CHAPTER- II

*Synthesis, Characterisation, Anti-microbial and Anti-angiogenic Activity of 3-(2-Chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one Derivatives*
2.0 Materials and Methods

2.1. Introduction

A brief description of purification of solvents, the analytical procedure followed for the different physico-chemical techniques for the characterization of the synthesized compounds and cell culture experiments are presented here.

2.1.1. Purification of organic solvents

The organic solvents such as Methanol, Chloroform, Dichloromethane, N,N-dimethyl formamide, Hexane, Toluene, Isopropyl alcohol, Acetonitrile, Ethyl acetate, Acetone, Dimethyl sulphoxide etc were purchased from various companies such as Merck, Ranbaxy, Qualigens, Sigma, Rankem and S. D. fine. Distilled water- double distilled water, were obtained by quartz distillation unit.

2.1.2. Reagents

Different amines, sulphonyl chlorides, benzoyl chlorides, isothiocyanates, aliphatic amides, aromatic amides, potassium carbonate, sodium sulphate, sodium carbonate, sodium chloride, sodium cyanoborohydride, phosphorus oxychloride etc., were obtained from standard commercial sources.

2.1.3. Analytical techniques

2.1.3.1. Thinlayer chromatography (TLC)

For TLC, Analtech silica gel GF 254 performed with 0.2 mm silica gel (E-merck, reagent No.017) with fluorescent indication and Merck made TLC plates. The following mobile phases were employed for TLC. Hexane: ethylacetate, methanol: ethylacetate in different ratio.

2.1.3.2. Column chromatography

For column chromatography silica gel (Merck Grade 60-120 mesh) was used.
2.1.3.3. Determination of melting point

Melting points were determined using SELACO-650 and Veego VMP- III model hot stage melting point apparatus and were uncorrected.

2.1.4. Instrumentation

The instrumental techniques employed for the characterization of the synthesized compounds include Infrared, $^1$H NMR and elemental analysis. The details of instrumentation are briefly given below.

2.1.4.1. pH measurements

The pH of the solutions was measured using digital pH meter, model APX 175 (Control Dynamics Instrumentation Pvt Ltd). The pH meter was standardized using buffer tablets of 9.2, 7.0 and 4.0 at 25°C.

2.1.4.2. Infrared Spectra

The Infrared spectra on KBr Pellets in the range of 4000-400 cm$^{-1}$ were recorded on Shimadzu 8300 and Jasco FT-IR 4100 series. FTIR Fourier transform spectrophotometer provided with KBr optics. The observed infrared bands were calibrated with standard frequencies of polystyrene.

2.1.4.3. $^1$H NMR Spectra

$^1$H NMR (400 MHz) spectra was recorded on CDCl$_3$, D$_2$O, DMSO solution in a 5mm tube on a BRUKER amx 400 Fourier transform spectrophotometer (at SIF, Indian Institute of Science, Bangalore, India) with tetramethylsilane (TMS) as internal standard. Chemical shifts were recorded in ppm relative to TMS.

2.1.4.4. Elemental analysis

Elemental analysis was carried out on Elemental Vario EL III. Oxygen was used for combustion and Helium as the mobile phase. The combustion chamber temperature was set at 1150°C and the reduction chamber temperature was at 850°C. Deleten used for
thermal conductance and the liberated SO$_2$ was detected at 140°C. The CO$_2$, N$_2$ and H$_2$O were detected at room temperature.

2.2. General procedure for the synthesis of 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 6(a-s)

A creamy white solid of 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin -4-one (5) (1.0 eq) in $N,N$-dimethyl formamide was taken, potassium carbonate (3.0 eq) was added to the reaction mixture and then the appropriate aliphatic/aromatic/heterocyclic amines (1.0 eq) were added and the reaction mixture was heated at 80°C for 8h. Progress of the reaction was monitored by TLC. Upon completion, the solvent was removed by water wash and extracted with ethyl acetate. The organic layer was washed with 10% ammonium chloride solution and finally water wash was given to organic layer and dried with anhydrous sodium sulphate. The solvent was evaporated to get crude product which was purified by column chromatography over silica gel (60-120 mesh) using hexane: ethyl acetate (8:2) as an eluent.

2.2.1. Synthesis of 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 6(a-s)

A series of novel 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 6(a-s) were prepared by the method summarized in Scheme 1. 2-Aminopyridine (1) and 3-acetyldihydrofuran-2(3H)-one (2) were refluxed in toluene in presence of catalytic amount of PTSA using Dean-Stark apparatus to give 3-(1-(pyridin-2-ylamino)ethylidene)dihydrofuran-2(3H)-one (3) in 80-85% yield. The structure of compound 3 was characterized by spectral and analytical methods. The compound 3 was cyclized to 3-(2-chloroethyl)-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one (4) having 3-chloroethyl side chain by refluxing in POCl$_3$. The cyclized compound 4 was characterized by IR, $^1$H-NMR and elemental analysis. Compound 4 underwent hydrogenation in presence of Pd/C under $H_2$
atmosphere in methanol and yielded 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4\textit{H}-pyrido[1,2-a]pyrimidin-4-one (5). The nucleophilic substitution reaction of 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4\textit{H}-pyrido[1,2-a]pyrimidin-4-one (5) with different aliphatic/aromatic/heterocyclic amines were carried out in the presence of potassium carbonate and N,N-dimethyl formamide as solvent at 80 °C with good yield ranging from 75-85% with good purity. Synthesized molecules 6(a-s) were characterised by IR, \textsuperscript{1}H NMR and elemental analysis. The products obtained were purified by column chromatography using hexane:ethylacetate (8:2) as eluent. The chemical structure and yield of the synthesized compounds are given in Table 1.
Reagents and reaction conditions: (i) Toluene, PTSA, 12 hr. (ii) POCl₃, 1 hr. (iii) H₂, Pd/C, MeOH, 24 hr. (iv) K₂CO₃, substituted aromatic/heterocyclic amines, DMF, 80 °C, 8hr.

2.2.2. Synthesis of 3-(1-(pyridin-2-ylamino)ethylidene)dihydropyran-2(3H)-one (3)

The mixture of 2-aminopyridine 1 (10 mmol) and 3-acetyldihydropyran-2(3H)-one 2 (10 mmol, 1.28 g, or 1.10 mL) was refluxed in toluene (30 mL) for 12 h in presence of catalytic amount of PTSA (0.02 g). The water separator was attached between the reaction flask and water condenser. Separation of equivalent amount of water indicates the completion of reaction. The solid obtained after cooling the reaction mixture was filtered and washed with toluene and then recrystallized in ethyl acetate. Yield 1.69 g (83%).

M. P: 87-88 °C (colorless flakes); ¹H NMR (CDCl₃), δ: 10.5 (bs, 1H, NH), 8.75 (d, 1H, -CH), 8.25 (dd, 1H, -CH), 6.83 (d, 1H, -CH), 6.75 (dd, 1H, -CH), 4.31 (t, 2H, -OCH₂),
2.92 (t, 2H, -CH$_2$), 2.50 (s, 3H, -CH$_3$). IR (KBr, cm$^{-1}$): 3200, 3050, 1690, 1640, 1600, 1570, 1500, 1480, 1410.

2.2.3. Synthesis of 3-(2-chloroethyl)-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one (4)

Compound 3 (10 mmol) was refluxed in phosphorus oxychloride (20 mL) for 1 h (TLC check). After completing the reaction, the excess phosphorus oxychloride was removed under reduced pressure. The residue obtained was stirred in ice-cold water (100 mL) for 30 min, then neutralized with solid sodium carbonate and further stirred overnight. The precipitated solid was filtered and washed with water and recrystallized using suitable solvent. Yield 1.60 g (72%), M. P: 140-142 °C (colorless needles). $^1$H NMR (CDCl$_3$), $\delta$: 9.07 (d, 1H, -CH), 7.78 (t, 1H, -CH), 7.69 (d, 1H, -CH), 7.15 (t, 1H, -CH), 3.88 (t, 2H, CH$_2$Cl), 3.23 (t, 2H, CH$_2$), 2.55 (s, 3H, CH$_3$). IR (KBr, cm$^{-1}$): 2950, 1670, 1640, 1580, 1540, 1480, 1430.

