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Novel benzofuroxan derivatives against multidrug-resistant *Staphylococcus aureus* strains: Design using Topliss' decision tree, synthesis and biological assay

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ABSTRACT

The aim of this study was the design of a set of benzofuroxan derivatives as antimicrobial agents exploring the physicochemical properties of the related substituents. Topliss' decision tree approach was applied to select the substituent groups. Hierarchical cluster analysis was also performed to emphasize natural clusters and patterns. The compounds were obtained using two synthetic approaches for reducing the synthetic steps as well as improving the yield. The minimal inhibitory concentration method was employed to evaluate the activity against multidrug-resistant *Staphylococcus aureus* strains. The most active compound was 4-nitro-3-(trifluoromethyl)[*N*′-(benzofuroxan-5-yl)methylene]benzhydrazide (MIC range 12.7–11.4 μg/mL), pointing out that the antimicrobial activity was indeed influenced by the hydrophobic and electron-withdrawing property of the substituent groups 3-CF_3 and 4-NO_2, respectively.

1. Introduction

Staphylococcus aureus is notorious for its ability to become resistant to antibiotics, and resistance turns it into an important hospital and communitarian pathogenic species of great concern.[1,2] Since the introduction of penicillin in the 1940s, *S. aureus* has developed resistance to all licensed antibacterial agents.[3,4] The selection of resistance is an inevitable consequence of the incorrect or inappropriate antibiotic use, and to address this challenge the search for novel antibiotics and/or new therapeutic strategies is needed. In this regard, a major scientific community mobilization has already begun for finding alternatives to treat these multidrug resistant strains.[5–9] Structure–activity relationship studies, which consider the chemical structure of a certain compound and its ability to exhibit a desirable biological effect, could assuredly contribute to the development of new promising synthetic derivatives.[10]

Topliss method is usually employed to guide the choice of substituents in the support of drug design efforts toward the identification of the most potent agents. This method was developed to maximize the chances of synthesizing the most potent compounds in the investigated set as early as possible. Furthermore, it was based upon the assumption that the biological activity depends on the hydrophobic and/or electronic effect of the substituent groups present in the aromatic ring.[11,12]

The Topliss decision tree considers the change in biological activity for suggesting the next substituent group to be explored. The introduction of a chlorine (4-Cl), for example, led to a more potent than a non-substituted compound, so a 3,4-Cl_2 disubstituted analogue should be synthesized. However, the decision of which compound between the 3,4-Cl_2 and 4-Cl analogues should be the next one in the investigation process will depend on their biological activity values. If the disubstituted compound presents the best activity, then the diagram suggests that the next most active compound would be the 4-NO_2, 3-CF_3 disubstituted derivative, considering the increment of both, the electronic (+σ) and hydrophobic (+π) effects. Otherwise, if the activity values are higher than or equal to the monosubstituted compound (4-Cl), the diagram will follow until the 4-NO_2 compound, suggesting the biological activity depends mainly upon the electronic effect. Coming back to the 4-Cl derivative, when the introduction of a chlorine led to an equipotent analogue in comparison to the parent compound, it can be interpreted as +π and −σ effect. Then, the Topliss scheme suggests the synthesis of a 4-CH_3 analogue, which presents +π and −σ values. Starting at the 4-CH_3 derivative, new branches are suggested to find the most active compounds. Finally, when the 4-Cl analogue is less active than the non-substituted compound, it might imply in the presence of steric hindrance, or the activity would be influenced by −π or −σ effect. In this case, a 4-OCH_3 substituted analogue should be explored.[11,12]

Benzofuroxan (benzofurazan oxide; benzo[1,2-c]1,2,5-oxadiazole *N*-oxide) is an interesting ring system which has attracted

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attention particularly due to its important biological activities, which are being extensively reviewed and reported.[13–15] Benzofuroxan derivatives have shown a wide spectrum of relevant biological activities, such as antiprotozoal, antifungal, platelet antiaggregatory and NO-releasing activity,[16] suggesting these compounds are quite promising for developing new potential lead drugs.

Recent studies developed by our research group have demonstrated that physicochemical properties as hydrophobicity (π) and electronic distribution (σ) can significantly influence the antibacterial activity of benzofuroxan derivatives, which are nifuroxazidés functional analogues.[9,17] The mechanism of action of nifuroxazide suggests that the antimicrobial activity of this compound is related to the reduction of the nitro group and formation of free radical toxic species.[18] Based on this assumption, the derivatives containing benzofuroxan as a pharmacophoric group would act as nifuroxazide, possibly reducing the *N*-oxide group and causing similar effects.[19–22]

So, in this study, the purpose was the identification of the most active compound against 3SP/R33 and VISA3 multidrug-resistant strains of *S. aureus* in a set of benzofuroxan derivatives designed by applying the Topliss' decision tree. The optimization of the synthetic pathway by reducing the synthetic steps and by improving the yield of the final compounds was also considered as a key point in this work.

