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ACID-BASE PROPERTIES AND AFFINITY TO DNA OF INDENOQUINOXALINECARBOXYLIC ACID DERIVATIVES

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Abstract

Three amide derivatives were synthesized on the basis of 11-oxoindeno[1,2-b]quinoxaline-6-carboxylic acid and amines. The structure of the synthesized compounds was confirmed by the methods of chromatography-mass spectrometry and ^{1}H NMR spectroscopy. Their ionization constants were determined by spectrophotometry. The intercalation of the obtained compounds into DNA was proved by two independent methods - competition with ethidium bromide and spectrophotometrically. Quantitatively, the affinity of the studied compounds to DNA was determined by the method of competition with ethidium bromide, and the association constants of this interaction were calculated. N-(2,6-dimethylpyrimidin-4-yl)-11-oxoindeno[2,1-b]quinoxaline-6-carboxamide was able to suppress the fluorescence of intercalated EB at concentrations close to the concentration of EB in the experiment, but at concentrations ten times higher all compounds significantly reduced the fluorescence intensity of EB. According to the results of the experiments, it was confirmed that the studied compounds are high-affinity DNA ligands, which allows them to be considered as APIs with antiviral and cytotoxic activity.

Keywords: nitrogen-containing heterocycles; indenoquinoxaline; ionization constant; spectrophotometry; DNA intercalator; biological activity.

КИСЛОТНІ-ОСНОВНІ ВЛАСТИВОСТІ ТА АФІНІТЕТ ДО ДНК ПОХІДНИХ ІНДЕНОХІНОКСАЛІНКАРБОНОВОЇ КИСЛОТИ

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Анотація

На основі 11-оксоіндено[1,2-b]хіноксалін-6-карбонової кислоти та амінів синтезовано три амідні похідні. Структуру синтезованих сполук підтверджено методами хроматомас-спектрометрії та 1Н ЯМР-спектроскопії. Методом спектрофотометрії визначено їх константи іонізації. Інтеркаляцію отриманих сполук до ДНК доведено двома незалежними способами – конкуренцією з етидієм бромідом та спектрофотометрично. Кількісно афінітет досліджених сполук до ДНК було визначено методом конкуренції з етидієм бромідом та розраховані константи асоціації цієї взаємодії. N-(2,6-диметилпіримідин-4-іл)-11-оксоіндено[2,1-b]хіноксалін-6-карбоксамід виявився здатним пригнічувати флуоресценцію інтеркальованого ЕБ в концентраціях, близьких до концентрації ЕБ в експерименті, але в удесятеро більших концентраціях всі сполуки значно зменшували інтенсивність флуоресценції ЕБ. За результатами експериментів підтверджено, що досліджувані сполуки є високоафінними лігандами ДНК , що дозволяє їх розглядати як АФІ із противірусною та цитотоксичною активністю.

Ключові слова: нітрогеновмісні гетероцикли, інденохіноксалін, константа іонізації, спектрофотометрія, інтеркалятор ДНК, біологічна активність

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Introduction

The indeno[1,2-b]quinoxaline framework serves as the basis for the synthesis of many derivatives [1–16] that exhibit biological activity and can potentially be the basis for the creation of medicinal products. The literature contains information about their inhibitory effect on acetylcholinesterase (AChE) [1], α-glucosidase [2], c-Jun N-terminal kinase (JNK) (JNK) [3–6], tryptophan-tRNA synthase (TrpRS) [7], antimicrobial effect [8; 9], anti-inflammatory [10] and antitumor activity [11–13]. Indenoquinoxalines can inhibit the growth of tumor cells, as well as inhibit mitogen-activating protein kinase [5]. Their pharmacological effect is explained by the mechanism of intercalation to DNA. Due to their flat structure, they are able to embed themselves in the DNA helix with a violation of its geometry, as a result of which they suppress the replication of viruses, cancer cells, etc.

The method and topology of side chain attachment are of great importance for the binding parameters of the intercalator with DNA. The intercalator, which is located in the binding site with a certain orientation with respect to base pairs, directs one or other exocyclic fragments into the small or large groove, and, as a result, the strength of the formed complex largely depends on the relative location of the side chain and non-indifferent nucleotide fragments. Considering the fact that the DNA molecule is a polyanion, and the highest density of the negative charge of DNA is observed in the region of the minor groove [17], and the latter, in turn, is a convenient "hydrophobic pocket" for side chains [18], it is desirable that the intercalator has a "short tail", which will be the carrier of a positive charge (Fig. 1).

