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A new class of nifuroxazide analogues: Synthesis of 5-nitrothiophene derivatives with antimicrobial activity against multidrug-resistant *Staphylococcus aureus*

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Abstract—Hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) has been an increasing problem worldwide since the initial reports over 40 years ago. To examine new drug leads with potential antibacterial activities, 14 *p*-substituted benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazides were designed, synthesized, and tested against standard and multidrug-resistant *S. aureus* strains by serial dilution tests. All compounds exhibited significant bacteriostatic activity and some of them also showed bactericidal activity. The results confirmed the potential of this class of compounds as an alternative for the development of selective antimicrobial agents.

1. Introduction

The resistance of bacteria against antimicrobial agents has become a widespread medical problem especially as nosocomial pathogens. Treatment options for these infections are often limited, especially in debilitated and immunocompromised patients.[1,2]

In the last decade, there has been a reemergence of Gram-positive bacteria, in particular *Staphylococcus aureus*, which is considered one of the main causes of nosocomial infections.[3–5] The infectious disease caused by MRSA (methicillin-resistant *Staphylococcus aureus*) is currently a serious problem because these bacteria show a multidrug-resistant phenotype, that is, resistance not only to methicillin but also to several other drugs except vancomycin and teicoplanin. Although potent antistaphylococcal drugs are available, this infection continues to present significant morbidity and mortality rates, justifying the need for the development of more effective compounds for its treatment.[6–9]

Nifuroxazide, Figure 1, a synthetic antimicrobial agent used as second or third choice in enteric infection treatments, presents excellent characteristics such as wide spectrum of activity, inability to promote significant bacterial resistance, and chemical structure favorable to synthetic modifications.[10–12]

In previous studies of nifuroxazide analogues accomplished by Tavares and co-workers,[13,14] the furanic ring was substituted by the thiophenic one and several *p*-aromatic substitutions were made, with a significant improvement of antibacterial activity being observed in the case of *p*-acetyl derivatives. The thiophenic analogues have the potential to be an ideal therapy for immunocompromised and hospitalized patients suffering from opportunistic infection and/or superinfection due to microbial substitution.

The physicochemical properties of a drug refer to any structural, physical or chemical properties that have an effect on its biological activity. The knowledge of these

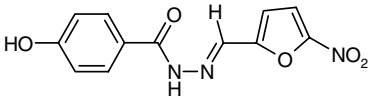

Figure 1. Nifuroxazide.

Keywords: Nifuroxazide; MRSA; MIC; Analogue preparation; Antimicrobials.
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properties allows the medicinal chemist to identify which features are important or not for biological activity resulting, in a way, in a successful lead development process. With the constant advancement of QSAR (quantitative structure–activity relationships) studies as a molecular modification approach, this procedure has been applied in several scientific areas.[10,15]

In order to use these medicinal chemistry advances to counter the high incidence of antibiotic-resistant microorganisms, this study is aimed at the design, synthesis, and determination of antimicrobial activity of *p*-substituted benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazides against *S. aureus* strains. The nifuroxazide phenolic hydroxyl was substituted by 14 groups conveniently selected according to physicochemical properties such as hydrophobicity and electronic distribution.[15,16] The choice of substituent groups was made in two steps. The first one involved the application of the Topliss'[17] operational scheme for aromatic substitution. In a second step, more derivatives were selected according to a previously determined hydrophobicity range. In addition to the substituent selection, the furanic ring was substituted by a thiophenic one resulting in increased hydrophobicity of the compounds.

The potency of compounds obtained was determined using serial dilution tests[18] against standard (ATCC 25923) and multidrug-resistant (3SP/R33)[19] *S. aureus* strains. Good antimicrobial activity values were observed for most of the compounds tested.

2. Results and discussion

2.1. Synthesis of *p*-substituted benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazides

Fourteen *p*-substituted benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazides were synthesized with good yields (90%) according to three standard methods: esterification reaction, ammonolysis reaction, and Schiff's base preparation.[13,14] The synthesis of target compounds is outlined in Fig. 2. The Schiff bases (D) were prepared by reacting the substituted benzhydrazides (B) and 5-nitro-2-thiophene carboxaldehyde in a solution with strongly acidic pH. This reaction condition is essential for the nucleophilic addition of the amino group to the carbonyl function of the aromatic aldehyde.[13,14] The compounds were structurally identified through IR, ^{1}H NMR and ^{13}C NMR spectra, melting point determination, and elemental analysis. All results obtained were consistent with the designed structures.

2.2. Antimicrobial activity determination

The minimal inhibitory concentration, MIC, was determined by serial dilution tests[18] in two sequential steps against standard (ATCC 25923) and multidrug-resistant (3SP/R33) strains of *S. aureus*. This microbiological method was previously adapted by Tavares and coworkers.[14] The 3SP/R33 strain of *S. aureus* is resistant to 19 antimicrobial agents in use (amoxicillin/clavulanic acid, ampicillin, cephazoline, cephotaxime, cephalotine, ciprofloxacin, clindamycin, erythromycin, gentamicin, imipenem, nitrofurantoin, norfloxacin, oxacillin, penicillin, rifampicin, trimethoprim/sulfametoxazole) and susceptible only to vancomycin. It was isolated from patients from hospitals of São Paulo City and was characterized by pulsed field gel electrophoresis (PFGE) by Mamizuka's research group (University of São Paulo), showing the same PFGE profile as the Brazilian Endemic Clone (BEC).[19]

