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Design, synthesis, antimicrobial activity and molecular modeling studies of novel benzofuroxan derivatives against *Staphylococcus aureus*

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ABSTRACT

Molecular modification is a quite promising strategy in the design and development of drug analogs with better bioavailability, higher intrinsic activity and less toxicity. In the search of new leads with potential antimicrobial activity, a new series of 14 4-substituted [*N*′-(benzofuroxan-5-yl)methylene]benzohydrazides, nifuroxazide derivatives, were synthesized and tested against standard and multidrug-resistant *Staphylococcus aureus* strains. The selection of the substituent groups was based on physicochemical properties, such as hydrophobicity and electronic effect. These properties were also evaluated through the lipophilic and electrostatic potential maps, respectively, considering the compounds with better biological profile. Twelve compounds exhibited similar bacteriostatic activity against standard and multidrug-resistant strains. The most active compound was the 4-CF_3 substituted derivative, which presented a minimum inhibitory concentration (MIC) value of 14.6–13.1 μg/mL, and a *Clog P* value of 1.87. The results highlight the benzofuroxan derivatives as potential leads for designing new future antimicrobial drug candidates.

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1. Introduction

Staphylococcus aureus has been recognized as an important pathogen of humans. Infections caused by this bacteria species can lead to serious consequences, especially in hospitalized patients. The appearance of methicillin resistant strains of *S. aureus*—MRSA—has been a concern all over the world. This situation becomes worse and more threatening regarding the ease of transmission of the pathogen among individuals, resulting in the dissemination of MRSA.[1] The resistance to a great number of antibiotics presented by MRSA makes it difficult to control in hospital environments.[2] In addition to cross-resistance to all beta-lactamic antibiotics, MRSA has also acquired resistance to macrolides, aminoglycosides, tetracycline, riphampicin and quinolones, remaining the glycopeptides, such as vancomycin, as some of the few therapeutical options.[3] Nevertheless, the extensive use of glycopeptides in the past has led to the emergence of glycopeptide-resistant organisms and, consequently, there are recommendations to restrict the use of these agents in the absence of any strong indications.[4,5] The first clinical high-level vancomycin-resistant *S. aureus*—VRSA—was isolated in 1998.[6]

The emergence of serious staphylococcal infections with reduced susceptibility to vancomycin highlights the need for more antimicrobial therapeutic alternatives with increased potency or enhanced bactericidal activity against MRSA, VISA (vancomycin-intermediate *S. aureus*) and VRSA. Only two new classes have been introduced over the past few decades: the oxazolidinones and the cyclic lipopeptides.[7] Considering the antimicrobial agents more recently approved for clinical use, just daptomycin (a cyclic lipopeptide), linezolid (an oxazolidinone), and tigecycline (a glycylcycline), present activity against Gram-positive organisms, including MRSA.[8]

In view of the scenario presented above, the search for new and effective antimicrobial agents, resistant to the mechanisms of defense of these bacteria, is of paramount importance. It is well known that the discovery of new drugs having completely new chemical structures is very expensive, involves years of research, and demands the effort of multi-disciplinary teams.[9,10] Therefore, the optimization of drugs already available can be a good and financially accessible alternative and could provide better and promising antimicrobial agents.[11]

Since the introduction of nitrofurazone, 2-((5-nitro-2-furanyl)methylene)hydrazine carboxamide, in the 40s,[12] hundreds of derivative compounds of 5-nitrofuran were synthesized and evaluated for the antimicrobial activity. These compounds have demonstrated a large spectrum of action, including Gram-negative and Gram-positive bacteria and also some protozoa.[13] It is important

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to point out that some of these compounds are not therapeutically employed owing to their undesirable side effects.[14,15] Nevertheless, more recently, a great interest in nitro-heterocyclic compounds has risen again and new compounds are being studied.[16,17]

The mechanism of action of nitrofuran compounds is not fully elucidated. Previous studies suggest that the biological activity of these compounds is related to the reduction of the nitro group with formation of free radical toxic species.[18] Nifuroxazide, a 5-nitrofuran compound, presents a chemical structure that favors chemical modifications by means of molecular rational planning. Therefore, one can envision this drug as a lead molecule for the development of new analogues. In this work, 14 compounds structurally analogous of nifuroxazide, (Fig. 1A), were designed and evaluated for antimicrobial activity against *S. aureus*. The replacement of the nitrofuran system by benzofuroxan (benzo[1,2-c]1,2,5-oxadiazole N-oxide) (Fig. 1B) was based on the pharmacophore similarity attributed to the nitro group, and also the possibility of the N-oxide moiety acts as a bioreducible group.[19–22]

A molecular modeling approach was also performed to verify possible qualitative property–activity relationships, considering the lipophilicity (LP) and electrostatic potential (EP) maps of the compounds which presented better biological profile. The lipophilicity or hydrophobic property is quite important to the diffusion of the compounds through the bacterial biological system, and is associated to the respective calculated log *P* (*C*log *P*) values. Otherwise, the EP is related to the electronic density distribution and considers the contribution of substituent groups to the whole molecular electronic density. The steric atomic hindrance was also calculated to verify the substituent volume contribution and its relationship with the biological data.

2. Results

2.1. Synthesis of the nifuroxazide analogues

The designed compounds were obtained as shown in Scheme 1. The choice of substituent groups was based on the influence of their physicochemical properties such as electronic effect (σ) and hydrophobicity (π), employing the Craig diagram.[23] Substituted benzoic acids **1a**–**n**, commercially available, were converted into the respective methyl esters **2a**–**n** by esterification. Ammonolysis reaction of **2a**–**n** with hydrazine yielded **3a**–**n**. Following, Schiff's bases were obtained reacting **3a**–**n** with 5-formylbenzofuroxan, given **4a–n**.

The 5-formylbenzofuroxan **7** employed in the last step of the synthesis of the analogues was synthesized by two different routes in order to evaluate which one would be better (Scheme 2). Route A, 4-chloro-3-nitrobenzaldehyde **5** was heated with sodium azide in dimethylsulfoxide to obtain 4-azido-3-nitrobenzaldehyde **6**. Cyclocondensation of this azide in boiling toluene yielded the 5-formylbenzofuroxan.[24] In another effort, route B, the intermediate was obtained starting from 4-chloro-3-nitrobenzaldehyde in a one-pot synthesis by nucleophilic substitution of chlorine by azido group followed by in situ cyclization under solid–liquid phase transfer catalysis conditions.[25] The aldehyde **7** was synthesized from the corresponding **5** by stirring them at 60 °C with a suspension of powdered sodium azide in dichloroethane in the presence of benzyl tributylammonium bromide as a phase-transfer catalyst.