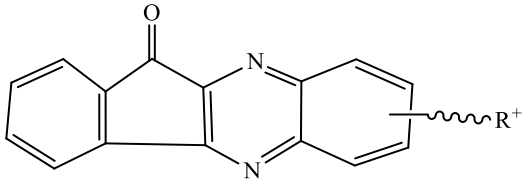

Fig. 1. Schematic illustration of the positively charged "side chain" (R) in the indenoquinoxaline molecule

Therefore, the aim of the presented research is the synthesis of new indenoquinoxaline derivatives as potential DNA intercalators, the establishment of their ionization constants to determine the influence of the acid-base properties of the compounds on the ability to intercalate, as well as the quantitative assessment of the affinity of the studied compounds to DNA.

Experimental part

All reagents used were qualified "for synthesis" by Merck (Germany). Control of reactions was carried out using thin-layer chromatography on «Silufol" and "Silufol UV-254" plates». ^{1}H NMR spectra were recorded in $CDCl_3$ and DMSO-d6 solutions relative to TMS on a Varian VXR-300 (Varian, USA) spectrometer (300 MHz), mass spectra were recorded on a VG 70-70 EQ (VG Instruments Inc., Danvers, MA) spectrometer. Ionization was carried out with a beam of Argon atoms with an energy of 10 kV (substances were dissolved in 3-nitrobenzyl alcohol). The melting points of synthesized compounds were measured in a soldered capillary. Electronic absorption spectra were recorded in the wavelength range of 210–340 nm in cuvettes with an absorbing layer thickness of 1 cm, on a spectrometer «Specord 250+» (Analytic Jena, Germany). Fluorescence spectra were recorded on the spectrofluorometer FL (Zagorsk Optical-Mechanical Plant, Russia) at a wavelength of 365 nm.

Synthesis method of 11-oxoindeno[1,2-b]quinoxaline-6-carboxylic acid derivatives with amines.

30 ml of chloroform was added to 1 g (3.5 mM) of 11-oxoindeno[1,2-b]quinoxaline-6-carboxylic acid [19] and cooled to 0 °C. 0.97 ml (7.0 mM) of triethylamine (TEA) and 0.34 ml (3.5 mM) of monochlorocarbonic acid ethyl ester were added to the cooled mixture. The mixture was stirred for approximately 1 h until the substance completely dissolved, then 3.5 mM of the appropriate amine was added and stirred for 1 day at room temperature. Precipitated products 1-3 were filtered. Purification was carried out using preparative column chromatography. The exception was compound (2), which was purified by recrystallization first from water and then from isopropyl alcohol.

N-[3-(dimethylamino)propyl]-11-oxoindeno[2,1-b]quinoxaline-6-carboxamide **(1)**. Yellow solid; 79% yield; m.p. 207-210 °C. ^{1}H NMR spectrum (300 MHz, DMSO-d_6), δ, ppm:

2.51 (6H, s, CH_3); 1.97 (2H, m, CH_2), 2.82 (2H, m, CH_2), 3.57 (2H, m, CH_2); 7.77 (1H, t), 7.92 (3H, m), 8.23 (1H), d), 8.31 (1H, d), 8.43 (1H, d) (J=8 Hz, arom.); 9.73 (1H, m, NH-CO). MS (EI), *m/z*: 360 $[M]^+$. Calculated $C_{21}H_{20}N_4O_2$: C 70.00, H 5.60, N 15.50. Found: C 70.1, H 5.66, N 15.59.

2-(Diethylamino)ethyl-4-[(11-oxoindeno[2,1-b]quinoxaline-6-carbonyl)amino]ɐenzoate **(2)**. Yellow solid; 75% yield; m.p. 199-202 °C. ^{1}H NMR spectrum (300 MHz, DMSO-d_6), δ, ppm: 0.99 (6H, t, CH_3); 2.57 (4H, k, CH_2), 2.79 (2H, t, N-CH_2), 4.32 (2H, t, O–CH_2); 7.77 (2H, m), 7.91 (2H, d), 7.93 (1H, d), 7.99 (2H, m), 8.04 (2H, m), 8.39 (1H , d), 8.43 (1H, d) (J=8 Hz, arom.); 11.78 (1H, br. s, NH-CO). MS (EI), *m/z*: 494 $[M]^+$. Calculated $C_{29}H_{26}N_4O_4$: C 70.40, H 5.30, N 11.30. Found: C 70.80, H 5.60, N 12.70.

