European Journal of Medicinal Chemistry 93 (2015) 349–359

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and antimicrobial activity of novel structural hybrids of benzofuroxan and benzothiazole derivatives

Elena Chugunova [a, **], Carla Boga [b, *], Ivan Sazykin [c], Silvia Cino [b], Gabriele Micheletti [b], Andrea Mazzanti [b], Marina Sazykina [c], Alexander Burilov [a], Ludmila Khmelevtsova [c], Natalia Kostina [c]

[a] A.E. Arbuzov Institute of Organic and Physical Chemistry, Kazan Scientific Center, Russian Academy of Sciences, Akad. Arbuzov st. 8, Kazan, Tatarstan 420088 Russia
[b] Department of Industrial Chemistry "Toso Montanari", Alma Mater Studiorum-University of Bologna, Viale del Risorgimento 4, 40136 Bologna, Italy
[c] The Research Institute of Biology, Southern Federal University (SFedU), 194/1 Stachki Ave., 344090 Rostov-on-Don, Russia

ARTICLE INFO

Article history:
Received 27 October 2014
Received in revised form
12 February 2015
Accepted 15 February 2015
Available online 17 February 2015

Keywords:
Benzothiazole
Benzofuroxan
Lux-biosensors
Ambident nitrogen reactivity

ABSTRACT

New compounds containing both benzofuroxan and benzothiazole scaffolds were synthesized through electrophile/nucleophile combination of nitrobenzofuroxan derivatives and 2-mercapto- or 2-aminobenzothiazole derivatives and their biological effect on the natural strain Vibrio genus and different bacterial lux-biosensors was studied. Among all the compounds synthesized, that obtained from 2-mercaptobenzothiazole and 7-chloro-4,6-dinitrobenzofuroxan was toxic for bacterial cells, and also able to activated the 1st type Quorum Sensing system. The reaction between 7-chloro-4,6-dinitrobenzofuroxan and 2-aminobenzothiazole derivatives gave two products, one bearing the benzofuroxan moiety linked to the exocyclic amino nitrogen, and the second derived from the attack of two molecules of electrophile to both the nitrogen atoms of the benzothiazole reagent. Their relative ratio is modifiable by tuning the reagents ratio and the reaction time.

1. Introduction

Furoxan and benzofuroxan derivatives represent an important class of heterocyclic compounds with interesting properties in many teoretical and applied fields [1–4]. In medicinal and biological fields growing interest has been devoted to this organic scaffold owing to its ability to release nitric oxide (NO) molecules under physiological conditions [5,6]. NO is considered the biologically important form of the endothelium-derived relaxing factor (EDRF), which endogenous formation plays an essential role in many bioregulatory systems, such as smooth muscle relaxation, platelet inhibition, neurotransmission and immune stimulation [7]. Due to the instability of aqueous solutions of NO, the interest to find compounds that are able to generate NO *in situ* (NO donors or NO releasing agents) is increasing. Benzofuroxan derivatives display typical NO-dependent activities both *in vitro* and *in vivo*, and the possibility of modulating NO release by changing the substituent on the ring makes them versatile tools in designing NO donor/drug hybrids [8]. Actually, the combination of a benzofuroxanyl moiety with another biologically active substructure in a single molecule has recently received particular attention. For example, the 1-oxybenzo[1,2,5]oxadiazol-5-ylmethyl[2-(2,6-dichloro-phenylamino)-phenyl]-acetate is a diclofenac derivative bearing a benzofuroxan moiety in its structure that showed anti-inflammatory activity and with better gastric tolerability with respect to that of native diclofenac, probably related to nitric oxide release ability [9].

The benzothiazole scaffold is prevalent in a variety of pharmacologically active synthetic and natural compounds exhibiting antimicrobial [10–15], anticancer [16–18], anthelmintic [19], and anti-diabetic [20] activity. They are widely found in bioorganic and medicinal chemistry with application in drug discovery [21].

Taking into consideration the above described beneficial effects of the nitric oxide, we realized that it would be of interest to synthesize novel structural hybrids containing both heterocyclic ring systems, benzofuroxan, able to release NO, and benzothiazole, a nucleus still receiving considerable attention in the drug field owing to the biological effects [22] related to its structure.

* Corresponding author.
** Corresponding author.
E-mail addresses: chugunova.e.a@gmail.com (E. Chugunova), carla.boga@unibo.it (C. Boga).

http://dx.doi.org/10.1016/j.ejmech.2015.02.023
0223-5234/

Herein, we report the synthesis of novel derivatives obtained linking a 4,6-dinitrobenzofuroxan ring to the amino- or mercapto-group of benzothiazole derivatives through electrophile/nucleophile combination and investigation of their biological properties with the natural strain *Vibrio* genus and bacterial lux-biosensors designed on the base of *Escherichia coli* MG1655. These biosensors are capable of assessing the compound integral toxicity degree and its ability to influence the damage of DNA and proteins, oxidative stress level and the 1st type Quorum Sensing system of bacterial cells.

2. Results and discussion

2.1. Chemistry

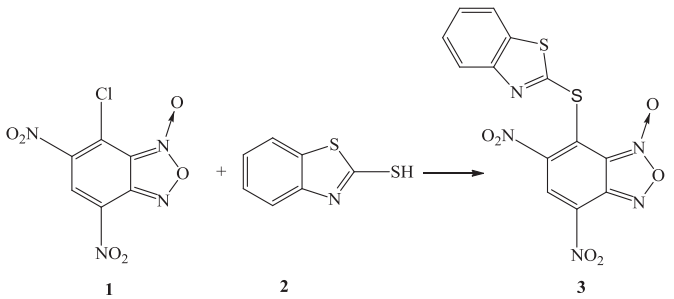
Since it was known that 7-chloro-4,6-dinitrobenzofuroxan (**1**, DNBF-Cl) is very prone to react with a variety of weak or very weak nucleophiles as water, alcohols, amines [23,24], and even with the poorly basic 2,4,6-trinitroaniline [25,26] we planned to use compound **1** to realize the electrophile/nucleophile combination with 2-thio- or 2-aminobenzothiazole derivatives. The reaction was carried out by mixing equimolar amounts of benzofuroxan **1** and 2-mercaptobenzothiazole **2** in acetonitrile and in presence of basic alumina, it was completed after 2 h at room temperature, and the product **3** was isolated in 86% yield. (see Scheme 1)

The high reactivity observed was expected on the basis of the following factors: i) the well known nucleophilic power of the sulphur charged nucleophiles; ii) the cumulation of the powerful activating effects exerted by the heteroannelated 5-membered ring and the electron-withdrawing groups attached to the 6-membered ring of the electrophile; iii) the low aromaticity of the neutral heteroaromatic 10π-system; iv) the good leaving group ability of the chloride ion.

This finding prompted us to try the reaction of **2** with a less electrophilic reagent, namely 4,6-dichloro-5-nitrobenzofuroxan (**4**). Recently, it has been shown that reactions of **4** with aliphatic and aromatic amines is going along with the substitution of chlorine atom in the fourth position of the carbocyclic ring of the benzofuroxan derivative [27,28]. The optimal condition for increase in product yield and pureness was the use of DMSO as a solvent [29]. The nitro-group and the chlorine atom in the 6-position were inactive under any conditions.

In contrast to these findings, the reaction of the benzofuroxan **4** with 2-mercaptobenzothiazole (**2**) gave a totally unexpected result. When compounds **4** and **2** were mixed in solvents such as chloroform, acetonitrile, and acetone, the reaction did not occur. Only the reaction in the more polar dimethyl sulfoxide at 80–90 °C leads to formation of a mixture of two products (Scheme 2).

On the basis of ^{1}H, ^{13}C NMR, mass spectrometry and, for compound **6**, X-ray diffraction analysis (Fig. 1), we have established the structure of the reaction products. Compound **5** was derived from a double nucleophilic attack with the displacement of the chlorine atom in the fourth position of the carbocyclic ring (in agreement with the previously reported reaction of **4** with amines) and that of the nitro group in position 5 (this latter resembled the displacement of a nitro group by mercaptide ions in dipolar aprotic solvents [30].



1 2 3

Scheme 1. Reaction between 7-chloro-4,6-dinitrobenzofuroxan and 2-mercaptobenzothiazole.

The formation of compound **6** is very unusual, in this case the replacement of the nitro group by chlorine might be explained by a mechanism involving radical species [31] or, as suggested by a reviewer, by reaction of compound **5** and chloride [32,33]. The same products were obtained in the reaction with sodium salt of 2-mercaptobenzothiazole.

Afterward, we tried the reaction between benzofuroxan **1** and the series of 2-aminobenzothiazoles **7a**–**f** (Scheme 3).

From the reaction between benzofuroxan **1** and 2-aminobenzothiazole derivatives **7a**–**d** we obtained a mixture of mono-adducts **8a**–**d** and di-adducts **9a**–**d**, while from compounds **7e** and **7f** only the mono-adducts were recovered.

About the structure of the mono-adduct, it is important to note that, in principle, due to the ambident nitrogen reactivity of 2-aminobenzothiazoles and their possibility of existence in different forms, structures **A** (and its tautomeric form) and **B** might be formed by reaction with **1**, as shown in Fig. 2.

It has been reported [34] that 2-aminothiazole (**10a**) and 4-methyl-2-aminothiazole (**10b**) act as bidentate nucleophiles toward 2,4-dinitrofluorobenzene (**11**) in dimethyl sulfoxide (Scheme 4). In particular, in the absence of steric hindrance, the endo aza nitrogen of 2-aminothiazole is the preferred reactive site in the nucleophilic aromatic substitution of 2,4-dinitrofluorobenzene (**11**, via a) while when the approach of the electrophile from the aza center is sterically hindered as in case of the product **10b**, the reaction takes place first at the amino nitrogen to give **13b** (via b). Because the second and much faster reaction occurs at the imino nitrogen of the monosubstituted product **12a**, the diadduct **14a** is obtained as the major product.

