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Original article

Synthesis and cytotoxicity evaluation of substituted pyridazino[4,5-*b*] phenazine-5,12-diones and tri/tetra-azabenzofluorene-5,6-diones

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Abstract

The substituted pyridazino[4,5-*b*]phenazine-5,12-diones and tri/tetra-azabenzo[*a*]fluorene-5,6-diones were synthesized from 6,7-dichlorophthalazine-5,8-dione and 6,7-dichloroquinoline-5,8-dione, respectively. The cytotoxic activities of the prepared compounds were evaluated by an SRB (Sulforhodamine B) assay against the following human cancer cell lines: A549 (lung), SK-OV-3 (ovarian), SK-MEL-2 (melanoma), XF 498 (CNS), and HCT 15 (colon). Almost all synthesized pyridazino[4,5-*b*]phenazine-5,12-diones (**7a–j**) presented higher cytotoxicity than that of doxorubicin ($IC_{50} = 0.097–0.225$ μM) against the cancer cell lines. In particular, the cytotoxicity of compounds **7f** (R_1 = Et) and **7h** (R_1, R_2 = Me) against all human cancer cell lines examined was about 10 times higher than that of doxorubicin. However, the cytotoxicities of several synthesized azabenzo[*a*]fluorene-5,6-diones (**12a**, **12c**, **12d**, **12e**, and **12g**) against the cancer cell lines *in vitro* were comparable to those of doxorubicin.

Keywords: Pyridazino[4,5-*b*]phenazine-5,12-diones; Azabenzo[*a*]fluorene-5,6-diones; Cytotoxicity

1. Introduction

The quinone moiety is involved in a wide variety of biochemical processes including electron transport and oxidative phosphorylation [1]. Various biological properties including enzyme inhibition, antibacterial, antifungal, and anticancer activities have been reported in quinones and quinone derivatives [2–9]. The antitumor activity of the quinone moiety has been thoroughly studied and it is known that they act as topoisomerase inhibitors via the DNA-intercalation [10–12] and the reduction of the quinone moiety by DT-diaphorase (quinone oxidoreductase) [13–15]. The intercalation of planar aromatic molecules with a DNA double helix has been known to be important in the medicinal action of antineoplastic drugs. Upon DNA intercalative binding, a separation between base pairs increases from 3.4 Å to 6–8 Å to accommodate a planar molecule and the intercalation is stabilized by hydrogen bond between the chromophore and the intercalation site base pair [16,17]. The antitumor activity of nitrogen containing heterocyclic quinones such as benzophenazinediones **1**, pyridophenazinediones **2**, quinoxalino[2,3-*b*]phthalazinediones **3** and triazabenzo[3,2-*a*]fluorene-5,6-diones **4** has been reported in our previous papers (Fig. 1) [18–20]. In our continuous effort to develop novel anticancer agents based on nitrogen containing heterocyclic quinones, a series of substituted pyridazino[4,5-*b*]phenazine-5,12-dione (**7a–j**), tetracyclic heteroquinone analogues with four nitrogen atoms were synthesized in this study and their cytotoxicity was compared with that of previously reported compounds which have the substituents in 8 position.

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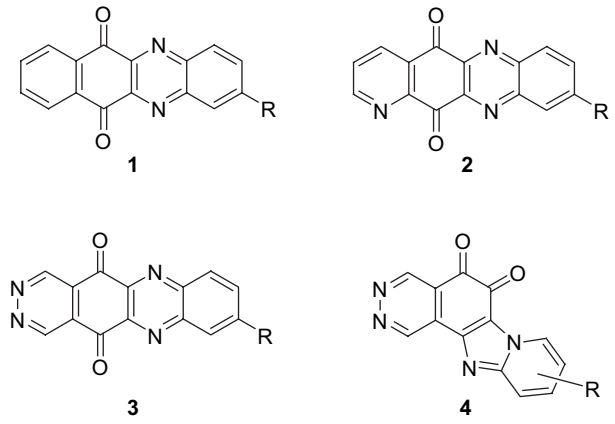


Fig. 1. The structures of nitrogen containing heterocyclic quinones.

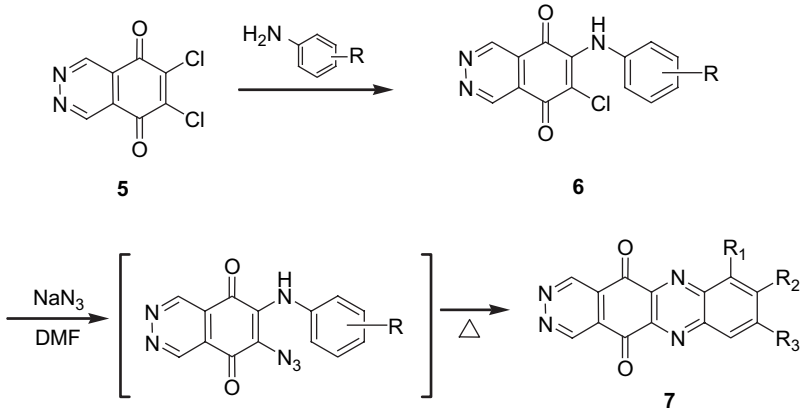
Also a series of tri/tetra-azabenzo[*a*]fluorene-5,6-dione (**12a–g**), tetracyclic quinone analogues with three or four nitrogen atoms were synthesized and evaluated in this study.