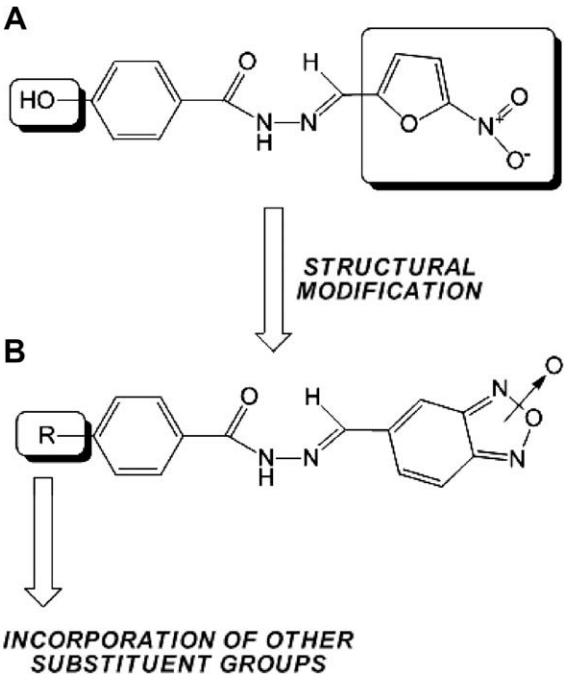


Figure 1. Chemical structures of nifuroxazide (A), pointing out the groups where chemical modifications were carried out in the present study, and of benzofuroxan derivatives (B).

2.2. Biological activity evaluation

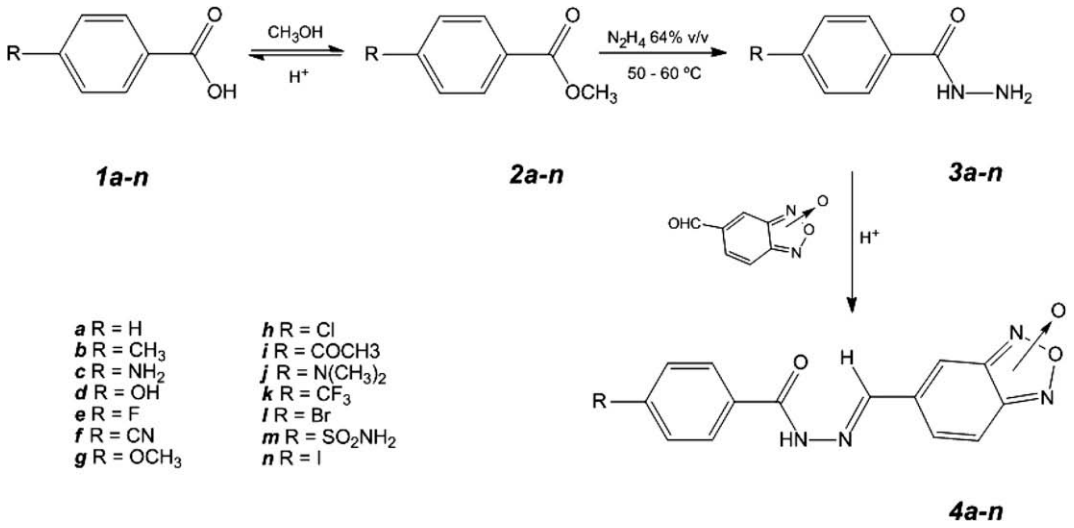
The minimal inhibitory concentration (MIC) was determined by the broth microdilution method against *S. aureus* standard strain ATCC25923 and multidrug-resistant strains 3SP/R33 and VISA3.[26] Table 1 presents the results MIC for the compounds synthesized.

3. Discussion

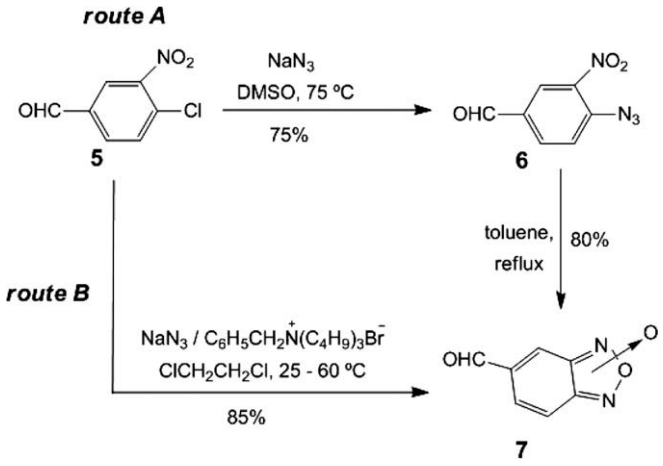
In this study, 14 new compounds were obtained and identified. All the compounds were synthesized in three steps starting from the corresponding benzoic acids, such as esterification, ammonolysis, and formation of Schiff's bases. The esterification step resulted in satisfactory yields for all the compounds, with values around 90%. However, lower yields, around 50%, were obtained for the ammonolysis step, owing to the distinct reactivity among methyl esters under the influence of the substituent groups, as well as to the difficulties associated with crystallization. The identification of methyl esters and benzhydrazides was carried out comparing the experimental melting points to the values previously described in the literature. Nevertheless, the yields obtained in these first stages were in agreement with previous results.[13,16,27] The classic method of Schiff's base formation was employed as the last step. This reaction is of ease execution, and yields around 85% were obtained. The aldehydes employed in this stage were previously synthesized as described in the literature.[24,25]

The compounds **4a**–**n** were isolated as mixture of tautomers at room temperature (Fig. 2A) that was observed through the corresponding ^{1}H NMR spectra which showed broad signals in the characteristic aromatic zone (Fig. 2B). The proportion of both tautomers depends on several factors (i.e., solvent, temperature, nature and position of the substituent of the ring).[28–31] The ^{1}H NMR and ^{13}C NMR spectra performed at higher temperature (Fig. 2C and D) makes it possible to determine which one of isomers predominate. The coupling constants and chemical shifts showed the presence of one isomer only, which is the 1,5-disubstituted heterocycle.

In order to evaluate the activity against *S. aureus*, the MIC of the compounds obtained were determined employing the successive microdilution method.[26] Owing to the low solubility of the compounds in the culture media, it was necessary to use DMSO as a solvent, but not exceeding 5% of the total. Concentrations of DMSO ranged from 5.0% to 0.01% in phase I and ranged from 2.5% to 1.0% in phase II. All derivatives presented MIC values with concentra-



Scheme 1. Synthesis of benzofuroxan derivatives.



Scheme 2. Synthesis of 5-formylbenzofuroxan by two different routes.

tions of DMSO much lesser than those needed to kill the microorganism (MIC = 12.5–11.0%, Table 1). Thus, the completely inhibition of *S. aureus* growth refers only to the intrinsic bacteriostatic activity of compounds, and any possibility of synergistic effect between the compounds and DMSO was discharged.

