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Synthesis and evaluation of antimicrobial activity of hydrazones derived from 3-oxido-1*H*-imidazole-4-carbohydrazides

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ABSTRACT

In this work we reported the synthesis and evaluation of *in vitro* antimicrobial activities of hydrazones **6** obtained from 3-oxido-1*H*-imidazole-4-carbohydrazides **4**. All new compounds were characterized by spectroscopic methods. Hydrazones **6** were tested for their *in vitro* antimicrobial activity against four Gram-positive and four Gram-negative strains of bacteria as well as one fungal species. Three of the tested compounds appeared to be promising agents against reference strains of *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. They were also tested against twelve clinical isolates of *S. aureus* and their cytotoxic effect on murine fibroblasts and HeLa human tumor cell line was determined.

1. Introduction

Infectious diseases caused by microorganisms are one of the main reasons of death in the world. The search for new antibacterial and antifungal drugs is like never-ending story because of the increasing resistance of microbial pathogens. It is desirable to find drugs with improved potency and wide activity spectrum. Acid hydrazides and the corresponding hydrazones are well known as a class of compounds with diverse biological activities [1–3]. Hydrazides of imidazolecarboxylic acids and their derivatives, e.g., hydrazones, thiosemicarbazides and 1,2,4-triazoles have been also reported to exhibit antibacterial, fungicidal, antiparasitic, anti-inflammatory, antitumor, antinociceptive, anticonvulsant, antihypertensive, and antidepressant properties [4–18]. Moreover, nitroimidazoles such as metronidazole, tinidazole, ornidazole are well known as antibacterial agents [19,20].

The aim of the present study was the synthesis of new hydrazones derived from 3-oxido-1*H*-imidazole-4-carbohydrazides and evaluation of their biological activity.

2. Results and discussion

2.1. Chemistry

In a series of recent reports, methods of synthesis and reactivity of new imidazole derivatives bearing 3-oxido function were described [21–26]. General strategy applied for the construction of the central imidazole ring is based on condensation of the corresponding α-hydroxyiminoketones with methylideneamines. Thus, starting with ethyl 2-hydroxyimino-3-oxobutanoate **1** and corresponding methylideneamines **2a**–**2c** expected ethyl 3-oxidoimidazole-4-carboxy late **3** can be smoothly obtained at room temperature in glacial acetic acid [22] (Scheme 1). Upon treatment with hydrazine hydrate, esters **3** were converted into carbohydrazides **4**, which after isolation and purification were used for reactions with aldehydes and ketones **5** [25].

Reactions of **4** with *p*-nitrobenzaldehyde and 5-nitrofurfural required heating in MeOH solution, whereas analogous reactions with benzaldehyde and other aromatic aldehydes containing electron donating substituent, were carried out at room temperature. In all cases expected hydrazones **6** were formed as single isomers, and isolated as crystalline materials in good to excellent yields (Table 1).

Their structures were confirmed based on spectroscopic data (^{1}H NMR, ^{13}C NMR, IR, HR-MS). For example, the ^{1}H NMR spectrum

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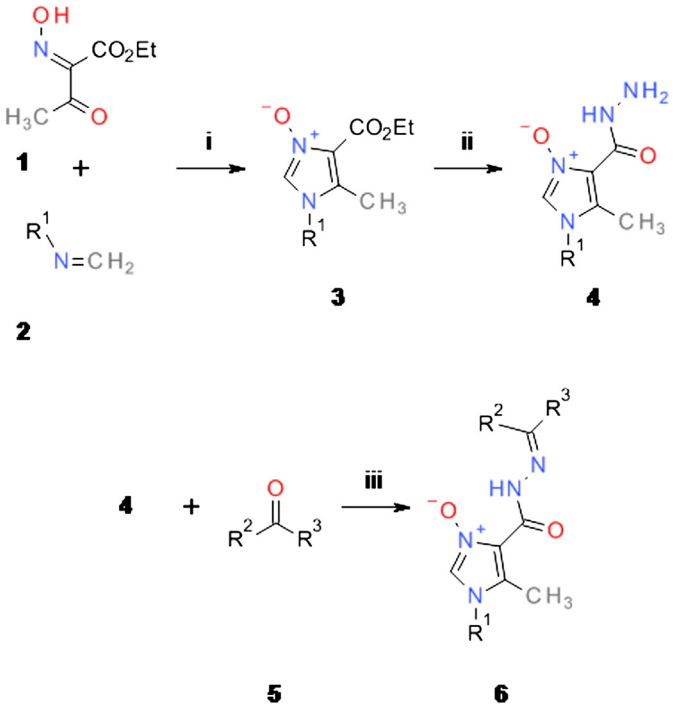

Scheme 1. Reagents and conditions: (i) AcOH, r. t., 16 h; (ii) $NH_2NH_2 \cdot H_2O$, r.t., 16 h; (iii) MeOH, r.t., 16 h or heating, 2 h.

of hydrazone **6i** reveals the presence of two characteristic signals at 8.76 and 8.41 ppm attributed to HC(2) of imidazole and HC=N protons, correspondingly. In the ^{13}C NMR spectra absorption signals of the C=O and C=N groups were found at 156.8 and 136.9 ppm, respectively. In all compounds typical signals of HC(2) protons of imidazole ring appeared between 8.55 and 8.78 ppm.

