ejpmr, 2018,5(1), 243-247

SJIF Impact Factor 4.161

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

STUDY OF NOVEL SUBSTITUTED 1, 2, 4-TRIAZOLES AS POTENT ANTI-TUBERCULAR AGENTS

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Article Received on 26/10/2017 **Article Revised on 16/11/2017** **Article Accepted on 07/12/2017**

ABSTRACT

A series of Mannich based substituted 1,2,4-triazoles were synthesized by condensation of 1,5 diphenyl 1,2-dihydro -3*H*- 1,2,4-triazoles-3-thione with different primary amines. The structures of the synthesized compounds were characterized by FT-IR, ^{1}H-NMR, ^{13}C-NMR and Mass spectral analysis. The synthesized compounds were docked against the target enzyme Pantothenate synthetase using Autodock 1.5.4 version to predict the interaction between the atoms of the synthesized compounds and the amino acid residues of the target enzyme. The compound 3c, 3d, 3e, 3f and 3g showed a significant binding affinity against the target enzyme and these compounds were evaluated for their Anti-tubercular activity using LRP (Luciferase Reporter Phage) Method. All the tested compounds showed 97-98% of inhibition at the concentration of 500 µg/ml.

KEYWORDS: Triazoles, Pantothenate synthetase, Anti-tubercular, Luciferace Reporter Phage.

INTRODUCTION

Nearly one third of the human population is infected with Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis (TB).[1] Despite the existence of approved drug regimens against TB, it continues to claim approximately 1.4 million lives every year.[1] In addition, drug resistant MTB, including multi-drug resistant tuberculosis (MDR-TB), has been found in all regions of the world.[2] Treatment for MDR-TB often requires the use of special drugs. And the emergence of increasingly drug resistant strains has made the need for improved therapies more urgent. Pantothenate synthetase enzyme involved in the pantothenate biosynthetic pathway is essential for the virulence and persistent growth of Mycobacterium tuberculosis. It is encoded by the panC gene, and has become an appropriate target for developing new therapeutics for tuberculosis. It is essential for the *in vitro* growth of M.tuberculosis. Pantothenate (vitamin B_5) is the essential precursor to coenzyme A and acyl carrier proteins. The de novo biosynthetic pathway to pantothenate is present in many bacteria, fungi and plants.[3] Bioinformatics analyses have identified this pathway as a potential target for antimicrobial agents.[4] The absence of each enzyme in mammals further suggests that inhibitors could be selective with a reduced risk of side effects.

Nitrogen containing heterocyclic derivatives have received considerable attention due to their significant biological importance. Various 1,2,4 -triazole derivatives have been reported to posses diversified biological properties such as Ribavirin (Anti-viral), Alprazolam (Anti-psychotic), Anastrazole (breast cancer), fluconazole (Anti-fungal). A detailed literature survey revealed that substituted 1,2,4 triazole possess interesting biological activities like anti-bacterial[5], anti-fungal,[6] anti-tubercular,[7-8] anti-cancer,[9] anti-convulsant[10] anti-viral,[11] etc. Hence, the present study deals with the synthesis and molecular docking study of novel substituted 1, 2, 4 -triazole derivatives and screened for their anti-tubercular activity.

EXPERIMENTAL

Materials and Methods

The Melting point were determined using open capillary tube method .The completion of the reaction was checked by thin layer chromatography using the solvent system Chloroform :Methanol(9:1). IR (cm^{-1}) spectra (in KBr pellets) were recorded on a Shimadzu FT-IR spectrophotometer. H^{1} NMR and C^{13}NMR spectra were recorded on Bruker Advance II using TMS as an internal standard. The Mass spectra were recorded on a ESI-MS system.

General procedure for the synthesis of 3-thione -1,5-diphenyl-1,2,4-triazole

To a solution of benzoyl chloride (0.01M) with 25 ml of dry acetone was taken in a 250 ml of volumetric flask, added Ammonium thiocyanate (0.01M) with stirring for 1-2hrs at room temperature. After completion of the

reaction, the formed precipitate was filtered. The filtrate contained benzoyl isothiocyanate **(1)** was treated with phenyl hydrazine (0.01M) and refluxed for 5- 7 hrs, the formed precipitate of 3- thione -1,5-diphenyl-1,2,4-triazole compound (2) was filtered, dried and recrystallized using ethanol.

