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Synthesis and Evaluation of Antitumor Activities of Novel Fused Uracil Derivatives

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RESEARCH ARTICLE

ABSTRACT

A simple one-pot synthesis of indenopyrrolopyrimidines and indolopyrrolopyrimidines through the cyclocondensation reaction of 6-aminouracils and ninhydrin and/or isatin in the presence of catalytic amounts of glacial acetic acid were described. On the other hand, 6-aminouracils undergo nitrosation followed by reduction afforded 5,6-diaminouracil derivatives which used as a direct starting material for the synthesis of indenopteridines and indolopteridines via the reaction with ninhydrin and isatin respectively. All the new synthesized compounds have been characterized by elemental analyses, IR, ^{1}H-NMR spectra and Mass spectral studies. The new synthesized compounds were evaluated for antitumor activity against human hepatocellular carcinoma cell line (Hep-G2) as well as the half maximal inhibitory concentration (IC_{50}). Some compounds showed a potent antitumor activity.

INTRODUCTION

In recent years pyrimidine derivatives have received significant attention for their diverse range of biological properties, particularly being antibacterial, antifungal, antitubercular, antitumor and herbicidal [1-3]. Kidder and Virginia [4] have noticed that the biological activity of pyrimidine changes with substitution. On the other side, Isatin is a core constituent of many alkaloids [5] and drugs [6,7]. Literature surveys show that various derivatives of isatin possess diverse activities such as antibacterial [8], antifungal [9], antiviral [10], anti-HIV [11], anti-mycobacterial, anticancer [12], anti-inflammatory [13] and anticonvulsant activities [14]. Derivatives of indane are also of much significance because of their wide range of biological activities such as antimicrobial, anti-inflammatory, and antagonistic inhibition [15-22]. The condensation of ninhydrin and/ or isatin with o-diamines leads to indeno-and/ or indolo-derivatives respectively which are of considerable interest as biologically active substances.

In continuation to our interest in the chemical and biological activities of fused uracil derivatives, we report herein the synthesis of a new series of fused uracils starting from 6-aminouracil derivative [23] which have been converted into the nitroso-analog by nitrosation. The latter compound has often been used as the precursors for 5,6-diaminouracil. 6-aminouracil and 5,6-diaminouracil have been used as a direct starting material for the preparation of indenopteridines and indolopteridines which have been developed and their biological effects are determined.

RESULTS AND DISCUSSION

Chemistry

The C-2 position of ninhydrin which is situated between two other carbonyl groups is more reactive towards nitrogen, oxygen, sulfur and carbon-based nucleophiles [24]. On the other hand, isatin has two carbonyl groups situated at C-2 and C-3 positions. C-3 is more reactive center towards nucleophiles [25]. Based on these facts, indenopyrrolopyrimidines 4-6, indolopyrrolopyrimidines

7-9, indenopteridines 10-14 and indolopteridines 18, 19 are synthesized from the starting material 6-aminouracils 1 and 5,6-diaminouracils 3 respectively **(Scheme 1).**
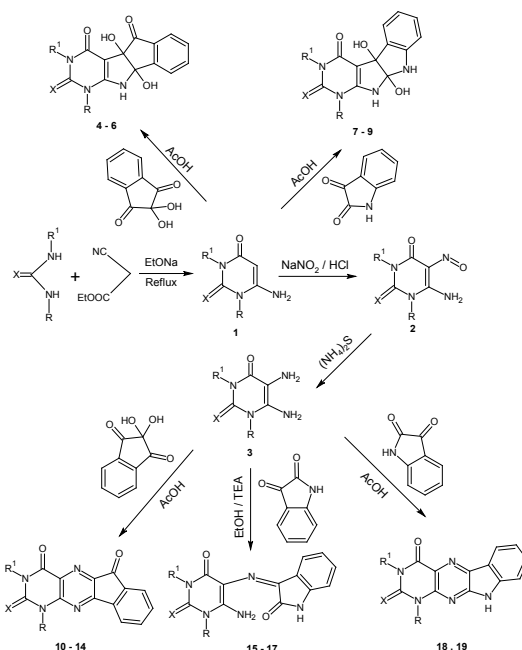
Compounds 4, 10, 16: X=O, R=R^1= H; compounds 5, 9, 11, 15, 20: X=O, R=CH$_3$, R^1= H; compounds 8, 13, 19: X=O, R= benz, R^1= H; compounds 6, 12, 17: X= S, R= CH$_3$, R^1= H; compounds 7, 14, 18: X=O, R= 2-Cl.benz, R^1= H.

Scheme 1. Reactions of 6-amino; 5,6-diaminouracil with ninhydrin and isatin.

