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Short communication

Synthesis, cytotoxicity, antiviral activity and interferon inducing ability of 6-(2-aminoethyl)-6H-indolo[2,3-b]quinoxalines

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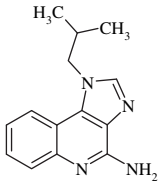
ABSTRACT

New 6-(2-aminoethyl)-6H-indolo[2,3-b]quinoxalines were synthesized with high yields using bromoethylisatin and 6-(2-bromoethyl)-6H-indolo[2,3-b]quinoxaline as intermediates. These compounds were screened for the cytotoxicity, antiviral activity and interferon inducing ability. It was shown, that tested 6-(2-aminoethyl)-6H-indolo[2,3-b]quinoxalines are low toxic potent interferon inducers and antivirals. Morpholine and 4-methyl-piperidine derivatives appeared as the most active antivirals and the least cytotoxic in the investigated series.

1. Introduction

Interferons (IFN) are ones among the most broadly used drugs for the human viral diseases treatment [1–4]. Though, side effects and sometimes low effectiveness are occurred, especially while long-term IFN introduction [2,5,6]. Inductors of the endogenous IFN are seemed as a good alternative for the exogenous IFN using [7]. Some of such inductors are widely used in the clinical practice. Thus, Imiquimod (**1**) as 5% cream is used for the viral and cancer diseases treatment [8]. Tilorone (**2**) [9,10] as Amixine [11] and carboxymethylacridone derivatives (**3a** and **3b**) as Cycloferon and Neovir [7] were proved in Ukraine and Russia as a highly active and tolerant human drugs for the prophylactics and treatment of different acute respiratory viral diseases, hepatitis (A and B), HSV-diseases, etc.



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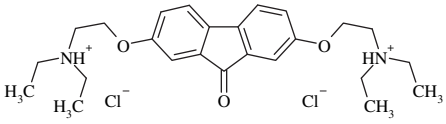

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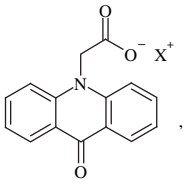

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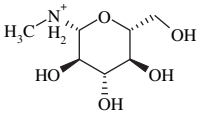

3a

Na^+

3b

3

Tilorone's ability to the DNA intercalation attributed to the presence of planar polycyclic system [12–14] is seemed as a main cause of it's IFN-inducing ability and antiviral action [15,16]. Furthermore, we speculated [16] that these properties are general

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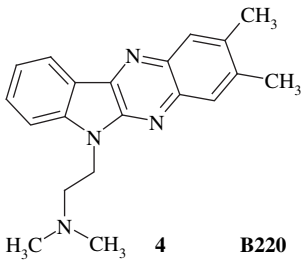
ones of the DNA intercalators. Parallelism of the intercalating ability on the one hand and IFN induction and antiviral activity on the other one demonstrated for fluorenes [15,17], dibenzofurans [18,19], anthraquinones [20], 9-aminoacridines [16,21], and many other compounds argue for this speculation validity.

Indoloquinoxalines are ones among DNA intercalators possessing highly defined anti-herpesvirus activity [22]. In respect to the mentioned above, we should wait that these compounds can demonstrate activity not only against herpes simplex, but against other viruses also, as well as interferon inducing properties. Coincidence in almost of the tilorone and 2,3-dimetyl-6(2-dimethylaminoethlyl)-6H-indolo[2,3-b]quinoxalin (B-220, **4**) activity types pays attention to itself: inhibition of Herpesviridae species' reproduction [9,22], inhibition of the spontaneous peroxide oxidation of lipids (POL) [23,24], antimutagenic action [25–27], antitumoral activity [28–30]. It is unlike, that such coincidence is occasional. Therefore we have supposed that indoloquinoxaline derivatives have also other properties like to tilorone, namely, the anticarcinogenic activity [26], IFN induction [31–33] and inhibition of cytochrome P-450 [34].

According above we have attempted the aminoethyl-6H-indolo[2,3-b]quinoxalines' (**9**–**20**) synthesis, and studying of their titled properties and SAR.



4 **B220**

2. Results and discussion

2.1. Chemistry

Targeted compounds were synthesized via 3-step procedure (Scheme 1) starting from isatin (**5**) through it's 1-bromoethyl-derivative (**6**) and 6-(2-bromoethyl)indoloquinoxaline (**7**) as intermediates. Final amination using of corresponding amines access leads us to aminoethyl-indoloquinoxalines (**8**–**19**).

1-(2-Bromoethyl)-indole-2,3-dion (**6**) was synthesized via isatin alkylation by excess of dibromoethane in DMF at room temperature in the presence of potassium carbonate. Potassium carbonate using instead of sodium hydride as described previously [35] leads both to the procedure simplifying and yield increasing up to 80%. The separation of the solution from the inorganic precipitate, the evaporation of the solution to dryness and the re-crystallization of the residue from the ethanol results in chromatographically pure **6** with 80% yield. Further condensation of **6** with 1,2-diaminobenzene under the boiling in acetic acid lead to the 6-bromoethyl-6H-indolo-[2,3-*b*]quinoxaline. Chromatographically homogenous product (with respect to the admixtures of the aromatic nature) falls out directly from the reacting mixture under the cooling down to room temperature. In some cases, when precipitated product contained uncertain admixtures, it was recrystallized from the glacial acetic acid or ethanol yielding 80%.

The compounds **9**–**19** were obtained by aminodebromination of **7** by excess secondary amines in boiling benzene. In contrast to them, compound **8** was obtained at room temperature in DMF.

Purity of all synthesized compounds was controlled by thin-layer chromatography on pre-coated silica gel F_{254} plates using eluents of different composition.

