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Synthesis and antimicrobial profile of *N*-substituted imidazolium oximes and their monoquaternary salts against multidrug resistant bacteria

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ABSTRACT

Two different series of *N*-substituted imidazolium oximes and their monoquaternary salts were synthesized and biologically tested with respect to their ability to inhibit growth a diverse panel of antibiotic susceptible Gram-positive and antibiotic resistant Gram-negative bacteria as well fungal strains. The newly synthesized compounds were analyzed by spectral studies to confirm their structure. The preliminary results showed that all compounds tested possess promising antimicrobial potential against both susceptible Gram-positive and antibiotic resistant Gram-negative isolates, exhibiting a wide range of MIC values from 0.14 to 100.0 μg/mL. The structure–activity relationship demonstrates that the *p*-methylphenyl and *p*-fluorophenyl groups in monoquaternary salts **6** and **7** attached directly to the imidazolium ring could be essential for observed remarkable inhibitory profiles against clinically important pathogens *Pseudomonas aeruginosa* (MIC = 0.14 μg/mL) and *Klebsiella pneumoniae* (MIC = 1.56 μg/mL). Furthermore, the broth microdilution assay was then used to investigate the antiresistance efficacy of compound **7** against fourteen extended-spectrum β-lactamase (ESBL)-producing strains in comparison to eight clinically relevant antibiotics. Compound **7** exhibited a remarkable antiresistance profiles ranging between 0.39 and 12.50 μg/mL against all of ESBL-producing strains, which leads to the suggestion that may be interesting candidate for development of new antimicrobials to combat multidrug resistant Gram-negative bacteria.

1. Introduction

The therapeutic treatment of bacterial and fungal diseases with antimicrobial drugs has had a remarkable and profound impact on human health around the world. The current worldwide emergence of resistance to extended spectrum cephalosporins, monobactams, and carbapenems among Gram-negative pathogenic bacteria constitutes an important growing public health threat. Infections caused by extended-spectrum β-lactamase (ESBL) producing pathogens have become a clinical and therapeutic problem because these organisms are resistant not only to β-lactam antibiotics but also to many other antimicrobial agents.[1] The dominant mechanisms for resistance to the Gram-negative bacteria are the production *of* clinically relevant β-lactamases, particularly extended-spectrum β-lactamases (ESBLs), such as class A TEM, SHV, and CTX-M β-lactamases.[2] Single amino acid substitutions in the SHV, TEM and CTX-M β-lactamases can drastically alter the substrate profiles of the enzymes and confer resistance to extended-spectrum cephalosporins and β-lactamase inhibitors.[1] Nonetheless, the recent increase and spread of acquired carbapenems resistance due to the production of metallo-β-lactamases (MBLs) such as SIM- and VIM-type enzymes already are starting to limit the clinical use of carbapenems.[3]

In addition to the infection control challenges that have arisen, infections caused by these bacterial and fungal pathogens present clinicians with serious treatment challenges, due to limited antibiotic options.[4] New strategies are therefore needed to identify and developed the next generation of drugs or agents with diverse chemical structures and novel mechanisms of action to control microbial infections. Thus, the search for effective molecules that have the ability to restore the susceptibility of multi-drug-resistant bacteria, such as clinical problematic ESBL- and MBL-containing strains, to clinically available antibiotics are a promising alternative to the development of novel antimicrobials.

In this regard, the prevention and treatment of these infectious diseases by applying heterocyclic imidazole derivatives as potential and promising sources of antimicrobial agents have been a focus of recent research for antibacterial drug discovery.[5,6] Among the numerous nitrogen heterocyclic compounds of biological and

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pharmacological interest, imidazole derivatives play very important role in chemistry as mediators for synthetic reactions primarily for drug discovery to a broad field of biological applications.[5,6] Moreover, imidazole derivatives are structural bioisosteres of naturally occurring nucleotides, which allows them to interact easily with the biopolymers of the living system, which is responsible for their promising biological activities and functions. In recent decades, research has indicated that the imidazole derivatives exhibiting a wide range of pharmacological activities, which included antitumor,[7] antimicrobial,[8] antiviral,[9] antitubercular activities.[10] Additionally, imidazoles are found to possess antifungal activity and form an essential part of the molecular structures of some antifungal agents.[11] A wide variety of imidazole containing compounds having high cytostatic and low cytotoxic effects on several cancer cell lines with different mechanisms of action have been developed over years.[5] They have also been utilized as a versatile template for the synthesis of compounds with potential the inhibition of β-lactamase enzymes.[12]

On the other hand, oximes represent versatile group of organic compounds that were extensively used as excellent ligands in pharmaceutical and synthetic applications as important precursor in new drug discovery, including some antibiotics.[13] Beside its antibacterial activity, oximes have been firstly reported as compounds with a great potential in the treatment of organophosphorus compounds poisoning including insecticides and nerve agents for acetylcholinesterase (AChE) reactivation.[14] From the view point of molecular design, more efficacious compounds can be designed by joining two or more biologically active system together in a single molecular framework. In the view of above mentioned facts we synthesized some *N*-substituted imidazolium oximes (series A) with phenyl, *p*-methyl, *p*-fluorophenyl or benzyl substituent on imadazolim ring. The most commonly used antibacterial reagents, benzalkonium chloride is monoquaternary ammonium salts containing benzyl moiety, so we also synthesized newly benzyl substituted compounds (series B) from *N*-substituted imidazolium oximes. It has been hoped that combination of these active groups in the new molecular design would lead to the development of adjuvants molecules that either directly target resistance mechanisms such as the inhibition of β-lactamase enzymes. Initially we wish to identify for the first time antimicrobial efficacy of each compounds (from series A and B) by using a disc diffusion assay as well as broth microdilution assay against a panel of both susceptible Gram-positive and clinically relevant multidrug resistant Gram-negative strains. Antifungal activity was also assessed against several fungal strains. In the final part of this study, we investigated the efficacy of the most potent newly synthesized antimicrobial compound to reverse antibiotic resistance in the clinical problematic ESBLs- and MBLs-containing strains and additionally explore its mechanism of action.

