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Short communication

Synthesis, antimicrobial activity, pharmacophore analysis of some new 2-(substitutedphenyl/benzyl)-5-[(2-benzofuryl)carboxamido]benzoxazoles

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Abstract

The synthesis and antimicrobial activity of a new series of 2-(substitutedphenyl/benzyl)-5-[(2-benzofuryl)carboxamido]benzoxazole derivatives **3–12** were described. The *in vitro* antimicrobial activity of the compounds was determined against some Gram-positive, Gram-negative bacteria and fungi and their drug-resistant isolates in comparison with standard drugs. Antimicrobial results indicated that the synthesized compounds possessed a broad spectrum of activity with MIC values 500–15.625 μg/ml. In the series, the most active compound against *Candida krusei* and *Candida albicans* isolate is **8** with MIC value 31.25 μg/ml. However, it is one dilution less potent than the compared fluconazole. Some of the screened compounds exhibit significant activity, having MIC value as 31.25 μg/ml in *Pseudomonas aeruginosa* having same activity as Rifampicin. Furthermore, considering the worth of developing new antibacterial agents against drug-resistant *P. aeruginosa* the present study explores the structure–activity relationship analysis of 2-(substitutedphenyl/benzyl)-5-[(2-benzofuryl)carboxamido]benzoxazoles using 3D-common features pharmacophore hypotheses approach.
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Keywords: Benzoxazole; Benzofuran; Antifungal activity; Antibacterial activity; Pharmacophore analysis

1. Introduction

The rising prevalance of multi-drug resistant microbial infections in the past few decades has become a serious health care problem. In particular, the emergence of multi-drug resistant strains of Gram-positive bacterial pathogens such as methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermis* and vancomycin-resistant *Enterococcus* is a problem of ever-increasing significance. In fact every day more common and uncommon bacteria previously susceptible to common antimicrobials are reported to have developed resistance to different antibiotics. Although these bacteria initially caused significant nosocomial infections and were the cause of major morbidity and mortality in hospitalized patients, more recently they have spread to community, causing severe illnesses in previously healthy and otherwise non-vulnerable patients [1–6]. Consequently, to prevent the emergence and dissemination of resistant bacteria, continuing efforts to develop new antibacterial agents are needed.

Benzofurans have drawn considerable attention over the last few years due to their profound physiological and chemotherapeutic properties as well as their widespread occurrences in nature. The antimicrobial activity of some 2-arylbenzofurans was investigated against vancomycin-resistant enterococci and methicillin-resistant *S. aureus* [7–12]. Besides, substituted-benzoxazoles possess diverse chemotherapeutic activities including antibiotic [13,14], antimicrobial [15–26], antiviral [27–31], topoisomerase I and II inhibitors [32–34],

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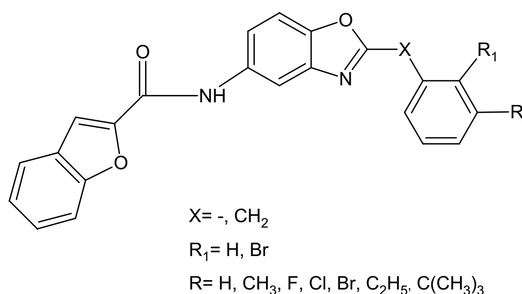


Fig. 1.

antitumor activities [35–38]. In our previous studies, various 2-(substitutedphenyl or benzyl)benzoxazole derivatives have been reported to be microbiologically active [15–24,20,39].

Because of the need of new antimicrobial drugs, we designed and synthesized new compounds having both benzoxazole and benzofuran binding via an amide bridge as a new class antimicrobial agents (Fig. 1). In this study, the strategy employed was to examine the effect of two isosteric heterocyclic nucleus connected with a carboxamide bridge together with the role of substituent on the second position of benzoxazole against some Gram-positive, Gram-negative bacteria, fungi and their drug-resistant isolates in comparison with the control drugs. The other goal at the outset of this research, was to compare how activity changed by attaching 2-benzofuryl ring instead of 2-furyl to carboxamido moiety of benzoxazole.

2. Results and discussion

2.1. Chemistry

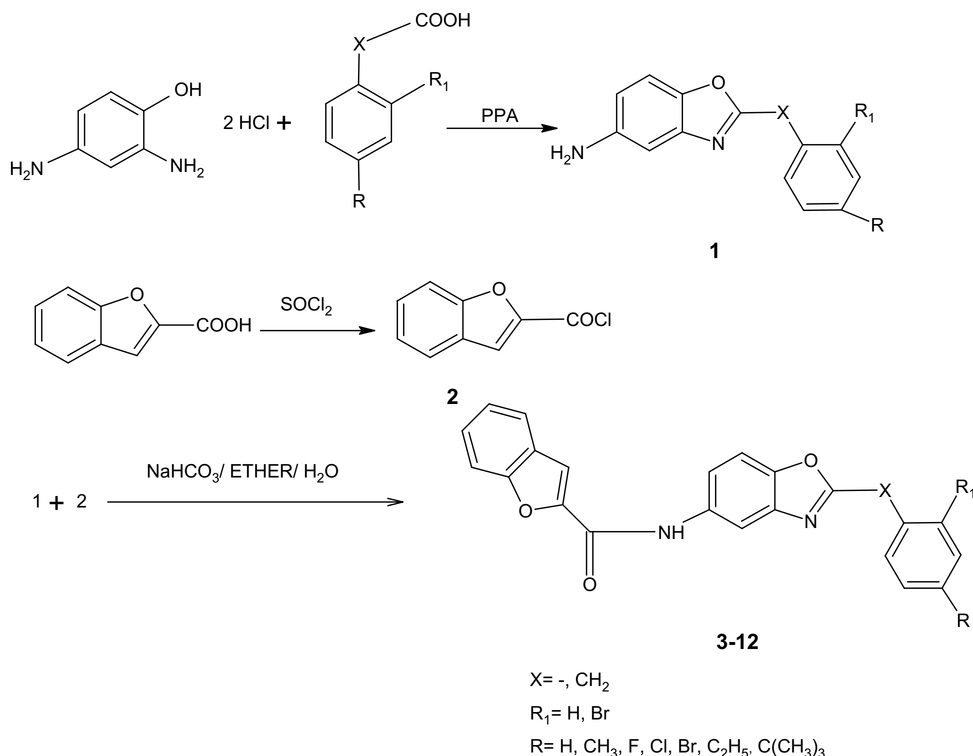
For the synthesis of compounds **3–12** firstly, 5-amino-2-[*p*-substitutedphenyl/benzyl]benzoxazoles and 5-amino-2-[*o*-bromophenyl]benzoxazole (**1**) were obtained by heating *p*-substitutedbenzoic acid and/or *p*-substitutedphenylacetic acid and/or *o*-bromobenzoic acid with 2,4-diaminophenol in PPA (polyphosphoric acid).