2.2.4. Synthesis of 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5)

Compound 3-(2-chloroethyl)-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one (4) (5 g, 22.45 mmol) was taken in methanol, added 0.5 g of 10 % Pd/C and maintained 3-4 kg pressure of H$_2$ for 24 hr. The reaction was monitored by TLC. After completion, the reaction mixture was filtered through celite bed and filtrate was concentrated under reduced pressure. The residue was dissolved in water to get a white precipitate, which was filtered, washed with water and dried to get creamy white solid. Yield: 4.6 g, (91.45 %). M. P: 173-175 °C. $^1$H NMR(CDCl$_3$), $\delta$: 7.55 (s, 1H, Ar-H), 7.12 (d, 1H, $J = 8.4$ Hz, Ar-H), 4.92 (dd, 1H, $J = 12.0$, 4.0 Hz, -CH), 4.58 (d, 2H, $J = 6.0$ Hz, -CH$_2$), 3.89 (s, 3H, -OCH$_3$). MS (ESI) m/z: 294.2. IR (KBr, cm$^{-1}$): 2955, 2853, 1635, 1463, 1377, 722.
Table 1. Chemical structures and yield of the synthesized compounds 6(a-s)

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2.2.4.1. Synthesis of 2-methyl-3-(2-(thiazol-2-yl-amino)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6a)

The product obtained was pink oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), thiazol-2-amine (0.07 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.41 (d, 1H, Ar-H), 6.87 (d, 1H, Ar-H), 5.11 (s, 1H, -NH), 4.31 (t, 2H, -CH$_2$), 3.32 (m, 2H, -CH$_2$), 3.01 (s, 3H, -CH$_3$), 2.20 (m, 2H, -CH$_2$), 1.91 (m, 2H, -CH$_2$), 1.71 (m, 2H, -CH$_2$), 1.52 (t, 2H, -CH$_2$). IR (KBr, cm$^{-1}$): 2923, 1746, 1656, 1536, 1462, 1346. Anal. Calculated. For C$_{14}$H$_{18}$N$_4$O$_2$ (in %): C-57.91, H-6.25, N-19.29, S-11.04. Found C-57.88, H-6.26, N-19.27, S-11.01.

2.2.4.2. Synthesis of 3-(2-(4-(5-fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6b)

The product obtained was brown oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), 6-fluoro-3-(piperidin-4-yl)benzo[d]isoxazole (0.07 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.85 (t, 1H, Ar-H), 7.51 (m, 1H, Ar-H), 7.28 (m, 1H, Ar-H), 4.28 (t, 2H, -CH$_2$), 3.51 (m, 2H, -CH$_2$), 3.12 (s, 3H, -CH$_3$), 2.78 (s, 1H, -CH), 2.6-1.71(m, 8H, (CH$_2$)$_4$), 2.12 (m, 2H, -CH$_2$), 1.91 (m, 2H, -CH$_2$), 1.71 (m, 2H, -CH$_2$), 1.53 (t, 2H, -CH$_2$). IR (KBr, cm$^{-1}$): 2854, 1735, 1660, 1536, 1460, 1376. Anal. Calculated. For C$_{23}$H$_{27}$FN$_4$O$_2$ (in %): C-67.30, H-6.63, N-13.65. Found C-67.27, H-6.60, N-13.62.
2.2.4.3. Synthesis of 3-(2-(4-(hydroxydiphenylmethyl)piperidin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6c)

The product obtained was orange oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), diphenyl(piperazin-1-yl)methanol (0.19 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.51 (m, 4H, Ar-H), 7.43 (m, 6H, Ar-H), 4.2 (t, 2H, -CH$_2$), 3.71 (s, 1H, -OH), 3.51 (m, 2H, -CH$_2$), 3.05 (s, 3H, -CH$_3$), 2.61 (m, 4H, (CH$_2$)$_2$), 2.38 (s, 1H, -CH), 2.05 (m, 2H, -CH$_2$), 1.85 (m, 2H, -CH$_2$), 1.71 (m, 4H, (CH$_2$)$_2$), 1.65 (m, 2H, -CH$_2$), 1.55 (t, 2H, -CH$_2$). IR (KBr, cm$^{-1}$): 2853, 2500, 1734, 1668, 1538, 1463. Anal. Calculated. For C$_{29}$H$_{35}$N$_3$O$_2$ (in %): C-76.12, H-7.71, N-9.18. Found C-76.10, H-7.74, N-9.20.

2.2.4.4. Synthesis of 3-(2-(furan-2-ylmethylamino)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6d)

The product obtained was pink oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), furan-2-yl-methanamine (0.07 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.91 (d, 1H, Ar-H), 7.02 (t, 1H, Ar-H), 6.75 (t, 1H, Ar-H), 4.31 (m, 2H, -CH$_2$), 3.25 (m, 2H, -CH$_2$), 3.01 (s, 3H, -CH$_3$), 2.71 (s, 3H, -CH$_3$), 2.25 (m, 2H, -CH$_2$), 1.91 (m, 2H, -CH$_2$), 1.71 (m, 2H, -CH$_2$), 1.55 (t, 2H, -CH$_2$). IR (KBr, cm$^{-1}$): 2514, 1730, 1656, 1536, 1462, 1340. Anal. Calculated. For C$_{16}$H$_{21}$N$_3$O$_2$ (in %): C-66.88, H-7.37, N-14.62. Found C-66.91, H-7.40, N-14.64.
2.2.4.5. Synthesis of 2-methyl-3-(2-((tetrahydrofuran-2-yl)methylamino)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6e)

The product obtained was pink oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), (tetrahydrofuran-2-yl) methanamine (0.07 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 5.05 (s, 1H, NH), 4.30 (m, 2H, -CH$_2$), 4.01 (s, 2H, -CH$_2$), 3.98 (t, 1H, -CH), 3.01 (s, 3H, -CH$_3$), 2.85 (m, 2H, -CH$_2$), 2.65 (m, 2H, -CH$_2$), 2.09 (m, 2H, -CH$_2$), 1.92 (m, 4H, (CH$_2$)$_2$), 1.88 (m, 2H, -CH$_2$), 1.71 (m, 2H, -CH$_2$), 1.55 (t, 2H, -CH$_2$). IR (KBr, cm$^{-1}$): 2924, 1735, 1656, 1536, 1460, 1340. Anal. Calculated. For C$_{16}$H$_{25}$N$_3$O$_2$ in (%): C-65.95, H-8.65, N-14.42. Found C-65.92, H-8.62, N-14.45.

2.2.4.6. Synthesis of 3-(2-(4-(2-fluorophenyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6f)

The product obtained was orange oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), 1-(2-fluorophenyl) piperazine (0.13 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.21 (m, 1H, Ar-H), 7.05 (d, 1H, Ar-H), 6.78 (m, 1H, Ar-H), 6.65 (m, 1H, Ar-H), 4.31 (m, 2H, -CH$_2$), 3.50 (m, 8H, (CH$_2$)$_4$), 3.42 (m, 2H, -CH$_2$), 3.05 (s, 3H, -CH$_3$), 2.09 (m, 2H, -CH$_2$), 1.86 (m, 2H, -CH$_2$), 1.65 (m, 2H, -CH$_2$), 1.53 (t, 2H, -CH$_2$). IR (KBr, cm$^{-1}$): 2956, 1732, 1665, 1580, 1462, 1376. Anal. Calculated. For C$_{21}$H$_{27}$FN$_4$O (in %): C-68.08, H-7.35, N-15.12. Found C-68.11, H-7.39, N-15.10.
2.2.4.7. Synthesis of 3-(2-(benzyl(methyl)amino)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6g)

The product obtained was black oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), N-methyl-1-phenylmethanamine (0.08 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol).