The synthesis of dihydroxyindenopyrrolopyrimidine derivatives 4-6 based on the addition reaction occurred in the reaction of ninhydrin and 6-aminouracils 1 in the presence of acetic acid. The cyclization presumably occurred via the formation of nonisolable acyclic intermediate. The latter might be formed via the attack of the more nucleophilic carbon at 5-position of uracil to the more reactive center at position-2 of ninhydrin. Cyclization could be affected via the addition of the amino group to the carbonyl at position 1 of ninhydrin moiety affording the final product 4-6 as shown in **Scheme 2.**
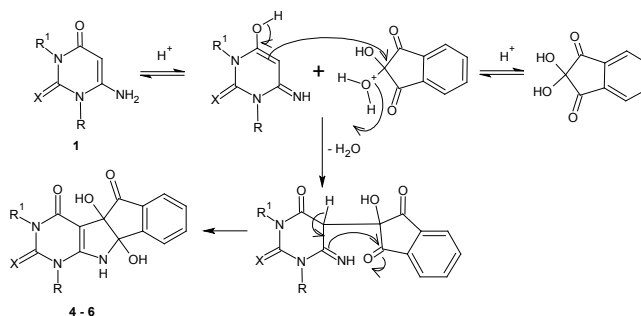
Scheme 2.

On the other hand, dihydroxyindolopyrrolopyrimidines 7-9 are obtained from the addition of isatin to 6-aminouracils in the presence of acetic acid. The formation of the nonisolated acyclic intermediate is based on the attack of the more nucleophilic carbon at 5-position of uracil to the more electrophilic center of isatin at position 3 followed by cyclization to afford the target compounds as shown in **Scheme 3.**
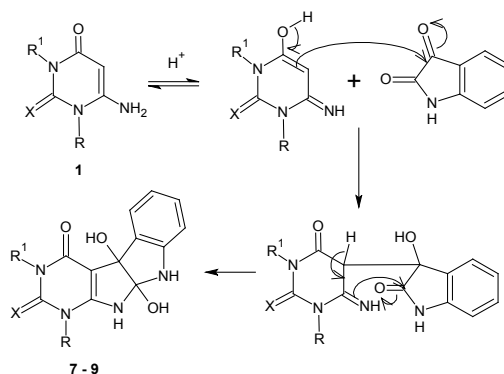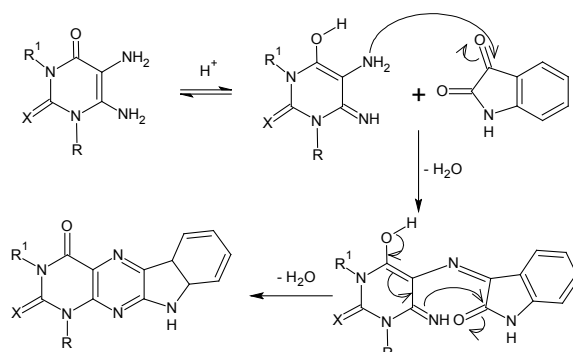

Scheme 3.

The structures of dihydroxyindenopyrrolopyrimidines 4-6 and dihydroxyindolopyrrolpyrimidines 7-9 were assigned on the basis of position of the hydroxyl groups and NH absorptions and the disappearance of NH_2 stretching in the IR spectra. Further support was given by ^{1}H-NMR spectra, which showed the integral band characterized for two OH groups of 4-6 around δ=6.89-6.56 ppm and δ= 6.08-5.82 ppm and around δ=11.11-10.89 and 10.73-10.66 ppm for 7-9. Also, the phenyl group obtained from isatin showed integral band around δ= 7.97-6.87 ppm. The disappearance of both NH_2 integral band around δ= 5.00-6.00 ppm at position C-5 and C-6 was further confirmed for the new compounds.

The reaction of ninhydrin with 5,6-diaminouracils 3 leads to the formation of indenopteridines 10-14 through the formation of the intermediate Schiff's base. The former reaction time (10-15 minutes) takes less time in comparison to isatin reaction time (2-3 hours). This fact can be explained by an increase in the positive charge on carbon atom at position 2 in ninhydrin due to elimination of H_2O in acidic medium which facilitates the nucleophilic attack of more basic amino group at this position as shown in **Scheme 4.**
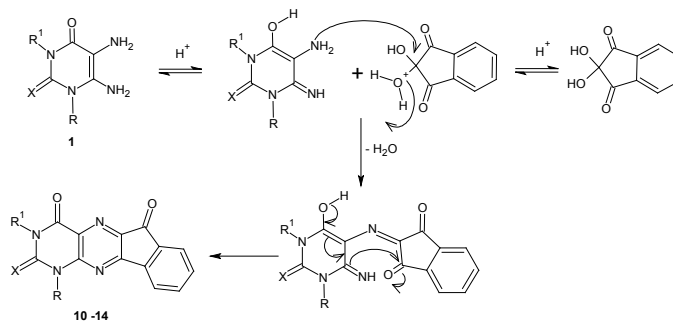
Scheme 4.

Refluxing of isatin with 5,6-diaminouracils 3 in ethanol in the presence of TEA involves the formation of Schiff's bases 15-17. On the basis of resonating structures of diaminouracils 3, the –NH_2 group at position C-5 is expected to be more basic attacking at the carbon atom at position 3 of isatin moiety. On the other hand, refluxing of the previous mixture in acetic acid instead of ethanol leads to isolation of indolopteridines 18, 19 via the intermediate formation of Schiff's bases followed by cyclodehydration, as shown in **Scheme 5.**

Scheme 5.