Compounds **3–12** were obtained from 5-amino-2-[*p*-substitutedphenyl/benzyl]benzoxazoles and 5-amino-2-[*o*-bromophenyl]benzoxazole with benzofuran-2-carboxylic acid chloride (**2**) obtained by treating benzofuran-2-carboxylic acid with thionyl chloride as given in Scheme 1 [20].

All the compounds **3–12** were prepared as new products. The structures of **3–12** were supported by spectral data. The IR, ^{1}H NMR, mass spectra and elemental analysis results are in agreement with the proposed structures. Additionally, ^{13}C NMR spectral results are given only for the compound **8** at the end of the experimental part. Physical and spectral data of the compounds are reported in Table 1.

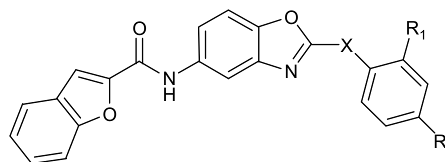
2.2. In vitro antibacterial and antifungal activity

All the synthesized 2-(substitutedphenyl/benzyl)-5-[(2-benzofuryl)carboxamido]benzoxazole derivatives **3–12** were assayed *in vitro* for antibacterial activity against *Klebsiella pneumoniae* RSHM 574, *Pseudomonas aeruginosa* ATCC



Scheme 1. Synthetic pathway of the target compounds **3–12**.

Table 1
Physical properties and spectral data of the compounds **3–12**



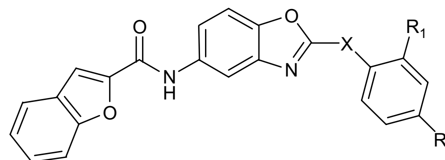
Compound no.	R_1	R	X	Formula: calculated/found	Mp	Yield (%)	IR (cm^{-1})	^1H NMR (δ ppm) J = Hz	MS (ESI^+) m/z (% X)
3	Br	H	–	$C_{22}H_{13}BrN_2O_3$: C: 60.99, H: 3.02, N: 6.47/C: 59.60, H: 3.02, N: 6.09	168–170	90	3509, 1690, 1623, 1553, 1481, 1345–1308, 1258, 1188–1109, 962–472	8.551 (1H, s), 8.247–8.242 (1H, d, J = 2.0), 8.084–8.060 (1H, dd, J = 8.0, J′ = 1.6), 7.773–7.680 (3H, m), 7.617–7.547 (3H, m), 7.467–7.428 (2H, m), 7.381–7.258 (2H, m)	433 (% 100) (M + H), 435 (% 100) (M + H+2)
4	H	H	–	$C_{22}H_{14}N_2O_3 \cdot 0.65HCl$: C: 70.00, H: 3.88, N: 7.42/C: 70.00, H: 3.70, N: 7.42	204–208	76	3400, 3077, 1671, 1621, 1563, 1487, 1354–1312, 1256, 1177–1111, 961–479	8.94 (1H, s), 8.270–8.235 (2H, dd, J = 6.8, J′ = 7.2), 8.161–8.156 (1H, d, J = 2.0), 7.723–7.697 (2H, dd, J = 1.6, J′ = 8.8), 7.624 (1H, s), 7.587–7.556 (2H, m), 7.483–7.441 (1H, dd, J = 8.0, J′ = 8.8), 7.347–7.310 (1H, dd, J = 7.6, J′ = 7.2), 7.257–7.193 (2H, dd, J = 8.4, J′ = 8,8)	355 (% 20) (M + H)
5	H	C_2H_5	–	$C_{24}H_{18}N_2O_3 \cdot 0.6H_2O$: C: 73.30, H: 4.92, N: 7.12/C: 73.30, H: 4.65, N: 6.77	202–206	60	3402, 2956, 1670, 1612, 1558, 1483, 1353–1291, 1255, 1175–1111, 960–475	8.504 (1H, s), 8.173–8.134 (2H, dd, J = 8.4, J′ = 7.2), 7.727–7.547 (5H, m), 7.475–7.436 (1H, dd, J = 7.2, J′ = 8.4), 7.364–7.303 (3H, m), 2.761–2.705 (2H, q), 1.307–1.269 (3H, t)	383 (% 100) (M + H)
6	H	F	–	$C_{22}H_{13}FN_2O_3 \cdot 0.9H_2O$: C: 68.00, H: 3.84, N: 7.21/C: 67.98, H: 3.57, N: 6.99	237–240	94	3400, 1671, 1621, 1559, 1487, 1354–1292, 1256, 1177–1111, 949–472	8.438 (1H, s), 8.183–8.148 (2H, m), 8.081 (1H, s), 7.647–7.609 (2H, dd, J = J′ = 7.6), 7.540 (1H, s), 7.490–7.469 (2H, m), 7.396–7.357 (1H, dd, J = 7.6, J′ = 8.0), 7.262–7.225 (1H, dd, J = 7.6, J′ = 7.2), 7.152–7.110 (2H, dd, J = J′ = 8.4)	373 (% 100) (M + H)
7	H	$C(CH_3)_3$	–	$C_{26}H_{22}N_2O_3$: C: 76.08, H: 5.40, N: 6.82/C: 75.99, H: 5.32, N: 6.81	223–226	37	3403, 2961, 1671, 1620, 1580, 1485, 1352–1290, 1255, 1176–1110, 962–476	10.73 (1H, s), 8.283–8.279(1H, d, J = 1.6), 8.121–8.100 (2H, d, J = 8.4), 7.828–7.713 (5H,m), 7.623–7.601 (2H, d, J = 8.8), 7.516–7.477 (1H, dd, J = 8.0, J′ = 7.6), 7.377–7.339 (1H, dd, J = 8.0, J′ = 7.2), 1.307 (9H, s)	411 (% 100) (M + H)
8	H	H	CH_2	$C_{23}H_{16}N_2O_3$: C: 74.99, H: 4.38, N: 7.60/C: 74.91, H: 4.37, N: 7.60	177–180	54	3270, 3078, 1669, 1625, 1569, 1482, 1354–1297, 1258, 1174–1144, 949–471	10.650 (1H, s), 8.185 (1H, s), 7.812–7.695 (4H, m), 7.643–7.621 (1H, d, J = 8.8), 7.501–7.463 (1H, dd, J = J′ = 7.6), 7.350–7.260 (6H, m), 4.313 (2H, s)	369 (% 100) (M + H)

