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Synthesis, Genotoxicity and UV-protective Activity of New Benzofuroxans Substituted by Aromatic Amines

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Abstract: The synthesis of new organic compounds possessing properties, which can be used for biological or pharmaceutical purpose, is very challenging and is a current trend in heterocyclic chemistry. So benzofuroxans are of great interest from a medical and combinatory chemistry point of view, being effective biologically active compounds. They are easily available, easily tuned and functionalized allowing the creation of databases in which a chemist can find proper informations to prepare the structurally appropriate compound with the requested selective effect. In this work, heterocyclic compounds are prepared on the basis of the interaction of 4,6-dichloro-5-nitrobenzofuroxan with aromatic amines and diamines. Their ability to suppress and prevent genotoxic effects of UV-radiation in the wavelength range between 300–400 nm has been studied. It has been shown that these compounds are able to protect bacterial cells from destructive effects of the UV-radiation. Comparing the results obtained for various benzofuroxans to those obtained for the natural antioxidant α - tocopherol (vitamin E) and for the synthetic antioxidant trolox, which are references in this domain, we have shown that some benzofuroxans quantitatively exhibit a similar protective effect, and that compounds prepared from the reaction between 4,6-dichloro-5-nitrobenzofuroxan and ethylenedianiline possess potent protective potential.

Keywords: Antioxidant; Benzofuroxan; Genotoxicity; lux-biosensor; S_NAr; UV-radiation.

INTRODUCTION

Dinitro - and nitro-benzofuroxans can be used for the synthesis of new biologically active substances and for the creation of medicinal active products. Many reviews dealing with the chemistry, the reactivity and the biological activity of benzofuroxans have been published in the past 40 years. For example, Gasco and Boulton [1] or Katritzky [2] have reported the synthesis and the chemical behaviour of benzofuroxans and furoxans. Other works dealing with the pharmacological properties of benzofurazans and benzofuroxans has been published between 1981 and 2005. In 1981, Gosh, Ternai and Whitehouse [3] are the first to publish a review in which the biochemical activity of benzofuroxans is extensively studied. More generally, benzofuroxan derivatives exhibit a broad spectrum of biological activity including antibacterial, antifungal, antileukemic, acaricide and immunodepressive properties. Recently, Cerecetto has published two reviews reporting the pharmaceutical properties of furoxans and benzofuroxans. [4, 5] These compounds are involved in many applications such as nitric oxide-releasing abilities or are known to be mutagenic and genotoxic agent. [6-9] The presence of NO-donor fragment increases the bioavailability of the drug, increasing its pharmacological activity and reducing its toxicity. Amines are a common fragment in biology and it has been demonstrated that many amino compounds exhibit high antioxidant and protective activities, as well as metabolic regulator properties. [10,11] Among them, antioxidants are known to protect cells from damage by near ultraviolet radiation, for example, allantoin and urate. [12] The *para*-aminobenzoic acid (PABA) is also a UV-B absorber used as a sunscreen component since the 1920's but recently withdrawn from the cosmetic market because of its photosensitivity. [13-15]

One of the main tasks of the biologist was the preliminary evaluation of the toxicity of the synthesized compounds by the SOS-lux test by means of various recombinant bioluminescent *E. Coli* strains [16-18] and also the evaluation of their ability to stimulate endocellular generation of reactive oxygen species (ROS) with the help of bacterial luminescent biosensor controls with soxS (oxidizing damages) or grpE (damage of proteins) promoters. [16]

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It has been well demonstrated that sunlight can result in damage to the human body, especially in connection with the skin. The fraction of sunlight of most concern is the long wave or near ultraviolet range, which is characterized by a wavelength range between 300-400 nanometers. All ranges of ultraviolet radiation (UV-R) are capable to cause genotoxic effects and various mechanisms can be envisioned during these processes. The primary products of DNA damage by UV-B and UV-C are basically cyclobutane dimers (UV-A are not absorbed by nucleic acids and provokes oxidation of DNA bases), starting a complicated succession of photoreactions, going along with the formation of by-products as hydrogen peroxide and singlet oxygen. According to recent ideas, these reactive oxygen species are basically responsible for genetic effects of the solar spectrum ultraviolet at the earth surface [19-22].

It has been recently shown in a paper, published by Mouret *et al*., [23] that the formation of cyclobutane dimers is induced by UV-A. However, it should be noted that the methods applied in most of these works, to identify and to highlight the formation of cyclobutane dimers, lead to ambiguous results. More particularly, methods, based on chromatographic analysis and mass spectrometry, allow to identify accurately photoproducts, and more particularly, thymine dimers. However, the use of cellular genetic material requires quite tough pre-treatments inducing damage to DNA molecules and a significant decrease of the accuracy of the results. Using more recent and accurate technique, based on the detection of specific antibodies combining with DNA dimers, using an atomic-force microscope, has allowed some authors to conclude that the role of pyrimidine dimers in the implementation of the effects of UV irradiation is still significantly overestimated [24]. Thus the ability of UV-A and UV-B to induce cyclobutane dimers should be checked thoroughly by means of more suitable methodologies.

Obviously, the solution of the problem of protection against negative effects of solar radiation should be based on the coordinated and integrated use of several different mechanisms. Thus, searching the best candidate able to protect a cell from ultraviolet genotoxic effects is definitely challenging. The larger the number of such substances is, the easier the possibility to create highly effective compounds is**.** Keeping in mind that hybrid structure containing both the benzofuroxan and the amine moieties could have potential anti-oxidant and UV-protective activities, we decided to study the genotoxicity of the compounds prepared upon the interaction of 4,6-dichloro-5-nitrobenzofuroxan with aromatic amines (aniline, p-anisidine) and diamines (*para*-phenylenediamine, methylenedianiline, ethylenedianiline) and their ability to suppress the destructive action of UV-radiation in the wavelength range between 300-400.

MATERIALS AND METHODS

Chemistry

General Comments

The ^{1}H NMR spectra were recorded on an AVANCE-600 NMR spectrometer (600 MHz) against residual proton signals of deuterated solvents ($CDCl_3$, acetone-d_6) using TMS (tetramethylsilane) as an internal reference.

The IR spectra were recorded on a Bruker Vector-22 Fourier spectrometer in the range 400-4000 cm^{-1}. Crystalline samples were studied as emulsions in vaseline oil.