2.2. Biological activities

The *in vitro* antimicrobial activities of the compounds **6a**–**6i**, at concentrations ranging from 1 to 600 μg/mL, were screened using the microdilution method against four Gram-negative and four Gram-positive reference species of bacteria and one fungal species. The results showed that compounds **6a**–**6h** were inactive against all tested bacteria and yeast (data not shown). Only compound **6i** showed strong antibacterial activity against some Gram-negative and Gram-positive bacteria. Due to the high activity of the tested compound **6i** an attempt was made to potentially improve its antimicrobial effect by introducing some modifications. Thus, on the basis of **6i** two more compounds were prepared, bearing 5-nitrofuryl group, differing from each other by substituent at N(1) atom in the imidazole ring. We selected small alcylic methyl group (**6j** R^1 = Me) and sterically more crowded cyclohexyl group (**6k** R^1 = cyclohexyl). It is worth mentioning that compounds bearing 5-nitrofuryl substituent have limited solubility in DMSO, which was increased by transferring compounds **6i** and **6j** into their hydrochloride salts. Such modification had no influence on their antibacterial activity. Compounds **6j** and **6k** were similarly tested against reference microorganisms and showed positive although slightly lower antibacterial activity than the initial one.

Table 1
Yields and melting points of the new hydrazones **6**.

	R^1	R^2	R^3	Yield [%]	mp [°C]
6a	Bn	H	Ph	92	232–234
6b	Bn	–$(CH_2)_5$–		98	220–222
6c	Bn	Me	Ph	76	222–226
6d	Bn	H	*p*-MeO-C_6H_4	90	221–222
6e	Bn	H	*p*-$(Me)_2N$–C_6H_4	89	252–254
6f	Bn	H	*o*-HO–C_6H_4	89	229–232
6g	Bn	H	Furyl	78	220–221
6h	Bn	H	*p*-NO_2–C_6H_4	97	264–265
6i	Bn	H	5-NO_2–furyl	87	237–240
6j	Me	H	5-NO_2–furyl	84	313–315
6k	cHex	H	5-NO_2–furyl	76	222–225

The *in vitro* results of antibacterial activity of the three compounds **6i**–**6k** are presented in Table 2 as a minimal inhibitory concentration (MIC) and a minimal bactericidal concentration (MBC). Among the Gram-positive species, the most sensitive to all of the active compounds was *Staphylococcus epidermidis*, and in case of **6i** and **6k** (MIC = 4 μg/mL) the results were similar to the control antibiotic vancomycin (MIC = 3 μg/mL). The most effective against both reference *Staphylococcus aureus* strains was compound **6i** (MIC = 11 μg/mL) although its activity was lower than that exhibited by vancomycin and oxacillin, which are the antibiotics commonly used in the therapy of staphylococcal infections. Among three tested compounds, **6i** was also the most effective against *Escherichia coli* reference strain (MIC = 11 μg/mL), which was higher activity than in case of nitrofurantoin but lower then for chloramphenicol (MIC = 16, and 0.25, respectively) – two antibiotics used in a treatment of *E. coli* infections.

Compounds that showed activity in this test were then examined using the disc diffusion method in order to determine growth inhibition zones of susceptible species of pathogens (Table 3). These results confirmed inhibitory activity of the tested compounds with the largest growth inhibition zones obtained for *S. epidermidis*.

Considering good activity of the tested **6i**, **6j**, and **6k** compounds against microorganisms of *Staphylococcus* spp., a set of 12 *S. aureus* clinical strains including the ones isolated from 2 typical sources such as naso-pharynx (carrier state) and ulcers/furuncles (skin and soft tissue infections), but also those from infected bones (invasive infections) was tested against these agents. Similarly to the reference *Staphylococcus* spp. strains clinical isolates displayed high level of susceptibility to the analyzed compounds (MIC ranging from 10 to 100 μg/mL) (Table 4). Compound **6i** again showed the strongest activity, with MIC being equal to 10 μg/mL for all of the tested clinical isolates, which for most of the strains was a better result than in case of vancomycin used as the positive control antibiotic. On the other hand, the second common antibiotic – oxacillin showed up to be the most active in almost all cases (MIC = 0.2–1.5 μg/mL), except for the two strains isolated from bones (*S. aureus* D15, and D17, MIC = 75 μg/mL) which were 7 times more sensitive to **6i** compound.

For all three tested compounds the MBC values were in the similar range, suggesting their bactericidal activity (MBC/MIC ≤ 4) (Table 4).

