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The Study of the Biological Activity of Amino-Substituted Benzofuroxans

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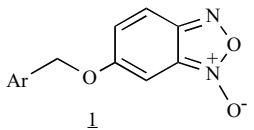
Abstract: As a part of our ongoing studies in developing new derivatives as dual antibacterial/antifungal agents, we describe the synthesis of novel amino substituted benzofuroxan derivatives. These compounds were tested for their antifungal activity against various strains. It is shown that their antimicrobial and antifungal activities depend on the structure of the amino moiety, on the position and on the nature of the substituents of the benzofuroxan ring. They displayed good bacteriostatic and fungistatic activities comparable to those of reference drugs. The more active benzofuroxan derivatives have been studied intraperitoneally in mice to get a first estimation of their toxicity. Preliminary results have also shown that some derivatives are able to inhibit enzymes such as GDH (glucose dehydrogenase).

Keywords: Bacteriostatic activity, Fungistatic activity, Intraperitoneal introduction, Amine substitution, Benzofuroxan.

INTRODUCTION



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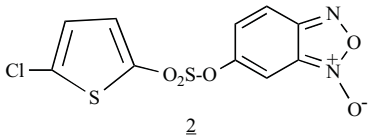

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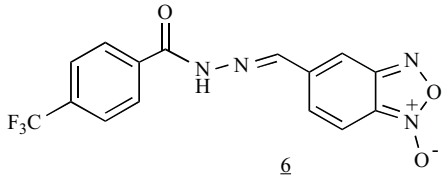
Many reviews dealing with the chemistry, the reactivity and the biological activity of benzofuroxans have been published in the past 40 years [1-3]. In 1981, Ghosh *et al.* [4] were the first to publish a review in which the biochemical activity of benzofuroxans was extensively studied. More generally, benzofuroxan derivatives exhibit a broad spectrum of biological activities including nitric oxide-releasing abilities, antibacterial, antifungal, antileukemic, acaricide and immunodepressive properties. Recently, Cerecetto [5, 6], and Goumont [7] have published two reviews reporting the biological and pharmaceutical properties of furoxans and benzofuroxans. Even if the first members of benzofuroxans have been synthesized at the end of the 19th century, papers in this major field of heterocyclic chemistry are still being published in 2013. Sartini *et al.* [8] reported the effective affect of benzofuroxan derivatives 1 against diabetic complications. These compounds act as an aldose reductase inhibitor, the enzyme which plays a key role in the pathogenesis of diabetic complications. Among twelve candidates, compound 2 was found to inhibit this enzyme through a screening campaign [9].

These anti-oxidant properties have also been recently confirmed by Chugunova [10]. Heterocyclic compounds 3-5 have been prepared on the basis of the interaction of 4,6-dichloro-5-nitrobenzofuroxan with aromatic amines and diamines (Scheme **1**). Their ability to suppress and prevent genotoxic effects of UV-radiation has been studied. Interestingly, novel benzofuroxan derivatives have been employed as drugs against multidrug-resistant *Staphylococcus aureus* strains. It has been shown that the more active compounds are benzylhydrazides 6-7 substituted by electron-withdrawing groups as CF_3 or nitro, pointing out that the antimicrobial activity was influenced by the hydrophobic and the electron-withdrawing properties of the substituents of the aromatic ring [11, 12]. It has to be noticed that if unsubstituted benzofuroxan 8 possesses moderate biological activity, the modification of the nature and of the position of the substituent of the carbocyclic ring of the benzofuroxan moiety allows a suitable design of new targets leading to appropriate biological properties [13].