All compounds were tested against the three strains, ATCC25923, SP3/R33 and VISA3. The SP3/R33 and VISA3 strains of *S. aureus* are resistant to 19 antimicrobial agents currently available in the market, differing only in susceptibility to vancomycin.[32,33] The SP3/R33 is susceptible to vancomycin (MIC $\leq$ 2 μg/mL), while the VISA3 strain presents intermediate resistance to vancomycin (MIC $\geq$ 8 μg/mL). The Clinical and Laboratory Standards Institute defines staphylococci requiring vancomycin concentrations lower than 4 μg/mL for growth inhibition as susceptible, those requiring 8–16 μg/mL as intermediate resistance, and those requiring concentrations over 32 μg/mL as resistant.[26] These two resistant strains used in our study were isolated from patients in hospitals of the city of São Paulo and were characterized by Pulsed Field Gel Electrophoresis (PFGE), showing the same PFGE profile as the Brazilian Endemic Clone (BEC).[32,33] The MIC results obtained are presented in Table 1. Accordingly, the most active compound was the 4-CF3 **(4k)** substituted derivative with MIC = 14.6–13.1 μg/mL, while the lowest activity was observed for 4-OH **(4d)** and 4-CN **(4f)** substituted derivatives with MIC = 29.1–26.2 μg/mL. The compounds 4-$N(CH_3)_2$ **(4j)** and 4-SO_2NH_2 **(4m)** substituted derivatives did not show any antimicrobial activity at the concentrations studied and it was not possible to increase the initial stock solution concentration of these compounds without increasing the concentration of dimethylsulfoxide used in the test.

The influence of the substituent group's physicochemical properties on the activity of the compounds was observed. More specifically, hydrophobicity was found to be directly related to the antimicrobial activity, in agreement with other studies carried out for a different series of nifuroxazide analogs.[13,16,27] This property is related to the ability of a compound to diffuse through the biological membranes and reach its site of action. The presence of hydrophobic substituents attached to a benzene moiety, such as 4-Cl **(4h)**, 4-CF_3 **(4k)**, and 4-Br **(4l)**, provided a positive influence on antimicrobial activity. The electronic effect also played a role in activity, as can be seen for the compounds having an electron donor character, such as 4-NH_2 **(4c)**, and 4-OCH_3 **(4g)**.

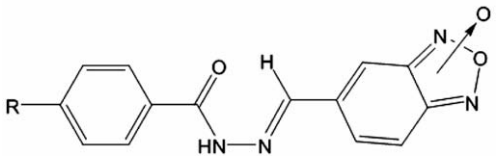
One interesting observation is that the two most active compounds, 4-CF_3 **4k** (MIC = 14.6–13.1 μg/mL) and 4-NH_2 **4c** (MIC = 17.0–15.3 μg/mL), possess completely distinct properties. While the –CF_3 substituent group presents a hydrophobic character and an electron withdrawing effect, the –NH_2 substituent group has a more hydrophilic character and an electron donor effect. The possible explanation to this fact would be based on the volume similarity of these two substituent groups. If the steric effect plays an important role in this portion of molecule, perhaps this could partially explain why the –NH_2 presents good activity despite it is hydrophilic character.

A molecular modeling approach was carried out to investigate the lipophilic, electronic, and steric hindrance properties of the two most active compounds, **4k** and **4c**. The LP and EP maps were calculated onto the molecular surfaces and are presented in Figures 3 and 4, respectively. The **4k** LP map shows a bigger brown region (hydrophobic), which is corroborated by its Clog*P* value (1.87) (see Fig. 3).

The EP maps are in agreement to the electronic effect of the –CF_3 and NH_2 substituent groups, already mentioned. The –CF_3 (**4k**) region presents higher electronic density (green/yellow color) than the –NH_2 (**4c**) (blue color) (see Fig. 4).

Considering the assumption that relates the better activity of **4k** and **4c** to the similar volume of their substituent groups, the atomic steric hindrance index was calculated. The visualization of the substituent group volumes are presented in Figure 5. The total ste-

Table 1
Minimal inhibitory concentration values of benzofuroxan derivatives and drug controls against *Staphylococcus aureus* standard strain ATCC25923 and multidrug-resistant strains 3SP/R33 and VISA3



Compound	R	ATCC25923 MIC (μg/mL)[c]		3SP/R33[a] MIC (μg/mL)[c]		VISA3[a,b] MIC (μg/mL)[c]	
		Phase I	Phase II	Phase I	Phase II	Phase I	Phase II
4a	H	20.0–10.0	18.0–16.2	20.0–10.0	18.0–16.2	20.0–10.0	18.0–16.2
4b	CH_3	40.0–20.0	23.3–21.0	40.0–20.0	23.3–21.0	40.0–20.0	23.3–21.0
4c	NH_2	20.0–10.0	17.0–15.3	20.0–10.0	17.0–15.3	20.0–10.0	17.0–15.3
4d	OH	40.0–20.0	29.1–26.2	40.0–20.0	29.1–26.2	40.0–20.0	29.1–26.2
4e	F	20.0–10.0	16.2–14.6	20.0–10.0	16.2–14.6	20.0–10.0	16.2–14.6
4f	CN	40.0–20.0	29.1–26.2	40.0–20.0	29.1–26.2	40.0–20.0	29.1–26.2
4g	OCH_3	20.0–10.0	18.0–16.2	20.0–10.0	18.0–16.2	20.0–10.0	18.0–16.2
4h	Cl	20.0–10.0	17.0–15.3	20.0–10.0	17.0–15.3	20.0–10.0	17.0–15.3
4i	$COCH_3$	40.0–20.0	23.6–21.2	40.0–20.0	23.6–21.2	40.0–20.0	23.6–21.2
4j[d]	$N(CH_3)_2$	>80.0	–	>80.0	–	>80.0	–
4k	CF_3	20.0–10.0	14.6–13.1	20.0–10.0	14.6–13.1	20.0–10.0	14.6–13.1
4l	BR	20.0–10.0	20.0–18.0	20.0–10.0	20.0–18.0	20.0–10.0	20.0–18.0
4m[d]	SO_2NH_2	>80.0	–	>80.0	–	>80.0	–
4n	I	40.0–20.0	26.2–23.6	40.0–20.0	26.2–23.6	40.0–20.0	26.2–23.6
Ampicilin		0.2–0.1		32.0–16.0		32.0–16.0	
Chloramphenicol		4.0–2.0		64.0–32.0		64.0–32.0	
Vancomycin		1.0–0.5		1.0–0.5		8.0–4.0	
DMSO (%)		12.5–11.0		12.5–11.0		12.5–11.0	

[a] Resistant to amoxicillin/clavulanic acid, ampicillin, cephazoline, cephotaxime, cephalotine, ciprofloxacin, clindamycin, erythromycin, gentamicin, imipenem, nitrofurantoin, norfloxacin, oxacillin, penicillin, rifampicin, trimethoprim/sulfametoxazole.
[b] Vancomycin-intermediate *Staphylococcus aureus* strain.
[c] Values corresponding to the average of 3 repetitions.

ric hindrance index found for $-CF_3$ and $-NH_2$ groups are 2.97 and 2.59, respectively, suggesting indeed some proximity of their volumes.

In order to better understand the influence of physicochemical properties on the activity of this new class of compounds, we are currently working on the synthesis of new analogues. The extension of the series will allow us, in a near future, to establish quantitative structure activity–relationships for this class and, therefore, help on the search of new analogues.