$^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.31 (m, 2H, Ar-H), 7.28 (d, 1H, Ar-H), 7.20 (m, 2H, Ar-H), 4.25 (m, 2H, -CH$_2$), 3.75 (s, 2H, -CH$_2$), 3.45 (m, 2H, -CH$_2$), 3.01 (s, 3H, -CH$_3$), 2.3 (s, 3H, -CH$_3$), 2.12 (m, 2H, -CH$_2$), 1.87 (m, 2H, -CH$_2$), 1.75 (m, 2H, -CH$_2$), 1.55 (t, 2H, -CH$_2$). IR (KBr, cm$^{-1}$): 2853, 1736, 1612, 1548, 1483, 1380. Anal. Calculated. For C$_{19}$H$_{25}$N$_3$O in (%): C-73.28, H-8.09, N-13.49. Found C-73.31, H-8.11, N-13.51.

2.2.4.8. Synthesis of 3-(2-(cyclopentylamino)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6h)

The product obtained was black oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), cyclopentanamine (0.06 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 5.11 (s, 1H, -NH), 4.28 (m, 2H, -CH$_2$), 3.01 (m, 2H, -CH$_2$), 2.98 (s, 3H, -CH$_3$), 2.71 (m, 1H, -CH), 2.05 (m, 2H, -CH$_2$), 1.87 (m, 2H, -CH$_2$), 1.75 (m, 4H, (CH$_2$)$_2$), 1.67 (m, 2H, -CH$_2$), 1.60 (m, 4H, (CH$_2$)$_2$), 1.52 (t, 2H, -CH$_2$). IR (KBr, cm$^{-1}$): 2854, 1787, 1632, 1598, 1462, 1340. Anal. Calculated. For C$_{16}$H$_{25}$N$_3$O (in %): C-69.78, H-9.15, N-15.26. Found C-69.81, H-9.18, N-15.30.

2.2.4.9. Synthesis of 3-(2-(4-(2-aminoethyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6i)

The product obtained was colourless oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), 2-(piperazin-1-yl)ethanamine (0.1 g, 0.77 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz)
δ: 5.21 (s, 2H, NH\textsubscript{2}), 4.31 (m, 2H, -CH\textsubscript{2}), 3.51 (m, 2H, -CH\textsubscript{2}), 3.01 (s, 3H, -CH\textsubscript{3}), 2.65 (m, 2H, -CH\textsubscript{2}), 2.58 (m, 2H, -CH\textsubscript{2}), 2.41 (m, 2H, -CH\textsubscript{2}), 2.12 (m, 2H, -CH\textsubscript{2}), 1.89 (m, 2H, -CH\textsubscript{2}), 1.72 (m, 2H, -CH\textsubscript{2}), 1.52 (t, 2H, -CH\textsubscript{2}). IR (KBr, cm\textsuperscript{-1}): 3200, 2854, 1746, 1600, 1536, 1460, 1340. Anal. Calculated. For C\textsubscript{17}H\textsubscript{29}N\textsubscript{5}O (in %): C-63.92, H-9.15, N-21.92. Found C-63.94, H-9.18, N-21.94.

2.2.4.10. Synthesis of 3-(2-(4-fluorobenzylamino)ethyl)-2-methyl-6,7,8,9-tetrahydro-4\textsubscript{H}-pyrido[1,2-a]pyrimidin-4-one (6j)

The product obtained was pink oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4\textsubscript{H}-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), (4-fluorophenyl)methanamine (0.1 g, 0.727 mmol) and K\textsubscript{2}CO\textsubscript{3} (0.27 g, 1.98 mmol).\textsuperscript{1}H NMR (DMSO-\textsubscript{d}\textsubscript{6}, 400 MHz) δ: 7.41 (m, 2H, Ar-H), 7.15 (m, 2H, Ar-H), 5.12 (s, 1H, -NH), 4.30 (m, 2H, -CH\textsubscript{2}), 3.81 (m, 2H, -CH\textsubscript{2}), 3.01 (s, 3H, -CH\textsubscript{3}), 2.65 (m, 2H, -CH\textsubscript{2}), 2.05 (m, 2H, -CH\textsubscript{2}), 1.90 (m, 2H, -CH\textsubscript{2}), 1.72 (m, 2H, -CH\textsubscript{2}), 1.51 (t, 2H, -CH\textsubscript{2}). IR (KBr, cm\textsuperscript{-1}): 2921, 1740, 1670, 1536, 1458, 1400, 1346. Anal. Calculated. For C\textsubscript{18}H\textsubscript{22}FN\textsubscript{3}O (in %): C-68.55, H-7.03, N-13.32. Found C-68.58, H-7.01, N-13.30.

2.2.4.11. Synthesis of 3-(2-((4-aminophenyl)(phenyl)amino)ethyl)-2-methyl-6,7,8,9-tetrahydro-4\textsubscript{H}-pyrido[1,2-a]pyrimidin-4-one (6k)

The product obtained was black oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4\textsubscript{H}-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), N\textsuperscript{1}-phenylbenzene-1,4-diamine (0.13 g, 0.727 mmol) and K\textsubscript{2}CO\textsubscript{3} (0.27 g, 1.98 mmol).\textsuperscript{1}H NMR (DMSO-\textsubscript{d}\textsubscript{6}, 400 MHz) δ: 7.41 (m, 2H, Ar-H), 7.29 (m, 2H, Ar-H), 6.81 (t, 1H, Ar-H), 6.48 (m, 2H, Ar-H), 6.39 (m, 2H, Ar-H), 6.21 (s, 2H, NH\textsubscript{2}), 4.30 (m, 2H, -CH\textsubscript{2}), 3.21 (m, 2H, -CH\textsubscript{2}), 3.05 (s, 3H, -CH\textsubscript{3}), 2.21 (m, 2H, -CH\textsubscript{2}), 1.89 (m, 2H, -CH\textsubscript{2}), 1.69 (m, 2H, -CH\textsubscript{2}), 1.50 (t, 2H, -CH\textsubscript{2}). IR (KBr, cm\textsuperscript{-1}): 2920, 1730, 1668, 1548, 1530, 1465, 1340. Anal.
Calculated. For $\text{C}_{23}\text{H}_{26}\text{N}_{4}\text{O}$ (in %): C-73.77, H-7.05, N-14.96. Found C-73.80, H-7.02, N-14.98.

2.2.4.12. Synthesis of 3-(2-(benzyl(2-hydroxyethyl)amino)ethyl)-2-methyl-6,7,8,9-tetrahydro-4$H$-pyrido[1,2-a]pyrimidin-4-one (6l)

The product obtained was orange oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4$H$-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), 2-(benzylamino)ethanol (0.11 g, 0.727 mmol) and $\text{K}_2\text{CO}_3$ (0.27 g, 1.98 mmol). $^1\text{H}$ NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.35 (m, 2H, Ar- H), 7.29 (d, 1H, Ar-H), 7.15 (m, 2H, Ar-H), 4.29 (m, 2H, -CH$_2$), 3.71 (s, 1H, -OH), 3.65 (m, 2H, -CH$_2$), 3.51 (m, 2H, -CH$_2$), 3.45 (m, 2H, -CH$_2$), 3.05 (s, 3H, -CH$_3$), 2.58 (m, 2H, -CH$_2$), 2.21 (m, 2H, -CH$_2$), 1.88 (m, 2H, -CH$_2$), 1.65 (t, 2H, -CH$_2$), 1.50 (m, 2H, -CH$_2$). IR (KBr, cm$^{-1}$): 2920, 1745, 1654, 1536, 1460, 1350. Anal. Calculated. For $\text{C}_{20}\text{H}_{27}\text{N}_{3}\text{O}_2$ (in %): C-70.35, H-7.97, N-12.31. Found C-70.31, H-7.99, N-12.30.