The structure of the synthesized compounds was proved by mass-spectrometry, IR spectroscopy and NMR-spectroscopy. Molecular ions peaks are present in electron impact mass-spectra and in mass-spectra with ionization method of fast atom bombardment (FAB). Fragment ions set correspond to suggested structures. Vibrations of aromatic C–H bonds are observed in IR spectrums of compounds **8**–**19** at 3064–3055 cm^{-1}, aliphatic – 2953–2773 cm^{-1}. Double bonds vibrations of heterocyclic fragments exhibit a band set at 1631–1440 cm^{-1}. The bands at 1240–10600 cm^{-1} are corresponds to CH_2–N vibrations.

Two "parts" corresponded to aromatic and aliphatic parts of molecule are observed in ^1H NMR-spectra of synthesized indoloquinoxalines derivatives. The "aliphatic parts"' spectrums are different according terminal amino-group structures and correspond to them. On the contrary, differences in the "aromatic parts"' of spectra are much less manifest. The complex multiplet with integral intensity 3H and five separate signals with integral intensity 1H each are present in "aromatic" fragments of spectrum. All detailed information is present in supplement. Details on mass-spectra and NMR signal assigning one can find in the supplement.

2.2. Biological activity

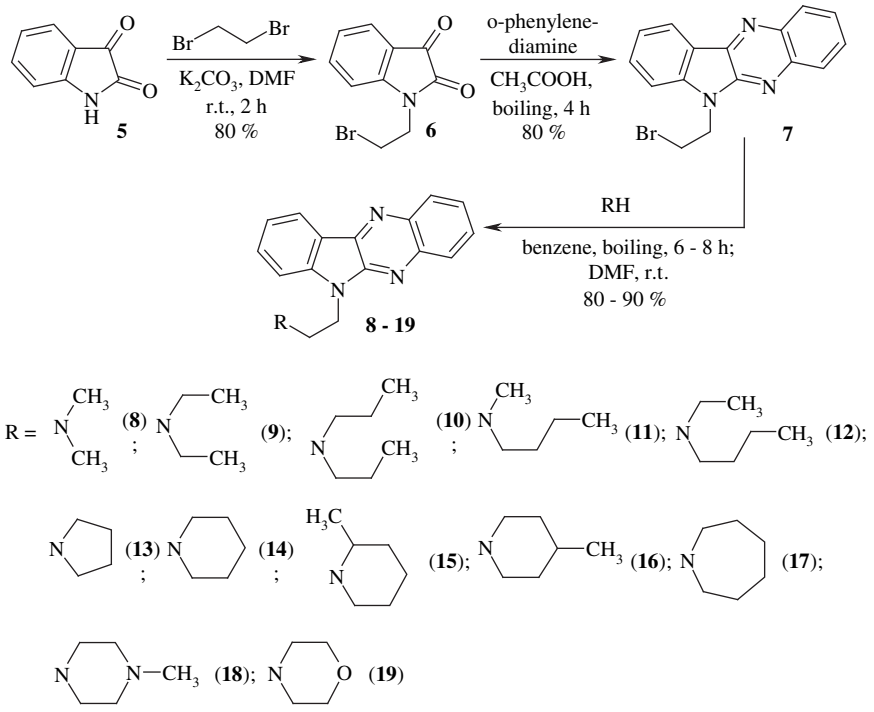
In vitro cytotoxicity, antiviral and IFN-inducing activities of all synthesized compounds **8**–**19** were tested using both murine fibroblasts (L929) and transferable piglet testicular (EPT) cells. Antiviral and IFN-inducing activities were determined against vesicular stomatitis virus (VSV). The obtained results are summarized in Tables 1–3.

As shown in Table 1 most of the synthesized 6H-indolo[2,3-b]quinoxaline derivatives showed low cytotoxicity in comparison with tilorone, their cytotoxicity for both cell lines differ insignificantly $P > 0.05$, using Van der Waerden's nonparametric (distribution-free) test X criteria [36,37]. The compounds with morpholine (**19**) and 4-methyl-piperidine (**16**) as terminal amino-group appeared as nontoxic (or slightly toxic) up to maximum test concentration of 1000 μM using both cell lines. On the contrary, pyrrolidine derivative (**13**) appears as the most cytotoxic. Cytotoxic concentration (CC_{50}) of this compound was found as 76 μM against L929 cells.

All synthesized compounds **8**–**19** demonstrate significant antiviral activity (Table 2) against vesicular stomatitis virus (VSV) using both cell lines. Their activity using L929 is higher ($P < 0.01$) than the same using EPT cell line for both preventive ($n = 24$, $X = 6.31$ vs $X_t = 5.55$) and therapeutic ($n = 23$, $X = 6.55$ vs $X_t = 5.40$) regimen of injection.

It should be noted that antiviral activity of the 6H-indolo[2,3-b]quinoxaline derivatives is higher ($P < 0.01$) when the tested compounds were added to the cell monolayer 24 h before virus infection (preventive regimen) than they were added immediately after virus infection (therapeutic regimen) for both L929 ($n = 24$, $X = 8.11$ vs $X_t = 5.55$) and EPT ($n = 23$, $X = 7.19$ vs $X_t = 5.40$). This is in accordance with an interferon-mediated mechanism of viral inhibition. Compound (**12**) contained ethyl-butyl-amine as a terminal amino-group, becomes as exception – its antiviral action was identical in both cases.

Compounds **9**–**18** when injected 24 h before virus to the L929 monolayer (preventive regimen) demonstrate activity similar to tilorone – no significant difference between their $-\lg IC_{50}$ values and the same one of tilorone is occurred. While EPT culture used, compounds **9**–**15** and **17** appeared less active then tilorone while compounds **8**, **16**, **18**, **19** demonstrated activity similar to tilorone.