2. Results and discussion

2.1. Chemistry

2.1.1. Design and synthesis

N-Substituted imidazolium oximes **1–4** were prepared in three steps according to the reported procedure. When the corresponding *N*-substituted imidazole (except *N*-benzylimidazole) is not commercially available, the first step in the synthesis is the arylation of nitrogen atoms in position 1 of imidazole ring with appropriate aryl halide with potassium carbonate as base.[15] The resulting *N*-substituted imidazoles can be translated into a 2-carbaldehyde by reacting with *n*-butyl lithium (*n*-BuLi), and then, without isolating the unstable organolithium compounds addition of *N*, *N*-dimethylformamide (DMF)[16] reaction with formaldehyde and then oxidation with SeO_2.[17] Imidazole-2-carbaldehyde is easy to translate the oxime in the usual manner by reaction with hydroxylammonium chloride in ethanol.[18] In the last step, the final monoquaternary imidazolium oximes **5–8** were prepared by the addition benzyl bromide in equimolar quantities to the solution of appropriate *N*-substituted imidazolium oximes in dry acetone,[19–21] Figure 1. The reaction mixtures were kept at room temperature without stirring in the dark for 2–3 days. The crystallization was spontaneous. Acetone was removed and white crystals were washed with dry diethyl ether and obtained in very good yields.

All synthesized monoquaternary oximes from series B, **5–8**, where identified by IR, 1D (^{1}H and ^{13}C (APT) and 2D (HETCOR) NMR spectroscopy. Analytical and spectral data of these oximes are given in Section 4. The synthesis and ^{1}H NMR spectra of compounds from series A, **1–4** were previously reported.[21–23]

2.1.2. Structural properties

The structures of compounds from series B, **5–8** were determined by spectral analyses and the spectroscopic properties were in accord with the proposed structures. The IR spectra showed intense absorption bands within 2690–3350 cm^{-1} range, 1428–1512 cm^{-1} range and 980–990 cm^{-1} that were attributed to O–H, C=N and N–O function vibrations, respectively. In the ^{1}H NMR spectra for the monoquaternary salts, the proton signals due to the N–OH group were recorded between 13.00 and 13.03 ppm as singlet. The proton signals belonging to the *CH*-NOH group appeared as singlet at 8.01–8.09 ppm. ^{1}H NMR data confirmed the quaternization of imidazole ring as expected, while the imidazole hydrogen at the 4 and 5 positions of compounds **1–4** showed peaks between 7.16 and 7.43 ppm, the imidazolium hydrogen at these positions in the final compounds **5–8** showed peaks to a higher frequency, between 8.19 and 8.21 ppm, which indicated that these compounds were quaternised with benzyl bromide. All the ^{13}C NMR findings and elemental analysis confirmed the structures proposed as indicated in the Section 4.

2.2. Biological evaluation

2.2.1. Antimicrobial activity

In this study, the antimicrobial efficacy of the imidazolium oximes from series A, **1–4** and newly synthesized monoquaternary compounds from series B, **5–8** were preliminary screened against a diverse panel of selected antibiotic susceptible Gram-positive bacteria including *Bacillus cereus* ATCC 11778, *Clostridium perfringens* FNSST 4999, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923 and antibiotic resistant Gram-negative bacteria namely *Pseudomonas aeruginosa* FNSST 014, *Escherichia coli* FNSST 982, *Klebsiella pneumoniae* FNSST 011 and *Aeromonas hydrophila* FNSST 118. Antifungal activity was also assessed on the yeast *Candida albicans* ATCC 6275 and filamentous fungi including *Penicillium funiculosum* FNSST 3724 and *Aspergillus fumigatus* FNSST 3833 by disc diffusion assay. Cefotaxime, gentamicine and amphotericin B were used as standard antibacterial as well as antifungal agents.

As shown in Table 1, the results of the disc diffusion assay indicate that all of the compounds tested exhibit the most potent and broad spectrum activity against bacterial and fungal strains tested. The mean zones of inhibition of the target compounds against all the bacterial strains tested were found in the range of 8.6 ± 1.1 to 20.7 ± 0.4 mm. It is noteworthy that target compounds are not only active against antibiotic susceptible Gram-positive bacteria, but also exhibit significant antibacterial effects against Gram-negative isolates with various antibiotic resistance profiles. Among the Gram-negative bacteria tested, two strains namely *Pseudomonas aeruginosa* and *Aeromonas hydrophila* showed relative high sensitivity towards the tested compounds with considerable zones

