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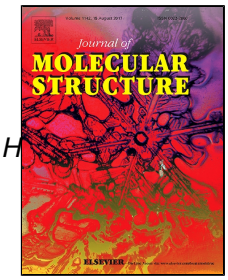

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Hamid Beyzaei, Reza Aryan, Mohammadreza Moghaddam-Manesh, Behzad Ghasemi, Pouya Karimi, Hojat Samareh Delarami, Mahmood Sanchooli

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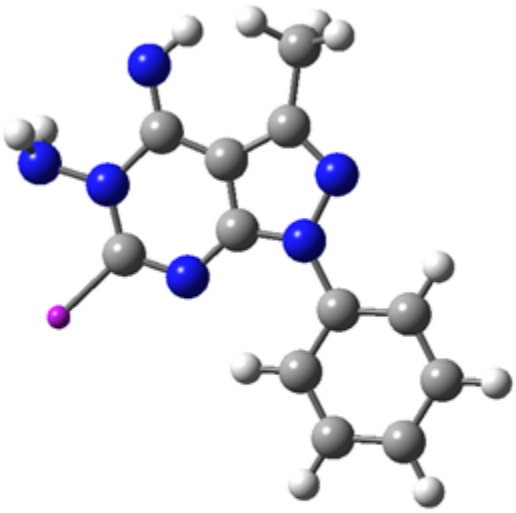

Computational Structure-activity Relationship Study

***In Vitro* Antibacterial Assay**

Highlights

- Five novel 4-imino-5*H*-pyrazolo[3,4-*d*]pyrimidin-5-amine derivatives were synthesized *via* a new and efficient method.
- The *in vitro* antimicrobial properties of all synthesized pyrazolo[3,4-*d*]pyrimidines were evaluated against eight Gram-positive and five Gram-negative pathogenic bacteria.
- The inhibitory activities of derivatives as IZD, MIC and MBC values were determined by disk diffusion and broth microdilution methods.
- Structural parameters such as dipole moment, atomic charge, molecular volume, frontier orbital energy, molecular surface area, hardness, softness and electrophilicity were calculated.
- The relatively strong relationships were found between hardness, softness, electrophilicity, amount of negative charge on N-3 atom and the sum of atomic charges on two heterocyclic rings of derivatives and the observed antibacterial activities.

Evaluation and structure-activity relationship analysis of a new series of 4-imino-5*H*-pyrazolo[3,4-*d*]pyrimidin-5-amines as potential antibacterial agents

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Abstract

The synthesis of pyrazolo[3,4-*d*]pyrimidine derivatives is important due to their presence in various biologically active compounds such as anticancer, antimicrobial, antiparasitic, anti-inflammatory and antidiabetic agents. In this project, a new and efficient approach for the synthesis of some novel 4-imino-5*H*-pyrazolo[3,4-*d*]pyrimidin-5-amines from reaction of 5-amino-pyrazole-4-carbonitrile with various hydrazides in ethanolic sodium ethoxide medium was reported. Antimicrobial activities of all synthesized derivatives were evaluated against eight Gram-positive and five Gram-negative pathogenic bacteria. The moderate to good inhibitory effects were observed based on inhibition zone diameter (IZD), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. In order to determine the reasonable relationship between antibacterial activities and physiochemical properties of the derivatives, computational studies were carried out in terms of geometry optimization, short-range van der Waals forces, dipole moments, atomic charges and frontier orbital energies. It was found that both short-range forces and covalent bonds are important in the observed inhibitory effects of the molecules. The results suggested that pyrazolo[3,4-*d*]pyrimidine derivatives prefer a soft nucleophilic attack on bio-macromolecular targets. Furthermore, our models proposed that the antibacterial activities of these derivatives can be improved by substituting large electron donating groups on the 6-phenyl rings.

Keywords Pyrazolo[3,4-*d*]pyrimidine, Hydrazide, New efficient synthesis, Antibacterial activity, Computational study, Quantitative structure-activity relationship.