Scheme 1.

Compound **12** appears as the least active one among investigated in both cases.

Difference in activity of the tested compounds is seemed as more prominent when injected in therapeutic regimen. In general, in this case the most of indoloquinoxalines appeared more active then tilorone using both cell cultures. Compounds **13** and **15** demonstrate the maximal antiviral activity among others.

All synthesized 6H-indolo[2,3-b]quinoxaline derivatives exhibited interferon inducing activity (Table 3), which different insignificantly ($P > 0.05$) using the EPT cells vs L929. The compounds **9**, **13**, **14** and **17** induced highest titers of interferon. It's have been noted, that active therapeutic agents induce as high interferon titers as tilorone or larger. On the other hand, compounds **14** and **17** (not the most active antivirals in both prophylactic and therapeutic regimens) appear as the most active IFN inducers exceeding tilorone by this property in the L929 cell line.

The selectively index (SI) value as the integral parameter of the antiviral effectiveness was determined as the ratio of the CC_{50} to the IC_{50} ($SI = CC_{50}/EC_{50}$). Compounds **16** and **19** appeared as potent antivirals and low cytotoxic. Thus, the SI (Table 4) for compounds **16** and **19** was 1783 and 1258, respectively, which is 20 and 15 times larger than the same for tilorone. Generally, SI of compounds for L929 and EPT cell lines don't differ significantly ($P > 0.05$) neither

Table 1
Cytotoxicity of synthesized 6H-indolo[2,3-b]quinoxaline derivatives.

Compound	L929		EPT	
	$-\lg CC_{50}$[a]	$\pm\epsilon$[b]	$-\lg CC_{50}$	$\pm\epsilon$
8	4.04	0.05	4.09	0.02
9	3.19	0.03	3.34	0.02
10	3.66	0.05	3.83	0.02
11	3.25	0.06	3.69	0.07
12	3.76	0.04	4.08	0.09
13	4.12	0.06	3.79	0.06
14	3.75	0.03	3.03	0.02
15	3.63	0.04	3.4	0.03
16	3.24	0.05	2.55[c]	–
17	3.84	0.09	3.88	0.05
18	4.01	0.02	4.09	0.03
19	2.97[c]	–	2.69[c]	–
Tilorone	3.95	0.03	3.97	0.04

[a] $-\lg CC_{50}$ – negative common logarithm of tested compound concentration (M) which leads to 50% cell monolayer destruction.
[b] Confidence interval value ($\pm\epsilon$) was calculated using $P < 0.05$.
[c] $-\lg CC_{50}$ value determination was failed (the compound in all investigated concentration range lead to cell monolayer destruction less than 50% cell); extrapolated value is given.

Table 2
Antiviral activity of synthesized 6H-indolo[2,3-b]quinoxaline derivatives.

Compound	L929				EPT			
	TE[a]		PE[b]		TE		PE	
	$-\lg IC_{50}$[c]	$\pm\epsilon$[d]	$-\lg IC_{50}$	$\pm\epsilon$	$-\lg IC_{50}$	$\pm\epsilon$	$-\lg IC_{50}$	$\pm\epsilon$
8	4.95	0.08	5.84	0.12	5.02	0.06	5.84	0.12
9	5.66	0.05	5.89	0.05	5.21	0.04	5.47	0.04
10	5.45	0.12	5.89	0.06	5.18	0.10	5.57	0.06
11	5.63	0.07	5.84	0.09	5.32	0.13	5.52	0.11
12	5.47	0.05	5.50	0.10	4.88	0.04	4.88	0.10
13	5.65	0.04	5.88	0.05	5.39	0.02	5.57	0.06
14	5.59	0.06	5.87	0.03	5.25	0.09	5.54	0.03
15	5.72	0.03	5.90	0.07	5.39	0.05	5.58	0.07
16	5.64	0.11	5.79	0.09	4.70	0.11	5.80	0.10
17	5.42	0.09	5.89	0.06	5.10	0.12	5.57	0.05
18	5.32	0.09	5.86	0.10	5.33	0.04	5.84	0.10
19	5.35	0.10	5.79	0.13	–[e]	–	5.79	0.13
Tilorone	4.86	0.06	5.84	0.08	4.87	0.06	5.88	0.08

[a] The tested compounds were added to cell monolayer immediately after virus infection (TE – "therapeutic effect").
[b] The tested compounds were added to cell monolayer 24 h before virus infection (PE – "preventive effect").
[c] $-\lg IC_{50}$ – negative common logarithm of the tested compound concentration (M) which lead to 50% cytopathic action of VVS inhibition.
[d] Confidence interval values ($\pm\epsilon$) were calculated using $P < 0.05$.
[e] $-\lg IC_{50}$ value was failed to determine (the compound in all investigated concentrations prevents less than 50% of VSV cytopathic action).

Table 3
IFN-inducing activity of synthesized 6H-indolo[2,3-b]quinoxaline derivatives.

Compound	L929	EPT
	T IFN at $C = 1.1 \times 10^{-6}$ M[a]	T IFN at $C = 1.5 \times 10^{-6}$ M
8	16	32
9	64	32
10	8	32
11	16	32
12	8	8
13	32	64
14	64	32
15	32	32
16	8	32
17	64	32
18	4	32
19	8	16
Tilorone	32	32

[a] T IFN – titer of the induced IFN – maximal IFN-containing cultural medium dilution, under which it's ability to the cytopathic action of VSV inhibition remains.

Table 4
Selectivity index value of synthesized 6H-indolo[2,3-b]quinoxaline.