2. Results and discussion

2.1. Synthesis

2.1.1. Synthesis of substituted pyridazino [4,5-b]phenazine-5,12-diones (7)

6,7-Dichlorophthalazine-5,8-dione **5** [21] was reacted with various *ortho* or *para*-substituted arylamines (such as *p*-aminobenzotrifluoride, *p*-trifluoromethoxyaniline, *N*,*N*-dimethyl-*p*-phenylenediamine, *o*-toluidine, *o*-anisidine, *o*-ethylaniline, *o*-ethoxyaniline, 3,4-dimethylaniline, 2,4-dimethoxyaniline and 3,4-(methylenedioxy) aniline) at room temperature to give 6-arylamino-7-chlorophthalazine-5,8-diones **6a–j**, a yield of 61–85%. The synthesized 6-arylamino-7-chlorophthalazine-5,8-diones **6a–j** were reacted with sodium azide in *N*,*N*-dimethylformamide (DMF) at 90–95 °C, which occurred *via* substitution of azide for chlorine *in situ* and an intramolecular cyclization, to yield the substituted pyridazino[4,5-*b*] phenazine-5,12-dione derivatives **7a–j** in 18–28% yield. Only α-substituted isomers (**7d–g**) are formed from the *ortho*-substituted arylamines and the *para*-substituted arylamines give the β-substituted ones (**7a–c**), while both regioisomers are formed from *meta*-substituted arylamines. On the other hand, 3,4-disubstituted arylamines are able to produce two regioisomeric cyclization compounds. The structures of regioisomers obtained from disubstituted compounds (**7h** and **7j**) were identified by the positions of protons on unsubstituted carbons (C-7 to C-10) on ^{1}H NMR. 7,8-Dimethylpyridazino[4,5-*b*]phenazine-5,12-dione **7h** showed 7.71 ppm (C-9) and 8.33 ppm (C-10) while 8,9-methylenedioxypyridazino[4,5-*b*]phenazine-5,12-dione **7j** showed 7.80 ppm (C-7 and C-10). The overall reaction strategy is outlined in Scheme 1 [19]. 6-Arylamino-7-chlorophthalazine-5,8-diones **6** obtained from the *ortho*-substituted arylamines showed lower yield than *para*-analogues due to steric hindrance. As the substituent size increased, the reaction time also increased. When **5** was treated with arylamine in refluxing ethanol, a by-product, in which both chlorine atoms were replaced by arylamine, was formed as well as in addition to the desired compounds **6**. In the reaction of 6-arylamino-7-chlorophthalazine-5,8-diones **6** with sodium azide, in either ethanol or dioxane, the attempts to synthesize **7** were unsuccessful even after 3 days of past leaving almost all the starting material **6** intact. In DMF, at low temperatures (60–70 °C), compound **7** was also not



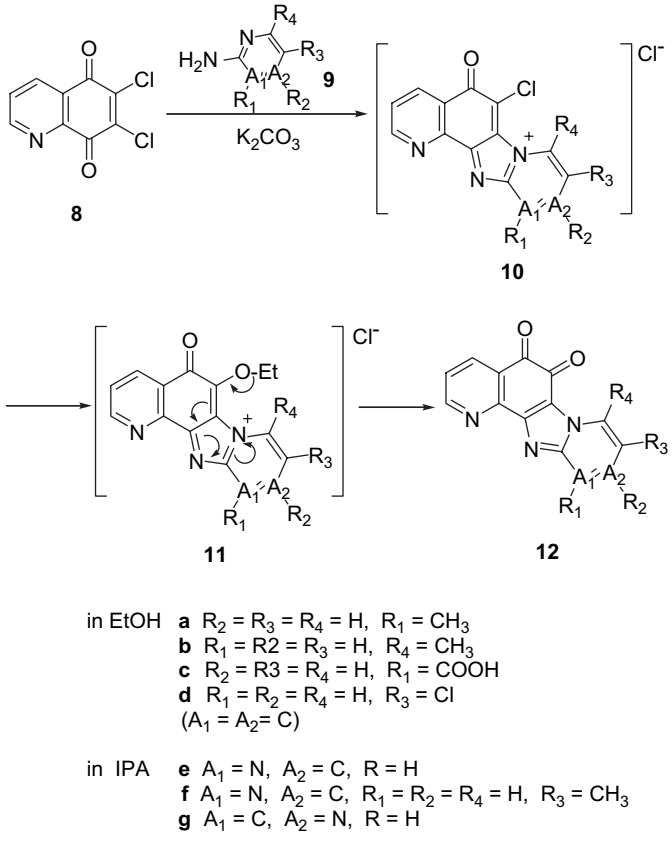
a R_1= R_3= H, R_2= CF_3
b R_1= R_3= H, R_2= OCF_3
c R_1= R_3= H, R_2= $N(CH_3)_2$
d R_2= R_3= H, R_1= CH_3
e R_2= R_3= H, R_1= OCH_3
f R_2= R_3= H, R_1= CH_2CH_3
g R_2= R_3= H, R_1= OCH_2CH_3
h R_1= R_2= CH_3, R_3=H
i R_2= H, R_1= R_3= OCH_3
j R_1= H, R_2+ R_3= OCH_2O