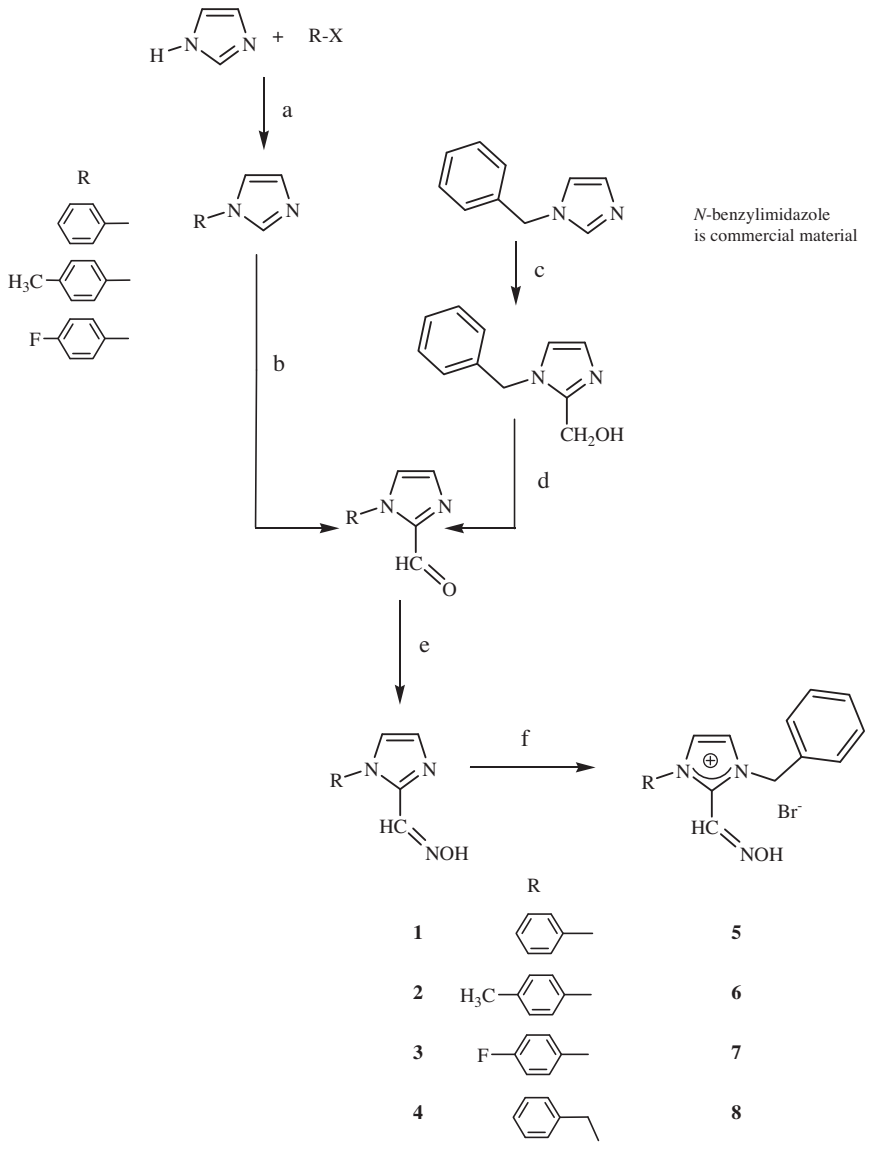


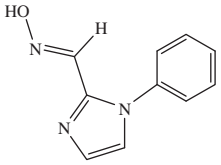
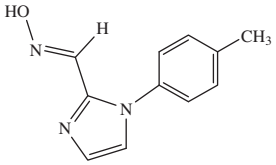
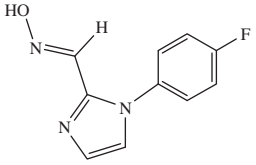
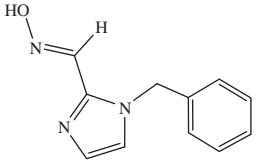
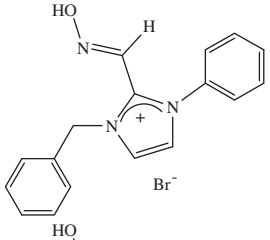
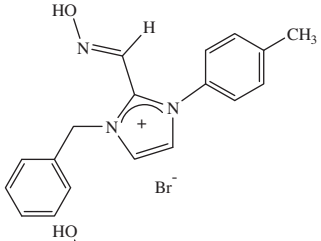
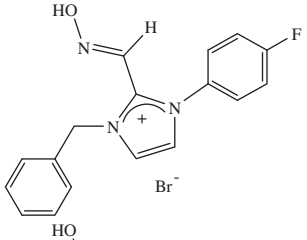
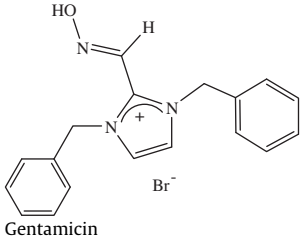
Figure 1. Reagents and conditions: (a) K_2CO_3, CuBr, nitrobenzen; (b) *n*-BuLi, DMF; (c) HCHO, Δ; (d) SeO_2, dioxane; (e) $NH_2OH{\cdot}HCl$, ethanol, pH 7–8; (f) $C_6H_5CH_2Br$, acetone.

Table 1
Antimicrobial activity of substituted imidazolium oximes (**1**–**4**) and their monoquaternary salts (**5**–**8**) by disc diffusion assay

Microorganisms	No strains	Diameters of the inhibition zone[a,c] (mm)									
		No Compound								Standard antibiotic[b,c]	
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	CTX	GEN
Gram-positive bacteria											
Bacillus cereus	ATCC11778	15.4 ± 0.5	14.6 ± 0.5	14.0 ± 0.2	17.5 ± 0.1	15.0 ± 0.3	12.2 ± 1.3	18.3 ± 0.5	15.5 ± 0.3	26.8 ± 0.4	18.2 ± 0.3
Enterococcus faecalis	ATCC 29212	15.7 ± 1.1	13.8 ± 0.7	12.2 ± 0.1	14.9 ± 0.3	13.3 ± 0.2	14.5 ± 0.2	19.6 ± 0.2	17.1 ± 0.8	23.5 ± 0.1	14.6 ± 1.4
Staphylococcus aureus	ATCC 25923	10.1 ± 0.7	18.5 ± 0.1	12.4 ± 0.4	10.8 ± 0.9	12.5 ± 0.7	18.4 ± 0.6	19.9 ± 0.7	14.2 ± 0.2	21.7 ± 0.4	23.9 ± 0.1
Clostridium perfringens	FNSST 4999	16.5 ± 0.5	12.6 ± 0.3	8.6 ± 1.1	15.9 ± 0.5	16.6 ± 1.2	20.7 ± 0.4	11.4 ± 0.4	18.3 ± 0.6	28.5 ± 0.7	21.7 ± 0.4
Gram-negative bacteria											
Escherichia coli	FNSST 982	17.8 ± 0.3	14.9 ± 0.2	14.8 ± 0.9	17.3 ± 1.1	12.3 ± 0.4	17.7 ± 0.3	15.3 ± 0.5	14.2 ± 0.4	22.8 ± 0.3	11.5 ± 1.2
Klebsiella pneumoniae	FNSST 011	17.2 ± 1.5	9.3 ± 0.1	10.3 ± 0.0	15.8 ± 0.3	15.5 ± 0.1	16.3 ± 0.3	14.7 ± 0.6	18.6 ± 0.2	21.4 ± 1.2	18.2 ± 0.6
Pseudomonas aeruginosa	FNSST 982	15.8 ± 1.5	10.9 ± 0.4	9.8 ± 0.3	17.9 ± 0.1	14.9 ± 0.3	20.6 ± 0.5	15.9 ± 0.4	12.4 ± 0.1	10.4 ± 0.9	9.2 ± 0.4
Aeromonas hydrophilla	FNSST 014	17.7 ± 1.5	15.7 ± 1.9	15.3 ± 0.1	10.1 ± 0.3	15.3 ± 0.8	16.6 ± 0.7	16.5 ± 0.2	17.1 ± 0.3	14.7 ± 0.4	12.6 ± 0.1
Fungi										AMPHB	
Candida albicans	ATCC 10231	16.4 ± 1.5	20.2 ± 0.4	18.9 ± 0.2	17.2 ± 0.2	18.1 ± 0.2	20.8 ± 0.9	21.9 ± 0.6	20.9 ± 0.8	21.6 ± 0.0	
Penicillium funiculosum	FNSST 3724	17.9 ± 1.5	15.4 ± 0.2	18.6 ± 0.5	17.6 ± 0.4	17.4 ± 0.8	18.4 ± 0.0	17.9 ± 2.2	17.6 ± 0.4	18.3 ± 0.3	
Aspergillus fumigatus	FNSST 3833	19.5 ± 1.5	16.3 ± 0.1	15.0 ± 0.4	11.5 ± 0.1	20.8 ± 0.7	20.3 ± 0.5	19. 4 ± 1.2	20.9 ± 0.2	19.2 ± 0.4	