Compound	L929		EPT	
	SI (TE)[a]	SI (PE)	SI (TE)	SI (PE)
8	58	63	9	56
9	79	499	76	139
10	158	169	23	55
11	41	388	43	68
12	27	54	6	6
13	468	59	40	60
14	224	132	164	323
15	74	185	98	149
16	78	358	142	1783
17	62	110	17	49
18	46	71	17	57
19	17	659	–	1258
Tilorone	10	80	10	80

[a] SI – selectivity index calculated as the ratio of CC_{50} to IC_{50}.

for therapeutic using ($n = 23$, $X = 3.26$ vs $X_t = 4.18$) nor for preventive one ($n = 24$, $X = 2.26$ vs $X_t = 4.29$). SI for the tested compounds was significantly ($n = 24$, $X = 4.42$ vs $X_t = 4.29$) higher under preventive regimen than under therapeutic one using L929 cell culture. Difference in the compounds SI under these regimens appeared as insignificant ($n = 23$, $X = 3.95$ vs $X_t = 4.18$) using EPT cell line.

Obtained results are seemed as promised regarding further investigations of indoloquinoxalines as perspective antivirals and interferon inducers.

3. Conclusions

New 6-(2-aminoethyl)-6H-indolo[2,3-b]quinoxalines where synthesized with high yields via developed scheme using bromoethylisatin and 6-(2-bromoethyl)-6H-indolo[2,3-b]quinoxaline as intermediates. These compounds were identified as a low toxic potent interferon inducers an antivirals through L929 and EPT cells based assays. Regarding compounds were more active as antivirals when they were added to the cell monolayer before virus infection than they were added immediately after virus infection we suppose, their antiviral action is mediated by interferon first. On the other hand, mode of their interferon inducing activity is still unassigned, but may be speculated as a DNA intercalation mediated. Thus, aminoethylquinoxalines **8**–**19** really demonstrate high interferon inducing activity according to the initial speculation. Additional studies to identify the mechanism of action of these compounds are in progress.

4. Experimental

4.1. Chemistry

Melting points are uncorrected. The ^{1}H NMR-spectra (300 MHz) of all compounds were recorded on a "Varian VXR-300" spectrometer in $CDCl_3$ solution using TMS as an internal standard. COSY and NOESY spectra were recorded using a "Varian Mercury-400" spectrometer. The mass-spectra of electron impact were recorded using MX-1321 spectrometer with straight sample introduction. The ionization energy of electrons was 70 eV, the source temperature was 220 °C. The mass-spectra with the fast atom bombardment (FAB) ionization were recorded using VG 70-70 EQ spectrometer. Ionization was realized using beam of argon-atoms with energy 10 kV (the compounds were dissolved in 3-nitrobenzyl alcohol). Thin-layer chromatography was performed on pre-coated silica gel F_{254} plates (Merck).

4.1.1. 1-(2-Bromoethyl)-1H-indole-2,3-dione (6)

Potassium carbonate (18.9 g, 0.102 mol) was added to a stirred solution of 1H-indole-2,3-dione (10 g, 0.068 mol) in 40 mL DMF, then 1,2-dibromo-ethane (255.5 g, 1.36 mol, 117.2 mL) was added. After stirring at room temperature for 2 h, the inorganic precipitate was filtered and washed with DMF 3 × 5 mL. Filtrate was evaporated under reduced pressure; the residue was washed with water and filtered. The product was purified by crystallization from ethanol. Yield: 80% (13.8 g); m.p. 132.8–133.4 °C (lit. [35] m.p. 131.0–132.0 °C from ethanol]). MW. 254.08. Mass-spectrum (electron impact) – *m*/*z* (I, %): 255 (21); 253 (20); 146 (100); 132 (55); 90 (7); 77 (10). ^{1}H NMR ($CDCl_3$) δ: 3.601 (t, 2H, Br*CH*$_2$CH$_2$N, $J = 6.9$ Hz); 4.136 (t, 2H, BrCH$_2$*CH*$_2$N, $J = 6.9$ Hz); 7.007 (d, 1H, arom, $J = 8.4$ Hz); 7.134 (t, 1H, arom, $J = 7.5$ Hz); 7.577–7.627 (m, 2H, arom). R_f 0.43 (benzene–triethylamine 10:1); R_f 0.63 (chloroform–acetone 10:1).

4.1.2. 6-(2-Bromoethyl)-6H-indolo[2,3-b]quinoxaline (7)

A mixture of 1-(2-bromoethyl)-1H-indole-2,3-dione (10 g, 0.04 mol), o-phenylenediamine (4.32 g, 0.04 mol) and acetic acid (60 mL) was boiled under reflux for 4 h. Precipitate formed after reaction mixture cooling was collected, washed with acetic acid (3 × 5 mL), and recrystallized from acetic acid. Yield: 80% (10.4 g); m.p. 169–170 °C. MW. 326.20. Mass-spectrum (electron impact) – *m*/*z* (I, %): 329 (14); 327 (21); 232 (4); 220 (5); 219 (96); 102 (8); 90 (15); 69 (6); 60 (32); 45 (100); 43 (58). ^{1}H NMR ($CDCl_3$) δ: 3.863 (t, 2H, Br*CH*$_2$CH$_2$N, $J = 7.2$ Hz); 4.843 (t, 2H, BrCH$_2$*CH*$_2$N, $J = 7.2$ Hz); 7.386 (t, 1H, arom, $J = 7.5$ Hz); 7.523 (d, 1H, arom, $J = 8.1$ Hz); 7.662–7.791 (m, 3H, arom); 8.132 (dd, 1H, $J = 8.4$ Hz, $J = 1.5$ Hz); 8.333 (dd, 1H, $J = 8.1$ Hz, $J = 1.5$ Hz); 8.503 (d, 1H, arom, $J = 7.5$ Hz). R_f 0.60 (benzene–triethylamine 10:1); R_f 0.84 (chloroform–acetone 10:1).