N-(2,6-dimethylpyrimidin-4-yl)-11-oxoindeno[2,1-b]quinoxaline-6-carboxamide **(3)**. Yellow solid; 70% yield; m.p. > 300°C. ^{1}H NMR spectrum (300 MHz, DMSO-d_6), δ, ppm: 2.41 (3H, s, CH_3); 2.54 (3H, s, CH_3), 7.73 (2H, m), 7.95 (1H, d), 8.06 (1H), t), 8.12 (1H, d), 8.25 (1H, d), 8.49 (1H, d) (J=8 Hz, arom.); 13.20 (1H, br. s, NH-CO). MS (EI), *m/z*: 381 $[M]^+$. Calculated $C_{22}H_{15}N_5O_2$: C 69.30, H 4.00, N 18.40. Found: C 69.41, H 4.08, N 18.47.

Determination of pK_a of the investigated compounds

Determination of the pK_a of indenoquinoxaline carboxylic acid derivatives 1-3 was carried out by the spectrophotometric method. A series of solutions of compounds 1–3 with concentrations of 30.5, 5.0, and 11.2 μM, respectively, were prepared in a wide pH range of 1–12. To create the required pH, solutions of hydrochloric acid, sodium hydroxide and universal buffer solutions were used. The light absorption spectra of the obtained solutions relative to the solution of the blank experiment were recorded. The obtained electronic absorption spectra were processed using the SpectroCalc-H5A program. The algorithm for calculating pK_a is based on the methods of iteration and multiple linear regression analysis using the method of least squares and is suitable for the study of substances even in the case of significant overlap of bands in the absorption spectra of individual forms [20].

Testing the ability of compounds to intercalate by spectral changes in the presence of DNA

Testing the ability of compounds to intercalate in DNA was carried out by spectrophotometry and fluorometry methods. For determination by the spectrophotometric method, solutions of compounds with a concentration of 11.2 μM were prepared in the presence of cattle thymus DNA concentrations, μM: 106; 79.5; 53; 35.4; 11.8; 0 in the buffer solution CH_3COONa (5 μM, pH = 5.5) and NaCl (37.3 μM).

Testing the ability of compounds to intercalate by spectral changes in the presence of DNA

The study of compounds affinity to DNA was carried out by the method of substitution with ethidium bromide [21] using DNA (C= 1.06×10^{-5} M); ethidium bromide (C = 1.27×10^{-5} M); NaCl (C = 1.87×10^{-2} M) ; sodium acetate (C = 4.00×10^{-3} M) , brought to pH = 5.5 by adding acetic acid; EDTA (C = 2.46×10^{-4} M).

The study of the dependence of the degree of inhibition of the fluorescence intensity of intercalated ethidium bromide on the logarithm of the concentration of the investigated compounds 1–3 was carried out by approximating the experimental values with the «Dose-effect» curve. From the average values obtained in this way, the values of the association constants were calculated according to equation in accordance with [21]:

$$lgK_x = 7 - lgC_{50} + lgC_e$$

where lgK_x $lg\ K_x$ – logarithm of compound association constant with DNA;

7 – logarithm of ethidium bromide association constant with DNA;

lgC_{50}– the logarithm of compound concentration that provides 50 % displacement of ethidium bromide;

lgC_E – the logarithm of ethidium bromide concentration in the experiment.

Results and discussion

The corresponding derivatives 1–3 were obtained by reaction of 11-oxoindeno[1,2-b]quinoxaline-6-carboxylic acid with amines (N,N-dimethylpropane-1,3-diamine, novocaine, 2,6-dimethylpyrimidine-4-amine) according to the scheme shown in Fig. 2. The carboxyl group was first converted to anhydride by adding monochlorocarbonic acid ethyl ester. The resulting anhydride reacted mildly at room temperature in the presence of TEA with the above amines to give the desired products in 70–79 % yields. All synthesized compounds are yellow amorphous powders that are poorly soluble in most organic solvents. The purity of the products was controlled by TLC, and their identification was carried out by the method of chromatography-mass spectrometry and ^{1}H NMR spectroscopy. In the ^{1}H NMR spectra of compounds 1–3, a tetracyclic indenoquinoxaline fragment of molecules with an almost identical set