Synthesis of Mannich base

Formaldehyde (0.01M) was added to a solution of compound **2** in ethanol with continuous stirring for 3-5 hrs at room temperature. The different primary amines(0.01M) was added. After completion of the reaction, the reaction mixture was poured into the crushed ice and the formed precipitate of substituted 1,2,4 triazoles (3a-3h) was filtered, dried and recrystallized using ethanol. (Scheme 1).

Synthesis of 4-{[2,3 diphenyl -5-thioxo-2,5 dihydro-1H-1,2,4 triazole-1yl)methyl]amino}Benzoic acid (3a)

Formaldehyde (0.01M) was added to a solution of compound **2** in ethanol and add 0.01M of Para Amino Benzoic Acid with continuous stirring for 3-5 hrs at room temperature. After completion of the reaction, the reaction mixture was poured into the crushed ice and the formed precipitate of substituted 1,2,4 triazole (3a) was filtered, dried and recrystallized with ethanol. white crystals. FT-IR (KBr); C= C (str) Aromatic – 1506; C=S(Str)- 686; OH (str)- 2338; -COOH -1478.

Synthesis of 4-{[2,3 diphenyl -5-thioxo-2,5 dihydro-1H-1,2,4 triazole-1yl)methyl]amino}Benzene sulfonic acid (3b)

Formaldehyde (0.01M) was added to a solution of compound **2** in ethanol and add 0.01M of Sulpanilic acid with continuous stirring for 3-5 hrs at room temperature. After completion of the reaction, the reaction mixture was poured into the crushed ice and the formed precipitate of substituted 1,2,4 triazole (3b) was filtered, dried and recrystallized with ethanol. white crystals. IR (KBr); C=C (str) Aromatic -1611; =NH – (str) amines-3048; OH –(str)acid- 2731; C=O(str) – 1687.H^1 NMR (400 MHz, $CDCl_3$): 2.0(s,1H, NH), 7.46-8.13 (m, Ar-H),12.9(SO_3H),1.5(s,CH2,2H);^{13}CNMR(100MHz,CDCl 3):76.69,77.01,77.21,124.54,126.32,128.90,129.32,131.4 0,137.63,149.66ESI;*m/z*–439(M+1).

Synthesis of 4-{[2,3 diphenyl -5-thioxo-2,5 dihydro-1H-1,2,4 triazole-1yl)methyl]amino} urea (3c)

Formaldehyde (0.01M) was added to a solution of compound **2** in ethanol and add 0.01M of urea with continuous stirring for 3-5 hrs at room temperature. After completion of the reaction, the reaction mixture was poured into the crushed ice and the formed precipitate of substituted 1,2,4 triazole(3c) was filtered, dried and recrystallized with ethanol. Pale white crystals. FT-IR (KBr); C=O (str) carbonyl – 1666; C-H (str) Aromatic -3047; NH(str) amides- 3360; C=C (str) Aromatic – 1477; C-H (methylene)- 1439.

Synthesis of 4-{[2,3 diphenyl -5-thioxo-2,5 dihydro-1H-1,2,4 triazole-1yl)methyl]amino} urea (3d)

Formaldehyde (0.01M) was added to a solution of compound **2** in ethanol and add 0.01M of Thiourea with continuous stirring for 3-5 hrs at room temperature. After completion of the reaction, the reaction mixture was poured into the crushed ice and the formed precipitate of substituted 1,2,4 triazole (3c) was filtered, dried and recrystallized with ethanol. Pale white crystals. IR (KBr); C=O (str) carbonyl – 1666; C-H (str) Aromatic -3047; NH(str) amides- 3360; C=C (str) Aromatic – 1477; C-H (methylene)- 1439.