The MIC values against the multidrug-resistant strains were similar to those observed against the ATCC standard strain. Therefore, we can suggest that the action mode of these compounds against the three strains tested is quite the same, and the multidrug-resistant strains did not develop any mechanisms of resistance to the benzofuroxan derivatives synthesized in this work. Although the MIC values of the compounds against the ATCC strain were higher than the antibiotics used as control, the benzofuroxan derivatives showed remarkable activity against both multidrug resistant strains, with MIC values higher than ampicilin and cloramphenicol, but lower than vancomycin. These findings reinforce the potential of these compounds as promising leads for the development of new drugs against multidrug-resistant *S. aureus*, as well as other pathogens.

4. Conclusions

In this study, 14 novel benzofuroxan derivatives were synthesized, identified and biological assayed. All synthesis reactions presented satisfactory yields and the melting point determinations attested the high degree of purity of the compounds. The benzofuroxan derivatives presented activity against standard and multidrug-resistant strains of *S. aureus*, except 4-$N(CH_3)_2$ **(4j)** and 4-SO_2NH_2 **(4m)**. The MIC results indicated that the antimicrobial activity of the investigated compounds is influenced by the physicochemical properties of the substituent group at position 4 in the benzene ring. More specifically, there seems to be a direct relationship between activity and hydrophobic property. The results highlight these novel benzofuroxan derivatives as potential leads for designing new antimicrobial drug candidates.

5. Experimental

5.1. Chemistry

IR spectra were recorded on a Shimadzu IR—470 spectrometer, using KBr pellets. NMR spectra were recorded on a BRUKER ADPX Advanced (300 MHz) spectrometer employing DMSO-d_6 solutions with tetramethylsilane as internal standard. Melting points were determined using Micro-Química MQAPF-301 apparatus and elemental analysis was performed on a Perkin–Elmer 24013 CHN Elemental Analyzer.

5.1.1. General procedure for the preparation of methyl esters (2a–n)

Each substituted benzoic acid **1a–n** (0.04 mol) was refluxed for 4 h in 50.0 mL (1.23 mol) of anhydrous methanol and 1.0 mL (2.0 mmol) of sulfuric acid. The solvent was evaporated and the product obtained washed with cold water.

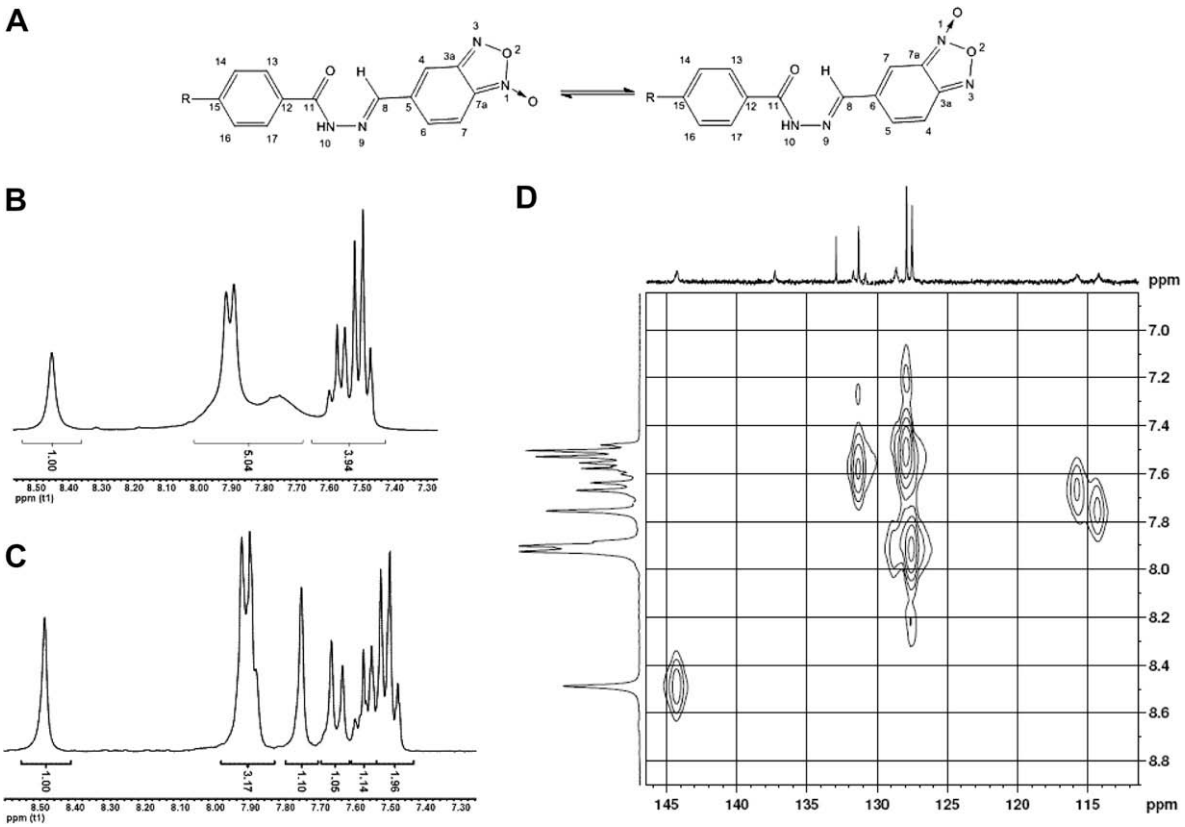


Figure 2. (A) Tautomeric equilibrium of benzofuroxan derivatives. (B) ^{1}H NMR spectra of **4a** in DMSO-d_6 at 298 K. (C) ^{1}H NMR spectra of **4a** in DMSO-d_6 at 348 K. (D) HETCOR spectra of **4a** in DMSO-d_6 at 348 K.