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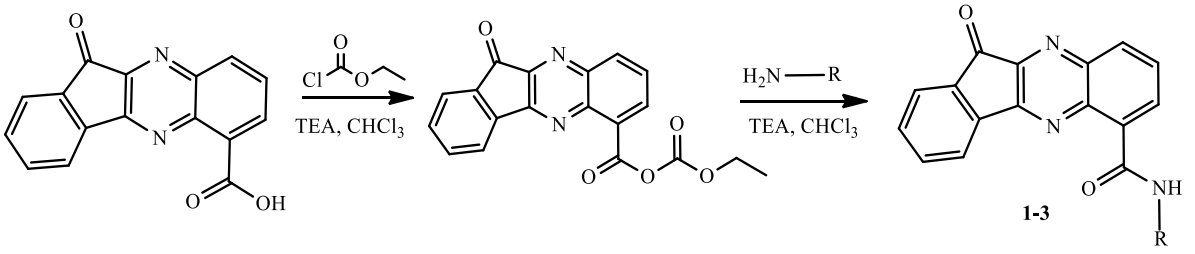
of seven separate signals of aromatic protons (δ = 7.73–8.49 ppm) in the form of doublets and triplets with spin-spin interaction constants of 8 Hz is clearly identified. Aliphatic fragments of products (1–3) are clearly represented in strong fields by characteristic signals of protons of methylene (δ = 1.97–4.32 ppm) and methyl (δ = 0.99–2.51 ppm) groups. In the weakest fields, signals of protons of amide groups were detected in all compounds (δ = 9.73–13.20 ppm).



R: **1** -$(CH_2)_3$-$N(CH_3)_2$ (79%); **2** - 4-C_6H_4COO-$(CH_2)_2$-$N(C_2H_5)_2$ (75%); **3** - 4(2,6-$(CH_3)_2$)Pyr (70%)

Fig. 2. Synthesis scheme of indenoquinoxaline-6-carboxylic acid derivatives with amines (1–3)

It is worth noting that in work [20], the synthesis of amide derivatives was carried out through the stage of formation of an intermediate product with 1,10-carbonyldiimidazole (CDI) (Fig. 3), which is a crystalline substance and requires considerable effort to separate it from the reaction medium. The final amides were obtained in the form of hydrochlorides with yields of 60–63 %. The method proposed by us does not require the separation of the intermediate compound and the use of additional solvents, which greatly simplifies the process of obtaining final substances with better yields.

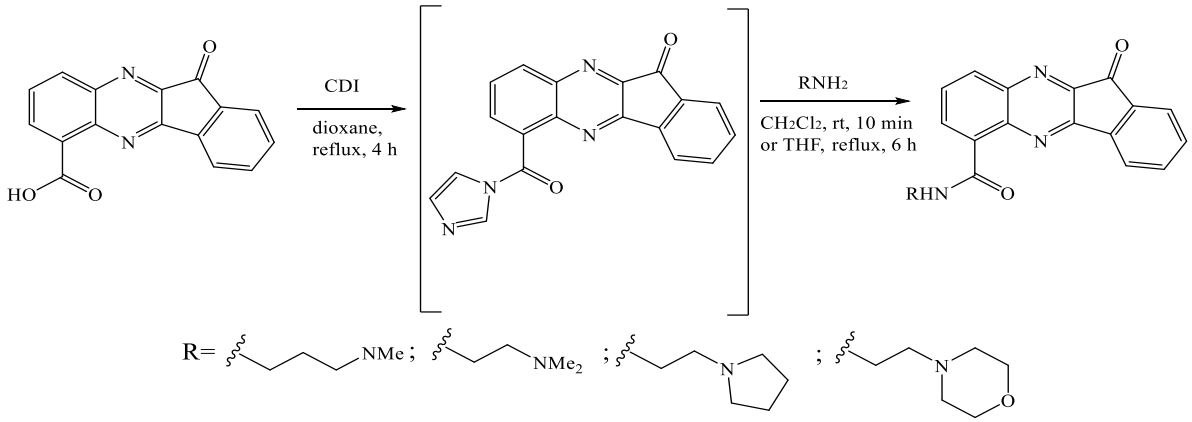


Fig. 3. Synthesis scheme of amide derivatives of indenoquinoxaline-6-carboxylic acid in work [11]

Electrostatic interactions are of great importance for the binding parameters of the intercalator with DNA, as they make an important contribution to the stabilization of the intercalator-DNA bond. The carrier of the positive charge in the intercalator molecules has been often side chains of a basic nature, as, for example, in amixin [22]. In our case, the tertiary amino group (for compounds 1, 2) or the pyrimidine ring (for compound 3), which are part of the side chain, will undergo protonation. Therefore, the ionization constant of these groups is an important parameter that affects the possibility of intercalation.