4.1.3. (2-Indolo[2,3-b]quinoxalin-6-yl-ethyl)-dimethylamine (8)

A 33% water solution of dimethylamine (0.225 mL, 0.005 mol) was added to a solution of 6-(2-bromoethyl)-6H-indolo[2,3-b]quinoxaline (0.81 g, 0.0025 mol) in 40 mL DMF. After stirring at room temperature for 6 h, a mixture was evaporated under reduced pressure. The residue was dissolved in 25 mL benzene and extracted with 10% acetic acid (3 × 20 mL). This acetous extract was neutralized with saturated solution of sodium carbonate to pH = 8–9. The obtained precipitate was collected, washed with water (3 × 5 mL) and dried. Yield: 78% (0.57 g); m.p. 95–95.5 °C (lit. [38] m.p. 88–90 °C from methyl acetate). MW. 290.37. Mass-spectrum (electron impact) – *m*/*z* (I, %): 290 (2); 232 (5); 219 (7); 71 (30); 58 (55). Mass-spectrum (FAB) – *m*/*z* (I, %): 291 (100) $[M + H]^+$; 246 (16); 232 (8); 219 (12); 72 (20); 57 (55). ^{1}H NMR ($CDCl_3$) δ: 2.408 (s, 6H, $(CH_3)_2$N); 2.878 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 6.9$ Hz); 4.613 (t, 2H,

$N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.2$ Hz); 7.355 (t, 1H, arom, $J = 7.5$ Hz); 7.520 (d, 1H, arom, $J = 7.8$ Hz); 7.625–7.752 (m, 3H, arom); 8.124 (dd, 1H, $J = 8.1$ Hz, $J = 1.2$ Hz); 8.289 (dd, 1H, $J = 8.1$ Hz, $J = 1.5$ Hz); 8.460 (d, 1H, arom, $J = 7.5$ Hz). R_f 0.46 (benzene–triethylamine 10:1); R_f 0.08 (chloroform–acetone 10:1).

4.1.4. Diethyl-(2-indolo[2,3-b]quinoxalin-6-yl-ethyl)-amine (9)

Diethylamine (0.36 g, 0.005 mol) was added to a solution of 6-(2-bromoethyl)-6H-indolo[2,3-b]quinoxaline (0.81 g, 0.0025 mol) in 40 mL benzene. A mixture was boiled under reflux for 6 h. After cooling the precipitate was filtered and washed with benzene 3 × 5 mL. Filtrate was evaporated under reduced pressure, the residue was dissolved in 25 mL benzene and extracted with 10% acetic acid (3 × 20 mL). This acetous extract was neutralized with saturated solution of sodium carbonate to pH = 8–9. The obtained precipitate was collected, washed with water (3 × 5 mL) and dried. The product was purified by crystallization from heptane. Yield: 83% (0.66 g); m.p. 107–107.5 °C (lit. [39] m.p. 108 °C from petroleum-ether). MW. 318.43. Mass-spectrum (electron impact) – *m/z* (I, %): 319 (1); 232 (4); 100 (12); 87 (100); 59(6). Mass-spectrum (FAB) – *m/z* (I, %): 319 (100) $[M + H]^+$; 246 (10); 232 (8); 100 (6); 86 (12); 69 (6); 53 (8). ^{1}H NMR ($CDCl_3$) δ: 1.029 (t, 6H, $(CH_3CH_2)_2N$, $J = 5.4$ Hz); 2.683 (q, 4H, $(CH_3CH_2)_2N$, $J = 3.9$ Hz); 2.980 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 6.9$ Hz); 4.51 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.5$ Hz); 7.360 (t, 1H, arom, $J = 7.5$ Hz); 7.360–7.760 (m, 4H, arom); 8.05 (d, 1H, $J = 8.1$ Hz); 8.21 (d, 1H, $J = 8.1$ Hz); 8.36 (d, 1H, arom, $J = 7.2$ Hz). R_f 0.62 (benzene–triethylamine 10:1); R_f 0.04 (chloroform–acetone 10:1).

The compounds **10**–**19** were obtained in a similar manner.

4.1.5. (2-Indolo[2,3-b]quinoxalin-6-yl-ethyl)-dipropylamine (10)

Yield: 82% (0.7 g); m.p. 75–76 °C. MW. 346.48. Mass-spectrum (electron impact) – *m/z* (I, %): 346 (1); 232 (2); 127 (13); 114 (100); 72 (6); 43 (8). Mass-spectrum (FAB) – *m/z* (I, %): 347 (100) $[M + H]^+$; 246 (20); 232 (6); 220 (9); 128 (17); 114 (83); 72 (5). ^{1}H NMR ($CDCl_3$) δ: 0.802 (t, 6H, $(CH_3CH_2CH_2)_2N$, $J = 7.2$ Hz); 1.339–1.463 (m, 4H, $(CH_3CH_2CH_2)_2N$); 2.504 (t, 4H, $(CH_3CH_2CH_2)_2N$, $J = 7.5$ Hz); 2.945 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.5$ Hz); 4.569 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.5$ Hz); 7.364 (t, 1H, arom, $J = 6.9$ Hz); 7.518 (d, 1H, arom, $J = 8.4$ Hz); 7.639–7.775 (m, 3H, arom); 8.136 (dd, 1H, $J = 8.4$ Hz, $J = 1.2$ Hz); 8.306 (dd, 1H, $J = 8.1$ Hz, $J = 1.2$ Hz); 8.476 (d, 1H, arom, $J = 7.8$ Hz). R_f 0.71 (benzene–triethylamine 10:1); R_f 0.09 (chloroform–acetone 10:1).