The X-ray structural analysis of **4** was carried out on automatic diffractometer "Bruker Smart APEX II CCD": graphite monochromator; λMoK_a = 0.71073 Å; ω-scanning; temperature 73 K. The semi empirical record of absorption by means of program SADABS was carried out [25]. Structures are deciphered by the direct method with SIR program [26] and are made more precise at first in isotropic, then in anisotropic approach with SHELXL-97 program [27]. All calculations are made by means of WinGX [28] and APEX2 [29] programs. The analysis of intermolecular contacts in crystals, figures of molecules and crystal packing are created with PLATON program. Coordinates of atoms, geometrical parameters of structures are deposited in Cambridge crystal structure database.

Element analyses were performed on a Carlo Erba Model EA-1108 elemental analyzer with an accuracy ±0.4% for C, H, Cl and N.

Melting points were determined on a melting point apparatus and are uncorrected.

Commercially available Dimethylsulfoxide (DMSO) was distilled at atmospheric pressure or under vacuum before use. Solvents were rectified and dehydrated by usual techniques [30]. Compounds **1-3** have been prepared using described procedures [31, 32].

General Procedure for the Synthesis of Compounds 4-7. Reactions of 4,6-dichloro-5-nitrobenzo [1,2-c] [1,2,5]-oxadiazol-1-oxide with Aromatic Amines and Diamines.

4-(4-aminophenylamino)-5-nitro-6-chlorobenzo[1,2-c][1,2,5]-oxadiazol-1-oxide (4).

To a solution of 4,6-dichloro-5-nitrobenzofuroxan **1 (**0.125 g, 0.0005 mole) in 5 ml DMSO at room temperature was added a solution of *para*-phenylenediamine (0.108 g, 0.001 mole) in 5 ml DMSO. The reaction mixture was stirred for 3-4 h (the reaction was monitored by thin layer chromatography). After verification of the completion of the reaction by TLC, the crude mixture is precipitated in distilled water and the purple solid was filtered off, washed with cold water and dried under vacuum (0.06 mm Hg) at 40°C temperature to constant weight. Yield: 0.11 g (75 %; Mp: > 300 °C; IR (KBr, cm^{-1}): 1614 (furoxan ring), 1559 (NO_2), 3086 (H), 3302 (NH); ^{1}H NMR ($CDCl_3$, 600 MHz): 5.52 (s, 2H, NH_2), 6.34 (d, J = 7.64 Hz, 2H), 6.92 (s,1H), 7.46 (d, J = 7.48 Hz, 2H, CH_{ap}), 8.17 (s, 1H; NH). Anal. calcd. for $C_{12}H_8ClN_5O_4$: C, 44.81; H, 2.51; Cl, 11.02; N, 21.77; Found: C, 44.71; H, 2.32; Cl, 11.11; N, 21.78.

1,1-bis(N-(5-nitro-6-chlorobenzo[1,2-c][1,2,5]-oxadiazol-1-oxide-4-yl)-4-aminophenyl)methane (5).

Compound **5** is prepared as above using 0.125 g (0.0005 mole) of 4,6-dichloro-5-nitrobenzofuroxan **1** and 0.148 g (0.00075 mole) of 4,4'-diaminodiphenylmethane. Compound **5** is obtained as an orange solid in a 91% yield (0.14 g). Mp:

112 °C; IR (KBr, cm^{-1}): 1561 (NO_2), 1615 (furoxan ring), 3087 (H), 3333 (NH); ^{1}H NMR (acetone-d_6, 600 MHz): 4.04 (s, 2H, CH_2), 7.12 (s, 2H), 7.22 (d, *J* = 8.07 Hz, 4H), 7.29 (d, *J* = 8.07 Hz, 4H), 9.12 (s, 2H; NH). Anal. calcd. for $C_{25}H_{14}Cl_2N_8O_8$: C, 48.02; H, 2.26; Cl, 11.34; N, 17.92; Found: C, 48.08; H, 1.94; Cl, 11.33; N, 17.91.

N-(4-(4-aminophenyl)-ethyl)-phenyl)-4--amino-5-nitro-6-chlorobenzo[1,2-c][1,2,5]-oxadiazol-1-oxide (6).

In a round bottomed flask, 0.125 g (0.0005 mole) of 4,6-dichloro-5-nitrobenzofuroxan **1** was dissolved in dioxane (5 ml). To this solution is added dropwise a solution of 0.212 g (0.001 mole) of 4,4'-diaminodiphenylethane in 5 ml of dioxane at room temperature. The reaction mixture is maintained 30 minutes at room temperature under stirring. The solvent was removed under reduced pressure and the resulting red solid is washed with water and dried under vacuum (0.06 mm Hg) at 40 °C to constant weight. Yield: 0.15 g (71 %); Mp: 171 °C; IR(KBr, cm^{-1}): 1563 (NO_2), 1620 (furoxan ring), 3099 (H), 3223, 3226 (NH_2), 3413 (NH); ^{1}H NMR ($CDCl_3$, 600 MHz): 2.83 (t, *J* = 7.54 Hz, 2H), 2.91 (t, *J* = 7.54 Hz, 2H), 3.57 (s, 2H; NH_2), 6.62 (d, *J* = 7.58 Hz, 2H), 6.85 (s, 1H), 6.91 (d, *J* = 8.41 Hz, 2H), 7.15 (s, 4H), 8.72 (s, 1H; NH); Anal. calcd. for $C_{20}H_{16}ClN_5O_4$: C, 56.41; H, 3.79; Cl, 8.33; N, 16.45; Found: C, 56.79; H, 3.89; Cl, 8.30; N, 16.47.

N-(4-(4-aminophenyl)-methyl)-phenyl)-4--amino-5-nitro-6-chlorobenzo[1,2-c][1,2,5]-oxadiazol-1-oxide (7).

Compound **7** is prepared as above using 0.125 g (0.0005 mole) of 4,6-dichloro-5-nitrobenzofuroxan **1** and 0.198 g (0.001 mole) of 4,4'-diaminodiphenylmethane to give 0.17 g (83 %) of **7** as an orange solid. Mp 185 °C; IR(KBr, cm^{-1}): 1622 (furoxan ring), 1560 (NO_2), 3086 (H), 3301, 3384 (NH_2), 3469 (NH); ^{1}H NMR ($CDCl_3$, 600 MHz): 3.78 (s, 2H, CH_2), 3.93 (s, 2H, NH_2), 6.63 (d, *J* = 8.54 Hz, 2H), 6.85 (s, 1H), 6.97 (d, *J* = 8.09 Hz, 2H), 7.16 (d, *J* = 8.09 Hz, 4H), 8.17 (s, 1H, NH); Anal. calcd. for $C_{19}H_{14}ClN_5O_4$: C, 55.42; H, 3.43; Cl, 8.61; N, 17.01; Found: C, 55.55; H, 3.56; Cll, 8.70; N, 17.06.