The acidity of the fluids inside the human body normally coincides with the acidity of the blood and is in the range from 7.35 to 7.45 pH, and therefore it is desirable that the synthesized compounds are able to protonate in this range. In this regard, on the basis of electronic absorption spectra (Fig. 4), in accordance with the methodology [11] pKa values were calculated for compounds 1-3, which were, respectively: 7.07 ± 0.03, 5.52 ± 0.01, 1.46 ±.0.01. It can be seen from the given results, that only compound 1 will be able to protonate under these conditions, and compounds 2 and 3 are weak and strong acids, respectively.

The changes observed in the electronic absorption spectra of solutions of compounds 1–3 are generally similar, so for the example in Fig. 3 shows the relevant data for compound 1.

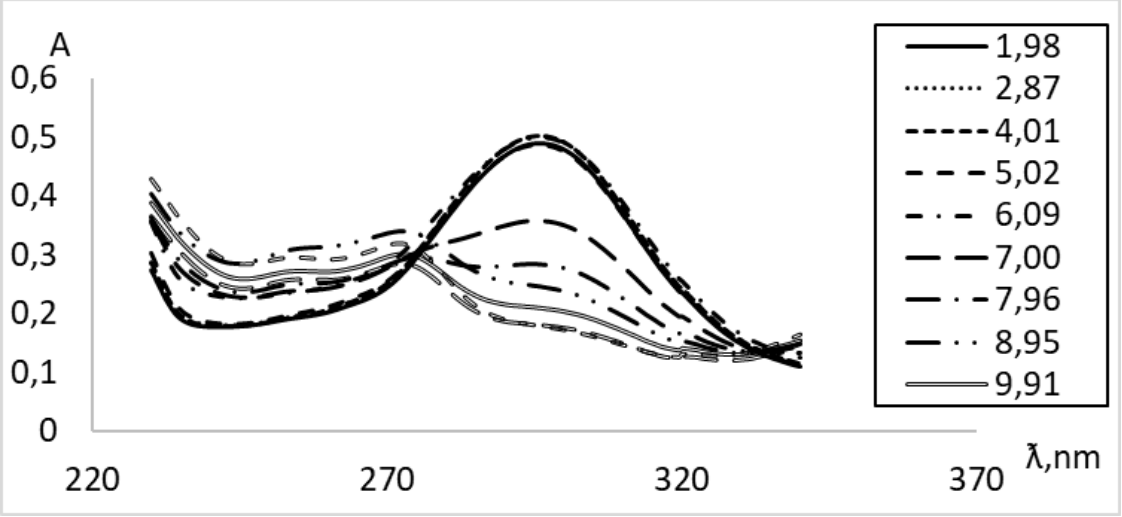


Fig. 4. Electronic absorption spectra of compound 1 at different Ph

One of the signs of planar ligands intercalation to DNA is the presence of characteristic changes in the electronic absorption spectra of their solutions, due to the stacking interaction of aromatic (or heteroaromatic) systems of planar parts of molecules with heteroaromatic systems of base pairs, namely, a bathochromic shift of the absorption band of the intercalator with simultaneous hypochromism [23]. Thus, on the basis of changes in absorption spectra, it is possible to assess the presence or absence of stacking interaction of the intercalator with DNA.

For this purpose, we registered the corresponding absorption spectra of compounds 1–3 in the presence of different concentrations of DNA. For compound 1, with an increase in DNA concentration, a decrease in the absorption maximum of the band in the region of 230–270 nm is observed at the same time as its shift by 2–5 nm (Fig. 5), which is characteristic of ligands whose center of positive charge is located in the side chain [24].