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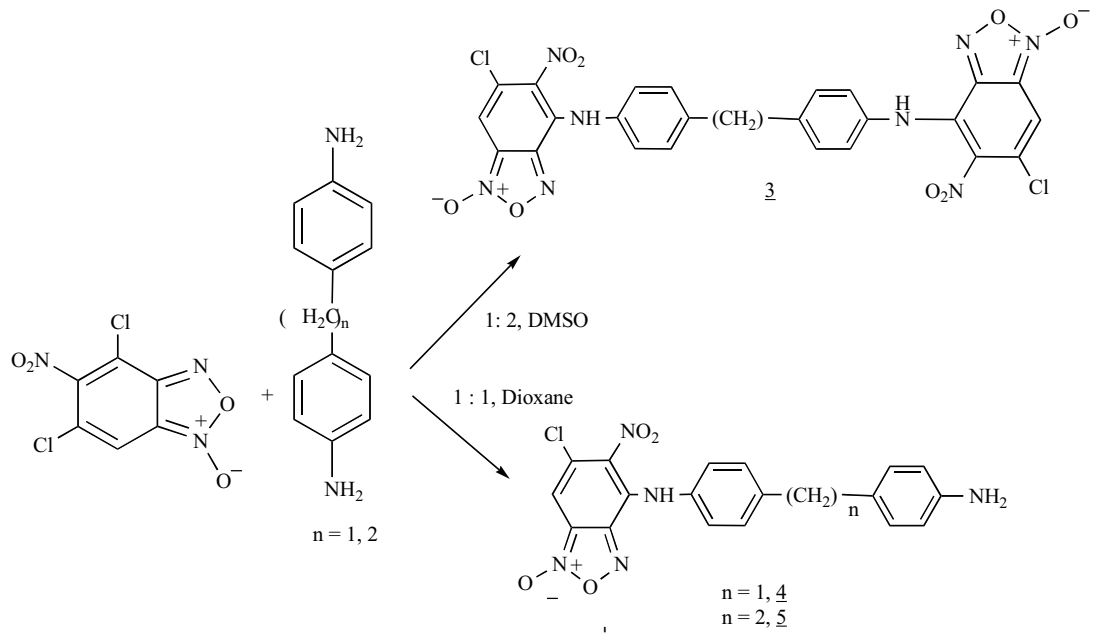
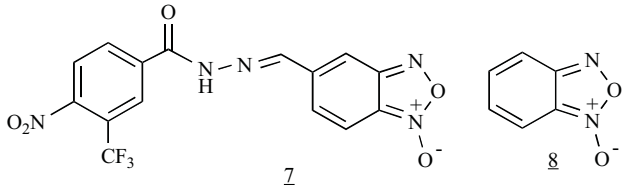
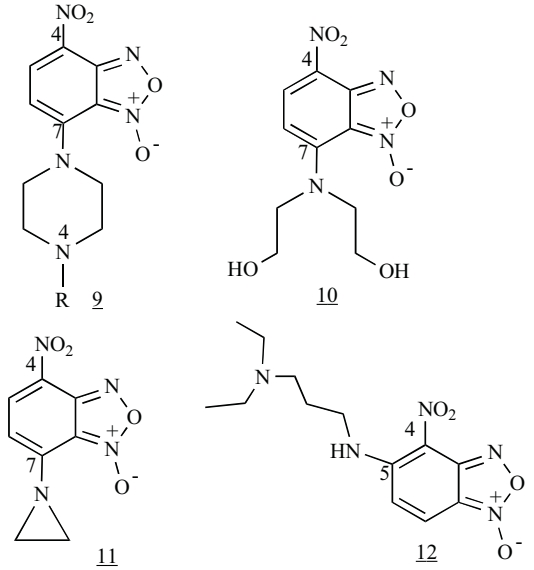
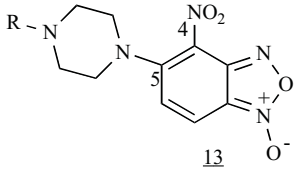


Scheme (1). Formation of benzofuroxans 3-5.



Amino-substituted 4-nitrobenzofuroxans are known to display a wide range of biological activities [14-16]. Compounds of type 9 sharing a piperazinyl residue with substitution at the N4 position have been shown to have a significant antileukemic activity in mice. This activity is also displayed by two further benzofuroxans in which the piperazinyl residue is replaced by 7-di(hydroxyethyl)amino 10 and aziridino 11 moieties, respectively. Similarly, benzofuroxan derivatives 12 and 13, bearing at position 5 a substituted amine moiety, have been tested for mutagenicity [17].





In order to overcome drug toxicity and drug resistance of some existing benzofuroxan derivatives and to obtain new lead structures, possessing antibacterial and/or antifungal activities, the synthesis of a new series of amino-substituted benzofuroxan derivatives has been investigated. Recently, a review pointed out that the development of new antifungal drugs is constantly required in clinical therapy, bringing some consistency to this study [18]. In the first part, this work will describe the synthetic pathways leading to new amino substituted benzofuroxans. Interestingly, the synthesis of 4-chlorobenzodifuroxan has been performed for the first time. In the second part, the biological activity, displayed by these derivatives, within *in vitro* investigation against several pathogenic representative Gram-negative bacteria (*Pseudomonas aeruginosa*9027 and *Escherichia coli* F-50), Gram-positive bacteria (*Staphylococcus aureus* 209p, *Bacillus cereus* 8035), moulds (*Aspergillusniger* BKMF-1119, *Trichophytonmentagrophytes var. gypseum* 1773) and yeast (*Candida Albicans* 885-653) will be reported.

MATERIALS AND METHODS

Chemistry

General Comments

Melting points were determined on a melting point apparatus and are uncorrected. The ^{1}H NMR spectra were recorded on BRUKER AVANCE-600 NMR spectrometer (600 MHz) using tetramethylsilane as an internal standard and the chemical shifts are reported in δ units. The IR spectra were

recorded on a Bruker Vector-22 Fourier spectrometer in the range 400-4000 cm^{-1}. Crystalline samples were studied as emulsions in vaseline oil. Elemental analyses were performed on a Carlo Erba Model EA-1108 elemental analyzer with an accuracy ±0.4% for C, H, Cl and N. Most chemicals were purchased from commercial sources. Solvents were of analytical grade and were used without further purification unless otherwise stated.

The X-ray structural analyses of 24d, 24f and 27 were carried out on automatic diffractometer "Bruker Smart APEX II CCD": graphite monochromator; λMoKa = 0.71073 Å; ω-scanning; temperature 73 K. The semiempirical record of absorption by means of program SADABS was carried out [19]. Structures are deciphered by the direct method with SIR program [20] and are treated at first in isotropic, then in anisotropic approach using SHELXL-97 program [21]. All calculations are made by means of WinGX [22] and APEX2 programs [23]. The analysis of intermolecular contacts in crystals, figures of molecules and crystal packing are created with PLATON program.

General Procedure for the Synthesis of Compounds

The benzofuroxan derivatives 17, 18, 20-22, 24 were prepared according to the published procedures [24-31].