Additionally, to explore the data related to the set of compounds, particularly regarding the physicochemical properties of the substituents chosen through the Topliss method and their respective contributions on bioactivity, a hierarchical cluster analysis (HCA) was also performed. The primary purpose of HCA is to present data in a manner which emphasizes natural groupings displaying them in the form of a dendrogram, which allows the visualization of such categories or clusters in a two-dimensional space.[23]

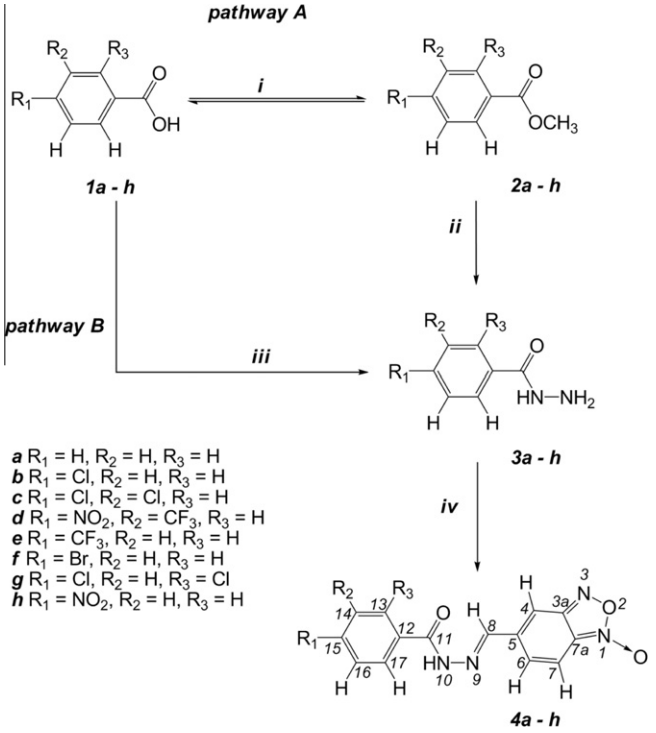
2. Methods

2.1. Synthesis

The designed compounds were obtained as shown in Scheme 1. As already mentioned, the choice of the substituent groups was based upon Topliss approach, aiming to obtain the most active compound in relationship to the initial non-substituted compound. The benzhydrazides used to obtain the final compounds were synthesized employing two synthetic approaches. In the pathway A, the substituted benzoic acids (**1a–h**) were converted into their methyl benzoates (**2a–h**) by Fischer esterification. The ammonolysis of intermediates **2a–h** with hydrazine hydrate resulted in benzhydrazides **3a–h**. Otherwise, in the pathway B, the benzhydrazides (**3a**–**h**) were obtained directly from benzoic acids in one pot synthesis, without the need of isolating their respective intermediate esters (**2a–h**). Further, benzofuroxan derivatives (**4a–h**) were obtained by reaction of the derivatives **3a–h** with 5-formylbenzofuroxan. The aldehyde used in the final stage of the synthesis of compounds **4a–h** was synthesized from 4-chloro-3-nitrobenzaldehyde as reported in reference.[24]

2.2. Hierarchical cluster analysis (HCA)

In HCA, distances between pairs of samples or variables are calculated and compared. When distances between samples are relatively small, this implies that the samples are similar, at least with respect to the measurements in hand. Dissimilar samples will be separated by relatively large distances. Thus, HCA groups data into clusters having similar attributes, which is known in biological sciences as numerical taxonomy.



Scheme 1. Synthesis of benzofuroxan derivatives. Reaction conditions: (i) CH_3OH, H_2SO_4/reflux; (ii) N_2H_4 64% aq/75 °C; (iii) CH_3OH, H_2SO_4/reflux; N_2H_4 80% aq/rt; (iv) 5-formylbenzofuroxan, H_2O, H_2SO_4, CH_3COOH, C_2H_5OH/reflux.

The data considered to perform HCA, in this study, were the Hammett (σ, electronic contribution) and Hansch (π, hydrophobic contribution) constant values of the substituents (R_1 and R_2) chosen in the Topliss approach for the eight benzofuroxan derivatives. Also, the van der Waals radii, which can express volume, were used for the R_3 substituent, since the *ortho* position in the aromatic ring is related to the steric effect. Biological data correspond to the dependent variable and their values were expressed as potency ($\log 1/C$) (see Table 1S in Supplementary data).

HCA was carried out using the Pirouette 3.11 program,[25] employing the complete linkage method and Euclidean distance. The distances between samples or variables were calculated and transformed into a similarity matrix whose elements correspond to the similarity indexes. The similarity scale ranges from zero to one, and the larger is the similarity index the smaller is the distance between any pair of samples or variables.[23] The results will be visualized as a dendrogram, which is a tree-shaped map constructed from the distances data.

Multivariate distance was computed on the independent variable block, the transformed and preprocessed (autoscaling) matrix **X**. The multivariate distance d_{ab} between two samples vectors, **a** and **b**, was determined by computing differences at each of the m variables:

$$d_{ab} = [\sum_{i}^{m}(x_{aj} - x_{bj})^{M}]^{1/M}$$

M is the order of the distance, and here corresponds to the Euclidean distance (M = 2).[25] Because inter-sample distances can vary with the type and number of measurements, it is customary to transform them onto a somewhat more standard scale of similarity:

$$\text{similarity}_{ab} = 1 - \frac{d_{ab}}{d_{max}}$$

The largest distance in the data set is d_{max}. As already mentioned, on this scale, a value of 1 is assigned to identical samples and a value of 0 to the most dissimilar samples.[25]

2.3. Biological activity evaluation

The minimum inhibitory concentration (MIC) method was used to evaluate the antibacterial activity of the synthesized compounds against 3SP/R33 and VISA3 multidrug-resistant strains of *S. aureus* and the procedure was performed in two steps. In the first step, called Phase I, the antibacterial activity was determined using the classic method of serial broth microdilution.[26] The second step, Phase II, was used for maximizing the sensitivity of the test by limitation of MIC values, which were defined in Phase I.[9]

3. Results and discussion

The Topliss operational scheme is used to provide a rational design of analogues, allowing the investigation of electronic, hydrophobic and even steric effects on bioactivity without the need of synthesizing a large number of compounds (see Fig. 1). The yield (%), melting point and MIC value ranges found for the benzofuroxan derivatives **4a**–**h** are presented in Table 1.