S. aureus permanently colonizes the epithelium of more than 20% of the population and also is one of the common human pathogens. These bacteria cause wide range of diseases: from acute infections involving staphylococcal toxins and enzymes (e.g. ulcers, furuncles, food poisoning, bacteremia, septic shock) to chronic infections, usually associated with biofilm formation (e.g. infections developed after invasive medical procedures using biomaterial devices, wounds infections, arthritis, osteomyelitis, endocarditis, bronchopulmonary infections in patients with cystic fibrosis) [27,28]. The emergence of multidrug-resistant *S. aureus* strains, including those resistant to β-lactams, glycopeptides, MLS-B (macrolides, lincosamides, streptogramin B), aminoglycosides, tetracyclines, fluoroquinolones, and even linezolid generate significant problem for the treatment of staphylococcal infections [29–31]. Moreover, these bacteria very easily form biofilm, which confers them severe

Table 2
In vitro antibacterial activity of **6i**, **6j**, **6k** expressed as a minimal inhibitory concentration (MIC) [μg/mL] and a minimal bactericidal concentration (MBC) [μg/mL].

Compound	**6i**		**6j**		**6k**		Vancomycin		Oxacillin		Chloramphenikol		Nitrofurantoin	
Tested strain	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli NCTC 8192	11	11	25	100	27	27	na[b]	na	na	na	0.25	0.5	16	32
S. aureus ATCC 6538	11	11	25	100	18	18	1.5	1.5	0.2	0.2	1	4	16	32
S. aureus ATCC 29213	11	11	25	100	18	18	1.5	3	0.2	0.2	1	4	16	32
P. vulgaris ATCC 49990	>600	nt	>600	nt	>600	nt	nt	nt	nt	nt	nt	nt	nt	nt
P. mirabilis ATCC 29906	>600	nt	>600	nt	>600	nt	nt	nt	nt	nt	nt	nt	nt	nt
E. faecalis ATCC 29212	>600	nt	>600	nt	>600	nt	nt	nt	nt	nt	nt	nt	nt	nt
S. epidermidis ATCC 12228	4	4	25	75	4	4	3	3	0.2	0.2	0.5	2	8	32
P. aeruginosa NCTC 6249	>600	nt	>600	nt	>600	nt	nt	nt	nt	nt	nt	nt	nt	nt
C. albicans ATCC 10231	>600	nt[a]	>600	nt	>600	nt	nt	nt	nt	nt	nt	nt	nt	nt

[a] nt – not tested.
[b] na – no activity.

resistance to antibiotics, primarily as the result of its complex structure, unique physiology and facilitated gene transfer within biofilm [28,32].

Within *E. coli* species, several strains are associated with serious infections, usually manifesting as inflammatory lesions in the intestines, but also cause systemic infections like hemolytic uremic syndrome with hemolytic anemia, thrombocytopenia and acute renal failure [33]. Enterobacteria resistant to antibiotics, such as extended-spectrum beta-lactamases (ESBL) producing *E. coli*, are very important therapeutic problem, particularly in hospitals. It is also worth mentioning that the long-term studies of trends in antimicrobial susceptibility performed by the European Antimicrobial Resistance Surveillance Network (EARS-Net) collecting data from 198 laboratories in 22 European countries in the period from 2002 to 2009, identified *E. coli* and *S. aureus* as two main causes of bloodstream infections and revealed significant rise of drug resistance in *E. coli* [34] In the light of increasing resistance of microorganisms to chemotherapeutic agents and the involvement of their biofilms in the development of pathological changes, it seems necessary to design new strategies for infection treatment.

The cytotoxic activities of **6i**–**6k** agents were assessed using L929 murine cell line (recommended by the International Standard ISO 10993:2009 for evaluation of cytotoxic activities) as well as HeLa human tumor cell line. The percentage of viability inhibition compared to the negative control in which cells were grown in the absence of tested compounds was estimated for concentrations ranging from 5 to 300 μg/mL of the compound (Figs. 1 and 2). The **6i** (LC_{50} = 277 μg/mL for L929 and LC_{50} > 300 μg/mL for HeLa) and **6j** (LC_{50} = 168 μg/mL for L929 and LC_{50} > 300 μg/mL for HeLa) showed weak cytotoxic effect on both tested cell lines. The toxicity of compound **6k** was more than four-fold higher for L929 line (LC_{50} = 38 μg/mL) and three-fold higher for HeLa cells (LC_{50} = 111 μg/mL).

3. Conclusion

The presented results show that 3-oxido-1*H*-imidazole-4-carbohydrazides **4** can be easily converted into hydrazones **6** via reactions with diverse electron-poor as well as electron-rich substituted aromatic aldehydes, and ketones **5**. The new synthesized hydrazones **6** were evaluated *in vitro* against bacterial and fungal species.

There are many known biological activities of hydrazones derived from carbohydrazides, however among nine products initially synthesized and tested in this study, only **6i** bearing 5-nitrofuryl group showed antibacterial activity. The evidence of the importance of this group in biological activity was confirmed by the fact that other tested compounds which differed only in the type of substituent in R^3 (Scheme 1) did not display any antibacterial activity. Among them, especially **6g** containing non-substituted furan ring proved that the presence of the nitro group was important for acquiring antibacterial activity. At the same time hydrazone **6h** containing *p*-nitrophenyl group instead of 5-nitrofuryl substituent did not inhibit growth of the tested microbes, what indicated that the nitro-substituted furan ring is essential for the antibacterial activity of the described hydrazones derived from 3-oxido-1*H*-imidazoles. In order to test this hypothesis two other compounds, **6j** and **6k** were synthesized, which similarly to the active **6i** contained 5-nitrofuryl substituent in R^3 and differed only in the type of R^1 (Table 1). Well-defined antibacterial activity of both compounds confirmed eventually the role of this group in conferring antibacterial activity on this type of imidazole *N*-oxides derivatives.

