Tetrazolo-Quinoxaline Analogues as Antibacterial Agents: Synthesis, Characterization and In Silico ADMET Study

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Abstract

Quinoxaline and their fused-ring systems are well known for their potential biological activities. In the present study new tetrazolo-quinoxaline analogues ($P5_{a-d}$) were synthesized and characterized by IR, NMR, Mass and elemental analyses. In silico ADMET and pharmacokinetic parameters of compounds ($P5_{a-d}$) were evaluated. Also, all the compounds were screened for their antibacterial activity against Pseudomonas aeruginosa, Bacillus subtilis and Escherichia coli strains. The results revealed that compounds ($P5_{a-d}$) have a significant biological activity against the tested microorganisms.

Keywords: Tetrazolo-quinoxaline, Antibacterial activity, ADMET, RO5, Tetrazole.

Introduction

During the ancient era the isolation of various compounds was done by the process of extraction. But this process was time consuming as well as laborious. Moreover, the yield was very low and large numbers of compounds were required. Today the process of isolation has been replaced by the synthetic routes. A large number of compounds can be synthesized by using a small amount of chemicals. Moreover, the synthetic routes take less amount of time and can easily be carried out. Quinoxaline derivatives hold a large bit of importance in today's world as they are being used for various chemical, clinical as well as biological aspects [1-2]. Medicinally quinoxaline has been used in various areas, especially as antimicrobial [3-5], antiprotozoal [3], antitubercular [5-7], antiviral [5], antiparasitic [5], antidiabetic [5], anti-inflammatory [8], antihyperglycemic [9], anticancer [5, 10], antiplasmodial [11], antileishmanial [11] and antiamoebic [12].

As a result of this research and for the purpose of obtaining new and more potent antibacterial agents that can improve the current chemotherapeutic antibacterial treatments, we have synthesized and characterized tetrazolo-quinoxaline analogues (**$P5_{a-d}$**). *In silico* pharmacokinetic parameters of compounds (**$P5_{a-d}$**) was evaluated to study the candidature of these molecules as drugs. Also, *in vitro* antibacterial activity against gram-positive (*Bacillus subtilis*) and gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*) bacterial strains was studied by agar diffusion method.

Result and Discussion

Chemistry

The synthesis of molecules (**P1-P4** and **$P5_{a-d}$**) are summarized in Schemes **I-V**. **Table 1** shows the type of substituent present on the target molecules **$P5_{a-d}$**. The starting material 1,4- dihydroquinoxaline-2,3-dione (**P1**) was prepared by refluxing the mixture of *o*-phenylenediamine and oxalic acid in the presence of

4N HCl. 1,4-dihydroquinoxaline-2,3-dione (**P1**) was stirred at room temperature with $POCl_3$ to get 2,3-dichloroquinoxaline **(P2)**. 2-chloro-3-hydrazineylquinoxaline (**P3**) was prepared by refluxing compound **P2** and hydrazine hydrate in ethanol for 3 h. The compound **P3** undergo cyclization reaction to form compound **P4**. The final tetrazolo quinoxaline analogues (**P5$_{a-d}$**) was prepared by reacting **P4** with different aldehydes.

Scheme I Method for the Preparation of 1,4-Dihydroquinoxaline-2,3-Dione (**P1**)

Scheme II Method for the Preparation of 2,3-Dichloroquinoxaline (**P2**)

Scheme III Method for the Preparation of 2 – Chloro -3-Hydrazineylquinoxaline (**P3**)

Scheme IV Method for the Preparation of 4 -Hydrazineyltetrazolo [1, 5-*a*] Quinoxaline (**P3**)

Scheme V Method for the Preparation of Tetrazolo Quinoxaline (**P5$_{a-d}$**)

Table 1. Scheme followed to designate the molecules synthesized (**P5$_{a-d}$**)

Sample Id(s)	R_1	R_2	R_3
P5$_a$	-H	$-OCH_3$	-OH
P5$_b$	-H	$-OCH_3$	$-OCH_3$
P5$_c$	-H	-H	$-OCH_3$
P5$_d$	-OH	-H	$-OCH_3$