Introduction

Pyrazolo[3,4-*d*]pyrimidines as purine analogues are important class of fused heterocyclic compounds due to their various pharmacological and biological activities. Commercial drugs or those are still under investigation such as allopurinol and its metabolite oxypurinol are known as inhibitors of xanthine oxidase. These drugs are being used for the treatment or prevention of hyperuricemia, acute uric acid nephropathy, gout, renal calculi and congestive heart failure. Pyrazolo[3,4-*d*]pyrimidine cores were also found in several natural products. Phidolopine is a purine derivative that was isolated from the bryozoan *Phidolopora pacifica* and is known as antifungal and antialgal agent [1]. A number of pyrazolo[3,4-*d*]pyrimidine derivatives were synthesized and studied for their anticancer [2], antiviral [3], antibacterial [4], antifungal [5], antiparasitic [6], herbicidal [7], radioprotective [8], anti-neoplastic [9], anti-inflammatory and analgesic [10] activities as well. Apoptotic effects of some synthesized pyrazolo[3,4-*d*]pyrimidines were proven on large granular lymphocyte leukemia cells *via* reduction of Fyn phosphorylation [11]. They were also used to treat hypertension [12], pyrexia and nociception [13]. The broad therapeutic scope for pyrazolo[3,4-*d*]pyrimidine derivatives has attracted a wide range of researches in the field of organic chemistry toward the design and synthesis of new derivatives. The synthetic strategies usually include reaction of pyrazole or pyrimidine derivatives with formamide [14, 15], *N*,*N*-dimethylphosgeniminium chloride [16], acetic acid [17], triethyl orthoalkanoate and ammonia [18], aldehydes [19], 1-benzyl-4-hydrazinylpiperidine dihydrochloride [20], alkyl carbazates [21] and hydrazines [22]. 5-Amino-pyrazole-4-carbonitriles are important synthetic precursors which were used as starting materials in the synthesis of these fused heterocycles [14, 16-18, 23] as well as pyrazolotriazines [24], azo dyes [25], azaindenes [26], pyrazolo[3,4-*b*]pyridine-5,6-dicarboxylates [27],

pyrazolo[3,4-*d*]pyridazines [28], pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines, pyrazolyl-tetrazoles [29] and pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines [30].

Nowadays, computational approaches are widely used for the assessment of structure-activity relationships. Some pyrazolo[3,4-*d*]pyrimidinones as antidiabetic agents were synthesized and their interactions with the DPP-IV enzyme were studied using surflex dock module of SYBYL X-2.0 [31]. The anti-inflammatory and neuroprotective properties of the synthesized pyrazolo[3,4-*d*]pyrimidine 4(5*H*)-ones were evaluated and the molecular operating environment module was used for docking experimentation [32]. The inhibitory activities of some pyrazolo[3,4-*d*]pyrimidine derivatives were proved against Bcr-Abl T315I mutant through both *in vitro* and *in vivo* tests and docking studies [33]. The antimicrobial properties of a series of pyrazolo[3,4-*d*]pyrimidine-6-thiols were studied and compared to the reference drugs *via* molecular modeling [34]. In another study, pyrazolo[1,5-*c*]pyrimidine-3-carboxylates were synthesized and the mechanism of formation, NBO analysis, Fukui functions and energies of HOMO and LUMO orbitals were calculated using B3LYP/6-31G (d,p) and B3LYP hybrid functional methods [35].

The wide range of pharmaceutical and biological properties of pyrazolo[3,4-*d*]pyrimidines encouraged us to a novel approach for the synthesis of these compounds. The *in vitro* antimicrobial activities of newly synthesized derivatives were evaluated against a number of pathogenic bacteria including Gram-positive and -negative standard strains of various genera, and the results were reported as IZD, MIC and MBC values. Finally, the correlation between biological activities and molecular properties of the novel derivatives were computationally studied.

Material and Methods

Chemicals

All chemicals and solvents were obtained from Merck and Sigma-Aldrich, were used without further purification. Antibiotics were purchased from Sigma-Aldrich. All yields refer to isolated products. Melting points were recorded on a Kruss type KSP1N melting point meter with no further correction. The reaction progress and the purity of the products were affected by aluminium TLC plates pre-coated with silica gel 60 using CH_2Cl_2:CH_3OH (8:2, v/v) as the desired mobile phase. The result TLC plates were visualized with iodine vapour. The IR spectra of the products were recorded on a Bruker Tensor-27 FT-IR spectrometer using KBr disks. The ^{1}H and ^{13}C-NMR spectra of DMSO-d_6 solutions were recorded on a Bruker FT-NMR Ultra Shield-400 spectrometer (400 and 100 MHz, resp.). Elemental analyses were performed for C, H and N on a Thermo Finnigan Flash EA microanalyzer. The concentration of bacterial suspension was determined using Jenway 6405 UV-Vis spectrophotometer. 5-Amino-3-methyl-1-phenyl-1*H*-pyrazole-4-carbonitrile (**4)** was prepared according to the previous literature report [36].

General procedure for the preparation of 6-substituted 4-imino-3-methyl-1-phenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*]pyrimidin-5-amines **6a-e**

Clean metallic sodium (10 mmol, 0.23 g) was added in a few portions to 20 mL of stirred and ice-water cooled absolute ethanol. Then, pyrazole derivative **4** (10 mmol, 1.98 g) and hydrazides **5a-e** (**5a**: 0.60 g, **5b**: 1.36 g, **5c**: 1.50 g, **5d**: 1.81 g, **5e**: 1.50 g; 10 mmol) were added and the mixture was heated to reflux for 10 h. The resulting solution was cooled to room temperature. A precipitate was crashed out upon addition of 20 mL of 0.5 M acetic acid. The

solid was filtered off, washed sequentially with water/acetone (2:1, v/v; 10 mL) and ethanol (10 mL), which then dried in an oven at 80 °C to afford pure compounds **6a-e**.