The structures of indenopteridines 10-14, indolopteridines 18, 19 were assigned on the basis of position of NH and carbonyl absorptions and the disappearance of NH_2 band in the IR spectra. Further support was given by ^{1}H-NMR spectra, which showed the intergral band around δ=8.23-7.14 ppm which is characteristic for phenyl group obtained from isatin. The disappearance of both NH_2 intergral band around δ=5.00-6.00 ppm at position C-5 and C-6 was further confirmed for the new compounds.

Biological investigation

Cytotoxic activity: The *in vitro* growth inhibitory rates (%) and inhibitory growth activity (as measured by IC_{50}) of the synthesized compounds were investigated in comparison with the well-known anticancer standard drugs 5-flourouracil (5-FU) and Imatinib (2-substituted aminopyrimidine derivative; Gleevec®), using crystal violet colorimetric viability assay. Data generated were used to plot dose response curves and presented in **Tables 1 and 2 and Figure 1 (A and B).** The results revealed that all the tested compounds showed high variation in the inhibitory growth rates and activities to the tumor cell line in a concentration dependent manner as shown in **Tables 1 and 2.**

Table 1. Growth inhibition rates of HepG2 cell line with compounds 4-6, 9, 10, 12, 14, 17-19 with different concentrations.

Compounds	Growth inhibition rates (%)								P value
	Compound concentration (µM)								
	0.062	0.125	0.25	0.5	1	2	4	8	
4	0	0	1.68	9.53	18.44	32.58	53.41	68.35	<0.001*

5	0	0.86	2.13	8.52	14.07	22.85	39.18	57.3	<0.001
6	3.62	12.85	20.58	32.79	45.16	61.84	75.03	84.57	<0.001
9	6.26	11.84	20.48	29.57	40.35	56.18	68.05	79.64	<0.001
10	1.15	3.41	7.57	16.33	31.09	44.68	54.86	63.03	<0.001
13	3.96	10.68	25.39	35.11	50.48	65.62	78.27	89.51	<0.001
14	0	0	0	0	0.98	2.82	10.59	26.64	<0.001*
17	0	1.28	5.82	13.54	21.88	34.97	62.59	71.81	<0.001
18	0	0	1.21	3.92	10.25	28.46	43.83	59.18	<0.001*
19	4.17	7.63	18.41	26.82	49.53	60.22	79.36	87.15	<0.001

Significant value: $P \leq 0.01$, *Chi- Square test conducted among inhibition rates of active concentrations only.

Table 2. The IC_{50} values represent the compound concentration (μM) required to inhibit tumor cell proliferation by 50%.

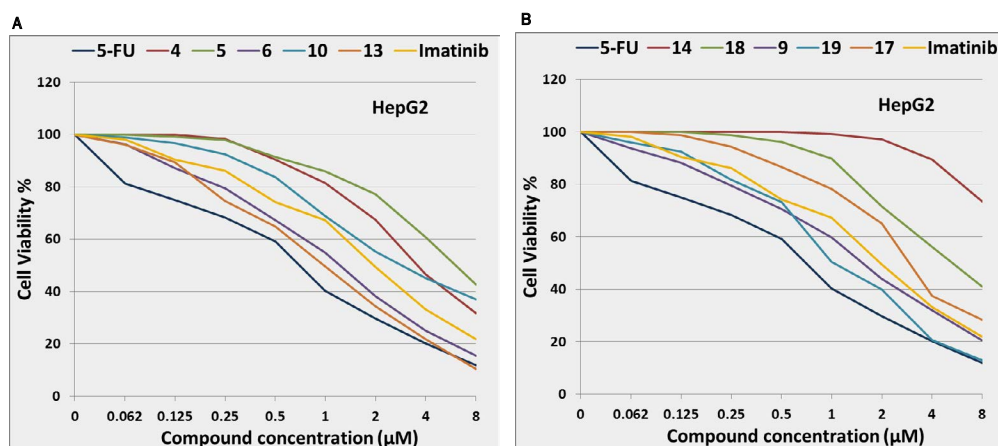| Compounds	IC$_{50}$ (μM)	Compounds	IC$_{50}$ (μM)
4	3.67± 0.15	13	0.98 ± 0.11
5	7.04 ± 0.22	14	>8
6	1.29 ± 0.07	17	3.09 ± 0.18
9	1.61 ± 0.13	18	5.61 ± 0.35
10	3.05 ± 0.19	19	1.04 ± 0.12
Imatinib	1.8 ± 0.13	5-FU	0.76 ± 0.14

Figure 1. Growth inhibition rates of HepG2 cell line: A) treated with compounds 4-6, 10, and 13 with different concentrations compared with reference drug imatinib and 5-FU. B) treated with compounds 9, 14, 17-19 with different concentrations compared with reference drug imatinib and 5-FU.

The results showed that compound 6 have good inhibitory activities with IC_{50} value of 1.29 μM. From the results in **Figure 1A**, it is clear that compounds 4, 6 and 13 were found to be very active at 8 μM against human hepatocellular carcinoma (HepG2) cell line with inhibition ratio values between 70% and 90%.