The high hydrophobicity of some compounds results in their low solubility in DMSO/TSB (dimethylsulfoxide/tryptic soy broth) solution. As alternative to this problem, ethyl alcohol was used as co-solvent in microbiological assays. Toxicity tests of the solvent, DMSO, and the co-solvent, ethyl alcohol, showed that the concentrations of ethyl alcohol used in antimicrobial activity assays did not interfere in the microorganism's growth. Nevertheless, the mixture of ethyl alcohol and DMSO (1:1, v/v) in concentrations higher than 25% inhibited the growth, confirming the results of toxicity studies of DMSO previously reported by Tavares

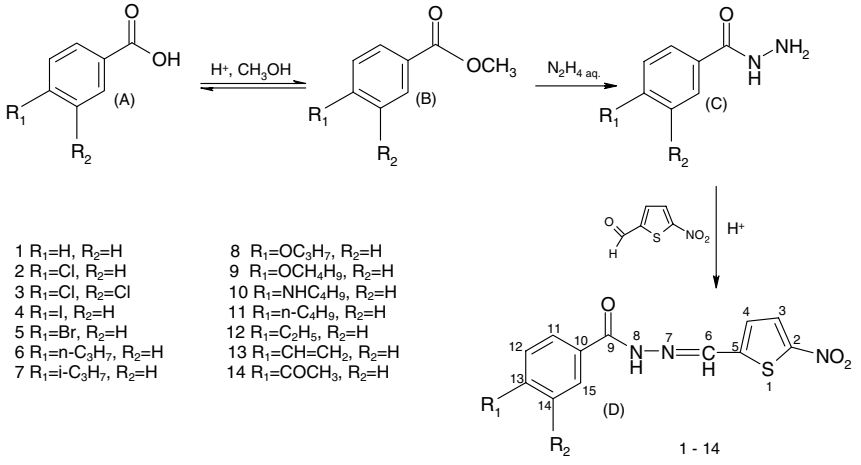


Figure 2. Synthesis of *p*-substituted benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide derivatives.

and co-workers.[14] Table 1 displays the MIC values of *p*-substituted benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazides against ATCC 25923 *S. aureus* strain.

The compounds showed marked activity against both strains of *S. aureus* and showed higher activity than nitrofurantoin (**15**), but only *p*-Cl (**2**), *m*,*p*-Cl_2 (**3**), *p*-Br (**5**), and *p*-$COCH_3$ (**14**) derivatives were more active than nifuroxazide (**16**), and chloramphenicol (**17**) (Table 1).

The most active compound against ATCC 25923 *S. aureus* strain was the *p*-$COCH_3$ (**14**) derivative with MIC = 0.14 μg/mL, while the lowest activity was observed for *p*-C_4H_9 (**11**) derivative with MIC = 13.50 μg/mL (Table 1). All derivatives had their MIC values determined at concentrations of DMSO ranging from 2% to 5%, i.e., much lower than those necessary to kill the microorganism. Thus, the complete inhibition of *S. aureus* growth refers only to the intrinsic bacteriostatic activity of the compounds.

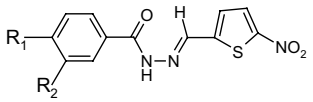
The MICs of the compounds against the multidrug-resistant (3SP/R33) strain of *S. aureus* were determined according to the same procedure employed for the microbiological assay against the standard strain (ATCC 25923). It was observed that the most active compound against 3SP/R33 *S. aureus* strain was the *p*-$COCH_3$ (**14**) derivative with MIC = 0.22–0.11 μg/mL, while the lowest activity was observed for *p*-C_4H_9 (**11**) derivative with MIC = 25.40–12.70 μg/mL. These results are in agreement with those previously determined for the ATCC 25923 strain.

The least bioactive compound, the *p*-C_4H_9 derivative (11) with MIC = 25.40–12.70 μg/mL, did not exhibit bactericidal activity at the solution concentrations employed in the microbiological test. Due to the high hydrophobicity of the *p*-C_4H_9 (**11**) derivative, the preparation of solutions with concentrations higher than 50.80 μg/mL was not possible. The same limitation was observed for *p*,*n*-C_3H_7 (**6**); *p*,*i*-C_3H_7 (**7**); *p*-I (**4**); *p*-OC_4H_9 (**9**); *p*-NHC_4H_9 (**10**); and *p*-C_2H_5 (**12**) derivatives.

The benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide (**1**), with MIC = 7.81–3.90 μg/mL, showed bactericidal effect at a relatively high concentration, MBC = 125 μg/mL, while the *p*-Cl (**2**) (MBC = 4.37–2.18 μg/mL), *p*-$COCH_3$ (**14**) (MBC = 0.22–0.11 μg/mL), *p*-Br (5) (MBC = 1.25–0.63 μg/mL), and *m*,*p*-Cl_2 (3) (MBC = 0.63–1.27 μg/mL) derivatives showed MBC values very similar to previously determined MIC values (Table 2).

Thus, experimental evidences suggested that the non-substituted derivative (**1**) showed mainly bacteriostatic activity, since the MBC value was higher than the MIC value. The *p*-Br (**5**), *p*-Cl (**2**), *p*,*m*-Cl_2 (**3**), and *p*-$COCH_3$ (**14**) derivatives were the most bioactive compounds of this series, since they exhibited excellent bacteriostatic and bactericidal activities.