Table 3
In vitro antibacterial activity of **6i**, **6j**, **6k** expressed as the growth inhibition zones (giz) [mm].

Tested strain	Compound		
	6i	**6j**	**6k**
E. coli NCTC 8192	7	8	7
S. aureus ATCC 6538	13	10	10
S. aureus ATCC 29213	15	10	10
S. epidermidis ATCC 12228	20	11	13

Table 4
In vitro activity of **6i**, **6j**, **6k** against clinical isolates of *S. aureus* expressed as the minimal inhibitory concentration (MIC) [μg/mL] and minimal bactericidal concentration (MBC) [μg/mL].

Compound	**6i**		**6j**		**6k**		Vancomycin		Oxacillin	
Tested strain	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Naso-pharynx isolates										
S. aureus C4	10	25	100	100	10	25	10	10	0.4	0.4
S. aureus C7	10	25	100	100	25	25	35	35	0.2	0.4
S. aureus C8	10	10	100	100	25	25	75	75	1.5	1.5
S. aureus C19	10	10	100	100	25	25	75	75	0.2	0.2
Ulcers/furuncles isolates										
S. aureus D12	10	10	100	100	25	110	75	75	0.2	0.4
S. aureus F1	10	10	100	200	25	25	75	75	0.8	0.8
S. aureus F7	10	25	100	100	25	25	35	35	0.2	0.2
S. aureus F12	10	10	100	100	25	110	10	10	0.2	0.4
Bone isolates										
S. aureus D14	10	10	100	100	25	110	75	75	0.2	0.2
S. aureus D15	10	10	100	100	25	25	75	75	75	150
S. aureus D17	10	10	100	100	25	110	75	75	75	150
S. aureus D20	10	10	100	100	25	25	75	75	0.4	0.4

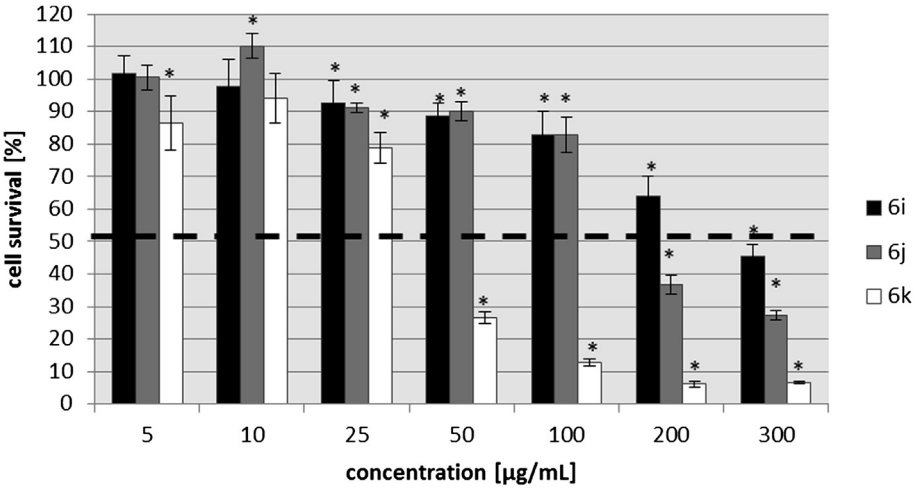

Fig. 1. Concentration-dependent cytotoxic activity of compound **6i** (black), **6j** (gray) and **6k** (white) for L929 cell line. * Denotes significant difference ($P < 0.05$) when compared with control.

Diverse compounds containing nitrofuran moiety are known for their antibacterial activity and are extensively reported in the literature. For example, nitrofurantoin (*N*-(5-nitro-2-furfurylidene)-1-aminohydantoin) is the common chemotherapeutic used in urinary tract infections, showing activity against Gram-positive as well as Gram-negative bacteria [35]. Similarly, nifuroxazide (4-hydroxy-*N*′-[(4-nitrocyclopenta-1,3-dienyl)methylene]benzohydrazide) is used in gastrointestinal tract infections. Biological activity of these drugs is conferred by the presence of nitrofuryl group which is reduced inside bacterial cells by nitroreductases and thereby generating hydroxy radicals. These highly reactive forms of oxygen are believed to inhibit protein biosynthesis [35,36]. Additionally, it has been shown that nitro group at R^2 α-position of the furan ring increases genotoxicity of furan derivatives leading to double strand breaks in DNA helix [37]. Thus, it is very likely that the mechanism of antibacterial activity of **6i**, **6j**, and **6k** compounds is similar.

