Original Article

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Molecular Docking and Antimicrobial Evaluation of Some Novel Pyrano[2,3-C] Pyrazole Derivatives

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Abstract

The extensive use of antifungal drugs and their resistance against fungal infections have led to develop novel antimicrobial compounds. In this research, 14 new pyranopyrazole compounds (D_1-D_{14}) which were synthesized before, screened for antimicrobial activity. These compounds consist of a pyranopyrazole scaffold with a phenyl substitution at the 4-position of the pyran ring. Antimicrobial evaluation of the above compounds were investigated against different species of fungi, gram positive and gram negative bacteria by broth micro dilution methods as recommended by CLSI. The specific binding mode or the binding orientation of the compounds to CYP51 active site, have been also performed by molecular modeling investigations. Our results implies that some of our compounds possess desirable inhibitory activity against the tested microorganisms. Our docking study results also showed that the binding free energy values of the compounds are in agreement with the corresponding experimental activity values. By comparison the relationship between chemical structures and biological activities revealed that the presence of a withdrawing substituent at4-position of phenyl group at para position of the pyran ring enhance the antimicrobial activity.

Keywords: Antifungal, Antibacterial, Molecular docking, Pyranopyrazoles

1. Introduction

Drug resistance to antimicrobial agents is considered as one of the major problems of global health (1). Despite extensive research and many efforts to control antimicrobial infectious, humans have still not succeeded to eliminate these problems (2). Increasing drug resistance among fungal species is due to the inappropriate use of antifungal drugs (3, 4). Therefore, it is necessary to develope and design new antibacterial and antifungal agents, with novel chemical structures, broad spectrum, low toxicity and low resistance which are helpful for overcoming drug resistance and improving the antimicrobial potency (4, 5). Recently synthesis of heterocyclic compounds has become one of the most essential planes of medicinal chemists (6). On the other hand, pyrazole ring that contains a five membered heterocyclic ring with two adjacent nitrogen atoms is a prominent heterocyclic scaffold in lots of bioactive molecules (7). Pyrazole and its fused heterocyclic derivatives are important substances and have gained widespread attention in pharmaceutical chemistry due to their effective biological activities (8). These heterocyclic compounds have attracted great attention considering their diverse biological properties such as analgesics (9), antifungal (10), antibacterial (7), anti-inflammatory (11), anticancer

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(12) and anticonvulsant (7). Following of our study on synthesis and biological evaluation of azole compounds (13, 14) here we are going to evaluate antifungal and antibacterial properties of a variety of some new pyrano[2,3-c] pyrazoles derivatives which were previously synthesized (15). Broth microdilution method was used for evaluation of their antimicrobial activity against different species of candida and filaments fungi and also gram positive and gram negative bacteria. Fluconazole and ciprofloxacin were used as positive controls for antifungal and antibacterial screening respectively. Molecular docking studied were applied on fourteen of these compounds to study the binding conformations and structural specificity of the antifungal agents.

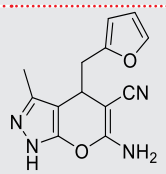
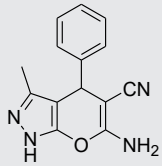
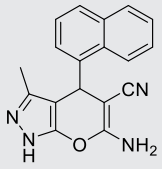
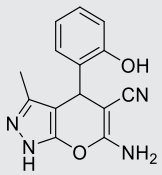
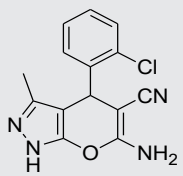
2. Material and Methods

All chemicals and solvents were purchased from Merck (Germany). Newly synthesized compounds were obtained from School of Chemistry, Yazd University, Iran (Table 1). The RPMI-1640 media were used from Sigma, St. Louis (USA). Serial dilutions (0.5-256 µl/mL) were prepared in the Muller-Hinton media and Sabouraud dextrose agar was produced from Merck, Darmstadt, Germany.

