Scientific paper

One-pot Synthesis, Molecular Modeling and *In Vitro* Antibacterial Activity of Novel 3-(1,3,4-Oxadiazol-2-yl) Quinoxaline 1,4-Dioxide and Pyrazolyl Analogs

Mohamed Attia Waly,[1,*] Laila Abou-zeid,[2] Sameh Ramadan El-Gogary[1,3] and Mona Abbas Shalaby[1]

[1] *Faculty of Science (Damietta), Chemistry Dept.,*

[2] *Pharm. Org. Chemistry, Faculty of Pharmacy, Mansoura University, P.O. 34517, Egypt*

[3] *Current address: Chemistry Department, Faculty of Science (Jazan), Jazan University, Kingdom of Saudi Arabia*

** Corresponding author: E-mail: mohamedwaly7@yahoo.com; Tel: 0020122792952, Fax: 0020572403868*

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Abstract

The new 1,3,4-oxadiazolylquinoxaline ring system was synthesized by the condensation of the quinoxaline 1,4-dioxide acid hydrazide **6** with acetyl chloride in one-pot synthesis. In which the hydrazide was refluxed in excess of acetyl chloride, acetic anhydride or acetic acid in the presence of phosphorus oxychloride forming the 1,3,4-oxadiazolylquinoxaline ring system. Molecular modeling studies have been performed to evaluate their recognition at the hDHFR binding-pocket as potential hDHFR inhibitors. The antibacterial properties of these compounds showed reasonable activities towards gram negative bacteria, *Escherichia coli*. Compound **20** performed appropriate complementarity study rather well; the conserved amino acids of hDHFR enzyme imitating the MTX docking pattern. Besides, compound **20** showed highest antibacterial activity against *E. coli* in comparison with the well known bacteriostatic chloramphenicol; the pyrazolyl analogues also have moderate affinity toward the applied bacteria.

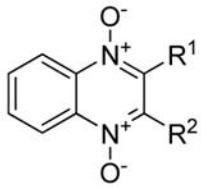
Keywords: Quninoxaline-1,4-dioxide carboxylic acid ester, molecular modeling, DHFR binding pocket recognition, antibacterial properties.

1. Introduction

The quinoxaline 1,4-dioxide (QDO) derivatives have shown very interesting biological properties; some of the first cases of bioactivity being described in the mid-1940s involving the QDO for its feed growth promotion capacity in the animal veterinary use,[1] i.e., compounds **1**–**4** (Scheme 1), and also for human antibacterial action of certain synthetic QDO derivatives.[2,3] Currently, the interest in bioactivity of QDO is still in progress[4] and there are a great number of patent registrations and commercial formulations concerned with the QDO use as antibacterial agents and animal feed additives.[5–9]

There are many findings that demonstrate the antibacterial activity of QDO derivatives **1**–**4** under anaerobic conditions acting as specific anti-microorganism agents. Anaerobic antibacterial mechanism of QDO involves DNA synthesis inhibition without effecting the RNA and protein syntheses.[10,11] Some of these findings regarding the biological action of QDO as an antibacterial and antifungal agents come from the results that 2-phenylsulfonyl-substituted QDO **5** and **6** and their analogues **7** and **8** (Scheme 2) were shown to be excellent scaffolds against the nosocomial *Enterococcus faecalis* and *Enterococcus faecium*.[11,12]

At the end of the 1990s Monge *et al*. described QDO derivatives with extensive substitution patterns possessing excellent anti-*Mycobacterium tuberculosis* $H_{37}Rv$ activity. In the first approach 3-amino-2-cyano-substituted quinoxaline 1,4-dioxide (i.e. **9**–**11**, Scheme 2) was identified as an antimycobacteria lead system.[13–15] Not only this structural architecture resulted in a significant antibacterial ac-

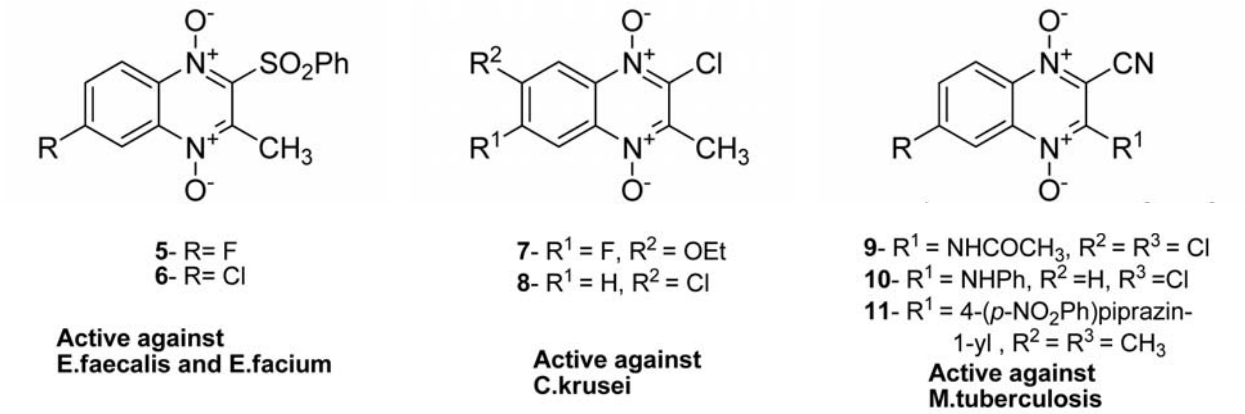


1- Quindoxin, grofas, celbar, $R^1 = R^2 = H$
2- carbadox, mecadox, $R^1 = CHNNHCO_2CH_3$, $R^2 = H$
3- Dioxidine, $R^1 = R^2 = CH_2OH$
4- Olaquindox, bayonox, $R^1 = CONHNHCH_2CH_2OH$, $R^2 = CH_3$

Scheme 1. First QDO patented as antibacterial agents and as animal-feed additives

tivity but also both *N*-oxide moieties played a role in the bioactivity, as proved by the finding that quinoxaline analogues were inactive.[15–17] Several 1,3,4-oxadiazole[18–20] and pyrazole[21] derivatives display various types of biological activity, including bactericidal activities.