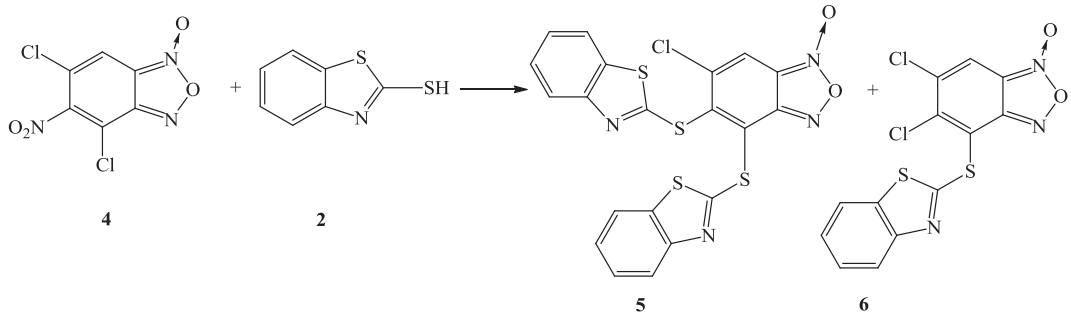
Recently, it has been reported that 2-aminobenzothiazole reacts with 2-((4-chloro-6-methylpyrimidin-2-ylthio)methyl)benzothiazole at the exocyclic amino group [10] while with glycidyl phenyl ether the reaction proceeds at both exo-and endocyclic nitrogen atoms, giving a diadduct [35].

As a result of our investigations we have found that the interaction between benzofuroxan **1** and 2-aminobenzothiazole derivatives **7** gave a mixture of mono- and di-adducts. The finding that compound **7e** does not produce the **9e** might be considered an indication that **7e** is in **A** form, probably preferred over form **B** due to the steric hindrance of the methoxy substituent. However, since all attempts to crystallize some mono-adducts failed, to gain further indications about the structure of compounds **8**, we prepared the methyl derivative of the mono-adduct derived from the reaction between compounds **1** and **7b** (Scheme 5) and carried out some NOESY-1D experiments on it.

The results obtained agreed with structure **15**, thus indicating that the benzofuroxan moiety in compounds **8** is bound to the exocyclic amino nitrogen atom.

Even if, on the basis of the above cited literature findings, the formation of the di-adduct **9** was not completely unexpected, we thought that the reaction course deserves to be deepened. We carried out the reaction between compounds **1** and **7a**–**e** directly in the NMR spectroscopy tube in acetone-d_6 at 25 °C and monitored its progress with time. The results obtained have been reported in Table 1.

Data of Table 1 for the reactions carried out using a 1:2 M ratio between **1** and 7 show that in the first reaction times (4 h) the diadducts **9a**–**d** are formed in greater amount with respect to the



Scheme 2. Reaction between 4,6-dichloro-5-nitrobenzofuroxan and 2-mercaptobenzothiazole.

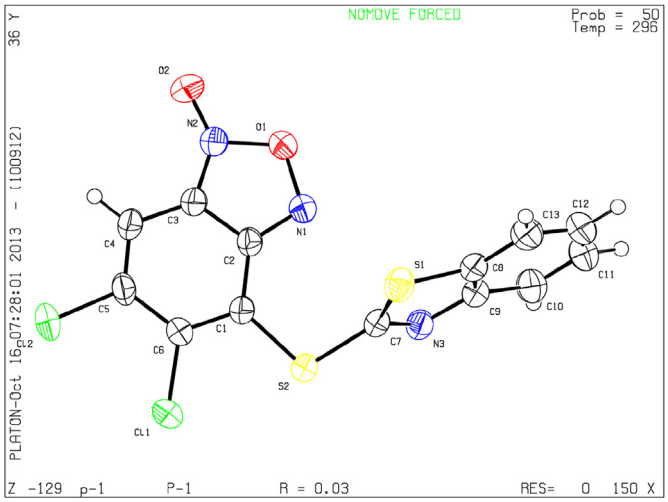


Fig. 1. The ORTEP drawing of compound **6** at 50% ellipsoid probability.

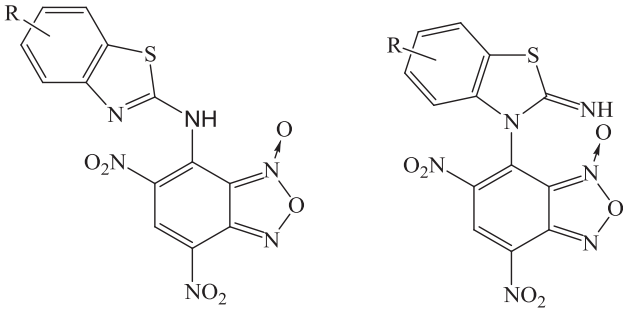
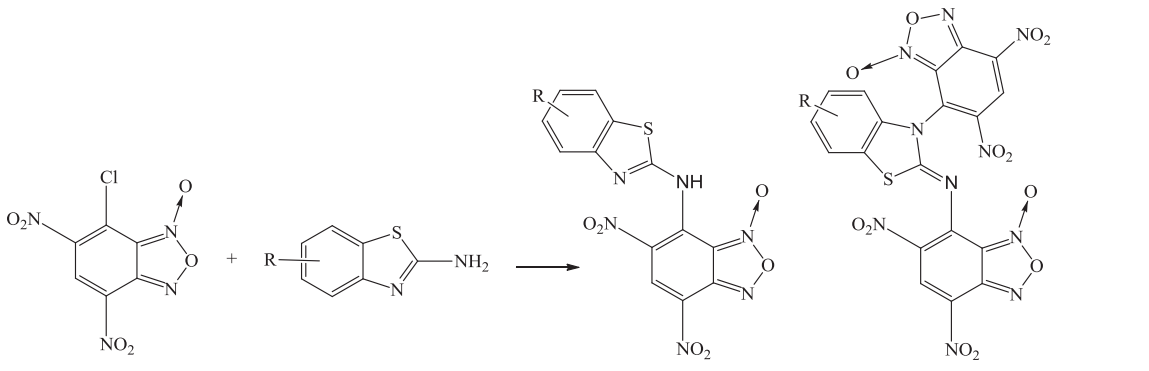


Fig. 2. Possible structures for mono-adducts **8** formed between compounds **1** and **7**.

respective monoadducts **8a**–**d**. With time, a gradual shift of the **8a**–**d**/**9a**–**d** relative ratio towards the monoadduct **8a**–**d** was observed, until to reach complete formation of this latter after about two weeks. This behavior suggests the occurrence, in the first reaction time, of a behavior similar to that already observed and above cited for the reaction between 2-aminothiazole and 2,4-dinitrofluorobenzene. In present case, after formation of the monoadduct, a second fast attack of **1** might occur thus giving the diadduct **9**. Then, the presence of further amount of 2-aminobenzothiazole derivative in the reaction mixture might induce formation of mono-adduct through the pathway proposed in Scheme 6. This hypothesis is supported by the fact that when the reaction is carried out with a 4:1 relative molar ratio between the benzothiazole derivative **7a**–**d** and the benzofuroxan **1**, the monoadducts **8a**–**d** were present as major products since the first reaction days and the relative **8**/**9** ratio became almost quantitative in favor of the first after a few days (compare the relative **8**/**9** ratios with those in brackets in Table 1).

Moreover, the pathway proposed and depicted in Scheme 6 was

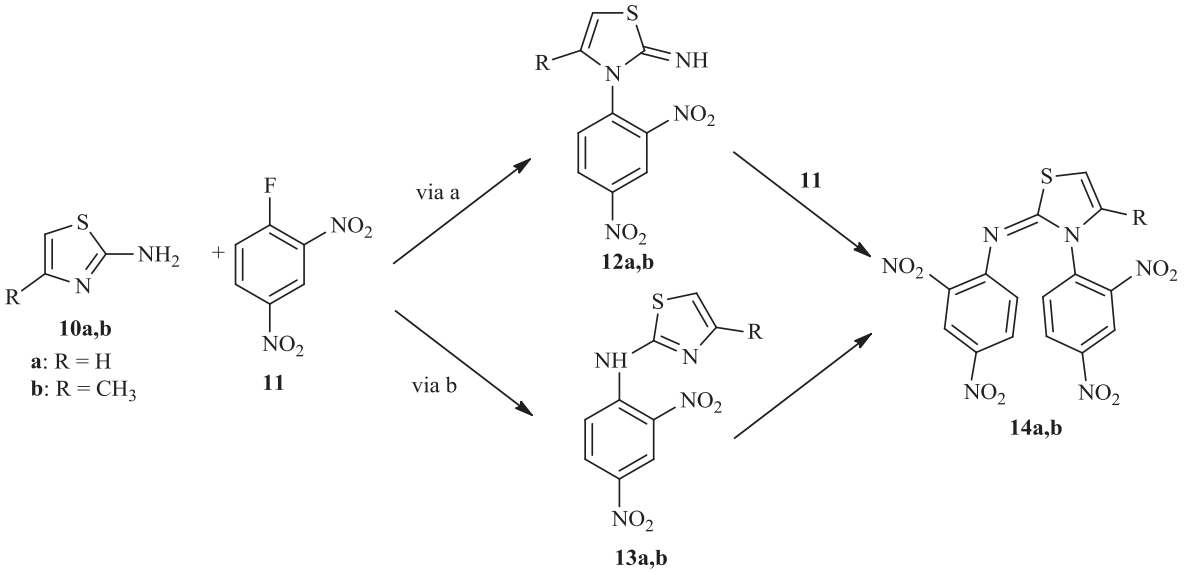


a: R = H **b**: R = 6-OC_2H_5
c: R = 6-CH_3 **d**: R = 6-Cl
e: R = 4-OCH_3 **f**: R = 5-NO_2

