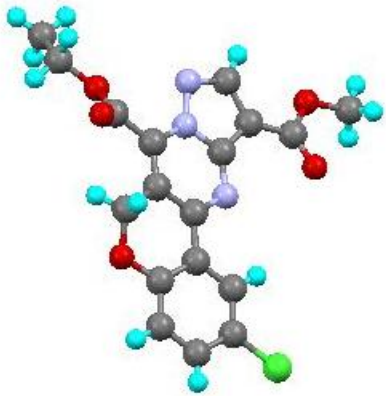


Fig.8B. 3-Chloro-6H-5-oxa-7a,8,11-triaza-cyclopenta[b]phenanthrene -7,10-dicarboxylic acid 7-ethyl ester 10-methyl ester (9a)

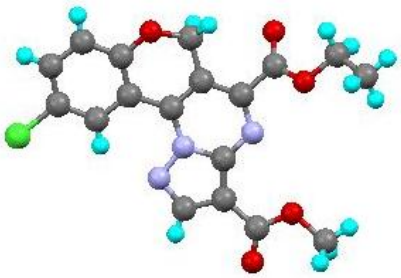


Fig.8C. 9- Chloro-6H-7-oxa-1,4,11c-triaza-cyclopenta[c]phenanthrene -3,5-dicarboxylic acid 5-ethyl ester 3-methyl ester (9b)

Anticancer activity

All compounds were screened for their in vitro anti-cancer activity against a representative human cancer cell line (HeLa cell line) using the 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. The MTT assay is a colorimetric assay that measures the

reduction of yellow MTT by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilized with an organic solvent (e.g., dimethylsulfoxide, isopropanol). The released solubilized formazan reagent can then be measured spectrophotometrically. Because reduction of MTT can only occur in metabolically active cells, the activity level is a measure of cell viability.

The MTT solution was prepared by dissolving 10 mg in 10 mL of Hank's balanced solution. The cell lines were maintained in 96-well microtiter plates containing minimum essential medium (MEM) supplemented with 10% heat inactivated fetal calf serum and containing 5% of a mixture of gentamycin, penicillin (100 Units/mL) and streptomycin (100 µg/mL) in the presence of 5% CO_2 at 37°C for 3 - 4 days. The MEM was then removed and replaced. The supernatant was then removed and the MEM media was replaced with Hank's balanced solution and the cells incubated overnight. The in vitro growth inhibition of test compounds was assessed using calorimetric or spectrophotometric methods to determine the conversion of MTT into formazan blue by living cells. The supernatant was again removed and fresh Hank's balanced salt solution was added. The supernatant was removed from the plate, fresh Hank's balanced salt solution was added and various concentrations of the compound (diluted in DMSO) was added. The commerical anticancer drug paclitaxel was tested as the reference compound in the assay. The control group contained only DMSO. After 24 h of incubation at 37°C in a humidified atmosphere of 5% CO_2, the medium was replaced with MTT solution (100 µL, 5 mg/mL in MEM) for another 4 h. The supernatant was then carefully aspirated and the precipitated crystals of Formazan blue were solubilized by adding DMSO (200 µL). The optical density was measured at a wavelength of 570 nm using ELISA microplate reader. The results are the means of triplicate experiments for each concentration. The percentage of lysed cells was calculated by comparing the OD of the sample to that of the control and by microscopic analysis. The concentration at which the OD of treated cells was reduced by 50% with respect to the untreated control was calculated.

RESULTS AND DISCUSSION

Generally, synthesis of ethyl 5-amino-1H-pyrazole-4-carboxylate **2** was achieved via a two-step protocol (Mangesh *et al*., 2011; Mc Call *et al*., 2012).[28,29] In the present work, we synthesized ethyl 5-amino-1H-pyrazole-4-carboxylate **2** in a single-pot reaction from

commercially available ethyl cyano acetate **1** using dimethylacetate and hydrazine hydrate in acetic acid under mild heating with dimethylformamide as the solvent.

(7-Chloro-4-oxo-chroman-3-yl)-oxo-acetic acid ethyl ester (**5**) was synthesized from 7-chloro-2,3-dihydrochromen-4-one (**3)** with lithium hexamethyldisilazide and diethyl oxalate (**4)** at -78°C. This chromenone ester was suspended in acetic acid with ethyl 5-amino-1H-pyrazole-4-carboxylate (**2**) and heated to reflux for 5 h to obtain the quadracyclic diester derivative **6**. The compound was purified by preparative HPLC.

To elucidate the formation of the above compound, we introduced the methyl and ethyl ester orthogonally by making the corresponding methyl 5-amino-1H-pyrazole-4-carboxylate (**8)** from methyl cyanoacetate (**7**). This methyl ester was suspended with (7-chloro-4-oxo-chroman-3-yl)-oxo-acetic acid ethyl ester (**5**) in acetic acid and heated to reflux to obtain **9a** and **9b,** instead of only **6** in the case of pyrazole ethyl ester. These compounds were purified by preparative HPLC to separate the regioisomers.