bioisosterically replace the pteridine ring in both classical and non classical antifolates.[22–24] In this context and for the synthesis of new quinoxalines with potential biological activity it appears desirable to combine the 1,3,4-oxadiazolyl or pyrazolyl rings with quinoxali-



5- R= F
6- R= Cl

Active against
E.faecalis and E.facium

7- R^1 = F, R^2 = OEt
8- R^1 = H, R^2 = Cl

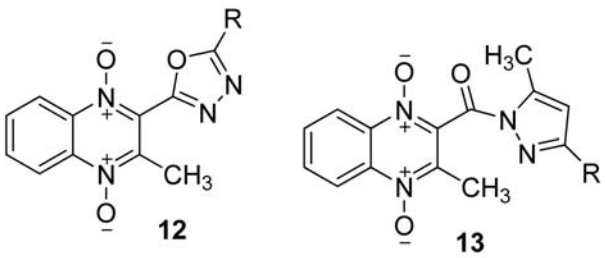
Active against
C.krusei

9- $R^1 = NHCOCH_3$, $R^2 = R^3 = Cl$
10- $R^1 = NHPh$, R^2 =H, R^3 =Cl
11- R^1 = 4-(*p*-NO_2Ph)piprazin-1-yl , $R^2 = R^3 = CH_3$

Active against
M.tuberculosis

Scheme 2. Noteworthy QDO with antibacterial and antifungal activities

Research was conducted in an attempt to develop new antibacterial agents targeted at human dihydrofolate reductase (DHFR) and tymidylate synthase (TS) (two specific enzymes involved in the biosynthesis of the nucleic acids). DHFR role is to regenerate folic acid into its reduced form of tetrahydrofolate and is therefore essential for bacteria, *Plasmodia* and normal and cancerous human cells. Inhibitors of DHFR have antibiotic, antimalarial and antineoplastic properties. We designed and synthesized novel quinoxaline derivatives based on the fact that quinazolin-4-ones exert their antibacterial activity through DHFR inhibition.[22] This has been done on the ground that the quinoxaline ring can



12 **13**

Scheme 3: Target structures

ne 1,4-dioxide moiety e.g. as in compound **12** and **13**, respectively (Scheme 3), and evaluate them as antibacterial agents.

2. Experimental

2. 1. Materials and Methods

All the chemicals used were of analytical grade and purified by standard methods prior to use. Thin layer and column chromatography were performed using silica gel type 60 (Merck 7731 and 7734, respectively). Melting points were determined on a Gallenkamp melting point apparatus. The IR spectra were recorded on a Jasco 4100 FTIR spectrophotometer as KBr discs (ν_{max} in cm^{-1}). The ^1H and ^{13}C NMR spectra were recorded in DMSO-d_6 or $CDCl_3$ on a Bruker DRX NMR spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C NMR. Chemical shift (δ) values are expressed in ppm and are referenced to the residual solvent signals of DMSO-d_6 or $CDCl_3$. The mass spectra were recorded on a Shimadzu GCMS-QP 1000 EX mass spectrometer at 70 eV. Elemental analyses were performed on Perkin–Elmer 2400 C, H, N, S Elemental analyzer.

2-Methyl-3-hydrazinocarbonylquinoxaline-1,4-dioxide (15).

A solution of quinoxaline carboxylic acid ester **14** (2 g, 0.008 mol) and hydrazine (4.03 g, 0.08 mol) in ethanol (20 mL) was refluxed for 1 h on a boiling water bath to precipitate white needles, which were collected by suction filtration (1.0 g). Evaporation of the filtrate gave additional product (0.5 g). Recrystallization of the acid hydrazide **15** in ethanol gave pure yellowish-white product **15** (1.3 g, 69%); m.p. 216–220 °C. IR (KBr) ν 1670 (CONH), 3278 (NH), 3344 cm^{-1} (NH_2). ^{1}H NMR (DMSO-d_6) δ 2.62 (3H, s, *CH*$_3$), 4.69 (2H, s, NH*NH*$_2$), 7.86–8.48 (4H, m, aromatic), 9.94 (1H, s, CO*NH*NH$_2$). MS *m/z* 234.11 (16%). Anal. Calcd for $C_{10}H_{10}N_4O_3$ (234.21): C, 51.28; H, 4.30; N, 23.92%; found: C, 49.96; H, 4.20; N, 23.66%.

2. 2. One-Pot Syntheses of Oxadiazolylquinoxaline Derivatives.

(i) Acetyl chloride: To a solution of acid hydrazide **15** (1.17 g, 0.005 mol) in acetonitrile (20 mL) and triethylamine (10 mL), acetyl chloride (10 mL) was added drop wise with stirring in ice bath. After the addition was completed, the reaction mixture was refluxed for 4 h. The solvent was removed *in vacuo* and the residue was purified by column chromatography and eluted by a mixture of dichloromethane, ethanol and ammonia in the ratio 300:8:1. The product **17** was recrystallized from ethanol giving pure **17** (0.87g, 58%); m.p. 245–247 °C. IR (KBr) ν 3421.1(N-OH) and 1735.62 (CHO). ^{1}H NMR (DMSO-d_6): δ 2.50 {3H, s, C(5')-CH_3}, 7.68-8.18 (4H, m, aromatic), 8.21 (1H, s, CHO), 10.67 {1H, br, exch., N(4)-OH}. and 11.38 {1H, br, exch., N(1)-OH}. ^{13}C NMR (DMSO-d_6): δ 21.01, 118.27, 129.99, 131.65, 136.32, 139.12, 141.79, 149.07, and 163.59. MS *m/z* 274.14 (33%). Anal. Calcd for $C_{12}H_{10}N_4O_4$ (274.23): C 52.56, H 3.68, N 20.43%; found: C 52.34, H 3.29, N 20.76%.

(ii) Acetic anhydride: A mixture of acid hydrazide **15** (1.17 g, 0.005 mol) and acetic anhydride was refluxed for 4 h. The reaction mixture was poured onto water and the separated solid was filtered off and subjected to column chromatography purification to give product **17** (1.0 g, and 66%).