The highest activity against human hepatocellular carcinoma (HepG2) cell line was measured for compound 13 with IC_{50} value 0.98 μM, compared with reference drug imatinib. However, compound 14 was almost inactive.

From the results in **Figure 1B**, it is clear that compounds 9, 17-19 were also found to be active at 8 μM against human hepatocellular carcinoma (HepG2) cell line with inhibition ratio values 87.15, 79.64, 71.81 and 59.18%, respectively.

The highest detected inhibitory activities against human hepatocellular carcinoma (HepG2) cell line was measured for compound 19 compared with reference drug imatinib followed by 9, then 17 with IC_{50} values of 1.04, 1.61 and 3.09 μM, respectively. The difference between inhibitory activities of all compounds with different concentrations was statistically significant P<0.001.

EXPERIMENTAL SECTION

General

All melting points were determined with an Electro thermal Mel.-Temp. II apparatus and are uncorrected. Element analyses were performed at Regional Center for Mycology and Biotechnology at Al-Azhar University. The infrared (IR) spectra were recorded using potassium bromide disc technique on Nikolet IR 200 FT IR. Mass spectra were recorded on DI-50 unit of Shimadzu GC/MS-QP 5050A at the Regional Center for Mycology and Biotechnology at Al-Azhar University. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on Bruker 400 MHz Spectrometer using DMSO-d$_6$ as a solvent, Applied Nucleic Acid Research Center, Zagazig University, Egypt. All reactions were monitored by TLC using precoated plastic sheets silica gel (Merck 60 F$_{254}$) and spots were visualized by irradiation with UV light (254 nm). The used solvent system was chloroform: methanol (9:1) and ethyl acetate: toluene (1:1).

4b, 9b-Dihydroxy-1-Substituted-9b, 10-indeno[2′, 1′:4,5]pyrrolo[2, 3-d]pyrimidine-2, 4, 5 (1H, 3H, 4bH)-triones4, 5) and 4b, 9b-Dihydroxy-1-methyl-9b, 10-dihydroindeno[2′,1′:4,5]pyrrolo[2, 3-d]pyrimidine-2(1H)-thio- 4, 5,(3H, 4bH)-dione (6).

A mixture of 6-amino-1-substituted-uracils (1) (1.2 mmol) and ninhydrin (1.2 mmol) in acetic acid (5 ml) was heated under reflux for 10-15 minutes. The formed precipitate was filteredon hot, washed with ethanol and crystallized from DMF/ethanol (1:3).

4b,9b-Dihydroxy-9b,10-dihydroindeno[2′,1′:4,5]pyrrolo[2,3-d]pyrimidine-2,4,5(1H,3H,4bH)-trione (4): Yield: 73%, m.p.=>300°C. IR=3300 (br, OH), 3210 (NH), 3064 (CH arom.), 2982 (CH aliph.), 1707, 1649 (C=O), 1593 (C=C). MS: m/z (%) =287 (M$^+$, 20), 283 (27), 271 (28), 245 (24), 181 (59), 168 (38), 125 (100), 105 (47), 76 (60). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 11.14 (s, 1H, NH), 10.05 (s, 1H, NH), 8.70 (s, 1H, NH), 7.97 – 7.91 (d, 1H, arom.), 7.81-7.77 (m, 1H, arom.), 7.68-7.66 (d, 1H, arom.), 7.57-7.53 (m, 1H, arom.), 6.56 (s, 1H, OH), 5.82 (s, 1H, OH). Anal. Calcd for C$_{13}$H$_9$N$_3$O$_5$ (287.22), Calcd.: C 54.36, H 3.16, N 14.63, Found: C 54.52, H 3.21, N 14.79.

4b,9b-Dihydroxy-1-methyl-9b,10-dihydroindeno[2′,1′:4,5]pyrrolo[2,3-d]pyrimidine-2,4,5(1H,3H,4bH)- trione (5): Yield: 79%, m.p.=>300°C. IR=3442, 3393 (br, OH), 3250, 3219 (NH), 3051 (CH arom.), 2990 (CH aliph.), 1700,1654 (C=O), 1549 (C=C). MS: m/z (%) =301 (M$^+$, 6), 283 (M –H$_2$O, 9), 244 (17), 168 (41), 104 (100), 76 (88). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 10.33 (s, 1H, NH), 9.24 (s, 1H, NH), 7.91 – 7.89 (d, 1H, arom.), 7.83-7.79 (m, 1H, arom.), 7.69-7.67 (d, 1H, arom.), 7.58-7.57 (m, 1H, arom.), 6.74 (s, 1H, OH), 5.89 (s, 1H, OH), 3.12 (s, 3H, CH$_3$). Anal. Calcd for C$_{14}$H$_{11}$N$_3$O$_5$ (301.25), Calcd.: C 55.82, H 3.68, N 13.95, Found: C 55.97, H 3.74, N 14.04.