Synthesis of 4-chlorobenzodifuroxan 27: To a solution of 4,6-dichloro-5-nitrobenzofuroxan 17 (0.250 g, 0.001 mole) in 5 ml acetone at room temperature was added a solution of sodium azide (0.062 g, 0.001 mole) was added in 1 ml of water. The reaction mixture was stirred for 1h (the reaction was monitored by thin layer chromatography). After completion of the reaction, the solvent was removed under reduced pressure, washed with cold water and dried in vacuum (0.06 mm Hg) at 40°C temperature to constant weight. Yield: 0.18 g (70 %); Mp = 60 ºC; IR (KBr, cm^{-1}): 2127 (N_3), 1614 (furoxan rings), 1559 (NO_2). ^{1}H NMR (acetone-d_6, 600 MHz): 7.74 (s, 1H). Anal. calcd. for $C_6HClN_6O_4$: C, 28.09; H, 0.39; Cl, 13.82; N, 32.76. Found: C, 28.07; H, 0.41; Cl, 13.84; N, 32.73.

This azide intermediate 26 was heated under reflux for 4h in 3 ml of acetic acid. After evaporation of the solvent, a brown solid (0.10 g) was obtained in a 65% yield. Mp = 83 ºC; IR (KBr, cm^{-1}): 1666, 1634 (furoxan rings). ^{1}H NMR (acetone-d_6, 600 MHz): 7.79 (s, 1H). Anal. calcd. for $C_6HClN_4O_4$: C, 31.53; H, 0.44; Cl, 15.51; N, 24.51. Found: C, 31.51; H, 0.47; Cl, 15.49; N, 24.52.

Study of the Biological Evaluation and of the Toxicity of Benzofuroxan Compounds

Antibacterial and Antifungal Activity

The *in vitro* antibacterial and antifungal activities of the benzofuroxan derivatives were investigated against several pathogenic representative Gram-negative bacteria (*Pseudomonas aeruginosa 9027, Escherichia coli F-50*), Gram-positive bacteria (*Staphylococcus aureus 209p, Bacillus cereus 8035*), moulds (*Aspergillus niger BKMF-1119, Trichophyton mentagrophytes var. gypseum 1773*) and yeast (*Candida Albicans 885-653*). Minimal inhibitory concentrations (MICs) were estimated by conventional dilution methods for bacteria and fungi [32, 33]. The antibacterial and antifungal assays were performed in nutrient broth (bacteria 3×10^5 cfu/ml) and Sabouraud dextrose broth (fungi 2×10^3-2×10^4 cfu/ml). Chloramphenicol, Ketoconazole and Amphotericin B were used as standard drugs. Positive growth control and standard drug controls were also run simultaneously. In the case of solutions of investigated compounds in DMSO the solvent was used as a negative control. The MICs were defined as the lowest concentrations that exhibited no growth and were recorded by visual observation, every 24 h during 5 days for bacteria and after incubation during 14 days for fungi. Assay tubes were filled with 1 ml of test compound solution in nutrient agar, with concentrations in a range from 5 to 500 μg/ml. Then, 1 ml of normal saline broth (bacteria 3 $\times10^5$ cfu/ml), was added to the tubes. After 4h, the inoculum was transferred from tubes onto Petri plates containing meal-peptone agar. Petri plates were incubated at 37 °C and minimum bactericidal concentration (MBC) recorded as the test compound dilution affecting the total cell death. For fungicidal activity determination, the tubes with the test compounds and fungi were incubated at 25 °C. After 6h, the inoculum was prepared in Sabouraud dextrose broth and incubated at 25 °C. Two replicas were done for each compound and the experiment was repeated twice.

Enzyme Inhibition Determination

Measurement of the dehydrogenase activity [34-35]: 0.5 ml of *Staphylococcus aureus* or *Candida Albicans* suspension and 0.5 ml of the solution of the benzofuroxan at the desired concentration were introduced into the Thunberg tube. Instead of the compound, a 0.5 ml of saline solution was poured into the control tube. The Thunberg tubes were incubated at 37.5ºC for 2 h, and then, the following reagents were introduced into each tube: 0.8 ml of phosphate buffer, pH 7.4, and 0.5 ml of 1% glucose solution. Methylene blue solutions (0.008%-0.0001%, 0.2 ml) were placed in the stopper of the Thunberg tubes, followed by a vacuum degassing of 2 minutes at about 1 Torr. Later, contents were placed into incubator for an equilibrium period of 2 minutes. Then methylene blue and the buffers were mixed, and this was considered as the beginning of the reaction. The decolorization time of the resulting mixtures was determined visually, and the control time was compared with one of the studied compounds. The mixtures in the Thunberg tubes were observed for 4 h in a water incubator. If no decolorization was observed, the tube was placed into an air incubator at 37ºC. If the color was retained for one day, this meant a 100% inhibition of GDH. The GDH activity was expressed as the ratio of the decolorization time of the control tube/decolorization time of the tube with the compound ×100%, and consequently the inhibition of the GDH activity was expressed as 100% minus the calculated GDH activity.