(continued on next page)

Table 1 (*continued*)

Compound no.	R_1	R	X	Formula: calculated/found	Mp	Yield (%)	IR (cm^{-1})	^1H NMR (δ ppm) J = Hz	MS (ESI^+) m/z (% X)
9	H	Br	CH_2	$C_{23}H_{15}BrN_2O_3$: C: 61.76, H: 3.39, N: 6.26/ C: 61.61, H: 3.39, N: 6.26	202−205	89	3268, 3078, 1683, 1669, 1625, 1589, 1486, 1354−1298, 1257, 1172−1108, 971−474	10.713 (1H, s), 8.201−8.197 (1H, d, J = 1.6), 7.854−7.834 (1H, d, J = 8.0), 7.790−7.733 (3H, m), 7.691−7.669 (1H, d, J = 8.8), 7.586−7.505 (3H, m), 7.405−7.361 (3H, m), 4.357 (2H, s)	447 (% 80) (M+), 449 (% 100) (M + 2)
10	H	CH_3	CH_2	$C_{24}H_{18}N_2O_3$: C: 75.38, H: 4.74, N: 7.32, C: 75.21, H: 4.38, N: 7.30	172−175	54	3077, 1670, 1626, 1567, 1482, 1354−1277, 1258, 1183−1144, 971−471	10.672 (1H, s), 8.173−8.168 (1H, d, J = 2.0), 7.819−7.800 (1H, d, J = 7.6), 7.760−7.699 (3H, m), 7.639−7.618 (1H, d, J = 8.4), 7.510−7.468 (1H, dd, J = J′ = 8.4), 7.371−7.332 (1H, dd, J = 8.0, J′ = 7.6), 7.247−7.226 (2H, d, J = 8.4), 7.148−7.129 (2H, d, J = 7.60), 4.256 (2H, s), 2.255 (3H, s)	383 (% 100) (M + H)
11	H	F	CH_2	$C_{23}H_{15}FN_2O_3$: C: 71.50, H: 3.91, N: 7.25/ C: 71.21, H: 3.63, N: 7.23	200−203	82	3270, 3077, 1670, 1625, 1571, 1482, 1355−1299, 1258, 1185−1109, 972−471	10.679 (1H, s), 8.176−8.171 (1H, d, J = 2.0), 7.820−7.801 (1H, d, J = 7.6), 7.759−7.700 (3H, m), 7.656−7.635 (1H, d, J = 8.4), 7.508−7.471 (1H, dd, J = 7.2, J′ = 7.6), 7.433−7.398 (2H, m), 7.371−7.333 (1H, dd, J = 8.0, J′ = 7.2), 7.194−7.151 (2H, dd, J = 8.4, J′ = 8.8), 4.327 (2H, s)	387 (% 100) (M + H)
12	H	Cl	CH_2	$C_{23}H_{15}ClN_2O_3$: C: 68.58, H: 3.75, N: 6.95/ C: 68.58, H: 3.67, N: 6.48	182−185	82	3268, 3077, 1670, 1625, 1569, 1482, 1354−1299, 1257, 1185−1109, 971−487	10.682 (1H, s), 8.177−8.172 (1H, d, J = 2.0), 7.817−7.799 (1H, d, J = 7.2), 7.761−7.698 (3H, m), 7.655−7.634 (1H, d, J = 8.4), 7.507−7.468 (1H, dd, J = 7.6, J′ = 8.0), 7.403−7.331 (5H, m), 4.40 (2H, s)	403 (% 100) (M + H)