4-Imino-3-methyl-1-phenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*]pyrimidin-5-amine (**6a**)

Yield 75%; mp 268-270 °C; IR (KBr) *v* 3419, 3244, 2973, 1558, 1436, 1222, 1174, 924, 584 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 2.69 (3H, s, CH_3), 7.50 (3H, m, H-3,4,5 Ph), 8.11 (1H, s, H-6), 8.16 (2H, d, *J* = 7.9 Hz, H-2,6 Ph), 8.44 (2H, s, NH_2), 12.00 (1H, s, C=NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 151.93 (C-4), 149.48 (C-3), 148.89 (C-6), 144.46 (C-7a), 140.30 (C-1 Ph), 129.91 (C-3,5 Ph), 125.08 (C-4 Ph), 124.40 (C-2,6 Ph), 113.93 (C-3a), 13.05 (CH_3). Anal Calcd. for $C_{12}H_{12}N_6$ (%): C, 59.99; H, 5.03; N, 34.98. Found (%): C, 59.96; H, 5.02; N, 35.02.

4-Imino-3-methyl-1,6-diphenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*]pyrimidin-5-amine (**6b**)

Yield 61%; mp 228-230 °C; IR (KBr) *v* 3353, 3180, 1680, 1600, 1552, 1445, 1287, 1142, 912, 540 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 2.63 (3H, s, CH_3), 7.47-7.63 (6H, m, H-3,4,5 Ph-1,6), 8.24 (2H, d, *J* = 7.6 Hz, H-2,6 Ph-1), 8.48 (2H, m, H-2,6 Ph-6), 8.74 (2H, s, NH_2), 11.87 (1H, s, C=NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 151.33 (C-4), 149.36 (C-6), 148.28 (C-3), 145.66 (C-7a), 139.69 (C-1 Ph-1), 129.90 (C-4 Ph-6), 129.36 (C-3,5 Ph-1), 128.99 (C-3,5 Ph-6), 128.10 (C-1 Ph-6), 127.59 (C-2,6 Ph-6), 125.66 (C-4 Ph-1), 123.59 (C-2,6 Ph-1), 112.78 (C-3a), 13.05 (CH_3). Anal Calcd. for $C_{18}H_{16}N_6$ (%): C, 68.34; H, 5.10; N, 26.56. Found (%): C, 68.31; H, 5.08; N, 26.61.

4-Imino-3-methyl-1-phenyl-6-(*p*-tolyl)-1,4-dihydro-5*H*-pyrazolo[3,4-*d*]pyrimidin-5-amine (**6c**)

Yield 73%; mp 219-221 °C; IR (KBr) *v* 3370, 3291, 1575, 1414, 1221, 1102, 967, 501 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 2.46 (3H, s, CH_3-Ph), 2.69 (3H, s, CH_3-pyrazole), 7.39 (2H, d, *J* = 7.4 Hz, H-3,5 Ph-6), 7.50 (3H, m, H-3,4,5 Ph-1), 7.54 (2H, d, *J* = 7.4 Hz, H-2,6 Ph-6), 8.20 (2H, d, *J* = 8.0 Hz, H-2,6 Ph-1), 8.56 (2H, s, NH_2), 11.55 (1H, s, C=NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 150.15 (C-4), 149.71 (C-3), 146.57 (C-6), 144.85 (C-7a), 140.00 (C-4 Ph-6), 139.71 (C-1 Ph-1), 131.34 (C-2,6 Ph-6), 129.90 (C-3,5 Ph-1), 128.04 (C-3,5 Ph-6), 125.68 (C-4 Ph-1), 124.39 (C-1 Ph-6), 122.27 (C-2,6 Ph-1), 112.66 (C-3a), 20.96 (CH_3-Ph), 13.05 (CH_3-pyrazole). Anal Calcd. for $C_{19}H_{18}N_6$ (%): C, 69.07; H, 5.49; N, 25.44. Found (%): C, 69.12; H, 5.46; N, 25.42.