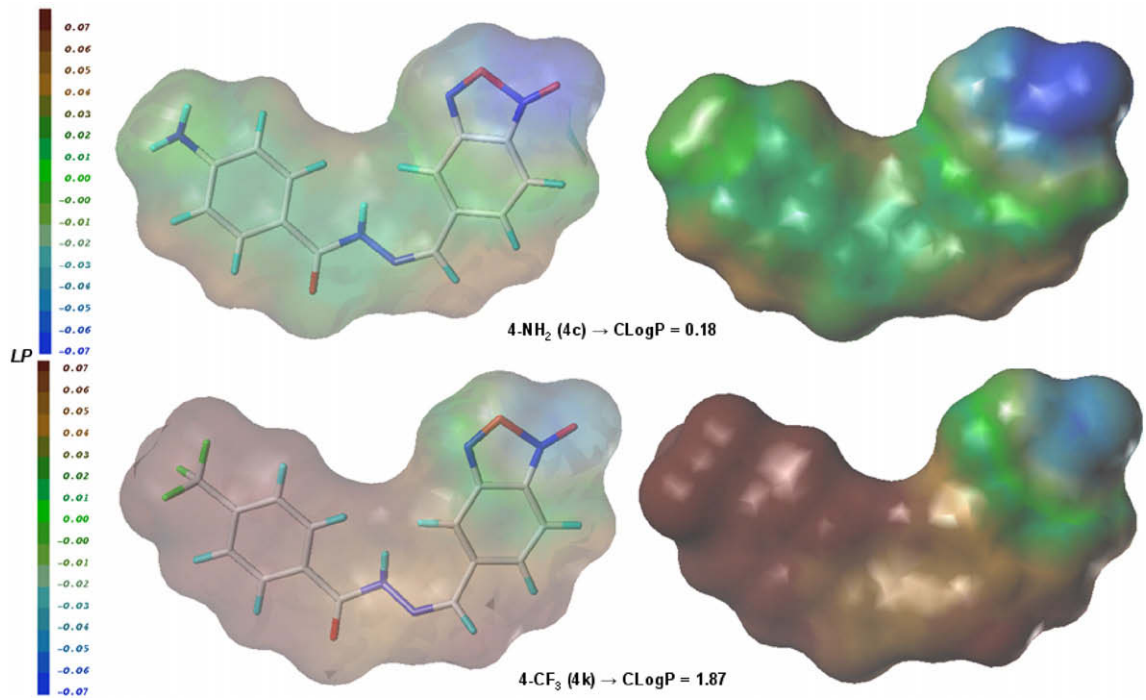


Figure 3. LP translucid and opaque colored maps of the benzofuroxan derivatives **4c** and **4k**, respectively, using SYBYL 8.0 (Tripos, Inc., 2007).[42] Brown color indicates hydrophobic regions and blue color denotes hydrophilic areas. The molecules are displayed in stick capped model (carbon atoms are in light gray color, oxygen in red, nitrogen in blue, fluorine in green, and hydrogen atoms are presented in cyan).

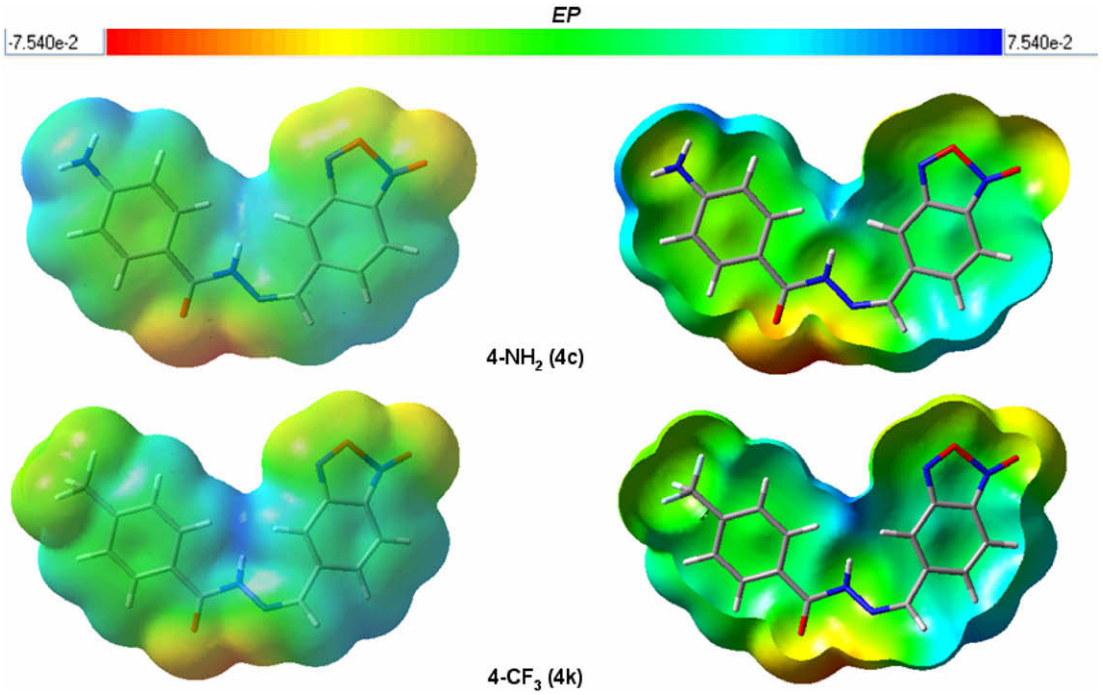


Figure 4. EP translucid and opaque zipped colored maps of the benzofuroxan derivatives **4c** and **4k**, respectively, using GAUSSVIEW 3.0 (Gaussian Inc. 1995–2003). Red color indicates negative values of EP (higher electronic density) while blue color denotes positive values (lower electronic density) in a color range from −7.54 to 7.54 e^{-2}. The molecules are displayed in stick capped model (carbon atoms are in gray color, oxygen in red, nitrogen in blue, fluorine in light blue, and hydrogen atoms are presented in white).

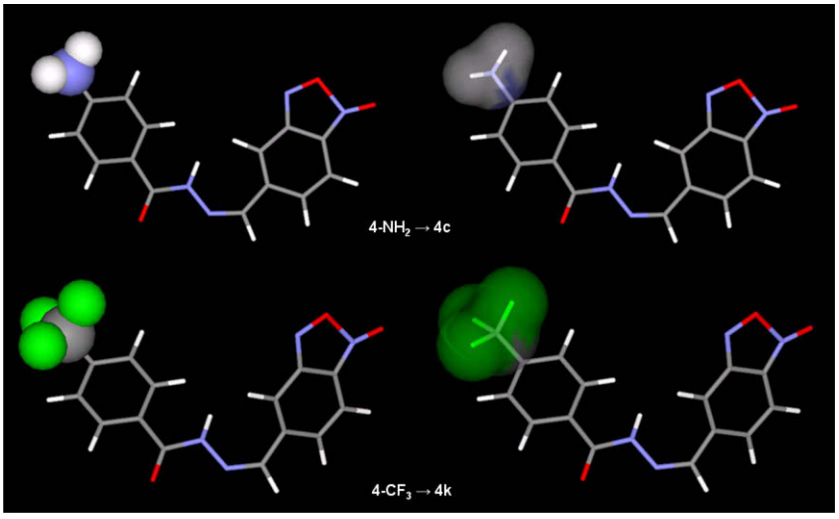


Figure 5. Visualization of the substituent groups' volumes for the benzofuroxan derivatives **4c** and **4k**, respectively (ViewerLite 5.0, Accelrys Inc.: 2002). The molecules are displayed in stick capped model (carbon atoms are in gray color, oxygen in red, nitrogen in blue, fluorine in green, and hydrogen atoms are presented in white). The substituent groups are showed as CPK models and van der Waals surfaces.

5.1.2. General procedure for the preparation of benzohydrazides (3a–n)

Hydrazine hydrate 64% (v/v) (30.0 mL, 0.33 mol) was heated up to 50–60 °C. The methyl ester **2a–n** (0.01 mol) was added and the mixture was refluxed for 10 min. The cooling was proceeded sequentially in a water bath, followed by ice bath and dry ice–ethanol bath. The solid was filtered and washed with cold water.