(iii) Acetic acid and phosphorous oxychloride: A mixture of acid hydrazide **15** (1.17 g, 0.005 mol), acetic acid (5 mL) and phosphorous oxychloride (10 mL) was heated gently at 70 °C for 30 min. After cooling, it was poured onto ice-water mixture (50 mL), the precipitate was collected and purified by column chromatography to give the oxadiazolylquinoxaline derivative **17** (0.77 g, 51%).

(iv) Formic acid and phosphorous oxychloride: A mixture of acid hydrazide **15** (1.0 g, 0.0042 mol), formic acid (5 mL) and phosphorous oxychloride (10 mL) was heated gently at 70 °C for 30 min. After cooling, it was poured onto ice-water mixture (50 mL) and the precipitate was collected and purified by column chromatography to give 1,4-dihydroxy-3-(1,3,4-oxadiazol-2-yl)-1,4-dihydroquinoxaline-2-carbaldehyde (**18**) (0.9 g, 87%); m.p. 220–222 °C. IR (KBr) ν 1735 (CHO), 3370 cm^{-1} (OH); ^{1}H NMR (DMSO-d_6) δ 7.96 (1H, s, C(5')-H), 8.16 (1H, s, CHO), 7.91–8.50 (4H, m, aromatic), 10.51 (1H, s, exch., N(1)-OH), 10.92 (1H, s, exch., N(4)-OH). ^{13}C NMR (DMSO-d_6): δ 118.14, 118.27, 129.87, 129.92, 129.96, 131.19, 131.39, 131.51, 149.18, 159.74, 163.22. MS *m/z* 246.01 (21%). Anal. Calcd for $C_{11}H_{10}N_4O_3$ (246.22): C, 53.66; H, 4.09; N, 22.75%; found: C, 53.45; H, 3.99; N, 22.50%.

(v) Carbon disulfide: A mixture of compound **15** (0.8 g, 0.0034 mol), a solution of methanolic KOH (1 g KOH and 20 mL MeOH) and carbon disulphide (2 mL, excess) was refluxed over night. After cooling, it was poured into water and ice mixture (50 mL), acidified with dilute HCl to pH 6 and the yellow precipitate was formed. The precipitate was filtered off and crystallized in ethanol to provide 2-(5-mercapto-1,3,4-oxadiazol-2-yl)-3-methylquinoxaline 1,4-dioxide (**20**) (0.13 g, 14%); m.p. 212–214 °C. IR (KBr) ν 3440 (NH), 1640 cm^{-1} (C=S); ^{1}H NMR (DMSO-d_6) δ 2.50 (3H, s, CH_3, quinoxaline), 7.89–8.46 (5H, m, aromatic and NH, exch.). ^{13}C NMR (DMSO-d_6): δ 14.07, 128.16, 129.11, 130.26, 130.53, 131.86, 132.25, 141.89, 152.46, 158.06, 177.46. MS *m/z* 276.20 (17%). Anal. Calcd for $C_{11}H_8N_4O_3S$ (276.27): C, 47.82; H, 2.92; N, 20.28; S, 11.61%; found: C, 47.55; H, 2.76; N, 20.56; S, 11.43%.

2-Methyl-3-[2-(phenylcarbamothioyl)hydrazinecarbonyl]quinoxaline-1,4-dioxide (21).

Phenylisothiocyanate (0.57 g, 0.0042 mol) was added to a solution of compound **15** (1 g, 0.0042 mol) in THF (20 mL) and was refluxed for 8 h. After cooling the yellow precipitate was filtered off. Crystallization from methanol gave compound **21**, as yellowish white crystals (1.0 g, 65%); m.p. 120 °C. IR (KBr) ν 1709 (CO), 3479, 3209 cm^{-1} (NH). ^{1}H NMR (DMSO-d_6) δ 3.07 (3H, s, CH_3, quinoxaline), 7.17–8.79 (11H, m, aromatic and 2NH, exch.), 10.75 (1H, br., NH(C=O)). ^{13}C NMR (DMSO-d_6): δ 14.01, 118.31, 126.10, 128.21, 129.53, 130.01, 131.60, 131.78, 136.22, 138.89, 139.01, 149.89, 165.15, 189.28. MS *m/z* 369.22 (0.9%). Anal. Calcd for $C_{17}H_{15}N_5O_3S$ (369.40): C, 55.27; H, 4.09; N, 18.96; S, 8.68%; found: C, 54.96; H, 4.22; N, 18.73; S, 8.56%.

2-Methyl-3-[5-(phenylamino)-1,3,4-oxadiazol-2-yl] quinoxaline-1,4-dioxide (22).

Compound **21** (0.4 g, 1.2 mmol) was dissolved in ethanolic solution of KOH (1.0 g KOH and 20 mL EtOH) and was refluxed for 8 h. After cooling, it was poured into water and ice mixture (50 mL) and acidified with dilute HCl to pH 6. The precipitate was filtered off and crystallized from aqueous ethanol to afford compound **22** (0.14 g, 42%); m.p. 250–252 °C. IR (KBr) ν 1624 (CO), 3417,

3278 cm^{-1} (NH). ^1H NMR (DMSO-d_6) δ 3.01 (3H, s, CH_3, quinoxaline), 7.04–8.48 (9H, m, aromatic), 11.10 (1H, s, exch., NH). ^{13}C NMR (DMSO-d_6): δ 13.91, 118.19, 118.89, 119.20, 119.61, 123.15, 130.10, 130.23, 131.11, 131.29, 133.87, 133.99, 140.18, 148.82, 153.91, 155.62, 158.77. MS *m/z* 335.15 (11%). Anal. Calcd for $C_{17}H_{13}N_5O_3$ (335.32): C, 60.89; H, 3.91; N, 20.89%; found: C, 60.66; H, 3.87; N, 20.66%.

(3,5-Dimethyl-1*H*-pyrazol-1-yl)(3-methylquinoxaline 1,4-dioxide-2-yl)methanone (24).