Scheme 1. The synthesis of 6-arylamino-7-chlorophthalazine-5,8-diones **6** and substituted pyridazino[4,5-*b*]phenazine-5,12-diones derivatives **7a–j**.

formed. However, when treated in DMF at 150 °C, the starting materials **6** seemed to appear to have been decomposed. Also, it is believed that the intramolecular and intermolecular reactions were entered in competition after the formation of 6-arylamino-7-azidophthalazine-5,8-dione. Moreover, in the preparation of the substituted pyridazino[4,5-*b*]phenazine-5,12-dione derivatives **7**, the yields did not appear to be affected by the position or the size of the substituents. The intramolecular cyclization did not occur when the substituent of arylamine was an electron-withdrawing group (*e.g.*, $R = NO_2$, F, Cl, Br). It is likely that the electron-withdrawing group lessens the nucleophilic substitution in intramolecular cyclization.

2.1.2. Synthesis of azabenzo[a]fluorene-5,6-diones (12)

A series of azabenzo[*a*]fluorene-5,6-dione derivatives **12**, the tetracyclic compounds, were newly designed and synthesized by reacting 6,7-dichloroquinoline-5,8-dione **8** [22], with a 1.5 equimolar ratio of various 2-aminopyridine derivatives in ethanol, in the presence of anhydrous K_2CO_3, as shown in Scheme 2. Compounds were formed in yields ranging from 20 to 50%. As the reaction requires higher temperature, isopropanol (IPA) was used instead of ethanol. Compounds **12e–g** were obtained in a low yield of 12–20%. The reaction of 6,7-dichloroquinoline-5,8-dione **8** with the 2-aminopyridine derivatives yielded azabenzo[*a*]fluorene-5,6-diones **12**. In the previous paper, the four feasible structures which include *ortho*- or *para*-quinones and their regioisomers were considered as possible product of the nucleophilic substitution reaction and 8-chloro-1,6*b*-11-tri-aza-benzo[*a*]fluorene-5,6-dione was identified as major product by X-ray crystallography [20,21]. The reaction mechanism has been previously reported [23]. Compounds **12a–g** have azabenzo[*a*]fluorene-5,6-dione ring and the NMR data of these compounds were compared with that of 8-chloro-1,6*b*-11-tri-aza-benzo[*a*]fluorene-5,6-dione.



Scheme 2. Synthesis of tri/tetra-azabenzo[*a*]fluorene-5,6-diones.

2.2. Cytotoxicity

The prepared substituted pyridazino[4,5-*b*]phenazine-5,12-diones **7a–j** and tri/tetra-azabenzo[*a*]fluorene-5,6-dione derivatives **12a–g** were evaluated for their cytotoxic activity against the cancer cell lines: A549 (lung), SK-OV-3 (ovarian), SK-MEL-2 (melanoma), XF 498 (CNS), and HCT 15 (colon) (Tables 1 and 2). The IC_{50} measurements reported were mean and standard error of two separate measurements which were each made of triple plates for measurement. All the compounds **7a–j** showed higher cytotoxic activity than that of doxorubicin against all cancer cell lines except for A549 (Table 1). In particular, the cytotoxicity of **7f** (R_1 = Et) and **7h** (R_1, R_2 = Me) against all human cancer cell lines examined was about 10 times higher than that of doxorubicin. Also the cytotoxicity of **7c** ($R_2 = N(CH_3)_2$), **7g** (R_1 = OEt), and **7i** (R_1, R_3 = OMe) was much higher (2–9 times) than that of doxorubicin.

The IC_{50} values of almost all azabenzo[*a*]fluorene-5,6-diones **12a–g**, with the exception of the compound **12b** and **12f**, on several human cancer cell lines *in vitro* were comparable to those of doxorubicin (Table 2). Among compounds **12a–g**, 1,6b,9,11-tetra-azabenzo[*a*]fluorene-5,6-dione **12g** exhibited highest cytotoxicity. This R_1 methyl substituted compound **12a** represented dramatically increased cytotoxicity than R_4 methyl substituted compound **12b**.

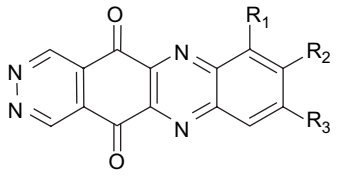
Johnson has reported that the number and positions of nitrogen atoms are important for cytotoxicity [24]. In the previous study, pyridophenazinediones and the substituted pyridazino[4,5-*b*]phenazine-5,12-diones with three or four nitrogen atoms showed much better cytotoxicity than benzophenazinediones possessing two nitrogen atoms [18,25]. In our study, the substituted pyridazino[4,5-*b*]phenazine-5,12-dione derivatives **7a–j**, which are linear and coplanar tetracyclic heteroquinones with four nitrogen atoms, showed more potent cytotoxicities than that of the *ortho*-quinone derivatives, benzo[*a*]fluorene-5,6-diones **12a–g**. The *in vitro* cytotoxic activity against human tumor cell lines of a series **7a–j** was much better than that of the similar series in our previous report [19].