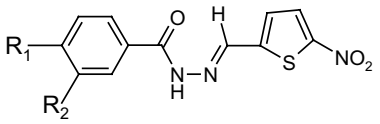
Table 1. Minimal inhibitory concentration, MIC, of *p*-substituted benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazides against ATCC 25923 *Staphylococcus aureus* strain



Compound	R_1	R_2	MIC (μg/mL)[a]		MIC (μM)	Potency Log(1/μM)
			Phase I	Phase II		
1	H	H	7.81–3.90	⩽4.59	⩽16.67	⩽4.78
2	Cl	H	2.18–1.09	⩽1.71	⩽5.52	⩽5.26
3	Cl	Cl	0.63–1.27	⩽0.76	⩽2.21	⩽5.66
4	I	H	11.10–5.55	⩽5.62	⩽14.01	⩽4.85
5	Br	H	1.25–0.63	⩽0.75	⩽2.12	⩽5.67
6	*n*-C_3H_7	H	14.50–7.25	⩽11.60	⩽36.55	⩽4.44
7	*i*-C_3H_7	H	10.04–5.02	⩽9.06	⩽28.55	⩽4.54
8	OC_3H_7	H	—[b]	⩽8.10	⩽24.29	⩽4.61
9	OC_4H_9	H	20.62–10.31	⩽12.30	⩽35.41	⩽4.45
10	NHC_4H_9	H	12.45–6.22	⩽7.75	⩽22.37	⩽4.65
11	*n*-C_4H_9	H	25.40–12.70	⩽13.50	⩽40.74	⩽4.39
12	C_2H_5	H	11.60–5.80	⩽6.75	⩽22.25	⩽4.65
13	$CH{=}CH_2$	H	6.75–3.38	⩽4.72	⩽15.66	⩽4.80
14	$COCH_3$	H	0.22–0.11	⩽0.14	⩽0.44	⩽6.36
15	Nitrofurantoin			16.00[c]		—
16	Nifuroxazide			3.60[d]		—
17	Chloramphenicol			3.10[c]		—

[a] Incubation at 35 °C for 18 h.
[b] MIC value was not determined in Phase I (Ref. 21).
[c] Ref. 14.
[d] Ref. 22.

Table 2. Minimal inhibitory and bactericidal concentrations, MIC and MBC, of *p*-substituted benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazides against 3SP/R33[a] *Staphylococcus aureus* strain



Compound	R_1	R_2	MIC (μg/mL)	MBC (μg/mL)
1	H	H	7.81–3.90	⩾125.00
2	Cl	H	2.18–1.09	4.37–2.18
3	Cl	Cl	0.63–1.27	0.63–1.27
4	I	H	11.10–5.55	>88.80[b]
5	Br	H	1.25–0.63	1.25–0.63
6	n-C_3H_7	H	14.50–7.25	>29.00[b]
7	i-C_3H_7	H	10.04–5.02	>40.16[b]
8	OC_3H_7	H	—	—
9	OC_4H_9	H	20.62–10.31	>41.24[b]
10	NHC_4H_9	H	12.45–6.22	>49.80[b]
11	n-C_4H_9	H	25.40–12.70	>50.80[b]
12	C_2H_5	H	11.60–5.80	>92.80[b]
13	$CH{=}CH_2$	H	6.75–3.38	>54.00[b]
14	$COCH_3$	H	0.22–0.11	0.44–0.22

[a] Ref. 19.
[b] MIC value was not determined due to high hydrophobicity of compound.

The presence of hydrophobic substituents attached to the benzene moiety exerts a positive influence on antibacterial activity. However, this influence is not unrestricted since the introduction of highly hydrophobic substituents, such as OC_4H_9 and n-C_4H_9, clearly decreased the potency of compounds. Consequently, the activity is not linearly correlated with this variable, although the hydrophobicity seemed to be the principal factor influencing the antibacterial activity. This property is certainly correlated with the ability of a compound to diffuse through the biological membranes to reach its site of action. While it is clear that hydrophobicity is perhaps the most important physicochemical property for determining the antibacterial activity, we believe that electronic and steric effects probably play a role in this activity.

In addition to this, the understanding of the physicochemical properties of this class of compounds deserves further investigation in order to clarify the mode of action at molecular level responsible for the observed activity. QSAR studies involving molecular descriptors of size and shape, electrostatic forces, and hydrogen bonds are in progress[20] in order to determine the physicochemical properties that represent a deeper insight into structure–activity relationships, and to optimize the effectiveness of this series of molecules.

3. Conclusions

Fourteen 5-nitro-2-thiophenylidene derivatives were synthesized, structurally identified, and tested against standard (ATCC 25923) and multidrug-resistant (3SP/R33) *S. aureus* strains. The most active compound was 4-acetylbenzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide and the least one was 4-butylbenzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide. The results obtained for the multidrug-resistant *S. aureus* strain were consistent with those determined for the ATCC 25923 strain, suggesting that this microorganism does not have yet mechanisms of resistance to this class of compounds. These results have shown the potential of nifuroxazide analogues as alternatives to the development of drugs for the treatment of infections caused by multidrug-resistant *S. aureus* strains.