Table 2
Antimicrobial activity results (MIC μg/ml) of newly synthesized compounds (**3**–**12**) with the standard drugs



Compound no	X	R_1	R	Kp*	Kp	Pa*	Pa	Ec*	Ec	Bs*	Bs	Sa*	Sa	Ca*	Ca	Ck
3	–	Br	H	125	62.5	62.5	62.5	125	62.5	125	500	31.25	125	62.5	62.5	250
4	–	H	H	125	62.5	62.5	62.5	125	62.5	250	125	62.5	62.5	62.5	62.5	62.5
5	–	H	C_2H_5	125	62.5	62.5	62.5	125	62.5	125	500	62.5	125	62.5	62.5	125
6	–	H	F	125	62.5	62.5	31.25	125	62.5	125	500	31.25	125	62.5	62.5	125
7	–	H	$C(CH_3)_3$	125	62.5	125	62.5	125	62.5	125	125	62.5	125	62.5	62.5	125
8	CH_2	H	H	62.5	62.5	62.5	31.25	62.5	62.5	125	125	62.5	125	31.25	62.5	31.25
9	CH_2	H	Br	62.5	62.5	15.625	31.25	125	62.5	125	125	62.5	125	62.5	62.5	62.5
10	CH_2	H	CH_3	125	62.5	125	62.5	125	62.5	125	125	62.5	125	62.5	62.5	250
11	CH_2	H	F	125	62.5	125	62.5	125	62.5	125	125	62.5	125	62.5	62.5	250
12	CH_2	H	Cl	125	62.5	125	31.25	125	62.5	125	125	125	125	62.5	62.5	125
Ampicillin trihydrate				15.625	0.48	>500	>500	>15.625	3.9	0.48	0.48	1.9	0.48	–	–	–
Gentamicin				7.8	0.24	62.5	31.25	15.625	0.48	0.12	0.24	7.8	0.48	–	–	–
Rifampicin				7.8	1.9	>500	>500	3.9	1.9	3.9	0,12	0.9	0.06	–	–	–
Ofloxacin				3.9	0.12	62.5	62.5	7.8	0.12	3.9	0.12	1.9	0.12			
Amphotericin B														0.24	0.48	1.9
Fluconazole														0.48	1.9	15.625

Kp*: *Klebsiella pneumoniae* isolate; Kp: *K. pneumoniae* RSHM 574; Pa*: *Pseudomonas aeruginosa* isolate; Pa: *P. aeruginosa* ATCC 25853; Ec*: *Escherichia coli* isolate; Ec: *E. coli* ATCC 25922; Bs*: *Bacillus subtilis* isolate; Bs: *B.subtilis* ATCC 6633; Sa*: *Staphylococcus aureus* isolate; Sa: *S.aureus* ATCC 25923; Ca*: *Candida albicans* isolate; Ca: *C. albicans* ATCC 10231; Ck: *C. krusei* ATCC 6258.

25853, *Escherichia coli* ATCC 25922, *K. pneumoniae* isolate [has Extended Spectrum β Lactamase (ESBL) enzyme], *E. coli* isolate [has Extended Spectrum β Lactamase (ESBL) enzyme] as Gram-negative bacteria, *Bacillus subtillis* ATCC 6633, *S. aureus* ATCC 25923, *B. subtillis* isolate (resistant to ceftriaxon), *S. aureus* isolate [resistant to meticillen (MRSA)] as Gram-positive bacteria and the antifungal activity was evaluated against *Candida krusei* ATCC 6258, *Candida albicans* ATCC 10231, *C. albicans* isolate (biofilm positive). The MIC values were determined by two-fold serial dilution technique in Mueller–Hinton broth and Sabouraud Dextrose agar for the antibacterial and antifungal assay, respectively. For comparison of the antimicrobial activity, rifampicin, ampicillin trihydrate, gentamycin sulfate, ofloxacine were used as the reference antibacterial agents and fluconazole, amphotericin B were employed as the reference antifungal agents. All the biological results of the tested compounds are given in Table 2.

According to Table 2, the synthesized compounds showed a broad spectrum of activity with MIC values 500–62.5 μg/ml against some Gram-positive bacteria such as *S. aureus* ATCC 25923, *B. subtillis* ATCC 6633. Interestingly, the antibacterial activity against meticillen resistant *S. aureus* (MRSA) of the tested compounds **3**–**12** was found to have more potent than against *S. aureus* strains. However, they were less active than the compared control drugs, rifampicin, gentamicin, ampicillin trihydrate and ofloxacin.

All of the new compounds **3**–**12** showed a narrow antibacterial activity against some *E. coli*, *K. pneumoniae*, *P. aeruginosa* as Gram-negative bacteria possessing MIC values between 62.5 and 31.25 μg/ml and they were found to have inhibitory effect with MIC values 125–15.625 μg/ml against their isolates.

All compounds were less active than standard drugs against *K. pneumoniae* and its isolate. Structurally differences on the substitution at position two of benzoxazole ring did not change the activity against *K. pneumoniae*. Newly synthesized compounds **6**, **8**, **9**, **12** showed higher activity against *P. aeruginosa* and its isolate when compared with all the standard drugs. Among the tested derivatives only **9** had a pretty good activity with a MIC value 15.625 μg/ml against drug-resistant *P. aeruginosa* even more potent than all standard drugs. Additionally, derivatives **6**, **8**, **9**, and **12** showed higher activity against *P. aeruginosa* than ampicillin, rifampicin and ofloxacin and had the same potency with gentamycin. The other compounds had a comparable effect with ofloxacin. It could be concluded that, this kind of structures might be a lead for *P. aeruginosa*. On the other hand, although the screened derivatives against *E. coli* were found to have a moderate potency, they showed very weak activity than the standard drugs. Among the molecules, only 2-benzyl-5-[(2-benzofuryl)carboxamido]benzoxazole **8**, has been found to be significantly active with a MIC value 62.5 μg/ml against drug-resistant *E. coli*.