4-Imino-3-methyl-6-(4-nitrophenyl)-1-phenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*]pyrimidin-5-amine (**6d**)

Yield 77%; mp 264-266 °C (decomp.); IR (KBr) *v* 3361, 3224, 2925, 1678, 1593, 1504, 1125, 869, 577 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 2.69 (3H, s, CH_3), 7.50 (2H, m, H-3,4,5 Ph-1), 8.04 (2H, d, *J* = 8.8 Hz, H-2,6 Ph-6), 8.13 (2H, d, *J* = 7.6 Hz, H-2,6 Ph-1), 8.33 (2H, d, *J* = 8.8 Hz, H-3,5 Ph-6), 8.63 (2H, s, NH_2), 12.20 (1H, s, C=NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 156.05 (C-6), 151.13 (C-4), 150.52 (C-4 Ph-6), 149.92 (C-3), 145.30 (C-7a), 139.41 (C-1 Ph-1), 135.81 (C-1 Ph-6), 130.34 (C-2,6 Ph-6), 129.73 (C-3,5 Ph-1), 125.86 (C-4 Ph-1), 124.39 (C-3,5 Ph-6), 122.97 (C-2,6 Ph-1), 112.66 (C-3a), 13.05 (CH_3). Anal Calcd. for $C_{18}H_{15}N_7O_2$ (%): C, 59.83; H, 4.18; N, 27.13. Found (%): C, 59.80; H, 4.19; N, 27.17.

6-Benzyl-4-imino-3-methyl-1-phenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*]pyrimidin-5-amine (**6e**)

Yield 74%; mp 212-214 °C; IR (KBr) *v* 3386, 3192, 1543, 1448, 1129, 865, 686 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 2.69 (3H, s, CH_3), 3.40 (CH_2), 7.15 (1H, m, H-4 Ph-6), 7.17 (2H, m, H-2,6 Ph-6), 7.19 (2H, m, H-3,5 Ph-6), 7.44 (3H, t, *J* = 7.5 Hz, H-3,4,5 Ph-1), 8.26 (2H, d, *J* = 8.0 Hz, H-2,6 Ph-1), 8.55 (2H, s, NH_2), 11.52 (1H, s, C=NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 150.68 (C-4), 148.91 (C-3), 147.23 (C-6), 143.82 (C-7a), 140.37 (C-1 Ph-1), 136.18 (C-1 Ph-6), 129.80 (C-3,5 Ph-1), 129.39 (C-2,6 Ph-6), 128.44 (C-3,5 Ph-6), 125.11 (C-4 Ph-1), 124.55 (C-4 Ph-6), 123.26 (C-2,6 Ph-1), 113.73 (C-3a), 36.41 (CH_2), 13.12 (CH_3). Anal Calcd. for $C_{18}H_{14}ClN_5$ (%): C, 64.38; H, 4.20; N, 20.86; Cl, 10.56. Found (%): C, 64.32; H, 4.13; N, 20.90; Cl, 10.65. Anal Calcd. for $C_{19}H_{18}N_6$ (%): C, 69.07; H, 5.49; N, 25.44. Found (%): C, 69.08; H, 5.52; N, 25.40.

In vitro antibacterial evaluation

In vitro inhibitory activity of heterocyclic compounds and antibiotics were evaluated against thirteen pathogenic bacteria including Gram-negative strains *Shigella flexneri* (PTCC 1234), *Proteus mirabilis* (PTCC 1776), *Proteus vulgaris* (PTCC 1079), *Salmonella enterica subsp. enterica* (PTCC 1709), *Salmonella typhi* (PTCC 1609) and Gram-positive strains *Streptococcus pyogenes* (PTCC 1447), *Streptococcus agalactiae* (PTCC 1768), *Streptococcus equinus* (PTCC 1445), *Streptococcus pneumoniae* (PTCC 1240), *Listeria monocytogenes* (PTCC 1297), *Staphylococcus epidermidis* (PTCC 1435), *Bacillus thuringiensis subsp. kurstaki* (PTCC 1494) and *Rhodococcus equi* (PTCC 1633). All strains were prepared from the Persian Type Culture Collection (PTCC), Tehran, Iran. The antibacterial activities were determined by employing broth microdilution and disk diffusion methods, according to CLSI (Clinical and Laboratory Standards Institute) guidelines M07-A9, M26-A and M02-A11 [37]. The stock solutions of all derivatives and antibiotics were respectively prepared in 10% DMSO and double-distilled water at concentrations of 9011 and 17.6 µg/mL. The IZD values were measured at these initial concentrations. All antibiogram tests were performed at least three times independently, and the results were reported as mean values ± SD. No standard deviation was observed at mean MIC and MBC values.