4.1.6. Butyl-(2-indolo[2,3-b]quinoxalin-6-yl-ethyl)-methylamine (11)

Yield: 80% (0.66 g); m.p.of hydrochloride 214.5–215 °C. MW. 332.45. Mass-spectrum (FAB) – *m/z* (I, %): 333 (100) $[M + H]^+$; 246 (8); 232 (6), 100 (12); 53 (5). ^{1}H NMR ($CDCl_3$) δ: 0.926 (t, 3H, $CH_3(CH_2)_3N_{(al)}CH_3$, $J = 7.5$ Hz); 1.281 (s, 1H, $CH_3(CH_2)_3N_{(al)}CH_3$); 1.302–1.491 (m, 2H, $CH_3CH_2(CH_2)_2N_{(al)}CH_3$); 1.694–1.977 (m, 2H, $CH_3CH_2CH_2CH_2N_{(al)}CH_3$); 2.921 (t, 2H, $CH_3(CH_2)_2CH_2N_{(al)}CH_3$, $J = 6.6$ Hz); 3.619 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 8.1$ Hz); 5.220 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.8$ Hz); 7.446 (t, 1H, arom, $J = 8.2$ Hz); 7.718–7.830 (m, 3H, arom); 8.126 (d, 1H, arom, $J = 7.8$ Hz); 8.193 (d, 1H, $J = 7.8$ Hz); 8.374 (dd, 1H, $J = 8.1$ Hz, $J = 1.2$ Hz); 8.512 (d, 1H, arom, $J = 8.1$ Hz). R_f 0.53 (benzene–triethylamine 10:1); R_f 0.01 (chloroform–acetone 10:1).

4.1.7. Butylethyl-(2-indolo[2,3-b]quinoxalin-6-yl-ethyl)-amine (12)

Yield: 84% (0.73 g); m.p. 53–54 °C. MW. 346.48. Mass-spectrum (electron impact) – *m/z* (I, %): 346 (1); 232 (2); 127 (13); 114 (100); 72 (10); 58 (9). Mass-spectrum (FAB) – *m/z* (I, %): 347 (100) $[M + H]^+$; 232 (6), 246 (8); 114 (10). ^{1}H NMR ($CDCl_3$) δ: 0.917 (t, 3H, $CH_3(CH_2)_3N_{(al)}CH_2CH_3$, $J = 7.2$ Hz); 1.243–1.404 (m, 5H, $CH_3CH_2(CH_2)_2N_{(al)}CH_2CH_3$); 1.758–1.915 (m, 2H, $CH_3CH_2CH_2CH_2N_{(al)}CH_2CH_3$); 3.047–3.126 (m, 2H, $CH_3(CH_2)_2CH_2N_{(al)}CH_2CH_3$); 3.184–3.315 (m, 2H, $CH_3(CH_2)_3N_{(al)}CH_2CH_3$); 3.460 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.8$ Hz); 5.075 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.5$ Hz); 7.414 (t, 1H, arom, $J = 7.8$ Hz); 7.677–7.799 (m, 3H, arom); 7.943 (d, 1H, arom, $J = 7.8$ Hz); 8.095 (dd, 1H, $J = 7.8$ Hz, $J = 1.5$ Hz); 8.324 (dd, 1H, $J = 8.1$ Hz, $J = 1.5$ Hz); 8.467 (d, 1H, arom, $J = 8.1$ Hz). R_f 0.68 (benzene–triethylamine 10:1); R_f 0.02 (chloroform–acetone 10:1).

4.1.8. 6-(2-Pyrrolidin-1-yl-ethyl)-6H-indolo[2,3-b]quinoxaline (13)

Yield: 79% (0.63 g); m.p. 93–95 °C. MW. 316.41. Mass-spectrum (FAB) – *m/z* (I, %): 317 (100) $[M + H]^+$; 247 (18); 233 (8); 221 (10); 84 (45); 54 (5). ^{1}H NMR ($CDCl_3$) δ: 1.698–1.875 (m, 4H, $(CH_2CH_2)_2N_{(al)}$); 2.616–2.831 (m, 4H, $(CH_2CH_2)_2N_{(al)}$); 3.049 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.8$ Hz); 4.657 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.8$ Hz); 7.346 (t, 1H, arom, $J = 7.5$ Hz); 7.553 (d, 1H, arom, $J = 8.1$ Hz); 7.619–7.751 (m, 3H, arom); 8.120 (dd, 1H, $J = 8.4$ Hz, $J = 1.8$ Hz); 8.284 (dd, 1H, $J = 8.1$ Hz, $J = 1.5$ Hz); 8.451 (d, 1H, arom, $J = 7.8$ Hz). R_f 0.46 (benzene–triethylamine 10:1); R_f 0.08 (chloroform–acetone 10:1).

4.1.9. 6-(2-Piperidin-1-yl-ethyl)-6H-indolo[2,3-b]quinoxaline (14)

Yield: 87% (0.72 g); m.p. 130–131 °C (lit. [39] m.p. 124–125 °C from petroleum-ether). MW. 330.44. Mass-spectrum (electron impact) – *m/z* (I, %): 330 (2); 232 (5); 112 (25); 98 (100); 55(5). Mass-spectrum (FAB) – *m/z* (I, %): 331 (100) $[M + H]^+$; 246 (17); 232 (7); 220 (9); 112 (20); 98 (60); 53(7). ^{1}H NMR ($CDCl_3$) δ: 1.432–1.480 (m, 2H, $CH_2(CH_2CH_2)_2N_{(al)}$); 1.580–1.615 (m, 4H, $CH_2(CH_2CH_2)_2N_{(al)}$); 2.451–2.603 (m, 4H, $CH_2(CH_2CH_2)_2N_{(al)}$); 2.865 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.2$ Hz); 4.647 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.2$ Hz); 7.354 (t, 1H, arom, $J = 7.2$ Hz); 7.561 (d, 1H, arom, $J = 8.1$ Hz); 7.629–7.760 (m, 3H, arom); 8.127 (dd, 1H, $J = 8.4$ Hz, $J = 1.5$ Hz); 8.298 (dd, 1H, $J = 8.1$ Hz, $J = 1.2$ Hz); 8.463 (d, 1H, arom, $J = 7.8$ Hz). R_f 0.59 (benzene–triethylamine 10:1); R_f 0.09 (chloroform–acetone 10:1).