Study of the Benzofuroxan Compounds Toxicity and of their Ability to Suppress Ultraviolet A genotoxic Effects

General Comments

All chemical preparations were of analytical purity: methylviologen («Sigma»), hydrogen peroxide («Ferrain»), MNNG (methylnitrosoguanidine) («Sigma»), ethanol («NeoSources Inc.»), tocopherol («AppliChem»), trolox («Fluka»), Tween («Amresco»), DMSO («Amresco»).

Test solutions were prepared in deionized water directly before use. Tocopherol solutions were prepared in 1 % Tween in ethanol. Benzofuroxans solutions were prepared in DMSO.

Bacterial Strains and Cultivation Conditions

E. coli MG1655 (pRecA-lux), *E. coli* MG1655 (pKatG-lux), *E. coli* MG1655 (pSoxS-lux), *E. coli* MG1655 (pGrpE-lux) (the strains kindly furnished by Manukhov I.V., Federal State Unitary Enterprise "GosNIIGenetika"), *E. coli* C600 (pPLS-lux) have been used.

Bacterial strains were cultivated in Luria-Bertani (LB) medium (Maniatis *et al.*, 1982), containing 100 mkg/ml of ampicillin. Cultures were grown overnight at constant stirring on ES-20 shaker («Biosan», Latvia), then diluted to $3x10^6$ cell/ml concentration by fresh LB medium, and were left to grow within 2,5 hours at 30°C. Then cells were immediately used for carrying out of tests [33].

Test System for Benzofuroxans Toxicity Evaluation

Culture samples of 190 μl were introduced into wells of plates. A part of wells were used as a control group (10 μl of distilled water or 10 μl of DMSO were added into them). 10 μl of toxicant solution (in case of positive control for promoter activation), or benzofuroxan compounds in various concentrations (ranging from $5x10^{-1}$ to $5x10^{-10}$ mg/ml) were introduced into other wells.

For control activation of the SoxS promoter methylviologen was used, for KatG promoter activation - hydrogen peroxide, for RecA promoter activation - MNNG («Sigma»), for grpE promoter - ethanol. Luminescence measurements were carried out on microtablet luminometer LM–01T (Immunotech, Czech Republic). Numerical values of a bioluminescence were expressed in arbitrary light units.

The induction factor, I, was defined as the relation of luminescence intensity of a lux-biosensor suspension, containing tested compound (Lc), to the luminescence intensity of a lux-biosensor control suspension (Lk): I=Lc/Lk.

Difference reliability of bioluminescence in experiment from control value was estimated by t-criterion with the help of Excel program. The conclusion about sample toxicity was made at $p < 0.05$. All the experiments were carried out three times independently.

Test System for Prooxidant Action Estimation of Ultra-Violet Radiation in the Wavelength Range Between 300–400 Nanometers

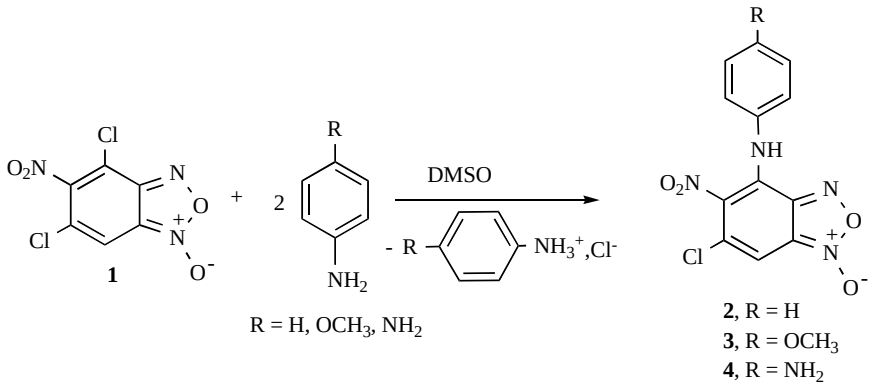
E. .coli MG1655 (pKatG-lux) biosensor was used as a basis of test system for prooxidant action estimation of ultra-violet radiation in the wavelength range between 300-400 nanometers.

45 μl of *E. coli* MG1655 (pKatG-lux) culture aliquots were transferred into microtiter wells and 5 μl of the tested substance of the required concentration in DMSO were added into them. 5 μl of DMSO were added into control wells. Thus two raws of wells were prepared. Only one raw of wells was exposed to the ultra-violet radiation.

The microtitration plate was irradiated in the wavelength range between 300-400 nanometers using UV-apparatus with a mercury lamp of low pressure (HG-125).

UV-1 apparatus [34] was designed in TTI SFedU especially for imitation of ultra-violet radiation of the Sun at a terrestrial surface at noon in summer [35]. The part of ultraviolet B is 3 % from the general capacity of a lamp (part of ultraviolet A - 97 %).

A lamp glass preventing the action of rigid radiation components in the lamp has been used. Measurements of radiation power with $\lambda < 300$ nanometers have been made by means of optical filters in the form of optical glasses BK-10,



Scheme 1. Synthesis of compounds **2-4**.

K-8. The lamp was placed near a parabolic reflecting mirror. In order to decrease the dispersion of the UV radiation, the radiation was transferred to irradiated object through an aluminium directing system. It allowed the generation of an axial and symmetrical uniform stream of radiation on a distance from 20 cm to 2 m in which the minimum area of an irradiated surface makes 80x80 mm, and non-uniformity (at 50 mm deviation from the axis) did not exceed 20 %. The dose of an irradiation during 1 minute was 600 J /m^2. The irradiation lasted for 10 minutes.

After the irradiation the plate with samples was placed into the luminometer and incubated at 30°C. Bioluminescence measurement was carried out for 2.5 hours. For the evaluation of the benzofuroxans protector properties in relation to the ultra-violet radiation in the wavelength range between 300-400 nanometers, the protector potential indicator (P, %) was used which was defined by the formula:

$$P = (1-I_a/Ip)100\%$$

where: I_a is the factor of lux-response induction of the investigated influence in the presence of the protector, I_p - the factor of lux-response induction of the investigated influence.

The protective activity indicators were calculated as mean values in three independent replications. The value of confidential intervals ($p < 0.05$) was calculated by means of the Excel program.