Furthermore, bearing 2-furyl [22] instead of 2-benzofuryl on the carboxamido moiety caused slightly better activity against Gram-positive bacteria *S. aureus* and *B. subtilis*. It could be concluded that if the hydrophobicity is higher, the potency gets lower. In contrast, attaching 2-benzofuryl instead of 2-furyl group at carboxamido moiety causes important enhancing inhibitory effect against Gram-negative bacterium *P. aeruginosa*. At this time, highly hydrophobic molecule could be needed for increasing activity.

In the past 10 years there has been a major expansion in the development of antifungal drugs, but there are still weaknesses in the range and scope of current antifungal chemotherapy [40]. New developments have included the modification of existing drug molecules to eliminate toxicity and improve activity. Therefore, we also tried to screen the antifungal activity besides their antibacterial activity to be able to discover new lead compounds.

All the newly synthesized derivatives showed the least activity than amphotericin B and fluconazole. The MIC range (250–31.25 μg/ml) of this series for *C. krusei* ATCC 6258 is larger than the MIC range (62.5–31.25 μg/ml) of *C. albicans* and its isolates. In one of our previous studies [39], 22-[*p*-substituted-benzyl]-5-[substitutedcarbonylamino]benzoxazole derivatives showed antifungal activity with MIC value of 6.25–100 μg/ml against *C. albicans* and *C. krusei*. The results of the previous study revealed that the *p*-substituted-benzyl moiety at position two of the benzoxazole ring slightly improved the antifungal activity. Therefore in this study, we got the best antifungal activity result with compound **8** carrying benzyl group at the second position of the benzoxazole ring. In addition, attaching a hydrogen or bromine on position R played a role for getting a good inhibitory potency. However, substituting position R_1 with the bromine atom, while the main structure was 2-phenylbenzoxazole decreased the inhibitory effect.

It seemed to be some more modifications on the benzofuran moiety are needed for the further study to get lead compounds. Moreover, the yielded result against *C. krusei* for derivative **8** was also quite encouraging due to its activity caused by only one-fold less dilution than fluconazole. As a result of our studies we could say that the derivatives with a phenyl ring at the fifth position of the benzoxazole ring, are more potent than the ones having a benzofuran ring at the same position against the *Candida* species.

For the 3D-common feature pharmacophore hypothesis approach, the hypothesis 1 that consists three HpArs and three HBAs as shown in Fig. 2 was generated as the common-feature functions to explain the specification of the antibacterial agents against drug-resistant *P. aureuginosa*. Fig. 3 depicted that the most active molecule is compound **9** showing a good fit with all the generated features than the rest of the compounds.

When the training compounds were mapped onto the common-feature functions generated by hypothesis 1, one of the HBA features slightly maps onto "N" of benzoxazole at the compounds **3**, **4**, **5**, **6**, showed a better match for derivative **8.** However, one of the HBA features slightly fitted into "O" of benzofuran at compound **8**. It could be concluded that "N" was more important than "O" of benzoxazole for increasing the potency.

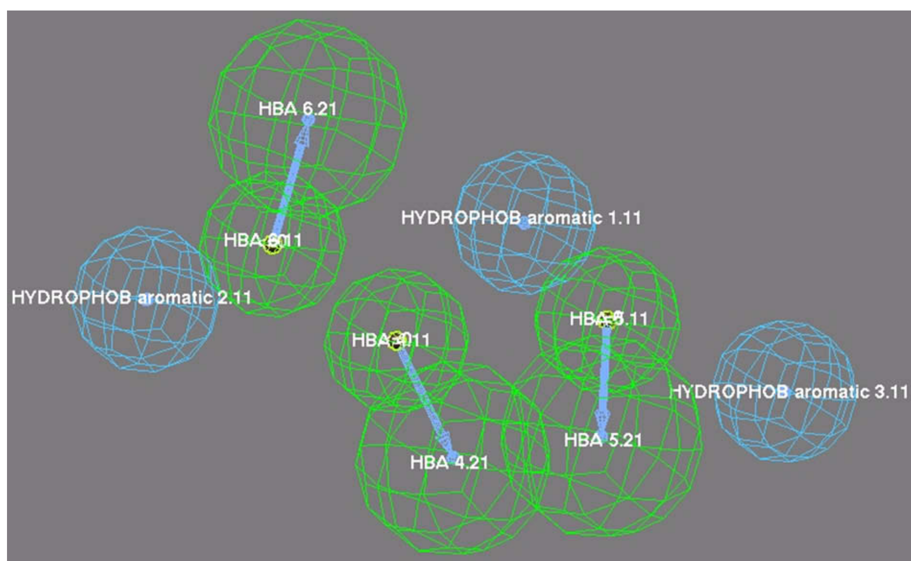


Fig. 2. Mapping of hypothesis 1, which contains three HpArs (blue), three HBAs (green) pharmacophore features (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

On the other side, when the less active compounds given in Table 1 mapped onto the generated hypothesis 1, it has been observed that they only showed a maximum match on five common features. While the ''O'' of carbonyl group at the compounds **10**, **11**, **12**, possessed a fit onto the HBA feature, the same group at the compound **7** did not show a match on this feature. The ''O'' of benzofuran of structure **7** fit into the HBA in contrast derivatives **10**, **11**, and **12**. It was noticed that, if the ''O'' of either benzofuran or benzoxazole placed at the same side played very important role for increasing the activity against drug-resistant *P*. *aeruginosa*.