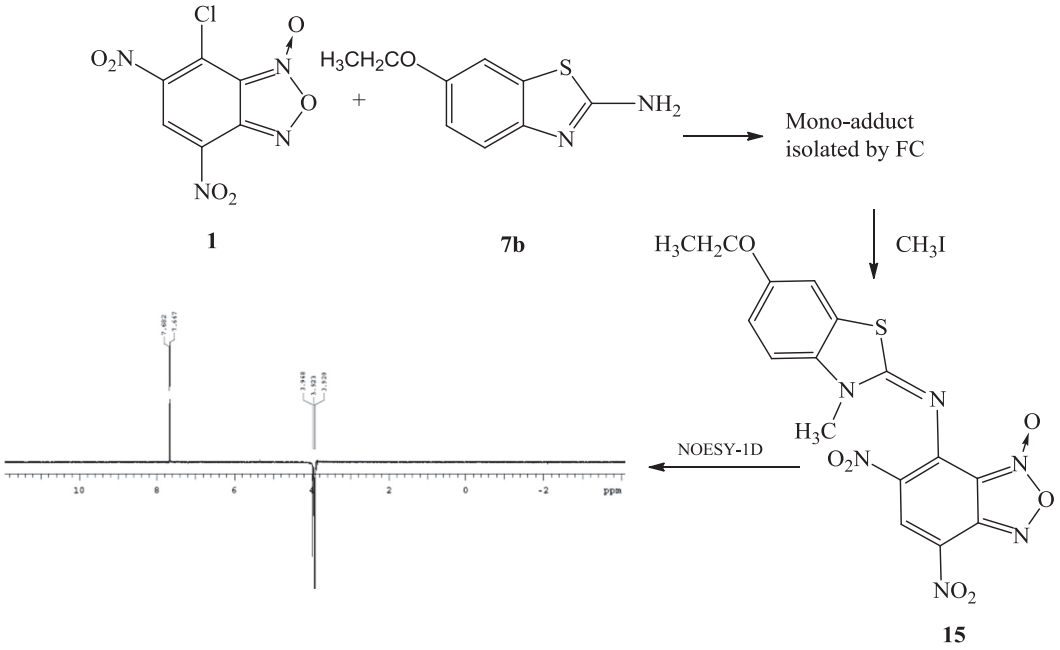
a: R = H **b**: R = 6-OC_2H_5
c: R = 6-CH_3 **d**: R = 6-Cl
e: R = 4-OCH_3 **f**: R = 5-NO_2

a: R = H **b**: R = 6-OC_2H_5
c: R = 6-CH_3 **d**: R = 6-Cl

Scheme 3. Reaction between **1** and aminobenzothiazole derivatives **7a**–**f**.



Scheme 4. Reported reaction between 2-aminothiazoles and 2,4-dinitrofluorobenzene [34].



Scheme 5. Methylation of the monoadduct **8b** and spectrum obtained from NOESY-1D experiment.

supported also by the observation that acetone-d_6 solution of the diadduct **9d**, monitored by ^{1}H NMR spectroscopy for a week, resulted unchanged (as well as after 40 days); after this time, **7d** was added to this solution, and the mono-adduct **8d** was present in 13% yield after one weak and in about 33% yield after about 40 days.

The only exception to this behavior was observed for 4-methoxy-2-aminobenzothiazole **7e** that gave exclusively the monoderivative **8e**. In this case probably the steric hindrance of the substituent hinders the formation of the diadduct. Also in the case of the aminobenzothiazole **7f**, with a nitro group in position 5, we obtained only the mono-adduct **8f**; in this case the presence of the nitro group might deactivate the second attack of the electrophile.

2.2. Biological activity

2.2.1. Investigation of the biological effects of the new structural hybrids *3*, *8a–c*, *8e–f*, *9d* by means of bacterial lux-biosensors

The evaluation of the biological effects of the studied compounds was carried out by means of bacterial luminescent biosensors which contain pBR322 vector with a set of genes luxCDABE from *Photorhabdus luminescens* ZM1 under control of the induced promotor, and also a natural luminescent strain. *Vibrio aquamarinus* VKPM B-11245 (*V. aquamarinus* DSM 26054) – the natural luminescent strain isolated from the Black Sea water and genetically engineered biosensor strains of *Escherichia coli* MG1655 (pXen7), *E. coli* MG1655 (pSoxS-lux), *E. coli* MG1655 (pKatG-lux), *E. coli* MG1655 (pRecA-lux), *E. coli* MG1655 (pColD-lux), *E. coli* MG1655

Table 1
Relative percentage of products[a] **8** and **9** dependent on the reaction time for the reaction between compounds **1** and **7a-e** in 1:2 and in 1:4[b] molar ratio.

R	Product	Reaction time				
		4 h	24 h	5 days	14 days	21 days
H	**8a**	29[c] (50)[c]	34 (86)	60 (100)	92	99
	9a	71 (50)	66 (14)	40 (0)	8	1
6-OC_2H_5	**8b**	35 (71)	55 (97)	72	91	99
	9b	65 (29)	45 (3)	28	9	1
6-CH_3	**8c**	10[c,d] (68)	43 (84)	73 (100)	88	100
	9c	90 (32)	57 (16)	27 (0)	12	–
6-Cl	**8d**	34 (35)[c]	19 (67)	62 (100)	99	[f]
	9d	66 (65)	81 (33)	38 (0)	1	[f]
4-OCH_3	**8e**	100[e]	100	[f]	[f]	[f]
	9e	–	–	[f]	[f]	[f]

[a] Calculated from the ^{1}H NMR spectrum recorded in acetone-d_6.
[b] In brackets.
[c] After this time the spectrum showed a singlet probably belonging to a benzofuroxanic species, that disappeared with time.
[d] The spectrum showed presence of ~6% of **1**.
[e] In this case the spectrum showed presence of **1** and **8e** in 25/75 relative ratio.
[f] Not measured.

(pGrpE-lux), *E. coli* MG1655 (pIbpA-lux), *E. coli* MG1655 (pVFR1-lux) were used in the experiments. By means of *E. coli* MG1655 (pXen7), a biosensor with a constitutive promotor, and *V. aquamarinus* VKPM B-11245, toxicity of substances can be defined.

Biosensors with RecA and ColD promotors fix the presence of the factors causing damage of DNA in a cell, the biosensor with the KatG promotor fixes production of hydroperoxides in a cell, and with the SoxS promotor - superoxide anion and NO [36–41]. Biosensor strains with GrpE and IbpA promotors respond to the substances damaging proteins [38,42]. The biosensor of *E. coli* MG1655 (pVFR1-lux) contains DNA fragment with luxR gene, the promotor and regulatory area of a lux-operon *Vibrio fischeri* inserted before genes of luxCDABE – it sensitively reacts to introduction of autoinducers of Quorum Sensing systems of the 1st type into the medium, but not of autoinducers of Quorum Sensing systems of the 2nd type [43].

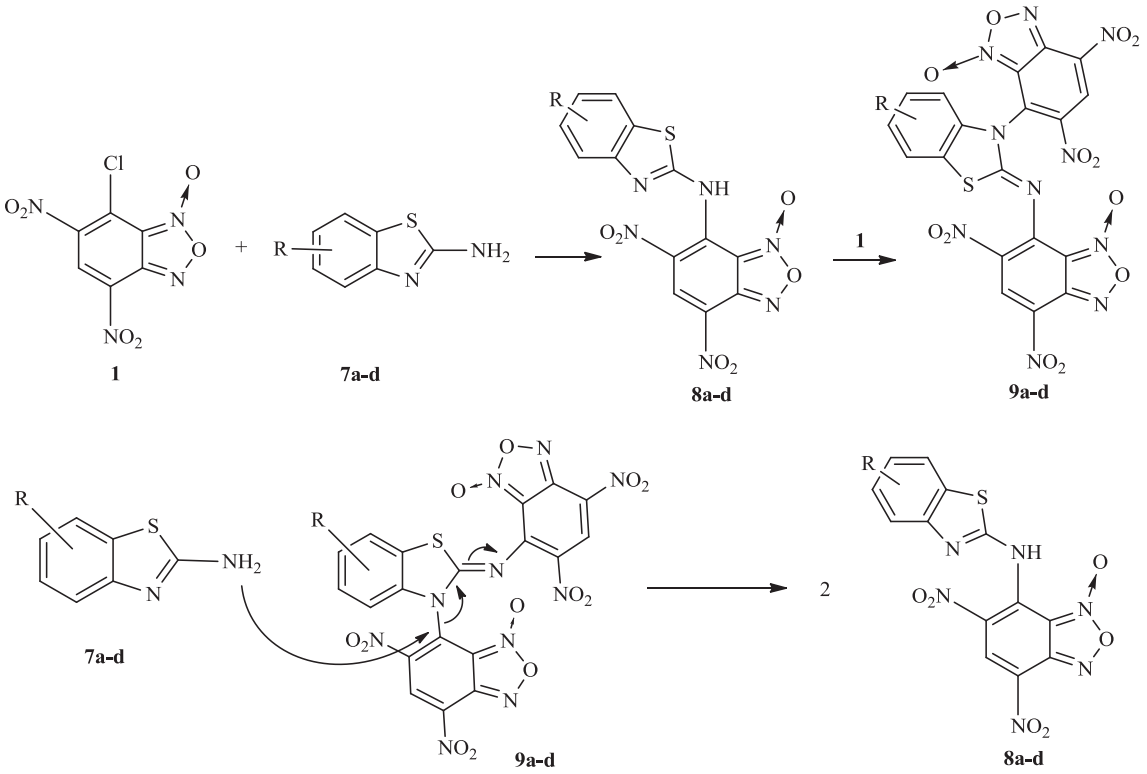
The assessment of the toxic influence of benzofuroxans **3**, **8a–c**, **8e–f**, **9d** on bacterial cells was researched by means of bacterial strains of *V. aquamarinus* VKPM B-11245 and *E. coli* MG1655 (pXen7). Benzofuroxan derivatives **8a–c**, **8e–f**, and **9d**, containing the 2-aminobenzothiazole fragment, did not show noticeable biological activity concerning damage of DNA both without and with metabolic activation [biosensors *E. coli* MG1655 (pRecA-lux) and *E. coli* MG1655 (pColD-lux)], biosensor *E. coli* MG 1655 (pSoxS-lux) did not cause oxidizing stress by increase of intracellular level superoxide anion radical and nitrogen oxide, and also peroxide compounds [biosensor *E. coli* MG 1655 (pKatG-lux)].