5.1.3. 5-Formylbenzofuroxan

Route A—a mixture of 4-chloro-3-nitrobenzaldehyde **5** (0.020 mol) and sodium azide (0.015 mol) in 30.0 mL of dimethylsulfoxide was heated at 75 °C during 30 min. The solution was cooled, poured into 100.0 mL of water, and extracted with ether. After drying with $MgSO_4$ and evaporating the solvent an yellow oil, which solidified at 0 °C, was obtained. It was crystallized from ethanol to give 4-azido-3-nitrobenzaldehyde **6**. Pale yellow plates (75%); 74.0–75.0 °C. The azide **6** (5 mmol) was refluxed for 30 min in 15 mL of toluene. The solvent was evaporated and the remaining oil was redissolved in 5.0 mL of boiling EtOAc. Petroleum ether (bp 60–80 °C) (20.0 mL) was added and the solution was cooled to give 5-formylbenzofuroxan **7** (80%). *Route B*—4-chloro-3-nitrobenzaldehyde **5** (0.01 mol) and sodium azide (0.01 mol) were added to a vigorously stirred solution of benzyltributylammonium bromide (1 mmol) in 50.0 mL of 1,2-dichloroeth-

ane at 25 °C. The mixture was slowly heated up to 60 °C and maintained at this temperature for 6 h. The reaction mixture was cooled to 28 °C and filtered. The filtrate was washed first with 100.0 mL of 1 N hydrochloric acid and subsequently with 50.0 mL of water. The organic layer was separated and dried with $MgSO_4$. The solvent was distilled and the residue was crystallized from ethanol to yield the aldehyde **7** (85%). Pale yellow needle crystal; mp 65.0–66.0 °C; IR ν_{max}: 1691, 1607, 1573, 1520; ^{1}H NMR(DMSO-d_6, 300 MHz): δ (ppm): 10.09 (s, 1H), 8.43 (s, 1H), 7.85 (d, 1H, J = 9.4 Hz), 7.78 (d, 1H, J = 9.4 Hz); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm): 191.1, 153.2, 136.8, 129.0, 122.1, 119.2, 114.8; Anal. Calcd for ($C_7H_4N_2O_3$): C, 51.27; H, 2.46; N, 17.07. Found: C, 51.57; H, 2.44; N, 16.93.

5.1.4. General procedure for the preparation of benzofuroxans derivatives (4a–n)

A mixture of 5-formylbenzofuroxan **7** (0.01 mol) and benzohydrazides **3a**–**n** (0.01 mol) in water, sulfuric acid, acetic acid and methanol (8:7:8:20 v/v) was heated under reflux for 1 h. After cooling, the mixture was poured into cold water to give **4a**–**n**.

5.1.4.1. *N′*-(Benzofuroxan-5-yl)methylene benzohydrazide (4a). Yellow solid (94%); mp 214.0–215.0 °C. ^{1}H NMR(DMSO-d_6, 300 MHz,): δ (ppm): 11.97 (s, 1H), 8.53 (s, 1H), 7.95 (d, 1H, J = 9.5 Hz), 7.94 (d, 2H, J = 6.9 Hz), 7.85 (s, 1H), 7.74 (d, 1H, J = 9.5 Hz), 7.58 (m, 3H); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm):163.8 (C11), 144.2 (C8), 137.3 (C5), 132.9 (C12), 131,7 (C7a), 131,3 (C15), 131.0 (C3a), 128.6 (C6), 127.9 (C14,C16), 127.5 (C13,C17), 115.7 (C7), 114.2 (C4); Anal. Calcd for ($C_{14}H_{10}N_4O_3$): C, 59.57; H, 3.57; N, 19.85. Found: C, 59.51; H, 3.58; N, 19.90.

5.1.4.2. 4-Methyl-[*N′*-(benzofuroxan-5-yl)methylene]benzohydrazide (4b). Yellow solid (93%); mp 217.0–218.0 °C. ^{1}H NMR(DMSO-d_6, 300 MHz,): δ (ppm): 11.87 (s, 1H), 8.51 (s, 1H), 7.93 (d, 1H, J = 9.5 Hz), 7.83 (d, 2H, J = 8.1 Hz), 7.80 (s, 1H), 7.71 (d, 1H, J = 9.5 Hz), 7.33 (d, 2H, J = 8.0 Hz), 2.39 (s, 3H); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm):164.4 (C11), 144.8 (C8), 142.3 (C15), 138.2 (C5), 131.2 (C12); 130.8 (C7a), 129.5 (C3a), 129.3 (C14,C16), 129.0 (C6), 128.4 (C13,C17), 116.5 (C7), 114.9 (C4), 21.3 (CH_3); Anal. Calcd for ($C_{15}H_{12}N_4O_3$): C, 60.81; H, 4.08; N, 18.91. Found: C, 61.01; H, 4.10; N, 18.93.

5.1.4.3. 4-Amino-[*N′*-(benzofuroxan-5-yl)methylene]benzohydrazide (4c). Yellow solid (90%); mp 226.0–227.0 °C. ^{1}H NMR(DMSO-d_6, 300 MHz,): δ (ppm): 11.59 (s, 1H), 8.48 (s, 1H), 7.96 (d, 1H, J = 9.5 Hz), 7.76 (s, 1H), 7.71 (d, 1H, J = 9.5 Hz), 7.70 (d, 2H, J = 8.5 Hz), 6.64 (d, 2H, J = 8.6 Hz), 5.61 (s, 2H); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm): 163.5 (C11), 158.9 (C15), 152.0 (C8), 142.6 (C5), 137.7 (C6), 129.3 (C13,C17), 128.8 (C7a), 128.4 (C3a), 119.2 (C12), 115.5 (C7), 113.3 (C4), 112.5 (C14,C16); Anal. Calcd for ($C_{14}H_{11}N_5O_3$): C, 56.56; H, 3.73; N, 23.56. Found: C, 56.51; H, 3.70; N, 23.43.

5.1.4.4. 4-Hydroxy-[*N′*-(benzofuroxan-5-yl)methylene]benzohydrazide (4d). Yellow solid (84%); mp 295.0–296.0 °C. ^{1}H NMR(DMSO-d_6, 300 MHz,): δ (ppm): 11.73 (s, 1H), 10.42 (s, 1H), 8.50 (s, 1H), 7.95 (d, 1H, J = 9.5 Hz), 7.84 (d, 2H, J = 8.4 Hz), 7.80 (s, 1H), 7.72 (d, 1H, J = 9.5 Hz), 6.89 (d, 2H, J = 8.4 Hz); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm):163.5 (C11), 161.4 (C15), 144.4 (C8), 136.9 (C5), 131.1 (C6), 130.4 (C13,C17), 129.9 (C7a), 127.6 (C3a), 123.8 (C12), 118.3 (C7), 115.5 (C14,C16), 113.4 (C4); Anal. Calcd for ($C_{14}H_{10}N_4O_4$): C, 56.38; H, 3.38; N, 18.78. Found: C, 55.83; H, 3.44; N, 18.31.