Computational studies

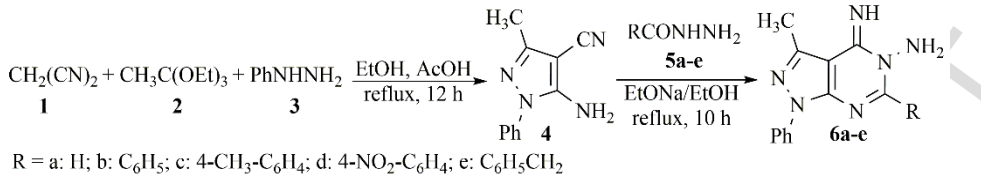
All geometries were optimized without any symmetry constraint at the B3LYP level of theory in conjunction with 6-311++G** basis set using Gaussian 09 program package [38]. Frequency calculations were performed at the aforementioned level of theory and the results showed that all geometries were corresponded to true minima with no imaginary frequency. The population analysis has been performed by the natural bond orbital (NBO) method at the B3LYP/6-311++G** level using NBO program implemented under Gaussian 09 program package [39].

Results and Discussion

Synthesis and characterization of pyrazolo[3,4-*d*]pyrimidines

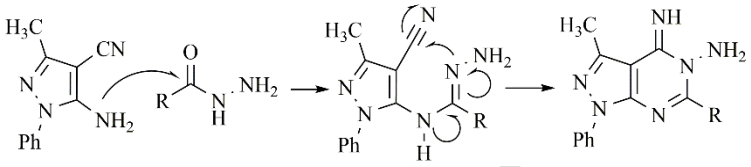
In this study, a new and efficient two step procedure was proposed for the synthesis of 6-substituted 4-imino-3-methyl-1-phenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*]pyrimidin-5-amines **6a-e** (Scheme 1). 5-Amino-pyrazole-4-carbonitrile **4** as starting material was prepared in one-pot procedure from the reaction of malononitrile (**1**), triethyl

orthoacetate (**2**), phenylhydrazine (**3**) and a few drops of acetic acid in ethanolic solution after refluxing for 12 h. The cyclocondensation of pyrazole **4** with hydrazides **5a-e** in 0.5 M NaOEt/EtOH medium produced pyrazolo[3,4-*d*]pyrimidines **6a-e** in moderate yields.



Scheme 1 Synthesis of pyrazolo[3,4-*d*]pyrimidines 6a-e

The reaction proceeded only in the presence of sodium ethoxide as catalyst. It seems that the nucleophilicity of the amino group was increased due to the formation of a strong hydrogen bonding with anionic oxygen of the ethoxide (Scheme 2). The proposed mechanism was based on the same transformation observed previously [40].



Scheme 2 The proposed mechanism for the formation of products 6a-e

The chemical structure of all compounds were deduced from their spectral and analytical data. In ^{1}H-NMR spectra, signals attributed to H-3,4,5 and H-1,6 of 1-phenyl ring appear within 7.44-7.50 and 8.13-8.26 ppm. In addition, the spectra showed singlet signals due to 3-CH_3, 5-NH_2 and 4-NH protons at 2.63-2.69, 8.44-8.74, 11.52-12.20 ppm. ^{13}C NMR spectra exhibited signals belonging to C-3, C-3a, C-4, C-6 and C-7a at 147.23-149.92, 112.66-113.93, 150.15-151.93, 146.57-156.05 and 143.82-145.66 ppm. The absorption bands appeared within ν = 3180-3291 and 3353-3419 cm^{-1} corresponded to stretching vibrations of NH and NH_2 groups. The purity of all derivatives also confirmed by the microanalytical data.

Interpretation of antibacterial activities

The *in vitro* antibacterial activities of the newly synthesized derivatives were evaluated against a wide range of Gram-positive and -negative pathogenic bacteria, which then compared with ceftriaxone and cefazolin antibiotics and reported as IZD, MIC and MBC values in Table 1.

Table 1 IZD, MIC and MBC values of pyrazolo[3,4-*d*]pyrimidines 6a-e

Bacteria \ Products		6a	6b	6c	6d	6e	Ceftriaxone	Cefazolin
1447	IZD	-	9.54±0.24	13.22±0.11	-	-	25.88±0.63	21.64±0.53
	MIC	-	128	64	-	-	0.5	4
	MBC	-	256	128	-	-	1	8
1297	IZD	9.26±0.32	-	-	7.13±0.31	7.48±0.41	8.27±0.26	21.43±0.65
	MIC	512	-	-	256	512	8	8
	MBC	2048	-	-	1024	2048	8	8
1	IZD	8.16±0.19	11.37±0.43	-	10.61±0.38	9.75±0.57	21.51±0.28	-
	MIC	1024	1024	-	1024	512	2	-