The structure of ethyl 5-amino-1H-pyrazole-4-carboxylate **2** was confirmed by spectral studies. FTIR spectrum showed a broad band at 3193.79 cm^{-1} indicating the presence of –NH stretch in the pyrazole ring. Peaks were also observed at 1615.86 cm^{-1} for the N-H bend and 1337.61 cm^{-1} for the C-N stretch. The ^{1}H NMR spectrum showed a singlet at δ 7.45 ppm for the proton in the pyrazole ring. In addition, the broad singlet at δ 11.84 ppm confirmed the presence of the –NH proton in the pyrazole ring. The signals at 94.00, 139.93 and 151.86 ppm in ^{13}C NMR spectrum represent the aromatic carbons in the pyrazole ring. The structure of compound **2** was further confirmed by its mass spectrum, which showed the molecular ion peak at 156.7 (M + H), corresponding to the molecular formula $C_6H_9N_3O_2$.

The structure of (7-chloro-4-oxo-chroman-3-yl)-oxo-acetic acid ethyl ester (**5)** was elucidated based on its IR, ^{1}H NMR, ^{13}C NMR and LC-MS values. The IR spectrum showed three strong bands at 1720.2 cm^{-1}, 1692.70 cm^{-1} and 1683.41 cm^{-1}, which correspond to the three carbonyl groups in the molecule. The presence of the ethyl ester group was further confirmed by the respective values in the ^{1}H-NMR. The appearance of a triplet at 1.53 ppm and a quartet at 4.46 ppm confirmed the finding. The singlet value observed at 5.46 ppm corresponds to the presence of $–OCH_2$ protons in the molecule. The signals at 163.42 ppm, 163.25 ppm and 161.24 ppm in the ^{13}C NMR spectra correspond to the presence of three carbonyl groups in the molecule. The structure of compound **2** was further confirmed by its

mass spectrum, which showed a molecular ion peak at 283.71 (M + H), corresponding to the molecular formula $C_{13}H_{11}ClO_5$.

The structure of 3-chloro-6H-5-oxa-7a,8,11-triaza-cyclopenta[b] phenanthrene-7,10-dicarboxylic acid diethyl ester **(6)** was elucidated based on its IR, ^{1}H NMR, ^{13}C NMR, and LC-MS values. The IR spectrum showed two strong bands at 1698.68 cm^{-1} and 1685.73 cm^{-1}, which correspond to the two carbonyl groups in the molecule. The presence of ethyl ester group was further confirmed by the respective values in the ^{1}H-NMR. The appearance of two triplets at 1.38 ppm and 1.39 ppm and two quartets at 4.34 ppm and 4.57 ppm confirmed the finding. The singlet value observed at 5.47 ppm corresponds to the presence of $–OCH_2$ protons in the molecule. The signals at 161.38 ppm and 158.61 ppm in the ^{13}C NMR spectra correspond to the presence of two carbonyl groups in the molecule. The structure of compound **6a** was further confirmed by its mass spectrum, which showed the molecular ion peak at 402.8(M + H), corresponding to the molecular formula $C_{19}H_{16}ClN_3O_5$.

The structure of methyl 5-amino-1H-pyrazole-4-carboxylate **8** was confirmed by spectral studies. The FTIR spectrum showed a broad band at 3193.79 cm^{-1} indicating the presence of an –NH stretch in the pyrazole ring. Peaks were also observed at 1615.86 cm^{-1} for the N-H bend and 1337.61 cm^{-1} for the C-N stretch. The ^{1}H NMR spectrum showed a singlet at δ 7.45 ppm for the proton in the pyrazole ring. In addition, the broad singlet at δ 11.84 ppm confirmed the presence of the –NH proton in the pyrazole ring. The signals at 94.00, 139.93, and 151.86 ppm in the ^{13}C NMR spectrum represent aromatic carbons in the pyrazole ring. The structure of compound **8** was further confirmed by its mass spectrum, which showed the molecular ion peak at 156.7 (M + H), corresponding to the molecular formula $C_6H_9N_3O_2$.

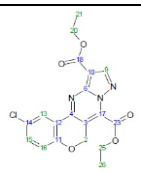
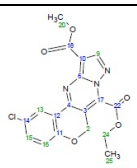
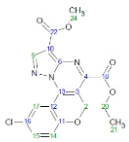
The structure of 3-chloro-6H-5-oxa-7a,8,11-triaza-cyclopenta[b]phenanthene“7,10” dicarboxylic acid 7-ethyl ester 10-methyl ester **(9a)** was elucidated based on its IR, ^{1}H NMR, ^{13}C NMR and LC-MS values. The IR spectrum showed two strong bands at 1698.68 cm^{-1} and 1685.73 cm^{-1}, corresponding to the two carbonyl groups in the molecule. The presence of the ethyl ester group was further confirmed by the respective values in the ^{1}H-NMR. The appearance of a triplet at 1.51 ppm and a quartet at 4.65 ppm confirmed the finding. The singlet at 4.00 ppm corresponds to the methyl ester group in the molecule. The singlet value observed at 5.32 ppm corresponds to the presence of $–OCH_2$ protons in the molecule. The signals at 162.66 ppm and 159.16 ppm in the ^{13}C NMR spectra correspond to the presence of two carbonyl groups in the molecule. The structure of compound **9a** was further confirmed

by its mass spectrum, which showed the molecular ion peak at 388.2 (M + H), corresponding to the molecular formula $C_{18}H_{14}ClN_3O_5$.