3. Conclusion

As a result of this study, some novel benzoxazole derivatives including 2-benzofuryl as antimicrobial agents have been discovered. It was found that structurally differences of the substituents at position two of the benzoxazole nucleus did not constitute a major role at their antimicrobial activity against Gram-positive bacteria. Consequently, this kind of structures might be guides for Gram-negative bacterium *P*. *aeruginosa*. In particular, compound **9** having 2-(*p*-bromobenzyl)benzoxazole group exhibited the highest activity with MIC value of 15.625 μg/ml against drug-resistant *P*. *aeruginosa*. Additionally, the result against *C*. *krusei* for **8** is quite encouraging due to its activity caused by only one-fold less dilution than fluconazole. 3D-common features pharmacophore hypothesis generation revealed that the conformational properties of the compounds are important for the activity against drug-resistant *P*. *aureuginosa* and the HBA features are more significant than the HpAr for increasing the activity. These observations provided us noteworthy predictions in order to design further antimicrobial active compounds prior to their synthesis.

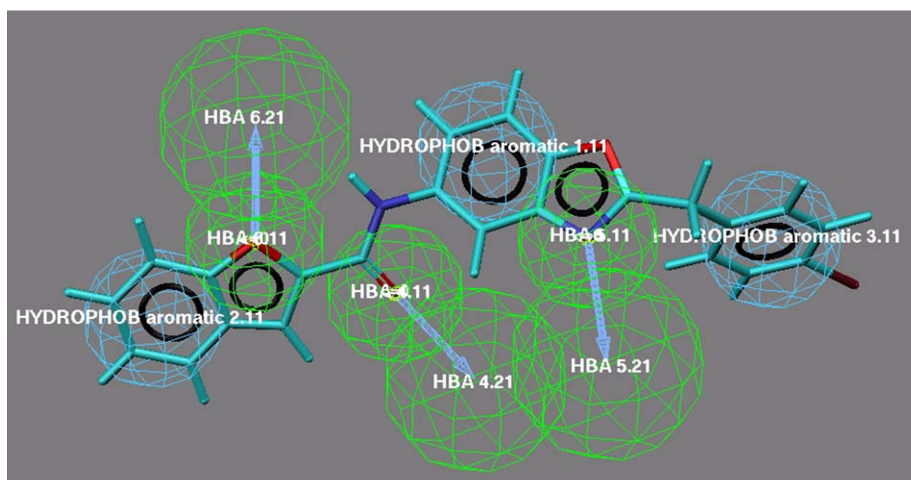


Fig. 3. Mapping of **9** onto hypothesis 1, which contain three HpArs (blue) and three HBAs (green) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

4. Experimental

The chemicals were purchased from the commercial venders and were used without purification. The reactions were monitored and the purity of the products was checked by thin layer choromatography TLC. Kieselgel HF 254 chromatoplates (0.3 mm) were used for TLC and the solvent systems were ethylacetate–*n*-hexane (5:5). All the melting points were taken on a Buchi SMP 20 capillary apparatus and are uncorrected. IR spectra were recorded on a Jasco FT/IR-420 spectrometer as KBr discs. ^{1}H NMR spectra were obtained with a Varian 400 MHz spectrometer in chloroform-*d* ($CDCl_3$) or dimethylsulfoxide-d_6 (DMSO-d_6) and tetramethylsilane (TMS) was used as an internal standard. Mass analyses were carried out with a Waters Micromass ZQ by using ESI^+ method. Elemental analysis were performed on LECO 932 CHNS (Leco 932, St. Joseph, MI, USA) instrument and were within ±0.4% of the theoretical values. All chemicals and solvents were purchased from Aldrich Chemical Co. or Fischer Scientific.

4.1. General procedure for the compounds **1**

5-Amino-2-[*p*-substitutedphenyl/benzyl]benzoxazoles and 5-amino-2-[*o*-bromophenyl]benzoxazole were synthesized by heating 0.01 mol 2,4-diaminophenol·2HCl with 0.01 mol *p*-substitutedphenyl acetic acid and/or *p*-substitutedbenzoic acid and/or *o*-bromobenzoic acid in 12.5 g polyphosphoric acid (PPA) and stirring for 1.5–2.5 h. At the end of the reaction period, the residue was poured into ice–water mixture and neutralized with excess of 10 M NaOH solution extracted with benzene and then this solution was dried over anhydrous sodium sulfate and evaporated under diminished pressure. The residue was boiled with 200 mg charcoal in ethanol and filtered. After the evaporation of solvent *in vacuo*, the crude product was obtained and recrystallized from ethanol [20].

4.2. General procedure for the compounds **2** and **3–12**

Appropriate carboxylic acid (0.005 mol) and thionyl chloride (1.5 ml) were refluxed in benzene (5 ml) at 80 °C for 3 h. Excess thionyl chloride was removed *in vacuo* for the compound **2**. The residue was dissolved in ether (10 ml) and this solution was added during 1 h to a stirred ice-cold mixture of 5-amino-2-[*p*-substitutedphenyl/benzyl]benzoxazoles and 5-amino-2-[*o*-bromophenyl]benzoxazole **1** (0.005 mol), sodium bicarbonate (0.005 mol), diethyl ether (10 ml) and water (10 ml). The mixture was kept stirred overnight at room temperature and filtered. The precipitate was washed with water, 2 N HCl and water, respectively, and finally with ether to give **3–12**. The products were recrystallized from ethanol–water as needles which are dried *in vacuo* [20]. The chemical, physical and spectral data of the compounds **3–12** are reported in Table 1. The result of the C-13 spectra of compound **8** is obtained as: 35.543, 110.770, 111.722, 112.027, 112.088, 118.146, 123.091, 124.143, 127.481, 127.603, 127.915, 129.081, 129.233, 134.087, 134.857, 142.142, 148.482, 148.634, 155.035, 156.910, 166.549.