Biological activities of hydrazones derived from 5-nitro-2-furylaldehyde and carbohydrazides are well known [1–3], but our data reports for the first time antimicrobial activities of imidazole-4-carboxylic acid hydrazide hydrazones containing the unique *N*-oxide function. Moreover, in the literature there are only few examples known reporting biological activities of imidazole *N*-oxides derivatives, such as antitumor [38], herbicidal [39] or antiprotozoal [40–42]. Derivatives **6i**–**6k** obtained from 5-nitro-2-furylaldehyde showed promising antimicrobial activity which, together with their low cytotoxicity, make them good candidates in the search for effective antibacterial drugs to combat most burdensome microorganisms, among which both *S. aureus* and *E. coli* but also *S. epidermidis* pose a significant threat to public health.

4. Experimental

4.1. Chemistry

General: Melting points were determined in a capillary using a Melt-Temp. II apparatus (Aldrich) or STUART SMP30 and are uncorrected. The IR Spectra were recorded on a NEXUS FT-IR spectrophotometer; absorptions (ν) in cm^{-1}. The ^1H- (600 MHz), $^{13}C\{^1H\}$- (150 MHz) spectra were measured on a Bruker Avance III instrument using solvent signals as reference. Chemical shifts (δ) are given in ppm and coupling constants J in Hz. Assignments of signals in ^{13}C NMR spectra were made on the basis of HMQC experiments. HR-ESI-MS: Bruker Esquire LC spectrometers.

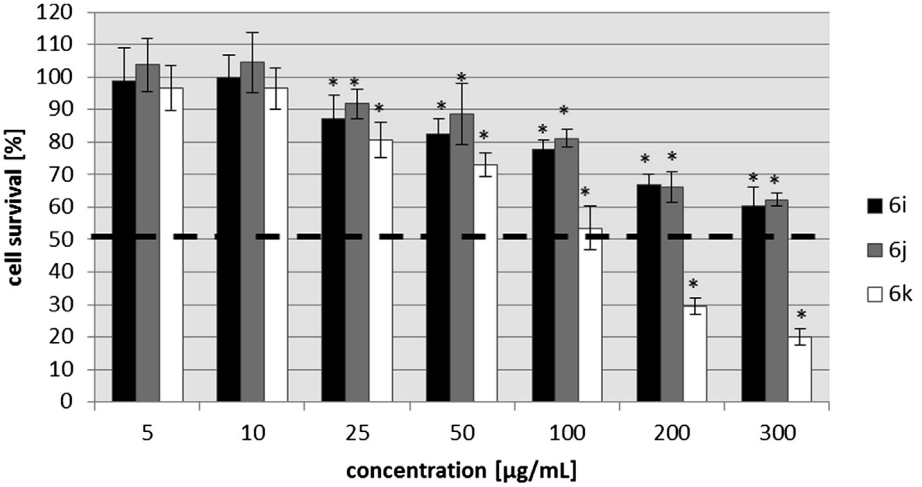

Fig. 2. Concentration-dependent cytotoxic activity of compound **6i** (black), **6j** (gray) and **6k** (white) for HeLa cell line. * Denotes significant difference ($P < 0.05$) when compared with control.

4.1.1. Starting materials

All solvents are commercially available and they were used as received. Ethyl 3-oxido-1*H*-imidazole-4-carboxylate **3** was obtained according to published procedure [22]. Carbohydrazides **4** were obtained from **3** by treatment with hydrazine hydrate following previously published protocol [25].

*4.1.2. General procedure for synthesis of hydrazones **6***

To a stirred solution of a hydrazide **4** (1 mmol) in MeOH (5 mL) at 20 °C, an equimolar quantity of the carbonyl component **5** was added slowly. The mixture was stirred for 16 h at room temperature (**6a**–**6g**), or refluxed for 2 h (**6h**–**6k**), the solution was concentrated, the resulting solid was treated with Et_2O, filtered, and crystallized from MeOH.

1-Benzyl-5-methyl-*N*′-[(*Z*)-phenylmethylidene]-1*H*-imidazole-4-carbohydrazide 3-oxide (**6a**), 1-Benzyl-*N*′-cyclohexylidene-5-methyl-1*H*-imidazole-4-carbohydrazide 3-oxide (**6b**), 1-Benzyl-5-methyl-*N*′-[(1*Z*)-1-phenylethylidene]-1*H*-imidazole-4-carbohydra zide 3-Oxide (**6c**), were described in our earlier paper [25].