Besides, significant level of protein damage in a bacterial cell was not registered [biosensor *E. coli* MG 1655 (pGrpE-lux)]. The investigated compounds also did not cause activation of Quorum Sensing system of the 1st type [biosensor *E. coli* MG1655 (pVFR1-lux)].

Among all the benzofuroxans containing the 2-aminobenzothiazole fragment, only compound **8e** showed the average level of toxicity for a bacterial cell in concentrations up to 10^{-7} M and only concerning *V. aquamarinus* VKPM B-11245. For other investigated benzofuroxans, the noticeable bacteriotoxic effect at concentration lower than 10^{-3}–10^{-4} M is revealed neither for a vibrio, nor for a constitutive biosensor on the basis of *E. coli* MG1655.

Introduction of mercaptobenzothiazole fragment instead of the aminobenzothiazole fragment leads to considerable strengthening of biological activity.

As it is clear from the data in Fig. 3, the benzofuroxan derivative **3** is highly toxic for *V. aquamarinus* VKPM B-11245 in the concentration range: 1×10^{-3} M–1×10^{-6} M.



Scheme 6. Proposed pathway to explain the observed time-dependence of the ratio between products **8a–d** and **9a–d**.

For *E. coli* MG1655 (pXen7), the substance is toxic in the concentration of 1×10^{-5} M and highly toxic in the concentration of 1×10^{-4} M and higher. Sensitivity of *V. aquamarinus* VKPM B-11245 to the studied substance was higher that is likely to be connected with more expressed sensitivity of this strain to toxic influences.

For researching possible mechanisms of the compound **3** influence on a bacterial cell, a number of experiments were carried out with genetically engineered luminescent biosensors of *Escherichia coli* MG1655 (pSoxS-lux), *E. coli* MG1655 (pKatG-lux), *E. coli* MG1655 (pRecA-lux), *E. coli* MG1655 (pColD-lux), *E. coli* MG1655 (pGrpE-lux), *E. coli* MG1655 (pIbpA-lux) and *E. coli* MG1655 (pVFR1-lux) that allow to reveal certain influence on bacterial cell homeostasis. Data on the biological effects of the studied substance **3** in various concentrations are presented in Fig. 4.

During the experiments with biosensors *E. coli* MG1655 (pKatG-lux), *E. coli* MG1655 (pRecA-lux), *E. coli* MG1655 (pColD-lux), *E. coli* MG1655 (pGrpE-lux), and *E. coli* MG1655 (pIbpA-lux), a significant response was not observed. Thus, it is possible to claim that during interaction of the studied substance (**3**) and bacterial cells there is no noticeable increase of peroxide compound level, damage of DNA and proteins.

On the other hand, for compound **3** a significant effect of superoxide-anion radical or NO level increase is registered in a bacterial cell ($I^s > 2$) in concentration of 1×10^{-4} M and a weak effect ($1.5 \leq I^s \leq 2$) in concentration of 1×10^{-3} M (in this case, probably, the biosensor luminescence suppression effect due to the toxicity of the studied substance manifests itself).

The most significant of the observed biological effects is expressed by 1st type Quorum Sensing system activation. For compound **3** effective activating concentrations are 1×10^{-6} M, 1×10^{-8} M, 1×10^{-10} M and 1×10^{-14} M ($I^s > 2$), weak activation is present at all other concentrations except for 1×10^{-4} M. The effect of benzofuroxan **3** on the 1st type Quorum Sensing system requires careful research for the purpose of studying the damage of pathogenic microorganisms biofilms formation.

The compounds influencing formation of bacterial biofilms, definitely deserve more careful research as for many pathogenic microorganisms an obligatory stage of infectious process development is biofilm formation. Even the substances not possessing their own antibacterial activity preventing biofilm formation by microorganisms, can be very useful as a part of joint therapy with antibiotics. Their application as a part of complex therapy can mitigate infectious diseases and accelerate treatment process.

3. Conclusion

The ability of benzofuroxan derivatives to release nitric oxide (NO) under physiological conditions and the bioactivity of many benzothiazole derivatives have inspired this research focused on the synthesis of novel structural hybrids bearing these two heterocyclic moieties and on the evaluation of their antibacterial activity. The new compounds have been synthesized through electrophile/nucleophile combination of nitrobenzofuroxan derivatives and 2-mercapto- or 2-aminobenzothiazole derivatives. The reaction between 4,6-dichloro-5-nitrobenzofuroxan and 2-mercaptobenzothiazole (or its sodium salt) gave two products, one deriving from a double nucleophilic attack with the displacement of both, the chlorine atom and the nitro group of the benzofuroxan reagent, and the second one implying an unexpected replacement of the nitro group by chlorine.

From the reaction between 7-chloro-4,6-dinitrobenzofuroxan and different 2-aminobenzothiazole derivatives two products have been isolated, one bearing the benzofuroxan moiety linked to the exocyclic amino nitrogen of the nucleophile, and the second derived from the attack of two molecules of electrophile to both the nitrogen atoms of the benzothiazole reagent. The reaction was monitored directly in the NMR spectroscopy tube and this experiment revealed that the relative ratio of the two products is time-dependent thus suggesting the possibility to tune the reaction depending on the product of interest.

The biological effect on the natural strain *Vibrio* genus and different bacterial lux-biosensors was studied. Among all the compounds synthesized, that were obtained by the reaction between 2-aminobenzothiazole and 7-chloro-4,6-dinitrobenzofuroxan only the compound **8e** displayed bacteriotoxic properties towards Vibrio in the concentration up to 10^{-7} M. Introduction of 2-mercaptobenzothiazole fragment into benzofuroxan molecules instead of the aminobenzothiazole fragment intensified the biological activity, actually, compound **3** displayed not only the bacteriotoxic effect but also activated the 1st type Quorum Sensing system effectively.

4. Experimental

4.1. General

The ^{1}H and ^{13}C NMR spectra were recorded with a Mercury 400 or a Inova 600 (Varian, Palo Alto USA) spectrometer operating at 400, or 600 MHz (for ^{1}H NMR) and 100.56, or 150.80 MHz (for ^{13}C NMR), respectively. Signal multiplicities were established by DEPT experiments. Chemical shifts were measured in δ (ppm) with reference to the solvent ($\delta = 1.96$ ppm and 118.10 ppm for CD_3CN; $\delta = 2.05$ ppm and 29.84 ppm for $(CD_3)_2CO$; $\delta = 7.26$ ppm and 77.00 ppm for $CDCl_3$, for ^{1}H and ^{13}C NMR, respectively). *J* values are given in Hz. Electron spray ionization mass spectra (ESI-MS) were recorded with a WATERS 2Q 4000 instrument. Elemental analyses

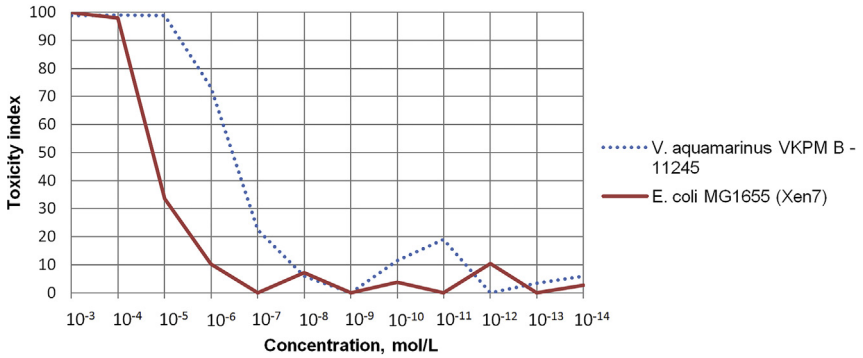


Fig. 3. Toxicity index of compound **3**, registered for natural and gene engineered strains.

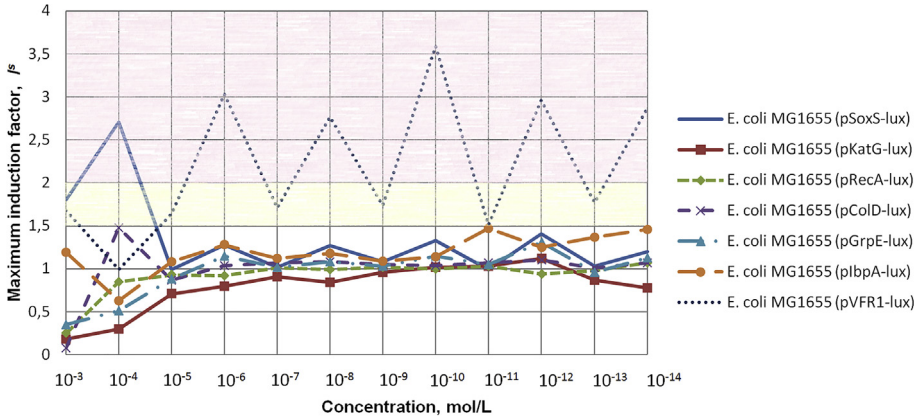


Fig. 4. The maximum induction factor obtained at 120 min incubation of bacterial biosensors with various concentrations of compound **3**.

were performed on a Carlo Erba Model EA-1108 elemental analyser. Chromatographic purifications (FC) were carried out on glass columns packed with silica gel (Merck grade 9385, 230–400 mesh particle size, 60 Å pore size) at medium pressure. Thin layer chromatography (TLC) was performed on silica gel 60 F254 coated aluminum foils (Fluka). Aluminum oxide used was activated, basic, Brockmann I, standard grade ca. 150 mesh. Melting points were measured on a Büchi 535 apparatus and are uncorrected; compounds **8** and **9** are red-brown solids that decompose in the melting tube above about 200 °C. 2-Mercaptobenzothiazole (**2**) and 2-aminobenzothiazoles **7a**–**f** were purchased from Sigma Aldrich (Milan, Italy). Benzofuroxans **1** and **4** were prepared using the methods reported in the literature [24,44]. Genetically engineered biosensor strains of *Escherichia coli* MG1655 (pXen7), *E. coli* MG1655 (pSoxS-lux), *E. coli* MG1655 (pKatG-lux), *E. coli* MG1655 (pRecA-lux), *E. coli* MG1655 (pColD-lux), *E. coli* MG1655 (pGrpE-lux), *E. coli* MG1655 (pIbpA-lux), *E. coli* MG1655 (pVFR1-lux) have been kindly furnished by Manukhov I.V., Federal State Unitary Enterprise "GosNIIGenetika". All chemical preparations for biological assays were of analytical purity: zinc sulfate (Aquatest, Russia), Dioxydin (Sigma–Aldrich), paraquat (Sigma–Aldrich), hydrogen peroxide (Ferrain, Russia), MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, Sigma–Aldrich), ethanol (NeoSources Inc.), 3-oxohexanoyl-homoserine lactone (Sigma–Aldrich).