4. Experimental—general methods

4.1. Synthesis and structural identification of *p*-substituted benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide derivatives

Target compounds were synthesized by reacting *p*-substituted benzoic acids with hydrazine and 5-nitro-2-thiophene carboxaldehyde (Fig. 2). Synthetic starting material, reagents, and solvents were purchased from Aldrich, Fluka or Labsynth. All solvents were of reagent grade and dried prior to use. Methods already described in the literature were used for the preparation of the compounds.[13,14,20] Initially, the methyl esters (B) were synthesized from their respective substituted benzoic acids (1 mmol) (A) and dried methyl alcohol (10 mmol). A small amount of concentrated sulfuric acid was added and the solution was refluxed for 4–5 h. Crystals were filtered off, washed with distilled water, and dried. Benzhydrazides (C) were synthesized from the above-mentioned methyl esters (1 mmol) and hydrazine 35% (30 mmol) under reflux for 30 min. The solid product obtained after cooling was filtered off, washed with distilled water, and dried. The target Schiff bases (D) were synthesized by reacting equimolar proportion of substituted benzhydrazides (C) and 5-nitro-2-thiophene carboxaldehyde in a solution of ethanol/acetic acid/sulfuric acid/water (20:8:7:8). The insoluble product was filtered off and purified by recrystallization in *N*,*N*-dimethylformamide.

The structural analysis of compounds is discussed as follows, where mp is melting point and EA represents elemental analysis. Melting points (°C) were determined in a Micro-Química MQAPF-301 digital melting point apparatus and elemental analysis was performed on a Perkin-Elmer 24013 CHN Elemental Analyzer. IR spectra were recorded, as KBr pellets, on a Shimadzu-470 spectrophotometer and the reported wavenumbers are given in cm^{-1}. NMR spectra were recorded on a Bruker DPX_{300} (300 MHz) spectrometer in DMSO-d_6 solutions. Chemical shifts are reported as δ (ppm) relative to TMS as internal standard.

4.1.1. Benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{12}H_9O_3N_3S$) (1). Dark-yellow amorphous solid. Yield: 93%; mp 246.5–247.3 °C.

IR (KBr): ν 3250 (ν_{N-H}); 3100 (ν_{C-H}); 1645 ($\nu_{C=O}$); 1595 and 1486 ($\nu_{C=C}$); 1542 (δ_{N-H}); 1521 ($\nu_{N=O\ ass.}$); 1330 ($\nu_{N=O\ sim.}$); 701 (δ_{C-H}); 628 (ν_{C-S}).

^{1}H NMR: δ 12.25 (s, 1H, H_8); 8.69 (s, 1H, H_6); 8.15 (d, 1H, H_4, J = 4.24 Hz); 7.91 (d, 2H, H_{11} and H_{15}, J = 7.03 Hz); 7.52–7.65 (m, 4H, H_3, H_{12}, H_{13}, and H_{14}).

^{13}C NMR: δ 163.14 (C_9); 147.54 (C_2); 142.02 (C_6); 133.64 (C_5); 133.06 (C_4); 131.39 (C_{10}); 130.57 (C_{13}); 129.47 (C_{11} and C_{15}); 128.59 (C_{12} and C_{14}); 114.74 (C_3).

EA: Calcd C, 49.14%; H, 3.09%; N, 14.32%. Experimental: C, 49.29%; H, 3.45%; N, 14.66%.

4.1.2. 4-Chloro-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{12}H_8O_3N_3SCl$) (2). Orange amorphous solid. Yield: 96%; mp 288.1–290.1 °C.

IR (KBr): ν 3255 (ν_{N-H}); 3100 (ν_{C-H}); 1646 ($\nu_{C=O}$); 1589 and 1487 ($\nu_{C=C}$); 1551 (δ_{N-H}); 1523 ($\nu_{N=O\ ass.}$); 1331 ($\nu_{N=O\ sim.}$); 1110 (ν_{C-Cl}); 725 (δ_{C-H}); 632 (ν_{C-S}).

^{1}H NMR: δ 12.28 (s, 1H, H_8); 8.68 (s, 1H, H_6); 8.14 (d, 1H, H_4, J = 4.25 Hz); 7.94 (d, 2H, H_{11} and H_{15}, J = 8.22 Hz); 7.59–7.64 (m, 3H, H_3, H_{12}, and H_{14}).

^{13}C NMR: δ 162.45 (C_9); 151.08 (C_6); 146.70 (C_2); 141.67 (C_{13}); 137.15 (C_5); 131.75 (C_{10}); 131.70 (C_4); 130.62 (C_{12} and C_{14}); 129.83 (C_{11} and C_{15}); 128.83 (C_3).

EA: Calcd C, 46.54%; H, 2.60%; N, 13.57%. Experimental: C, 46.44%; H, 2.97%; N, 13.48%.

4.1.3. 3,4-Dichloro-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{12}H_7O_3N_3SCl_2$) (3). Orange amorphous solid. Yield: 98%; mp 222.1–225.5 °C.

IR (KBr): ν 3360 (ν_{N-H}); 3185 (ν_{C-H}); 1640 ($\nu_{C=O}$); 1595 and 1497 ($\nu_{C=C}$); 1564 (δ_{N-H}); 1526 ($\nu_{N=O\ ass.}$); 1332 ($\nu_{N=O\ sim.}$); 1069 (ν_{C-Cl}); 742 (δ_{C-H}); 673 (ν_{C-S}).

^{1}H NMR: δ 12.30 (s, 1H, H_8); 8.58 (s, 1H, H_6); 8.14 (d, 1H, H_4, J = 4.18 Hz); 7.80 (d, 2H, H_{11} and H_{15}, J = 8.12 Hz); 7.79–7.81 (m, 1H, H_{12}), 7.56 (d, 1H, H_3, J = 4.18 Hz).