3. Experimental

3.1. Materials and methods

All melting points were taken in Pyrex capillaries using electrothermal digital melting point apparatus (Büchi) and were not corrected. The IR spectra were recorded on an

Table 1
In vitro cytotoxic potential of substituted pyridazino[4,5-*b*]phenazine-5,12-dione derivatives



7a~j

Compounds				IC_{50} (μM ± S.E.)				
	R_1	R_2	R_3	A549	SK-OV-3	SK-MEL-2	XF 498	HCT 15
Doxorubicin				0.118 ± 0.018	0.181 ± 0.033	0.097 ± 0.004	0.112 ± 0.002	0.225 ± 0.036
7a	H	CF_3	H	0.174 ± 0.003	0.079 ± 0.004	0.116 ± 0.008	0.116 ± 0.004	0.195 ± 0.013
7b	H	OCF_3	H	0.134 ± 0.031	0.063 ± 0.017	0.085 ± 0.002	0.098 ± 0.000	0.074 ± 0.019
7c	H	$N(CH_3)_2$	H	0.051 ± 0.012	0.031 ± 0.004	0.088 ± 0.012	0.070 ± 0.002	0.024 ± 0.001
7d	Me	H	H	0.029 ± 0.007	0.070 ± 0.019	0.034 ± 0.007	0.021 ± 0.001	0.018 ± 0.001
7e	OMe	H	H	0.109 ± 0.011	0.039 ± 0.008	0.029 ± 0.002	0.065 ± 0.012	0.093 ± 0.002
7f	Et	H	H	0.010 ± 0.002	0.034 ± 0.010	0.019 ± 0.002	0.013 ± 0.002	0.012 ± 0.001
7g	OEt	H	H	0.053 ± 0.020	0.032 ± 0.008	0.015 ± 0.001	0.029 ± 0.007	0.030 ± 0.001
7h	Me	Me	H	0.013 ± 0.003	0.019 ± 0.003	0.012 ± 0.000	0.013 ± 0.002	0.010 ± 0.001
7i	OMe	H	OMe	0.044 ± 0.009	0.070 ± 0.002	0.038 ± 0.006	0.033 ± 0.006	0.026 ± 0.002
7j	H	OCH_2O		0.138 ± 0.025	0.041 ± 0.006	0.151 ± 0.006	0.118 ± 0.010	0.116 ± 0.006

FT-Infrared spectrometer (Bio-Rad Co., USA) using KBr pellets. ^{1}H NMR spectra were recorded on a 400 MHz Varian FT-NMR spectrometer facility by using tetramethylsilane as an internal standard. Samples were dissolved in DMSO-d_6 or $CDCl_3$. Elemental analyses were performed using Thermo Quest (CE Instruments) EA 1110. Most of the reagents were purchased from Aldrich Chemical Company and Merck Company.

3.2. Synthesis

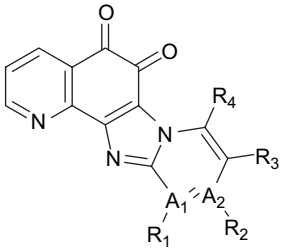
3.2.1. General procedure for the preparation of 6-arylamino-7-chlorophthalazine-5,8-diones (*6a–j*)

To a solution of 6,7-dichlorophthalazine-5,8-dione **5**, prepared according to the literature [21] (500 mg, 2.19 mmol) in ethanol (20 mL), arylamine (4.37 mmol) was added and stirred at room temperature. The reaction mixture was cooled and then filtered. The filtered precipitate was washed with cold 95% ethanol.

3.2.2. General procedure for the preparation of pyridazino[4,5-b]phenazine-5,12-diones (*7a–j*)

To the solution of **6a–j** (2.0 mmol) in DMF (100 mL), sodium azide (200 mg, 3.08 mmol) which was suspended in little amount of distilled water was added. The mixture was heated at 90–95 °C on a steam bath overnight. The reaction mixture was cooled and the filtered precipitate was extracted with ethylacetate. The organic layer was washed with water, dried with anhydrous $MgSO_4$, concentrated, and then the residue was purified by recrystallization or by column chromatography.

Table 2
In vitro anticancer activity of azabenzo[*a*]fluorene-5,6-diones



12a~g

Compounds							IC_{50} (μM ± S.E.)				
	A_1	A_2	R_1	R_2	R_3	R_4	A549	SK-OV-3	SK-MEL-2	XF 498	HCT 15
12a	C	C	Me	H	H	H	0.112 ± 0.007	0.051 ± 0.017	0.122 ± 0.015	0.086 ± 0.013	0.123 ± 0.011
12b	C	C	H	H	H	Me	1.277 ± 0.130	1.653 ± 0.015	1.360 ± 0.209	2.568 ± 0.215	1.452 ± 0.008
12c	C	C	CO_2H	H	H	H	0.117 ± 0.010	0.056 ± 0.011	0.125 ± 0.008	0.095 ± 0.019	0.122 ± 0.005
12d	C	C	H	H	Cl	H	0.126 ± 0.002	0.046 ± 0.002	0.141 ± 0.001	0.124 ± 0.008	0.134 ± 0.002
12e	N	C	H	H	H	H	0.160 ± 0.018	0.027 ± 0.004	0.139 ± 0.003	0.137 ± 0.001	0.301 ± 0.040
12f	N	C	H	Me	H	H	0.745 ± 0.069	0.183 ± 0.005	0.286 ± 0.002	0.382 ± 0.055	0.978 ± 0.091
12g	C	N	H	H	H	H	0.098 ± 0.005	0.018 ± 0.003	0.114 ± 0.012	0.092 ± 0.013	0.123 ± 0.010