4.1.10. 6-[2-(2-Methylpiperidin-1-yl)-ethyl]-6H-indolo [2,3-b]quinoxaline (15)

Yield: 85% (0.75 g); m.p. 113–115 °C. MW. 344.46. Mass-spectrum (FAB) – *m/z* (I, %): 345 (100) $[M + H]^+$; 247 (17); 233 (5); 221 (7); 126 (15); 113 (63); 54 (8). ^{1}H NMR ($CDCl_3$) δ: 1.120 (d, 3H, $CH(CH_3)N_{(al)}$, $J = 6.3$ Hz); 1.307–1.434 (m, 2H, $CH_2(CH_2)_2CH_2CH$); 1.634–1.813 (m, 4H, $CH_2(CH_2)_2CH_2CH$); 2.486–2.548 (m, 2H, $CH_2(CH_2)_2CH_2CH(CH_3)N_{(al)}$); 2.834–2.925 (m, 1H, $CH_2CH(CH_3)N_{(al)}$); 3.098–3.270 (m, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$); 4.607–4.688 (m, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$); 7.350 (t, 1H, arom, $J = 7.5$ Hz); 7.577 (d, 1H, arom, $J = 7.8$ Hz); 7.647–7.756 (m, 3H, arom); 8.114 (dd, 1H, $J = 8.4$ Hz, $J = 1.5$ Hz); 8.291 (dd, 1H, $J = 8.4$ Hz, $J = 1.8$ Hz); 8.454 (d, 1H, arom, $J = 7.8$ Hz). R_f 0.19 (benzene–triethylamine 10:1); R_f 0.02 (chloroform–acetone 10:1).

4.1.11. 6-[2-(4-Methylpiperidin-1-yl)-ethyl]-6H-indolo[2,3-b]quinoxaline (16)

Yield: 80% (0.70 g); m.p. 141–142 °C. MW. 344.46. Mass-spectrum (electron impact) – *m/z* (I, %): 344 (1); 232 (4); 125 (27); 112 (100); 69 (5); 44 (11). Mass-spectrum (FAB) – *m/z* (I, %): 345 (100) $[M + H]^+$; 246 (15); 232 (8); 126 (17); 112 (75); 69 (5); 53(8). ^{1}H NMR ($CDCl_3$) δ: 0.916 (d, 3H, CH_3CH, $J = 5.7$ Hz); 1.234–1.346 (m, 3H, $CH_3CH(CH_2CH_2^A)_2N_{(al)}$); 1.619–1.662 (m, 2H, $CH_3CH(CH_2^ACH_2)_2N_{(al)}$); 2.156–2.227 (m, 2H, $CH_3CH(CH_2CH_2^B)_2N_{(al)}$); 2.910 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.2$ Hz); 3.094–3.131 (m, 2H, $CH_3CH(CH_2^BCH_2)_2N_{(al)}$); 4.651 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.2$ Hz); 7.353 (t, 1H, arom, $J = 6.9$ Hz); 7.585 (d, 1H, arom, $J = 8.1$ Hz); 7.624–7.757 (m, 3H, arom); 8.116 (dd, 1H, $J = 8.4$ Hz, $J = 1.2$ Hz);

8.289 (dd, 1H, $J = 8.4$ Hz, $J = 1.2$ Hz); 8.454 (d, 1H, arom, $J = 7.5$ Hz). R_f 0.62 (benzene–triethylamine 10:1); R_f 0.08 (chloroform–acetone 10:1).

4.1.12. 6-(2-Azepan-1-yl-ethyl)-6H-indolo[2,3-b]quinoxaline (17)

Yield: 84% (0.73 g); m.p. 75–76 °C. MW. 344.46. Mass-spectrum (FAB) – *m/z* (I, %): 345 (100) $[M + H]^+$; 246 (16); 232 (7); 219 (8); 126 (17); 112 (52); 69 (17); 53 (35). ^{1}H NMR ($CDCl_3$) δ: 0.822–0.888 (m, 4H, $(CH_2CH_2CH_2)_2N_{(al)}$); 1.563–1.592 (m, 4H, $(CH_2CH_2CH_2)_2N_{(al)}$); 2.940–3.055 (m, 4H, $(CH_2CH_2CH_2)_2N_{(al)}$); 3.160 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.2$ Hz); 4.710 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.2$ Hz); 7.381 (t, 1H, arom, $J = 7.2$ Hz); 7.624 (d, 1H, arom, $J = 8.1$ Hz); 7.649–7.781 (m, 3H, arom); 8.136 (dd, 1H, $J = 8.1$ Hz, $J = 1.8$ Hz); 8.311 (dd, 1H, $J = 8.1$ Hz, $J = 1.2$ Hz); 8.477 (d, 1H, arom, $J = 7.8$ Hz). R_f 0.56 (benzene–triethylamine 10:1); R_f 0.01 (chloroform–acetone 10:1).