9-Chloro-6H-7-oxa-1,4,11c-triaza-cyclopenta[c]phenanthene-3,5-dicarboxylic acid 5-ethyl ester 3-methyl ester (**9b)** was elucidated based on its IR, ^{1}H NMR, ^{13}C NMR, and LC-MS values. The IR spectrum showed two strong bands at 1698.68 cm^{-1} and 1685.73 cm^{-1}, which correspond to the two carbonyl groups in the molecule. The presence of the ethyl ester group was further confirmed by the respective values in the ^{1}H-NMR. The appearance of a triplet at 1.39 ppm and a quartet at 4.45 ppm confirmed the finding. The singlet at 3.86 ppm corresponds to the methyl ester group in the molecule. The singlet value observed at 5.58 ppm corresponds to the presence of $–OCH_2$ protons in the molecule. The signals at 163.53 ppm and 161.64 ppm in the ^{13}C NMR spectra correspond to the presence of two carbonyl groups in the molecule. The structure of compound **9b** was further confirmed by its mass spectrum, which showed the molecular ion peak at 388.1(M + H), corresponding to the molecular formula $C_{18}H_{14}ClN_3O_5$.

Crude **6** was purified to 99% purity with a mass of 402.1. **9a** and **9b** were separated and purified to obtain greater than >98% purity. The mass of these two isomers was 388.0. The FTIR spectra of all three compounds revealed two strong characteristic absorption bands at 1725 and 1716, 1721 and 1728.5 and 1720.5 and 1698.5 cm-1, indicating the presence of two carbonyl groups each in **6**, **9a** and **9b**. The proton–carbon correlations of all three compounds were established using edited HSQC spectra and are shown in Table 8.

Table 8: Correlation observed in the HSQC spectra of 6, 9a, and 9b

6	**9a**	**9b**
		
(Atom #),δppm	**(Atom #),δppm**	**(Atom #),δppm**
(2) 5.448-63.99	(2) 5.327-64.433	(2) 5.59-63.99
(9) 8.647-147.555	(9) 8.580-148.234	(9) 8.94-148.47
(13) 8.114-124.405	(13) 8.404-125.866	(14) 7.23-119.34
(15) 7.575-133.769	(15) 7.401-134.091	(15) 7.67-134.16
(16) 7.147- 119.869	(16) 6.983-119.125	(17) 9.21-128.86
(20) 4.329-59.865	(20) 3.997-51.780	(20) 4.46-62.49
(21) 1.372-14.249	(24) 4.652-64.061	(21) 1.39-13.94
(25) 4.570-63.794	(25) 1.515-14.141	(24) 3.86-51.43
(26) 1.392-13.855		

Anticancer evaluation

The synthesized diester derivatives were screened for their in vitro anticancer activity against a representative human cervical cancer cell line HeLa. Paclitaxel was used as the reference standard. The data generated from this study (**Table 9)** reveal that all of the synthesized derivatives exhibited good potency for inhibiting HeLa cell growth. Interestingly, among these compounds, the in vitro anticancer activity of compounds **6** and **9a** was superior to that of paclitaxel.

The anticancer activity of these novel compounds suggests that compounds **6** and **9a** in which the ester groups are placed on the same side have better anticancer activity compared to regioisomer **9b** in which the ester groups are placed on the opposite side.

Table 9: Anticancer activity

Sl.No.	Compound	Concentration (µM/mL)	Percentage of Growth Inhibition	IC_{50} (µM)
1	**6**	10	87.89	0.023 ± 0.002
		20	88.43	
		30	89.28	
2	**9a**	10	88.48	0.031 ± 0.003
		20	90.08	
		30	90.27	
3	**9b**	10	53.91	0.104 ± 0.017
		20	54.55	
		30	47.38	

Values are expressed as the mean of three independent determinations and are within ±10%
IC50- Half maximal inhibitory concentration – It is the half maximal (50%)inhibitory concentration (IC) of a Substance (50% IC, or IC50).

CONCLUSION

A set of two novel quadracyclic regioisomers comprising pyrazolo[1,5-a]pyrimidine derivatives was designed and synthesized. The synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, ESI-MS and IR. The compounds were tested for their in vitro anti-proliferative activity by MTT assay. All four derivatives exhibited good anticancer activity in a HeLa cell line using paclitaxel as the reference standard. Compounds **6** and **9a** had IC_{50} values of 23 nM and 31 nM respectively, that were superior to that of paclitaxel (IC_{50}30 nM), which is used clinically as an anticancer agent.

The results from in vitro anticancer studies suggested these derivatives are very potent against the HeLa cervical cancer cell line. Further structure-activity relationship studies on these compounds are in progress in our laboratory.

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