Copies of ^{1}H and ^{13}C NMR spectra of compounds **3**, **5**, **6**, **8a**–**e**, **9a**–**d**, and **15** and tabulated data related to Figs. 3 and 4 are reported in Supporting Information.

4.2. Synthesis of 7-(1,3-benzothiazol-2-ylthio)-4,6-dinitro-2,1,3-benzoxadiazole 1-oxide (3)

To a magnetically stirred solution of 7-chloro-4,6-dinitrobenzofuroxan (**1**, 0.020 g, 0.077 mmol) dissolved in $CHCl_3$ (10 mL) was added an equimolar amount of 1,3-benzothiazole-2-thiol (**2**, 0.013 g, 0.077 mmol) and 0.08 g of basic aluminum oxide, at room temperature. Immediately after mixing the solution turned from pale yellow to red. The solution was stirred for 1 h and the progress of the reaction was monitored by TLC (eluent: dichloromethane) and ^{1}H NMR analysis. After filtration and removal of the solvent in vacuum, the product **3** was washed with a little amount of Et_2O then *n*-hexane was added and compound **3** precipitated as dark red solid (0.026 g, 86%). The purification by FC (eluent: dichloromethane) gave **3** in lower yield probably because of its partial decomposition on silica gel. ^{1}H NMR (600 MHz, $CDCl_3$, 25 °C) δ (ppm): 7.41–7.47 (m, 2H, H-5 and H-6 benzothiazolyl), 7.69–7.71 (m, 1H, H-4 or H-7 benzothiazolyl), 7.86–7.89 (m, 1H, H-4 or H-7 benzothiazolyl), 8.98 (m, 1H, H-5); ^{13}C NMR (150.80 MHz ($CDCl_3$, 25 °C) δ (ppm): 115.8, 121.6 (CH), 122.7 (CH), 126.3 (CH), 126.7 (CH), 127.0 (CH), 130.5, 135.6, 136.1, 144.2, 145.6, 152.1, 158.9. Anal. calcd for $C_{13}H_5N_5O_6S_2$: C 39.90, H 1.29, N 17.90; found: C 40.00, H 1.30, N 17.94; ESI-MS (ES^+): m/z = 414 $[M+Na]^+$.

4.3. Reaction between 4,6-dichloro-5-nitrobenzofuroxan (4) and 1,3-benzothiazole-2-thiol (2)

To a solution of 4,6-dichloro-5-nitrobenzofuroxan **4** (0.125 g, 0.0005 mol) in 5 mL of DMSO at room temperature was added a solution of 2-mercaptobenzothiazole (**2**, 0.166 g, 0.001 mol) in 5 mL of DMSO. The reaction mixture was heated at 80–90 °C for 5–6 h (the reaction was monitored by TLC). After verification of the completion of the reaction by TLC, distilled water was added to the crude reaction mixture and a yellow solid precipitated. It was filtered off, washed with water and dried under vacuum (0.06 mm Hg) at 40 °C until to constant weight. The mixture of products **5** and **6** was separated by column chromatography, using ethyl acetate as eluent. The same results were obtained using an equimolar ratio of the reagents.

4.3.1. 4,5-bis(benzo[d]thiazol-2-ylthio)-6-chlorobenzo[c][1,2,5] oxadiazole 1-oxide (5)

Yellow oil, 45% yield; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C), δ (ppm): 7.32–7.37 (m, 2H, benzothiazolyl), 7.41–7.45 (m, 2H, benzothiazolyl), 7.64 (s, 1H, H-7), 7.74–7.78 (m, 2H, benzothiazolyl), 7.84–7.86 (m, 2H, benzothiazolyl); ^{13}C NMR (100.56 MHz, $CDCl_3$, 25 °C), δ (ppm): 113.7 (CH), 114.2, 116.7 (CH), 121.1 (CH), 121.2 (CH), 122.5 (CH), 122.9 (CH), 125.2 (CH), 125.6 (CH), 125.8, 126.5 (CH), 132.7, 133.0, 135.8, 136.5, 138.5, 152.8, 152.9, 153.0, 160.2; ESI-MS (ES^+): m/z = 523, 525 $[M+Na]^+$.

4.3.2. 4-(benzo[d]thiazol-2-ylthio)-5,6-dichlorobenzo[c][1,2,5] oxadiazole 1-oxide (6)

Yellow solid, 52% yield; M.p.: 199–201 °C (CH_2Cl_2/n-hexane); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C), δ (ppm): 7.38 (t, J = 7.78 Hz, 1H, H-5 or H-6 benzothiazolyl), 7.46 (t, J = 7.78 Hz, 1H, H-5 or H-6 benzothiazolyl), 7.65 (s, 1H, H-7), 7.57 (dm, J = 8.06 Hz, 1H, H-4 or H-7 benzothiazolyl), 7.88 (br.d, J = 8.01 Hz, 1H, H-4 or H-7 benzothiazolyl); ^{13}C NMR (100.56 MHz, $CDCl_3$, 25 °C), δ (ppm): 113.2, 116.2 (CH), 118.3, 121.3 (CH), 123.2 (CH), 125.5, 126.0 (CH), 126.8 (CH), 136.6, 149.2, 152.8, 153.9, 157.4; Anal. calcd for $C_{13}H_5Cl_2N_3O_2S_2$: C 42.17, H 1.36, N 11.35; found: C 42.19, H 1.36, N 11.34; ESI-MS (ES^+): m/z = 392, 394 $[M+Na]^+$.

*4.3.3. Crystal data for 4-(benzo[d]thiazol-2-ylthio)-5,6-dichlorobenzo[c][1,2,5]oxadiazole 1-oxide (**6**)*

Molecular formula: $C_{13}H_5Cl_2N_3O_2S_2$; M_r = 370.22, Triclinic, space group P-1 a = 5.7888 (7), b = 11.2709 (14), c = 11.7634 (15) Å, α = 67.9960 (19), β = 94.385 (2), γ = 89.0390 (10); V = 708.91 (15) Å^3, T = 298 (2) K, Z = 2, ρ_c = 1.734 g cm^{-3}, $F(000)$ = 372, graphite-monochromated $Mo_{K\alpha}$ radiation (λ = 0.71073 Å), $\mu(Mo_{K\alpha})$ = 0.761 mm^{-1}, colourless brick (0.30 × 0.15 × 0.15 mm^3), empirical absorption correction with SADABS (transmission factors: 0.8944–0.8039), 2400 frames, exposure time 15 s, $1.87 \le \theta \le 28.77$, $-7 \le h \le 7$, $-15 \le k \le 15$, $-15 \le l \le 15$, 8264 reflections collected, 3358 independent reflections (R_{int} = 0.0203), solution by direct methods (SHELXS97) and subsequent Fourier syntheses, full-matrix least-squares on F_o^2 (SHELXTL 2008-4), hydrogen atoms refined with a riding model. Data/restraints/parameters = 3358/0/199, $S(F^2)$ = 1.061, $R(F)$ = 0.0381 and $wR(F^2)$ = 0.0843 on all data, $R(F)$ = 0.0336 and $wR(F^2)$ = 0.0809 for 3012 reflections with $I > 4\sigma(I)$; weighting scheme $w = 1/[\sigma^2(F_o^2) + (0.0357P)^2 + 0.000P]$ where $P = (F_o^2 + 2F_c^2)/3$; largest difference peak and hole 0.301 and −0.307 e Å^{-3}. Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 1028845. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB21EZ, UK (fax: (+44) 1223 336 033; e-mail: deposit@ccdc.cam.ac.uk).

*4.4. General procedure for the synthesis of compounds **8a–f** and **9a–d***

To a solution of 4,6-dinitro-7-chlorobenzofuroxan **1** (0.025 g, 0.0001 mol) in 5 mL of acetonitrile or chloroform at room temperature was added a solution of 2-aminobenzothiazole **7** (0.0002 mol) in 5 mL of acetonitrile or chloroform. The reaction mixture was stirred for 2–24 h; the reaction products and their relative yield depend from the reaction time, with the increase of time amount of mono-substituted product increases (see Table 1). The reaction was carried out also with a 1:4 M amount of **1**:**7**, and the results obtained are reported in Table 1. After removal of the solvent under reduced pressure, the products were separated by column chromatography, using ethyl acetate as eluent.

*4.4.1. 7-(benzo[d]thiazol-2-ylamino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (**8a**)*

^{1}H NMR (400 MHz, CD_3CN, 25 °C), δ (ppm): 7.23 (td, J = 8.41 Hz, J = 1.2 Hz, 1H, H-5 or H-6 benzothiazolyl), 7.36 (td, J = 8.41 Hz, J = 1.2 Hz, 1H, H-5 or H-6 benzothiazolyl), 7.60 (dd, J = 8.2 Hz, J = 0.6 Hz, 1H, H-4 or H-7 benzothiazolyl), 7.81 (dd, J = 8.0 Hz, J = 0.78 Hz, 1H, H-4 or H-7 benzothiazolyl), 8.89 (s, 1H, H-7); ^{13}C NMR (100.56 MHz, DMSO-d_6, 25 °C), δ (ppm): 112.0, 115.8, 120.6 (CH), 121.5 (CH), 123.1 (CH), 125.7, 125.8 (CH), 133.7, 134.0, 134.1 (CH), 142.0, 147.5, 150.8; Anal. calcd for $C_{13}H_6N_6O_6S$: C 41.72, H 1.62, N 22.45; found: C 41.89, H 1.63, N 22.52; ESI-MS (ES$^-$): m/z = 373 [M−H]$^-$.

