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 Burilova, 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

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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 theoretical 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 bio-regulatory 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.
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 basis 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 10-π-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 1H, 13C 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].

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-aminothioanilide (10a) and 4-methyl-2-aminothioanilide (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 azomethine nitrogen of 2-aminothioanilide 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 azo 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 amino nitrogen of the nonsubstituted 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-d6 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
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
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 week 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
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: $1 \times 10^{-3}$ M–$1 \times 10^{-6}$ M.

![Scheme 6](image)

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 effects.

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 (pIbpA-lux), *E. coli* MG1655 (pGrpE-lux), and *E. coli* MG1655 (pVRFl-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 (*P > 2*) in concentration of 1 × 10^{-4} M and a weak effect (*1.5 < P < 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 (*P > 2*), weak activation is present at all other concentrations except for 1 × 10^{-8} M. The effect of benzofoxuran 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 benzofoxuran 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 nitrobenzofoxuran derivatives and 2-mercapto- or 2-aminothiazole derivatives. The reaction between 4,6-dichloro-5-nitrobenzofoxan and 2-mercaptothiazole (or its sodium salt) gave two products, one deriving from a double nucleophilic attack with the displacement of both, the chlorine atom and the nitrogen group of the benzofoxuran reagent, and the second one implying an unexpected replacement of the nitrogen group by chlorine.

From the reaction between 7-chloro-4,6-dinitrobenzofoxan and different 2-aminothiazole derivatives two products have been isolated, one bearing the benzofoxuran 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 benzofoxuran 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-aminothiazole and 7-chloro-4,6-dinitrobenzofoxan only the compound 8e displayed bacteriotoxic properties towards *Vibrio* in the concentration up to 10^{-7} M. Introduction of 2-mercaptothiazole fragment into benzofoxan molecules instead of the aminothiazole 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 400 and 1H NMR spectra were recorded with a Mercury 400 or an Inova 600 (Varian, Palo Alto USA) spectrometer operating at 400, or 600 MHz (for 1H NMR) and 100,56, or 150.80 MHz (for 13C NMR), respectively. Signal multiplicities were established by DEPT experiments. Chemical shifts were measured in δ (ppm) with reference to the solvent (δ = 1.96 ppm and 118.10 ppm for CD3CN; δ = 2.05 ppm and 29.84 ppm for (CD3)2CO; δ = 7.26 ppm and 77.00 ppm for CDCl3, for 1H and 13C NMR, respectively). J values are given in Hz. Electron spray ionization mass spectra (ESI-MS) were recorded with a WATERS Q 4000 instrument. Elemental analyses

![](image-url) Fig. 3. Toxicity index of compound 3, registered for natural and gene engineered strains.
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-aminothiazoles 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 (pSoxS-lux), E. coli lux), E. coli (pRecA-lux), E. coli MG1655 (pIbpA-lux), E. coli MG1655 (pColD-lux), MG1655 (pKatG-lux), MG1655 (pVFR1-lux) have been kindly furnished by Manukhov I.V., Federal State Unitary Enterprise "GosNILGenetika". All chemical preparations for biological assays were of analytical purity: zinc sulfate (Aquatest, Russia), "Enterprise", at room temperature. Immediately after mixing the solution of 2-mercaptobenzothiazole (2, 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. 4.5-bis[benzo[d]thiazol-2-ylthio]-6-chlorobenzol[c]1,2,5] oxadiazole 1-oxide (5)

Yellow oil, 45% yield; 1H NMR (400 MHz, CDCl3, 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); 13C NMR (100.56 MHz, CDCl3, 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-dichlorobenzol[c]1,2,5] oxadiazole 1-oxide (6)

Yellow solid, 52% yield; M.p.: 199–201 °C (CH2Cl2/n-hexane); 1H NMR (400 MHz, CDCl3, 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); 13C NMR (100.56 MHz, CDCl3, 25 °C), δ (ppm): 113.2, 116.2 (CH), 118.3, 121.3 (CH), 122.3 (CH), 125.5, 126.0 (CH), 126.8 (CH), 136.6, 149.2, 152.8, 153.9, 157.4; Anal. calced for C13H23Cl2N2O2S2: C 42.19, H 1.36, N 11.34; ESI-MS (ES+): m/z = 392, 394 [M+Na]+.
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, 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-(benz[d]thiazol-2-ylamino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (8a)

1H NMR (400 MHz, CD3CN, 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); 13C NMR (100.56 MHz, CD3CN, 25 °C), δ (ppm): 112.0, 115.8, 120.6 (CH), 121.5 (CH), 123.1 (CH), 125.7, 125.8 (CH), 127.3, 134.0, 134.1 (CH), 142.0, 147.5, 150.8; Anal. calcd for C14H14N5O4S: C 41.72, H 1.62, N 22.45; found: C 41.89, H 1.63, N 22.52; EI-MS (ES+): m/z = 373 [M−H]+.

4.4.2. 7-(6-ethoxybenz[d]thiazol-2-ylamino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (8b)

1H NMR (400 MHz, CD3CN, 25 °C), δ (ppm): 1.38 (t, J = 6.95 Hz, H3(CH2CH3)), 4.09 (q, J = 6.95 Hz, 2H, CH2CH3), 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); 13C NMR (100.56 MHz, CD3CN, 25 °C), δ (ppm): 152.0 (CH3), 64.6 (CH3), 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 C14H14N5O4S: C 43.07, H 2.41, N 20.09; found: C 43.24, H 2.42, N 20.07; EI-MS (ES+): m/z = 417 [M−H]+.