4b,9b-Dihydroxy-1-methyl-9b,10-dihydroindeno[2′,1′:4,5]pyrrolo[2,3-d]pyrimidine-2(1H)-thio-4,5(3H,4bH)-dione (6): Yield: 71%, m.p.=>300°C. IR=3400 (br, OH), 3198 (NH), 3052 (CH arom.), 2959 (CH aliph.), 1695, 1647 (C=O), 1537 (C=C). MS: m/z (%) =317 (M$^+$, 31), 315 (6), 299 (M –H$_2$O,25), 256 (24), 240 (20), 224 (32), 192 (46), 184 (40), 160 (51), 128 (35), 104 (100), 76 (96). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 11.84 (s, 1H, NH), 9.43 (s, 1H, NH), 7.93 – 7.91 (d, 1H, arom.), 7.85-7.81 (m, 1H, arom.), 7.71-7.69 (d, 1H, arom.), 7.60-7.56 (m, 1H, arom.), 6.89 (s, 1H, OH), 6.08 (s, 1H, OH), 3.53 (s, 3H, CH$_3$). Anal. Calcd for C$_{14}$H$_{11}$N$_3$O$_4$S (317.32), Calcd.: C 52.99, H 3.49, N 13.24, Found: C 53.08, H 3.53, N 13.37.

Dihydroxy-1-substituted-indolopyrrolopyrimidine (7-9): A mixture of 6-amino-1-substituted-uracil (1) (1.2 mmol) and isatin (1.2 mmol) in acetic acid (5 ml) was heated under reflux for 2-2.30 hours. The formed precipitate was filteredon hot, washed with ethanol and crystallized from DMF/ethanol (1:3).

1-(2-chlorobenzyl)-4b,9a-dihydroxy-4b,9,9a,10-tetrahydropyrimido[5′,4′:4,5]pyrrolo-[2,3-b]indolo-2,4(1H,3H)-dione (7): Yield: 66%, m.p. >300°C. IR=3406 (OH), 3177 (NH), 3055 (CH arom.), 2808 (CH aliph.), 1710, 1652 (C=O), 1526 (C=C), 754 (o-substituted phenyl). MS: m/z (%) =400 (M$^+$2, 1), 398 (M$^+$, 2), 380 (M–H$_2$O, 2), 342 (72), 271 (14), 181 (31), 125 (100), 102 (24), 76 (14). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 11.69 (s, 1H, NH), 11.20 (s, 1H, NH), 11.01 (s,1H, OH), 10.66 (s,1H, OH), 9.42 (s,1H, NH), 7.56 – 6.97 (m, 8H, arom.), 5.39-5.24 (dd, 2H, NCH$_2$). Anal. Calcd for C$_{19}$H$_{15}$ClN$_4$O$_4$ (398.79), Calcd.: C 57.22, H 3.79, N 14.05, Found: C 57.43, H 3.81, N 14.13.

1-benzyl-4b,9a-dihydroxy-4b,9,9a,10-tetrahydropyrimido[5′,4′:4,5]pyrrolo-[2,3-b]indolo-2,4(1H,3H)-dione (8): Yield: 68%, m.p.=>300°C. IR=3579, 3339 (OH), 3154 (NH), 3065 (CH arom.), 2860 (CH aliph.), 1720, 1654 (C=O), 1527 (C=C). MS: m/z (%) =364 (M$^+$, 5), 348 (4), 302 (7), 276 (9), 119 (100), 106 (19), 92 (60). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 11.95 (s, 1H, NH), 11.69 (s, 1H, NH), 11.11 (s,1H, OH), 10.89 (s,1H, OH), 9.39 (s, 1H, NH), 7.40 – 6.87 (m, 9H, arom.), 5.42 – 5.27 (dd, 2H, NCH$_2$). Anal. Calcd for C$_{19}$H$_{16}$N$_4$O$_4$ (364.35), Calcd.: C 62.63, H 4.43, N 15.38, Found: C 62.80, H 4.49, N 15.47.

4b,9a-dihydroxy-1-Methyl-4b,9,9a,10-tetrahydropyrimido[5′,4′:4,5]pyrrolo-[2,3-b]indolo-2,4(1H,3H)-dione (9): Yield: 59%, m.p.=>300°C. IR=3524, 3385 (OH), 3159 (NH), 3025 (CH arom.), 2939, 2846 (CH aliph.), 1746, 1688 (C=O), 1592 (C=C). MS: m/z (%) =288 (M$^+$, 8), 270 (M–H$_2$O, 8), 188 (9), 119 (52), 92 (36), 61 (100). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 11.95 (s, 1H, NH), 11.48 (s, 1H, NH), 10.89 (s,1H, OH), 10.73 (s,1H, OH), 9.25 (s, 1H, NH),7.26-6.93 (m, 4H, arom.), 3.42 (s, 3H, CH$_3$). Anal. Calcd for C$_{13}$H$_{12}$N$_4$O$_4$ (288.26), Calcd.: C 54.17, H 4.20, N 19.44. Found: C 54.25, H 4.26, N 19.58.

1-Substituted-2H-indeno[2,1-g]pteridine-2,4,6-(1H,3H)-triones(10-14): A mixture of 5,6-diamino-1-substituteduracil (3) (1.00 mmol) and ninhydrin (1.00 mmol) in acetic acid (5 ml) was heated under reflux for 10-15 minutes. The formed precipitate was filteredon hot, washed with ethanol and crystallized from DMF/ethanol (1:3).