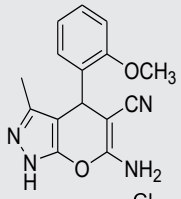
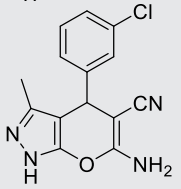
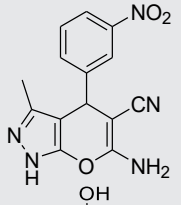
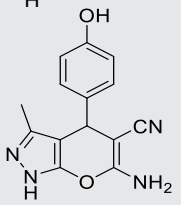
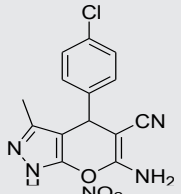
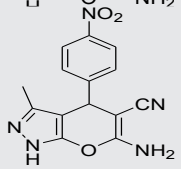
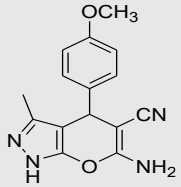
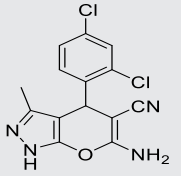
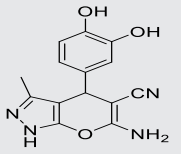
2.1. Microorganisms

In this study the sensitivity of the compounds based on the proposed protocol by CLSI which is accurate and valid was done. Antifungal activities of the synthetic compounds against some American Type Culture Collection (ATCC) strains of fungi, including *Candida neoformans* (ATCC 9011), *Candida dubliniensis* (ATCC 8500), *Candida albicans* (ATCC 10261), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258), *Candida tropicalis* (ATCC 750), *Candida parapsilosis* (ATCC 4344), *Exophiala dermatiti-*

Table 1. Synthesized pyrano[2,3-c] pyrazoles derivatives, which were tested against fungi and bacteria.

Entry	Chemical structures	Chemical names	M.W.	M.P. (˚C)	Log P
D1		6-amino-4-(furan-2-ylmethyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	256	228-230	1
D2		6-Amino-3-methyl-4-phenyl-2,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	252	242-244	2.11
D3		6-amino-3-methyl-4-(naphthalen-1-yl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	302	206-208	3.1
D4		6-amino-4-(2-hydroxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	268	223-225	1.72
D5		. 6-Amino-3-methyl-4-(2-chlorophenyl)-2,4-dihydropyrano[2,3-c] pyrazole-5-carbonitrile	286	147-148	2.66

Continued Table 1.

Entry	Chemical structures	Chemical names	M.W.	M.P. (˚C)	Log P
D6		6-Amino-3-methyl-4-(2-methoxyphenyl)-2,4 dihydropyrano[2,3-c]pyrazole-5-carbonitrile	282	226-228	1.98
D7		6-amino-4-(3-chlorophenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	286	223-224	2.66
D8		6-amino-3-methyl-4-(3-nitrophenyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	297	210-211	1.11
D9		6-amino-4-(4-hydroxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	268	222-224	1.72
D10		6-Amino-3-methyl-4-(4-chlorophenyl)-2,4-dihydropyrano[2,3-c] pyrazole-5-carbonitrile	286	233-234	2.66
D11		6-Amino-3-methyl-4-(4-nitrophenyl)-2,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	297	242-244	1.11
D12		6-amino-4-(4-methoxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	282	218-219	1.98
D13		6-amino-4-(2,4-dichlorophenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	320	223-225	3.22
D14		6-amino-4-(3,4-dihydroxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c] pyrazole-5-carbonitrile	284	225-227	1.33

dis (ATCC 109136), *Aspergillus clavatus* (CBS 514.65), *Aspergillus fumigatus* (ATCC 14110), *Aspergillus flavus* (ATCC 64025), *Pseudallescheria boydii* (ATCC 36282) were determined. The susceptibility of all clinical isolates of fungi against selected antibiotics was examined by microdilution method. The antibacterial activities of the above compounds against standard species of gram-positive and gram-negative bacteria including *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeroginosa* (ATCC 27853) were also determined in this study. In order to evaluate the biological activity of the synthesized compounds microdilution method was used. The microorganisms were cultured in Brain Heart Infusion (BHI) broth overnight and stored in glycerol/Media (20%) at -70 ºC. The pure cultures of microorganisms were sub-cultured in BHI broth at 37 °C in a shaker incubator 24h before experiment. The antibacterial evaluation was done using following methods.