2.2.4.13. Synthesis of 3-(2-(hexylamino)ethyl)-2-methyl-6,7,8,9-tetrahydro-4$H$-pyrido[1,2-a]pyrimidin-4-one (6m)

The product obtained was black oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4$H$-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), hexan-1-amine (0.07 g, 0.727 mmol) and $\text{K}_2\text{CO}_3$ (0.27 g, 1.98 mmol). $^1\text{H}$ NMR (DMSO-$d_6$, 400 MHz) $\delta$: 5.12 (s, 1H, -NH), 4.28 (m, 2H, -CH$_2$), 3.01 (s, 3H, -CH$_3$), 2.68 (m, 2H, -CH$_2$), 2.52 (m, 2H, -CH$_2$), 2.10 (m, 2H, -CH$_2$), 1.90 (m, 2H, -CH$_2$), 1.68 (m, 2H, -CH$_2$), 1.50 (t, 2H, -CH$_2$), 1.38-1.31 (m, 8H, (CH$_2$)$_4$), 0.88 (t, 3H, -CH$_3$). IR (KBr, cm$^{-1}$): 2916, 1736, 1666, 1537, 1462, 1303. Anal. Calculated. For $\text{C}_{17}\text{H}_{29}\text{N}_{3}\text{O}$ (in %): C-70.06, H-10.03, N-14.42. Found C-70.10, H-10.05, N-14.44.
2.2.4.14. Synthesis of 3-(2-(cyclohexylamino)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6n)

The product obtained was orange oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), cyclohexanamine (0.07 g, 0.72 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 5.15 (s, 1H, -NH), 4.35 (m, 2H, -CH$_2$), 3.02 (s, 3H, -CH$_3$), 2.65 (m, 2H, -CH$_2$), 2.57 (m, 1H, -CH), 2.02 (m, 2H, -CH$_2$), 1.87 (m, 2H, -CH$_2$), 1.71 (m, 4H, (CH$_2$)$_2$), 1.65 (m, 2H, -CH$_2$), 1.52 (m, 2H, -CH$_2$), 1.50 (t, 2H, -CH$_2$), 1.11 (m, 4H, (CH$_2$)$_2$). IR (KBr, cm$^{-1}$): 2852, 1740, 1667, 1536, 1462, 1320. Anal. Calculated. For C$_{17}$H$_{27}$N$_3$O (in %): C-70.55, H-9.40, N-14.52. Found C-70.58, H-9.43, N-14.54.

2.2.4.15. Synthesis of 3-(2-(4-benzhydrylpiperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6o)

The product obtained was brown oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), 1-benzhydrylpiperazine (0.19 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$:7.41 (m, 4H, Ar-H), 7.38 (m, 4H, Ar-H), 7.29 (m, 2H, -CH$_2$), 5.14 (s, 1H, -CH), 4.30 (m, 2H, -CH$_2$), 3.53 (m, 2H, -CH$_2$), 3.01 (s, 3H, -CH$_3$), 2.41 (m, 8H, (CH$_2$)$_2$), 2.08 (m, 2H, -CH$_2$), 1.89 (m, 2H, -CH$_2$), 1.69 (m, 2H, -CH$_2$), 1.55 (t, 2H, -CH$_2$). IR (KBr, cm$^{-1}$): 2923, 1746, 1644, 1540, 1462, 1380. Anal. Calculated. For C$_{28}$H$_{34}$NO (in %): C-75.98, H-7.74, N-12.66. Found C-75.94, H-7.72, N-12.64.

2.2.4.16. Synthesis of 1-(2-(2-methyl-4-oxo-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-3-yl)ethyl)-1H-indole-3-carbaldehyde (6p)

The product obtained was brown oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), 1H-indole-3-carbaldehyde (0.15 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$HNMR
(DMSO- $d_6$, 400 MHz) $\delta$: 9.81 (s, 1H, -CHO), 8.57 (d, 1H, Ar-H), 8.31 (s, 1H, Ar-H), 7.61 (m, 1H, Ar-H), 7.45 (m, 2H, Ar-H), 4.31 (m, 2H, -CH$_2$), 3.71 (m, 2H, -CH$_2$), 3.01 (s, 3H, -CH$_3$), 2.52 (m, 2H, -CH$_2$), 1.89 (m, 2H, -CH$_2$), 1.68 (m, 2H, -CH$_2$), 1.50 (t, 2H, -CH$_2$). IR (KBr, cm$^{-1}$): 2924, 1740, 1654, 1598, 1462, 1345. Anal. Calculated. For C$_{20}$H$_{21}$N$_3$O$_2$ (in %): C-71.62, H-6.31, N-12.53. Found C-71.60, H-6.34, N-12.56.

2.2.4.17. Synthesis of 2-methyl-3-(2-(2-methyl-1H-imidazol-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6q)

The product obtained was pink oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), 2-methyl-1H-imidazole (0.06 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.01 (d, 1H, Ar-H), 6.82 (d, 1H, Ar-H), 4.32 (m, 2H, -CH$_2$), 3.82 (m, 2H, -CH$_2$), 3.02 (s, 3H, -CH$_3$), 2.61 (s, 3H, -CH$_3$), 2.52 (m, 2H, -CH$_2$), 1.89 (m, 2H, -CH$_2$), 1.69 (m, 2H, -CH$_2$), 1.50 (t, 2H, -CH$_2$). IR (KBr, cm$^{-1}$): 2973, 1787, 1670, 1533, 1457, 1377. Anal. Calculated. For C$_{15}$H$_{20}$N$_4$O (in %): C-66.15, H-7.40, N-20.57. Found C-66.12, H-7.42, N-20.58.

2.2.4.18. Synthesis of 3-(2-(1-(3,4-diethoxybenzyl)-5,6-diethoxyisoindolin-2-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6r)

The product obtained was brown oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), 1-(3,4-diethoxybenzyl)-5,6-diethoxyisoindoline (0.29 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.01-6.83 (m, 5H, Ar-H), 4.32 (t, 1H, -CH), 4.29 (m, 2H, -CH$_2$), 4.12 (m, 8H, (CH$_2$)$_4$), 3.67 (m, 2H, -CH$_2$), 3.12 (m, 2H, -CH$_2$), 3.03 (s, 3H, -CH$_3$), 2.61 (m, 2H, -CH$_2$), 2.21 (m, 2H, -CH$_2$), 1.89 (m, 2H, -CH$_2$), 1.68 (m, 2H, -CH$_2$), 1.50 (t, 2H, -CH$_2$), 1.32 (m, 12H, (CH$_2$)$_6$). IR (KBr, cm$^{-1}$): 2926, 2856, 1759, 1670, 1566, 1458, 1366. Anal. Calculated. For C$_{34}$H$_{45}$N$_3$O$_5$ (in %): C-70.93, H-7.88, N-7.30. Found C-70.95, H-7.90, N-7.33.
2.2.4.19. Synthesis of 3-(2-(6-ethoxybenzo[d]thiazol-2-ylamino)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (6s)

The product obtained was brown oily from 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5) (0.15 g, 0.661 mmol), 6-ethoxybenzo[d]thiazol-2-amine (0.14 g, 0.727 mmol) and K$_2$CO$_3$ (0.27 g, 1.98 mmol). $^1$H NMR (DMSO-d$_6$, 400 MHz) $\delta$: 7.55 (d, 1H, Ar-H), 7.53 (d, 1H, Ar-H), 6.98 (t, 1H, Ar-H), 5.14 (s, 1H, -NH), 4.23 (m, 2H, -CH$_2$), 4.12 (m, 2H, -CH$_2$), 3.21 (m, 2H, -CH$_2$), 3.05 (s, 3H, -CH$_3$), 2.21 (m, 2H, -CH$_2$), 1.88 (m, 2H, -CH$_2$), 1.65 (m, 2H, -CH$_2$), 1.53 (t, 2H, -CH$_2$), 1.32 (s, 3H, -OCH$_3$). IR (KBr, cm$^{-1}$): 2920, 1746, 1650, 1545, 1486, 1310. Anal. Calculated. For C$_{20}$H$_{24}$N$_4$O$_2$S (in %): C-62.48, H-6.29, N-14.57, S-8.34. Found C-62.50, H-6.31, N-14.55, S-8.3.