^{13}C NMR: δ 161.98 (C_9); 151.93 (C_6); 147.16 (C_2); 142.73 (C_{13}); 135.82 and 131.88 (C_{12} and C_{14}); 134.04 (C_5); 132.35 (C_{10}); 131.37 and 130.98 (C_{11} and C_{15}); 130.46 (C_4); 128.97 (C_3).

EA: Calcd C, 39.79%; H, 1.95%; N, 11.60%. Experimental: C, 40.01%; H, 2.62%; N, 11.82%.

4.1.4. 4-Iodo-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{12}H_8O_3N_3SI$) (4). Dark-yellow amorphous solid. Yield: 95%; mp 276.5–277.9 °C.

IR (KBr): ν 3160 (ν_{N-H}); 3025 (ν_{C-H}); 1643 ($\nu_{C=O}$); 1590 and 1495 ($\nu_{C=C}$); 1557 (δ_{N-H}); 1530 ($\nu_{N=O\ ass.}$); 1320 ($\nu_{N=O\ sim.}$); 1005 (ν_{C-I}); 728 (δ_{C-H}); 680 (ν_{C-S}).

^{1}H NMR: δ 12.15 (s, 1H, H_8); 8.67 (s, 1H, H_6); 8.14 (d, 1H, H_4, J = 4.23 Hz); 7.94 (d, 2H, H_{11} and H_{15}, J = 8.20 Hz); 7.69 (d, 2H, H_{12} and H_{14}, J = 8.20 Hz); 7.60 (d, 1H, H_3, J = 4.23 Hz).

^{13}C NMR: δ 162.70 (C_9); 146.50 (C_6); 146.48 (C_{13}); 141.45 (C_5); 137.36 (C_2); 130.51 (C_{12} and C_{14}); 129.94 (C_{10}); 129.53 (C_{11} and C_{15}); 126.01 (C_4); 125.05 (C_3).

EA: Calcd C, 35.92%; H, 2.01%; N, 10.47%. Experimental: C, 36.03%; H, 1.92%; N, 10.42%.

4.1.5. 4-Bromo-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{12}H_8O_3N_3SBr$) (5). Dark-yellow amorphous solid. Yield: 92%; mp 274.4–276.5 °C.

IR (KBr): ν 3345 (ν_{N-H}); 3160 (ν_{C-H}); 1653 ($\nu_{C=O}$); 1602 and 1490 ($\nu_{C=C}$); 1554 (δ_{N-H}); 1528 ($\nu_{N=O\ ass.}$); 1333 ($\nu_{N=O\ sim.}$); 1037 (ν_{C-Br}); 812 (δ_{C-H}); 630 (ν_{C-S}).

^{1}H NMR: δ 12.10 (s, 1H, H_8); 8.58 (s, 1H, H_6); 8.00 (d, 1H, H_4, J = 4.22 Hz); 7.81 (d, 2H, H_{11} and H_{15}, J = 8.20 Hz); 7.67 (d, 2H, H_{12} and H_{14}, J = 8.20 Hz); 7.56 (d, 1H, H_3, J = 4.22 Hz).

^{13}C NMR: δ 163.30 (C_9); 151.81 (C_6); 147.41 (C_2); 142.35 (C_{13}); 132.65 (C_{12} and C_{14}); 131.40 (C_5); 130.69 (C_{10}); 129.58 (C_{11} and C_{15}); 126.88 (C_4); 126.29 (C_3).

EA: Calcd C, 40.69%; H, 2.28%; N, 11.86%. Experimental: C, 41.12%; H, 2.23%; N, 12.42%.

4.1.6. 4-propyl-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{15}H_{15}O_3N_3S$) (6). Dark-yellow amorphous solid. Yield: 93%; mp 200.5–201.6 °C.

IR (KBr): ν 3210 (ν_{N-H}); 3110 (ν_{C-H}); 1650 ($\nu_{C=O}$); 1611 and 1509 ($\nu_{C=C}$); 1556 (δ_{N-H}); 1534 ($\nu_{N=O\ ass.}$); 1466 (δ_{CH_2}); 1375 (δ_{CH_3}); 1341 ($\nu_{N=O\ sim.}$); 814 (δ_{C-H}); 690 (ν_{C-S}).

^{1}H NMR: δ 12.14 (s, 1H, H_8); 8.65 (s, 1H, H_6); 8.08 (d, 1H, H_4, J = 4.29 Hz); 7.80 (d, 2H, H_{11} and H_{15}, J = 8.04 Hz); 7.52 (d, 1H, H_3, J = 4.29 Hz); 7.32 (d, 2H, H_{12} and H_{14}, J = 8.04 Hz); 2.59 (t, 2H, CH_2, J = 7.30 Hz); 1.60 (sextet, 2H, CH_2, J = 7.30 Hz); 0.87 (t, 3H, CH_3, J = 7.30 Hz).

^{13}C NMR: δ 163.32 (C_9); 150.76 (C_6); 140.85 (C_5); 146.90 (C_2); 146.83 (C_{13}); 130.48 (C_{12} and C_{14}); 129.49 (C_{10}); 128.49 (C_{11} and C_{15}); 127.81 (C_4); 125.48 (C_3); 64.25 (CH_2); 25.89 (CH_2); 10.28 (CH_3).

EA: Calcd C, 56.77%; H, 4.76%; N, 13.24%. Experimental: C, 57.10%; H, 4.90%; N, 13.33%.

4.1.7. 4-Isopropyl-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{15}H_{15}O_3N_3S$) (7). Dark-yellow amorphous solid. Yield: 90%; mp 251.3–252.7 °C.