A mixture of compound **15** (1 g, 0.0042 mol) and acetyl acetone (0.43 g, 0.0042 mol) in DMF (20 mL), was refluxed over night. The mixture was allowed to cool and the crystals were formed. The precipitate was filtered and recrystallized from DMF giving **24** (0.2 g, 16%); m.p. 230–232 °C. IR (KBr) ν 1647 cm^{-1} (CO); ^1H NMR (DMSO-d_6) δ 1.82 (3H, s, C(5')-CH_3, pyrazole), 1.94 (3H, s, C(3')-CH_3, pyrazole), 2.45 (3H, s, C(3)-CH_3, quinoxaline), 6.92 (1H, s, C(4')-H, pyrazole), 7.83–8.48 (4H, m, aromatic). Anal. Calcd for $C_{15}H_{14}N_4O_3$ (298.3): C, 60.40; H, 4.73; N, 18.78%; found: C, 60.66; H, 4.39; N, 17.74%.

(3-Hydroxy-5-methyl-1*H*-pyrazol-1-yl)(3-methylquinoxalin-1,4-dioxide-2-yl)methanone (25).

A mixture of compound **15** (1 g, 0.0042 mol) and ethyl acetoacetate (3 g, 0.023 mol) was refluxed for 8 h. After cooling and triturating with methanol, the white precipitate was filtered off and recrystallized from DMF giving **25** (0.18 g, 14%); m.p. 266 °C. IR (KBr) ν 1612 (CO), 3220 cm^{-1} (OH-pyrazole). ^1H NMR (DMSO-d_6) δ 2.76 (3H, s, C(3)-CH_3, quinoxaline), 7.91–9.52 (5H, m, aromatic and C(4')-H, pyrazole), 11.06 (1H, s, OH pyrazole). MS *m/z* 300.10 (M^+, 0.44), 301.10 (M^++1, 0.42). Anal. Calcd for $C_{14}H_{12}N_4O_4$ (300.27): C, 56.00; H, 4.03; N, 18.66%; found: C, 55.81; H, 4.22; N, 18.14%.

2. 3. Antibacterial Activity Experiment

Two different concentrations (10 and 50 mg/L) of the applied compounds (Table 1) were prepared and loaded (200 μL) into a hole prepared previously into the medium inoculated with the different tested bacteria to study their effects. After incubation at 30 °C at for 24–48 days, the zones of inhibition were measured in mm.

2. 4. Bacterial Growth Effect

Nutrient Agar-NaCl (NA-NaCl) medium was used for culturing the tested bacteria and for studying the effect of the compounds on the bacterial growth. NA-NaCl medium containing (g/L), peptone, 5.0; beef extract, 1.5; NaCl, 5.0 and agar 20.0 and the pH 7.0, was used for screening the effect of the compounds on the microbial growth.

2. 5. Molecular Modeling and Docking Methods

The molecular modeling study was conducted with Hyperchem 6.03 package from Hypercube and MOE programs running on a PC computer[28,29] to distinguish compounds possessing higher binding affinities for the enzyme. Hydrogen bonds with a bond length up to 3 Å were considered. Once a reasonable complex was formed, the energy of interaction of each ligand with the enzyme was calculated. In measuring degree of complementarity using energy calculations, the more negative the energy change caused upon the docking of a given ligand into the enzyme active site, the more favorable the interaction and the more stable the complex.

2. 6. Enzyme Structure

Starting coordinates of the human dihydrofolate reductase enzyme in a tertiary complex with nicotinamide

Table 1: Antibacterial effect of the novel quinoxaline analogs

Comp. No.	Zone of inhibition (mm)							
	10 mg/L				50 mg/L			
	Gram-negative		Gram-positive		Gram-negative		Gram-positive	
	K. pneumonia	*E. coli*	*B. cereus*	*B. subtilis*	*K. pneumonia*	*E. coli*	*B. cereus*	*B. subtilis*
15	12	9	6	3	14	11	13	10
17	5	13	11	10	9	20	17	14
18	7	14	6	7	8	20	11	13
20	17	30	11	15	27	37	10	23
21	4	2	3	5	4	2	2	3
22	2	22	10	11	11	31	16	12
24	15	19	16	18	22	23	21	20
25	11	17	10	15	19	20	10	14
chloramphenicol	17	16	20	21	27	24	25	28

Degree of activity is measured by the zone of inhibition: – no inhibition, resistant, not sensitive; 10–15 mm, slightly active; 15–20 mm, moderately active; 20–25 mm, highly active; > 25 mm, very active.

adenosine diphosphate (NADPH) and MTX (code ID DLS) was obtained from the Protein Data Bank of Brookhaven National Laboratory.[30]

2. 7. Molecular Structure of Quinoxalines

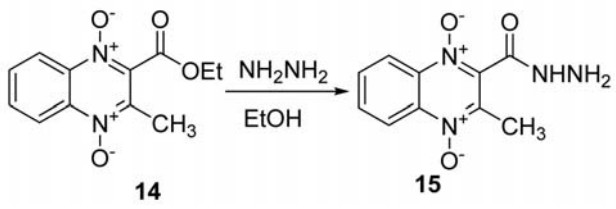
The quinoxaline analogs were constructed from fragment libraries in the Hyperchem program followed by energy minimization using the »Amber force field«. The partial atomic charges for each analog were assigned with the semiempirical mechanical calculation method »AM1« implemented in Hyperchem 6.03. Conformational search was performed around all the rotatable bonds with an increment of 10° using conformational search module as implemented in HyperChem6. All the conformers were minimized until the Rms deviation was 0.01 kcal/mol Å.

3. Results and Discussions

3. 1. One-Pot Synthesis of the 1,3,4-Oxadiazolyl Ring

In the present paper, the quinoxaline 1,4-dioxide acid hydrazide **15** was synthesized as the starting material,[24,25] which was used as a versatile intermediate for the synthesis of various 3-(1,3,4-oxadiazol-2-yl)quinoxaline 1,4-dioxide derivatives. These compounds will be investigated for their *in vitro* antibacterial activities.

The quinoxaline 1,4-dioxide acid hydrazide derivative **15** was synthesized by refluxing the quinoxaline carboxylic ester **14**[25] with hydrazine hydrate in ethanol with 69% yield (Scheme 4). The 1,4-dioxide moieties were stable during the reaction with hydrazine and appeared around 1350 cm^{-1} in their IR data, in spite of N. C. Romeiro *et al.*[25f] stating the reduction of *N*-dioxide groups seems to occur simultaneously to the formation of hydrazide.