*3.2.3. 8-(Trifluoromethyl)pyridazino[4,5-b]phenazine-5, 12-dione (**7a**)*

The general procedure was followed for 30 h with 6-(4-(trifluoromethyl)phenylamino)-7-chlorophthalazine-5,8-dione **6a** (710 mg) added as arylamine and the concentrated residue was purified by column chromatography (hexane/ethylacetate, 1:8) to give a pale yellow solid (170 mg, 26%): mp > 300 °C; IR (KBr, cm^{-1}) 1697 (C=O); ^1H NMR (400 MHz, DMSO-d_6) δ 10.08 (br s, 2H, C-1, C-4), 8.94 (s, 1H, C-7), 8.68 (s, 1H, C-10), 8.41 (br s, 1H, C-9). Anal. Calcd. for $C_{15}H_5F_3N_4O_2$: C, 54.56; H, 1.53; N, 16.97. Found: C, 54.87; H, 1.52; N, 16.79.

*3.2.4. 8-(Trifluoromethoxy)pyridazino [4,5-b]phenazine-5,12-dione (**7b**)*

The general procedure was followed for 34 h with 6-(4-(trifluoromethoxy)phenylamino)-7-chlorophthalazine-5,8-dione **6b** (740 mg) added as arylamine and the concentrated residue was purified by column chromatography (hexane/ethylacetate, 1:4) to give a yellow solid (150 mg, 23%): mp > 300 °C; IR (KBr, cm^{-1}) 1697 (C=O); ^1H NMR (400 MHz, DMSO-d_6) δ 10.08 (br s, 2H, C-1, C-4), 8.63 (d, $J = 9.6$ Hz, 1H, C-10), 8.47 (s, 1H, C-7), 8.15 (br s, 1H, C-9). Anal. Calcd. for $C_{15}H_5F_3N_4O_3$: C, 52.04; H, 1.46; N, 16.18. Found: C, 52.49; H, 1.39; N, 15.83.

*3.2.5. 8-(Dimethylamino)pyridazino[4,5-b]phenazine-5, 12-dione (**7c**)*

The general procedure was followed for 31 h with 6-(4-dimethylamino)phenylamino)-7-chlorophthalazine-5,8-dione **6c** (740 mg) added as arylamine and the concentrated residue was purified by column chromatography (hexane/ethylacetate, 1:9) to give a violet solid (110 mg, 19%): mp > 300 °C; IR (KBr, cm^{-1}) 1691 (C=O); ^1H NMR (400 MHz, DMSO-d_6) δ 9.95 (s, 1H, C-4), 9.93 (s, 1H, C-1), 8.16 (d, $J = 9.2$ Hz, 1H, C-10), 7.86 (dd, $J = 9.2$ and 3.2 Hz, 1H, C-7), 7.22 (d, $J = 3.2$ Hz, 1H, C-9), 3.26 (s, 6H, $-N(CH_3)_2$). HR-FABMS Calcd. for $C_{16}H_{11}N_5O_2$ ($M^+ + H$): 306.0991. Found: 306.0992.

*3.2.6. 7-Methylpyridazino[4,5-b]phenazine-5,12-dione (**7d**)*

The general procedure was followed for 23 h with 6-(2-methylphenylamino)-7-chlorophthalazine-5,8-dione **6d** (600 mg) added as arylamine and the concentrated residue was purified by recrystallization with methanol to give a yellow solid (110 mg, 20%): mp > 300 °C; IR (KBr, cm^{-1}) 1697 (C=O); ^1H NMR (400 MHz, DMSO-d_6) δ 10.05 (s, 1H, C-4), 10.03 (s, 1H, C-1), 8.29 (d, 1H, C-10), 8.07 (m, 2H, C-8, C-9), 2.89 (s, 3H, $-CH_3$). Anal. Calcd. for $C_{15}H_8N_4O_2$: C, 65.22; H, 2.92; N, 20.28. Found: C, 65.09; H, 2.97; N, 19.66.

*3.2.7. 7-Methoxypyridazino[4,5-b]phenazine-5, 12-dione (**7e**)*

The general procedure was followed for 26 h with 6-(2-methoxyphenylamino)-7-chlorophthalazine-5,8-dione **6e** (630 mg) added as arylamine and the concentrated residue was purified by column chromatography (ethylacetate) to give a orange colored solid (120 mg, 21%): mp > 300 °C; IR (KBr, cm^{-1}) 1692 (C=O); ^1H NMR (400 MHz, DMSO-d_6) δ 10.11 (br s, 2H, C-1, C-4), 7.81–8.22 (m, 3H, C-8, C-9, C-10), 4.13 (s, 3H, $-OCH_3$). Anal. Calcd. for $C_{15}H_8N_4O_3$: C, 61.65; H, 2.76; N, 19.17. Found: C, 61.86; H, 2.78; N, 18.87.