The IR spectra were obtained, was correlated with the standard IR frequency of the vibration modes given by Silverstein [13]. The IR spectra showed absorption bands at ~3050 cm^{-1}, ~1460 cm^{-1} and ~750 cm^{-1} for all the compounds (**P1-P4** and **P5$_{a-d}$**) due to stretching of -CH, C=C and bending of -CH in aromatic ring respectively. The peak at ~720 cm^{-1} was attributed to C-Cl stretching in compounds **P2** and **P3**. The final moieties (**P5$_{a-d}$**) showed peak at ~1260 cm^{-1} due to stretching of Ar-O-CH_3 group. The analogues **P5$_a$** and **P5$_d$** showed a broad peak at ~3423 cm^{-1} due to stretching of -OH group. The mass spectrum of molecules (**P1-P4** and **P5$_{a-d}$**) showed molecular ion peak (M+1) corresponding to their respective molecular weights, which additionally confirmed by the molecular framework. Aromatic hydrogens of all the compounds (**P1-P4** and **P5$_{a-d}$**) appeared between ~6.55-7.99 ppm in ^{1}H-NMR. The -NH group in compounds **P3**, **P4** and **P5$_{a-d}$** appeared in-between ~8.21-9.26 ppm as 1H singlet. In the ^{13}C-NMR, the presence of aromatic carbon showed characteristic peaks between ~115-160 ppm for compounds **P1-P4** and **P5$_{a-d}$**. The data from MS, IR, ^{1}H-NMR, ^{13}C-NMR and elemental analysis for all the compounds (**P1-P4** and **P5$_{a-d}$**) are reported in the experimental section.

In Silico Pharmacokinetic Study

In order to better understand the drug likeness properties of the compounds (**P5$_{a-d}$**), the parameters such as logP, PSA, molecular weight, volume, water solubility, %HIA, BBB, %PPB etc. were calculated using ADMET and Molsoft ICM 3.5 software and the data are presented in **Table 2** and **Table 3**.

Table 2. Evaluation of pharmacokinetic parameters of molecules

Sample ID(s)	Mol. Wt.[a]	HBA[b]	HBD[c]	RotB[d]	Drug Likeness	logP[e]	logS[f] log(mol/l)	PSA[g] (°A^2)	Volume (°A^3)
$P5_a$	335.11	7	2	4	0.25	2.18	-5.46	90.78	299.66
$P5_b$	349.13	7	1	5	0.25	2.52	-5.75	81.86	319.66
$P5_c$	319.12	6	1	4	0.11	2.55	-5.88	74.15	288.24
$P5_d$	335.11	7	2	4	0.03	2.18	-5.39	90.69	298.84

a = Molecular Weight ≤ 500 (g/mol) [14]; **b** = Hydrogen Bond Acceptor ≤ 10 [14]; **c** = Hydrogen Bond Donor ≤ 5 [15]; **d** = Rotatable Bonds ≤ 10 [15]; **e** = ClogP ≤ 5 [15]; **f**; Water Solubility range -0.5 to -6.5 (mol/l) [16]; **g** = Polar Surface Area ≤ 140°A^2 [16].

From the data presented in **Table 2**, it was remarkable to note that all the compounds (**$P5_{a-d}$**) showed significant values for the various parameters analyzed and showed good drug likeness characteristics based on Lipinski's rule of five [14-16]. The data obtained for all the analogues (**$P5_{a-d}$**) was within the range of accepted values. None of the molecules had violated the Lipinski's rule of five. The value of polar surface area (PSA) for compounds (**$P5_{a-d}$**) indicated good oral bioavailability.