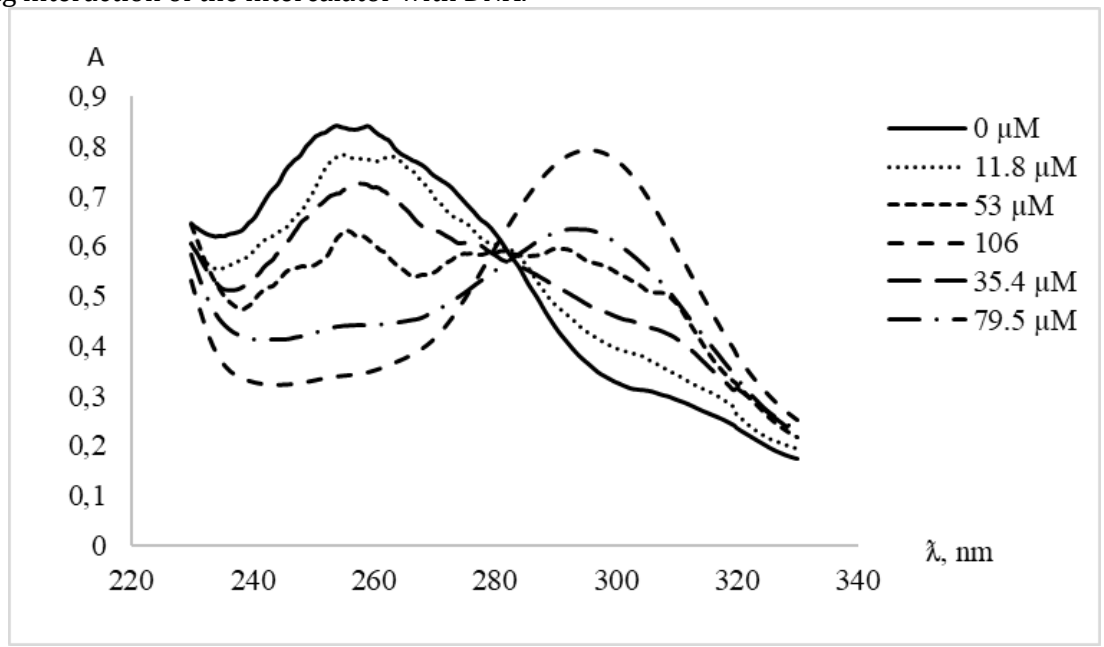


Fig. 5. Absorption spectra of compound 1 with a concentration of 11.2 μM in the presence of different DNA concentrations

For the solution of compound 2, it turned out to be impossible to register the spectra, because when DNA was added, a precipitate immediately began to fall out, which, on the one hand, complicates the determination of the way the substance binds to DNA, but on the other hand, it proves its fundamental ability to interact with it, since it has already been shown that due to the formation of ion pairs as a result of electrostatic interactions between DNA anions and amino cations of the ligand, hydrophilicity is lost, which leads to the collapse of the DNA conformation, as

a result of which it loses its helical structure and precipitates from the solution [25]. For compound 3, with increasing DNA concentration, only a bathochromic shift was observed without a decrease in optical density, which may indicate that the molecule can bind to DNA in different ways at once. Therefore, on the basis of the spectroscopic data for compounds 2 and 3, it is possible to confidently assert the presence of their interaction with DNA.

Quantitatively, the affinity of the investigated compounds was determined by the method of competition with ethidium bromide (EB) [21], which consists in the ability of EB to form strong fluorescent complexes with DNA. Therefore, when the compounds under study bind to DNA, they must squeeze out EB, thereby suppressing fluorescence, since the complexes of these compounds themselves are not capable of fluorescence. By approximating the experimental data on the dependence of the degree of fluorescence suppression (DFS) on the logarithm of compound concentration, the "Dose-effect" curves were obtained, which are presented in Fig. 6, from the average values (lgC_{50}) of which the association constants (lgK_x) of compounds 1–3 with DNA were calculated (table).