4.1.13. 6-[2-(4-Methylpiperazin-1-yl)-ethyl]-6H-indolo [2,3-b]quinoxaline (18)

Yield: 88% (0.76 g); m.p. 143–144 °C. MW. 345.45. Mass-spectrum (FAB) – *m/z* (I, %): 346 (100) $[M + H]^+$; 247 (13); 233 (8); 221 (10); 114 (26); 70 (22). ^{1}H NMR ($CDCl_3$) δ: 2.270 (s, 3H, CH_3N); 2.347–2.443 (m, 4H, $CH_3N(CH_2CH_2)_2N_{(al)}$); 2.598–2.696 (m, 4H, $CH_3N(CH_2CH_2)_2N_{(al)}$); 2.874 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 7.2$ Hz); 4.583 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 6.9$ Hz); 7.350 (t, 1H, arom, $J = 7.8$ Hz); 7.473 (d, 1H, arom, $J = 8.1$ Hz); 7.621–7.753 (m, 3H, arom); 8.094 (dd, 1H, $J = 8.4$ Hz, $J = 1.5$ Hz); д.д. 8.288 (dd, 1H, $J = 8.4$ Hz, $J = 1.5$ Hz); 8.460 (d, 1H, arom, $J = 7.8$ Hz). R_f 0.19 (benzene–triethylamine 10:1); R_f 0.02 (chloroform–acetone 10:1).

4.1.14. 6-(2-Morpholin-4-yl-ethyl)-6H-indolo[2,3-b]quinoxaline (19)

Yield: 93% (0.78 g); m.p. 125–126 °C (lit. [40] m.p. 135 °C from ethyl acetate). MW. 332.41. Mass-spectrum (FAB) – *m/z* (I, %): 333 (100) $[M + H]^+$; 247 (22); 233 (10); 220 (14); 100 (43); 77 (6) ^{1}H NMR ($CDCl_3$) δ: 2.600 (t, 4H, $O(CH_2CH_2)_2N_{(al)}$, $J = 4.5$ Hz); 2.864 (t, 2H, $N_{(al)}CH_2CH_2N_{(ar)}$, $J = 6.9$ Hz); 3.613 (t, 4H, $O(CH_2CH_2)_2N_{(al)}$, $J = 4.8$ Hz); 4.613 (t, 2H, $N_{(al)}CH_2\ CH_2N_{(ar)}$, $J = 6.9$ Hz); 7.377 (t, 1H, arom, $J = 7.2$ Hz); 7.502 (d, 1H, arom, $J = 8.1$ Hz); 7.643–7.774 (m, 3H, arom); 8.113 (dd, 1H, $J = 7.8$ Hz, $J = 1.2$ Hz); 8.292 (dd, 1H, $J = 7.8$ Hz, $J = 1.5$ Hz); 8.487 (d, 1H, arom, $J = 7.8$ Hz). R_f 0.53 (benzene–triethylamine 10:1); R_f 0.29 (chloroform–acetone 10:1).

4.2. Biological assays

4.2.1. Cytotoxicity assay

Lines of murine fibroblast L929 and established piglet testicular EPT cells were obtained from the Research Institute of Veterinary Medicine, Ukrainian Academy of Agriculture, Kiev, Ukraine. EPT cells were cultivated in Medium 199 supplemented with 10% of calf embryo serum (Sigma) and 50 μg/mL kanamycin (Virion, Russian Federation) while L929 cells were cultivated in Eagle's Minimum Essential medium supplemented with the same components as above plus 300 μg/mL glutamine by standard techniques [41].

The in vitro cytotoxicity of synthesized compounds was determined against monolayer cell cultures EPT and L929 using 96-well microtiter plates (FaCCon) at 37 °C in 5% CO_2. The culture medium was substituted for cell maintenance medium (supplemented with 5% of newborn calf serum) with compounds in concentration 1–1000 μM. Toxic action of different compound concentrations was registered in 24–48 h using device Labsystem Myltiscan MS ($\lambda = 540/620$) past stained with 0.5% crystal violet in 70% ethanol. LC_{50}, concentration (*M*) which lead to 50% cell monolayer destruction, was determined using regression analysis method (Microsoft Excel).

4.2.2. Antiviral activity assay

Vesicular stomatitis virus (VSV) was grown in EPT and L929 cells to titers of about 10^6 ID_{50}/0.1 mL. Assay of antiviral effects of compounds was based on evaluation of virus cell pathogen effects for VSV. The tested compounds properly diluted in appropriate media were added to cell monolayer grown in 96-well microtiter plates (FaCCon) at 37 °C in 5% CO_2 (0.2 mL per well) either 24 h before ("preventive effect") or immediately after virus infection ("therapeutic effect"). Concentrations range 0.1–100 μM of compounds was used. Triplicate wells were employed as a rule. The multiplicity of infection was 0.1 ID_{50}/cell for VSV. Virus yields (virus cytodestructive action) were estimated 24 h post infection. EC_{50}, concentration (*M*) which lead to 50% cytopathic action of VVS inhibition, was determined using regression analysis method (Microsoft Excel).

Statistical evaluation of results was made by Student's *t*-test under $P < 0.05$.

4.2.3. IFN-inducing activity assay

IFN-inducing activity was tested in vitro on murine fibroblast (L929) cells and piglet testicular (EPT) cells line. The supernatant was took after 24 and 48 h cultivation at 37 °C in 5% CO_2 and activity of IFN was determined in it. Titers of the IFN induced by compounds were determined on L929 and EPT cell cultures. The tested compound samples (0.1 mL) in serial double dilution were added to cell monolayer. The culture medium (0.1 mL) was added in control well. VSV suspension (0.05 mL) was added in each well after 24 h incubation 37 °C in 5% CO_2. Plates were cultivated in the same condition for 24 h, result estimate was determined by microscopical method.

Maximal dilution of the interferon contained media allowed the cell culture protection against cytopathic action of the VSV 100 CPA50 was taken as interferon titer.

Appendix. Supplementary data

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.ejmech.2009.12.014.

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