Measurement of the the lipase activity [36-37], a mixture of 1.5 ml of *Staphylococcus aureus* or *Candida Albicans* suspension with 1.5 ml of solution of the benzofuroxan at the desired concentration was added to the assay substrate containing 1.3 ml of olive oil, 2 ml of Twin-80 and 1ml of phosphate buffer, pH 7.4. The resulting mixture was shaken and incubated at 37°C for 24 h. To stop the reaction, 15 ml ethanol was added to the reaction mixture. The liberated fatty acids were titrated with 0.1mol/L NaOH. In the control ex-

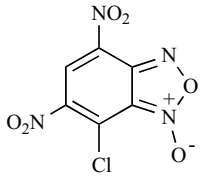
periment the procedure was the same except that 1.5 ml of saline was added instead of the compound solution. The absence of liberated fatty acids in the broth meant complete (100%) inhibition of lipase. Extracellular lipase activity was expressed as the difference between the volume of NaOH (in ml) in the control tube and the volume of NaOH (in ml) in the experiment in the presence of the benzofuroxan multiplicated by 0.31. This value is the ratio of the volume of the resulting mixture (22.3 ml) and the product of incubation time (24 h) with the volume of the mixture of microbial culture and the investigated compound (3 ml). The inhibition of the lipase activity was expressed as 100% minus the calculated lipase activity.

Toxicity of Subsituted Benzofuroxans

Toxicity tests were carried out through intraperitoneal introduction of benzofuroxan derivatives aqueous solutions with the addition of 0.2 % of the twin-80 in acute tests on white outbreed mice of both sexes, the weight of these mice being in the range 16-20 g. The observation period was 72 hours. Average lethal doses were used – LD50- as the criteria of toxicity. To measure these values each compound was introduced to 5 groups of mice (10 mice per dose; n=50). Gramicidin S, amphotericin B and ketoconazole were used as standard drugs. The results were processed using the program ToxCalc™v.5.0.23E (Tidepool Scientific Software; USA) [38].

RESULTS AND DISCUSSION

Chemistry



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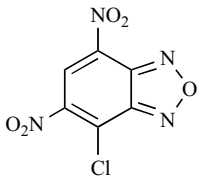

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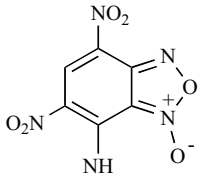

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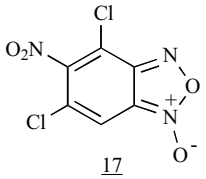

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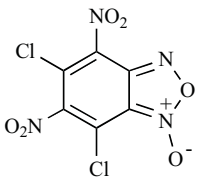

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As it was mentioned before, many amino-substituted benzofuroxans displayed biological activity. To develop the synthesis of benzofuroxans bearing amine moieties is very challenging. It has been shown that 7-chloro-4,6-dinitrobenzofuroxan (DNBF-Cl) and – benzofurazan (DNBZ-Cl) are very prone to react with a variety of weak or very weak nucleophiles as water, alcohols or amines. The great reactivity of DNBF-Cl 14 and DNBZ-Cl 15 is of interest towards a variety of weak amine nucleophiles [39-40]. This can be emphasized by the reaction of DNBF-Cl with the poorly basic 2,4,6-trinitroaniline which takes place smoothly in methanol to give 7-(2,4,6-trinitrophenylamino)-4,6-dinitrobenzofur-oxan 16, a compound exhibiting interesting thermal and explosive properties [41, 42]. In these instances, the good leaving group ability of the chloride anion could be explained by two major factors: the first one is 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 and the second one is the low aromaticity of the neutral heteroaromatic 10π-systems.

These results prompted us to use structurally similar chloro nitro benzofuroxan derivatives, namely 4,6-dichloro-5-nitrobenzofuroxan 17, 5,7-dichloro-4,6-dinitrobenzofuroxan 18 in the reaction with amines. Compounds 17 and 18 were easily obtained in three steps from 2,6-dichloro-4-nitronitrosobenzene [24] and 1,5-dinitro-2,4,6-trichlorobenzene [25], respectively, in moderate to good yields. Several synthetic pathways have been used to synthesize the derivatives reported in Table **1**. The synthesis of coumpounds depicted in Scheme **2** has been previously discussed and reported in literature [25-27].

Interestingly, within the reaction of equimolar amounts of benzofuroxan 17 and piperazine, compound 22 is isolated in a 70% yield. The formation of 22 could be accounted for in terms of reaction of both nitrogen atom of the piperazine with derivative 17, leading to the disubstituted compound 22**,** whose structure has been confirmed by an X-ray analysis (Scheme **3**) [28].

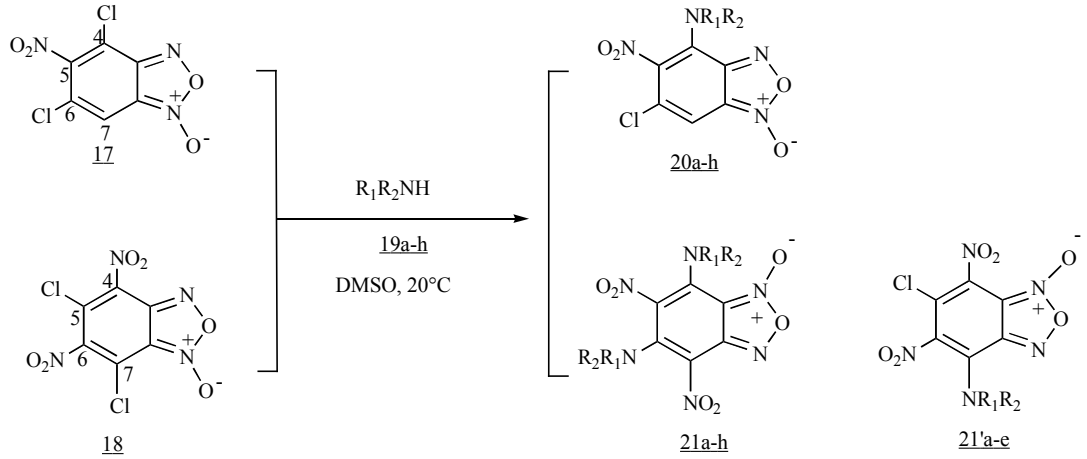
Compounds 24a-g have been obtained upon the interaction between amino acids and nitrate of aminoalcohols 23a-g with nitrobenzofuroxan 17. The reactions have been performed in boiling methanol in the presence of sodium bicarbonate to neutralize hydrogen chloride (Scheme **4**) [29-31]. The use of DMSO instead of methanol and the use of tertiary amines, such as triethylamine or pyridine instead of sodium bicarbonate, are going along with a large decrease in the yield of the target compounds. This could be explained by the fact that tertiary amines are able to substitute chlorine atom, through S_NAr process, leading to unstable positively charged compounds.