2.3. Anti-microbial sactivity of 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 6(a-s)

2.3.1. Introduction to anti-microbial activity

The discovery of antibiotics and other antimicrobial agents in the 1930’s is arguably the most significant therapeutic advance in medical chemistry. Over 50 years ago the golden age of antibiotics dawned with considerable achievements in the discovery and development of the penicillin and streptomycin. This success was followed by the characterization of the tetracyclines, macrolides, glycopeptides, cephalosporins and nalidixic acid [1-3]. Most of these compounds are either derived from natural products or produced by the synthetic modification of natural products. The compounds from this time period have provided the basic scaffold for medicinal chemistry modifications to expand the spectrum and/or potency of improved analogs in subsequent years [1]. In the past 20 years, over 50 antimicrobial drugs have been developed and large pharmaceutical companies have supplied generation after generation of improved antibiotics characterized
by these original classes of drug to meet the existing medical need for novel agents with antibiotic activity [4-10]. However, these numbers are dwarfed by the number of new antibiotics introduced in the preceding 20 years when antibiotics were the mainstay of every large pharmaceutical company.

Most microbes belong to one of four major groups: bacteria, viruses, fungi or protozoa. Microbiologists distinguish two groups of anti-microbial agents used in the treatment of infectious diseases. Antibiotics, which are natural substances, produced by certain groups of microorganisms, and chemotherapeutic agents, which are chemically synthesized. A hybrid substance is a semisynthetic antibiotic, wherein a molecular version produced by the microbe is subsequently modified by the chemist to achieve the desired properties.

Table 2. Targets and classes of anti-bacterial drugs

<table>
<thead>
<tr>
<th>Drug target</th>
<th>Drug classes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall synthesis</td>
<td>β-Lactams, bacitracin, cyclocerine, fosfomycin, and glicopeptides</td>
</tr>
<tr>
<td>Cell membrane integrity</td>
<td>Deptomycin and polymyxins</td>
</tr>
<tr>
<td>Nucleotide biosynthesis</td>
<td>Sulphanamides and trimethoprim</td>
</tr>
<tr>
<td>DNA replication</td>
<td>Quinolines nitrofurans and nitroimidazoles</td>
</tr>
<tr>
<td>RNA synthesis</td>
<td>Rifamycins</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Aminoglycosides, chloramphenicol, fusidic acid, ketolides, macrolides, oxazolidinones, streptogramins, tetracyclines, and mupirocin</td>
</tr>
</tbody>
</table>
2.3.1.2. A clinically useful antimicrobial drug should have the following characteristics

- It should have a wide spectrum of activity with the ability to destroy or inhibit many different species of pathogenic organisms.
- It should be non-toxic to the host and without side effects.
- It should be non-allergic to the host.
- It should destroy the normal flora of the host.
- It should be able to reach the part of the human body where the infection is occurring.
- It should be inexpensive and easy to manufacture in large scale.
- It should be chemically stable (have a long shelf-life).
- Microbial resistance is uncommon and unlikely to develop.

2.3.1.3. Antimicrobial drugs classification based on chemical structure

**Sulphanamides and related drugs:** Sulphadiazine and others, Sulfones-Dapsone (DDS), Paraaminosalicylic acid (PAS)

**Diaminopyrimidenes:** Tremethoprim, Pyrimethamine.

**Quinolones:** Nalidixic acid, Norfloxacin, Ciprofloxacin, Gatifloxacin, e.t.c.

**β-Lactam antibiotics:** Penicillins, Cephalospoins, Monobactims, Carbapenems.

**Tetracyclins:** Oxytetracycline, Doxycycline, e.t.c.

**Nitrobenzene derivatives:** Chloramphenicol.

**Aminoglycosides:** Streptomycin, Gentamycin, Amikacin, Neomycin, e.t.c.

**Macrolide antibiotics:** Erythromycin, Clarithromycin, Azithromycin, e.t.c.

**Linocosamide antibiotics:** Lincomycin, Clindamycin.

**Oxazolidinone:** Linezolid.

**Glycopeptide antibiotics:** Vancomycin, Teicoplanin.

**Polypeptide antibiotics:** Polymyxin-B, Colistin, Bacitracin, Tyrothricin.
Nitrofuran derivatives: Nitrofurantoin, Furazolidone.

Nitroimidazoles: Metronidazole, Tinidazole, e.t.c.

Nicotinic acid derivatives: Isoniazid, Pyrazinamide, Ethionamide.

Polyene antibiotics: Nystatin, Amphotericin-b, Hamycin.

Azole derivatives: Miconazole, Clotrimazole, Ketoconazole, Fluconazole.

Others: Rifampin, Spectinomycin, Cycloserine, Viomycin, Ethambutol,
Thiacetazone, Clofazime.

2.3.1.4. Some example of antimicrobial agents

- **Amikacin**: An amino glycoside antimicrobial agent is active against a wide range of Gram-positive organisms including strains to other antimicrobial. Susceptible pathogens include pseudomonas, *E-coli*, *proteus* and *staphylococcus*.

- **Ampicillin**: Amino penicillin antimicrobial agent exerts bactericidal action on both Gram-positive and Gram-negative organisms. Its spectrum includes *staphylococci* spp. *Peptococcus, B. arthracis, Clostridium tetani* and *C. botulinum*

- **Ciprofloxacin**: A fluoroquinolone antimicrobial agent, bactericidal by inhibition of DNA supercoiling in the bacteria. Its spectrum includes Gram-negative aerobic bacteria viz. *Enterobacteriaceae, Haemophilus, Neiseriae* and *Pseudomonas aeruginosa*.

- **Clotrimazole**: Clotrimazole is an antifungal medication commonly used in the treatment of fungal infections of both humans and animals. CANDID 1% cream, gel, lotion, powder, 100 mg vaginal tab.

- **Fluconazole**: Fluconazole is a triazole antifungal drug used in the treatment and prevention of superficial and systemic fungal infections. TRICAN (Pfizer) 50, 100, 150, 200 mg caps, 200 mg/100 mL i.v. infusion, 0.3% eye drops.
Gentamycin: Gentamycin is an aminoglycoside anti-bacterial agent. This is given by injection. They are bactericidal broad-spectrum antibiotics but with low activity against anaerobes, streptococci and pneumococci.

Ketoconazole: Ketoconazole is a synthetic antifungal drug used to prevent and treat skin and fungal infections. It is similarly to imidazole, lipophilic and interferes with the fungal synthesis of ergosterol, a constituent of cell membranes, as well as certain enzymes. KETOVATE 200 mg tab, cream, lotion, shampoo, etc.

Miconazole: Miconazole is an imidazole antifungal agent, commonly applied to the skin or mucus membranes to cure fungal infections. It works by inhibiting the synthesis of ergosterol, a critical component of fungal cell membranes.

Penicillin: Penicillin constitutes one of the most important groups of antibiotics. These are bactericidal and act by interfering with the synthesis of bacterial peptidoglycan cell wall. Penicillin-G and Penicillin-V have a useful antimicrobial spectrum against streptococci, pneumococci, gonococci, meningococci; also effective in anthrax, diphtheria, gas gangrene, leptospirosis, syphilis, and tetanus.

Streptomycin: An aminoglyciside antibacterial agent is active against a wide range of Gram-negative and some Gram-positive organisms. It is also active against Mycobacterium tuberculosis.

Sodium fusidate: Steroidal antibiotic is mainly active against Gram-positive bacteria staphylococci, colostridal strains and Nocardia asteroids. This acts by inhibiting bacterial protein synthesis.