2.2. Determination of Minimum Inhibitory Concentrations

MICs were determined using the broth micro dilution method recommended by the CLSI with some modifications (16, 17). Briefly, for determination of antimicrobial activities against fungi, serial dilutions of the synthetic compounds (1-1024 µg/mL) were prepared in 96-well micro titer plates using RPMI-1640 media buffered with MOPS. Stock inoculums were prepared by suspending three colonies of the examined yeast in 5 mL sterile 0.85% NaCl, and adjusting the turbidity of the inoculums to 0.5 McFarland standards at 530 nm wavelengths (this yields stock suspension of $1\text{-}5\times10^6$ cells/mL). For moulds (*Aspergillus* spp. and *Dermatophytes*), conidia were recovered from the 7-day old cultures grown on potato dextrose agar by a wetting loop with tween-20. The collected conidia were transferred in sterile saline and their turbidity was adjusted to OD=0.09-0.11 that yields $0.4\text{-}5\times10^6$ conidia/mL. Working suspension was prepared by making a 1/50 and 1/1000 dilution with RPMI of the stock suspension for moulds and yeasts, respectively. Working inoculums (0.1 mL) were added to the micro titer plates, which were incubated in a humid atmosphere at 30 ºC for 24-48 h. Uninoculated medium (200 mL) was included as a sterility control (blank). In addition, growth controls (medium with inoculums but without antibiotics or the synthetic compounds) were also included. The growth in each well was compared with that of the growth in the control well.

2.3. Bactericidal and fungicidal assessment of the compounds

To measure the minimum bactericidal concentrations (MBCs), the media from wells, in which no bacteria had growth, was cultured on triptych soy agar and incubated for 24 h at 37 °C. The MBC values were the lowest concentration of compounds to reduce the viability of the initial bacterial inoculums by ≥99.9%; so that less than 4 countable colonies can be detected after 24 h incubation at 37 °C in agar plates. Minimum fungicidal concentrations (MFCs) measurement was the same except in culture media that was Sabouraud-dextrose agar in this experiment.

2.4. Docking procedures

An in house batch script (DOCK-FACE) for automatic running of AutoDock 4.2 was used to carrying out the docking simulations (18) in a parallel mode, using all system resources as described before (19). To prepare the receptor structure, the complex of Mycobacterium tuberculosis-CYP51 enzyme with Fluconazole (PDB ID: 1EA1) was acquired from Protein Data Bank (PDB data base; http://www.rcsb.org) (20) and water molecules and co-crystal ligand were removed from the structure. The PDB were then checked for missing atom types with MODELLER 9.17 (21).The ligand structures were made by using HyperChem software package (Version 7, Hypercube Inc). For geometry optimization, Molecular Mechanic (MM+), followed by semi empirical AM1 method was performed. The prepared Ligands were given to 100 independent genetic algorithm (GA) runs with 150 population size, a maximum number of 2,500,000 energy evaluations and 27,000 maximum generations. The grid points of 40, 40, and 40 in x, y, and z directions were used. A grid spacing of 0.375 A° was built centered on hem group in the

catalytic site of the receptor. Number of points in x, y and z was -17, -3 and 65 respectively. All visualization of protein ligand interaction was evaluated using VMD software14.