IR (KBr): ν 3270 (ν_{N-H}); 3080 (ν_{C-H}); 1652 ($\nu_{C=O}$); 1605 and 1495 ($\nu_{C=C}$); 1566 (δ_{N-H}); 1524 ($\nu_{N=O\ ass.}$); 1381 (δ_{CH_3}); 1331 ($\nu_{N=O\ sim.}$); 708 (δ_{C-H}); 670 (ν_{C-S}).

^{1}H NMR: δ 12.08 (s, 1H, H_8); 8.59 (s, 1H, H_6); 8.04 (d, 1H, H_4, J = 4.32 Hz); 7.75 (d, 2H, H_{11} and H_{15}, J = 8.12 Hz); 7.32 (d, 2H, H_{12} and H_{14}, J = 8.12 Hz);

7.49 (d, 1H, H_3, $J = 4.32$ Hz); 2.88 (septet, 1H, CH, $J = 6.87$ Hz); 1.14 (d, 6H, $2CH_3$, $J = 6.87$ Hz).

^{13}C NMR: δ 164.15 (C_9); 153.80 (C_6); 151.62 (C_2); 147.73 (C_{13}); 141.76 (C_5); 131.39 (C_{10}); 131.31 (C_{12} and C_{14}); 130.40 (C_{11} and C_{15}); 128.76 (C_4); 127.36 (C_3); 34.32 (CH); 24.45 ($2CH_3$).

EA: Calcd C, 56.77%; H, 4.76%; N, 13.24%. Experimental: C, 57.02%; H, 4.81%; N, 13.29%.

4.1.8. 4-Propoxy-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{15}H_{15}O_4N_3S$) (8). Dark-yellow amorphous solid. Yield: 93%; mp 195.7–197.2 °C.

IR (KBr): ν 3290 (ν_{N-H}); 3105 (ν_{C-H}); 1659 ($\nu_{C=O}$); 1607 and 1507 ($\nu_{C=C}$); 1571 (δ_{N-H}); 1528 ($\nu_{N=O\ ass.}$); 1470 (δ_{CH_2}); 1391 (δ_{CH_3}); 1337 ($\nu_{N=O\ sim.}$); 1256 (ν_{C-O}); 729 (δ_{C-H}); 680 (ν_{C-S}).

^{1}H NMR: δ 12.12 (s, 1H, H_8); 8.67 (s, 1H, H_6); 8.13 (d, 1H, H_4, $J = 4.29$ Hz); 7.90 (d, 2H, H_{11} and H_{15}, $J = 8.54$ Hz); 7.07 (d, 2H, H_{12} and H_{14}, $J = 8.54$ Hz); 7.56 (d, 1H, H_3, $J = 4.29$ Hz); 4.02 (t, 2H, CH_2, $J = 6.94$ Hz); 1.76 (sextet, 2H, CH_2, $J = 6.94$ Hz); 0.99 (t, 3H, CH_3, $J = 6.94$ Hz).

^{13}C NMR: δ 161.80 (C_9); 150.67 (C_6); 147.04 (C_2); 140.50 (C_{13}); 130.60 (C_5); 129.80 (C_{10}); 129.41 (C_{12} and C_{14}); 124.63 (C_{11} and C_{15}); 124.58 (C_4); 114.25 (C_3); 69.28 (CH_2); 21.95 (CH_2); 10.35 (CH_3).

EA: Calcd C, 53.04%; H, 4.54%; N, 12.60%. Experimental: C, 52.08%; H, 4.60%; N, 12.31%.

4.1.9. 4-Butoxy-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{16}H_{17}O_4N_3S$) (9). Dark-yellow amorphous solid. Yield: 97%; mp 200.5–201.6 °C.

IR (KBr): ν 3360 (ν_{N-H}); 3075 (ν_{C-H}); 1643 ($\nu_{C=O}$); 1607 and 1507 ($\nu_{C=C}$); 1570 (δ_{N-H}); 1529 ($\nu_{N=O\ ass.}$); 1466 (δ_{CH_2}); 1384 (δ_{CH_3}); 1339 ($\nu_{N=O\ sim.}$); 1256 (ν_{C-O}); 729 (δ_{C-H}); 679 (ν_{C-S}).

^{1}H NMR: δ 12.08 (s, 1H, H_8); 8.64 (s, 1H, H_6); 8.11 (d, 1H, H_4, $J = 4.18$ Hz); 7.87 (d, 2H, H_{11} and H_{15}, $J = 8.39$ Hz); 7.04 (d, 2H, H_{12} and H_{14}, $J = 8.39$ Hz); 7.54 (d, 1H, H_3, $J = 4.18$ Hz); 4.03 (t, 2H, CH_2, $J = 6.80$ Hz); 1.70 (quintet, 2H, CH_2, $J = 6.80$ Hz); 1.40 (sextet, 2H, CH_2, $J = 6.80$ Hz); 0.92 (t, 3H, CH_3, $J = 6.80$ Hz).

^{13}C NMR: δ 161.79 (C_9); 150.65 (C_6); 147.03 (C_2); 140.46 (C_{13}); 130.58 (C_5); 129.76 (C_4); 129.56 (C_{10}); 129.40 (C_{12} and C_{14}); 124.62 (C_{11} and C_{15}); 114.24 (C_3); 67.51 (CH_2); 30.61 (CH_2); 18.69 (CH_2); 13.69 (CH_3).

EA: Calcd C, 55.32%; H, 4.93%; N, 11.50%. Experimental: C, 56.09%; H, 5.16%; N, 11.57%.