Synthesis of 4-{[2,3diphenyl -5-thioxo-2,5 dihydro-1H-1,2,4 triazole-1yl)methyl]amino}Benzene sulfonamide(3e)

Formaldehyde (0.01M) was added to a solution of compound **2** in ethanol and add 0.01M of Sulphanilamide with continuous stirring for 3-5 hrs at room temperature. After completion of the reaction, the reaction mixture was poured into the crushed ice and the formed precipitate of substituted 1,2,4 triazole (3d) was filtered, dried and recrystallized with ethanol. White crystals. IR (KBr); C-H (str) Aromatic -3065; N-H (str) Amines – 3361; C=C (str)Aromatic- 1477; C-H methylene(str)-2897; C- C (str) -1596.

Synthesis of 4-{[2,3 diphenyl -5-thioxo-2,5 dihydro-1H-1,2,4 triazole-1-yl)methyl]amino}acetic acid (3f)

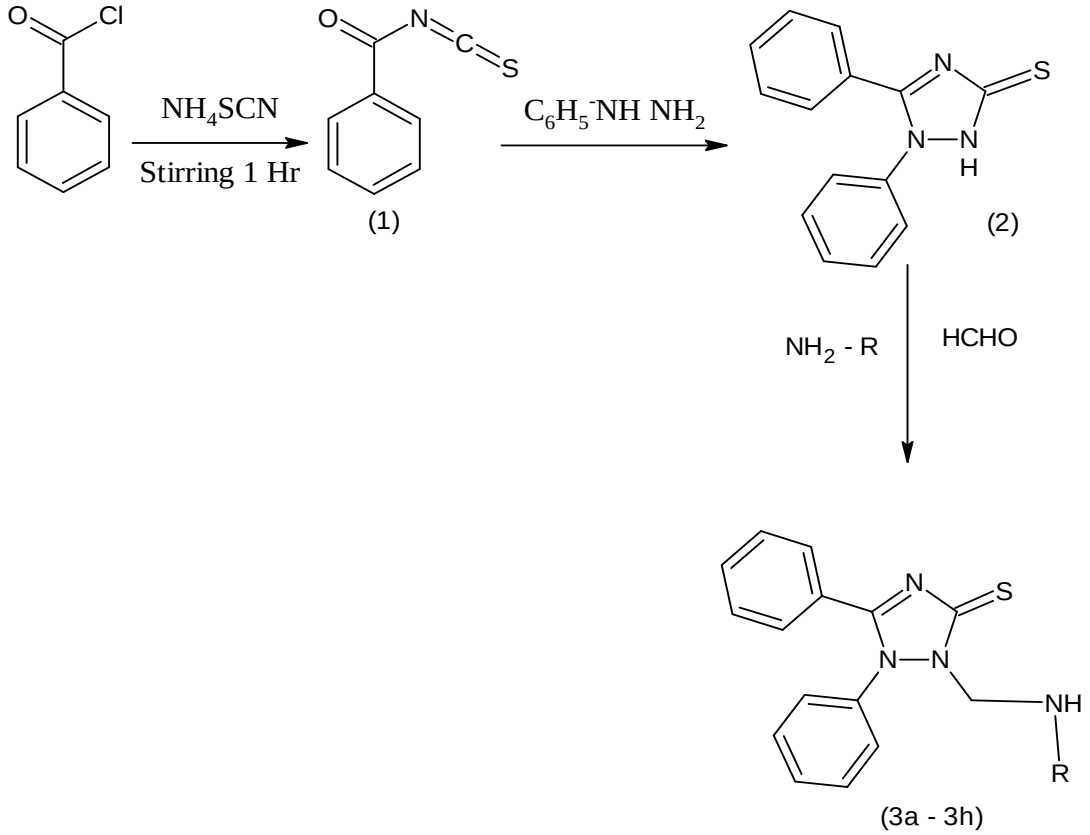
Formaldehyde (0.01M) was added to a solution of compound **2** in ethanol and add 0.01M of Glycine with continuous stirring for 3-5 hrs at room temperature. After completion of the reaction, the reaction mixture was poured into the crushed ice and the formed precipitate of substituted 1,2,4 triazole (3f) was filtered, dried and recrystallized with ethanol. Brownish crystals. IR (KBr); N-H (str) Amines -3310; OH (str) Acid– 2562; C=C (str) Aromatic – 1600; C= S(str)- 664; N-H(str) Aromatic – 3048.