All experiments for protective activity estimation were carried out in three independent replications.

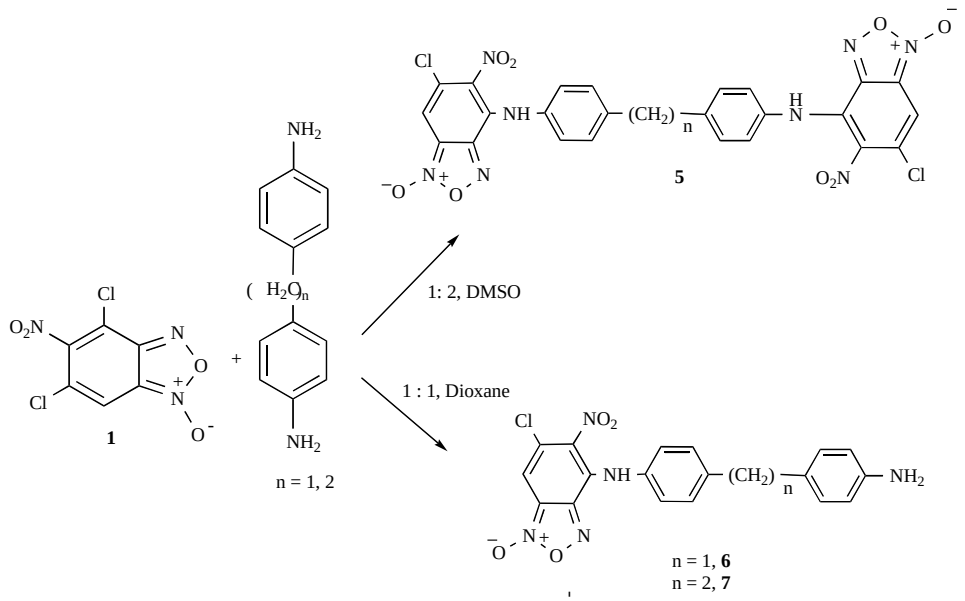
RESULTS AND DISCUSSION

Chemistry

It has been now well demonstrated that 4,6- dichloro-5-nitrobenzofuroxan **1**, which possesses fungicide activity can be also used for its biological activity. Recently, we have shown that reactions of 4,6-dichloro-5-nitrobenzofuroxan **1** with aliphatic amines (methylamine, dimethylamine, 2,2,-dimethoxythylmethyl- amine) is going along with the substitution of chlorine atom in the fourth position of the carbocyclic ring of the benzofuroxan derivative.[36] We tried to find the appropriate experimental conditions to perform the substitution of both chlorine atoms. The interactions between 4,6-dichloro-5-nitrobenzofuroxan and amines have been performed in DMSO, IPA (isopropyl alcohol), dioxane and chloroform. Unfortunately, the only substitution of the chlorine atom in the fourth position has been observed. One could explain that the electron releasing effect of the amine moiety is slowing down a second S_NAr process making it kinetically unfavourable.[37] Nevertheless, these results clearly show that DMSO is the more appropriate solvent to isolate the substitution product in a high yield and with a high purity. DMSO is a polar aprotic solvent and this solvent is known to favor S_NAr processes with the stabilization of σ-adducts type-intermediates.[37] For example, the interaction of **1** with aniline and *para*-anisidine in DMSO leads to the formation of compounds **2** and **3** (Scheme **1**) in high yield (70-90%) [31].

We have previously mentioned that the interaction of 4,6-dichloro-5-nitrobenzofuroxan with *para*-phenylenediamine is going along with the substitution of one chlorine atom and is leading to the formation of compound **4** (Scheme **1**). When the amine is located at the fourth position of the benzofuroxan ring, the second amine group is not involved in a second substitution process. A reasonable explanation may be the electron withdrawing effect exerts by the benzofuroxan ring, reducing the nucleophilicity of the second amine group of the aromatic ring. In fact, the nucleophily of the remaining amino group (see structures **6** and **7**) seems to be higher in DMSO than in dioxane leading to the diadduct **5**.

The radiocrystallographic analysis of compound **4** packing Fig. **(1)** shows that, in this compound, the 3D supramolecular structure is formed on the basis of classical hygrogen bonds of N–H···N- and N–H···O-type, and also of short contacts of C-H···O-type. It is interesting to note that compound **4** displays only one absorption band at 3302 cm^{-1} in the IR-spectrum. This is characteristic for secondary amines and could be explained by the formation of numerous hydrogen bonds between molecules. The reactions of 4,6-dichloro-5-nitrobenzofuroxan with aromatic diamines (4,4'-methylenedianiline (n=1), 4,4'-ethylenedianiline (n=2)) in various solvents lead to the formation of two types of



Scheme 2. Synthesis of compounds **5-7**.

products: a 1:1 isomer, when one molecule of benzofuroxan is reacting with one molecule of diamine and a 2:1 isomer when two molecules of benzofuroxan are reacting with one molecule of diamine. It is important to note that using the appropriate solvent and the appropriate excess of amine allows a selective synthesis of the substitution product. It appears very easy to perform the synthesis and the isolation of compounds, containing one or two benzofuroxan fragments.

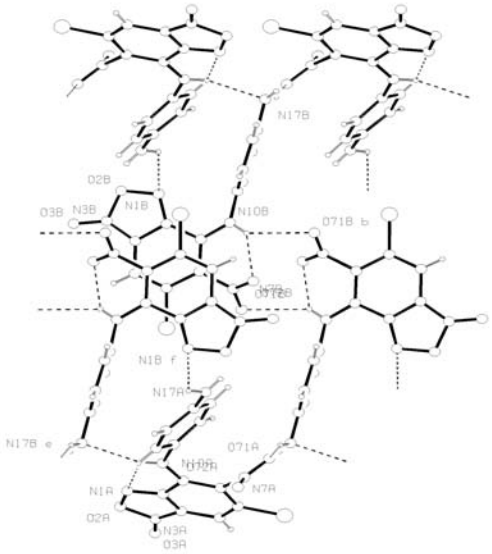


Fig. (1). A fragment of molecules packing in compound **4** crystal.

Study of the Destructive Effect of Benzofuroxans Using Bacterial Lux-Biosensor Controls

The bioluminescent response of lux-biosensor controls to action of various benzofuroxans is presented in table **1**.

Compound **3** displayed the effect expressed in strengthening of endocellular superoxide anion generation, in concentration $1.5x10^{-4}$mmol/ml at testing with biosensor control *E. coli* MG 1655 (pSoxS-lux). An induction factor of 2.5 units has been determined.