The initial compounds synthesized were the non-substituted (R_1 = H; π = 0.0, σ = 0.0; **4a**) and 4-Cl substituted (R_1 = Cl, $\pi_{4\text{-Cl}}$ = 0.70, $\sigma_{4\text{-Cl}}$ = 0.23; **4b**) compound, which presented MIC value ranges from 18.0 to 16.2 μg/mL (potency = 4.19) and from 17.0 to 15.3 μg/mL (potency = 4.27), respectively (Table 1). The higher potency showed by **4b** suggest that the bioactivity improvement can be related to the hydrophobic (π+) and/or electron-withdrawing character (σ+) of the substituent chlorine (R_1).

According to the Topliss diagram approach (see Fig. 1), the next compound synthesized was 3,4-dichloro disubstituted (R_1 = Cl, $\pi_{4\text{-Cl}}$ = 0.70, $\sigma_{4\text{-Cl}}$ = 0.23, R_2 = Cl, $\pi_{3\text{-Cl}}$ = 0.76, $\sigma_{3\text{-Cl}}$ = 0.37; **4c**). This

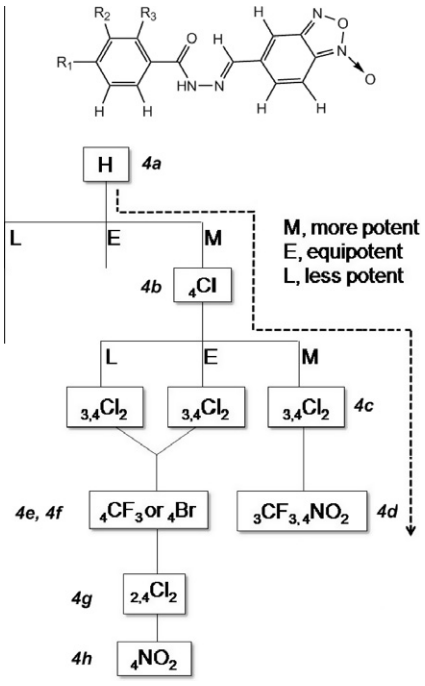


Figure 1. Sequence of the potential antibacterial benzofuroxan compounds planned from non-substituted derivative **4a** employing the Topliss method. Dashed line indicates the most active sequence in this study. [Adapted with permission from *J. Med. Chem.*, 15, Topliss, J. G. Utilization of operational schemes for analog synthesis in drug design, 1006–1011 Copyright (1972) American Chemical Society.]

MIC values varied from 13.1 to 11.8 μg/mL (potency = 4.43, Table 1), and the hydrophobic (π) and electronic (σ) contributions also increased because these physicochemical properties are additive (R_1 = R_2 = Cl).

Following the selected branch, the 4-NO_2, 3-CF_3 disubstituted compound (R_1 = NO_2, $\pi_{4\text{-NO2}}$ = 0.24, $\sigma_{4\text{-NO2}}$ = 0.78, R_2 = CF_3, $\pi_{3\text{-CF3}}$ = 1.21, $\sigma_{3\text{-CF3}}$ = 0.43, **4d**) would be even more active. Then, it was synthesized (Fig. 1). The intermediated compounds (**2d** ester and **3d** benzhydrazide) used to synthesize **4d** compound are unpublished, and their chemical structures were confirmed by ^{1}H and ^{13}C NMR spectroscopy. Centesimal analysis was employed as reference for testing the purity degree (details in Experimental Section). The **4d** compound, which showed a MIC value range from 12.7 to 11.4 μg/mL (potency = 4.49), was the most active of this series. The hydrophobic (π+) and electron-withdrawing (σ+) substituent groups have certainly presented influence on the antimicrobial effectiveness. Regarding the Hammett (σ) and Hansch (π) constant values, the 4-NO_2 substituent has a more relevant contribution on the electronic effect ($\sigma_{4\text{-NO2}}$ = 0.78) whereas the 3-CF_3 substituent is more efficient on the hydrophobic feature ($\pi_{3\text{-CF3}}$ = 1.21). Anyway, as already mentioned, these physicochemical properties have an additive character. The summation of the contributions of these substituents to the whole molecule is 1.75 and 1.21 regarding both, the hydrophobic (π) and electronic (σ) character, respectively. This balance of the hydrophobic-electronic contribution could be considered quite suitable to the antimicrobial activity.

Regarding the Topliss' decision tree, the best compound of the investigated set was already found. However, to generate more findings by exploring the Topliss diagram, observing the series behavior, and optimizing synthetic pathways, some compounds near to the right branch of the decision tree (see Fig. 1) whose activities would be σ-dependent were also synthesized.