Table 3. Evaluation of ADMET parameters of molecules

Sample ID(s)	BBB	Caco2 (nm/sec)	% HIA	%PPB	MDCK (nm/sec)	hERG inhibition
$P5_a$	0.1313	14.6543	93.1388	85.8682	0.1346	Medium risk
$P5_b$	0.2045	13.5446	96.7792	88.2571	0.1278	Medium risk
$P5_c$	0.2354	8.4476	96.8529	93.3806	12.1980	Medium risk
$P5_d$	0.0984	15.9294	93.1393	85.1666	5.9175	Medium risk

BBB (Blood Brain Barrier) [17]: High absorption CNS >2.0, Middle absorption CNS 2.0-0.1, Low absorption to CNS <0.1; **Caco2** [17]: High permeability >70, Middle permeability 4-70, Low permeability <4; **%HIA** (Human Intestinal Absorbance) [18]: Well absorbed compounds 70-100%, Moderately absorbed compounds 20-70%, Poorly absorbed compounds 0-20%; **%PPB** (Plasma Protein Binding) [19]: Strongly Bound >90%, Weakly Bound <90%, **MDCK** [20]: Higher permeability >500, Medium Permeability 25-500, lower permeability <25.

Calculations related to protein binding, blood–brain barrier (BBB), MDCK cell permeability, Caco-2 cell permeability and human oral absorption in the gastrointestinal tract showed that these values for the derivatives (**$P5_{a-d}$**) fell within the standard ranges generally observed for drugs (**Table 3**). hERG is best known for its contribution to the electrical activity of the heart that coordinates the heart's beating. In the present investigation, all the molecules (**$P5_{a-d}$**) had a moderate risk suggest that these analogues (**$P5_{a-d}$**) were good drug candidates.

Antibacterial Activity

In the present study, the final analogues (**$P5_{a-d}$**) were screened for antibacterial activity against gram-positive and gram-negative bacterial strains by agar diffusion method. **Table 4** represents the result of antibacterial screening data in terms of zone of inhibition (mm) for each of the compounds. *In vitro* antibacterial activity of any compound is directly correlated with their zone of inhibition on the medium. More than 20 mm of inhibition zone is called high activity, more than 10 mm of inhibition is called good activity, inhibition zone between 6-9 mm moderate activity and least activity is conducted between 1-5

mm of inhibition zone [21].

Table 4. Zone of growth inhibition of synthesized compounds

Sample Id(s)	Zone of Inhibition (mm)		
	Gram-positive	***Gram-negative***	
	BS	***PA***	***EC***
$P5_a$	17	NA	13
$P5_b$	21	NA	14
$P5_c$	12	NA	13
$P5_d$	22	NA	16
Ciprofloxacin	44	NA	30
DMSO	NA	NA	NA

BS: *Bacillus subtilis*; ***PA***: *Pseudomonas aeruginosa*; ***EC***: *Escherichia coli*; **DMSO**: Dimethyl sulfoxide (Positive Control); **NA**: Not active

The antibacterial activity of all the compounds (**$P5_{a-d}$**) showed moderate to high activity against *B. subtilis* and *E. coli*. It is to be noted that compounds (**$P5_{a-d}$**) and standard drug (**Ciprofloxacin**) in this study were found to be inactive against *P. aeruginosa* strain. Quinoxaline derivatives (**$P5_{a-d}$**) showed poor zone of inhibition compared to **Ciprofloxacin**. Analogues **$P5_b$** and **$P5_d$** were exhibited high activity against *B. subtilis* strain compared to **$P5_a$** and **$P5_c$**.

Experimental

The raw materials used of synthetic grade from Sigma Aldrich & Loba chemicals Ltd. Solvents used were distilled and dried. The purity of the compounds was monitored by ascending thin-layer chromatography (TLC) on silica gel-G coated on aluminium plates. Developing solvents used in TLC were ethyl acetate: hexane (1:1 %v/v) and the plates were viewed under UV light 254 nm and 265 nm respectively. Melting points were determined by the open capillary tubes equipped with electro thermal melting point apparatus. All the melting points are uncorrected. The yields of all the compounds reported are in crystallized form. The elemental analysis was studied by C, H, N analyser on Perkin Elmer (U.S.A, 2400 Series II). Infrared (IR) spectra were recorded for the compounds on Perkin Elmer Spectrum GX using KBr pellet disc technique. The structure will be elucidated by recording its mass spectra on Shimadzu LCMS 2010 eV. NMR was recorded in Torrent Pharmaceuticals Ltd Research Centre on Bruker Avance FT- NMR 400 Hz.