The bioluminescent response of biosensor control *E. coli* MG 1655 (pSoxS-lux) have also been induced by compound **5** in concentrations ranging from $8x10^{-12}$ to $8x10^{-4}$mmol/ml, compound **4** in concentrations ranging from $3x10^{-3}$ to $1.5x10^{-4}$mmol/ml and compound **1** (4,6-dichloro-5-nitrobenzofuroxan) - in concentrations ranging from $4x10^{-4}$ to $2x10^{-5}$mmol/ml. Induction factors thus varied within 1.5-2.0 units, 3.0-5.0 units, 1.4-7.4 units, respectively.

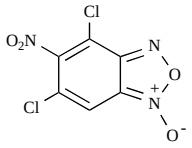
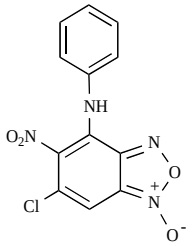
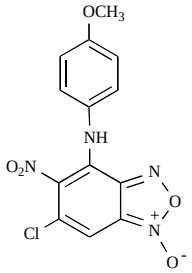
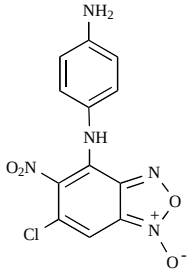
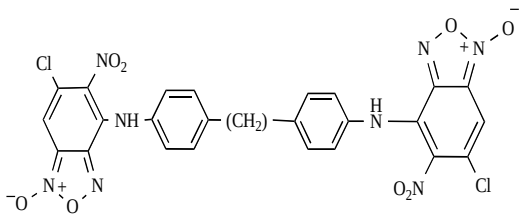
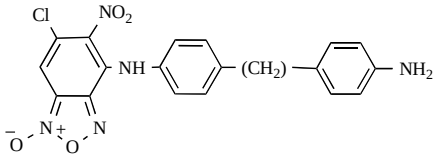
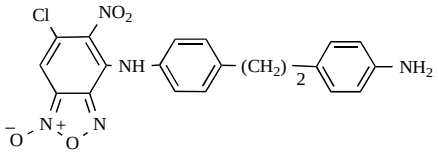
It should be pointed out that almost all concentrations of compound **5** produced the response of biosensor control *E. coli* MG 1655 (pSoxS-lux).

Thus, this result allows to assume that the mechanism of action of compounds **3, 4, 5** and 4,6-dichloro-5-nitrobenzofuroxan on the cell is superoxide anion generation that leads to oxidizing stress condition.

Compound **4**, besides, has produced the response of biosensor controls *E. coli* MG1655 (pRecA-lux) and *E. coli* MG1655 (pGrpE-lux) in concentration $1.5x10^{-4}$mmol/ml that testifies that this concentration is genotoxic (the induction factor is 2.5 units), causes damage of proteins in bacterial cells (the induction factor is 3.0 units) and cannot be used in pharmacological conditions.

Except for compound **4,** during biotesting of other benzofuroxans the response of the lux-biosensor controls reacting to DNA and protein damage (both with use of metabolic activation and without it), was not detected. It implies that compounds **2, 3, 5, 6, 7** and 4,6-dichloro-5-nitrobenzofuroxan do not belong to the class of the substances damaging DNA and proteins. During biotesting of compounds **2, 6, 7** no response of lux-biosensor controls was observed, indicating that these compounds do not belong to the class of substances damaging DNA, proteins, and causing generation of superoxide anion.

Table 1. The toxicity of the compounds 1-7 in the experiments with bacterial lux-biosensors.

No.	Compound structure	Toxic effect characteristics		
		biosensor control type	induction factor value[a]	range of effective concentrations, mmol/ml
1		*E. coli* MG1655 (pSoxS-lux)	1.4–7.4[b]	$4x10^{-4}$–$2x10^{-5}$
2		no effect		
3		*E. coli* MG1655 (pSoxS-lux)	2.5[b]	$1.5x10^{-4}$
4		*E. coli* MG1655 (pSoxS-lux)	3.0–5.0[b]	$3x10^{-3}$–$1.5x10^{-4}$
		E. coli MG1655 (pRecA-lux)	2.5[b]	$1.5x10^{-3}$
		E. coli MG1655 (pGrpE-lux)	3.0[b]	$1.5x10^{-3}$
5		*E. coli* MG1655 (pSoxS-lux)	1.5–2.0[b]	$8x10^{-12}$–$8x10^{-4}$
6		no effect		
7		no effect		

[a] differences from control are statistically significant, t-criterion; $p < 0.05$. [b] when one value is specified, the effect was detected at only one concentration.