*4.1.2.1. 1-Benzyl-5-methyl-N′-[(Z)-(4-methoxyphenyl)methylidene]-1H-imidazole-4-carbohydrazide 3-oxide (**6d**).* Colorless crystals; yield: 90%; mp 221–222 °C (MeOH). IR (ν_{max}, cm^{-1}, KBr): 3083*m*, 1663*vs* (C=O), 1607*vs*, 1510*m*, 1254*m*, 1170*m*. ^{1}H NMR (600 MHz, DMSO-d_6, δ, ppm): 14.24 (1H, br. s, NH); 8.72 (1H, s, HC(2)); 8.28 (1H, s, HC=N); 7.69, 7.01 (4H, *AA′BB′*, J_{AB} = 9.0, Ar–H); 7.42–7.26 (5H, m, Ar–H); 5.25 (2H, s, CH_2); 3.80 (3H, s, OMe); 2.48 (3H, s, Me). ^{13}C NMR (150 MHz, ()DMSO-d_6, δ, ppm): 161.4 (C=O); 156.3, 135.8, 127.4 (3 arom. C); 148.6 (C=N); 131.8, 120.9 (C(4), C(5)); 129.5, 129.3, 128.6, 127.7, 114.8 (9 arom. CH); 126.9 (C(2)); 55.8 (OMe); 48.8 (CH_2); 9.9 (Me). HR-ESI-MS: 365.1608 ([M + H]$^+$, $C_{20}H_{21}N_4O_3^+$; calc. 365.1606).

*4.1.2.2. 1-Benzyl-5-methyl-N′-[(Z)-(4-dimethyloaminophenyl)methylidene]-1H-imidazole-4-carbohydrazide 3-oxide (**6e**).* Colorless crystals; yield: 89%; mp 252–254 °C (MeOH). IR (ν_{max}, cm^{-1}, KBr): 3088*m*, 2906*m*, 1664*vs* (C=O), 1603*vs*, 1524*m*, 1368*m*, 1181*m*. ^{1}H NMR (600 MHz, DMSO-d_6, δ, ppm): 14.10 (1H, br. s, NH); 8.71 (1H, s, HC(2)); 8.15 (1H, s, HC=N); 7.56, 6.74 (4H, *AA′BB′*, J_{AB} = 9.0, Ar–H); 7.42–7.26 (5H, m, Ar–H); 5.25 (2H, s, CH_2); 2.97 (6H, s, Me_2N); 2.48 (3H, s, Me). ^{13}C NMR (150 MHz, ()DMSO-d_6, δ, ppm): 160.0 (C=O); 152.1, 135.8, 122.0 (3 arom. C); 149.4 (C=N); 131.5, 121.1 (C(4), C(5)); 129.5, 129.1, 128.6, 127.7, 112.2 (9 arom. CH); 126.9 (C(2)); 48.7 (CH_2); 40.5 (Me_2N); 9.9 (Me). HR-ESI-MS: 378.1923 ([M + H]$^+$, $C_{21}H_{24}N_5O_2^+$; calc. 378.1924).

*4.1.2.3. 1-Benzyl-5-methyl-N′-[(Z)-(2-hydroxyphenyl)methylidene]-1H-imidazole-4-carbohydrazide 3-oxide (**6f**).* Colorless crystals; yield: 89%; mp 229–232 °C (MeOH). IR (ν_{max}, cm^{-1}, KBr): 3140*m*, 1670*vs* (C=O), 1616*s*, 1590*m*, 1277*m*. ^{1}H NMR (600 MHz, DMSO-d_6, δ, ppm): 14.55 (1H, br. s, NH); 8.76 (1H, s, HC(2)); 8.58 (1H, s, HC=N); 7.54–6.90 (9H, m, Ar–H); 5.27 (2H, s, CH_2); 2.49 (3H, s, Me). ^{13}C NMR (150 MHz, DMSO-d_6, δ, ppm): 157.9 (C=O); 156.3, 135.7, 120.5 (3 arom. C); 149.6 (C=N); 132.0, 119.1 (C(4), C(5)); 132.2, 130.3, 128.7, 119.8, 116.9 (9 arom. CH); 127.1 (C(2)); 48.8 (CH_2); 9.9 (Me). HR-ESI-MS: 351.1449 ([M + H]$^+$, $C_{19}H_{19}N_4O_3^+$; calc. 351.1452).

*4.1.2.4. 1-Benzyl-5-methyl-N′-[(Z)-2-furylmethylidene]-1H-imidazole-4-carbohydrazide 3-oxide (**6g**).* Colorless crystals; yield: 78%; mp 220–221 °C (MeOH). IR (ν_{max}, cm^{-1}, KBr): 3138*m*, 1656*vs* (C=O), 1627*s*, 1593*s*, 1276*m*. ^{1}H NMR (600 MHz, DMSO-d_6, δ, ppm): 14.30 (1H, br. s, NH); 8.73 (1H, s, HC(2)); 8.25 (1H, s, HC=N); 7.84, 6.89, 6.62 (3H, 3d, J = 3.6, furyl Ar–H); 7.42–7.25 (5H, m, Ar–H); 5.25 (2H, s, CH_2); 2.48 (3H, s, Me). ^{13}C NMR (150 MHz, DMSO-d_6, δ, ppm): 156.5 (C=O); 149.9, 135.8, (2 arom. C); 138.6 (C=N); 132.0, 120.8 (C(4), C(5)); 145.8, 129.5, 128.6, 127.7, 114.7, 112.6 (8 arom. CH); 127.0 (C(2)); 48.8 (CH_2); 9.9 (Me). HR-ESI-MS: 325.1295 ([M + H]$^+$, $C_{17}H_{17}N_4O_3^+$; calc. 325.1295).