2.3.1.5. Mechanism of antimicrobial action

An ideal anti-microbial drug exhibits selective inhibition. Selective inhibition usually depends on the inhibition of biochemical processes that exist in or are essential to the parasite but not to the host. For many of the anti-microbial drugs, the mechanism of action
is not completely understood. However, it is convenient to present anti-microbial mechanisms under four distinct headings [11-13].

(i) Inhibition of cell wall synthesis.

(ii) Alteration in the permeability of cell membrane or active transport across cell membrane.

(iii) Inhibition of protein synthesis (i.e. inhibition of translation and transcription of genetic material).

(iv) Inhibition of nucleic acid synthesis.

2.3.1.6. Anti-microbial action through inhibition of cell wall synthesis

In contrast to animal cell, bacteria possess a rigid outer layer, the cell wall that completely surrounds the cytoplasmic cell membrane. The cell wall contains a chemically distinct complex cross-linked polymer, peptidoglycan, consisting of polysaccharides and polypeptides. The polysaccharides regularly contain the amino sugars N-acetylglucosamine and acetylmurmaic acid. The later is found only in bacteria. The final rigidity of the cell wall is imparted by cross linking of the peptide chains (e.g., through pentaglycine bonds) as a result of transpeptidation reactions catalyzed by several enzymes. All pencillins and all cephalosporins (beta-lactam antibiotics) are selective inhibitors of bacterial cell wall synthesis [14, 15]. The initial step in drug action consists of binding of the drug to cell receptors. After a beta-lactam drug has attached to its receptors, the transpeptidation reaction is inhibited and peptidoglycan synthesis is blocked. Inhibition of the transpeptidation enzymes by the pencillins and cephalosporins may be due to a structural similarity of these drugs to acyl-D-alanyl-D-alanine. The transpeptidation reaction involves loss of a D-alanine from the pentapeptide. The remarkable lack of toxicity of beta-lactam antibiotics to mammalian cells must be attributed to the absence of
a bacteria type cell wall, with its peptidoglycan, in animal cells. E.g., Penicillins, Cephalosporins, Cycloserine, Vancomycin, Bacitracin.

2.3.1.7. Anti-microbial action through inhibition of cell membrane function

The cytoplasm of all living cells is bounded by the cytoplasmic membrane, which serves as a selective permeability barrier, performs active transport functions, and thus controls the internal composition of the cell. If the functional integrity of the cytoplasmic membrane is disrupted, macromolecules and ions escape from the cell, and cell damage or death ensures. The cytoplasmic membrane of certain bacteria and fungi can be more readily disrupted by animal cells. Consequently, selective chemotherapeutic activity is possible. Polymyxins acting on Gram-negative bacteria (polymyxins selectively act on membranes rich in phosphatidyl ethanolamine and act like cationic detergents) and the polyene antibiotics acting on fungi. However, polymyxins are inactive against fungi, and polyenes are inactive against bacteria. This is because ergosterol is present in the fungal cell membrane and absent in the bacterial cell membrane. Polyenes require ergosterol in the fungal cell membrane to exert their effect. Bacterial cell membranes do not contain that sterol and (presumably for this reason) are resistant to polyene action, a good example of cell individuality and of selective inhibition [16, 17]. E.g., Amphotericin B, Azoles, Polyenes, Polymyxins.

2.3.1.8. Anti-microbial action through inhibition of protein synthesis

It is established that Aminoglycosides, Tetracyclines, Chloramphenicol, Macrolides, and Lincomycins can selectively inhibit protein synthesis [18, 19] through an action on ribosomes in bacteria. Bacteria have 70’s ribosomes, where as mammalian cells have 80’s ribosomes. The subunits of each type of ribosome, their chemical composition, and their functional specificities are sufficiently different to explain why anti-microbial drugs can inhibit protein synthesis in bacterial ribosomes without having a major effect on
mammalian ribosomes. E.g., Aminoglycosides, Tetracyclines, Erythromycins, Chloramphenicol, Lincomycins.

2.3.1.9. Anti-microbial action through inhibition of nucleic acid synthesis

Drugs such as the Actinomycin are effective inhibitors of DNA synthesis. Actually, they form complexes with DNA by binding to the deoxyguanosine residues. The DNA-Actinomycin complex inhibits the DNA dependent RNA polymerase and blocks mRNA formation. Actinomycin also inhibits DNA virus replication. Mitomycins inhibit bacterial as well as animal cells but are not sufficiently selective to be employed in anti-bacterial chemotherapy. E.g., Quinolones, Pyrimethamine, Rifampincin, Sulfonamides, Trimethoprim.

2.3.1.10. Antibacterial therapy-a success story

The research and development of antibacterial agents during the past 50 years has been an immense success story. The rate of mortality caused by bacterial infections has dropped precipitously, since the pre-penicillin days of the 1930’s [20, 21]. Although antibacterial agents, improved hygiene, vaccines and an awareness of the bacterial cause of various disease states are all believed to have contributed to a lower morbidity and mortality has been observed in the industrialized world, where drug supplies have been readily available. In 1967 and 1969, the US Surgeon General, William H. Stewart, was reported to have commented: ‘…that we had essentially defeated infectious diseases and could close the book on them [infectious diseases]…’ [22, 23], and the popular consensus of the time was that the unmet.
2.3.2. Important Anti-bacterial agents

2.3.2.1. SULPHANAMIDES

The discovery of the antibacterial activity of sulphanilamides in early 1930’s marked the beginning of the present era of chemotherapy. Following Prontosil’s dramatic successes, a cascade of sulphanilamide derivatives began to be synthesized and tested more than 4,500 by 1948 alone [24]. From these, only about two dozen actually have been used in clinical practice.

Chemistry: The term “sulphanamide” is commonly used to refer to antibacterial that are (1) aniline-substituted sulphanamides, the “sulfanilamide” (2) prodrugs that produce sulfanilamides (e.g., sulfasalazine); and (3) non-aniline sulfonamides (e.g., mafenide). However, several other widely used drugs are also sulfonamides as sulfanilamides. Included among these non-antibacterial sulfonamides are tolbutamide (an oral diabetic drug), furosemide (a potent diuretic) and chlorthiazide (also a diuretic). The following generalizations regarding structure-activity relationships arrived at early in the development of sulphanamides.

1. The amino and sulfonyl radicals on the benzene ring should be in a 1,4 disposition for the compounds to show activity; the amino group should be unsubstituted or have a substituent that is readily removed in vivo.

2. Replacement of the benzene ring by other ring systems, or the introduction of additional substituents on it, decreases or abolishes the activity.
3. Exchange of the \( \text{SO}_2\text{NH}_2 \) by \( \text{SO}_2\text{C}_6\text{H}_4-p\text{-NH}_2 \) retains the activity, while exchange by \( \text{CONH}, \text{COC}_6\text{H}_4-p\text{-NH}_2 \) markedly reduces it.

4. \( N^1 \)-Monosubstitution (on the amide \( N \)) results in more active compounds with greatly modified pharmacokinetic properties; \( N^1 \)-disubstituted in general leads to inactive compounds.

**Anti-microbial activity:** Sulphanamides are bacteriostatic in nature. However, in large doses they may also act as bactericidal. It is now accepted that sulphanamide sensitive micro-organisms require \( p \)-amino benzoic acid (PABA) for the synthesis of folic acid which is essential for the synthesis of DNA and RNA and, therefore, for the growth and multiplication of bacteria. Sulphanamides can compete with PABA and prevent the utilization of PABA and therefore by the synthesis of bacterial DNA and RNA. Through mammals require folic acid too but they use perform folic acid from diet. Thus the sulphanamides have selective effect on the sensitive bacteria. Today, a few sulphanamides and, especially, sulphanamide-trimethoprim combinations are used extensively for the opportunistic infections in patients with AIDS, [25] urinary tract infections and burn therapy [26].