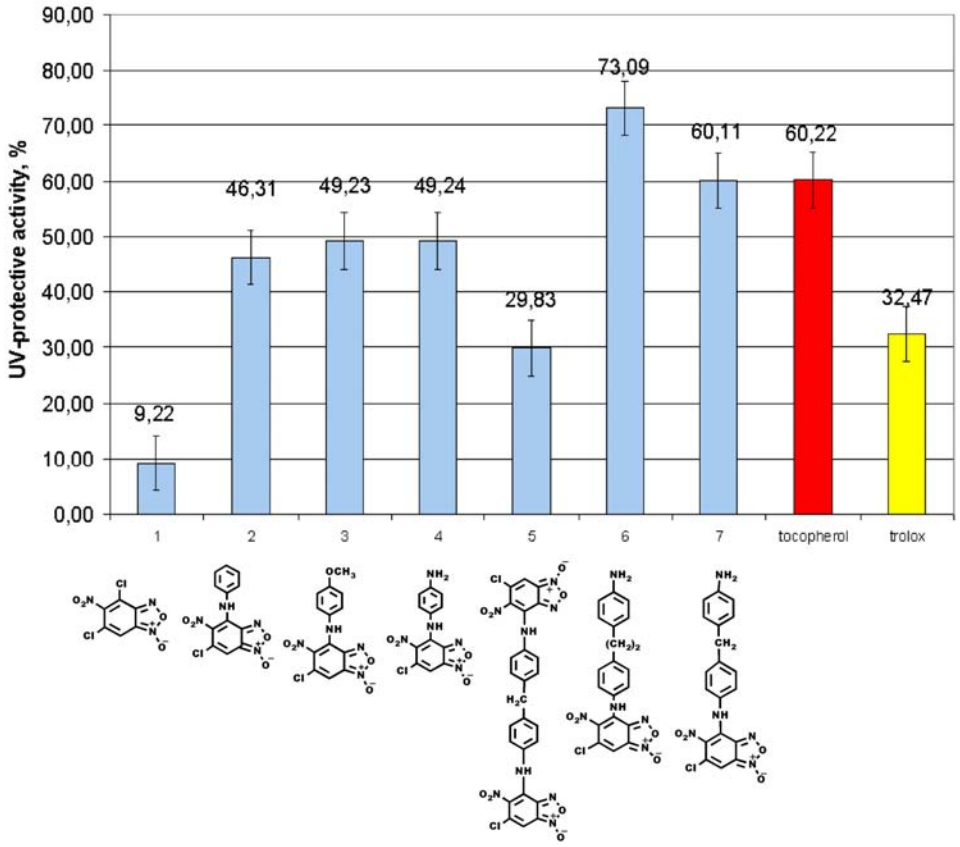


Fig. (2). The maximum protective effect of benzofuroxans **1-7** and antioxidants on *E. coli* MG1655 (pKatG-lux) in the wavelength range between 300-400 nanometers was reported at the following concentrations: **1** : 10^{-6} mmol/ml, **2**: 10^{-4} mmol /ml; **3** : 10^{-6} mmol /ml; **4** : 10^{-7} mmol /ml; **5** : 10^{-8} mmol /ml; **6** : 10^{-7} mmol /ml; **7** : 10^{-5} mmol/ml; **α–tocopherol** : 10^{-3} mmol /ml; **trolox** : 10^{-4} mmol /ml.

Preliminary systemic toxicity of all synthesized compounds was estimated with a bacterial lux-biosensor with a constitutive promoter (data is not given). In further experiments for protective effect estimation only nontoxic concentrations were used.

Safety of the benzofuroxans derivatives was exclusively studied for the modeled object – various bacterial biosensors. To draw a final conclusion about toxicity and genotoxicity of the compounds, the range of tests for pharmaceutical preparations should include not only tests with the use of bacteria, but also tests based on bio-cytoculture of mammals.

The most promising compounds in terms of UV protection will be later used in a battery of tests.

The Ability of Benzofuroxans to Suppress Destructive UV Radiation in the Wavelength Range Between 300-400 Nanometers

The test system for the estimation of pro-oxidant action of ultra-violet radiation in the wavelength range between 300-400 nanometers, which has been chosen, is the biosensor control *E. coli* MG1655 (pKatG-lux) [15]. This biosensor control is a highly specific method and only responses due to the presence of hydrogen peroxide and organic peroxides in a cell are detected. After the exposure to different doses of UV-radiation in the wavelength range between 300-400 nanometers, the biosensor control reacted with stable luminescence induction. The maximum bioluminescent *E. coli* MG1655 (pKatG-lux) response was recorded after UV irradiation (see above) using a dose of 6000 J/m^2; this UV dose was used for revealing UV-protective effects.

Thus, bioluminescence induction of the given biosensor control after influence of UV-radiation in the wavelength range between 300-400 nanometers, is characteristic of the formation of hydrogen peroxide or organic peroxides generation in a cell during this exposure.

Fig. (**2**). shows the maximum UV protective effect observed in the case of benzofuroxans and both tocopherol and trolox. In the caption are mentioned, for each compound, the needed concentrations to obtain the maximum UV-protective effect. For all other concentrations (both higher and lower), the molecules have a protective effect but to a lesser extent.

As shown in Fig. (**2**), the parent molecule – 4,6-dichloro-5-nitrobenzofuroxan 1, exhibits the weaker effect, and the substitution of the chlorine atom by nitrogen-substituted fragments is going along with an increase of the potential protective effect.

The larger protective effect (73 %) is displayed by compound **6**. If the results obtained for various benzofuroxans are compared with those of classical antioxidant as α-tocopherol (vitamin E) and trolox, which are references in this domain, it appears obvious that

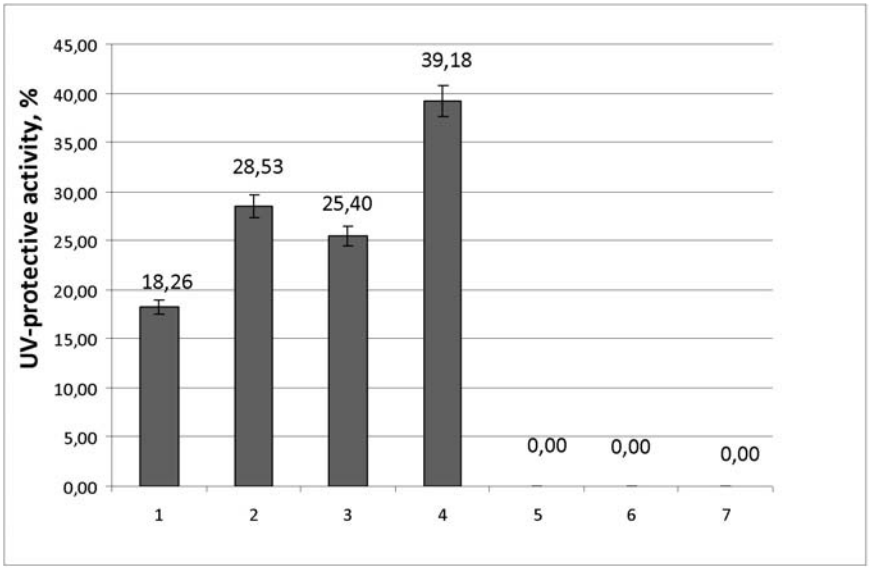


Fig. (3). Ability of benzofuroxan **6** at various concentrations to suppress UV genotoxicity (strain *E. coli* AB1157 (pRecA-lux)): Entry 1 : 10^{-10} mmol/ml; Entry 2 : 10^{-9} mmol /ml; Entry 3 : 10^{-8} mmol /ml; Entry 4 : 10^{-7} mmol /ml; Entry 5 : 10^{-6} mmol /ml; Entry 6 : 10^{-5} mmol /ml; 7 : 10^{-4} mmol /ml.

benzofuroxans show similar protective effect and that compound **6** even possesses a higher protective effect. As benzofuroxans protect bacterial cells from prooxidant effect of UV-radiation, these substances may be used for the formulation of highly effective UV-protective cosmetics.

Compound **6**, which exhibits the higher protective effect against the action of UV radiation in the wavelength range between 300–400 nanometers, in experiments with *E. coli* MG1655 (pKatG-lux), was also studied to estimate its ability to suppress UV genotoxicity. For this purpose, *E. coli MG1655 (pRecA-lux)* strain responding to the action of DNA-tropic agents was also exposed to UV-radiation in the presence of compound **6** and not. The results are summarized in fig. (**3**).