*3.2.8. 7-Ethylpyridazino[4,5-b]phenazine-5,12-dione (**7f**)*

The general procedure was followed for 30 h with 6-(2-ethylphenylamino)-7-chlorophthalazine-5,8-dione **6f** (630 mg) added as arylamine and the concentrated residue was purified by column chromatography (hexane/ethylacetate, 1:2) to give a yellow solid (150 mg, 26%): mp > 300 °C; IR (KBr, cm^{-1}) 1702 (C=O); ^1H NMR (400 MHz, DMSO-d_6) δ 10.05 (s, 2H, C-1, C-4), 8.29 (d, $J = 7.6$ Hz, 1H, C-10), 8.09 (br s, 1H, C-9), 7.99 (br s, 1H, C-8), 3.38 (q, $J = 7.2$ Hz, 2H, $-CH_2$), 1.39 (t, $J = 7.2$ Hz, 3H, $-CH_3$). HR-FABMS Calcd. for $C_{16}H_{10}N_4O_2$ ($M^+ + H$): 291.0882. Found: 291.0887.

*3.2.9. 7-Ethoxypyridazino[4,5-b]phenazine-5,12-dione (**7g**)*

The general procedure was followed for 25 h with 6-(2-ethoxyphenylamino)-7-chlorophthalazine-5,8-dione **6g** (630 mg) added as arylamine and the concentrated residue was purified by column chromatography (hexane/ethylacetate, 1:9) to give an orange colored solid (170 mg, 28%): mp > 300 °C; IR (KBr, cm^{-1}) 1697 (C=O); ^1H NMR (400 MHz, DMSO-d_6) δ 10.05 (br s, 2H, C-1, C-4), 7.81–8.19 (m, 2H, C-9, C-10), 7.55 (d, $J = 8.0$ Hz, 1H, C-8), 4.21 (q, $J = 6.8$ Hz, 2H, $-CH_2$), 1.56 (t, $J = 6.8$ Hz, 3H, $-CH_3$). Anal. Calcd. for $C_{16}H_{10}N_4O_3$: C, 62.74; H, 3.29; N, 18.29. Found: C, 62.29; H, 3.35; N, 17.77.

*3.2.10. 7,8-Dimethylpyridazino[4,5-b]phenazine-5, 12-dione (**7h**)*

The general procedure was followed for 29 h with 6-(3,4-dimethylphenylamino)-7-chloro-5,8-phthalazinedione **6h** (630 mg) added as arylamine and the concentrated residue was purified by column chromatography (hexane/ethylacetate, 1:4) to give a yellow solid (110 mg, 19%): mp > 300 °C; IR (KBr, cm^{-1}) 1703 (C=O); ^1H NMR (400 MHz, DMSO-d_6) δ 10.01 (s, 2H, C-1, C-4), 8.33 (s, 1H, C-10), 7.71 (s, 1H, C-9), 2.93 (s, 3H, $-CH_3$), 2.78 (s, 3H, $-CH_3$). Anal. Calcd. for $C_{16}H_{10}N_4O_2$: C, 66.20; H, 3.47; N, 19.30. Found: C, 65.71; H, 3.49; N, 18.83.

*3.2.11. 7,9-Dimethoxypyridazino[4,5-b]phenazine-5, 12-dione (**7i**)*

The general procedure was followed for 25 h with 6-(2,4-dimethoxyphenylamino)-7-chlorophthalazine-5,8-dione **6i** (630 mg) added as arylamine and the concentrated residue was purified by column chromatography (hexane/ethylacetate, 1:4) to give a yellow solid (110 mg, 19%): mp > 300 °C; IR (KBr, cm^{-1}) 1692 (C=O); ^1H NMR (400 MHz, DMSO-d_6) δ 10.02 (br s, 2H, C-1, C-4), 8.35 (d, $J = 9.2$ Hz, 1H, C-10), 7.84 (s, 1H, C-8), 4.12 (s, 6H, $-OCH_3$, $-OCH_3$). Anal. Calcd. for $C_{16}H_{10}N_4O_4$: C, 59.63; H, 3.13; N, 17.38. Found: C, 59.35; H, 2.80; N, 17.86.

*3.2.12. 8,9-Methylenedioxypyridazino[4,5-b]phenazine-5, 12-dione (**7j**)*

The general procedure was followed for 25 h with 6-(3,4-methylenedioxyanilino)-7-chlorophthalazine-5,8-dione **6j** (660 mg) added as arylamine and the concentrated residue was purified by column chromatography (hexane/ethylacetate, 1:10) to give a dark yellow solid (110 mg, 18%): mp > 300 °C; IR (KBr, cm^{-1}) 1683 (C═O); ^1H NMR (400 MHz, DMSO-d_6) δ 9.99 (br s, 2H, C-1, C-4), 7.80 (s, 2H, C-7, C-10), 6.81 (s, 2H, $-OCH_2O-$). Anal. Calcd. for $C_{15}H_6N_4O_4$: C, 58.84; H, 1.97; N, 18.30. Found: C, 58.64; H, 2.04; N, 17.88.