Procedure for Synthesis of 1,4-Dihydroquinoxaline-2,3-Dione (P1)

The mixture of *o*-phenylenediamine (0.025 mol), oxalic acid (0.03 mol) and 4N HCl (15 ml) in 50 ml round bottom flask was refluxed for 1 h and then cooled at room temperature. The residue of **P1** separated was filtered, washed and recrystallised with rectified spirit. Yield: 86%; White Solid; M.P.: >300°C; IR (KBr) (cm^{-1}): 775, 1472, 1680, 3049, 3159; EIMS (m/z): 162.9 (M+1); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.10 (4H, m, Ar-H), 11.93 (2H, s, -NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 125.6, 125.9, 133.3, 159.9; Anal. Calc. for $C_8H_6N_2O_2$: C, 59.25; H, 3.08; N, 17.28; Found: C, 59.00; H, 2.98; N, 17.10.

Procedure for Synthesis of 2,3-Dichloroquinoxaline (P2)

The mixture of 1,4-dihydroquinoxaline-2,3-dione (**P1**) (0.01 mol) and phosphorous oxytrichloride (0.01 mol) stirred at room temperature for 2 h. The resulting mixture purified by recrystallised from rectified spirit to obtain product **P2**. Yield: 82%; Grey Solid; M.P.:

268°C; IR (KBr) (cm^{-1}): 733, 765, 1378, 1447, 3047; EIMS (m/z): 200.1 (M+1); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.72 (4H, m, Ar-H); ^{13}C NMR (DMSO-d_6) δ (ppm): 126.8, 131.2, 137.5, 152.9; Anal. Calc. for $C_8H_4Cl_2N_2$: C, 48.73; H, 2.03; N, 14.21; Found: C, 48.35; H, 2.01; N, 14.07.

General Procedure for Synthesis of 2-Chloro-3-Hydrazineylquinoxaline (P3)

2, 3-dichloroquinoxaline (**P2**) (0.015 mol) was added to the mixture of hydrazine hydrate (0.015 mol) and ethanol (10 ml). The content was refluxed for 3 h. The solid precipitate of **P3** obtained was filtered and re-crystallised from rectified spirit. Yield: 84%; Brown Solid; M.P.: 190°C; IR (KBr) (cm^{-1}): 704, 754, 1390, 1472, 3051, 3159, 3315, 3419; EIMS (m/z): 194.9 (M+1); ^{1}H NMR (DMSO-d_6) δ (ppm): 4.20 (2H, s, -NHNH$_2$), 7.73 (4H, m, Ar-H), 8.21 (1H, s, -NHNH$_2$); ^{13}C NMR (DMSO-d_6) δ (ppm): 122.7, 124.6, 125.9, 129.7, 133.1, 136.2, 150.8, 158.3; Anal. Calc. for $C_8H_7ClN_4$: C, 49.48; H, 3.61; N, 28.86. Found: C, 49.21; H, 3.05; N 28.11.

Procedure for Synthesis of 4-Hydrazineyltetrazolo [1, 5-*a*] Quinoxaline (P4)

2-chloro-3-hydrazinylquinoxaline (**P3**) (0.02 mol) was added to the sodium azide (0.02 mol) in methanol and the resultant mixture was refluxed for 3 h with stirring. The reaction mixture was cooled to room temperature, then poured into crushed ice and finally 10 ml ethyl acetate was added to the mixture. The organic layer was separated, dried over anhydrous sodium sulphate and concentrated to get 4-hydrazinyl tetrazolo[1,5-*a*] quinoxaline (**P4**). Yield: 83%; Brown Solid; M.P.: 189°C; IR (KBr) (cm^{-1}): 760, 1385, 1432, 1452, 3048, 3145, 3321, 3408; EIMS (m/z): 202.1 (M+1); ^{1}H NMR (DMSO-d_6) δ (ppm): 4.46 (2H, s, -NHNH$_2$), 7.99 (4H, m, Ar-H), 8.91 (1H, s, -NHNH$_2$); ^{13}C NMR (DMSO-d_6) δ (ppm): 123.4, 126.9, 129.8, 130.3, 136.1, 142.8, 152.6, 160.3; Anal. Calc. for $C_8H_7N_7$: C, 48.24; H, 3.52; N, 49.25. Found: C, 47.85; H, 3.03; N, 48.12.