The general tendency of protective activity strengthening with the dose growth is characteristic for concentrations 10^{-10}–10^{-7} mmol/ml. The effects of concentrations 10^{-9} and 10^{-8} mmol/ml are not statistically significantly different, i.e. in this area of concentrations there is apparently a plateau of the dose/effect ratio. The compounds in concentrations higher than 10^{-7} mmol/ml do not possess the protective effect.

As shown in fig. (**3**) for experiments 1–4, compound **6** possesses the ability to reduce the level of DNA damage in the course of UV-irradiation in the wavelength range between 300-400 nanometers. The extent of the antimutagenic effect of benzofuroxan **6** is varying in a range from 18.3 to 39.2 %. For concentration 10^{-1} mg/ml considerable bioluminescence suppression of *E. coli MG1655 (pRecA-lux)* strain was observed, as in the experiment with *E. coli* MG1655 (pKatG-lux) (data are not given) in comparison to reference that indicates the toxic character of this concentration. The maximum protective effect is recorded for 10^{-4} mg/ml concentration and is equal to 39.18 %.

From these results, it is evident to assume that compound **6** has a double effect – acting in the reduction of the peroxide compounds generation (experiment with *E. coli* MG1655 (pKatG-lux)), and also acting in the reduction of the proportion of DNA damage (experiment with *E. coli* MG1655 (pRecA-lux) that occur in the course of the exposition to UV radiation in the wavelength range between 300-400 nanometers. Its ability to suppress UV genotoxicity is obviously based on antioxidant activity.

Thus, some representative members of benzofuroxans are shown to protect bacterial cells from destructive effects of ultraviolet in the wavelength range between 300–400 nanometers. These compounds are potentially useful for the creation of highly effective UV-protective products (already mentioned above). Compounds **6**, **2** and **7** are of greatest interest from this viewpoint, because they displayed a complete absence of destructive effects during the lux-biosensor control tests.

These results obtained during this study, allow to assume that benzofuroxan compounds will exhibit a protective effect not only in experiments on bacteria, but also in experiments with more complex multicellular organisms.

In this work, we have shown that there are no *in vitro* destructive effects induced by benzofuroxan derivatives during the lux-biosensor control tests. These primary results are the first evidences showing that these compounds are potentially of low toxicity and genotoxicity. *In vivo* experiments (preclinical studies) are performed with mice and daphnia. The preliminary results show that these compounds belong to the toxicity Class 2: moderately hazardous (LD50 are found to be in the range 50-500 mg/kg). Further toxicity tests will be performed to get more informations, but nevertheless, benzofuroxan derivatives could be used for cosmetic formulae as sunscreen for external use.

In this paper, it has been shown that the interaction of 4,6-dichloro-5-nitrobenzofuroxan with aromatic amines and diamines are leading to products only deriving from the replacement of one chlorine atom in the fourth position of the benzene ring. It has also been shown that this interaction is depending on experimental conditions producing 1:1 or 2:1 isomers.

Biotesting has been performed using of bacterial lux-biosensor controls (*E. Coli* strains, responding to specific metabolism changes by luminescence strengthening). It has been shown that these derivatives and particularly, compounds **1, 3, 4, 5** in a number of concentrations exhibited a strengthening of generation of endocellular superoxide anion. The use of compound **4** is causing proteins damage in bacterial cells and DNA and cannot be employed in pharmacological use.

Compounds **6, 7, 2** do not display any destructive biological effects in the given experimental conditions and are of great interest for formulation of pharmacological preparations and cosmetics.

It has been demonstrated that these compounds protect bacterial cells from genotoxic effects of an UV radiation in the wavelength range between 300–400 nanometers. The weakest effect is displayed by the parent substance – 4,6-dihloro-5-nitrobenzofuroxan whereas the more important protective effect (73 %) is displayed by compound **6**.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

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REFERENCES

[1] Gasco, A.; Boulton, A.J. Furoxans and Benzofuroxans. *Adv. Heterocycl. Chem*., **1981**, *29*, 251-340.

[2] Katritzky, A.R.; Gordeev, M.F. Heterocyclic Rearrangements of Benzofuroxans and Related Compounds. *Heterocycles*, **1993**, *35*, 483-518. Ghosh, P., Ternai, B.; Whitehouse, M. Benzofurazans and benzofuroxans: Biochemical and pharmacological properties. *Med. Res. Rev*., **1981**, *1*, 159-187.

[3] Ghosh, P.; Ternai, B.; Whitehouse, M. Benzofurazans and benzofuroxans: Biochemical and pharmacological properties. *Med. Res. Rev*., **1981**, *1*, 159-187.

[4] Cerecetto, H.; Porcal, W. Pharmacological properties of furoxans and benzofuroxans: recent developments. *Mini-Rev. Med. Chem*., **2005**, *5*, 57-71.

[5] Cerecetto, H. and Gonzalez, M. Benzofuroxan and Furoxan. Chemistry and Biology. *Top. Heterocycl. Chem.*, **2007**, *10*, 265-308.

[6] Olea-Azar, C.; Rigol, C.; Mendizabal, F.; Cerecetto, H.; Di Maio, R.; González, M.; Porcal, W.; Morello, A.; Repetto, Y.; Maya, J.D. Novel benzo[1,2-c]1,2,5-oxadiazole N-oxide derivatives as antichagasic agents: chemical and biological studies. *Lett. Drug. Des. Discovery*, **2005**, *2*, 294-301.

[7] Porcal, W.; Hernandez, P.; Aguirre, G.; Boiani, L.; Boiani, M.; Merlino, A.; Ferreira, A.; Di Maio, R.; Castro, A.; González, M.; Cerecetto, H. Second generation of 5-ethenylbenzofuroxan derivatives as inhibitors of Trypanosoma cruzi growth: Synthesis, biological evaluation, and structure-activity relationships. *Bioorg. Med. Chem*., **2007**, *15***,** 2768-2781.

[8] Jorge, S.D.; Masunari, A.; Rangel-Yagui, C.O.; Pasqualotoa, K.F.M.; Tavares, L.C. Design, synthesis, antimicrobial activity and molecular modeling studiesof novel benzofuroxan derivatives against Staphylococcus aureus. *Bioorg. Med. Chem*., **2009**, *17*, 3028-3036.