[a] Diameter of inhibition zone (values in mm) around the disc: 250 μg/disc.
[b] Standard antibiotics disc: CTX, cefotaxime (30 μg/disc), GEN, gentamicine (15 μg/disc), AMPHB, amphotericin B (10 μg/disc).
[c] Values are expressed as mean ± SE.

Table 2
Minimum inhibitory concentration of *N*-substituted imidazolium oximes (**1**–**4**) and their monoquaternary salts (**5**–**8**)

Compound	Structure	MIC (μg/mL)							
		Gram-positive bacteria				Gram-negative bacteria			
		Bacillus cereus	*Enterococcus faecalis*	*Staphylococcus aureus*	*Clostridium perfringens*	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Aeromonas hydrophilla*
1		50.00	6.25	100.0	100.0	50.00	100.0	25.00	50.00
2		50.00	50.00	32.00	100.0	25.00	100.0	50.00	25.00
3		50.00	25.00	100.0	100.0	25.00	100.0	50.00	25.00
4		6.25	6.25	6.25	50.00	25.00	25.00	12.50	25.00
5		25.00	25.00	12.50	25.00	3.12	31.20	25.00	25.00
6		12.50	25.00	6.25	25.00	3.12	12.50	0.14	12.50
7		6.25	6.25	6.25	25.00	12.50	1.56	25.00	12.50
8		75.00	12.50	12.50	25.00	100.0	42.0	62.50	50.00
	Gentamicin	4.00	4.00	1.00	0.50	32.00	8.00	64.00	8.00
	Cefotaxime	0.25	0.50	0.50	0.10	0.50	0.50	16.00	8.00

of growth inhibition and were even more effective than the cefotaxime and gentamicin which were used as positive controls. Moreover, the antifungal profiles of the these compounds was found to be the most potent in inhibiting fungal growth, with mean diameter of inhibition zones ranging from 11.5 ± 0.1 to 21.9 ± 0.6 mm. It was also worth noting that monoquaternary imidazolium oximes were active against the fungal strains tested, which indicates a promising antifungal potential on important pathogen such as *Candida albicans* ATCC 6275 when compared with standard antifungal agent amphotericin B.

2.2.2. Minimum inhibitory concentrations (MICs)

The antimicrobial activity was also investigated by a broth microdilution method to determine the minimum inhibitory concentrations (MICs) of the series A of substituted imidazolium oximes and their quaternary salts, series B, necessary to inhibit the bacterial growth. As a comparison, we also tested the MICs of relevant conventional antimicrobial agents that are currently used in clinical treatment, such as gentamicin and cefotaxime. The results presented in Table 2 revealed that the all compounds tested showed remarkable selectivity and effective activities against tested microorganisms with MIC values from 0.14 to 100.0 μg/mL. In general, compounds of series A (**1**–**4**), *N*-substituted imidazolium oximes bearing phenyl, *p*-methylphenyl, *p*-fluorophenyl and benzyl substituent on the imidazolium ring, displayed more potent activity against the Gram-positive (6.25–100.0 μg/mL), then against the Gram-negative bacteria tested which is in according to the previous literature.[24] Differences in the composition of the cell wall of Gram-positive and Gram-negative bacteria and a possible relationship with the lipophilic characteristics of the target molecules could be helpful to explain the difference of these antibacterial compounds. Interestingly, compound **4** among these uncharged compounds, with benzyl substitutent demonstrated excellent in vitro activity against Gram-positive and common Gram-negative pathogens. Compared to other compounds from these series, compounds **4** we found that to be 5- to 16-fold more potent than other *N*-substituted imidazolium oximes against clinically relevant pathogens such as *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923, with equal MIC values of 6.25 μg/mL. The structure activity analysis suggested that the presence of a benzyl ring on the imidazolium ring in the compound **4** could be significant for observed promising inhibitory profiles against clinically important pathogens, including Gram-negative *P. aeruginosa*, with a MIC fivefold lower than the MIC of standard antibiotic gentamicin.