4.4.3. 7-(6-methylbenz[d]thiazol-2-ylamino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (8c)

1H NMR (400 MHz, acetone-d6, 25 °C), δ (ppm): 2.39 (s, 3H, CH3), 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); 13C NMR (100.56 MHz, acetone-d6, 25 °C), δ (ppm): 21.4 (CH3), 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 C14H14N5O4S: C 43.30, H 2.08, N 21.64; found: C 43.50, H 2.09, N 21.60; EI-MS (ES+): m/z = 387 [M−H]+.

4.4.4. 7-(4-methoxybenz[d]thiazol-2-ylamino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (8d)

1H NMR (400 MHz, acetone-d6, 25 °C), δ (ppm): 3.92 (s, 3H, OCH3), 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); 13C NMR (100.56 MHz, acetone-d6, 25 °C), δ (ppm): 56.6 (OCH3), 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 C14H14N5O4S: C 41.59, H 1.99, N 20.79; found: C 41.73, H 2.01, N 20.78; EI-MS (ES+): m/z = 403 [M−H]+.

4.4.5. 6,6-Dinitro-7-((5-nitrobenzo[d]thiazol-2-yl)amino)benzo[c][1,2,5]oxadiazole 1-oxide (8e)

1H NMR (400 MHz, acetone-d6, 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-7 benzothiazolyl), 7.85 (d, J = 8.75 Hz, 1H, H-7 benzothiazolyl), 8.95 (s, 1H, H-7); 13C NMR (100.56 MHz, acetone-d6, 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 C14H14N5O4S: C 37.24, H 1.20, N 23.38; found: C 37.27, H 1.21, N 23.36; HRMS (ESI-TOF): m/z = 417 [M−H]+.

4.4.6. 7-((3-(4,6-dinitro-1-oxobenz[d]thiazol-2-yl)imidazol-2(3H)-ylidene)amino)-4,6-dinitrobenzo[c][1,2,5]oxadiazole 1-oxide (8f)

Brown oil, 1H NMR (400 MHz, CDCl3, 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, benzenesulfonyl); 13C NMR (100.56 MHz, CD2CN, 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 C14H12N4O5S: C 38.14, H 1.01, N 23.41; found: C 38.12, H 1.00, N 23.38; EI-MS (ES+): m/z = 599 [M−H]+, 621 [M+Na]+.
9.00 (s, 1H, benzo fur oxa nyl), 9.25 (s, 1H, benzof uryl), 7.67 (d, J = 8.8 Hz, 1H, H-4 benzothiazolyl), 7.52 (dd, J = 2.5 Hz, 1H, H-7 benzothiazolyl), 7.42 (d, J = 8.8 Hz, 1H, H-4 benzothiazolyl), 7.26-7.33 (m, 2H, H-4 benzothiazolyl), 7.11 (dd, J = 2.5 Hz, 1H, H-7 benzothiazolyl), 7.02 (s, 1H, benzo fur oxa nyl), 9.02 (s, 1H, benzo fur oxa nyl), 9.25 (s, 1H, benzo fur oxa nyl); 13C NMR (100.56 MHz, acetone- d6, δ (ppm): 77.2-77.4 (m, 1H, H-7 benzothia zolyl), 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 C14H10N2O3S: C 36.06, H 0.68, N 22.13; found: C 36.20, H 0.80, N 22.09; ESI-MS (ESI⁺): m/z = 615 [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 mL (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; 1H NMR (600 MHz, acetone-d6, 25 °C): δ (ppm): 1.39 (t, J = 6.8 Hz, 3H, CH3CH2), 3.92 (s, 3H, NCH3), 4.13 (q, J = 6.8 Hz, 2H, CH2CH3), 7.21 (dd, J = 8.9 Hz, J = 2.5 Hz, 1H, H-7 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); 13C NMR (150.80 MHz, acetone-d6, 25 °C): δ (ppm): 14.9 (CH3), 33.0 (NCH3), 65.0 (CH2), 108.6 (CH), 113.3, 115.0 (CH), 170.0 (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 benzo fur oxa nyl 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 microsomes 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 (pSosX-lux) – Dioxynine (2,3-bis[(hydroxymethyl]quinoline-1,4-di-Oxido), 2.25 × 10⁻⁵ M; or paraquat, 1 × 10⁻⁵ M; E. coli MG1655 (pKatG-lux) – hydrogen peroxide, 1 × 10⁻³ M; E. coli MG1655 (pCoID-lux) – N-methyl-N′-nitro-N′-nitrosoguanidine (MNNG), 1 × 10⁻³ M; E. coli MG1655 (pGrpE-lux) – ethanol, 3.5% (V/V); E. coli MG1655 (pIpBA-lux) – ethanol, 3.5% (V/V); E. coli MG1655 (pVRK1-lux) – N-(3-Oxohexanoyl)-l-homoserine lactone, 1 × 10⁻² M.

Plates with lux-biosensor cells were placed in a microplate luminometer LM-0117 (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 in 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.

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The quantitative assessment of the test reaction parameter is shown by the expression of operons the induction factor (Ie) was calculated according to the Formula (1):

\[ I_e = \frac{L_e}{L_k} \]  

where: \( L_e \) is the intensity of the control sample luminescence and \( L_k \) 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 shown by the expression of operons the induction factor (Ie) was calculated according to the Formula (2):

\[ T = \frac{100(L_e - L_c)}{L_c} \]  

where \( L_e \) and \( L_c \) 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 than that of the control sample. In that case irrespective of the size of negative 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.

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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