6	MBC	2048	4096	-	4096	2048	2	-
1776	IZD	-	-	-	8.43±0.17	-	33.91±0.44	17.38±0.49
	MIC	-	-	-	256	-	0.063	0.25
	MBC	-	-	-	512	-	1	2
1494	IZD	-	-	-	7.52±0.40	-	-	-
	MIC	-	-	-	1024	-	-	-
	MBC	-	-	-	2048	-	-	-
1079	IZD	7.90±0.16	-	7.41±0.26	7.83±0.29	7.06±0.17	-	8.24±0.54
	MIC	512	-	2048	512	2048	-	8
	MBC	1024	-	4096	1024	4096	-	32
1709	IZD	-	-	-	8.64±0.55	-	32.64±0.37	23.25±0.22
	MIC	-	-	-	2048	-	2	2
	MBC	-	-	-	4096	-	8	2
1768	IZD	-	10.36±0.29	-	8.18±0.38	-	-	36.08±0.67
	MIC	-	256	-	512	-	-	8
	MBC	-	512	-	1024	-	-	8
1234	IZD	-	-	-	7.06±0.13	-	34.08±0.26	21.88±0.61
	MIC	-	-	-	512	-	2	2
	MBC	-	-	-	1024	-	4	2
1609	IZD	-	-	10.76±0.14	8.53±0.49	-	30.43±0.64	25.14±0.56
	MIC	-	-	2048	1024	-	0.063	0.063
	MBC	-	-	4096	2048	-	0.125	0.125
1445	IZD	-	-	-	7.87±0.12	-	8.09±0.48	21.18±0.24
	MIC	-	-	-	2048	-	8	16
	MBC	-	-	-	4096	-	16	64
1240	IZD	-	-	-	7.55±0.33	-	-	-
	MIC	-	-	-	1024	-	-	-
	MBC	-	-	-	2048	-	-	-
1435	IZD	-	-	-	9.64±0.25	-	18.54±0.23	25.90±0.37
	MIC	-	-	-	1024	-	0.5	1
	MBC	-	-	-	2048	-	2	2

IZD (mm), MIC (μg/mL), MBC (μg/mL)
(-): Indicates no noticeable antibacterial effect at initial concentrations

The derivatives showed moderate to good inhibitory activities against thirteen Gram-positive and -negative bacterial strains of various genera. The inhibitory effects against all tested *Streptococcus* and *Proteus* species were observed from at least one heterocyclic compound. Pyrazolo[3,4-*d*]pyrimidine **6d** was known as the most broad-spectrum antibacterial agent due to inhibitory properties on twelve bacterial strains. Structural study of compound **6d** indicated that the presence of nitro moiety at position 4 of 6-phenyl ring as antimicrobial functional group was the main reason for the observed difference in the inhibitory activity [41]. To the best of our knowledge, compound **6c** showed the highest inhibitory effect against *Streptococcus pyogenes* with MIC and MBC values of 64 and 128 μg/mL in comparison to others. There for, it is considered as the most effective antibacterial agent.

Theoretical calculations

Quantum mechanical studies were performed to shed some light on the correlation between antibacterial activities and the molecular properties of fused heterocycles **6a-e**. A general structure of heterocycles **6a-e** is illustrated in Fig. 1. The results in Table 2 reflect the calculated parameters including energy, orbital energy, dipole moment, molecular volume and surface area.

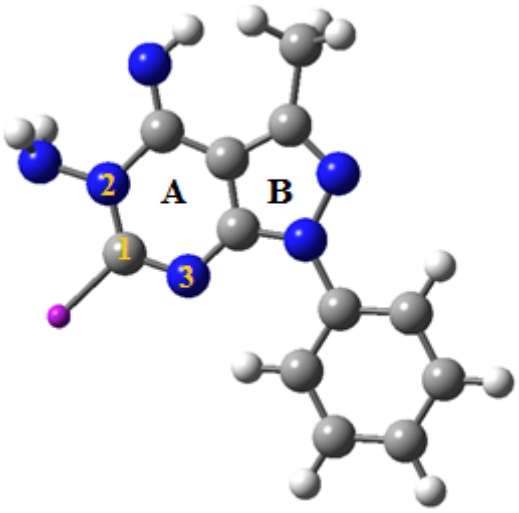


Fig. 1 Typical structure of the pyrazolo[3,4-*d*]pyrimidines

Table 2 Quantum mechanical parameters of pyrazolo[3,4-*d*]pyrimidines

molecule	Energy[a]	Dipole moment[b]	HOMO[a]	LUMO[a]	Molecular volume[c]	Molecular surface area[d]
6a	-793.187	0.631	-0.23174	-0.05577	702.18	432.75
6b	-1024.294	1.652	-0.22389	-0.06622	912.13	541.81
6c	-1063.622	2.264	-0.22168	-0.06371	962.50	565.86
6d	-1228.847	0.965	-0.23354	-0.12399	975.48	579.46
6e	-1063.615	6.052	-0.22149	-0.05288	953.39	567.80

a, b, c and d are in Hartree, Debye, Å^3, and Å^2, respectively.