4.3. Microbiology

4.3.1. Materials

Mueller–Hinton Agar (MHA) (Merck), Mueller–Hinton Broth (MHB) (Merck), Sabouraud Dextrose Agar (SDA) (Merck), RPMI-1640 medium with L-glutamine (Sigma), 3-[*N*-morpholino]-propan-sulfonic acid (MOPS) (Sigma), 96-well microplates (Falcon), Transfer pipette (Biohit), Rifampicin (Kocak), Ampicillin trihydrate (Paninkret Chem. Pharm.), Gentamicin sulfate (Deva Ilac Sanayii), Ofloxacin (Zhejiang Huangyan East Asia Chemical Co.), Fluconazole (Nobel), Amphotericin B (Bristol Myers Squibb), Ethanol (Riedel de Haen), Dimethylsulphoxide (DMSO) (Riedel de Haen).

4.3.2. Microorganisms

4.3.2.1. Isolates. *K. pneumoniae* isolate [has Extended Spectrum β Lactamase (ESBL) enzyme], *P. aeruginosa* isolate (resistant to gentamicin), *E. coli* isolate [has Extended Spectrum β Lactamase (ESBL) enzyme], *B. subtilis* isolate (resistant to ceftriaxon), *S. aureus* isolate [resistant to meticillen (MRSA)] and *C. albicans* isolate (biofilm positive).

4.3.2.2. Standard strains. *K. pneumoniae* RSHM 574 (Refik Saydam Hygiene Center Culture Collection), *P. aeruginosa* ATCC 25853 (American Type Culture Collection), *E. coli* ATCC 25922, *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *C. albicans* ATCC 10231, *C. krusei* ATCC 6258.

4.3.3. Method

Standard strains of *K. pneumoniae* RSHM 574, *P. aeruginosa* ATCC 25853, *E. coli* ATCC 25922, *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *C. albicans* ATCC 10231 and clinical isolates of these microorganisms that are known to be resistant to various antimicrobial agents were included in the study. Resistance was determined by Kirby Bauer Disk Diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) in the clinical isolates [41].

Standard powders of rifampicin, ampicillin trihydrate, gentamicin sulfate, ofloxacin, fluconazole, and amphotericin B were obtained from the manufacturers. Stock solutions were dissolved in dimethylsulphoxide (ofloxacin), methanol (rifampicin), pH 8 phosphate-buffered saline (PBS) (ampicillin trihydrate), and distilled water (gentamicin sulfate, fluconazole, and amphotericin B).

All bacterial isolates were subcultured in MHA plates and incubated overnight at 37 °C and all *Candida* isolates were subcultured in SDA plates at 35 °C for 24–48 h. The microorganisms were passaged at least twice to ensure purity and viability. The solution of the newly synthesized compounds and standard drugs were prepared at 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.8, 3.9, 1.95, 0.98, 0.48, 0.24, 0.12, 0.06 μg/ml concentrations in the wells of microplates by

diluting in the liquid media. Bacterial susceptibility testing was performed according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) M100-S16 [42]. The bacterial suspensions used for inoculation were prepared at 10^5 cfu/ml by diluting fresh cultures at MacFarland 0.5 density (10^7 cfu/ml). Suspensions of the bacteria at 10^5 cfu/ml concentration were inoculated to the two-fold diluted solution of the compounds. There were 10^4 cfu/ml bacteria in the wells after inoculations. MHB was used for diluting the bacterial suspension and for two-fold dilution of the compound. DMSO (80%) and EtOH (20%), methanol, DMSO, PBS, pure microorganisms, and pure media were used as control wells. A 10 µl bacteria inoculum was added to each well of the microdilution trays. The trays were incubated at 37 °C in a humid chamber and MIC endpoints were read after 24 h of incubation. All organisms were tested in triplicate in each run of the experiments. The lowest concentration of the compound that completely inhibits macroscopic growth was determined and minimum inhibitory concentrations (MICs) were reported.

All *Candida* isolates were subcultured in SDA plates, incubated at 35 °C for 24–48 h prior to antifungal susceptibility testing, and passaged at least twice to ensure purity and viability. Susceptibility testing was performed in RPMI-1640 medium with L-glutamine buffered, pH 7, with MOPS and culture suspensions were prepared through the guideline of CLSI M27-A [43]. The yeast suspensions used for inoculation were prepared at 10^4 cfu/ml by diluting fresh cultures at MacFarland 0.5 density (10^6 cfu/ml). Suspensions of the yeast at 10^4 cfu/ml concentration were inoculated to the two-fold diluted solution of the compounds. There were 10^3 cfu/ml yeasts in the wells after inoculations. A 10 µl yeast inoculum was added to each well of the microdilution trays. The trays were incubated at 35 °C in a humid chamber and MIC endpoints were read after 48 h of incubation. All organisms were tested in triplicate in each run of the experiments. The lowest concentration of the compound that completely inhibits macroscopic growth was determined and minimum inhibitory concentrations (MICs) were reported.