3. Results and Discussion

3.1. Antimicrobial activities of the compounds

Table 2 summarizes the inhibitory activities of the synthetic compounds against yeasts and filamentous fungi. MIC values of the synthetic compounds showed D_1, D_7 and D_{11}-D_{14} exhibited inhibitory activity against *C. neoformans* at concentrations ranging from 0.5-128 µg/ml. Compound D_{11} exhibited inhibitory activity against *C. neoformans*, *C. dubliniensis*, *C. albicans*, *C. krusei* and *A. flavusat* concentrations ranging from 0.5-256 µg/ml. Compound D_{14} exhibited inhibitory activity against *C. neoformans*, *C. tropicalis* and *E. dermatitidis* at concentrations 32 µg/ml. Consideration on chemical structure of the active compounds showed variation of the substitutions on the 4-position of pyran ring could influence on the biological activities. Increasing in lipophilicity of the compounds may also be another reason for improving activity. It should be noted that higher lipophilicity can affect the solubility of the compounds, so a suitable hydrophilicity/lipophilicity balance in the compounds can cause the best final biological effect (21, 22).

MIC values of the synthetic compounds against gram positive and gram negative bacteria are summarized in Table 3. The results showed compounds D_3 and D_{13}, exhibited strong inhibitory activities against *S. aurous* at concentrations ranging from 16-64 µg/ml. Compounds D_{11} and D_{13} showed inhibitory effect on *E. fecalis* at con-

Table 2. MIC and MFC values (µg/mL) of the tested compounds against different yeasts and filamentous fungi

Microorganism		D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9	D_{10}	D_{11}	D_{12}	D_{13}	D_{14}	Flu*
C.	MIC	8	G	G	G	G	G	32	G	G	G	0.5	4	128	16	0.25
neoformans	MFC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	ND
C.	MIC	G	G	G	G	G	G	G	G	G	G	32	G	G	G	0.25
dubliniensis	MFC	G	G	G	G	G	G	G	G	G	G	128	G	G	G	ND
C.	MIC	G	G	G	G	G	G	G	G	G	G	16	G	G	G	4
albicans	MFC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	ND
C.	MIC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	1
glabrata	MFC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	ND
C.	MIC	G	G	G	G	G	G	G	G	G	G	8	G	G	G	64
krusei	MFC	G	G	G	G	G	G	G	G	G	G	256	G	G	G	ND
C.	MIC	G	G	G	G	G	G	G	G	G	G	G	G	G	8	2
tropicalis	MFC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	ND
C.	MIC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	0.25
parapsilosis	MFC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	ND
Exophilia	MIC	G	G	G	G	G	G	G	G	G	G	G	G	G	128	2
	MFC	G	G	G	G	G	G	G	G	G	G	G	G	G	256	ND
A.	MIC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	8
clavatus	MFC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	ND
A .	MIC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	8
fumigatus	MFC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	ND
A.	MIC	G	G	G	G	G	G	G	G	G	32	16	G	G	G	16
flavus	MFC	G	G	G	G	G	G	G	G	G	G	32	G	G	G	ND
Pseudallesch-	MIC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	4
era boydii	MFC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	ND

Flu*: Fluconazole

Table 3. MIC and MBC values (µg/mL) of the tested compounds against the examined bacteria.

Microorganism		D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9	D_{10}	D_{11}	D_{12}	D_{13}	D_{14}	Cip*
S. aurous	MIC	G	G	64	G	G	G	G	G	G	G	G	G	16	G	0.5
	MBC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
E. fecalis	MIC	G	G	G	G	G	G	G	G	G	G	128	G	32	G	
	MBC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	
E. coli	MIC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	0.025
	MBC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
P. aeruginosa	MIC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	2
	MBC	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G

Cip*:Ciprofloxacin

centration of 32-128 µg/ml.

3.2. Docking studies

The docking study was also performed to study and predict the binding mode of the synthesized compounds in active site of the target enzyme cytochrome P450 lanosterol 14α-demethylase of *C. albicans*. All the docking protocols were performed on validated structures, with RMSD values below 2Å. The conformation with the lowest binding energies was considered as the best docking result. Binding energy of all compounds were showed in Table 4. All investigated complexes showed better docking binding energies than the co-crystal ligands (fluconazole). Compounds D_1, D_3, D_{10}, D_{11}, D_{13} and D_{14} which displayed higher biological activities also had lower binding energies in docking studies. This may cause by their strong binding affinity to the *C. albicans* lanosterol 14α-demethylase enzyme active site.