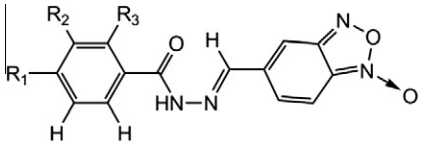
Thus, 4-CF_3 substituted (R_1 = CF_3, $\pi_{4\text{-CF3}}$ = 0.54, $\sigma_{4\text{-CF3}}$ = 1.07, **4e**) and 4-Br substituted (R_1 = Br, $\pi_{4\text{-Br}}$ = 1.19, $\sigma_{4\text{-Br}}$ = 0.23; **4f**) compounds were synthesized and presented MIC value ranges from 14.6 to 13.1 μg/mL (potency = 4.38), and from 20.0 to 18.0 μg/mL (potency = 4.26) (Table 1), respectively. The bioactivity of **4f** compound is more influenced by the hydrophobic contribution of the substituent.

After, a 2,4-dichloro disubstituted derivative (R_1 = Cl, $\pi_{4\text{-Cl}}$ = 0.70, $\sigma_{4\text{-Cl}}$ = 0.23, R_2 = H, π = 0.00, σ = 0.00, R_3 = Cl, **4g**) was synthesized from the **3g** intermediate compound, which was obtained following *pathway B* (Scheme 1). The MIC values varied from 19.7 to 17.7 μg/mL (potency = 4.25). The presence of 2-Cl instead of 2-H confers a bigger steric hindrance on that region, since the *ortho* position is primarily related to the steric effect of the substituent. This kind of molecular modification seems to be not favorable to the bioactivity, since the **4g** compound is less potent than the **4c** analogue (3,4-dichloro disubstituted) (see Table 1). Additionally, the potency of the **4g** compound is similar to the monosubstituted compound (4-Cl), **4b**. Regarding the synthetic procedure, the 2,4-methyl dichlorobenzoate ester (**2g**) was not obtained by *pathway A* because the purification steps have failed.

The 4-NO_2 substituted compound (R_1 = NO_2, $\pi_{4\text{-NO2}}$ = 0.24, $\sigma_{4\text{-NO2}}$ = 0.78, **4h**) was the last compound synthesized, and also the less active in this set. The MIC value ranged from 24.3 to 21.9 μg/mL (potency = 4.16) (Table 1). This data can indicate that the π value of 4-NO_2 substituent is far from π-*optimum*, since the antimicrobial potency decreased in comparison to the unsubstituted compound (**4a**), and it was not compensated by the electronic effect (σ).

The biological data found for the derivatives in this branch of the decision diagram indicated that when the substituent group presents σ and π values out of the considered optimum values, the antimicrobial activity decreases in the following direction: ***4e*** >> ***4f*** >> ***4g*** >> ***4h***.

Table 1
Experimental data found for the synthesized benzofuroxan derivatives



	R_1	R_2	R_3	MW	Yield (%)	Melting point (°C)	MIC (μg/mL)[a] 3SP/R33[b], VISA3[c]		C^d (10^{-5}) (M)	Potency log $1/C^d$
							Phase I	Phase II		
4a	H	H	H	282.08	94	214.0–215.0	20.0–10.0	18.0–16.2	6.38	4.19
4b	Cl	H	H	316.04	91	212.0–213.0	20.0–10.0	17.0–15.3	5.38	4.27
4c	Cl	Cl	H	350.00	91	205.0–206.0	15.0–7.5	13.1–11.8	3.74	4.43
4d	NO_2	CF_3	H	395.05	73	191.0–192.0	15.0–7.5	12.7–11.4	3.21	4.49
4e	CF_3	H	H	350.06	82	201.0–202.0	20.0–10.0	14.6–13.1	4.17	4.38
4f	Br	H	H	359.99	90	210.0–211.0	20.0––10.0	20.0–18.0	5.56	4.26
4g	Cl	H	Cl	350.00	73	163.0–164.0	30.0–15.0	19.7–17.7	5.62	4.25
4h	NO_2	H	H	327.06	94	262.0–263.0	30.0–15.0	24.3–21.9	7.43	4.16
Ampicilin				32.0–16.0 (3SP/R33)				32.0–16.0 (VISA3)		
Vancomycin				1.0–0.5 (3SP/R33)				8.0–4.0 (VISA3)		

[a] Values corresponding to the average of triplicates.
[b] Resistant to 19 antibiotics.
[c] Vancomycin-intermediate *Staphylococcus aureus* strain.
[d] Regarding the potency determination (log 1/*C*), the highest C value of phase II range was converted into molarity, which is based upon the compounds molecular weight (MW).

HCA can focus on samples or variables. Clustering of samples reveals similarities among the samples while clustering of variables pinpoints intervariable relationships. In this study, HCA was couched in terms of samples (compounds) clustering and the findings are presented in Figure 2.

HCA showed that the designed compounds were grouped into two main groups (see Fig. 2). The group A has the unsubstituted compound (**4a**, similiarity index = 0.27) and two subgroups (A′ and A″), which correspond to the monosubstituted compounds. The subgroup A′ is composed by the compounds whose bioactivities are mostly σ-dependent (**4h** and **4f**) whereas subgroup A″ contains the compounds in which the substituents present a more pronounced hydrophobic character (**4g** and **4b**). The similarity indexes found for the compounds in the subgroups A′ and A″ were 0.75 and 0.71, respectively. The group B contains the R_1 and R_2 disubstituted compounds, including the more active compound (B′, **4d**). The similarity index between **4d** e **4c** is 0.52 (subgroup B′). Although the **4e** compound is part of group B, its similarity index is 0.18.