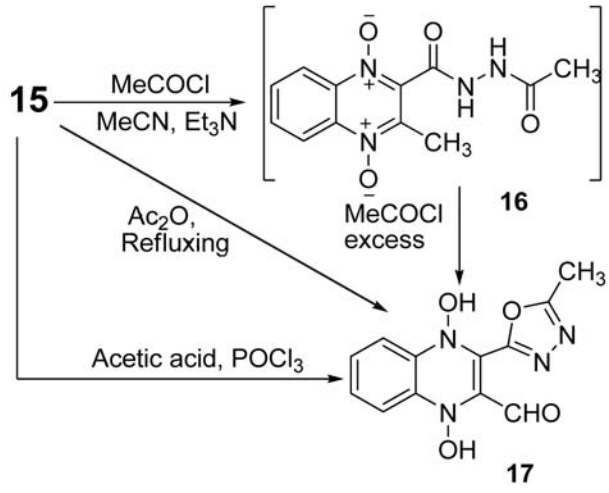
Scheme 4: Synthesis of quinoxaline acid hydrazide

Compound **15** melted at 216–220 °C, consistent with its recorded value of 217–218 °C given in the literature.[24] IR spectrum of **15** showed absorption bands at 3344 cm^{-1} and 1670 cm^{-1} for NH_2 and amide carbonyl, respectively. The ^{1}H NMR data for **15** showed the presence of the exchangeable hydrazide protons at δ 4.69 (NH_2) and 9.94 (NH) ppm.

One of the methods used in the synthesis of the 1,3,4-oxadiazole ring was the reaction of acid hydrazide with acetyl chloride forming (in one step) the diamide derivative, followed, in the second step, by the treatment with phosphorus oxychloride to cause dehydration.[26]

In our work, heating of the acid hydrazide **15** with an excess of acetyl chloride in acetonitrile and triethylamine, afforded a product that was inconsistent with the expected diamide derivative **16**, as was shown by spectral data. In addition, the refluxing of the hydrazide **15** in acetic anhydride gave the same product as previously. Another confirmation for this short synthetic path was accomplished by heating of the hydrazide derivative **15** with acetic acid and an excess of phosphorus oxychloride (Scheme 5).

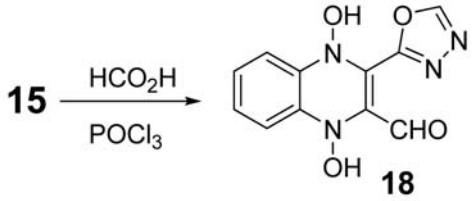
The IR spectrum of the product stemming from **15** showed the presence of absorption at 1736 cm^{-1}, characterizing the presence of a formyl carbonyl group, and the absence of the diamide carbonyl absorption. The ^{1}H NMR data for this product showed the presence of a signal at δ 2.50 ppm related to C(5')-CH_3 and the disappearance of the quinoxaline C(3)-CH_3 signal. An additional proton at δ 8.21 ppm, characterizing the CHO proton and two exchangeable OH protons at δ 10.97 and 11.38 ppm for N(4)-OH and N(1)-OH were detected, respectively. ^{13}C NMR data showed the presence of a methyl group at δ 21.01 ppm, in addition to the C=O carbon at δ 163.59 ppm. These spectral data, in addition to the mass spectrum for the product, are consistent with the new 1,3,4-oxadiazolylquinoxaline structure **17**, obtained ***in a new one-pot synthesis for the 1,3,4-oxadiazole ring*** shown in Scheme 5. The formation of the N-OH and CHO groups was attributed to the redox reactions during the reaction course.



Scheme 5: One-pot synthesis of the 1,3,4-oxadiazolylquinoxaline

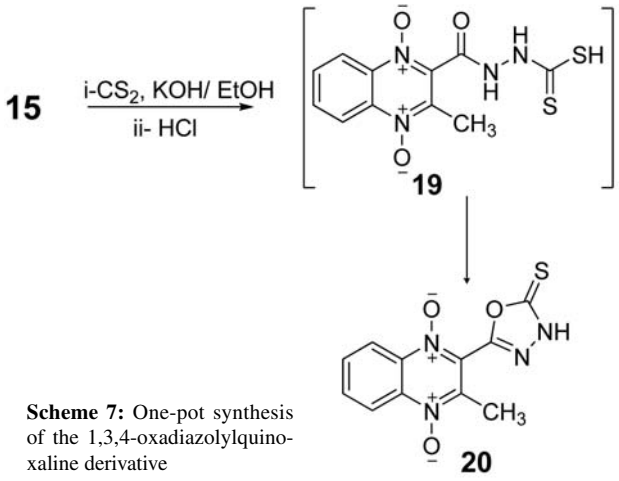
Refluxing the acid hydrazide **15** with formic acid in an excess of phosphorus oxychloride afforded in a one-pot synthesis the 1,3,4-oxadiazolylquinoxaline derivative **18** (Scheme 6). The ^{1}H NMR data for **18** shown the reduction

of both N(1) and N(4)-oxides and gave two exchangeable N-OH protons at δ 10.51 and 10.92 ppm, respectively. The C(5')-H and CHO protons appeared at δ 7.96 and 8.16 ppm, respectively. The IR spectra for **18** showed the appearance of a broad OH absorption at 3370 cm^{-1}, and CHO group carbonyl at 1735 cm^{-1}. ^{13}C NMR for **18** showed the C=O signal at δ 163.22 ppm and the molecular mass (*m/z* 260.25) is consistent with its calculated molecular weight (260.21).