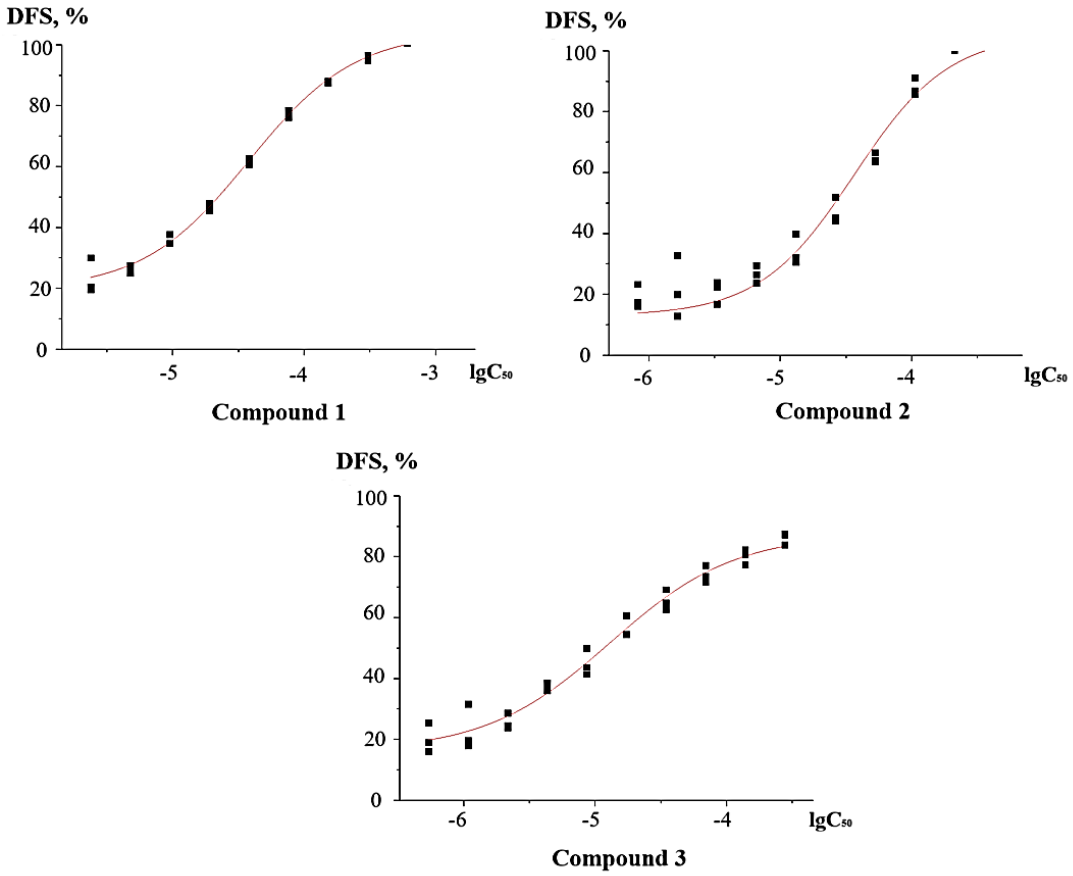


Fig. 6. Graphic illustration of the approximation of the experimental data of the "dose-effect" curve for the determination of the average values of lgC_{50}

Table

Values of compounds 1-3 concentrations, which ensure the displacement of 50% of intercalated ethidium bromide from its complex with DNA and the stability constants of their associates

Compound	lgC_{50}	$C_{50}\ 10^{-5}$ M	lgK_x	$K_x \cdot 10^6$, M^{-1}
1	-4.42±0.01	3.78	6.52	3.31·
2	-4.44±0.03	3.63	6.54	3.47·
3	-4.89±0.03	1.30	6.99	9.77·

In the test for the ability of compounds 1–3 to compete with EB for binding sites in DNA, only compound 3 was able to suppress the fluorescence of intercalated EB at concentrations close to 12.5 μM (the concentration of EB in the experiment), but at concentrations ten times higher, all compounds significantly reduced EB fluorescence intensity. The large value of the DNA association constant of compound 3 and the absence of hypochromism when studying

intercalation by spectral changes may indicate that the compound can bind to DNA in several ways at once.

Conclusions

Three amide derivatives of the indenoquinoxaline series were synthesized and identified: N-[3-(dimethylamino)propyl]-11-oxoindeno[2,1-b]quinoxaline-6-carboxamide, 2-(Diethylamino)ethyl-4-[(11-oxoindeno[2,1-b]quinoxaline-6-carbonyl)amino]benzoate, N-(2,6-dimethylpyrimidin-4-yl)-11-oxoindeno[2,1-b]quinoxaline-6-carboxamide, the last two were synthesized for the first time. The proposed method of synthesis is simpler compared to that described in the literature. The determined values of their ionization constants are: 7.07±0.03, 5.52±0.01, 1.46±0.01. Spectrophotometric and fluorescence methods have proven the possibility of binding the obtained compounds to DNA, which allows them to be considered as potential antiviral and antitumor agents.

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