4.1.10. 4-Butylamine-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{16}H_{18}O_3N_3S$) (10). Dark-red amorphous solid. Yield: 93%; mp 249.1–250.9 °C.

IR (KBr): ν 3350 (ν_{N-H}); 3020 (ν_{C-H}); 1657 ($\nu_{C=O}$); 1598 and 1474 ($\nu_{C=C}$); 1571 (δ_{N-H}); 1542 ($\nu_{N=O\ ass.}$); 1453 (δ_{CH_2}); 1373 (δ_{CH_3}); 1340 ($\nu_{N=O\ sim.}$); 729 (δ_{C-H}); 645 (ν_{C-S}).

^{1}H NMR: δ 11.86 (s, 1H, H_8); 8.63 (s, 1H, H_6); 8.11 (d, 1H, H_4, $J = 4.35$ Hz); 7.72 (d, 2H, H_{11} and H_{15}, $J = 7.50$ Hz); 6.60 (d, 2H, H_{12} and H_{14}, $J = 7.50$ Hz); 7.51 (d, 1H, H_3, $J = 4.35$ Hz); 6.41 (t, 1H, NH, $J = 5.22$ Hz), 3.07 (dd, 2H, CH_2, $J = 6.91$ Hz, $J' = 5.22$ Hz); 1.54 (quintet, 2H, CH_2, $J = 6.91$ Hz); 1.39 (sextet, 2H, CH_2, $J = 6.91$ Hz); 0.91 (t, 3H, CH_3, $J = 6.91$ Hz).

^{13}C NMR: δ 164.18 (C_9); 153.18 (C_6); 151.16 (C_2); 148.47 (C_{13}); 139.86 (C_5); 131.43 (C_{10}); 130.52 (C_{12} and C_{14}); 129.62 (C_{11} and C_{15}); 126.29 (C_4); 119.18 (C_3); 42.87 (CH_2); 31.50 (CH_2); 20.63 (CH_2); 14.64 (CH_3).

EA: Calcd C, 52.74%; H, 4.98%; N, 15.37%. Experimental: C, 52.85%; H, 4.69%; N, 15.47%.

4.1.11. 4-Butyl-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{16}H_{17}O_3N_3S$) (11). Dark-yellow amorphous solid. Yield: 98%; mp 175.8–179.2 °C.

IR (KBr): ν 3250 (ν_{N-H}); 3110 (ν_{C-H}); 1652 ($\nu_{C=O}$); 1610 and 1490 ($\nu_{C=C}$); 1557 (δ_{N-H}); 1534 ($\nu_{N=O\ ass.}$); 1465 (δ_{CH_2}); 1393 (δ_{CH_3}); 1339 ($\nu_{N=O\ sim.}$); 729 (δ_{C-H}); 656 (ν_{C-S}).

^{1}H NMR: δ 12.18 (s, 1H, H_8); 8.67 (s, 1H, H_6); 8.14 (d, 1H, H_4, $J = 4.27$ Hz); 7.83 (d, 2H, H_{11} and H_{15}, $J = 7.90$ Hz); 7.36 (d, 2H, H_{12} and H_{14}, $J = 7.90$ Hz); 7.58 (d, 1H, H_3, $J = 4.27$ Hz); 2.65 (t, 2H, CH_2, $J = 7.63$ Hz); 1.57 (quintet, 2H, CH_2, $J = 7.63$ Hz); 1.32 (sextet, 2H, CH_2, $J = 7.63$ Hz), 0.90 (t, 3H, CH_3, $J = 7.63$ Hz).

^{13}C NMR: δ 163.03 (C_9); 151.61 (C_6); 147.92 (C_2); 147.73 (C_{13}); 141.71 (C_5); 131.41 (C_{10}); 131.12 (C_{12} and C_{14}); 130.43 (C_{11} and C_{15}); 129.33 (C_4); 128.67 (C_3); 35.56 (CH_2); 33.69 (CH_2); 22.59 (CH_2); 14.61(CH_3).

EA: Calcd C, 57.99%; H, 5.17%; N, 12.07%. Experimental: C, 58.32%; H, 5.30%; N, 12.77%.

4.1.12. 4-Ethyl-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{14}H_{13}O_3N_3S$) (12). Dark-yellow amorphous solid. Yield: 94%; mp 207.2–208.5 °C.

IR (KBr): ν 3310 (ν_{N-H}); 3105 (ν_{C-H}); 1649 ($\nu_{C=O}$); 1611 and 1503 ($\nu_{C=C}$); 1567 (δ_{N-H}); 1544 ($\nu_{N=O\ ass.}$); 14652(δ_{CH_2}); 1376 (δ_{CH_3}); 1342 ($\nu_{N=O\ sim.}$); 730 (δ_{C-H}); 660 (ν_{C-S}).

^{1}H NMR: δ 12.19 (s, 1H, H_8); 8.68 (s, 1H, H_6); 8.13 (d, 1H, H_4, $J = 4.30$ Hz); 7.84 (d, 2H, H_{11} and H_{15}, $J = 7.80$ Hz); 7.38 (d, 2H, H_{12} and H_{14}, $J = 7.80$ Hz); 7.58 (d, 1H, H_3, $J = 4.30$ Hz); 2.50 (q, 2H, CH_2, $J = 7.54$ Hz); 1.21 (t, 3H, CH_3, $J = 7.54$ Hz).