The biological assays were performed in triplicate in two stages, phase I and II, increasing the results' reliability. Dimethyl sulfoxide (DMSO) was used as solvent at minimal concentration, which did not show sinergic effect with the compounds tested, due to the low solubility of the bezofuroxan derivatives into culture media. Ampicilin and vancomicyn, frequently used in antimicrobial therapeutic, were used as standard drugs.

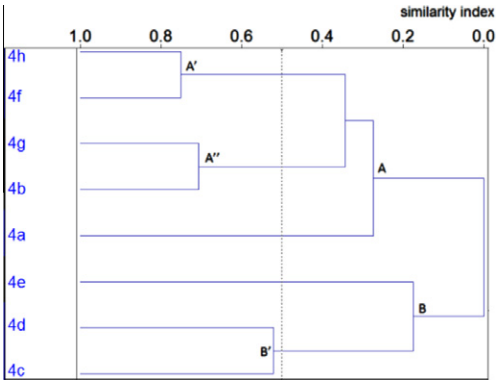


Figure 2. Dendrogram found for the eight antimicrobial agents (**4a**–**h**) applying HCA. The cursor (dashed line) is pointing out a similarity index of 0.50.

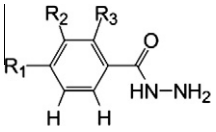
The MIC intervals determined were the same for the two multidrug-resistant strains of *S. aureus*. The two strains were chosen based upon their multidrug resistance for nineteen antibiotics currently used in therapeutic to treat *S. aureus* infections. They differ only in their vancomycin vulnerability.[27,28] The 3SP/R33 strain is sensitive to vancomycin (MIC $\leqslant$ 2 μg/mL), and resistant to amoxicyllin/clavulanic acid, ampicillin, cefazolin, cefotaxime, cefalotin, ciprofloxacin, clindamicin, erythromycin, gentamicin, imipinem, nitrofurantoin, norfloxacin, oxacilin, penicillin, rifampicin and trimethoprim/sulfamethoxazole. The VISA3 strain shows intermediate resistance to vancomicyn (MIC $\geqslant$ 4 μg/mL), besides the antibiotics already cited.

The *Clinical and Laboratory Standards Institute* of United States considers as susceptible to vancomycin those *S. aureus* strains that present MIC values $\leqslant$ 2 μg/mL.[26,29] If the MIC values are between 4 μg/mL and 8 μg/mL, the strains are called as vancomycin intermediate resistant (VISA), and the strains are considered resistant to vancomycin (VRSA) when the MIC values are $\geqslant$ 16 μg/mL. Vancomycin does not work for patients infected with *S. aureus* strains for which the MIC values are $\geqslant$8 μg/mL.[26]

Among the eight compounds synthesized and identified in this study, there are four new chemical entities, **4c**, **4d**, **4g**, **4h** (experimental section). The identification of compounds **4a**, **4b**, **4e** and **4f** was carried out through the comparison of the experimental melting point values to those previously reported in the literature (see Table 5S, in Supplementary data), and also using ^1H and ^{13}C NMR spectroscopy.

The designed compounds were prepared employing two synthetic pathways, A and B (Scheme 1). For the pathway A, the final compounds were synthesized from the corresponding benzoic acids in three steps: esterification, ammonolysis, and preparation of Schiff's bases. The esterification step provided satisfactory yields for all compounds (81–96%). However, lower yields (51–90%) were obtained from the ammonolysis step probably due to the difference of reactivity among methyl esters under the influence of

Table 2
Global yields obtained in benzhydrazides synthesis by pathway A and B



	R_1	R_2	R_3	Pathway A (η%)			Pathway B (η%)	Ratio B:A (%)
				Methyl esters	Benzhydrazides	Global[a]		
3a	H	H	H	89	67	60	88	48
3b	Cl	H	H	96	88	84	85	1
3c	Cl	Cl	H	88	90	79	90	14
3d	NO_2	CF_3	H	89	66	59	73	24
3e	CF_3	H	H	81	59	48	81	69
3f	Br	H	H	98	82	80	95	18
3g	Cl	H	Cl	85[b]	88[c]	74	80	8
3h	NO_2	H	H	96	51	49	76	55
Average				90	74	67	84	22

[a] η% global = η% methyl esthers * η% benzhydrazides/10^2.
[b] Ref. 31.
[c] Ref. 32.

substituent groups as well as some difficulties associated to the crystallization procedure. The identification of methyl esters and benzhydrazides was performed through the comparison of the experimental melting point values to those previously reported in the literature (see Tables 2S and 3S in Supplementary data).

As a trial to improve the yield, benzhydrazide synthesis was developed in one step pot (pathway B, Scheme 1). The esters dissolution into ethanol,[30] skipping the isolation step after esterification, simplified the amonolysis reaction. For this reaction, hydrazine hydrate was added to the system. In the first step, the alcohol and acid contributed favorably to the ester carbon protonation, becoming it more susceptible to the hydrazine nucleophilic attack, thus, the reaction was driven to the formation of benzhydrazides.