*3.2.13. General procedure for the preparation of azabenzo[a]fluorene-5,6-diones (**12a–g**)*

To a mixture of 6,7-dichloro-5,8-quinolinedione **8**, prepared according to the literature[22], (1.0 g, 4.7 mmol) and anhydrous K_2CO_3 (0.6 g, 4.4 mmol) in ethanol or isopropanol (50 mL), 2-aminopyridine derivatives **9a–g** (6.2 mmol) were added. The reaction mixture was refluxed, cooled, and the filtered precipitate was extracted with CH_2Cl_2. The organic layer was washed with water, dried with anhydrous $MgSO_4$, concentrated, and then the residue was purified by recrystallization or by column chromatography.

*3.2.14. 10-Methyl-1,6b,11-triaza-benzo[a]fluorene-5, 6-dione (**12a**)*

The general procedure was followed for 48 h with 2-amino-3-methylpyridine (0.7 mL, 6.2 mmol) added and the concentrated residue was purified by recrystallization with ethanol to give an orange colored solid (350 mg, 28%): mp > 300 °C; IR (KBr, cm^{-1}) 1650 (C═O); ^1H NMR (400 MHz, $CDCl_3$) δ 9.17 (br d, $J = 6.8$ Hz, 1H, C-2), 8.90 (dd, $J = 4.8$ and 1.2 Hz, 1H, C-4), 8.35 (dd, $J = 7.6$ and 1.2 Hz, 1H, C-7), 7.44 (m, 2H, C-3, C-9), 7.12 (t, $J = 6.8$ Hz, 1H, C-8), 2.75 (s, 3H, $-CH_3$). Anal. Calcd. for $C_{15}H_9N_3O_2$: C, 68.44; H, 3.45; N, 15.96. Found: C, 68.58; H, 3.45; N, 15.61.

*3.2.15. 7-Methyl-1,6b,11-triaza-benzo[a]fluorene-5, 6-dione (**12b**)*

The general procedure was followed for 40 h with 2-amino-6-methylpyridine (0.7 g, 6.2 mmol) added and the concentrated residue was purified by recrystallization with ethanol to give a dark yellow solid (620 mg, 50%): mp > 300 °C; IR (KBr, cm^{-1}) 1649 (C═O); ^1H NMR (400 MHz, $CDCl_3$) δ 9.01 (dd, $J = 4.8$ and 1.6 Hz, 1H, C-2), 8.55 (dd, $J = 8.0$ and 1.6 Hz, 1H, C-4), 7.77 (d, $J = 8.8$ Hz, 1H, C-3), 7.64 (dd, $J = 8.0$ and 4.8 Hz, 1H, C-10), 7.54 (dd, $J = 8.8$ and 7.2 Hz, 1H, C-9), 6.99 (d, $J = 7.2$ Hz, 1H, C-8), 3.11 (s, 3H, $-CH_3$). Anal. Calcd. for $C_{15}H_9N_3O_2$: C, 68.44; H, 3.45; N, 15.96. Found: C, 67.97; H, 3.47; N, 15.69.

*3.2.16. 5,6-Dioxo-5,6-dihydro-1,6b,11-triaza-benzo[a] fluorene-10-carboxylic acid (**12c**)*

The general procedure was followed for 38 h with 2-aminonicotinic acid (0.9 g, 6.2 mmol) added and the concentrated residue was purified by recrystallization with ethylacetate to give a dark yellow solid (270 mg, 20%): mp > 300 °C; IR (KBr, cm^{-1}) 1659 (C═O); ^1H NMR (400 MHz, $CDCl_3$) δ 11.88 (br s, 1H, −OH), 9.45 (d, $J = 8.0$ Hz, 1H, C-2), 8.91 (d, $J = 3.6$ Hz, 1H, C-7), 8.35 (dd, $J = 7.6$ and 1.2 Hz, 1H, C-4), 8.19 (d, $J = 7.2$ Hz, 1H, C-3), 7.45 (dd, $J = 8.0$ and 7.6 Hz, 1H, C-9), 7.28 (m, 1H, C-8). HR-FABMS Calcd. for $C_{15}H_7N_3O_4$ ($M^+ + H$): 294.0515. Found: 294.0518.

*3.2.17. 8-Chloro-1,6b,11-triaza-benzo[a]fluorene-5, 6-dione (**12d**)*

The general procedure was followed for 34 h with 2-amino-5-chloropyridine (0.7 g, 6.2 mmol) added and the concentrated residue was purified by recrystallization with ethanol to give an orange colored solid (600 mg, 45%): mp > 300 °C; IR (KBr, cm^{-1}) 1649 (C═O); ^1H NMR (400 MHz, $CDCl_3$) δ 9.32 (dd, $J = 2.0$ and 0.6 Hz, 1H, C-2), 8.88 (dd, $J = 4.8$ and 1.6 Hz, 1H, C-7), 8.36 (dd, $J = 7.6$ and 1.6 Hz, 1H, C-4), 7.84 (dd, $J = 9.2$ and 0.6 Hz, 1H, C-10), 7.61 (dd, $J = 9.2$ and 2.0 Hz, 1H, C-3), 7.46 (dd, $J = 7.6$ and 4.8 Hz, 1H, C-9). Anal. Calcd. for $C_{14}H_6ClN_3O_2$: C, 59.28; H, 2.13; N, 14.81. Found: C, 59.00; H, 2.11; N, 14.56.