4.4. Common features pharmacophore hypotheses generation

All computational experiments were conducted on a Silicon Graphics O2, running under the IRIX 6.5 operating system. Hypotheses generation was applied against previously described data sets by using Catalyst/HipHop (version 4.9) from Accelrys [44]. Molecules were edited using the Catalyst 2D/3D visualizer. Catalyst automatically generated conformational models for each compound using the Poling Algorithm [45–47]. The "best conformer generation" procedure was applied to provide the best conformational coverage for a maximum number of conformers generated defaulted to 250 in a 0–25 kcal/mol range from the global minimum. The conformations generated were used to align common molecular features and generate pharmacophore hypothesis. HipHop is used to the conformations generated to align chemically important functional groups common to the molecules in the study set. A pharmacophoric hypothesis was then generated from these aligned structures.

HipHop provides feature-based alignment of a collection of compounds without considering the activity. It matches the chemical features of a molecule, against drug candidate molecules. HipHop takes a collection of conformational models of molecules and a selection of chemical features, and produces a series of molecular alignments in a variety of standard file formats. HipHop begins by identifying configurations of features common to a set of molecules. A configuration consists of a set of relative locations in 3D space and associated feature types. A molecule matches the configurations if it possesses conformations and structural features that can be superimposed within a certain tolerance from the corresponding ideal locations. HipHop also maps partial features of molecules in the alignment set. This provision gives the option to use partial mapping during the alignment. Partial mapping allows to identify larger, more diverse, more significant hypotheses and alignment models without the risk of missing compounds that do not have to map to all of the pharmacophore features.

In this research, HipHop common feature hypotheses were generated to explain the specification of the antibacterial agents against drug-resistant *P. aureuginosa*, which are the best significant results among the screened microorganisms. This tool builds hypotheses (overlays common features) for which the fit of individual molecules to a hypothesis can be correlated with activity of the molecule. A set of six active compounds from Table 2 was selected as the target training set (Table 3). Among the selected six molecules, the most active molecule, compound **9**, was chosen as the reference, which should allow to map all features on the generated hypotheses for the antibacterial activity against drug-resistant *P. aureuginosa*.

The geometry of each compound was built with a visualizer and optimized by using the generalized CHARMm-like force

Table 3
Selected active compounds and characteristics for the common feature hypothesis run

Compounds	Confs[a]	Features/confs[a]	Principal[b]	MaxOmitFeat[c]
3	32	9.75	1	2
4	27	9.85	1	2
5	36	9.83	1	2
6	13	9.85	1	2
8	49	9.82	1	2
9	71	9.41	**2**	**0**

[a] Confs, number of conformers; Features/confs, total number of features divided by the number of conformers (summed over the entire family of conformers).

[b] Principal = **1** means that this molecule must map onto the hypothese generated by the search procedure. Partial mapping is allowed. Principal = **2** means that this is a reference compound. The chemical feature space of the conformers of such a compound is used to define the initial set of potential hypotheses.

[c] The MaxOmitFeat column specifies how many hypothesis features must map to the chemical features in each compound. A **0** in this column forces mapping of all features, a **2** allows hypotheses to which no compound features map.

Table 4
Results of the common feature hypothesis run

Hypotheses	Feature[a]	Rank score	Direct hit[b]	Partial hit[b]
1	HpAr HpAr HpAr HBA HBA HBA	102.164	111111	000000
2	HpAr HpAr HpAr HBA HBA HBA	102.028	111111	000000
3	HpAr HpAr HpAr HBA HBA HBA	100.904	111111	000000
4	HpAr HpAr HpAr HBA HBA HBA	98.9037	111111	000000
5	HpAr HpAr HBD HBA HBA	91.3093	111111	000000
6	HpAr HpAr HBD HBA HBA	90.4743	111111	000000
7	HpAr HpAr HpAr HBA HBA	88.8775	111111	000000
8	HpAr HpAr HBA HBA HBA	88.4734	111111	000000
9	HpAr HpAr HBD HBA HBA	87.7719	111111	000000
10	HpAr HpAr HBD HBA HBA	87.2921	111111	000000

[a] HpAr, Hydrophobic aromatic; HBA, Hydrogen-bond acceptor; HBD, Hydrogen-bond donor.
[b] Direct hit, all the features of the hypothesis are mapped. Direct hit = 1 means yes; partial hit, partial mapping of the hypothesis. Partial hit = 0 means no. Each number refers to a molecule in Table 5 (same order).

field implemented in the program. A preparative test was performed with hydrogen-bond acceptor (HBA), hydrogen-bond acceptor lipid (HBAI), hydrogen-bond donor (HBD), hydrophobic (Hp), hydrophobic aromatic (HpAr), hydrophobic aliphatic (HpAl), negative ionizable (NI), positive ionizable (PI) and Ring Aromatic (R) [48]. NI and PI were used rather than negative charge and positive charge in order to broaden the search for deprotonated and protonated atoms or groups at physiological pH. By using conformational poling [47], a representative family of conformers was generated, within a 25 kcal/mol range of the computed minimum, for each molecule. Potential hypothesis models were produced with the minimum permitted interfeature spacing of 2.00 Å generating alignments of common features [49] that included the projected points of HpAr, HBA and HBD [48]. The characteristics of the generated potential 10 hypotheses are listed in Table 4 and first four hypotheses contain six features concerning of three HpAr and three HBA with the ranking score of 102.164. The rest hypotheses have five features. Consequently, because of showing the highest ranking score and having more features, hypothesis 1 has been chosen for the further evaluation among the other generated potential hypotheses.

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