*4.1.2.5. 1-Benzyl-5-methyl-N′-[(Z)-(4-nitrophenyl)methylidene]-1H-imidazole-4-carbohydrazide 3-oxide (**6h**).* Yellowish crystals; yield: 97%; mp 264–265 °C (MeOH). IR (ν_{max}, cm^{-1}, KBr): 3081*m*, 1665*vs* (C=O), 1614*m*, 1519*s*, 1342*s*. ^{1}H NMR (600 MHz, DMSO-d_6, δ, ppm): 14.63 (1H, br. s, NH); 8.78 (1H, s, HC(2)); 8.52 (1H, s, HC=N); 8.30, 7.99 (4H, *AA′BB′*, J_{AB} = 9.0, Ar–H); 7.42–7.27 (5H, m, Ar–H); 5.27 (2H, s, CH_2); 2.50 (3H, s, Me). ^{13}C NMR (150 MHz, DMSO-d_6, δ, ppm): 156.8 (C=O); 146.6 (C=N); 141.2, 135.7, 122.0 (3 arom. C); 132.5, 120.7 (C(4), C(5)); 129.5, 128.7, 128.5, 127.7, 124.5 (9 arom. CH); 127.1 (C(2)); 48.9 (CH_2); 9.9 (Me). HR-ESI-MS: 380.1350 ([M + H]$^+$, $C_{19}H_{18}N_5O_4^+$; calc. 380.1353).

*4.1.2.6. 1-Benzyl-5-methyl-N′-[(Z)-(5-nitro-2-furyl)methylidene]-1H-imidazole-4-carbohydrazide 3-oxide (**6i**).* Yellowish crystals; yield: 89%; mp 237–240 °C (MeOH). IR (ν_{max}, cm^{-1}, KBr): 3161*m*, 1680*vs* (C=O), 1607*s*, 1474*s*, 1362*m*, 1254*m*. ^{1}H NMR (600 MHz, DMSO-d_6, δ, ppm): 14.71 (1H, br. s, NH); 8.76 (1H, s, HC(2)); 8.41 (1H, s, HC=N); 7.77, 7.17 (2H, 2d, J = 4.2, furyl Ar–H); 7.42–7.27 (5H, m, Ar–H); 5.27 (2H, s, CH_2); 2.49 (3H, s, Me). ^{13}C NMR (150 MHz, DMSO-d_6, δ, ppm): 156.8 (C=O); 152.2, 135.7, 127.7 (3 arom. C); 136.9 (C=N); 132.7, 120.6 (C(4), C(5)); 129.5, 128.7, 127.7, 116.3, 115.0 (7 arom. CH); 127.2 (C(2)); 48.9 (CH_2); 10.0 (Me). HR-ESI-MS: 370.1145 ([M + H]$^+$, $C_{17}H_{16}N_5O_5^+$; calc. 370.1146).

*4.1.2.7. 1,5-Dimethyl-N′-[(Z)-(5-nitro-2-furyl)methylidene]-1H-imidazole-4-carbohydrazide 3-oxide (**6j**).* Yellowish crystals; yield: 84%; mp 313–315 °C (MeOH). IR (ν_{max}, cm^{-1}, KBr): 3149*m*, 1680*vs* (C=O), 1603*m*, 1477*s*, 1355*s*, 1269*m*. ^{1}H NMR (600 MHz, DMSO-d_6, δ, ppm): 14.77 (1H, br. s, NH); 8.55 (1H, s, HC(2)); 8.39 (1H, s, HC=N); 7.77, 7.16 (2H, 2d, J = 4.2, furyl Ar–H); 3.59 (3H, s, NMe); 2.54 (3H, s, Me). ^{13}C NMR (150 MHz, DMSO-d_6, δ, ppm): 158.8 (C=O); 152.2, 136.7 (2 arom. C); 136.9 (C=N); 132.5, 120.7 (C(4), C(5)); 116.2, 115.1 (2 arom. CH); 127.2 (C(2)); 32.5 (NMe); 9.7 (Me). HR-ESI-MS: 294.0830 ([M + H]$^+$, $C_{11}H_{12}N_5O_5^+$; calc. 294.0833).

*4.1.2.8. 1-Cyclohexyl-5-methyl-N′-[(Z)-(5-nitro-2-furyl)methylidene]-1H-imidazole-4-carbohydrazide 3-oxide (**6k**).* Yellowish crystals; yield: 74%; mp 222–225 °C (MeOH). IR (ν_{max}, cm^{-1}, KBr): 3105*m*, 2939*m*, 2856*m*, 1675*vs* (C=O), 1590*s*, 1472*s*, 1350*m*, 1266*m*. ^{1}H NMR (600 MHz, DMSO-d_6, δ, ppm): 14.81 (1H, br. s, NH); 8.80 (1H, s, HC(2)); 8.40 (1H, s, HC=N); 7.77, 7.16 (2H, 2d, J = 4.2, furyl Ar–H); 4.14–4.09 (1H, m, CH); 2.61 (3H, s, Me); 1.96–1.80 (4H, m); 1.67–1.39 (5H, m); 1.21–1.13 (5H, m). ^{13}C NMR (150 MHz, DMSO-d_6, δ, ppm): 157.1 (C=O); 152.2, 133.7 (2 arom. C); 136.7 (C=N); 132.1, 119.9 (C(4), C(5)); 116.2, 115.0 (2 arom. CH); 124.9 (C(2)); 55.3 (CH); 32.9, 25.3, 24.9 (5 cyclohexyl CH_2); 9.7 (Me). HR-ESI-MS: 362.1455 ([M + H]$^+$, $C_{16}H_{20}N_5O_5^+$; calc. 362.1459).