The X-ray structures reveal that the benzofuroxan ring, which is made of the 6 membered carbocyclic ring and of the 5 membered furoxan ring, is planar, the chlorine atom being located in the plane of this fragment. Nitro group is located out of this plane with torsion angles greater than 30 degrees, being far from the hydrocarbon moiety. During the study of the molecular interactions, in the crystal pertaining to compound 24f, classical hydrogen bonds have not been observed, but intermolecular hydrogen bonds of C-H ... O and C-H ...

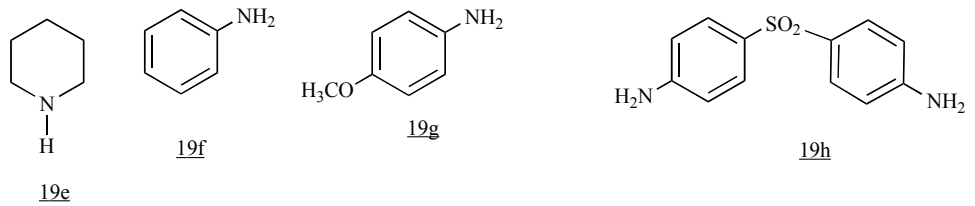
Table 1. ***In Vitro*** **Bacteriostatic and Fungistatic Activity of Benzofuroxan Derivatives[a].**

Compound	Minimal Inhibitory Concentration (MICs) µg/ml				
	Sa	Bc	An	Tm	Ca
17	3.1	15.6	125	3.9	0.78
18	3.1	7.8	500	31.25	3.1
20c	250	>500	>500	500	62.5
21'c	500	>500	>500	250	31.3
20d	31.3	250	>500	62.5	7.8
20e	31.3	250	>500	31.3	3.9
20g	15.6	125	125	15.6	1.9
20h	15.6	>500	>500	>500	>500
24a	15.6	62.5	>500	62.5	7.8
24d	31.25	125	>500	125	15.6
24f	15.6	62.5	>500	62.5	7.8
Chloramphenicol	62.5	62.5	-	-	-
Ketoconazole	-	-	-	3.9	3.9

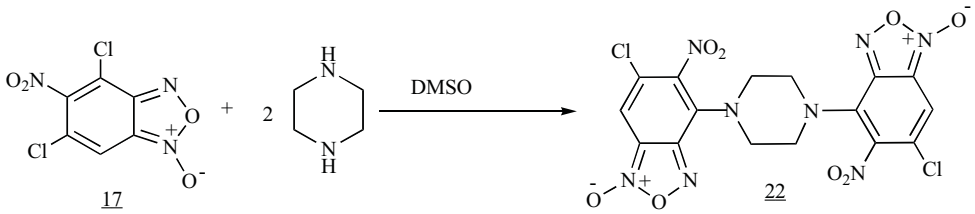
[a]The tests were performed in duplicate; Pa, Pseudomonas aeruginosa; Bc, Bacillus cereus; An, Aspergillusniger; Tm, Trichophytonmentagrophytes; Ca, Candida albicans.