2H-Indeno[2,1-g]pteridine-2,4,6-(1H,3H)-trione (10): Yield: 81%, m.p.=>300°C. IR=3360, 3193 (NH), 3089 (CH arom.), 2813 (CH aliph.), 1714, 1642 (C=O), 1562 (C=C). MS: m/z (%) =266 (M$^+$, 1), 239 (27), 182 (8), 154 (13), 135 (100), 98 (59), 76 (25), 74(52). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 11.35 (s, 1H, NH), 10.29 (s, 1H, NH), 7.87 – 7.81 (m, 3H, arom.), 7.74-7.70 (m, 1H, arom). Anal. Calcd for C$_{13}$H$_6$N$_4$O$_3$ (266.21), Calcd.: C 58.65, H 2.27, N 21.05. Found: C 58.83, H 2.24, N 21.19.

1-Methyl-2H-indeno[2,1-g]pteridine-2,4,6-(1H,3H)-trione (11): Yield: 79%, m.p.=>300°C. IR=3361, 3176 (NH), 3071 (CH arom.), 2827 (CH aliph.), 1684, 1568 (C=O), 1500 (C=C). MS: m/z (%) =280 (M$^+$, 97), 209 (66), 182 (100), 154 (51), 130 (32), 102 (28), 76 (23). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 10.54 (s, 1H, NH), 7.99 – 7.97 (d, 1H, arom.), 7.86-7.82 (m, 2H, arom.), 7.74 – 7.71 (m, 1H, arom.), 3.59 (s, 3H, CH$_3$). Anal. Calcd for C$_{14}$H$_8$N$_4$O$_3$ (280.24), Calcd.: C 60.00, H 2.88, N 19.99, Found: C 60.14, H 2.91, N 20.04.

1-Methyl-2H-indeno[2,1-g]pteridine-2-thio-4,6-(1H,3H)-dione (12): Yield: 82%, m.p. >300 °C. IR=3326, 3210 (NH), 3093 (CH arom.), 2900 (CH aliph.), 1702, 1599 (C=O), 1567 (C=C). MS: m/z (%) =296 (M$^+$, 65), 280 (15), 236 (35), 209 (34), 182 (100), 154 (77), 126 (54), 102 (55), 75 (30). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 12.22 (s, 1H, NH), 8.03 – 7.73 (m, 4H, arom.), 3.68 (s, 3H, CH$_3$). Anal. Calcd for C$_{14}$H$_8$N$_4$O$_2$S (296.30), Calcd.: C 56.75, H 2.72, N 18.91, Found: C 56.89, H 2.70, N 19.07.

1-Benzyl-1H-indeno[2,1-g]pteridine-2,4,6(3H)-trione (13): Yield: 77%, m.p.=>300 °C. IR=3390, 3168 (NH), 3024 (CH arom.), 2843 (CH aliph.), 1692, 1601 (C=O), 1563 (C=C). MS: m/z (%) =356 (M$^+$, 18), 284 (11), 232 (13), 230 (17), 104 (8), 91 (100). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 10.21 (s, 1H, NH), 7.95 – 7.93 (d, 1H, arom.), 7.84 – 7.80 (m, 2H, arom.), 7.73 – 7.69 (m, 1H, arom.), 7.50 – 7.48 (d, 2H, arom.), 7.34 – 7.25 (m, 3H, arom.), 5.44 (s, 2H, NCH$_2$). Anal. Calcd for C$_{20}$H$_{12}$N$_4$O$_3$ (356.33), Calcd.: C 67.41, H 3.39, N 15.72, Found: C 67.59, H 3.45, N 15.81.

1-(2-Chlorobenzyl)methyl-2H-indeno[2,1-g]pteridine-2,4,6-(1H,3H)-trione (14): Yield: 83%, m.p. >300 °C. IR=3303, 3175 (NH), 3059 (CH arom.), 2842 (CH aliph.), 1695, 1659 (C=O), 1557 (C=C), 751 (o-substituted phenyl). MS: m/z (%) =392 (M+2, 1.63), 390 (M+, 0.62), 355 (100), 318 (9), 284 (11), 154 (11), 125 (94), 102 (20), 76 (21). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 10.52 (s, 1H, NH), 7.82 – 7.69 (m, 2H, arom.), 7.53 – 7.50 (m, 1H, arom.), 7.34 – 7.22 (m, 5H, arom.), 5.49 (s, 2H, NCH$_2$). Anal. Calcd for C$_{20}$H$_{11}$ClN$_4$O$_3$ (390.78), Calcd.: C 61.47, H 2.84, N 14.34, Found: C 61.61, H 2.83, N 14.51.

6-Amino-1-substituted-5-{[(3Z)-2-oxo-1,2-dihydro-3H-indol-3-ylidene]amino}pyrimidine-2,4(1H,3H)-diones (15-17): A mixture of 5,6-diamino-1-substituteduracils (3) (1.00 mmol) and isatin (1.00 mmol) in ethanol (20 ml) in the presence of TEA as a base was heated under reflux for 4-5 hours. The formed precipitate was filtered on hot, washed with ethanol and crystallized from DMF/ethanol (1:3) afforded the desired compounds 15-17.