Furthermore, the data indicate that, in general, the monoquaternary derivatives of substituted imidazolium oximes are more potent with broad spectrum antimicrobial activity compared to their uncharged analogs, with the exception of compound **8**. These results suggested that the quaternization nitrogen atom of the imidazolium ring can yield differences in both potency and spectrum of antimicrobial activity and that is in accordance with previous studies that confirmed that the higher electron density, the higher the antimicrobial activity of quaternary ammonium compounds.[25] Minimum inhibitory concentrations demonstrated that benzyl derivatives **5**–**7** were found to be the most superior compounds, especially against Gram-negative bacteria exhibited range of MIC values from 0.14 to 25.0 μg/mL, when compared to the their uncharged analogs (**1**–**3**). All these compounds showed good antimicrobial activities especially for *Escherichia coli* which is 2.5 to 10-fold better than gentamicin. Monoquaternary imidazolium oximes **6** and **7** were found to be the most potent compounds in both series, with MIC values ranging from 0.14 to 25.00 μg/mL and 1.56 to 25.00 μg/mL, respectively. However, compounds **6** with *p*-methylphenyl substituent on imidazolium ring exhibited promising antimicrobial effect on *Pseudomonas aeruginosa* (MIC = 0.14 μg/mL) it is known to have high level of intrinsic resistance to virtually all known antibiotics. In comparison with relevant antibiotic compound **6** is 450-fold better than the gentamicin and 115-fold better than the cefotaxime. Methyl group have been added incrementally to the phenyl ring with the intention of increasing lipophilicity and as a consequence improving the activity. This compound and compound **5** also showed excellent in vitro activity against *Escherichia coli* at MIC values of 3.12 μg/mL, which was 10-fold more potent than the gentamicin. It is interesting to note that benzyl derivatives **7** with substitution of *p*-fluorophenyl of imidazole ring exhibited excellent in vitro activity against a broad spectrum of clinically important resistant Gram-negative pathogens such as *Klebsiella pneumoniae* (MIC = 1.56 μg/mL) *Escherichia coli, Aeromonas hydrophila* (MIC = 12.5 μg/mL) and *Pseudomonas aeruginosa* (MIC = 25.00 μg/mL) which is 2.5 to 5-fold better antimicrobial activity than the gentamicin. Moreover, this compound also displayed excellent activity against Gram-positive bacteria *Bacillus cereus*, *Enterococcus faecalis* and *Staphylococcus aureus*, and MICs values were fourfold decrease (MIC = 6.25–25.00 μg/mL) when compared to their analog **3** (MIC = 25.00–100.0 μg/mL). This result may be substantiated on the basis of previous publications which demonstrated that the substitution of fluorine atom at the *p*-position of the phenyl ring also increases the positive charge density on the imidazolium cation. Also, fluorine atom with high electronegativity, relatively small size, and low polarisability of the C–F bond and, in many cases, increased lipophilicity of fluorine containing organic molecules can have considerable influence their therapeutic potency.[26] The results suggested that introduction of a substituent, either electron-donating (**6**) or electron-withdrawing (**7**), to the phenyl ring could significantly enhance the antimicrobial potential.

Among quaternary oximes, compound **8** was less in vitro effective against growth of wide spectrum pathogens, with MIC values ranging between 12.50 and 100.00 μg/mL. The compounds **8** is symmetrical 1,3-disubstituted imidazolium salts containing two benzyl groups exhibited 2- to 12-fold decreased antibacterial efficacy then their uncharged analogue **4**. That two hydrophobic benzyl groups on imidazolium ring enhances not improve the antibacterial activity indicate that the symmetry of the molecule or other factors such as electronic properties or aromatic interaction[27] could be contribute to reduced activity might be to play a role in the antimicrobial efficacy of this compound. Whether differences in antimicrobial activities are due to differential uptake or different intramolecular interactions requires further investigation.

2.2.3. Antiresistance profiles

Among the synthesized imidazolium oximes, compound **7** demonstrated the strongest efficacy against growth of broad spectrum clinically relevant Gram-negative bacterial strains, with MICs values in the range from 6.25 to 25.00 μg/mL. Therefore, it was selected to verify its ability to inhibit the growth of fourteen clinically and environmental multidrug isolates resistant to third-generation cephalosporins and carbapenems via expression of molecular classes A and B β-lactamase enzymes. The antiresistance profiles of compound **7** was investigated by the determination of the minimum inhibitory concentrations (MICs) in comparison with different classes of antibiotics including ceftazidime, cefotaxime, ciprofloxacin, gentamicin, tetracycline, tobramycin, imipenem and meropenem. As shown in Table 3, compound **7** treatment significantly reduced resistance level in a variety of ESBL and MBL-producing isolates, with MICs values ranging from 0.39 to 12.50 μg/mL when compare to the activity of eight broad-spectrum antibiotics. The promising antiresistance efficacy was obtained against *Pseudomonas aeruginosa* 197 producing GIM-1 metallo β-lactamase (MIC = 0.39 μg/mL),

Table 3
Minimum inhibitory concentration of monoquaternary imidazolium oxime (**7**) (μg/mL) against multidrug resistant Gram-negative ESBL and MBL-producing isolates

Isolates	Compound **7** MIC (μg/mL)	ESBL genotype	MBL genotype	Standard antibiotics MIC[a] (μg/mL)							
				CAZ	CTX	CIP	GEN	TET	TOB	IMP	MEM
Stenotrophomonas maltophilia 125	12.50	CTX-15		128	32	0.25	2	128	8.0	0.12	128
Klebsiella pneumoniae 303	12.50	TEM-1, SHV-12		128	64	2.00	32	256	2.0	0.12	0.12
Enterobacter cloacae 62	6.25	TEM-1, CTX-M-15		>256	>256	0.25	4	16	32	0.25	0.50
Enterobacter cloacae 306	6.25	TEM-1, SHV-12		>256	>128	0.12	256	16	16	0.25	0.25
Enterobacter cloacae 51	6.25	CTX-15		16	32	1.00	128	64	>32	8	0.25
Enterobacter intermedius 243	6.25	TEM-1, SHV-12, CTX 15		8	8	0.50	2	16	>32	0.12	0.12
Aeromonas hydrophila 268	12.50	TEM-1, SHV-12		64	256	2.00	4	8	16	0.06	0.06
Aeromonas caviae 258	12.50	CTX-15		4	258	0.50	12.8	64	32	0.25	0.125
Aeromonas caviae 260	12.50	TEM-1, CTX-M-15		128	256	1.00	32	128	32	2.00	1.00
Escherichia coli 4024	1.56	CTX-15		16	256	0.25	64	16	32	0.25	0.12
Escherichia coli 237	3.12	TEM-1, SHV-12		8	>256	0.50	8	128	64	0.125	1.00
Escherichia coli 235	6.25	CTX-15		64	>256	0.50	16	32	16	0.06	0.12
Acinetobacter baumannii 170	0.78		SIM-1	>256	>256	0.50	16	64	16	8	16
Pseudomonas aeruginosa 197	0.39		GIM-1	256	256	>4	>8	>8	16	>8	>8

[a] MIC values highlighted by shading represent resistance according to the breakpoints established by CLSI (M100-S17, 2010). Abbreviations of standard antibiotics: CAZ, ceftazidime; CTX, cefotaxime; CIP, ciprofloxacin; GEN, gentamicin; TET, tetracycline; TOB, tobramycin; IMP, imipenem; MEM, meropenem.

which was 20-fold more potent than the imipenem and meropenem, as well as against *Acinetobacter baumannii* 170 producing SIM-1 metallo β-lactamase (MIC = 0.78 μg/mL). Moreover, the significant antiresistance potential of compound **7** was also seen against three isolates of *Escherichia coli* ESBL-producing with MIC values ranging from 1.56 to 6.25 μg/mL. Compound **7** also demonstrated good activity by inhibiting the β-lactamases produced by strains of the *Enterobacter* species including *Enterobacter intermedius* 243, *Enterobacter cloacae* 62, 306, and 51 at concentration of 6.25 μg/mL.