Docking studies revealed that these amino acid residues of enzyme. His259, Met79, Arg96, Thr 260 and Ala256 were observed to play an active role in the interaction with pyrano[2,3-c] pyrazoles derivatives. The interaction modes of D_3 and D_{11}-D_{13} those with the best antimicrobial activities are shown in Figure1.

As it can be seen, there is a close interaction between substitutions of 4-position at pyran ring and pocket of heme iron of CYP51. This ori-

Table 4. The bonding energies (kcal/mol) of the tested compounds with 1EA1 using AutoDock.

Compounds	Binding Energy (kcal/mol)
D_1	-7.2
D_2	-6.79
D_3	-8.63
D_4	-6.89
D_5	-7.31
D_6	-6.9
D_7	-7.46
D_8	-7.71
D_9	-7.06
D_{10}	-7.56
D_{11}	-7.61
D_{12}	-7.2
D_{13}	-7.87
D_{14}	-7.59
TPF	-6.89

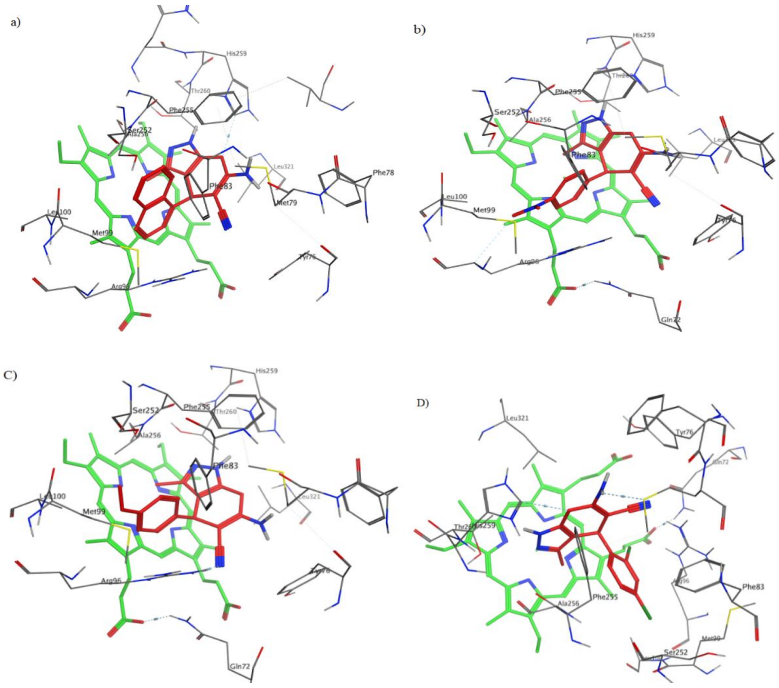


Figure 1. The docked configuration of D_3(a), D_{11} (b), D_{12} (C) and D_{13} (D) in the binding site of lanosterol 14-α-demethylase (CYP51).

entation with respect to the hydrogen bonding and hydrophobic interactions may be in favor of antimicrobial activity.

4. Conclusion

All tested compounds in this study (D_1-D_{14}) contain a pyranopyrazole moiety with substitution at 4-position at the pyran ring. This substitution is phenyl ring in all analogues except D_1. Our results indicate that adding an electron-withdrawing group on the para-position of the phenyl ring could improve antimicrobial activities. For example compound D_{11} with 4-NO_2 substitution on phenyl ring at para position of the pyran showed antifungal activity comparable to fluconazole against *C. krusei*. The best inhibitory effects of the compounds D_3, D_{11} and D_{13} on bacteria, might be due to the increase in their lipophilicity (log P). The biological behavior of these compounds seems to be related to the ratio of their lipophilicity and electronegativity. For some cases increase in lipophilicity (log P), causes an increase in the penetrations into the cells to exert their intracellular effects. The docking studies of synthesized compounds with lanosterol 14α-demethylase (CYP51) modeled protein showed good binding interactions with enzyme. It indicated that there is a good correlation between the docking studies and the antimicrobial activity results.

Acknowledgments

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Conflict of Interest

None declared.

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