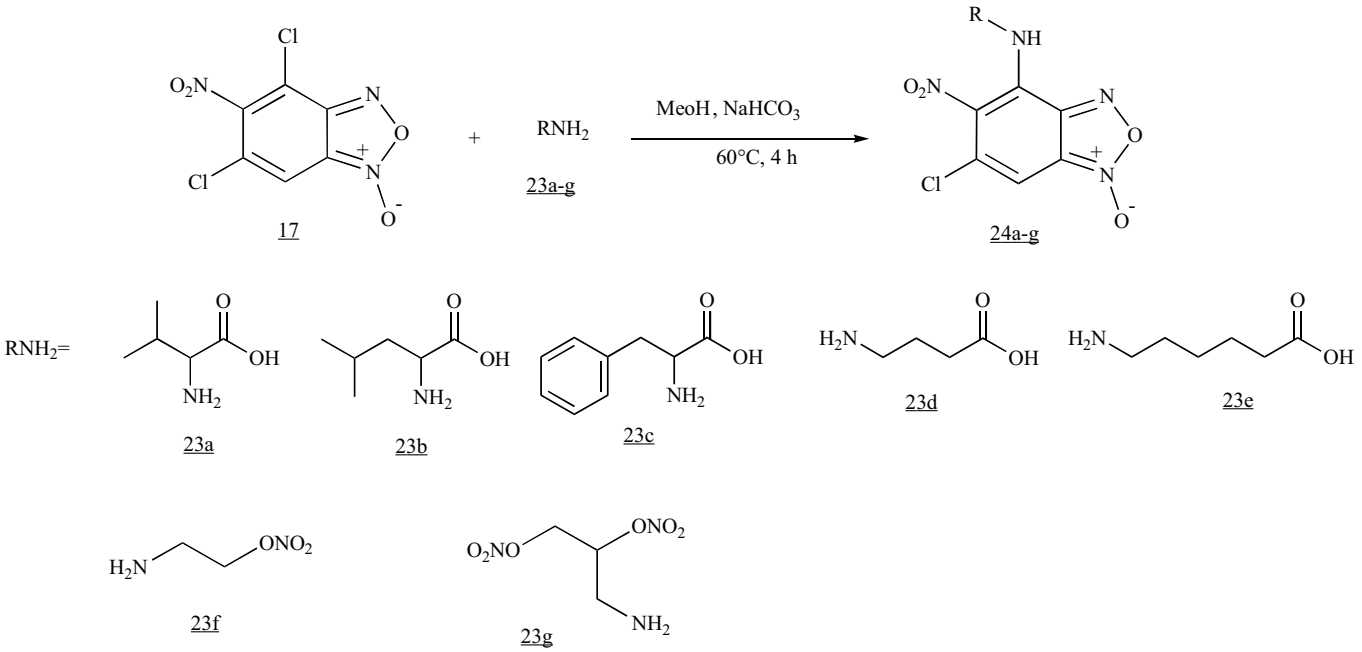
19a :CH_3-NH_2 ; 19b : $(CH_3)_2NH$; 19c: CH_3-NH-CH_2-$CH(OCH_3)_2$;19d : $(CH_3O)_2CH$-CH_2-NH_2



Scheme (2). Global scheme of the synthetic pathways leading to 20, 21a-h and 21'a-e.



Scheme (3). Formation of 22.



Scheme (4). General synthesis of 24a-g.

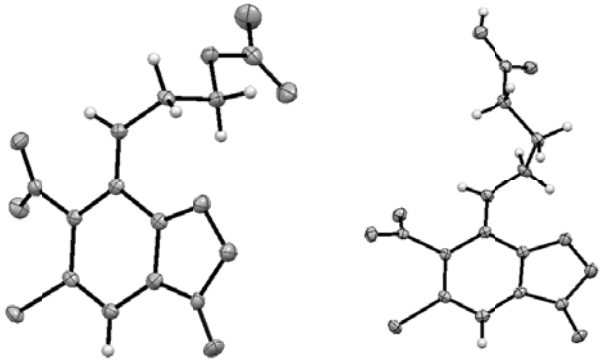
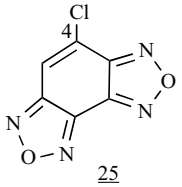


Fig. (1). ORTEP views of compounds 24d and 24f.

N-types have been found to lead to the formation of complex three-dimensional networks. In the case of 24d, the nearly planar centrosymmetric dimers in the crystal are linked by OH..O hydrogen bonds, with a distance of 2.668(3) Å.

It has been found that benzodifurazan derivatives exhibited bacteriostatic, fungistatic, and acaricide properties. Among these compounds, the benzodifurazan exhibiting the most potent biological activity is 4-chlorobenzodifurazan 25 [43]. In this context, it would also be interesting to study the fungistatic and bacteriostatic activity of substituted benzodifuroxan as 4-chlorobenzodifuroxan 27.



The 4-chlorobenzodifuroxan has been synthesized for the first time in two steps from 4,6-chloro-5-nitrobenzofuroxan 17. First, the substitution of the chlorine atom at the position 4 by sodium azide is achieved smoothly at room temperature leading to the azide compound 26. Then, the heating of 26 in acetic acid for 4 hours allows the isolation of 4-chlorobenzodifuroxan 27 in good yield (70%).

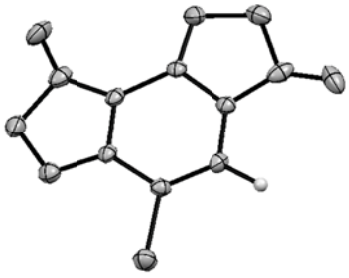
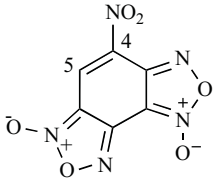
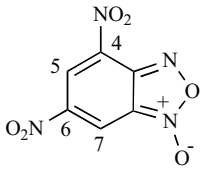


Fig. (2). ORTEP view of compound 27.

This compound has been fully characterized through ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry, and elemental analysis. Interestingly, the structure of 27 has been confirmed through a radio crystallographic study (Fig. **2**).



NBDF, 28



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This study has clearly shown that the compound 27 is planar with the chlorine atom being located in the plane of this fragment. Intermolecular interactions of C-H ... O-type form an infinite one dimensional zigzag chain along one of the crystallographic axes. Interestingly, the length of the double bond C_4-C_5 is found to be 1.350(6) Å. This bond length is reminiscent to that of NBDF 28 possessing a bond length of 1.339Å[44-46]. This is typical of a nitro-olefinic fragment and in contrast with the situation reported for DNBF 29 where values of 1.37 and 1.40 Å have been measured for the two potentially reactive nitro-activated C_6-C_7 and C_4-C_5 double bonds, respectively [47, 48].