Benzhydrazides were identified through the melting point procedure and ^1H and ^{13}C NMR spectroscopy. The findings were compared to those previously reported in the literature (see Table 4S in Supplementary Material). The average yield found for this synthetic pathway was 84% (73–95%), and the data are presented in Table 2. For comparison, the information regarding the conditions used to obtain the compounds methyl 2,4-dichlorobenzoate **2g** and 2,4-dichlorobenzhydrazide **3g** are from the literature.[31,32] The one pot synthesis showed greater yield values in comparison to a multi-step synthesis, and indeed can be consider as a better synthetic pathway.

The synthesis methodology was modified to obtain 4-nitro-3-(trifluoromethyl)benzhydrazide, **3d** and 4-nitrobenzhydrazide **3h**. Hydrazine hydrate acts as reducing agent, which is widely used in the reduction of nitro aromatic groups.[33] The reduction process is favoured by the use of catalysts, such as, metallic ferrous, iron (III) hydroxide, or graphite.[34,35] However, despite the time of reaction used in this stage has not been so long, the convertion through reduction of the nitro group to amino in the aromatic ring ocurred for the both intermediates. This fact was confirmed by ^1H NMR spectra (see Fig. 3), considering the chemical deviation (δ) related to the internal standard reference (tetrametilsilane). It was observed a singlet with two protons integration at δ 6.0 ppm (**2d**) and δ 5.3 ppm (**2h**) regions, respectively, indicating the presence of NH_2 group at *para* position in the aromatic ring.[36] One alternative to avoid the reduction of these compounds is to perform the reactions in a controlled temperature of -3 °C and 2 °C.

The products obtained by *pathway B* (Scheme 1) presented a crystal structure and they could be separated from the reaction

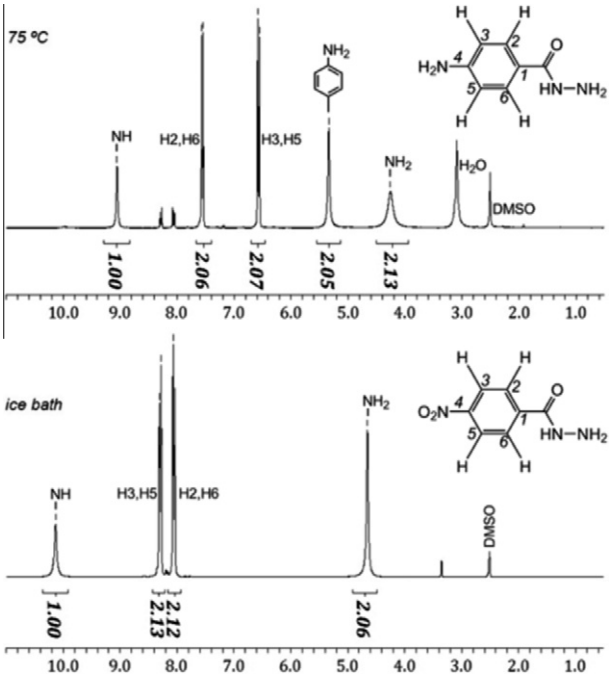
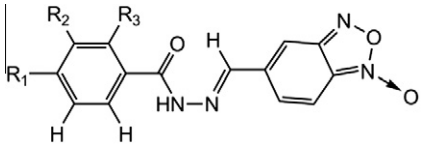


Figure 3. ^1H NMR spectra of the isolated products when different temperatures were employed in the ammonolysis of compound **2h** (using pathway A) or **1h** (using the pathway B).

medium using a simple filtration step, avoiding an additional recrystallization procedure. It is noteworthy that the differences in the structure of the crystals depend upon the temperature in which the system is maintained after reaction. The crystals habits tend to be larger when the system is kept at room temperature than at temperatures below 0 °C.

Finally, the classic method of Schiff's base preparation was employed as the last step reaction. This reaction is easy to perform, and the resulting yields were around 86% (73%–94%). The partial and total yields obtained by the pathways *A* and *B* are presented in Table 3. The synthesis of benzofuroxan derivatives was performed in three steps using the *pathway A* (Scheme 1), and the average global yield was 57%. Otherwise, when the eight

Table 3
Global yields obtained in pathway A and B



	R_1	R_2	R_3	Pathway A (η%)				Pathway B (η%)			Ratio B:A (%)
				Methyl esters	Benzhydrazides	Schiff's bases	Global[a]	Benzhydrazides	Schiff's bases	Global[b]	
4a	H	H	H	89	67	94	56	88	94	83	48
4b	Cl	H	H	96	88	91	77	85	91	77	1
4c	Cl	Cl	H	88	90	91	72	90	91	82	14
4d	NO_2	CF_3	H	89	66	73	43	73	73	53	24
4e	CF_3	H	H	81	59	73	35	81	73	59	69
4f	Br	H	H	98	82	82	66	95	82	78	18
4g	Cl	H	Cl	85[c]	88[d]	90	67	80	90	72	7
4h	NO_2	H	H	96	51	94	46	76	94	71	55
Average				90	74	86	57	84	86	72	25

[a] η% global = η% methyl esthers * η% benzhydrazides * η% Schiff's bases/10^4.
[b] η% global = η% benzhydrazides * η% Schiff's bases/10^2.
[c] Ref. 31.
[d] Ref. 32.

compounds were synthesized using *pathway B* (Scheme 1) the average global yield obtained was 72%, meaning a gain around 25% in comparison to the *pathway A*. Thus, to increase the global yield, the ammonolysis reaction was performed subsequently to esterification, skipping the ester isolation step.