Scheme 6: One-pot synthesis of the unsubstituted 1,3,4-oxadiazolylquinoxaline

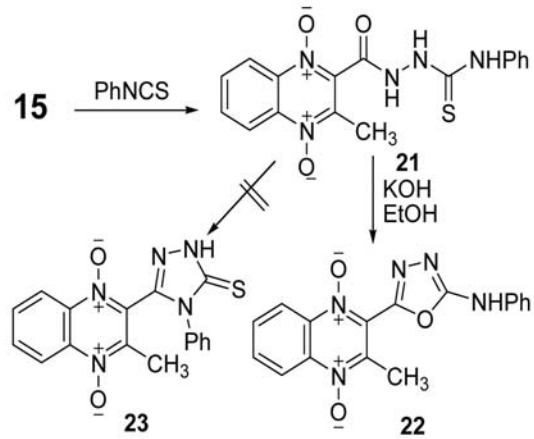
Synthesis of the 5'-substituted 1,3,4-oxadiazolylquinoxaline derivative **20** was performed by refluxing the acid hydrazide **15** with carbon disulfide in the presence of alcoholic potassium hydroxide as shown in Scheme 7, in a procedure resembling the triazole ring synthesis.[19c] The NH proton in ^1H NMR appeared at δ 7.89 ppm and was shown to be an exchangeable signal. The IR spectrum for **20** showed the presence of NH stretching as a broad band at 3440 cm^{-1} and C=S at 1640 cm^{-1}. We propose the reaction path via the formation of the thiocarbazoic acid **19** as an intermediate, which was under the reaction conditions cyclized to give the 1,3,4-oxadiazolylquinoxaline derivative **20** (Scheme 7).



Scheme 7: One-pot synthesis of the 1,3,4-oxadiazolylquinoxaline derivative

3. 2. Two-Pot Synthesis of the 1,3,4-Oxadiazolyl Ring

The reaction of acid hydrazide **15** with phenyl isothiocyanate provided the phenylcarbamothioylhydrazide derivative **21**, which in its ^1H NMR showed three exchangeable NH protons at δ 7.45, 8.79 and 10.75 ppm for C(=S)-NH, C(=S)-NH-Ph and C(=O)-NH, respectively. On treating **21** with alcoholic potassium hydroxide a product inconsistent with the published data for 1,3,4-triazoloquinoxaline **23**[27] was obtained (Scheme 8). The elemental analysis for this product indicated the absence of sulfur, and was consistent with the structure of the 1,3,4-oxadiazolylquinoxaline derivative **22** (Scheme 8). The ^1H



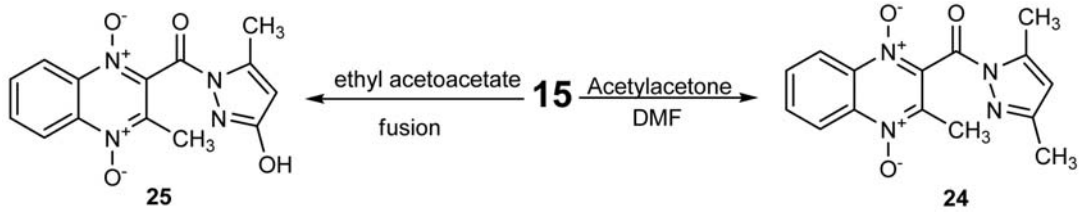
Scheme 8: Two-pot synthesis of the 5'-phenyamino 1,3,4-oxadiazolylquinoxaline

3. 3. Syntheses of Pyrazolylquinoxaline 1,4-Dioxide Derivatives

Refluxing the quinoxaline hydrazide **15** either with acetylacetone in DMF or its fusion with ethyl acetoacetate yielded the pyrazolyl derivatives **24** or **25**, respectively (Scheme 9). The pyrazolyl product **24** was precipitated in DMF after cooling. The ^1H NMR data for **24** showed that the protons characterizing hydrazide part have disappeared and instead of them the proton of the pyrazole ring appeared at δ 6.92 ppm; additionally three methyl group protons at δ 1.82, 1.94 and 2.45 ppm appeared. The ^1H NMR for **25** showed the presence of an exchangeable hydroxyl proton at δ 11.06 ppm and the pyrazolyl ring proton appearing at δ 7.91–9.52 with the quinoxaline ring protons signal.

3. 4. Molecular Modeling Study

The proposed target compounds **15**–**23** have been comparatively evaluated in terms of their mode of binding to human dihydrofolate reductase (hDHFR) pocket. Molecular docking has been performed for the proposed compounds to evaluate their recognition profiles at the h-DHFR binding-pocket. The idea behind this concept is based on the finding that synthetic hDHFR inhibitors fit at the enzyme pocket in a highly comparable manner to the dihydrofolate inhibitor MTX (3) (Figure 1).