6-Amino-1-methyl-5-{[(3Z)-2-oxo-1,2-dihydro-3H-indol-3-ylidene]amino}pyrimidine-2,4(1H,3H)-dione (15): Yield: 85%, m.p.=>300 °C. IR=3349, 3309, 3260, 3146 (NH$_2$ and NH), 3054 (CH arom.), 2846 (CH aliph.), 1693, 1634 (C=O), 1575 (C=C). MS: m/z (%) =285 (M$^+$,1.13), 267 (M-H$_2$O, 1.15), 156 (100), 139 (46), 98 (15), 84 (21), 57 (51). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 11.78 (s, 1H, NH), 10.54 (s, 1H, NH), 8.22 – 8.20 (d, 1H, arom.), 7.63 – 7.56 (m, 2H, arom.), 7.42 – 7.38 (m, 1H, arom.), 6.11 (s, 2H, NH$_2$), 3.56 (s, 3H, CH$_3$). Anal. Calcd for C$_{13}$H$_{11}$N$_5$O$_3$ (285.25), Calcd.: C 54.74, H 3.89, N 24.55, Found: C 54.91, H 3.94, N 24.67.

6-Amino-5-{[(3Z)-2-oxo-1,2-dihydro-3H-indol-3-ylidene]amino}pyrimidine-2,4(1H,3H)-dione (16): Yield: 88%, m.p.=>300 °C. IR=3459, 3407, 3360, 3194 (NH$_2$ and NH), 3093 (CH arom.), 2842 (CH aliph.), 1704, 1647 (C=O), 1564 (C=C). MS: m/z (%) =271 (M$^+$, 3), 253 (M-H$_2$O, 6), 266 (20), 142 (100), 97 (67), 71 (32), 43 (50). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 12.37 (s, 1H, NH), 10.90 (s, 1H, NH), 10.26 (s, 1H, NH), 8.22 – 8.20 (d, 1H, arom.), 7.69 – 7.35 (m, 3H, arom.), 5.54 (s, 2H, NH$_2$). Anal. Calcd for C$_{12}$H$_9$N$_5$O$_3$ (271.23), Calcd.: C 53.14, H 3.34, N 25.82, Found: C 53.42, H 3.23, N 26.07.

(3Z)-3-[(6-Amino-1-methyl-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-yl)imino]-1,3-dihydro-2H-indol-2-one (17): Yield: 87%, m.p.=>300 °C. IR=3315, 3292, 3150 (NH$_2$ and NH), 3098 (CH arom.), 2942 (CH aliph.), 1694, 1623 (C=O), 1570 (C=C). MS: m/z (%) =301 (M$^+$, 2.24), 283 (M- H$_2$O, 3), 239 (9), 185 (16), 147 (67), 132 (16), 129 (29), 119 (100), 104 (33), 98 (36), 97 (26), 92 (82), 76 (44), 57 (16), 55 (28). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 11.89 (s, 1H, NH), 10.20 (s, 1H, NH), 8.30–8.28 (d, 1H, arom.), 7.78 – 7.37 (m, 3H, arom.), 6.19 (s, 2H, NH$_2$), 3.73 (s, 3H, CH$_3$). Anal. Calcd for C$_{13}$H$_{11}$N$_5$O$_2$S (301.32), Calcd.: C 51.82, H 3.68, N 23.24, Found: C 51.98, H 3.71, N 23.43.

1-Substituted-indolopteridine (18-20)

Method A: A mixture of 5,6-diamino-1-substituteduracil (3) (1.00 mmol) and isatin (1.00 mmol) in acetic acid (5 ml) was heated under reflux for 2-3 hours. The formed precipitate was filtered on hot, washed with ethanol and crystallized from DMF/ethanol (1:3) to give 18 and 19.

Method B: Compound 15 (1.00 mmol) in acetic acid (5 ml) was heated under reflux for 2 hours. The formed precipitate was filtered on hot, washed with ethanol and crystallized from DMF/ethanol (1:3) to give 20.

1-(2-Chlorobenzyl-1,10-dihydro-2H-indolo[3,2-g]pteridine-2,3(3H)-dione (18): Yield: 68% (method A), m.p.=>300 °C. IR=3250, 3183 (NH), 3050 (CH arom.), 2814 (CH aliph.), 1705, 1638 (C=O), 1562 (C=C), 756 (o-substituted phenyl). MS: m/z (%) =379 (M+2, 12), 377 (M$^+$, 17), 361 (M-H$_2$O, 25), 287 (19), 166 (23), 104 (21), 63 (27), 51 (100). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 12.16 (s, 1H, NH), 10.79 (s,1H, NH), 8.23 – 8.21 (d, 1H, arom.), 7.60 – 7.14 (m, 7H, arom.), 5.41 (s, 2H, NCH$_2$). Anal. Calcd for C$_{19}$H$_{12}$ClN$_5$O$_2$ (377.78), Calcd.: C 60.41, H 3.20, N 18.54, Found: 60.53, H 3.18, N 18.71.