*4.4.2. 7-((6-ethoxybenzo[d]thiazol-2-yl)amino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (**8b**)*

^{1}H NMR (400 MHz, acetone-d_6, 25 °C), δ (ppm): 1.38 (t, J = 6.95 Hz, 3H, CH_3CH_2), 4.09 (q, J = 6.95 Hz, 2H, CH_2CH_3), 6.92 (dd, J = 8.8 Hz, J = 2.4 Hz, 1H, H-5 benzothiazolyl), 7.36 (d, J = 2.4 Hz, 1H, H-7 benzothiazolyl), 7.46 (d, J = 8.8 Hz, 1H, H-4 benzothiazolyl), 8.92 (s, 1H, H-7); ^{13}C NMR (100.56 MHz, acetone-d_6, 25 °C), δ (ppm): 15.2 (CH_3), 64.6 (CH_2), 106.1 (CH), 112.8, 114.6, 115.5 (CH), 122.2 (CH), 134.8 (CH), 136.5, 142.7, 146.6, 148.8, 156.5, 169.3; Anal. calcd for $C_{15}H_{10}N_6O_7S$: C 43.07, H 2.41, N 20.09; found: C 43.24, H 2.42, N 20.07; ESI-MS (ES$^-$): m/z = 417 [M−H]$^-$.

*4.4.3. 7-((6-methylbenzo[d]thiazol-2-yl)amino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (**8c**)*

^{1}H NMR (400 MHz, acetone-d_6, 25 °C), δ (ppm): 2.39 (s, 3H, CH_3), 7.13 (dd, J = 8.35, J = 1.97 Hz, 1H, H-5 benzothiazolyl), 7.45 (d, J = 8.35 Hz, 1H, H-4 benzothiazolyl), 7.60–7.57 (m, 1H, H-7 benzothiazolyl), 8.93 (s, 1H, H-7); ^{13}C NMR (100.56 MHz, acetone-d_6, 25 °C), δ (ppm): 21.4 (CH_3), 112.8, 121.4 (CH), 121.6, 121.8 (CH), 127.4, 127.6 (CH), 133.5, 134.8 (CH), 135.5, 142.9, 148.7, 150.5, 170.6; Anal. calcd for $C_{14}H_8N_6O_6S$: C 43.30, H 2.08, N 21.64; found: C 43.50, H 2.09, N 21.60; ESI-MS (ES$^-$): m/z = 387 [M−H]$^-$.

*4.4.4. 7-((6-chlorobenzo[d]thiazol-2-yl)amino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (**8d**)*

^{1}H NMR (400 MHz, acetone-d_6, 25 °C), δ (ppm): 7.30 (dd, J = 8.68 Hz, J = 1.74 Hz, 1H, H-5 benzothiazolyl), 7.52 (dd, J = 8.68 Hz, J = 1.74 Hz, 1H, H-4 benzothiazolyl), 7.83–7.88 (d, 1H, J = 1.74 Hz, H-7 benzothiazolyl), 8.93 (s, 1H, H-7); ^{13}C (100.56 MHz $(CD_3)_2CO$, 25 °C) δ, ppm: 112.8, 116.8, 121.5 (CH), 122.7 (CH), 126.6 (CH), 126.8, 128.3, 134.9 (CH), 137.2, 143.2, 148.6, 151.9, 172.3; Anal. calcd for $C_{13}H_5ClN_6O_6S$: C 38.20, H 1.23, N 20.56; found: C 38.21, H 1.23, N 20.55; ESI-MS (ES$^-$): m/z = 407, 409 [M−H]$^-$.

*4.4.5. 7-((4-methoxybenzo[d]thiazol-2-yl)amino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (**8e**)*

^{1}H NMR (400 MHz, acetone-d_6, 25 °C), δ (ppm): 3.92 (s, 3H, OCH_3), 6.90 (d, J = 8.20 Hz, 1H, H-5 or H-7 benzothiazolyl), 7.15 (t, J = 8.20 Hz, 1H, H-6 benzothiazolyl), 7.38 (d, J = 8.20 Hz, 1H, H-5 or H-7 benzothiazolyl), 8.93 (s, 1H, H-7); ^{13}C NMR (100.56 MHz, acetone-d_6, 25 °C), δ (ppm): 56.6 (OCH_3), 108.7 (CH), 112.8, 114.5 (CH), 117.1, 124.8 (CH), 126.4, 135.1 (CH), 136.2, 142.0, 143.4, 148.7, 152.7, 170.7; Anal. calcd for $C_{14}H_8N_6O_7S$: C 41.59, H 1.99, N 20.79; found: C 41.73, H 2.01, N 20.78; ESI-MS (ES$^-$): m/z = 403 [M−H]$^-$.

*4.4.6. 4,6-Dinitro-7-((5-nitrobenzo[d]thiazol-2-yl)amino)benzo[c][1,2,5]oxadiazole 1-oxide (**8f**)*

^{1}H NMR (400 MHz, acetone-d_6, 25 °C), δ (ppm): 7.41 (dd, J = 8.75, J = 2.33 Hz, 1H, H-6 benzothiazolyl), 7.81 (d, J = 2.33 Hz, 1H, H-4 benzothiazolyl), 7.85 (d, J = 8.75 Hz, 1H, H-7 benzothiazolyl), 8.95 (s, 1H, H-7); ^{13}C NMR (100.56 MHz, acetone-d_6, 25 °C), δ (ppm): 111.2, 112.7, 116.3 (CH), 117.0, 124.6, 127.1 (CH), 129.9 (CH), 135.6 (CH), 141.8, 146.2, 149.0, 153.4; Anal. calcd for $C_{13}H_5N_7O_8S$: C 37.24, H 1.20, N 23.38; found: C 37.27, H 1.21, N 23.36; HRMS (ESI-TOF) m/z: [M−H]$^-$ Calcd for [M−H]$^-$ $C_{13}H_4N_7O_8S$- 417.98475; Found 417.984.

*4.4.7. 7-((3-(4,6-dinitro-1-oxidobenzo[c][1,2,5]oxadiazol-7-yl)benzo[d]thiazol-2(3H)-ylidene)amino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (**9a**)*

Brown oil, ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C), δ (ppm): 6.85–6.87 (m, 1H, benzothiazolyl), 7.43–7.46 (m, 2H, benzothiazolyl), 7.64–7.66 (m, 1H, benzothiazolyl), 9.06 (s, 1H, benzofuroxanyl), 9.13 (s, 1H, benzofuroxanyl); ^{13}C NMR: (100.56 MHz, CD_3CN, 25 °C) δ, ppm: 113.0, 113.1 (CH), 115.0, 123.3, 124.5 (CH), 125.2, 126.7 (CH), 128.7 (CH), 129.1 (CH), 130.2, 131.2 (CH), 132.2, 137.3, 139.0, 141.1, 144.6, 146.1, 146.7, 162.1; Anal. calcd for $C_{19}H_6N_{10}O_{12}S$: C 38.14, H 1.01, N 23.41; found: C 38.12, H 1.00, N 23.38; ESI-MS (ES$^+$): m/z = 599 [M+H]$^+$, 621 [M+Na]$^+$.

*4.4.8. 7-((3-(4,6-dinitro-1-oxidobenzo[c][1,2,5]oxadiazol-7-yl)-6-ethoxybenzo[d]thiazol-2(3H)-ylidene)amino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (**9b**)*

^{1}H NMR (400 MHz, acetone-d_6, 25 °C), δ (ppm): 1.38 (t, J = 6.77 Hz, 3H, CH_3), 4.12 (q, J = 6.77 Hz, 2H, CH_2), 7.04 (dd, J = 8.8 Hz, J = 1.8 Hz, 1H, H-5 benzothiazolyl), 7.46 (d, J = 8.8 Hz, 1H, H-4 benzothiazolyl), 7.53 (d, J = 1.8 Hz, 1H, H-7 benzothiazolyl),

9.00 (s, 1H, benzofuroxanyl), 9.25 (s, 1H, benzofuroxanyl); ^{13}C NMR (100.56 MHz, acetone-d_6, 25 °C), δ (ppm): 15.0 (CH_3), 65.1 (CH_2), 109.5 (CH), 114.3 (CH), 115.1, 116.7 (CH), 125.0, 125.5, 128.9 (CH), 129.0, 130.0, 131.2, 131.4 (CH), 132.0, 139.1, 141.6, 144.9, 146.3, 146.9, 158.3, 162.7; Anal. calcd for $C_{21}H_{10}N_{10}O_{13}S$: C 39.26, H 1.57, N 21.80; found: C 39.41, H 1.58, N 21.77; ESI-MS (ES^+): $m/z = 665$ $[M+Na]^+$.

4.4.9. 7-((3-(4,6-dinitro-1-oxidobenzo[c][1,2,5]oxadiazol-7-yl)-6-methylbenzo[d]thiazol-2(3H)-ylidene)amino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (**9c**)

^{1}H NMR (400 MHz, acetone-d_6, 25 °C), δ (ppm): 2.43 (s, 3H, CH_3), 7.26–7.33 (m, 2H, H-4, H-5 benzothiazolyl), 7.72–7.74 (m, 1H, H-7 benzothiazolyl), 9.02 (s, 1H, benzofuroxanyl), 9.25 (s, 1H, benzofuroxanyl); ^{13}C NMR (100.56 MHz, acetone-d_6, 25 °C), δ (ppm): 21.1 (CH_3), 113.1 (CH), 115.0, 123.7, 124.5 (CH), 125.5, 128.9 (CH), 129.9 (CH), 130.3, 131.3 (CH), 132.2, 135.5, 137.1, 139.1, 141.5, 144.9, 146.3, 146.9, 162.7, 164.5; Anal. calcd for $C_{20}H_8N_{10}O_{12}S$: C 39.22, H 1.32, N 22.87; found: C 39.20, H 1.34, N 22.82; HRMS (ESI-TOF) *m/z*: $[M+Na]^+$ Calcd for $[M+Na]^+$ $C_{20}H_8N_{10}O_{12}SNa^+$ 634.99361; Found 634.994. ESI-MS (ES^+): $m/z = 613$ $[M+H]^+$, 635 $[M+Na]^+$, 651 $[M+K]^+$.