Synthesis of 4-{[2,3diphenyl-5-thioxo-2,5dihydro-1H-1,2,4triazole 1yl)methyl]amino}propanoic acid (3g)

Formaldehyde (0.01M) was added to a solution of compound **2** in ethanol and add 0.01M of Alanine with continuous stirring for 3-5 hrs at room temperature. After completion of the reaction, the reaction mixture was poured into the crushed ice and the formed precipitate of substituted 1,2,4 triazole (3e) was filtered, dried and recrystallized with ethanol. Brown colour crystals. IR (KBr);N-H (str) Amines -3084; OH – (str) Acid – 2564; C=C (str) Aromatic – 1601; C=S (str) – 653;-CH_3(methyl group)-1452.. H^1 NMR (400 MHz$CDCl_3$):1.23(s, CH_3,3H), 7.46-8.13 (m, Ar-H, CH), 2.0(s, NH).NMR (100MHz,CDCl3):77.34,77.02,128.50,129.35,130.22,17 1.93.ESI; *m/z*-354(M+1).

Synthesis of 5-amino-6-{[2,3diphenyl -5-thioxo-2,5 dihydro-1H-1,2,4 triazole-1-yl)methyl] -1,3- dimethyl pyrimidine- 2,4(1H, 3H)- dione (3h)

Formaldehyde (0.01M) was added to a solution of compound **2** in ethanol and add 0.01M of 6-Amino 2,3 dimethyl uracil with continuous stirring for 3-5 hrs at room temperature. After completion of the reaction, the reaction mixture was poured into the crushed ice and the formed precipitate of substituted 1,2,4 triazole (3h) was filtered, dried and recrystallized with ethanol. White crystals. IR (KBr); C-H (str) -3045; -CH_2 (methylene)- 1267; C=C (str) Aromatic- 1650 ; C-N (Aromatic)- 1066.



Scheme 1: Synthesis of substituted 1,2,4 triazoles (3a- 3h).

Table 1: Physicochemical parameters of the synthesized compounds.

S. No.	Compound Code	Molecular Formula	Molecular Weight (Gms)	%Yield	Melting Point (◦C)	Log P	Rf Value
1	3a	$C_{22}H_{17}O_2N_4S$	401	56	198	4.28	0.88
2	3b	$C_{21}H_{18}O_3N_4S_2$	438.53	58	190	1.36	0.92
3	3c	$C_{16}H_{15}O_1N_5S$	325.40	70	200	1.95	0.71
4	3d	$C_{16}H_{15}N_5S_2$	341.46	68	192	2.49	0.72
5	3e	$C_{21}H_{18}O_2N_5S_2$	437.55	58	194	3.07	0.80
6	3f	$C_{17}H_{16}O_2N_4S$	340.41	72	90	1.01	0.83
7	3g	$C_{18}H_{18}O_2N_4S$	354.41	65	98	0.80	0.73
8	3h	$C_{21}H_{20}N_6O_2S$	420.50	75	195	2.02	0.78

Molecular docking study

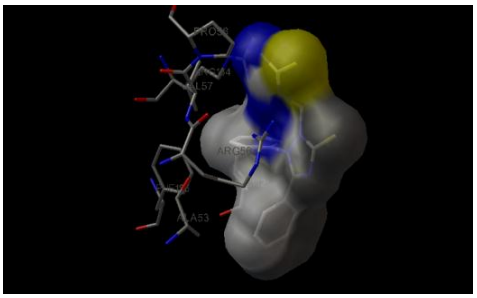
Protein Preparation

The crystallographic structure of Pantothenate Synthetase which were retrieved from the RCSB Protein Data Bank (PDB code 1MOP) serves as docking receptor and all the synthesized compounds are selected as ligand molecules. Before docking the screened ligands in to the protein active site, the protein was prepared by deleting the substrate cofactor as well as the crystallographically observed water molecules and then protein was defined for generating the grid.