4.2. Antibacterial assay

The *in vitro* antimicrobial activity of newly synthesized compounds was evaluated against the reference strains of Gram-negative (*E. coli* NCTC 8192, *Proteus vulgaris* ATCC 49990, *Proteus mirabilis* ATCC 29906, *Pseudomonas aeruginosa* NCTC 6249), Gram-positive (*S. aureus* ATCC 6538, *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *S. epidermidis* ATCC 12228) bacterial and fungal (*Candida albicans* ATCC 10231) species. Moreover, the most active (against the reference strains) compounds were examined against a set of twelve clinical isolates of *S. aureus* from the collection belonging to the Chair of Immunology and Infectious Biology,

University of Łódź. These strains were isolated from 3 sources: naso-pharynx of young patients hospitalized at Children's Hospital in Łódź ($n = 4$), ulcers and furuncles from adult patients of Dermatological Clinic in Łódź ($n = 4$), and from infected bones of patients hospitalized at Oncological Hospital in Łódź ($n = 4$). All strains were kept frozen at −80 °C on Tryptic Soy Broth with 15% of glycerol until testing. Before using, *S. aureus* strains were sub-cultured on blood agar and identified by routine methods (catalase, coagulase and clumping factor). Minimal inhibitory concentration (MIC) was determined as the lowest concentration of the compound preventing growth of the tested microorganism using microdilution method. The inoculum density was adjusted to 0.5 McFarland standard. All of the tested compounds were dissolved in dimethyl sulfoxide (DMSO). Concentration of the agents evaluated in Mueller–Hinton broth ranged from 1 to 600 μg/mL. DMSO at the final concentration in the medium had no influence on growth of the tested microorganisms. The incubation was carried out at 37 °C for 18 h and optical density (OD_{600}) measurements were determined for bacterial cultures in the presence and absence of the tested compounds. Vancomycin and oxacillin, widely used in the treatment of infectious diseases, were used as positive control antimicrobial agents for Gram-positive strains. Chloramphenicol was used as positive control for *E. coli*. Moreover, nitrofurantoin was used because of bearing 5-nitrofuryl group as our **6i**–**6k** compound. Minimal bactericidal concentration (MBC), defined as the lowest concentration of a compound that resulted in >99.9% reduction in CFU of the initial inocula (2×10^8 cfu) was assessed only for compounds **6i**–**6k**. MBC was determined by a broth microdilution technique followed by plating out the contents of the wells that showed no visible growth of bacteria onto Mueller–Hinton agar plates and incubating at 35 °C for 18 h. Both MIC and MBC values were performed in triplicates and are given in μg/mL, according to CLSI reference [43].

The disc diffusion method was used to determine activity of compound **6i**–**6k**. Sterile filter paper discs (6 mm in diameter) were soaked in compound solution to load 500 μM of compound per disc and dried. Dry discs were placed on the surface of Mueller–Hinton agar medium. Diameter of the growth inhibition zones was read after 18 h of incubation at 35 °C.

4.2.1. Cytotoxicity assay

Cytotoxic effect of compounds **6i**–**6k** on host cells was detected by determining cellular viability using MTT reduction assay. Mouse fibroblast cells L929 (ATTC® catalog no. CCL-1, mouse fibroblasts) or human tumor cells HeLa (ATTC® catalog no. CCL-2™, human epithelial cells) were plated in 96-well microplates at a density of 1×10^4 cells/mL (100 μL per well) and cultivated in Iscove's modified Dulbecco's medium (IMDM), supplemented with 100.0 U/mL penicillin and streptomycin, 5×10^{-5} M 2-mercapto ethanol and enriched with 10% fetal bovine serum (FBS). After overnight incubation at 37 °C, the growth medium was removed and 100 μL of medium supplemented with different concentrations of synthetic compounds in the range of 1–300 μg/mL were added. Cells were further incubated for 24 h with tested agents. At the end of the incubation time, the medium was removed and MTT was added to each well at a final concentration of 0.5 mg/mL and plates were incubated for another 2 h at 37 °C. Then, formazan crystals were solubilized in 150 μL DMSO. The optical density was measured at 550 nm. The results of experiments were shown as mean arithmetic values of eight repeats (two experiments), the percentage of viability inhibition compared to control wells was calculated for each concentration of the tested compounds, and LC_{50} values were determined. Analysis of statistical significance was performed using Mann–Whitney *U* test. During statistical verification, significance levels of $p < 0.05^*$ were considered.

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