**2.3.2.2. \( \beta \)-LACTAM ANTIBIOTICS**

Antibiotics that contain the \( \beta \)-lactam (a four membered cyclic amide) ring structure constitute the dominant class of agents currently employed for the chemotherapy of bacterial infections. The first antibiotic to be used in therapy, penicillin (penicillin G or benzyl penicillin), and a close biosynthetic relative, phenoxyethyl penicillin (penicillin V), remain the agents of choice for the treatment of infections caused by most species of Gram-positive bacteria. The discovery of a second major group of \( \beta \)-lactam antibiotics, the cephalosporins, and chemical modifications of naturally occurring penicillins and cephalosporins have provided semi-synthetic derivatives that are variously effective
against bacterial species known to be resistant to penicillin, in particular, penicillinase producing *Staphylococci* and Gram-negative *Bacilli*.

2.3.2.3. PENICILLINS

Commercial production of biosynthetic penicillins today depends chiefly on various strains of *Penicillium notatum* and *P. chrysogenum*. Over 30 penicillins have been isolated from fermentation mixtures. Some of these occur naturally; others have been biosynthesized by altering the culture medium to provide certain precursors that may be incorporated as acyl groups.

**Chemistry:** All penicillins share the basic structure in fig. There is a thiazolidine ring (A) attached to be a β-lactam ring (B) that carries a secondary amino group (R-NH-). Acidic radicals can be attached to the amino group and cleaved by bacterial and other amidases. The structural integrity of the 6-aminopenicillanic acid nucleus is essential to the biological activity of the molecules. If the β-lactam ring is enzymatically cleaved by bacterial β-lactamases (penicillinases), the resulting product, penicilloic acid, is devoid of anti-bacterial activity.

However, it carries an antigenic determinant of the penicillins, which acts as sensitizing structure when attached to host proteins, and can be used as skin-testing material when attached to peptide chains. Products of alkaline hydrolysis of the penicillins also contribute to sensitization. The attachment of different radicals R to the amino group of 6-aminopenicillanic acid determines the essential pharmacologic properties of the resulting molecules.
Anti-bacterial activity: β-Lactam agents share general mechanisms of anti-bacterial action that involve damage to the cell wall of bacteria. These mechanisms [27] are (1) attachment to specific penicillin-binding proteins (PBPs) that serve as drug receptors on bacteria, (2) inhibition of cell wall synthesis by blocking transpeptidation of peptidoglycan, and (3) activation of autolytic enzymes in the cell wall, which result in lesions that cause bacterial death.

2.3.2.4. CEPHALOSPORINS

Cephalosporium fungi yielded several antibiotics that resembled penicillins but were resistant to β-lactamase and were active against both Gram-positive and Gram-negative bacteria. Methods were eventually developed for the large-scale production of the common nucleus, 7-aminoscephalosporanic acid. This made possible the synthesis of a vast array of cephalexins with varying properties.

Chemistry: The chemical structure of the 7-aminoscephalosporanic acid is shown in the figure. The nucleus of the cephalosporins, 7-aminoscephalosporanic acid, bears a close resemblance to 6-aminopenicillanic acid and also the nucleus of the cephalexin antibiotics. The intrinsic anti-microbial activity of natural cephalosporins is low, but the attachment of various R₁ and R₂ groups has yielded drugs of good therapeutic activity and low toxicity. The cephalosporins have molecular weight of 400-450. They are soluble in water and relatively stable with respect to pH and temperature changes. They vary in resistance to β-lactamases. The sodium salt of cephalothin contains 2.4 meq Na⁺ per gram.
Anti-microbial activity: The mechanism [28] of action of cephalosporins is analougs to that of penicillins: (1) binding to specific penicillin-binding proteins (PBPs) that serve as drug receptors on bacteria; (2) inhibition of cell wall synthesis by blocking transpeptidation of peptidoglycan and (3) activation of autolytic enzymes in the cell wall, which results in lesions that cause bacterial death.

2.3.2.5. CHLORAMPHENICOL

Chloramphenicol was first isolated from cultures of *Streptomyces venezuelae* in 1947 and was synthesized in 1949, the first completely synthentic antibiotic of importance to be produced commercially. It is the only available representative of its chemical type.

Chemistry: Crystalline chloramphenicol is a neutral, stable compound. The molecular structure is shown in figure. It is highly soluble in alcohol and poorly soluble in water. Saturated aqueous solutions (0.25%) keep their activity for many months at refrigerator or room temperatures if protected from light. Chloramphenicol succinate is highly soluble in water and is hydrolysed in tissues, with liberation of free chloramphenicol; it is used for parental administration.

Anti-microbial Activity: Chloramphenicol is a potent inhibitor of microbial protein synthesis and has little effect on other microbial metabolic functions. Chloramphenicol
binds reversibly to receptor site on the 50’s subunit of the bacterial ribosome. There it interferes markedly with the incorporation of amino acids into newly formed peptides by blocking the action of peptidyl transferase. Chloramphenicol also inhibits mitochondrial protein synthesis in mammalian bone marrow cells but does not greatly affect intact cells.

### 2.3.2.6. TETRACYCLINES

The tetracyclines [29] are a large group of drugs with a common basic structure and activity. Chlorotetracycline, isolated from *Streptomyces aureofaciens*, was introduced in 1948. Oxytetracycline, derived from *Streptomyces rimosus*, was introduced in 1950. Tetracycline, obtained by catalytic dehalogenation of chlorotetracycline, has been available since 1953. Demeclocycline was obtained by demethylation of chlorotetracycline. More recently developed tetracyclines have emphasized good absorption combined with prolonged blood levels.

**Chemistry:** Free tetracyclines are crystalline amphoteric substances of low solubility. They are available as hydrochlorides, which are more soluble. Such solutions are acidic and, with the exception of chlortetracycline, fairly stable. Tetracyclines combine firmly with divalent metal ions, and this chelation can interfere with absorption and activity of the molecule. Tetracyclines fluoresce bright yellow in ultraviolet light of 360 nm wavelength.

![Basic structures of tetracyclines](image)

**Anti-microbial activity:** Tetracyclines are the prototype broad-spectrum [30] anti-microbial drugs. They are bacteriostatic for many Gram-positive and Gram-negative bacteria including some anaerobes; for rickettsiae, chlamydiae, mycoplasmas, and L. forms; and for some protozoa, eg. Amebas. Equal concentrations of tetracyclines in body
fluids or tissues have approximately equal anti-microbial activity. Most differences in activity claimed for individual tetracycline drugs are of small magnitude and limited importance. Differences in clinical efficacy are attributable largely to features of absorption, distribution, and excretion of individual drugs.

2.3.2.7. AMINOGLYCOSIDES

Aminoglycosides are group of bactericidal drugs originally obtained from various *Streptomyces* species and sharing chemical, anti-microbial, pharmacologic, and toxic characteristics. At present, the group includes streptomycin, neomycin, kanamycin, amikacin, gentamycin, tobramycin, sisomicin, netilmicin, and others. All of these agents inhibit protein synthesis in bacteria and suffer the disadvantage of multiple types of resistance.

2.3.2.8. STREPTOMYCIN

Streptomycin was isolated from a strain of *Streptomyces griseus* by Waksman and his associates in 1944. Dihydrostreptomycin can be produced by catalytic reduction of streptomycin trihydrochloride. Both have similar chemical and identical anti-microbial properties.