Scheme 9: Synthesis of the pyrazoly-lquinoxaline derivatives

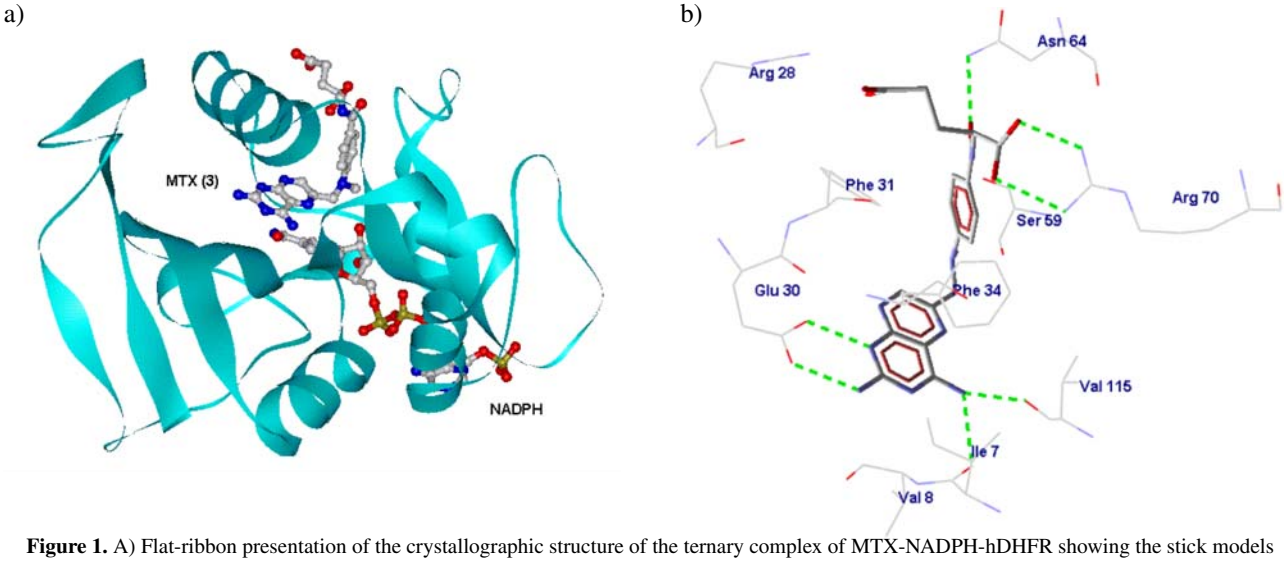


Figure 1. A) Flat-ribbon presentation of the crystallographic structure of the ternary complex of MTX-NADPH-hDHFR showing the stick models of MTX (3) and the cofactor NADPH (C, gray; N, blue; O, red, P, orange). B) Crystal structure of MTX (3) at the hDHFR binding pocket showing H-bonding interactions with the key residues. (Colored-stick, C, gray; N, blue; O, red).

Molecular mechanics studies indicated that hydrogen bonding interactions and conformational changes of the amino acid residues of the active site can be considered the main recognition elements for DHFR-ligand systems.

Molecular dynamic studies of the docked quinoxaline analogs into the enzyme active site indicated that in compounds **15**–**23**, the quinoxaline oxygen atoms at N1 and N4 atoms were able to stabilize the H bonding interactions with Glu30 and Val115; the crucial amino acids at the binding pocket. The N3 of the oxadiazole was involved in H-bonding with Ser59 which is one of the essential conserved amino acids responsible for the recognition at the binding pocket.

However, in compound **20**, the most active compound, presence of a terminal thiol group at C2 provides proper recognition with both Ser59 and Thr56. On the other hand, the quinoxaline ring remained geometrically oriented with the formation of the van der Waals interactions with both Phe31 and Phe34 at the active site. The essential cofactor NADP at the binding site that enhanced the enzymatic interactions enabled H bonding interaction with both N1 of quinoxaline and O1 of the oxadiazole rings (Figure 2).

In compound **22** the phenylamino substituent at C2 of the oxadiazole ring considerably changed the conformational orientation and in turn maintains the interaction with Ser59. The oxadiazole ring and its phenylamino group were accommodated by embedding between two electrophilic branches including Lys55 and Thr56 at one side and Gly20, Asp21 and Tyr22 from the other side, inducing preferable electrostatic recognition. However the bulkiness of the phenylamino group dramatically influences the position of Ser59, deviating from its original configuration leading to the formation of a moderate H bond. All these features together give a good explanation for the high level of antibacterial effect that was shown with these two analogs.

Comparing the performance of compounds **17** and **18** revealed a favorable binding for the C2-methylated derivative due to the lipophilic interaction of this methyl group in the hydrophobic binding pocket of the enzyme. However, this methyl group hinders the H-bonding interaction with the conserved amino acid Thr56. In compound **18** due to the absence of C2 substitution, there was no chance to perform a binding interaction with Thr56 (Figure 3).

In compound **15** the shortening of the hydrazinocarbonyl side chain negatively interferes with the formation

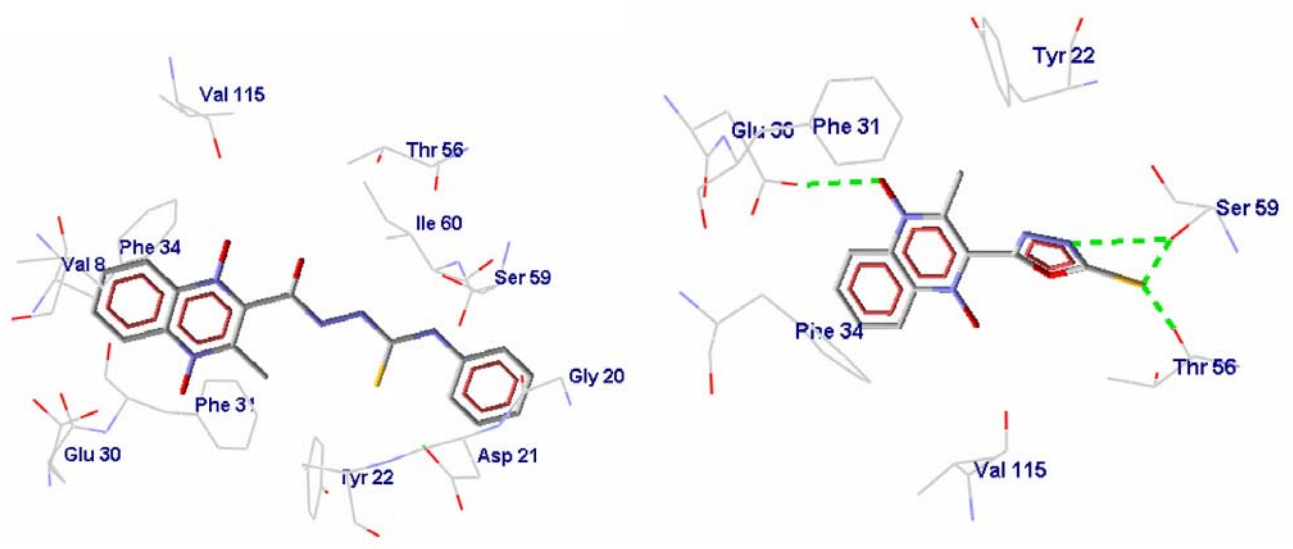


Figure 2. The binding mode and residues involved in the recognition of the most inactive compound **21** and the most active compound **20** docked and minimized in the hDHFR binding pocket

of electrostatic and hydrogen bonding interactions with the crucial amino acid Ser59 that is located far away from the recognition site. On the other hand, in compound **21** substitution of the hydrazinocarbonyl chain with the phenylthiocarboxamide group leads to an unfavorable expanding of the side chain (5 carbon spacer) that pushed the phenyl group out of the binding pocket and oriented it away from the amino acid residues by a distance that is not sufficient to provide a recognizable hydrogen bonds in turn missing the chance for recognition with Ser59. In compounds **15** and **21** poor conformational recognition with the surrounding key amino acids can explain their loss of the antibacterial potency (Figure 2).