5.1.4.5. 4-Fluoro-[*N′*-(benzofuroxan-5-yl)methylene]benzohydrazide (4e). Orange solid (82%); mp 196.0–197.0 °C. ^{1}H NMR(DMSO-d_6, 300 MHz,): δ (ppm): 11.97 (s, 1H), 8.49 (s, 1H), 8.00 (d, 2H, J = 7.0 Hz), 7.92 (d, 1H, J = 9.4 Hz), 7.84 (s, 1H), 7.71 (d,1H, J = 9.4 Hz), 7.34 (d, 2H, J = 7.9 Hz); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm):166.4 (C15), 163.1 (C11), 145.4 (C8), 136.6 (C5), 131.05 (C13,C17), 130.9 (C12), 130.1 (C7a), 129.8 (C3a), 125.6 (C6), 115.8 (C14,C16), 113.9 (C7), 112.3 (C4); Anal. Calcd for ($C_{14}H_9FN_4O_3$): C, 56.00; H, 3.02; N, 18.66. Found: C, 55.73; H, 3.15; N, 18.19.

5.1.4.6. 4-Cyano-[*N′*-(benzofuroxan-5-yl)methylene]benzohydrazide (4f). Dark yellow solid (92%); mp 204.0–205.0 °C. ^{1}H NMR(DMSO-d_6, 300 MHz,): δ (ppm): 12.14 (s, 1H), 8.48 (s, 1H), 8.04 (d, 2H, J = 7.6 Hz), 7.96 (d, 2H, J = 8.2 Hz), 7.83 (d, 1H, J = 9.4 Hz), 7.79 (s, 1H), 7.70 (d, 1H, J = 9.4 Hz); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm): 159.8 (C11), 145.4 (C8), 136.8 (C12), 132.8 (C5), 129.6 (C14,C16), 129.3 (C7a), 129.0 (C3a), 128.6 (C13,C17), 127.7 (C6), 118.1 (CN), 117.1 (C15), 116.7 (C7), 115.2 (C4); Anal. Calcd for ($C_{15}H_9N_5O_3$): C, 58.63; H, 2.95; N, 22.79. Found: C, 58.59; H, 3.01; N, 22.62.

5.1.4.7. 4-Methoxy-[*N′*-(benzofuroxan-5-yl)methylene]benzohydrazide (4g). Yellow solid (93%); mp 211.0–212.0 °C. ^{1}H NMR(DMSO-d_6, 300 MHz,): δ (ppm): 11.81 (s, 1H), 8.48 (s, 1H), 7.92 (d, 2H, J = 8.7 Hz), 7.90 (d, 1H, J = 9.4 Hz), 7.79 (s, 1H), 7.70 (d, 1H, J = 9.4 Hz), 7.04 (d, 2H, J = 8.7 Hz), 3.84 (s, 3H); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm): 164.0 (C15), 162.7 (C11), 144.5 (C8), 138.2 (C5), 130.7 (C7a), 130.3 (C13,C17), 130.1 (C3a), 129.5 (C6), 125.8 (C12), 116.5 (C7), 114.7 (C4), 114.1 (C14,C16), 55.9 (CH_3); Anal. Calcd for ($C_{15}H_{12}N_4O_4$): C, 57.69; H, 3.87; N, 17.94. Found: C, 57.19; H, 3.88; N, 17.31.

5.1.4.8. 4-Chloro-[*N′*-(benzofuroxan-5-yl)methylene]benzohydrazide (4h). Yellow solid (91%); mp 212.0–213.0 °C. ^{1}H NMR(DMSO-d_6, 300 MHz,): δ (ppm): 11.97 (s, 1H), 8.46 (s, 1H), 7.90 (d, 2H, J = 8.6 Hz), 7.89 (d, 1H, J = 9.4 Hz), 7.81 (s, 1H), 7.68 (d, 1H, J = 9.4 Hz), 7.55 (d, 2H, J = 8.6 Hz); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm): 159.8 (C11), 145.3 (C8), 137.9 (C15), 137.2 (C5), 132.4 (C12), 130.3 (C13,C17), 130.2 (C7a), 129.4 (C3a), 128.8 (C14,C16), 127.6 (C6), 116.6 (C7), 115.3 (C4); Anal. Calcd for ($C_{14}H_9ClN_4O_3$): C, 53.09; H, 2.86; N, 17.69. Found: C, 53.20; H, 2.83; N, 17.76.

5.1.4.9. 4-Acetyl-[*N′*-(benzofuroxan-5-yl)methylene]benzohydrazide (4i). Yellow solid (87%); mp 229.0–230.0 °C. ^{1}H NMR(DMSO-d_6, 300 MHz,): δ (ppm): 12.06 (s, 1H), 8.51 (s, 1H), 8.06 (d, 2H, J = 7.9 Hz), 8.01 (d, 2H, J = 7.4 Hz), 7.84 (s, 1H), 7.77 (d, 1H, J = 9.5 Hz), 7.71 (d, 1H, J = 9.5 Hz), 2.62 (s, 3H); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm): 197.6 (C=O), 162,5 (C11), 145.5 (C8), 139.1 (C15), 136.6 (C12), 132.0 (C5), 131.1 (C7a), 129.9 (C3a), 128.2 (C14,C15), 128.1 (C13,C17), 126.0 (C6), 117.3 (C7), 114.1 (C4), 26.9 (CH_3); Anal. Calcd for ($C_{16}H_{12}N_4O_4$): C, 59.26; H, 3.73; N, 17.28. Found: C, 59.42; H, 3.74; N, 17.36.

5.1.4.10. 4-Dimethylamino-[*N′*-(benzofuroxan-5-yl)methylene]benzohydrazide (4j). Orange solid (82%); mp 214.0–215.0 °C. ^{1}H NMR(DMSO-d_6, 300 MHz,): δ (ppm): 11.64 (s, 1H), 8.48 (s, 1H), 7.93 (d, 1H, J = 9.6 Hz), 7.82 (d, 2H, J = 9.0 Hz), 7.74 (s, 1H), 7.69 (d, 1H, J = 9.6 Hz), 6.75 (d, 2H, J = 9.0 Hz), 2.99 (s, 6H); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm): 164.1 (C11), 153.2 (C15), 143.6 (C8), 138.5 (C5), 129.9 (C13,C17), 129.6 (C7a), 127.9 (C3a), 126.4 (C6), 119.8 (C12), 116.3 (C7), 114.3 (C4), 111.3 (C14,C16), 41.2 ($N(CH_3)_2$); Anal. Calcd for ($C_{16}H_{15}N_5O_3$): C, 59.07; H, 4.65; N, 21.53. Found: C, 59.25; H, 4.63; N, 21.32.

5.1.4.11. 4-Trifluoromethyl-[*N′*-(benzofuroxan-5-yl)methylene]benzohydrazide (4k). Yellow solid (82%); mp 201.0–202.0 °C. ^{1}H

NMR(DMSO-d_6, 300 MHz,): δ (ppm): 12.13 (s, 1H), 8.49 (s, 1H), 8.15 (d, 1H, J = 9.6 Hz), 8.09 (d, 2H, J = 7.7 Hz), 7.87 (d, 2H, J = 8.9 Hz), 7.86 (s, 1H), 7.71 (d, 1H, J = 9.6 Hz); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm): 159.8 (C11), 145.9 (C8), 137.8 (C12), 137.5 (C15), 135.4 (C5), 132.6 (C7a), 131.9 (C3a), 129.2 (C13,C17), 126.1 (C6), 125.7 (C14,C16), 122.5 (CF_3), 116.7 (C7), 115.5 (C4); Anal. Calcd for ($C_{15}H_9F_3N_4O_3$): C, 51.44; H, 2.59; N, 16.00. Found: C, 50.95; H, 2.44; N, 15.99.