The compound that significantly showed the efficiency of synthesis optimization was the 4-trifluoromethyl-[*N*′-(benzofuroxan-5-yl)methylene]benzhydrazide, **4e.** When three synthetic steps were used the global yield was 35% whereas when just two synthetic steps were employed the global yield raised to 59%, meaning a real yield improvement of 70%. Then, the exclusion of one synthetic step can also reflect in economy of time, quantity of raw material and solvent.

4. Conclusions

In this study, the synthesis, identification, and biological assay of a set of benzofuroxan derivatives was explored. The choice of substituent groups was based upon the Topliss approach, which has indicated as more active compound of the set, the 4-nitro-3-trifluoromethyl-[*N*′-(benzofuroxan-5-yl)methylene]benzhydrazide (**4d**), showing a MIC value $\leqslant$ 12.7 μg/mL against the multidrug-resistant *S. aureus* strains.

HCA divided the designed compounds in two main groups according to the substitution patterns, and the subgroups regarding the influence of the electronic and hydrophobic character of the substituent on bioactivity.

All synthesis reactions have presented satisfactory yields, and the melting point determination procedures have attested the high purity degree of the resulting compounds. Additionally, the one-step synthesis of benzhydrazides was considered more efficient than the same synthesis using two steps, besides to be an easier method and less time consuming.

5. Experimental

5.1. Chemistry

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 (2)

Each substituted benzoic acid **1a–h** (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 was washed with cold water.

5.1.1.1. 4-Nitro-3-(trifluoromethyl)benzoic acid methyl ester (2d). Pale white needle crystal (89%); mp 68.0–69.0 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm): 8.46–8.41 (m, 1H, H6), 8.35 (s, 1H, H2), 8.31–8.25 (m, 1H, H5), 3.96 (s, 3H, CH_3). ^{13}C NMR {H} (DMSO-d_6, 75 MHz): 164.9 (C=O), 149.9 (C4), 135.5 (C1), 134.1 (C6), 128.7 (C2), 126.5 (C5), 122.3 (C3), 121.8 (CF_3), 53.5 (CH_3). Anal. Calcd for ($C_9H_6F_3NO_4$) C, H, N: calc: C, 43.39; H, 2.43; N, 5.62. exp: C, 43.26; H, 2.48; N, 5.59.

5.1.2. General procedure for the preparation of benzhydrazides (3)

Pathway A—Hydrazine hydrate 64% (v/v) (30.0 mL, 0.33 mol) was heated up to 50–60 °C. The methyl ester previously isolated (0.01 mol) was added and the mixture was refluxed during 10 min. The cooling down 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. Different conditions were needed to obtain 4-nitro-3-(trifluoromethyl)benzhydrazide (3d) and 4-nitrobenzhydrazide (3 h). Hydrazine hydrate 64% (v/v) (30.0 mL, 0.33 mol) was cooled down in ice bath to −3 to 2 °C. The respective methyl ester (0.01 mol) was added and the mixture was stirred during 1 hour. The cooling down was proceeded in dry ice – ethanol bath. The solid was filtered and washed with cold water. *Pathway B*—each substituted benzoic acid (0.01 mol) was refluxed during 4 h in 20.0 mL (0.50 mol) of anhydrous methanol and 0.5 mL (1.0 mmol) of sulfuric acid. The reaction mixture was cooled down to room temperature. and the hydrazine hydrate 80% (v/v) (10.0 mL, 0.11 mol) was added. The system was maintained into vigorously stirring for more 30 minutes. In the case of compounds with 4-nitro and 4-nitro-3-trifluoromethyl substituent groups attached in the benzene moiety, after the addition of hydrazine hy-

drate 80% (v/v) at room temperature, the reaction mixture was cooled down in ice bath and maintained into stirring during 1 hour. After these periods, the mixture was maintained at cold temperature to give **3**.

5.1.2.1. 4-Nitro-3-(trifluoromethyl)benzhydrazide (3d). White solid (pathway A—66%; pathway B—73%); mp 166.0–167.0 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm): 10.25 (s, 1H, NH), 8.33 (s, 1H, H2), 8.30 (d, 1H, $J_{(6,5)}$ = 8.3 Hz, H6), 8.21 (d, 1H, $J_{(5,6)}$ = 8.3 Hz, H5), 4.66 (s, 2H, NH_2). ^{13}C NMR {H} (DMSO-d_6, 75 MHz): δ (ppm): 162.8 (C=O), 148.8 (C4), 137.7 (C1), 133.3 (C6), 127.0 (C2), 126.2 (C5), 122.1 (C3), 121.7 (CF_3); Anal. Calcd for ($C_8H_6F_3N_3O_3$) C, H, N: calc: C, 38.57; H, 2.43; N, 16.87. exp: C, 38.67; H, 2.30; N, 16.87.

5.1.3. General procedure for the preparation of benzofuroxans derivatives (4a–h)

A mixture of 5-formylbenzofuroxan (1.0 mmol) and benzhydrazides (1.0 mmol) in water, sulfuric acid, acetic acid and methanol (8:7:8:20 v/v) was heated under reflux during 1 h. After cooling down, the mixture was poured into cold water to give **4**.