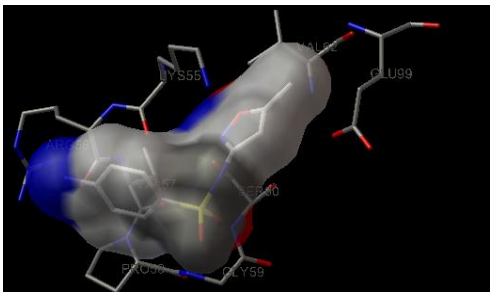
Ligand Preparation

ChemSketch, the chemically intelligent drawing interface freeware (http://www.acdlabs.com/download) was used to draw the structures of triazole derivatives, followed by generation of 3D structure in PDB format using Marvin sketch. Automated docking was used to locate the appropriate binding orientations and conformations of various inhibitors into the 1MOP binding pocket. To perform the task, the powerful genetic algorithm method implemented in the program Auto Dock 4.0.1 was employed. Grid maps were

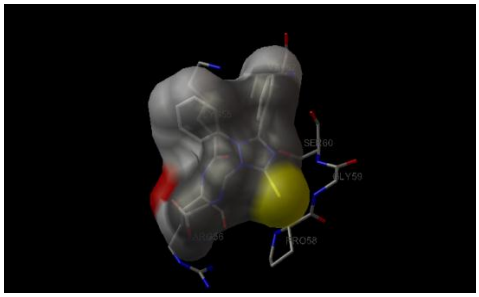
generated by AutoGrid program. Each grid was centered at the crystal structure of the corresponding 1MOP. Lamarckian Genetic Algorithm was employed as the docking algorithm. The grid dimensions were 60 Å X 60 Å X 60 Å with points separated by 0.375 Å. For all ligands, random starting positions, random orientations and torsions were used. During docking, grid parameters were specified for x, y and z axes as 60, 60 and 60 respectively. The Docking parameters Number of Genetic Algorithm (GA) runs: 25, Population size: 150, Maximum number of evaluation: 2,500,000, Maximum number of generation: 27,000 were used for this study. The structure with the lowest binding free energy and the most cluster members was chosen for the optimum docking conformation.



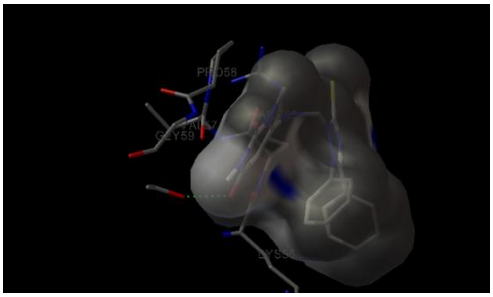
Compound 3b



Compound 3e



Compound 3f



Compound 3h

Figure 1: Binding mode of compounds in the active site of the protein Pantothenate Synthetase.

Table 2: Interactions of the synthesized compounds in the active site of the target protein Pantothenate Synthetase (PDB ID: 1MOP).

Sl. No.	Compound Code	Vdw desolvation energy	Amino acids involved in van der waals interactions	Binding energy (kcal/mol)
1	3a	6.57	Ala53, Phe156, Arg56, Val57, Arg154, Pro 58	-5.92
2	3b	6.9	Lys55, Ser60, Val 62, Glu 99, Arg 56	-6.57
3	3c	6.25	Val 57, Pro58, Ala53, Phe156, Arg154	-7.19
4	3d	7.2	Arg52, Lys55, Ser 60, Pro58, Gly59	-6.9
5	3e	8.16	Lys 55,Val 62, Glu99,Ser60, Pro 56, Gly59	-7.29
6	3f	8.59	Pro 58, Arg 56, Gly 59, Ser 60, Val 62	-7.65
7	3g	9.2	Lys 55, Ser 60, Pro 58, Arg 56	- 7.46
8	3h	10.1	Pro 58, Val 57, Gly59, Lys 55, Arg 56	-7.69