General Procedure for Synthesis of Tetrazolo Quinoxaline (P5$_{a-d}$)

The compound **P4** (0.01 mol) and various aromatic aldehydes (0.01 mol) in 5 ml DMF was refluxed for 2 h. Then the reaction mixture was cold at room temperature and poured into crushed ice. The crude of tetrazolo quinoxaline (**P5$_{a-d}$**) were filtered, dried and crystallized using methanol.

2-methoxy-4-((2-(tetrazolo[1,5-*a*]quinoxalin-4-yl)hydrazineylidene)methyl)phenol(P5$_a$) :

Yield: 72%; Yellow Solid; M.P.: 296°C; IR (KBr) (cm^{-1}): 756, 1034, 1275, 1390, 1445, 1473, 1614, 3050, 3160, 3423; EIMS (m/z): 336.2 (M+1); ^{1}H NMR (DMSO-d_6) δ (ppm): 3.81 (3H, s, -OCH$_3$), 7.01 (1H, d, Ar-H), 7.24 (1H, d, Ar-H), 7.33 (1H, s, Ar-H), 7.75 (4H, m, Ar-H), 8.88 (1H, s, -CH=N), 9.01 (1H, s, -NH), 9.98 (1H, s, -OH); ^{13}C NMR (DMSO-d_6) δ (ppm): 55.8, 116.2, 119.3, 121.8, 122.5, 123.4, 125.5, 128.8, 130.2, 139.2, 143.8, 145.9, 146.6, 152.3, 156.8, 159.9; Anal. Calc. for $C_{16}H_{13}N_7O_2$: C, 57.63; H, 3.90; N, 29.42. Found: C, 57.22; H, 3.55; N, 29.13.

4-(2-(3,4-dimethoxybenzylidene)hydrazineyl)tetrazolo [1,5-*a*]quinoxaline (P5$_b$) :

Yield: 77%; Brown Solid; M.P.: 284°C; IR (KBr) (cm^{-1}): 758, 1033, 1249, 1391, 1418, 1474, 1681, 3048, 3162; EIMS (m/z): 349.8 (M+1); ^{1}H NMR (DMSO-d_6) δ (ppm): 3.74 (3H, s, -OCH$_3$), 3.87 (3H, s, -OCH$_3$), 6.99 (1H, d, Ar-H), 7.29 (1H, d, Ar-H), 7.38 (1H, s, Ar-H), 7.79 (4H, m, Ar-H), 8.96 (1H, s, -CH=N), 9.11 (1H, s, -NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 55.4, 55.7, 115.6, 120.1, 121.3, 123.7, 123.9, 126.6, 127.8, 132.3, 138.4, 144.9, 146.1, 147.6, 154.2, 157.1, 160.2; Anal. Calc. for $C_{17}H_{15}N_7O_2$: C, 58.76; H, 4.32; N, 28.23. Found: C, 58.40; H, 4.10; N, 28.02.

4-(2-(4-methoxybenzylidene)hydrazineyl)tetrazolo[1, 5-*a*]quinoxaline (P5$_c$):

Yield: 81%; Yellow Solid; M.P.: >300°C; IR (KBr) (cm^{-1}): 755, 1035, 1250, 1390, 1419, 1473, 1682, 3052, 3161; EIMS (m/z): 319.6 (M+1); ^{1}H NMR (DMSO-d_6) δ (ppm): 3.80 (3H, s, -OCH$_3$), 7.05 (2H, d, Ar-H), 7.39 (2H, d, Ar-H), 7.69 (4H, m, Ar-H), 8.76 (1H, s, -CH=N), 9.24 (1H, s, -NH); ^{13}C NMR

(DMSO-d_6) δ (ppm): 55.6, 118.2, 119.9 122.2, 124.1, 124.4, 126.5, 128.8, 134.8, 137.9, 145.3, 156.1, 158.6, 161.8; Anal. Calc. for $C_{16}H_{13}N_7O$: C, 60.54; H, 4.09; N, 30.90. Found: C, 59.41; H, 3.92; N, 30.28.