3. Conclusions

In summary, we have investigated antimicrobial activity of some *N*-substituted imidazolium oxime **1**–**4** and their monoquaternary salts **5**–**8**. The final synthesized compounds characterized by spectral data (IR, 1D and 2D NMR). Among uncharged compounds (**1**–**4**), it was found that the benzyl group as substituent on imidazolium ring had the specific activity against Gram-positive bacteria, including Gram-negative *Pseudomonas aeruginosa* with MIC fivefold lower than MIC of gentamicin. Further, the results of the present study indicated that monoquaternary oximes showed better antimicrobial activity than they uncharged analogs with exception of symmetrical compounds **8** with two benzyl groups on imidazolium ring. Remarkable activity was found in quaternary compounds carrying a *p*-methylphenyl or *p*-fluorophenyl substituent on the imidazolium ring. The MICs of the most active derivatives (**6** and **7**) were shown to be law as 0.14 μg/mL against *P. aeruginosa* and 1.56 μg/mL against *Klebsiella pneumoniae*. A simple inspection of Table 2 indicates that the presence of electron-donating (CH_3) or electron-withdrawing (F) groups at C-4 of the phenyl ring is mandatory for the broad spectrum antimicrobial activity and with exception of *P. aeruginosa* and *K. pneumoniae*, the resulting activity is superior of the standards gentamicin and cefotaxime, for example compound **6** is 450-fold better than gentamicin and 115-fold better than cefotaxime.

These results clearly indicate that compound **7** possess the ability to inhibit the growth of fourteen clinically and environmental β-lactamases producing isolates resistant to third-generation cephalosporins and carbapenems. However, further work is needed to optimize these compounds for specificity; efficacy and experimental validation which may serve as adjuvant for the treatment of multidrug resistant bacterial infections.

4. Experimental section

4.1. Materials and general methods

Reagents and solvents used for synthesis were purchased from Aldrich, USA. Reactions were monitors by thin-layer chromatography using DC-Alufolien Aluminiumoxide 60 F254 plates (Merck) with 9:1 chloroform-methanol as the eluent. The detection of spots was achieved by UV light and by the reversible absorption of iodine. Melting points were determined in open capillaries using a Stuart Scientific digital melting point apparatus SMP30 and are uncorrected. FTIR spectra were recorded on a Bruker VECTOR 22FT/IR spectrometer. All samples were prepared by mixing FTIR-grade KBr (Sigma–Aldrich) with 1% (w/w) salt and grinding to a fine powder. Spectra were recorded over the 400–4000 cm^{-1} range without baseline corrections. Characteristic absorptions are given in cm^{-1}. ^1H and ^{13}C NMR spectra were recorded in DMSO-d_6 solution on a Bruker AV 300 spectrometer at room temperature. Chemical shifts were measured in with TMS as internal reference. Chemical shifts are reported as δ values in ppm using TMS as an internal standard. Abbreviations for data quoted are: s, singlet; d, doublet; m, multiplet. Imidazole hydrogen was indicated as Im, phenyl as Ph while benzyl hydrogen indicated as Bnl. Elemental analysis were performed with a Perkin-Elmer PE 2400 Series II CHNS/O Analyzer.

4.2. Synthesis

4.2.1. General procedure for synthesis of quaternary derivatives 5–8

N-substituted imidazolium oxime (1.00 mmol) and benzyl bromide (1.00 mmol) were stirred in acetone (15 mL) at room temperature for 24 or 36 h in the dark. The precipitate was filtered and washed with ether.

4.2.1.1. *N*-Benzyl-2-hydroxyiminomethyl-3-phenylimidazolium bromide (5). Yield 93%; mp: 190.3–191.5 °C; IR (ν, cm^{-1}): 990 (N–O), 1595 (C=N), 2700–3300 (O–H); ^1H NMR δ: 5.73 (s, 2H, CH_2 Bnl), 7.73–7.75 (m, 5H, Ph), 8.08 (s, 1H, C*H*=NOH), 8.14–8.21 (m, 7H, 2H Im and 5H Bnl), 13.00 (s, 1H, CH=NO*H*); ^{13}C NMR δ: 52.09 (CH_2 Bnl), 124.35 (C-4 Im), 126.10 (C-2 and C-6 Ph), 126.37 (C-5 Im), 127.96 (C-4 Bnl), 128.51 (C-4 Ph), 129.89 (C-3 and C-5 Bnl), 130.70 (C-2 and C-6 Bnl), 134.35 (C-1 Bnl),

134.54 (C-3 and C-5 Ph), 134.60 (C-1 Ph), 140.23 (C-2 Im), 151.17 (CH=NOH) ppm. Anal. Calcd for $C_{17}H_{16}N_3OBr$ (M: 358.23) C, 56.99; H, 4.50; N, 11.73. Found: C, 57.05; H, 4.54; N, 11.80.

4.2.1.2. *N*-Benzyl-2-hydroxyiminomethyl-3-(*p*-methyl)phenylimidazolium bromide (6). Yield 90%; mp: 211.5–212.6 °C; IR (ν, cm^{-1}): 990 (N–O), 1595 (C=N), 2700–3300 (O–H); ^1H NMR δ: 3.32 (s, 3H, CH_3-Ph), 5.74 (s, 2H, C*H*$_2$ Bnl), 7.46–7.65 (m, 5H, Bnl), 8.09 (s, 1H, C*H*=NOH), 8.15–8.16 (m, 4H, Ph), 8.19–8.20 (m, 2H, 2H, Im), 13.01 (s, 1H, CH=NO*H*); ^{13}C NMR δ: 21.20 (CH_3-Ph), 52.53 (CH_2 Bnl), 121.12 (C-4 Im), 126.61 (C-2 and C-6 Ph), 128.44(C-5 Im), 128.99 (C-4 Bnl), 129.35 (C-3 and C-5 Bnl), 130.75 (C-2 and C-6 Bnl), 132.62 (C-1 Ph), 134.89 (C-1 Bnl), 135.06 (C-3 and C-5 Ph), 137.60 (C-4 Ph), 141.12 (C-2 Im), 152.70 (CH=NOH) ppm. Anal. Calcd for $C_{18}H_{18}N_3OBr$ (M: 372.25) C, 58.07; H, 4.87; N, 11.29. Found: C, 58.20; H, 4.90; N, 11.35.