*3.2.18. 1,6b,10,11-Tetra-azabenzo[a]fluorene-5, 6-dione (**12e**)*

The general procedure was followed for 48 h with 2-aminopyrimidine (0.6 g, 6.3 mmol) added and the concentrated residue was purified by column chromatography (hexane/ethylacetate/methanol, 4:4:1) to give a yellow solid (140 mg, 12%): mp > 300 °C; IR (KBr, cm^{-1}) 1649 (C═O); ^1H NMR (400 MHz, $CDCl_3$) δ 9.49 (dd, $J = 6.4$ and 1.2 Hz, 1H, C-2), 8.94 (dd, $J = 4.8$ and 1.2 Hz, 1H, C-7), 8.89 (dd, $J = 8.0$ and 1.2 Hz, 1H, C-9), 8.38 (dd, $J = 8.0$ and 4.8 Hz, 1H, C-4), 7.49 (dd, $J = 6.4$ and 4.0 Hz, 1H, C-3), 7.28 (dd, $J = 6.4$ and 4.0 Hz, 1H, C-8). Anal. Calcd. for $C_{13}H_6N_4O_2$: C, 62.40; H, 2.42; N, 22.39. Found: C, 62.25; H, 2.36; N, 22.14.

*3.2.19. 8-Methyl-1,6b,10,11-tetra-azabenzo[a]fluorene-5, 6-dione (**12f**)*

The general procedure was followed for 38 h with 2-amino-5-methylpyrimidine (0.7 g, 6.2 mmol) and the concentrated residue was purified by recrystallization with ethanol to give an orange colored solid (200 mg, 16%): mp > 300 °C; IR (KBr, cm^{-1}) 1655 (C═O); ^1H NMR (400 MHz, $CDCl_3$) δ 9.30 (d, $J = 6.8$ Hz, 1H, C-2), 8.91 (dd, $J = 5.2$ and 2.0 Hz, 1H, C-7), 8.35 (dd, $J = 8.0$ and 2.0 Hz, 1H, C-9), 7.45 (dd, $J = 7.6$ and 6.8 Hz, 1H, C-4), 7.11 (d, $J = 7.6$ Hz, 1H, C-3), 1.48 (s, 3H, $-CH_3$). HR-FABMS Calcd. for $C_{14}H_8N_4O_2$ ($M^+ + H$): 265.0726. Found: 265.0722.

*3.2.20. 1,6b,9,11-Tetra-azabenzo[a]fluorene-5, 6-dione (**12g**)*

The general procedure was followed for 50 h with 2-aminopyrazine (0.6 g, 6.2 mmol) added and the concentrated residue was purified by column chromatography (hexane/ethylacetate/methanol, 3:3:1) to give a yellow solid (150 mg,

12%): mp > 300 °C; IR (KBr, cm^{-1}) 1649 (C=O); ^1H NMR (400 MHz, $CDCl_3$) δ 9.43 (d, $J = 1.6$ Hz, 1H, C-2), 9.13 (dd, $J = 4.4$ and 1.6 Hz, 1H, C-7), 8.98 (dd, $J = 4.8$ and 1.6 Hz, 1H, C-10), 8.44 (dd, $J = 8.0$ and 1.6 Hz, 1H, C-8), 8.40 (d, $J = 4.4$ Hz, 1H, C-4), 7.55 (dd, $J = 8.0$ and 1.6 Hz, 1H, C-3). Anal. Calcd. for $C_{13}H_6N_4O_2$: C, 62.40; H, 2.42; N, 22.39. Found: C, 62.87; H, 2.55; N, 22.34.

3.3. In vitro cytotoxic activity evaluation by SRB assay

The newly obtained derivatives were evaluated for cytotoxic activity to cancer cell lines at the Korea Research Institute of Chemical Technology by using the sulforhodamine B (SRB) assay [26,27]. This method was developed for measuring cellular culture protein content, and involves tumor cell lines representing five different cancer types, namely, A549 (human lung), SK-OV-3 (human ovarian), SK-MEL-2 (human melanoma), XF 498 (human CNS), and HCT 15 (human colon). The cells were maintained as stocks in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), and cultures were passaged once or twice a week using trypsin–EDTA to detach the cells from their culture flasks. The rapidly growing cells were harvested, counted, and incubated at the appropriate concentration ($1–2 \times 10^4$ cells/well) in 96-well microplates. After incubation for 24 h, the compounds, dissolved in the culture medium, were applied to the culture wells in triplicate and incubated for 48 h at 37 °C under a 5% CO_2 atmosphere. The cultures were fixed with cold TCA and stained with 0.4% SRB dissolved in 1% acetic acid. After dissolving the bound stain with 10 mL of the unbuffered tris base solution (pH 10.5) using a gyratory shaker, absorbance at 520 nm was measured using a microplate reader (Molecular Devices E-max, Sunnyvale, USA). The cytotoxic activity was evaluated by measuring the concentration needed to inhibit protein synthesis by 50% (*i.e.*, IC_{50}) as comparison. Each value represents the mean of triplicate experiments.

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