5.1.4.12. 4-Bromo-[*N*′-(benzofuroxan-5-yl)methylene]benzohydrazide (4l). Yellow solid (90%); mp 210.0–211.0 °C. ^{1}H NMR(DMSO-d_6, 300 MHz,): δ (ppm): 11.97 (s, 1H), 8.46 (s, 1H), 7.88 (d, 1H, J = 9.1 Hz), 7.83 (d, 2H, J = 8.5 Hz), 7.81 (s, 1H), 7.70 (d, 2H, J = 8.2 Hz), 7.68 (d, 1H, J = 9.1 Hz); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm): 159.8 (C11), 145.4 (C8), 138.0 (C5), 132.9 (C12), 131.8 (C14,C16), 130.6 (C7a), 130.4 (C13,C17), 129.9 (C3a), 129.4 (C6), 126.0 (C15), 116.4 (C7), 115.4 (C4); Anal. Calcd for ($C_{14}H_9BrN_4O_3$): C, 46.56; H, 2.51; N, 15.51. Found: C, 46.61; H, 2.63; N, 15.56.

5.1.4.13. 4-Sulfamoyl-[*N*′-(benzofuroxan-5-yl)methylene]benzohydrazide (4m). Yellow solid (97%); mp 272.0–273.0 °C. ^{1}H NMR(DMSO-d_6, 300 MHz,): δ (ppm): 12.00 (s, 1H), 8.46 (s, 1H), 8.03 (d, 2H, J = 8.1 Hz), 7.94 (d, 2H, J = 8.1 Hz), 7.79 (s, 1H), 7.77 (d, 1H, J = 9.3 Hz), 7.65 (d, 1H, J = 9.3 Hz), 7.28 (s, 2H); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm): 164.2 (C11), 148.5 (C15), 147.4 (C8), 136.7 (C12), 135.5 (C5), 132.6 (C7a), 131.9 (C3a), 129.0 (C13,C17), 126.1 (C14,C16), 123.9 (C6), 116.7 (C7), 115.4 (C4); Anal. Calcd for ($C_{14}H_{11}N_5O_5S$): C, 46.54; H, 3.07; N, 19.38. Found: C, 46.45; H, 3.05; N, 19.62.

5.1.4.14. 4-Iodo-[*N*′-(benzofuroxan-5-yl)methylene]benzohydrazide (4n). Yellow solid (87%); mp 228.0–229.0 °C. ^{1}H NMR (DMSO-d_6, 300 MHz,): δ (ppm): 11.98 (s, 1H), 8.47 (s, 1H), 7.90 (d, 2H, J = 8.4 Hz), 7.88 (d, 1H, J = 9.7 Hz), 7.82 (s, 1H), 7.70 (d, 1H, J = 9.7 Hz), 7.68 (d, 2H, J = 8.2 Hz); ^{13}C NMR {H} (DMSO-d_6, 300 MHz): δ (ppm): 159.8 (C11), 145.4 (C8), 138.0 (C12), 137.8 (C14,C16), 133.2 (C5), 131.8 (C7a), 130.3 (C13,C17), 130.0 (C3a), 129.4 (C6), 116.7 (C7), 115.3 (C4), 99.5 (C15); Anal. Calcd for ($C_{14}H_9IN_4O_3$): C, 41.20; H, 2.22; N, 13.73. Found: C, 41.68; H, 2.36; N, 13.71.

5.2. Biology

Phase I—Minimal Inhibitory Concentration (MIC) of the compounds was determined with 96-well microtiter plates containing twofold serial dilutions of the compounds in Tryptic Soy Broth (TSB-Sigma®) medium.[26] Stock solutions of the compounds were prepared in DMSO/TSB 1:10 v/v. Concentrations ranged from 0.1 to 80 μg/mL, using ampicilin, chloramphenicol and vancomycin as drug controls. Bacterial suspensions were prepared by turbidity adjustment to a density of 0.5 on the McFarland scale and further dilution in sterile physiologic saline solution and TSB. The plates were incubated at 35 °C for 18 h, and the lowest concentration of compound at which there was no visible growth was considered the MIC. Readings at 24 and 48 h were carried out for sterility control. Experiments were performed in triplicate.

Phase II—this phase aimed at narrowing the MIC values that were defined in phase I. Stock solutions (1 mL) were prepared using twofold the MIC value determined in phase I. A volume of 0.1 mL of this solution was added to the column 1 of microplate. Then 0.1 mL of TSB was added to the initial stock solution diluting it up to 10%. After mixed, 0.1 mL of this new solution was added to column 2. Then 0.1 mL of TSB was added to the solution diluting it once more up to 10%. This procedure was repeated till the 11th column. For the positive growth control, 0.1 mL of TSB was added to column 12. Bacterial suspensions were prepared by the same procedure described in phase I and 0.1 mL of inoculum was transferred to each well, except for the column 11. The plates were mixed and incubated at 35 °C for 18 h.

5.3. Molecular modeling

The three-dimensional structures the benzofuroxan derivatives or ligands, which presented better biological profile (**4c** and **4k**), in their neutral forms were constructed using the HYPERCHEM 7 software.[34] The Cartesian coordinates of NX crystallized structure were retrieved from Cambridge Structural Database (CSD)[35] (entry code LEQTAC (*R*-factor 0.11)[36] and used as a geometry reference in the building up of the ligands. Each structure was energy-minimized using the following methods: MM+ force field (derived from MM2),[37] AM1 semiempirical method[38] in HYPERCHEM 7,[34] and Hartree–Fock (basis set 6–31G**) ab initio method in GAUSSIAN 03W, v.6,[39] without any restriction. Electrostatic partial atomic charges (Chelpg) were computed employing HF/6-31G** method, also implemented in the GAUSSIAN 03W program.

The structures modeled as described above were taken as the initial structures for computing the LP property onto a Connolly molecular surface,[40,41] using a sphere probe of 1.4 Å radii (SYBYL 8.0 package).[42] The LP property and the *Clog P* values were calculated employing the Ghose and co-workers method[43] and Sybyl Line Notation (SLN) (SYBYL 8.0 package).[42] Properties that are applied to a surface can be a useful analytic tool in visually identifying areas of interest on the surface. The resulting LP maps were analyzed according to the color ramps, which range from brown (highest lipophilic area of the molecule) to blue (highest hydrophilic area). The color scheme is easy to interpret if the blue is associated with water and the brown as oil/fat.

The EP property was computed in a cube surface using an isovalue of 0.0004 employing the GAUSSIAN 03W program.[39] Negative values of EP (higher electronic density) are denoted in red while positive values are mapped in blue (lower electronic density) on the molecular surfaces.

The atomic steric hindrance of the ligands **4c** and **4k** were calculated from the covalent radii values and geometrical distances, using the MARVIN VIEW program.[44] This property is additive, which means the total steric hindrance of a substituent group corresponds to the sum of each atomic steric hindrance calculated.[44]

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