4.2.1.3. *N*-Benzyl-2-hydroyximinomethyl-3-(*p*-fluoro)phenylimidazolium bromide (7). Yield 91%; mp: 227.4–228.5 °C; IR (ν, cm^{-1}): 990 (N–O), 1595 (C=N), 2700–3300 (O–H); ^1H NMR δ: 5.73 (s, 2H, C*H*$_2$ Bnl), 7.38–7.54 (m, 5H, Bnl), 8.01 (s, 1H, C*H*=NOH), 8.15–8.20 (m, 6H, 2H, Im and 4H, Ph), 13.01 (s, 1H, CH=NO*H*); ^{13}C NMR δ: 52.50 (CH_2 Bnl), 117.14 (C-4 Im), 117.45 (C-3 and C-5 Ph), 128.42 (C-5 Im), 129.01 (C-2 and C-6 Ph), 129.37 (C-4 Bnl), 129.48 (C-3 and C-5 Bnl), 129.60 (C-2 and C-6 Bnl), 134.83 (C-1 Ph), 135.07 (C-1 Bnl), 141.82 (C-2 Im), 152.20 (CH=NOH), 163.12 (C-4 Ph) ppm. Anal. Calcd for $C_{17}H_{15}N_3OBrF$ (M: 376.22) C, 54.27; H, 4.02; N, 11.17. Found: C, 54.30; H, 4.10; N, 11.31.

4.2.1.4. *N*-Benzyl-2-hydroxyiminomethyl-3-benzylimidazolium bromide (8). Yield 94%; mp: 215.2–216.8 °C; IR (ν, cm^{-1}): 990 (N–O), 1595 (C=N), 2700–3300 (O–H); ^1H NMR δ: 5.66 (s, 4H, C*H*$_2$ Bnl), 7.32–7.43 (m, 12H, 2H Im and 10H Bnl) 8.02 (s, 1H, C*H*=NOH), 13.03 (s, 1H, CH=NO*H*); ^{13}C NMR δ: 52.23 (2× CH_2 Bnl), 124.61 (C-4 and C-5 Im), 128.14 (2× C-4 Bnl), 129.55 (2× C-3 and C-5 Bnl), 130.05 (2× C-2 and C-6 Bnl), 138.22 (2× C-1 Bnl), 143.12 (C-2 Im), 151.70 (CH=NOH) ppm. Anal. Calcd for $C_{18}H_{18}N_3OBr$ (M: 372.25) C, 58.07; H, 4.87; N, 11.29. Found: C, 58.11; H, 4.93; N, 11.37.

4.3. Biological methods

All of the newly synthesized target compounds were evaluated for their in vitro antibacterial activity. The tested microorganisms were obtained from the culture collection at the American Type Culture Collection (ATCC) (Rockville, MD, USA) and at the Microbiology laboratory, Department of Biology, Faculty of Natural Science, University of Split, Croatia (FNSST). The assayed collection included four Gram-positive bacteria *Bacillus cereus* (ATTC 11778), *Enterococcus faecalis* (ATCC 29212), and *Staphylococcus aureus* (ATCC 25923) *Clostridium perfringens* (FNSST 4999) and four Gram-negative ampicillin-resistant bacterial strains *Escherichia coli* (FNSST 982), *Klebsiella pneumoniae* (FNSST 011), *Pseudomonas aeruginosa* (FNSST 982) and *Aeromonas hydrophilla* FNSST 014. Antifungal activity was assessed on the yeast *Candida albicans* (ATCC 10231) and fungal strains *Penicillium funiculosum* (FNSST 3724) and *Aspergillus fumigatus* (FNSST 3833). Bacterial strains were cultured overnight at 37 °C in tryptic soy broth (TSB) and fungi were cultured overnight at 30 °C in Sabouraud dextrose broth (SDB) to achieve optical densities corresponding to 10^6 colony forming units (cfu/mL) for bacteria and 10^4 CFU/mL for fungal strains.

4.3.1. Disc diffusion assay

In order to investigate the antimicrobial activities of the synthesized compounds, a disc diffusion assay was employed according to the CLSI guidelines. Briefly, 100 μL of suspension containing 10^6 - colony-forming units (cfu)/mL of bacterial cells and 10^4 cfu/mL spore for fungal strains was spread on a Mueller Hinton agar (Becton Dickinson, Sparks, MD) and Sabouraud dextrose agar (SDA; Becton Dickinson, Sparks, MD). The stock solutions of synthesized compounds were prepared by dissolving in 96% ethanol to a final concentration of 10 mg/mL. The sterile filter discs (6 mm) were individually loaded with 25 μL of the stock solution, equivalent to a final concentration at 250 μg/disc of synthesized compounds and then placed on the nutrient agar that had been previously inoculated with the target microbial strains. Additionally, 96% ethanol was used as a negative control, ampicillin (30 μg) gentamicin (15 μg) and commercial fungicide amphotericin B (10 μg) were used as positive controls. The plates were incubated for 24 h at 37 °C for bacterial strains and 48 h or 72 h at 30 °C for yeast and mold isolates, respectively. Antibacterial activity was assessed by measuring the diameter of the inhibition zone in millimeters, including disc diameter for the test isolates, compared to the controls. Samples were assayed in triplicate for each condition and the diameter of inhibition zones were presented as mean ± SE values.