[9] Medana, C.A.; Stilo, Di; Visentin, S.; Fruttero, R.; Gasco, A.; Ghigo, D.; Bosia, A. NO donor and biological properties of different benzofuroxans. *Pharm. Res*., **1999**, *16*, 956-960.

[10] Hervé R.; Bernadette L. Amine, amide, sulphonamide and carbamate derivatives of benzalmalonic salts and photoprotective cosmetic composition comprised thereof. US Patent No 7326407, 2006.

[11] Farng, L.O.; Horodysky A.G. Amine-containing hindered phenolic antioxidant lubricant additives and compositions thereof, US Patent No 5169547, 1978.

[12] Sazykina, M.A.; Chistyakov, V.A.; Kolenko, M.A.; Azarin, K.V. Allantoin and urate as suppressors of genotoxic effect of ultraviolet radiation with wavelength 300 - 400 nm. *Ecological genetics***, 2009**, *7*,44-46.

[13] Macie, B.S.; Macie, L.E. The PABA story, *Australas J. Dermatol*, **1999**, *40*, 51-53.

[14] Ambrosio, M. I prodotti solari, *Cosmesi Dermatologica*, **1992,** *41*, 7-41.

[15] Cantrell, T.; Jussel, N.; Morris, S. Sun damage and skin protection, *Pharmaceutics*, **2002**, *425***,** 1-8.

[16] Zavilgelsky, G.B.; Kotova, V.Yu; Manukhov, I.V. Action of 1,1-dimethylhydrazine on bacterial cells is determined by hydrogen peroxide. *Mutation Res*., **2007**, *634*, 172-176.

[17] Ptitsyn, L.R.;, Horneck, G.; Komova, O.; Kozubek, S.; Krasavin, E.A.; Bonev, M.; Rettberg, P. A biosensor for envi-ronmental genotoxin screening based on an SOS lux assay in recombinant Escherichia coli cells. *Appl. Environ. Microbiol.* **1997**, *63*, 4377-4384.

[18] Frey, J.; Ghersa, P.; Palacios, P.G.; Belet, M. Physical and genetic analysis of the ColD plasmid. *J. Bacteriol.*, **1986**, *166*, 15-19.

[19] Timares, L.; Katiyar, S.K.; Elmets, C.A. DNA damage, apoptosis and langerhans cells – activators of UV-induced immune tolerance. *Photochem. Photobiol*., **2008**, *84*, 422-436.

[20] Pfeifer, G.P.; You, Y.H.;Besaratinia, A. Mutations induced by ultraviolet light. *Mutat. Res*., **2005,** *571*(1-2),19-31.

[21] Rünger, T.M.; Epe, B.; Möller, K. Processing of directly and indirectly ultraviolet-induced DNA damage in human cells. *Recent Results Cancer Res*., **1995**, *139*, 31-42.

[22] Cadet, J.; Berger, M.; Douki, T.; Morin, B.; Raoul, S.; Ravanat, J.L.; Spinelli, S. Effects of UV and visible radiation on DNA-final base damage. *Biol Chem.*, **1997**, *378*, 1275-1286.

[23] Mouret, S.; Baudouin, C.; Charveron, M.; Favier, A.; Cadet, J.; Douki, T. Cyclobutane pyrimidine dimers are predominant DNA lesions in whole human skin exposed to UV-A radiation. *Proc. Nat. Acad. Sci. USA*, **2006**, *103*, 13765-13770.

[24] Jiang, Y.; Ke, C.; Mieczkowskiy, P.A.; Marszalek, P.E. Detecting Ultraviolet Damage in Single DNA Molecules by Atomic Force Microscopy. *Biophys. J*. **2007**, *93*, 1758-1767.

[25] Sheldrick, G.M. SADABS. Bruker AXS Inc, Madison, WI-53719, USA, 1997.

[26] Altomare, A.; Cascarano, G.; Giacovazzo, C.; Viterbo, D. E-map improvement in direct procedures. *Acta Crystallogr. Sec. A***., 1991**, *47*, 744-748.

[27] Sheldrick, G.M. SHELX-97. Programs for crystal structure analysis Germany: University of Gottingen. 1, 2, 1997.

[28] Farrugia, L.J. WinGX suite for small-molecule single-crystal crystallography. *J. Appl. Crystallogr*., **1999**, *32*, 837-838.

[29] APEX2 (Version 2.1), SAINTPlus. Data Reduction and Correction Program (Version 7.31A, Bruker Advansed X-ray Solutions, BrukerAXS Inc., Madison, Wisconsin, USA, **2006**.

[30] Weissberger, A.; Proskauer, E.S.; Riddick, J.A.; Toops, E.E. *Organic solvents. Physical properties and methods of purification*. Interscience publishers, Inc. New York Interscience publishers LTD., London, **1955**.

[31] Burilov, A.R.; Kasymova, E.M.; Chugunova, E.A.; Yusupova, L.M.; Zobov, V.V. New benzofuroxans possessing fungicidal and bactericidal activities. RU Patent No. 228419, 2009.

[32] Yusupova, L.M.; Molodykh, Z.V.; Buzykin, B.I.; Falyakhov, I.F.; Anisimova, N.N.; Sharnin, G.P.; Bulidorov, V.V.; Sviridov, S.I.;

Levinson, F.S. 4- or 6-nitro-5,7-dichlorobenzofuroxan having fungicidal activity. RU Patent 2032678, 1955.

[33] Maniatis, T.; Fritsch, E.F.; Sambrook, J. *Molecular Cloning: a Laboratory Manual*. 4th Edition Cold Spring Harbor Laboratory, New York, **1992**.

[34] Chistiakov, V.A.; Sazykina, M.A.; Kolenko, M.A.; Cherviakov, G.G.; Usatov, A.V. Methylene blue as a supressor of the genotoxic effect of ultraviolet radiation with a wavelength of 300-400 nm. Genetika. **2009**, *45(3)*, 349-353.

[35] Ohnaka, T. Health effects of ultraviolet radiation. *Ann. Physiol. Anthropol.*, **1993**, *12*, 1-10.

[36] Chugunova, E.A.; Kasymova, E.M.; Burilov, A.R.; Krivolapov, D.B.; Yusupova, L.M.; Pudovik, M.A. Reaction of dichloronitrobenzofuroxanes with amines and their derivatives. *Russ. J. Gen. Chem.*, **2009**, *79*, 2207-2211.

[37] Terrier, F. *Nucleophilic Aromatic Displacement"*; Feuer, H, Ed.; VCH: New York, **1991.**

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