4.4.10. 7-(6-Chloro-2-((4,6-dinitro-1-oxidobenzo[c][1,2,5]oxadiazol-7-yl)imino)benzo[d]thiazol-3(2H)-yl)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (**9d**)

^{1}H NMR (400 MHz, acetone-d_6, 25 °C), δ (ppm): 7.42 (d, $J = 8.8$ Hz, 1H, H-4 benzothiazolyl),7.52 (dd, $J = 8.8$ Hz, $J = 1.7$ Hz, 1H, H-5 benzothiazolyl), 8.03 (d, 1H, $J = 1.7$ Hz, H-7 benzothiazolyl), 9.02 (s, 1H, benzofuroxanyl), 9.25 (s, 1H, benzofuroxanyl); ^{13}C NMR (100.56 MHz, acetone-d_6, 25 °C) δ (ppm): 113.1, 114.6 (CH), 115.1, 124.4 (CH), 125.0, 125.4, 128.9 (CH), 129.2 (CH), 130.8, 131.1 (CH), 131.5, 132.6, 136.6, 139.3, 141.0, 145.1, 146.3, 146.8, 162.0; Anal. calcd for $C_{19}H_5ClN_{10}O_{12}S$: C 36.06, H 0.80, N 22.13; found: C 36.20, H 0.80, N 22.09; ESI-MS (ES^+): $m/z = 655$ $[M+Na]^+$, 657 $[M+Na]^+$.

4.5. Synthesis of 7-((6-ethoxy-3-methylbenzo[d]thiazol-2(3H)-ylidene)amino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (**15**)

To a solution (15 mg, 0.036 mmol) of the monoadduct derived from the reaction between **1** and **7b**, dissolved in 3 mL of anhydrous THF, 150 μL (2.4 mmol) of methyl iodide was added. The reaction mixture was heated to reflux under nitrogen atmosphere for 24 h. The solvent was removed and flash chromatography on silica gel (eluent: ethyl acetate) of the residue gave compound **15** as dark violet solid (64% yield): m.p.: 187.5–188.7 °C; ^{1}H NMR (600 MHz, acetone-d_6, 25 °C): δ (ppm): 1.39 (t, $J = 6.8$ Hz, 3H, $C\underline{H}_3CH_2$), 3.92 (s, 3H, NCH_3), 4.13 (q, $J = 6.8$ Hz, 2H, $CH_3C\underline{H}_2$), 7.21 (dd, $J = 8.9$ Hz, $J = 2.5$ Hz, 1H, H-5 benzothiazolyl), 7.47 (d, $J = 2.5$ Hz, 1H, H-7 benzothiazolyl), 7.67 (d, $J = 8.9$ Hz, 1H, H-4 benzothiazolyl), 9.06 (s, 1H, H-7); ^{13}C NMR (150.80 MHz, acetone-d_6, 25 °C), δ (ppm): 14.9 (CH_3), 33.0 (NCH_3), 65.0 (CH_2), 108.6 (CH), 113.3, 115.0 (CH), 117.0 (CH), 125.5, 125.7, 128.3, 133.0 (CH), 133.9, 144.5, 147.6, 158.0, 166.3. NMR experiment carried out by irradiating methyl signal NOE effect on the H-4 proton of the benzothiazole moiety, indicating that compound **15** bears the benzofuroxan moiety bound to the 2-aminobenzothiazole exocyclic nitrogen atom (see Scheme 5).

4.6. Biological essays

E. coli biosensor cells, the containing recombinant plasmids, were cultivated on complete Luria–Bertani's (LB) [45] medium with addition of ampicillin antibiotic (100 μg/mL). The biosensor strain culture was grown in the liquid LB medium over the night. The night culture was diluted with the same medium (LB) to the density 0.1 McFarland unit and was grown at 37 °C for 2 h.

180 μL of biosensor cell suspension and 20 μL of toxicant solution in various concentrations were introduced into the wells of the 96-well plate. During genotoxicity evaluation with use of metabolic activation 160 μL of culture, 10 μL of toxicant solution and 10 μL the activating mix containing S-9 fraction of the rat microsome enzymes (Almalab) were introduced into the wells [46]. 20 μL of distilled water were introduced as negative control.

Standard toxicants or mutagens in the concentrations causing the expressed luminescent response were used as positive control for biosensor strains: *V. aquamarinus* VKPM B-11245 – zinc sulfate, 1.86×10^{-6} M; *E. coli* MG1655 (pXen7) – zinc sulfate, 2.48×10^{-5} M; *E. coli* MG1655 (pSoxS-lux) – Dioxydine (2,3-bis(hydroxymethyl)quinoxaline-1,4-di-*N*-oxide), 2.25×10^{-5} M; or paraquat, 1×10^{-6} M; *E. coli* MG1655 (pKatG-lux) – hydrogen peroxide, 1×10^{-3} M; *E. coli* MG1655 (pRecA-lux) – *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), 1×10^{-3} M; *E. coli* MG1655 (pColD-lux) – *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, 1×10^{-3} M; *E. coli* MG1655 (pGrpE-lux) – ethanol, 3.5% (V/V); *E. coli* MG1655 (pIbpA-lux) – ethanol, 3.5% (V/V); *E. coli* MG1655 (pVFR1-lux) – *N*-(3-Oxohexanoyl)-L-homoserine lactone, 1×10^{-7} M.

Plates with lux-biosensor cells were placed in a microplate luminometer LM-01T (Immunotech) and bioluminescence intensity was measured with intervals of 10 min during 120 min at 37 °C.

Cultures of strains were grown within 18–20 h at the temperature of 37 °C (*V. aquamarinus* VKPM B-11245 – at the temperature of 25 °C). Then part of daily culture was diluted with the culture medium to the density determined in advance that provides the optimum luminescent cell response of the biosensor by means of the densitometer (DEN-1B, Biosan, Latvia) and was placed into a thermostat for 2 h.

The studied substances were diluted with DMSO to concentration of 10^{-2} M, and then to concentration of 10^{-3} M with DMSO and ethanol (1:1) mix, further dilutions were obtained by addition of deionized water. As control solutions similar dilutions of DMSO/ethanol mix in the deionized water were used.

For the experiments 190 culture μL and 10 solution μL of the studied substance were placed in the wells of a 96-well plate.

Measurement of the bioluminescence level was carried out within 2 h by means of microplate thermostatically controlled Luminoscan Ascent luminometer (Termo Electron, USA).

For the assessment of the studied factors influence on the expression of operons the induction factor (I^s) was calculated according to the Formula (1):

$$I^s = L_e/L_k \quad (1)$$

where: L_k is the intensity of the control sample luminescence and L_e is the luminescence intensity of the proof sample.

Statistically reliable excess of L_e over L_k estimated according to the t-criterion was considered to be the sign of statistical importance of the induction effect [47].

The criterion of toxic influence is bioluminescence intensity change of the test object in the researched sample in comparison with that for the sample with the solution not containing the studied substances. Change of bioluminescence intensity is proportional to the toxic effect.

Strong toxic influence of the studied toxicant on bacteria is evaluated according to the inhibition of their bioluminescence for 30 min exposition period.

The quantitative assessment of the test reaction parameter is reflected as a dimensionless number – the toxicity index "T", calculated according to the Formula (2):

$$T = 100(I_c - I_e)/I_c \quad (2)$$

where I_e and I_c are the intensity of bacteria luminescence in proof

and control samples respectively at fixed exposition time of the studied solution with test object.

In some cases a situation is possible when bioluminescence intensity of an analyzed sample is higher that of the control sample. In that case irrespective of the size of negative T value the conclusion about absence of the sample toxicity is drawn, and the toxicity index equals zero.

The technique allows three threshold levels of the toxicity index:

- Admissible degree of toxicity: the toxicity index is less than 20.
- The sample is toxic: the toxicity index is equal or more than 20 and less than 50.
- The sample is highly toxic: the toxicity index is equal or more than 50.

All the experiments were carried out in three independent replications. Average sizes and variation indicators (error of averages representativeness) of the protector effects were calculated according to three independent experiments.

Acknowledgments

The authors are grateful, for the financial support, to Alma Mater Studiorum – Università di Bologna (RFO funds), Russian Scientific Foundation (grant 14-50-00014), the Southern Federal University grant № 213.01-07-2014/12 PCVG and the leading scientific school of the Russian Federation grant № NS-2449.2014.4. Authors thank Mr. Luca Zuppiroli for running the mass spectra.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.02.023.