4.3.2. Minimum inhibitory concentration assay

In addition, antimicrobial activities of the synthesized compounds were tested by a broth microdilution assay in 96 well plates. The standard two fold serial microdilution assay described by the Clinical and Laboratory Standards Institute was performed for the assessment of the minimum inhibitory concentrations (MICs). Bacteria and fungi were grown overnight in Mueller-Hilton broth (MHB) at 37 °C and Sabouraud dextrose broth (SDB) at 30 °C to the stationary phase. The microbial cultures were diluted in fresh MHB and SDB to a final concentration of 10^6 CFU/mL for bacteria and 10^4 CFU/mL for fungal strains. The synthesized compounds was first dissolved in DMSO and incorporated into aqueous nutrient medium to obtain a concentration of 10 mg/mL. The stock solution was then serially two fold diluted to obtain concentrations ranging from 400 to 0.09 μg/mL in sterile plates containing Mueller Hinton broth (MHB) for bacterial as well as Sabouraud dextrose broth (SDB) for the fungi. Serial dilutions of the synthesized compounds were added to the microtiter plates in a volume of 100 μL. Each well was additionally inoculated with 10 μL of inoculums of the target microorganism and incubated at 37 °C for 18–24 h for bacterial and at 30 °C for 48 h for fungal strains. The MIC value was determined as the lowest concentration of the sample at which the tested microorganisms did not demonstrate any visible growth after incubation. As an indicator of bacterial growth, 50 μL of 0.2 mg/mL *p*-iodonitrotetrazoliumchloride (INT; Sigma-Aldrich Co. Ltd, Poole, UK) was added to the wells and incubated at 37 °C for 30 min. Following addition of INT and incubation, the MIC was determined as the lowest sample concentration at which no pink color appeared. Cefotaxime, gentamicin and amphotericin B were used as positive controls. Minimum inhibitory concentrations of the commercial antibiotics were determined by the *E*-test (AB Biodisk, Solna, Sweden). Each assay in this experiment was replicated three times. The MIC was interpreted as the point of intersection of the inhibition ellipse with the *E*-test strip edge. In this study, no bioactivity was defined as a MIC >1000 μg/mL, mild bioactivity as a MIC in the range 512–1000 μg/mL, moderate bioactivity as a MIC in the range 128–512 μg/mL, good bioactivity as a MIC in the range 32–128 μg/mL, strong bioactivity as a MIC in the range 10–32 μg/mL and very strong bioactivity as a MIC <10 μg/mL.

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References and notes

1. Rodriguez-Bano, J.; Picon, E.; Gijon, P.; Hernandez, J. R.; Cisneros, J. M.; Pena, C.; Almela, M.; Almirante, B.; Grill, F.; Colomina, J. *J. Clin. Microbiol.* **2010**, *48*, 1726.
2. Bush, K.; Jacoby, G. A. *Antimicrob. Agents Chemother.* **2010**, *54*, 969.
3. Walsh, T. R. *Curr. Opin. Infect. Dis.* **2008**, *21*, 367.
4. Bush, K.; Fisher, F. *Annu. Rev. Microbiol.* **2011**, *65*, 455.
5. De Luca, L. *Curr. Med. Chem.* **2006**, *13*, 1.
6. Anderson, E. B.; Long, T. E. *Polymer* **2010**, *51*, 2447.
7. Congiu, C.; Cocco, M. T.; Onnis, V. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 989.
8. Nagarapu, L.; Satyender, A.; Rajashaker, B.; Srinivas, K.; Rani, P. R.; Radhika, K.; Subhashini, G. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1167.
9. Xue, F.; Luo, X.; Ye, C.; Ye, W.; Wang, Y. *Bioorg. Med. Chem.* **2011**, *19*, 2641.
10. Gadad, A. K.; Noolvi, M. N.; Karpoormath, R. V. *Bioorg. Med. Chem.* **2004**, *12*, 5651.
11. Fromtling, R. P. *Clin. Microbial. Rev.* **1988**, *1*, 187.
12. Venkatesan, A. M.; Gu, Y.; Dos Santos, O.; Abe, T.; Agarwal, A.; Yang, Y.; Petersen, P. J.; Weiss, W. J.; Mansour, T. S.; Nukaga, M.; Hujer, A. M.; Bonomo, R. A.; Knox, J. R. *J. Med. Chem.* **2004**, *47*, 6556.
13. Fukuoka, T.; Ohya, S.; Narita, T.; Katsuta, M.; Iijima, M.; Masuda, N.; Yasuda, H.; Trias, J.; Nikaido, H. *Antimicrob. Agents Chemother.* **1993**, *37*, 322.
14. Dawson, R. M. *J. Appl. Toxicol.* **1994**, *14*, 317.
15. Požarski, A. F.; Marcoha, B. K.; Simonov, A. M. *Zh. Obshch. Khim.* **1963**, *33*, 1005.
16. Gebert, U.; Kerekjarto, B. *Ann. Chem.* **1974**, 644.
17. Jones, R. G. *J. Am. Chem. Soc.* **1949**, *71*, 383.
18. Fournari, P.; de Cointet, P.; Laviron, E. *Bull. Soc. Chim. Fr.* **1968**, *6*, 2438.
19. Grifantini, M.; Martelli, S.; Stein, M. L. *J. Pharm. Sci.* **1972**, *61*, 631.
20. Galoši, A.; Deljac, A.; Deljac, V.; Binenfeld, Z. *Acta Pharm.* **1988**, *38*, 23.
21. Mesić, M.; Deljac, A.; Deljac, V.; Binenfeld, Z.; Kilibarda, V.; Maksimović, M.; Kovačević, V. *Acta Pharm. Jugosl.* **1991**, *41*, 203.
22. Mesić, M.; Deljac, A.; Deljac, V.; Binenfeld, Z.; Maksimović, M.; Kilibarda, V. *Acta Pharm.* **1992**, *42*, 169.
23. Mesić, M.; Rončević, R.; Radić, B.; Fajdetić, A.; Binenfeld, Z. *Acta Pharm.* **1994**, *44*, 145.
24. Iwai, N.; Nakayama, K.; Kitazume, T. *Bioorg. Chem. Lett.* **2011**, *21*, 1728.
25. Türkmen, H.; Ceyhan, N.; Ülkü Karabay Yavaşoğlu, N.; Özdemir, G.; Çetinkaya, B. *Eur. J. Med. Chem.* **2011**, *46*, 2895.
26. Filler, R.; Saha, R. *Future Med. Chem.* **2009**, *1*, 777.
27. Çetinkaya, E.; Denizci, A.; Özdemir, I.; Ozturk, H. T.; Karaboz, I.; Çetinkaya, B. *J. Chemother.* **2002**, *14*, 241.