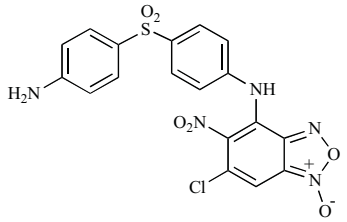
Biological Activity

Antibacterial and Antifungal Activity

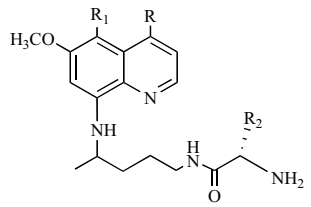
All the target compounds were evaluated for their antibacterial and antifungal activities against several pathogenic representative Gram-negative bacteria (*Pseudomonas aeruginosa*(Pa) 9027 and *Escherichia coli* (Ec) F-50), Gram-positive bacteria (*Staphylococcus aureus* (Sa) 209p, *Bacillus cereus* (Bc) 8035), moulds (*Aspergillusniger* (An) BKMF-1119, *Trichophytonmentagrophytes var. gypseum* (Tm) 1773) and yeast (*Candida Albicans* (Ca) 885-653). The Minimal Inhibitory Concentration (MIC) was determined by the broth dilution methods against the aforementioned strains and the activities of the more active compounds are collected in Table **1** along with those of reference drugs Chloramphenicol and Ketoconazole. It has to be noted that all the compounds are totally inactive against the both strains *Pseudomonas aeruginosa* and *Aspergillusniger* (the results pertaining to these two strains have been omitted in Table **1**).

The parent benzofuroxan compound 17 (MIC = 0.78 μg/mL) displays excellent fungistatic activity against *Candida Albicans* strain and is 5x more active compared to Ketoconazole (MIC = 3.9 μg/mL). Compound 17 (MIC 3.1 μg/mL) is 20x more active than the reference drug (62.5 μg/mL) against *S. aureus*. Interestingly, adding one nitro group and switching the position of the chlorine atoms in benzofuroxan 18, is going along with feeble fungistatic activity but an excellent bacteriostatic activity against gram-negative bacteria *Escherichia coli.* Surprisingly, benzofuroxan 14 displays no biological activity.

The substitution of a chlorine atom in molecules 17 and 18 by an aliphatic amine fragment leads to the loss of both activities. The class of the amine is of primary importance. If the benzofuroxan is substituted by a secondary amine, case of compounds 20d, the fungistatic activity is slightly enhanced compared to that of the benzofuroxan substituted by a tertiary amine 20c but remains lower than that of parent compounds.



20h



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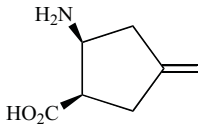
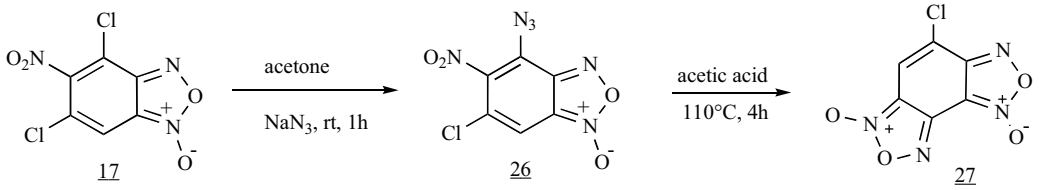


32

In the case of benzofuroxans substituted by cyclic and aromatic amines (compounds 20e-h, 22), the activity is worst with that of benzofuroxans 17 and 18. The synthesis of 20h and 21h has been performed upon the reaction of 4,6-dichloro-5-nitrobenzofuroxan 17 and 5,7-dichloro-4,6-dinitrobenzofuroxan 18 with 4-[(4'-aminophenyl)sulfonyl]-aniline, known as dapsone, respectively. Dapsone is an antibacterial most commonly used in combination with rifampicin and clofazimine as multidrug therapy for the treatment of *Mycobacterium leprae* infections. Even if both benzofuroxan derivative and dapsone have antibacterial and antifungal activities, assuming that the combination of the two partners in a single entity could lead to a highly active compound, both compounds 20h and 21h exhibit lower activity than benzofuroxan parents.

It has been largely demonstrated in literature than compounds bearing amino acids exhibit a broad range of biological activity. For example, 8-quinolinamines 31 conjugated



Scheme (5). Synthesis of chlorobenzodifuroxan 27.

with amino acids are exhibiting potent blood-schizontocidal antimalarial activities and the novel antifungal-β-amino acids 32 are displaying activity against *Candida albicans* [49-50]. As it is shown by the data summarized in Table **1**, benzofuroxans bearing amino acids (compounds 24a, 24d) exhibit medium biological activity and it appears clearly that this activity depends on the length of the carbon chain between the nitrogen atom and the carbocyclic acid moiety. The compound 20e does not display any activity (it is not presented in Table **1**). The shorter this chain is, the more active the benzofuroxan derivative (compound 24a) is. The isovaleric amino acid exhibits the best activity among all benzofuroxan derivatives containing an amino acid.