References

[1] G.N. Nikonov, S. Bobrov, 1,2,5-Oxadiazoles, in: A.R. Katritzky, C.A. Ramsden, E.F.V. Scriven, R.J.K. Taylor (Eds.), Comprehensive Heterocyclic Chemistry III, Five-membered Rings: Triazoles, Oxadiazoles, Thiadiazoles and Their Fused Carbocyclic Derivatives, vol. 5, Elsevier, 2008, pp. 315–395.
[2] F. Terrier, Modern Nucleophilic Aromatic Substitution, Wiley VCH, Weinheim, 2013.
[3] F. Terrier, Rate and equilibrium studies in Jackson-Meisenheimer complexes, Chem. Rev. 82 (1982) 77–152.
[4] E. Buncel, J.M. Dust, F. Terrier, Rationalizing the regioselectivity in polynitroarene anionic sigma-adduct formation. Relevance to Nucleophilic Aromatic Substitution, Chem. Rev. 95 (1995) 2261–2280.
[5] H. Cerecetto, W. Porcal, Pharmacological properties of furoxans and benzofuroxans: recent developments, Mini Rev. Med. Chem. 5 (2005) 57–71.
[6] C. Medana, A. Di Stilo, S. Visentin, R. Fruttero, A. Gasco, D. Ghigo, A. Bosia, NO donor and biological properties of different benzofuroxans, Pharm. Res. 16 (1999) 956–960.
[7] P.G. Wang, T.B. Cai, N. Taniguchi (Eds.), Nitric Oxide Donors: for Pharmaceutical and Biological Applications, Wiley-VCH, Weinheim, 2005.
[8] A. Gasco, R. Fruttero, G. Sorba, A. Di Stilo, R. Calvino, NO donors: focus on furoxan derivatives, Pure Appl. Chem. 76 (2004) 973–981.
[9] P.S. Carvalho, M. Maróstica, A. Gambero, J. Pedrazzoli, Synthesis and pharmacological characterization of a novel nitric oxide-releasing diclofenac derivative containing a benzofuroxan moiety, Eur. J. Med. Chem. 45 (2010) 2489–2493.
[10] D. Seenaiah, P.R. Reddy, G.M. Reddy, A. Padmaja, V. Padmavathi, N.S. Krishna, Synthesis, antimicrobial and cytotoxic activities of pyrimidinyl benzoxazole, benzothiazole and benzimidazole, Eur. J. Med. Chem. 77 (2014) 1–7.
[11] J.K. Malik, F.V. Manvi, B.K. Nanjwade, S. Singh, P. Purohit, Review of the 2-amino substituted benzothiazoles: different methods of the synthesis, Pharm. Lett. 2 (2010) 347–359.
[12] N.K. Sharma, Priyanka, K.K. Jha, Benzothiazole: the molecule of diverse biological activities, Int. J. Curr. Pharm. Res. 2 (2010) 1–6.
[13] S. Rostamizadeh, S.A.G. Housaini, Microwave-assisted preparation of 2-substituted benzothiazoles, Phosphorus Sulfur Silicon Relat. Elem. 180 (2005) 1321–1326.
[14] S.S. Patil, V.D. Bobade, Simple and efficient one-pot synthesis of 2-substituted benzoxazole and benzothiazoles, Synth. Commun. 40 (2010) 206–212.
[15] F. Al-Qalaf, R.A. Mekheimer, K.U. Sadek, Cerium (IV) ammonium nitrate (CAN) catalyzed one-pot synthesis of 2-arylbenzothiazoles, Molecules 13 (2008) 2908–2914.
[16] H.Y. Guo, J.C. Li, Y.L. Shang, A simple and efficient synthesis of 2-substituted benzothiazoles catalyzed by H_2O_2/HCl, Chin. Chem. Lett. 20 (2009) 1408–1410.
[17] D. Azarifar, B. Maleki, A. Setayeshnazar, A simple, microwave-assisted, and solvent-free synthesis of 2-arylbenzothiazoles by acetic acid-promoted condensation of aldehydes with 2-aminothiophenol in air, Phosphorus Sulfur Silicon Relat. Elem. 184 (2009) 2097–2102.
[18] U.R. Pratap, J.R. Mali, D.V. Jawale, R.A. Mane, Baker's yeast catalyzed synthesis of benzothiazoles in an organic medium, Tetrahedron Lett. 50 (2009) 1352–1354.
[19] V.P. Devmurari, T.J. Ghodasara, Synthesis and antibacterial activity of some substituted 2-phenyl benzothiazole, Arch. Appl. Sci. Res. 2 (2010) 198–203.
[20] D.L. Boger, A convenient preparation of 2-substituted benzothiazoles, J. Org. Chem. 43 (1978) 2296–2297.
[21] S.L. Khokra, K. Arora, H. Mehta, A. Aggarwal, M. Yadav, Common methods to synthesize benzothiazole derivatives and their medicinal significance, Int. J. Pharm. Sci. Res. 2 (2011) 1356–1377.
[22] J. Jena, Significance of benzothiazole moiety in the field of cancer, Int. J. Pharm. Pharm. Sci. 6 (2014) 16–22.
[23] S. Kurbatov, P. Rodriguez-Dafonte, R. Goumont, F. Terrier, Carbon nucleophilicities of indoles in S_NAr substitutions of superelectrophilic 7-chloro-4,6-dinitrobenzofuroxan and –benzofurazan, J. Org. Chem. 74 (2009) 3305–3315.
[24] W.P. Norris, A. Chafin, Synthesis and thermal rearrangement of 5-chloro-4,6-dinitrobenzofuroxan, Heterocycles 22 (1984) 271–274.
[25] Mehilal, A.K. Sikder, R.B. Salunke, N. Sikder, Nitro-anilinodinitrobenzofuroxans-synthesis, characterisation, thermal stability and explosive properties, New J. Chem. 25 (2001) 1549–1552.
[26] Mehilal, A.K. Sikder, R.B. Salunke, N. Sikder, Synthesis, characterization and explosives properties of 7-(1H-1,2,4-triazol-3-amino)-4,6-dinitrobenzofuroxan (TADNB) and 7-(1H-1,2,3,4-tetrazol-5-amino)-4,6-dinitrobenzofuroxan (TEADNBF), J. Energ. Mater. 20 (2002) 39–51.
[27] E.A. Chugunova, E.M. Kasymova, A.R. Burilov, D.B. Krivolapov, L.M. Yusupova, M.A. Pudovik, Reaction of dichloronitrobenzofuroxanes with amines and their derivatives, Russ. J. Gen. Chem. 79 (2009) 2207–2211.
[28] E.M. Gibadullina, E.A. Chugunova, E.V. Mironova, D.B. Krivolapov, A.R. Burilov, L.M. Yusupova, M.A. Pudovik, Reaction of 4,6-Dichloro-5-nitrobenzofuroxan with aromatic amines and nitrogen-containing heterocycles, Chem. Heterocycl. Compd. 8 (2012) 1228–1234.
[29] E.A. Chugunova, M.A. Sazykina, E.M. Gibadullina, A.R. Burilov, I.S. Sazykin, V.A. Chistyakov, R.E. Timasheva, D.B. Krivolapov, R. Goumont, Synthesis, genotoxicity and UV-protective activity of new benzofuroxans substituted by aromatic amines, Lett. Drug Des. Discov. 10 (2013) 145–154.
[30] F. Terrier, Nucleophilic Aromatic Displacement: the Influence of the Nitro Group, Wiley-VCH, New York, 1991, pp. 157–206.
[31] C.R. Everly, J.G. Traynham, Formation and rearrangement of ipso intermediates in aromatic free-radical chlorination reactions, J. Am. Chem. Soc. 100 (1978) 4316–4317.
[32] P.H. Gore, S.D. Hammond, D.F.C. Morris, Nucleophilic replacement os a nitro-group in an aromatic compound by chlorine, Tetrahedron Lett. 32 (1970) 2747–2748.
[33] B. Andersson, B. Lamm, Studies in nucleophilic aromatic substitution reactions IV. Replacement of the nitro group in 2,6-dichloro-4-nitro- and 2,4-dichloro-6-nitrobenzenediazonium ions by chloride ion, Acta Chem. Scand. 23 (1969) 2983–2988.
[34] L. Forlani, P. De Maria, E. Foresti, G. Pradella, Reactivity of 2-aminothiazole toward 2,4-dinitrofluorobenzene. Products and structures, J. Org. Chem. 46 (1981) 3178–3181.
[35] L.P. Kosmacheva, R.F. Ambartsumova, Reaction of 2-aminobenzothiazoles with glycidyl phenyl ether, Chem. Heterocycl. Compd. 22 (1986) 683.
[36] G.B. Zavilgelsky, V. Yu Kotova, I.V. Manukhov, Action of 1,1–dimethylhydrazine on bacterial cells is determined by hydrogen peroxide, Mutat. Res. 634 (2007) 172–176.
[37] A.C. Vollmer, S. Belkin, D.R. Smulski, T.K. Van Dyk, R.A. La Rossa, Detection of DNA damage by use of Escherichia coli carrying recA'::lux, uvrA'::lux, or alkA'::lux reporter plasmids, Appl. Environ. Microbiol. 63 (1997) 2566–2571.
[38] T.K. Van Dyk, W.R. Majarian, K.B. Konstantinov, R.M. Young, P.S. Dhurjati, R.A. La Rossa, Rapid and sensitive pollutant detection by induction of heat shock gene-bioluminescence gene fusions, Appl. Environ. Microbiol. 60 (1994) 1414–1420.
[39] S. Belkin, Microbial whole-cell sensing systems of environmental pollutants, Curr. Opin. Microbiol. 6 (2003) 206–212.
[40] H.J. Lee, M.B. Gu, Construction of a sodA::luxCDABE fusion Escherichia coli: comparison with a katG fusion strain through their responses to oxidative stresses, Appl. Microbiol. Biotechnol. 60 (2003) 577–580.
[41] S. Belkin, D.R. Smulski, A.C. Vollmer, T.K. Van Dyk, R.A. La Rossa, Oxidative stress detection with Escherichia coli harboring a katG′::lux fusion, Appl. Environ. Microbiol. 62 (1996) 2252–2256.
[42] T.K. Van Dyk, T.T. Reed, A.C. Vollmer, R.A. La Rossa, Synergistic induction of the heat shock response in *Escherichia coli* by simultaneous treatment with chemical inducers, J. Bacteriol. 177 (1995) 6001–6004.
[43] S.A. Khrulnova, I.V. Manukhov, G.B. Zavil'gel'skii, "Quorum sensing" regulation

and the structure of *lux* the Operon in marine bacteria *Aliivibrio logei*, Russ. J. Genet. 47 (2011) 1415–1421.
[44] L.M. Yusupova, Z.V. Molodykh, B.I. Buzykin, I.F. Falyakhov, N.N. Anisimova, G.P. Sharnin, V.V. Bulidorov, S.I. Sviridov, F.S. Levinson, 4- or 6-nitro-5,7-dichlorobenzofuroxan having fungicidal activity, RU Patent 2032678, 1995.
[45] T. Maniatis, E.F. Fritsch, J. Sambrook, Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1982.
[46] P. Gerhardt, R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Kieg, G.B. Philips, Manuals of Methods for General Bacteriology, American Society of Microbiology, Washington, DC, 1981.
[47] G.F. Lakin, Biometria, Vischaja shcola, Moskva, 1990.