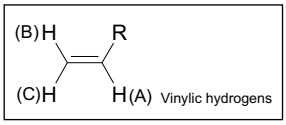
^{13}C NMR: δ 164.09 (C_9); 151.61 (C_6); 149.30 (C_2); 147.72 (C_{13}); 141.76 (C_5); 131.42 (C_{10}); 131.11 (C_{12} and C_{14}); 130.44 (C_{11} and C_{15}); 128.82 (C_4); 126.30 (C_3); 28.97 (CH_2); 16.19 (CH_3).

EA: Calcd C, 52.33%; H, 4.08%; N, 13.08%. Experimental: C, 53.01%; H, 4.14%; N, 13.95%.

4.1.13. 4-Vinyl-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{14}H_{11}O_3N_3S$) (13). Dark-yellow amorphous solid. Yield: 90%; mp 202.1–204 °C.

IR (KBr): ν 3300 (ν_{N-H}); 3105 (ν_{C-H}); 1658 ($\nu_{C=O}$); 1608 and 1496 ($\nu_{C=C}$); 1573 (δ_{N-H}); 1529 ($\nu_{N=O\ ass.}$); 1328 ($\nu_{N=O\ sim.}$); 1003 e 903 ($\delta_{C-H,vinyl}$); 729 (δ_{C-H}); 655 (ν_{C-S}).

^{1}H NMR: δ 12.18 (s, 1H, H_8); 8.65 (s, 1H, H_6); 8.10 (d, 1H, H_4, J = 4.24 Hz); 7.85 (d, 2H, H_{11} and H_{15}, J = 7.63 Hz); 7.60 (d, 2H, H_{12} and H_{14}, J = 7.64 Hz); 7.54 (d, 1H, H_3, J = 4.24 Hz); 6.77 (dd, 1H, H_A, J_{AB} = 10.95 Hz, J_{AC} = 6.70 Hz); 5.91 (d, 1H, H_B, J_{BC} = 1.70 Hz); 5.37 (d, 1H, H_C, J_{CB} = 1.70 Hz).



^{13}C NMR: δ 161.62 (C_9); 147.63 (C_6); 142.01 (C_2); 141.59 (C_{13}); 136.66 (C_5); 136.01 (CH); 131.42 (C_{10}); 130.55 (C_{12} and C_{14}); 129.03 (C_{11} and C_{15}); 127.08 (C_4); 126.29 (C_3), 117.76 (CH_2).

EA: Calcd C, 52.66%; H, 3.47%; N, 13.16%. Experimental: C, 52.87%; H, 3.60%; N, 13.31%.

4.1.14. 4-Acetyl-benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazide ($C_{12}H_{11}O_4N_3S$) (14). Dark-yellow amorphous solid. Yield: 90%; mp 228.1–233 °C.

IR (KBr): ν 3355 (ν_{N-H}); 3095 (ν_{C-H}); 1676 ($\nu_{C=O}$); 1607 and 1494 ($\nu_{C=C}$); 1584 (δ_{N-H}); 1522 ($\nu_{N=O\ ass.}$); 1329 ($\nu_{N=O\ sim.}$); 727 (δ_{C-H}); 672 (ν_{C-S}).

^{1}H NMR: δ 12.29 (s, 1H, H_8); 8.60 (s, 1H, H_6); 7.92 (d, 1H, H_4, J = 4.20 Hz); 7.76–7.67 (m, 4H, H_{11}, H_{12}, H_{14} and H_{15}); 3.26 (s, 3H, CH_3).

^{13}C NMR: δ 163.45 (C_9); 158.20 (C=O); 153.18 (C_6); 147.34 (C_2); 144.63 (C_{13}); 142.61 (C_5); 135.44 (C_{10}); 131.34 (C_{12} and C_{14}); 130.81 (C_{11} and C_{15}); 129.92 (C_4); 128.93 (C_3); 27.86 (CH_3).

EA: Calcd C, 52.99%; H, 3.49%; N, 13.24%. Experimental: C, 53.97%; H, 3.83%; N, 14.12%.

4.2. Microbiological assay—antibacterial activity in vitro

The inoculum was prepared with fresh cultures of bacterial strains, cultured on plate count agar (PCA—Merck, Germany) for 18 h at 35 °C. The density of the inoculum was adjusted according to Mac Farland n.1 scale.[18]

The minimal inhibitory concentration, MIC, was determined by the broth twofold macro-dilution method in Tryptic Soy Broth (TSB—Difco Laboratories, Detroit, USA), using the serial dilution tests[18,23] in two sequential steps against standard (ATCC 25923) and multidrug-resistant (3SP/R33)[19] *S. aureus* strains. Initially, stock solutions of *p*-substituted benzoic acid [(5-nitro-thiophen-2-yl)-methylene]-hydrazides were prepared in dimethylsulfoxide (DMSO—Merck, Germany) and then diluted in culture medium, TSB. The tubes were inoculated with a standardized number of microorganisms and incubated at 35 °C for 18 h, after which the tubes were examined for visible signs of bacterial growth. MIC was defined as the lowest concentration of a compound that completely inhibited the bacterial growth. All experiments were performed in quadruplicate.

The minimal bactericidal concentration (MBC) was determined according to the Kirby–Bauer method[24] using Müller–Hinton agar medium (Difco Laboratories, Detroit, USA). The MBCs were measured by subculturing an aliquot of 10 μL from each sample that remained clear in the MIC determination tubes. Each aliquot was spread across the entire surface of 15-cm diameter plates containing 70 mL of Müller–Hinton agar medium. The plates were incubated at 35 °C for 24 h.

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