3. 5. In Vitro Antibacterial Activity

The compounds **15**–**25** synthesized in this study were tested *in vitro* for their growth inhibitory activities against two different types of bacteria with respect to chloramphenicol as an antibacterial standard reference.[31] Two Gram-negative bacteria namely, *Klebsiella pneumoniae*, and *Escherichia coli*, and two Gram-positive (*Bacillus cereus* and *Bacillus subtilis*) which have been selected for this study. It was found that the applied compounds were active toward *Escherichia coli* (Table 1), and varied in their potency toward the other bacteria members. Compound **20** is more active against these bacteria. Compound

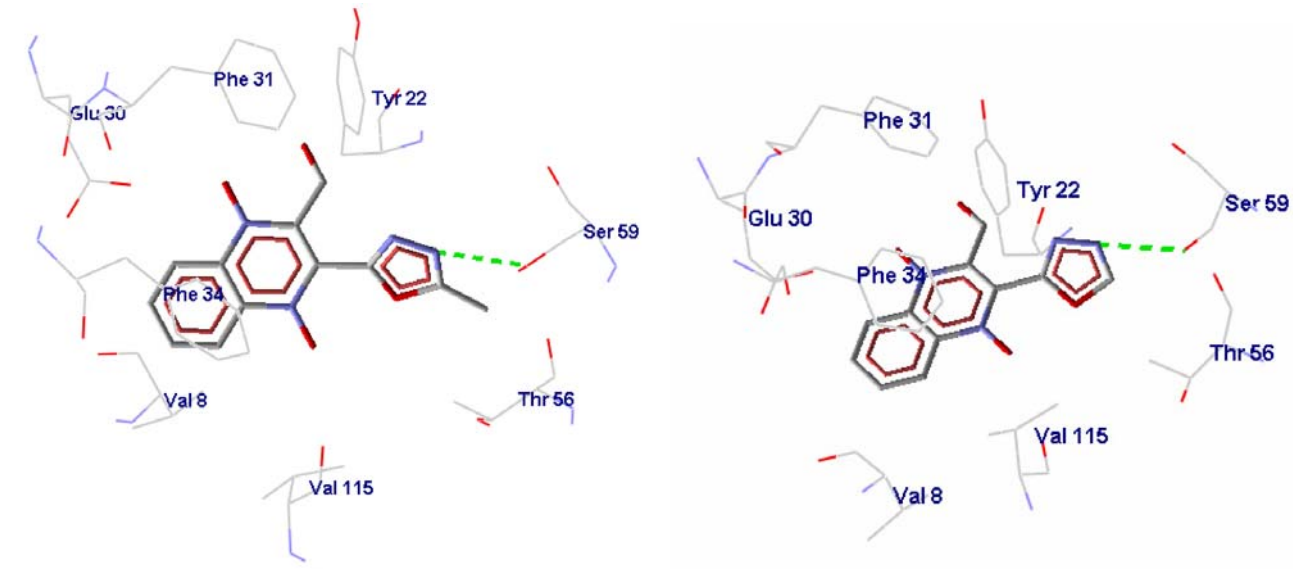


Figure 3. The binding mode and residues involved in the recognition of the moderately active compounds **17** and **18** docked and minimized in the hDHFR binding pocket

21 is not the 1,3,4-oxadiazolylquinoxaline derivatives and appeared inactive toward *Escherichia coli* (Table 1). The applied pyrazole derivatives **24** and **25** have a moderate efficiency toward these bacteria in comparison with the applied 1,3,4-oxadiazole derivatives.

4. Conclusions

It could be concluded that a modified synthesis of 1,3,4-oxadiazole ring was established in a one-pot synthesis instead of two separated steps. The acid hydrazide was treated either with an excess of acetyl chloride, acetic anhydride or carboxylic acid with *phosphorus oxychloride* to give the 1,3,4-oxadiazole ring in a one-pot synthesis. The overall outcome of this molecular modeling study revealed that:

1. Quinoxaline ring is an essential backbone for hDHFR inhibition as it carries the recognition feature with the key amino acid residues in the enzyme pockets, namely Glu30 and Val115.
2. The presence of electrophilic substituents at C2 of the oxadiazole ring is essential for improving recognition at the binding pocket.
3. The carbon spacer linked to the quinoxaline ring should not exceed 4–5 carbon atoms to keep the chance of substitution to be accommodated within the enzyme binding pocket without steric hindrance.
4. The compound containing 2-thiol group (i.e. **20**) is more efficient in recognition of the surrounding amino acids than the 2-methyl counterpart (i.e. **17**), which diminishes the recognition with the active site residues.
5. Additionally, the presence of phenylthiocarboxamide group (in **21**) impairs the interaction with the binding pocket.

5. Acknowledgement

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Povzetek

S kondenzacijo kislinskega hidrazida kinoksalin 1,4-dioksida **6** z acetil kloridom smo z »one-pot« sintezo pripravili 1,3,4-oksadiazolilkinoksalinski obročni sistem. Pri tem postopku smo hidrazid refluktirali s prebitnim acetil kloridom, acetanhidridom ali ocetno kislino v prisotnosti fosforjevega oksiklorida pri čemer so nastali 1,3,4-oksadiazolilkinoksalinski obročni sistemi. Izvedli smo tudi študije molekularnega modeliranja, s katerim smo želeli določiti sposobnost prepoznavanja hDHFR vezavnega mesta kot možnost za inhibicijo hDHFR. Antibakterijske lastnosti pripravljenih spojin so kazale zmerno učinkovitost proti gram negativnim bakterijam, kakršna je npr. *Escherichia coli*. Spojina **20** se je izkazala tudi pri ustreznih študijah komplementarnosti; ohranjeno zaporednje aminokislin v encimu hDHFR namreč dobro posnema vzorec vezave na MTX. Poleg tega je spojina **20**, v primerjavi z znanim bakteriostatikom kloramfenikolom, pokazala najvišjo antibakterijsko aktivnost proti *E. coli*. Tudi pirazolilni analogi so kazali zmerno aktivnost proti proučevanim bakterijam.