***Invitro* Anti tubercular Activity**

Screening of Anti-tubercular activity by LRP (Luciferase Reporter Phage) method

Luciferase can be used to monitor promoter response activity in bacteria, cultured cells and transgenic plants or animals. The luciferase reporter mycobacterio phage technique has been described as an efficient system to decrease the time required for diagnosis and drug susceptibility testing of M. tuberculosis and other mycobacteria. Compounds are usually screened at a concentration of100µg/ ml concentration against a set of standard strains including M. tuberculosis $H_{37}RV$, SHRE sensitive clinical isolate and SHRE resistant clinical isolate.

Standard strain $H_{37}RV$, a clinical sensitive strain was grown in Middlebrook 7H9 complete medium 12 with and without test compounds for 3 days at 37°C. Luciferase Reporter Phage Assay was done using different concentration of test compounds. DMSO was used as the solvent control. LRP phage AETRC21 was added and the samples were incubated for four hours. Equal volume of the cell phage mixture was mixed with 0.3 Mm D-Luciferin in 0.05 M sodium citrate buffer of pH 4.5 and light output was immediately measured as RLU (Relative light units) in the luminometer at 10 seconds integration. Compounds exhibiting a reduction of 50% or more in RLU in the test vials compared to that

of the control were considered to have anti-mycobacterial activity.

Luciferase Reporter phage (LRP) Assay

Arrange 4 cryovials per set (one for control, another one for solvent control, third vial for sample concentration of 500µg/ml, last one for sample concentration of 100µg/ml). Transfer 400 µl of G7H9 broth in to the first vials and 350 µl in the other 3 vials. Add 50 µl of 10% sterile DMSO in the second vial (solvent control). Add 50 µl of stock 1 to the 3rd vial (500µg/ml). Add 50 µl of stock 2 to the 4th vial (100 µg/ml). Add 100 µl of *M. tuberculosis* H37Rv cell suspension all vials. Prepare similar sets for the clinical isolates (SHRE sensitive and SHRE resistant strains). Incubate all the vials at 37^0C for 72 hours. After incubation, add 50 µl of phage phAETRC21 and 40 µl of 0.1M $CaCl_2$ into all the vials. Incubate all the vials at 37^0C for 4 hours.

Transfer 100 µl of cell-phage mixture to a luminometer cuvette and add 100 µl of D-luciferin in sodium citrate buffer. Read immediately in the luminometer at 10S integration. Calculate the percentage of reduction in relative light units (RLU) of the test compared to control by using the following formula:

$$\% \text{ RLU Reduction} = \frac{\text{Control RLU} - \text{Test RLU}}{\text{Control RLU}} X\ 100$$

Table 3: Anti-tubercular activity of the synthesized compound by LRP method.

Sl. No.	Compound code	RLU Value		% of Inhibition	
		100 µg/ml	500 µg/ml	100 µg/ml	500 µg/ml
1	3c	1821	168	84.7%	98.59%
2	3d	1671	317	86.02%	97%
3	3e	191	162	98.4.%	98.6%
4	3f	183	179	98.4%	98.5%
5	3g	175	182	98%	98.1%
6	3h	3322	205	72.2%	98.2%

RESULTS AND DISCUSSION

In the present work, novel substituted 1, 2, 4-triazole derivatives have been synthesized. The structure of the synthesized compounds was characterized using FT-IR, ^{1}H-NMR, ^{13}C-NMR and Mass spectral analysis. The synthesized compounds were docked against the target enzyme Pantothenate synthetase using Autodock 1.5.4 version to predict the interaction between the atoms of the synthesized compounds and the amino acid residues of the target enzyme. Based on the binding energy, the compound 3c, 3d, 3e, 3f, 3g and 3h were evaluated for their *in vitro* anti-tubercular activity by LRP method using H_{37}RV strains of M. tuberculosis. All the tested compounds showed significant percentage of inhibition at concentration of 100 and 500 µg/ml (97-98.6 %).

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