Good structure-activity relationships were observed between the IZD values and molecular volumes/surface areas respectively within r = 0.97 and r = 0.95 values, except for the highly active molecule **6a**.

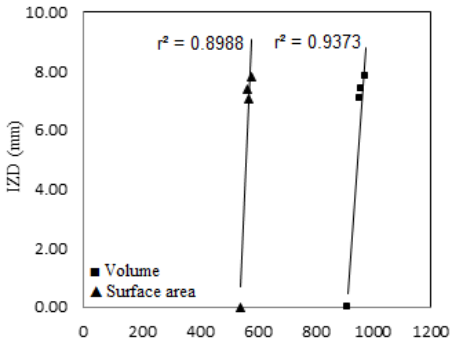


Fig. 2 Correlation between the IZD values and molecular volumes (in Å^3) and surface areas (in Å^2)

The molecular volumes/surface areas were directly proportional to the IZD values. No significant relationship was observed between dipole moments and the IZD values. These results implied that dispersion forces play a considerable role in inhibitory activity of the molecules **6b-e**. Nevertheless, molecule **6a** with lowest volume and surface area showed the highest antibacterial activity. Such observations may imply that other interactions, beside the van der Waals dispersion forces, could be involved. Recent studies indicate that reactive nucleophilic or electrophilic species cause discrete types of injuries to microbial cells because of redox stress [42]. Accordingly, our

attempts were focused on the construct a correlation between orbital energies and the IZD values of the molecules. In reality, we have not found a straightforward correlation between HOMO, LUMO and HOMO-LUMO energy gaps with the IZD values.

More investigations revealed that the IZD values can be correlated to the inverse of HOMO and LUMO values pretty well, as shown in Fig. 3. It can be deduced that beside short-distance dispersion forces, covalent bonding might be involved in the action mechanism of the antibacterial activity of compounds **6a-e**.

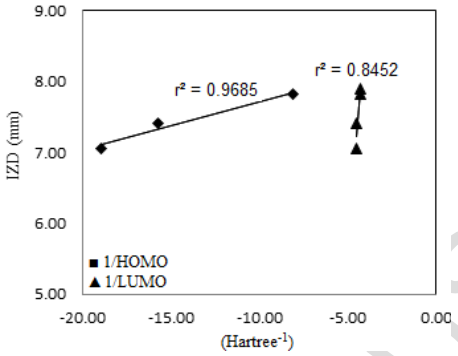


Fig. 3 Correlation between antibacterial effects and the inverse of HOMO and LUMO values

It is difficult to interpret the interaction of probable chemical bonding according to the inverse of frontier orbital energies. Many drug-receptor chemical interactions such as SN2, Schiff base formation and Michael addition involve reaction of electrophiles with nucleophilic sites on biological macromolecules. The significant degree of selectivity that occurs in electrophile-nucleophile interactions was predicted by Pearson's Hard and Soft, Acids and Bases (HSAB) theory [43-45]. This principle implies that electrophilic molecules will preferentially react with similar hard or soft biological targets. Hardness (HD), softness (SOF) and electrophilicity (EPH) are three important electronic descriptors obtained from HOMO and LUMO energies. They describe stability, reactivity and a measure of energy lowering due to maximal electron flow between the donor and acceptor [44]. These electronic features can be calculated as HD = (LUMO-HOMO)/2, SOF = 1/HD, EPH = $-(LUMO+HOMO)^2/(8HD)$ [46-48]. These chemical indices were calculated for all molecules **6a-e**, as shown in Table 3.

Table 3 Chemical descriptors including hardness, softness, and electrophilicity

Molecule	HD[a]	SOF[b]	EPH[c]
6a	0.088	11.37	-0.117
6b	0.158	6.34	-0.067
6c	0.158	6.33	-0.064
6d	0.110	9.13	-0.146
6e	0.169	5.93	-0.056

[a]Hardness (in Hartree), [b]softness (in $Hartree^{-1}$), [c]electrophilicity (in Hartree)

According to Figures 4 and 5, there are relatively strong relationships between the aforementioned descriptors and the non-zero IZD values. The IZD values correlate reasonably with EPH, SOF, and HD within linear regression coefficients of 0.90, 0.91 and 0.96, respectively. In addition to, direct and inverse correlations between softness and hardness parameters and antibacterial effects are quite obvious.