Multitarget drugs, namely, compounds capable of interacting with more than one target simultaneously could be useful tools in the therapy of complex diseases such as cancer and, AIDS. Designing drugs possessing antifungal activity together with NO-release ability have also been also envisioned. It has been reported in literature that benzofuroxan derivatives are spontaneous exogenous nitric oxide donor, i.e. they are able to release NO without interaction with enzymes or amino acids [51]. Keeping in mind, that organic alcohol nitrates can be used as NO-donor pharmacophore, compounds 24f-g have been synthesized. The NO donor ability of these compounds has not yet been determined but will be the object of another paper. Compound 24f exhibits an average bacteriostatic and fungistatic activity, which is lower than the activity of parent compound.

The substitution of a nitro group by a furoxan moiety is going along with the total loss of the biological activity displayed by compound 27. Interestingly, this could imply that the benzofuroxan derivative has to bear a nitro group to display some biological activity. More important, on going from the 4-chlorobenzodifurazan 25 to the 4-chlorobenzodifuroxan 27, the biological activity is suppressed leaving no doubt that two furazan groups are important to give bacteriostatic and fungistatic activity.

After a global survey of the biological activity of benzofuroxan derivatives, substituted by various substituents, it could be concluded that the position and the number of nitrogroup and/or chlorine atoms are very important for displaying the appropriate biological activity (compounds 16-18).

Enzyme Inhibition

It is assumed that benzofuroxan derivatives exhibit a specific mechanism of action towards gram-positive bacteria and fungi. To elucidate the antimicrobial activity of benzofuroxan compounds, a preliminary study has been performed to determine what kind of enzyme is implied in this process.

The efficacy of the inhibition of glucose dehydrogenase (GDH) and extracellular triglyceride lipase was determined from *Staphylococcus aureus* and *Candida Albicans* after treating the cells with the above compounds by decolorization time measurements of methylene blue in the absence of oxygen [40, 41] and the titrimetric method with olive oil as a substrate [42, 43], respectively. Some results involving the most active benzofuroxans 17, 18 and 20g are presented in Table **2**. As shown in Table **2**, the significant inhibition of GDH from *Staphylococcus aureus* occurs already at bacteriostatic and fungistatic concentrations (near 0.5-50 μg/mL), while the inhibition of GDH from *Candida Albicans* is observedat bactericidal and fungicidal concentrations (near 500μg/mL). The inhibition of lipase is observed at bacteriostatic and fungistatic concentrations. These data are in quite good agreement with the MICs of the compounds against *Staphylococcus aureus* and *Candida Albicans*, which are in the same range of concentrations. This preliminary study allowed to assume that the benzofuroxans exhibit some efficiency against bacteria and fungi inhibiting enzymes such as glucose dehydrogenase (GDH) and extracellular triglyceride liase.

Compounds with the more potent bacteriostatic and fungistatic efficacy were further screened to determine their bactericidal and fungicidal activity. Benzofuroxan derivatives (20d-20h) do not display bactericidal and fungicidal activity (concentration was more than 500 μg/ml). Compounds 17 and 18 exhibit an inhibitory effect at 50μg/ml against yeast *C.albicans* and *T.Mentagrophytes*. Importantly, these minimum fungicidal concentration (MFC) values are equal or lower than those reported for the antifungal drug Amphotericin B.

Toxicity

The toxicity of the more effective benzofuroxan derivatives has been determined in mice (Table **3**). According to levels of acute toxicity for mammals, the benzofuroxan derivatives can be considered as moderately toxic compounds. It should be noted that the most active compound 20g against microbes possesses the smallest toxicity in mice. Compound 20g exhibiting the highest activity with the smallest toxicity will be used in further clinical trials.

We have described the antibacterial and antifungal activity of a series of benzofuroxans substituted by different amine moieties. This screening has shown that benzofuroxans exhibit excellent activity towards gram-positive bacteria especially against *Staphylococcus aureus,* gram-negative bacteria *Escherichia coli* and fungi especially *Candida albicans* with the MIC values being comparable to those

Table 2. Inhibition of GDH and Lipase Activities.

Compound	Concentration, g/mL	Inhibition of GDH Activity, %		Inhibition of Lipase Activity,%	
		Sa	*Ca*	*Sa*	*Ca*
17	500	100	25	93	100
	50	88	0	75	92
	5	82	0	57	79
	0,5	55	0	0	48
18	500	100	35	92	100
	50	78	0	71	93
	5	71	0	48	87
	0,5	48	0	0	55
20g	500	88	13	92	92
	50	45	0	68	48
	5	15	0	0	15
	0,5	0	0	0	0

Table 3. Toxicity of Benzofuroxan Derivatives.

Compound	LD50, mice, mg/kg[a]
17	50.0 (35.2-61.0)
18	25.0 (17.9-30.7)
20a	55.7 (36.3-60.9)
20d	20.2 (15.1-26.4)
20e	90.0 (84.2-110.2)
20f	150.0 (127.7-183.8)
20g	250.0 (219.0-290.2)
20h	150.0 (126.5-173.1)
26	50.75 (34.1-59.9)
Gramicidin S	28.0 (19.5-33.8)
Ketoconazole	618
Chloramphenicol	1500

[a]Average values (in parenthesis, for each compound, are reported the range of experimental results).

pertaining to Chloramphenicol, Ketoconazole and Amphotericin B. It has been found that highly diluted benzofuroxan solutions induce the inhibition of GDH and decrease the extracellular lipase activity. The location and the number of nitro-group and/or of chlorine atom are very important for biological activity of benzofuroxan derivatives. Compound 20g, exhibiting the highest activity with the smallest toxicity, will be used in further pre-clinical trials.

CONFLICT OF INTEREST

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

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