5.1.3.1. 3,4-Dichloro-[*N*′-(benzofuroxan-5-yl)methylene]benzhydrazide (4c). Yellow solid (91%); mp 205.0–206.0 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm): 11.96 (s, 1H, H10), 8.36 (s, 1H, H8), 8.01 (d, 1H, $J_{(13,17)}$ = 1.5 Hz, H13), 7.79 (d, 1H, $J_{(17,16)}$ = 8.3 Hz, H17), 7.77 (d, 1H, J = 9.4 Hz, H6), 7.73 (s, 1H, H4), 7.65 (d, 1H, $J_{(16,17)}$ = 8.3 Hz, H16), 7.58 (d, 1H, J = 9.4 Hz, H7); ^{13}C NMR {H} (DMSO-d_6, 75 MHz): δ (ppm): 162.1 (C11), 145.9 (C8), 137.9 (C5), 137.8 (C15), 135.2 (C12), 134.0 (C14), 131.9 (C16), 131.6 (C13), 130.3 (C7a), 130.2 (C17), 129.3 (C3a), 128.6 (C6), 116.7 (C7), 115.5 (C4); Anal. Calcd for ($C_{14}H_8Cl_2N_4O_3$): C, 47.89; H, 2.30; N, 15.96. Found: C, 47.87; H, 1.72; N, 15.56.

5.1.3.2. 4-Nitro-3-(trifluoromethyl)-[*N*′-(benzofuroxan-5-yl)methylene]benzhydrazide (4d). Yellow solid (73%); mp 191.0–192.0 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm): 12.20 (s, 1H, H10), 8.38 (s, 1H, H8), 8.34 (s, 1H, H13), 8.31 (d, 1H, J = 9.2 Hz, H6), 8.18 (d, 1H, $J_{(16/17)}$ = 8.1 Hz, H16), 7.79 (s, 1H, H4), 7.71 (d, 1H, $J_{(17/16)}$ = 8.3 Hz, H17), 7.60 (d, 1H, J = 9.4 Hz, H7); ^{13}C NMR {H} (DMSO-d_6, 75 MHz): δ (ppm): 163.7 (C11), 149.3 (C15), 147.3 (C8), 137.7 (C12), 137.3 (C5), 133.5 (C17), 130.5 (C7a), 129.7 (C3a), 129.3 (C6), 128.3 (C13), 127.0 (C16), 122.6 (C14), 121.8 (CF_3), 117.4 (C7), 114.4 (C4); Anal. Calcd for ($C_{15}H_8F_3N_5O_5$): C, 45.58; H, 2.04; N, 17.71. Found: C, 45.71; H, 1.98; N, 17.32.

5.1.3.3. 2,4-Dichloro-[*N*′-(benzofuroxan-5-yl)methylene]benzhydrazide (4g). Yellow solid (73%); mp 163.0–164.0 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm): 12.34 (s, 1H, H10), 8.35 (s, 1H, H8), 8.15 (s, 1H, H14), 7.96 (s, 1H, H4), 7.86 (d, 1H, J = 9.2 Hz, H6), 7.76 (d, 1H, J = 9.2 Hz, H7), 7.66 (d, 1H, $J_{(17,16)}$ = 8.1 Hz, H17), 7.60–7.52 (m, 1H, H16); ^{13}C NMR {H} (DMSO-d_6, 75 MHz): δ (ppm): 162.4 (C11), 146.8 (C8), 135.9 (C13), 135.0 (C5), 134.1 (12), 132.2 (C15), 131.4 (C17), 131.2 (C14), 130.7 (C7a), 129.8 (C3a), 129.1 (C16), 128.0 (C6), 118.6 (C7), 114.6 (C4); Anal. Calcd for ($C_{14}H_8Cl_2N_4O_3$): C, 47.89; H, 2.30; N, 15.96. Found: C, 47.56; H, 1.65; N, 15.39.

5.1.3.4. 4-Nitro-[*N*′-(benzofuroxan-5-yl)methylene]benzhydrazide (4h). Yellow solid (94%); mp 262.0–263.0 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm): 12.22 (s, 1H, H10), 8.50 (s, 1H, H8), 8.35 (d, 2H, J = 8.8 Hz, H14, H16), 8.14 (d, 2H, J = 8.5 Hz, H13, H17), 7.95 (d, 1H, J = 9.4 Hz, H6), 7.88 (s, 1H, H4), 7.72 (d, 1H, J = 9.4 Hz, H7); ^{13}C NMR {H} (DMSO-d_6, 75 MHz): δ (ppm): 162.5 (C11), 149.5 (C15), 146.3 (C8), 139.4 (C12), 137.7 (C5), 129.9 (C13, C17), 129.7 (C7a), 129.6 (C3a), 129.4 (C6), 123.8 (C14, C16), 116.7 (C7), 115.6 (C4); Anal. Calcd for ($C_{14}H_9N_5O_5$): C, 51.38; H, 2.77; N, 21.40. Found: C, 51.60; H, 2.79; N, 21.00.

5.2. Biology

Phase I—Minimal Inhibitory Concentration (MIC) of the compounds was determined with 96-well microliter plates containing two-fold serial dilutions of the compounds in Tryptic Soy Broth (TSB-Sigma®) medium. 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 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 during 18 h. The lowest concentration of compound—that in which there was no visible growth—was considered as the MIC value. Readings at 24 and 48 h were carried out for sterility control. Experiments were performed in triplicate.

Phase II—Stock solutions (1 mL) were prepared using two-fold 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 column, except to the column 11. The plates were mixed and incubated at 35 °C during 18 h.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.06.034.

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