5-methoxy-2-((2-(tetrazolo[1,5-*a*]quinoxalin-4-yl)hydrazineylidene)methyl)phenol ($P5_d$):

Yield: 75%; Yellow Solid; M.P.: 291°C; IR (KBr) (cm^{-1}): 755, 1254, 1388, 1418, 1472, 1681, 3045, 3163, 3422; EIMS (m/z): 335.8 (M+1); ^{1}H NMR (DMSO-d_6) δ (ppm): 3.87 (3H, s, -OC<u>H</u>$_3$), 6.55 (1H, s, Ar-<u>H</u>), 6.78 (1H, d, Ar-<u>H</u>), 7.38 (1H, d, Ar-<u>H</u>), 7.80 (4H, m, Ar-<u>H</u>), 9.05 (1H, s, -C<u>H</u>=N), 9.26 (1H, s, -N<u>H</u>), 10.10 (1H, s, -O<u>H</u>); ^{13}C NMR (DMSO-d_6) δ (ppm): 56.1, 117.3, 121.6, 122.4, 123.7, 124.9, 125.1, 129.2, 131.7, 140.3, 143.3, 145.5, 147.8, 154.6, 159.2, 162.2; Anal. Calc. for $C_{16}H_{13}N_7O_2$: C, 57.63; H, 3.90; N, 29.42. Found: C, 57.22; H, 3.55; N, 29.13.

In Silico Pharmacokinetic Evaluation

In silico pharmacokinetic parameters of the compounds (**$P5_{a-d}$**) were obtained using programs ADMET and Molsoft ICM 3.5 software. These programs computed pharmacokinetic parameters such as logP, PSA, molecular weight, volume, water solubility, %HIA, BBB, %PPB etc.

Antibacterial Study

Culture of microorganisms: Bacterial samples used for primary screening of antibacterial activity for the compounds. Bacteria strains were supplied from ARIBAS, namely *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*. They were maintained by periodical transfer on a fresh nutrient agar slant.

Preparation of media: The antibacterial activities were carried out by using Mueller-Hinton agar plate. The composition of the medium was Nutrient broth 13 g and Agar-Agar powder 30 g. Both ingredients were completely dissolved in 1 L of distilled water and the pH of the medium was adjusted to 7.4. The medium was sterilized in an autoclave at 121°C for 15 min. It was then cooled down to 45°C and 20 ml was poured in each sterilized Petri dish.

Preparation of inoculums: A fresh microbial seed was prepared separately by sub culturing into nutrient broth medium and incubated at 37°C.

Antibacterial screening test: The antibacterial activity was performed by taking a fixed concentration (0.02 g/mL) of compounds ($P5_{a-d}$) and standard drug using the cup method [22]. Minimum inhibitory concentration is the lowest substance concentration at which no sign of bacterial growth was detectable microscopically. The test compounds were dissolved in DMSO to produce (0.02 g/mL) for minimum inhibitory concentration (MIC). Different compounds on different microorganisms were placed in each cup. The plates were first placed in a refrigerator for 30 min and incubated at 37°C for 24 h. The results were recorded by measuring the zone of inhibition in the plate and recorded for further calculation.

Conclusions

Tetrazolo-quinoxaline analogues (**$P5_{a-d}$**) were synthesized and characterized by IR, MS, NMR and elemental analysis. The results of *in silico* pharmacokinetic parameters and ADMET data suggested that the compounds synthesized (**$P5_{a-d}$**) were good drug candidates. Further, the antibacterial activity of compounds (**$P5_{a-d}$**) was studied on three different types of bacterial strains. The result of antibacterial activity suggested the compounds **$P5_b$** and **$P5_d$** to be potent and used for further research.

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