1-benzyl-1,10-dihydro-2H-indolo[3,2-g]pteridine-2,3(3H)-dione (19): Yield: 65% (method A), m.p.=>300 °C. IR=3270, 3136 (NH), 3029 (CH arom.), 2920 (CH aliph.), 1712, 1642 (C=O), 1533 (C=C). MS: m/z (%) =343 (M$^+$, 8), 271 (10), 181 (15),125 (12), 102 (12), 91 (100). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 12.63 (s, 1H, NH), 11.89 (s, 1H, NH), 8.21 – 8.20 (d, 1H, arom.), 7.63 – 7.59 (m, 1H, arom.), 7.54 – 7.52 (d, 1H, arom.), 7.40 – 7.21 (m, 6H, arom.), 5.43 (s, 2H, NCH$_2$). Anal. Calcd for C$_{19}$H$_{13}$N$_5$O$_2$ (343.34), Calcd.: C 66.47, H 3.82, N 20.40, Found: C 66.63, H 3.79, N 20.62.

1,10-Dihydro-1-methyl-2H-indolo[3,2-g]pteridine-2,3(3H)-dione (20): Yield: 82% (method B), m.p.=>300 °C. IR=3265, 3140 (NH), 3044 (CH arom.), 2890(CH aliph.), 1698, 1635 (C=O), 1558 (C=C). MS: m/z (%) =267 (M$^+$, 99), 196 (100), 181 (24),156 (73), 102 (11), 58 (47). ^{1}H-NMR (DMSO-d$_6$) δ ppm: 11.89 (s, 1H, NH), 11.54 (s, 1H, NH), 8.18 – 7.35 (m, 4H, arom.), 3.55(s, 3H, CH$_3$). Anal. Calcd for C$_{13}$H$_9$N$_5$O$_2$ (267.24), Calcd.: C 58.43, H 3.39, N 26.21, Found: C 58.70, H 3.43, N 26.35.

Biological investigation

Evaluation of the antitumor activity

Mammalian cell lines: The cell line that used in this study was human hepatocellular carcinoma cell line (HepG2 cells) was obtained from tissue culture Unit, VACSERA, Cairo, Egypt. The mammalian cells were propagated in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 depending on the type of cell line supplemented with 10% heat-inactivated fetal bovine [26,27] serum, 1% L-glutamine, HEPES buffer and 50 μg/ml gentamycin. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured two times a week along experimentation.

(I) Antitumor activity evaluation using viability assay: Antitumor activity assay was carried according to the method described literature [28]. All the experiments concerning the cytotoxicity evaluation were performed and analyzed by tissue culture unit at the Regional Center for Mycology and Biotechnology RCMB, Al-Azhar University, Cairo, Egypt.

(II) Procedure: The tumor cell lines were seeded in 96-well plate in 100 μl of growth medium at a cell concentration of 1 × 10^4 cells per well. After 24 h of seeding, the monolayers were then washed with sterile phosphate buffered saline (0.01 M, pH 7.2) and simultaneously the cells were treated with 100 μl from different dilutions of the test sample in fresh maintenance medium and incubated at 37°C. Different two-fold dilutions of the tested compound (8, 4. 2, 1, 0.5, 0.25, 0.125 and 0.062 μM) were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO_2 for a period of 24 h. Untreated cells were served as controls. Three independent experiments were performed each containing six replicates for each concentration of the tested samples. The cytotoxic effects of the tested compounds were then measured using crystal violet staining viability assay. Briefly, after 24h of treatment, the medium was removed, 100 μL of 0.5% of crystal violet in 50% methanol was added to each well and incubated for 20 minutes at room temperature and subsequently excess dye was washed out gently by distilled water. The plate was allowed to dry then the viable crystal violet-stained cells were lysed using 33% glacial acetic acid solution. Absorbance at 570 nm was then measured in each well using microplate reader (SunRise, TECAN, Inc, USA). 5-fluorouracil and imatinib were used as positive control. The absorbance is proportional to the number of surviving cells in the culture plate. Thus, using this colorimetric procedure, the tested compounds-mediated cell lysis and the cytotoxic effect of 5-FU and imatinib (used as a positive control) were measured and compared to the viability of untreated cells. Because the stock solutions to prepare the different concentrations from the tested compounds were solubilized in DMSO, controls with DMSO alone were performed in parallel for each concentration.

Data analysis: The percentage cell viability was calculated using the Microsoft Excel®. Percentage cell viability was calculated as follows: according to the following calculation: the percentage of cell viability=[1−(ODt/ODc)] × 100%, where ODt is the mean optical density of wells treated with the tested compound and ODc is the mean optical density of untreated cells. The test compounds were compared using the IC_{50} value, i.e., the concentration of an individual compound leading to 50% cell death that was estimated from graphical plots of surviving cells versus compound concentrations.

CONCLUSIONS

In summary, new compounds indenopyrrolopyrimidines, indolopyrrolopyrimidines, indenopteridines and indolopteridineshave beensynthesized and their *in vitro* anticancer activities against **HepG2 cell line** have been evaluated. These compounds exhibited good anticancer activities towards **HepG2 cell line**. Compounds 13 and 19 with an IC_{50} of 0.98 μM and 1.04 μM respectively were found to be the most active.

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