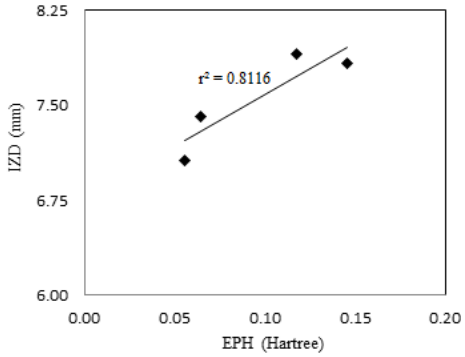


Fig. 4 Relationship between the IZDs and absolute values of electrophilicity

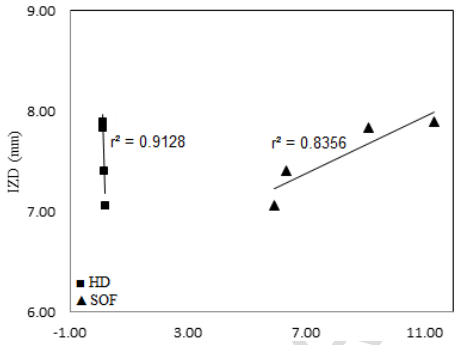


Fig. 5 Correlation of the IZD values with hardness (in Hartree) and softness (in Hartree^{-1}) coefficients

The obtained results illustrated that the antibacterial activities of molecules **6a-e** wererelated to redox interactions as well as van der Waals dispersion forces. To find more verification on chemical interactions, charges on the most important atoms in molecules were calculated. It was found that an increase in negative charge on N-3 atom of the molecules, except for molecule **6b**, was correlated to the increase of the IZD values (Fig. 6). Therefore, it would be plausible to consider the N-3 atom connected to the amino group contributes to the electron flow between donors and acceptors. Furthermore, sum of the atomic charges on the rings A and B were calculated for the molecules **6a-e** (Fig. 7). The inhibitory properties of molecules (except for molecule **6b**) were improved by increasing negative atomic charges on these two rings. It seems that charges flow from nucleophilic site especially N-3 atom to electrophilic centers on the biological macromolecules. It can be concluded that the covalent bonding occurs when the electron density flows between the reactive species of nucleophiles and electrophiles *via* their frontier molecular orbitals (HOMO and LUMO). Therefore, based on HSAB theory, the nucleophilic molecules **6a-e** (due to electron rich N-3 atom) react preferentially with biological sites with similar hardness or softness. Due to the increase in antibacterial activities of nucleophiles **6a-e** with their softness, they prefer to interact with soft electrophilic receptors.

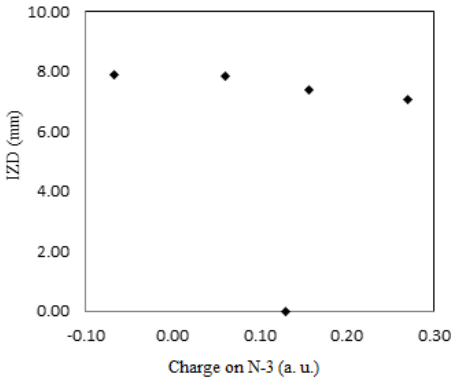


Fig. 6 The IZD values versus charge on N-3 atom in the pyrazolo[3,4-*d*]pyrimidines

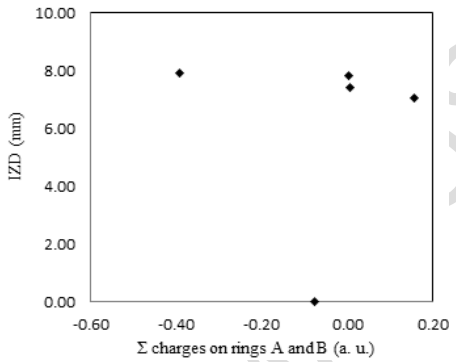


Fig. 7 Relationship between the IZD values and the sum of atomic charges on the rings A and B

Conclusions

Some new pyrazolo[3,4-*d*]pyrimidines were synthesized through a novel and efficient method. Antibacterial activities of the synthesized derivatives were evaluated against thirteen pathogenic bacteria and reported as IZD, MIC and MBC values. Theoretical studies such as energy data and NBO analysis were carried out to determine structure-activity relationships. Some plausible theoretical evidences proved that both physical (short-distance forces) and chemical (covalent bonding) interactions are responsible in the observed antibacterial activities of the pyrazolo[3,4-*d*]pyrimidine derivatives. Except for the smallest molecule **6a** with the highest inhibitory activity, it is concluded that van der Waals interactions might contribute to the action mechanism of these molecules. The direct relationship between absolute values of the EPH and the inhibitory activities accentuates the role of the charge flow. It appeared that the charge flows from nucleophilic N-3 atom on the molecules **6a-e** to electrophilic sites on biological macromolecule. Since the IZD values of the nucleophiles **6a-e** increase with their softness, they interact preferably with soft electrophilic receptors. Our models suggest that the soft pyrazolo[3,4-*d*]pyrimidines derivatives with the higher electrophilicity should be considered for the synthesis of antibacterial potentials in the future. Consequently, large electron donating substitutions on both rings A and B are recommended for the higher biological activities.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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