However, dihydrostreptomycin is more ototoxic than streptomycin and has been abandoned. The Anti-microbial activity of streptomycin [31] is typical of that of other aminoglycosides, as are the mechanisms of resistance. Resistant microorganisms have emerged in species, severly limiting the current usefulness of streptomycin. The emergence of resistance in an apparently susceptible isolate tends to be rapid, so that treatment with streptomycins as the sole drug is usually limited to 5 days.
2.3.2.9. A snapshot of the antibacterial agents currently available

Examination of the current status of potential novel antibacterial drugs indicates that there are only a few compounds in development by the large pharmaceutical companies (Table 3), with the majority of candidates coming from the smaller biotechnology pharmaceutical companies (Table 3) [32, 33]. In the past 30 years, the only truly novel agents that have been launched are linezolid (Pharmacia and Pfizer) and daptomycin (Cubist) [32]. Concomitant with the development of these novel agents, there has been a decrease in the number of analogs generated of the classical antibacterials, predominantly penicillins, carbapenems, cephalosporins, tetracyclines, macrolides, and quinolones [34-39]. Between 1983 and 2001, 47 new antibiotics won approval by the US FDA or the Canada Health Ministry. Only nine new antibiotics have been approved since 1998, of which just two had a novel mechanism of action. In 2002, there were no new antibacterials in the list of almost 90 drugs approved by the FDA and, in 2003, there were just two antibacterials approved. Of the ~550 drugs currently in development, only six are novel antibiotics (Table 3) [40, 41].
Table 3. Anti-bacterials currently in clinical development by large pharmaceutical companies

<table>
<thead>
<tr>
<th>Drug name (Company)</th>
<th>Class</th>
<th>Target</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABT 492 (Wakunaga)</td>
<td>Quinolone</td>
<td>DNA gyrase and topo IV</td>
<td>Phase I&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WCK 771A (Wockhardt)</td>
<td>Quinolone</td>
<td>DNA gyrase and topo IV</td>
<td>Phase I&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PNU 288034 [Pfizer (Pharmacology)]</td>
<td>Oxazolidinone</td>
<td>Protein synthesis</td>
<td>Phase I&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Garenoxacin [BMS284756 Schering-Plough and Toyama] and Pennisula Pharma</td>
<td>Quinolone</td>
<td>DNA gyrase and topo IV</td>
<td>PhaseIII&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Doripenem (Shionogi and Pennisula Pharma)</td>
<td>Carbapenem</td>
<td>Cell wall</td>
<td>PhaseIII&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CS-023 (Sankyo and Roche)</td>
<td>Carbapenem</td>
<td>Cell wall</td>
<td>Phase II&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tigecycline [GAR 936 (Wyeth)]</td>
<td>Tetracycline</td>
<td>Protein synthesis</td>
<td>PhaseIII&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCO2479 [RWJ54428, RWJ442831&lt;sup&gt;a&lt;/sup&gt;]</td>
<td>Cephalosporin</td>
<td>Cell wall and transpeptidation</td>
<td>Phase I&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Drug Code/Brand</td>
<td>Chemical Class</td>
<td>Cellular Target</td>
<td>Development Stage</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------------</td>
<td>-----------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>MC04546</td>
<td>Cephalosporin</td>
<td>Cell wall and transpeptidation</td>
<td>Phase I&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VRC 4887</td>
<td>Hydroxamate</td>
<td>Peptide deformylase</td>
<td>Phase I&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BB83698</td>
<td>Hydroxamate</td>
<td>Peptide deformylase</td>
<td>Phase I&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ramoplanin</td>
<td>Glycolipodepsipeptide</td>
<td>Transglycosylation and Lipid II</td>
<td>Phase II-III&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oritavancin</td>
<td>Glycopeptide</td>
<td>Cell wall</td>
<td>Phase III&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rifalazil</td>
<td>Benzoxazinorifamycin</td>
<td>RNA polymerase</td>
<td>Phase II&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BaL 5788</td>
<td>Cephalosporin</td>
<td>Cell wall</td>
<td>Phase II&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MC04, 124</td>
<td>Peptide</td>
<td>Efflux pump inhibitor</td>
<td>Preclinical&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MP601, 205</td>
<td>Peptide</td>
<td>Efflux pump inhibitor</td>
<td>Preclinical&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dalbavancin</td>
<td>Glycopeptide</td>
<td>Cell wall</td>
<td>Phase III&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TD6424</td>
<td>Lipoglycopeptide</td>
<td>Cell wall</td>
<td>Phase II&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Information acquired from: <sup>a</sup>Investigational drugs data base; and <sup>b</sup>company website, press release or analyst meeting. Abbreviation: Topo, topoisomerase.

### 2.3.5. Combinatorial chemistry in anti-microbial research

Over the past decade, drug resistance has become a growing problem in the treatment of infectious diseases caused by bacteria, fungi, parasites and viruses. In particular, resistance of bacterial pathogens to current antibiotics has emerged as a major health problem. This is especially true in hospitals and chronic care facilities, which provide strong selection...
pressure for the emergence of resistance, because of the large qualities and the variety of antibiotics used in these environments. As a result, infections such as pneumonia, meningitis and tuberculosis that would once have been easily treated with antibiotics are no longer so readily treated. Hospital- acquired infections caused by methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin resistant enterococci (VRE) are especially difficult to treat. At present, all widely used antibiotics, including some of the newer agents, such as the Streptogramins and the new-generation fluoroquinolones, are subject to bacterial resistance. The urgent need to tackle the resistance problem and the lack of a robust pipeline of innovative antimicrobial substances has led to a dramatic increase in antibacterial research in academic, government and industrial laboratories. There are currently four principle approaches being pursued to challenge antibiotic resistance and identify new antibacterial drugs:

- Screening of compound libraries;
- Modification of known antibiotics;
- Protection of known classes by resistance mechanism inhibitors; and
- Discovery of new agents through rational selection of novel targets

2.3.6. Microbiology: In vitro evaluation of anti-microbial activity

The standard strains were procured from the (MTCC), and the pathological strains were procured from the Department of Studies in Microbiology, University of Mysore, Mysore, India. The antibacterial activity of the synthesized compounds was tested against the following standard bacterial strains: Bacillus subtilis (MTCC 121), Staphylococcus epidermidis (435), Xanthomonas campestris (7903) and Pseudomonas aeruginosa (MTCC 7908).
2.3.6.1. Paper disc diffusion method

Preliminary screening for antibacterial activity was performed by the agar diffusion method using a paper disc. The sterilized (autoclaved at 120 °C for 30 min), liquified Muller Hinton agar (40-50 °C) was inoculated (1 mL/100 mL of medium) with the suspension of the microorganism (matched to McFarland barium sulfate standard) and poured into a petri dish to give a depth of 3-4 mm. The paper discs impregnated with the test compounds (500 µg/mL in dimethyl sulfoxide) were placed on the solidified medium. The plates were refrigerated at 4 °C for 2 h and then incubated at 37 °C for 24 h. The observed zones of (diameter) of inhibition (in mm) are presented in Table 4.

2.3.6.2. Minimum inhibitory concentration

A series of glass tubes containing different concentration of the synthesized compounds (1-500 µg/mL in dimethyl sulfoxide) with Muller Hinton broth was inoculated with the required amount of the inoculum to obtain a suspension of microorganism, which contained 105 colony-forming units per mL. One growth control tube was prepared with the addition of the compound and one blank tube was prepared without the addition of any microorganism. The tubes were incubated at 37 °C for 24 h. The turbidity produced in each tube was recorded by using a UV-visible spectrometer. The minimum inhibitory concentration (MIC-µg/mL) was the lowest concentration which exhibited the same turbidity as the blank tube. The observed (MIC-µg/mL) are presented in Table 5.
Table 4. Inhibition zone (diameter mm) of the synthesized compounds 6(a-s) against tested bacterial strains by paper disc diffusion method

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Gram-positive bacteria</th>
<th>Gram-negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacillus subtilis</td>
<td>Staphylococcus epidermidis</td>
</tr>
<tr>
<td>6a</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>6b</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td>6c</td>
<td><strong>42</strong></td>
<td><strong>31</strong></td>
</tr>
<tr>
<td>6d</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>6e</td>
<td>21</td>
<td>18</td>
</tr>
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<td>6f</td>
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<td>6h</td>
<td>13</td>
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<td>6i</td>
<td><strong>45</strong></td>
<td><strong>37</strong></td>
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<td>6j</td>
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<tr>
<td>6r</td>
<td>32</td>
<td>28</td>
</tr>
<tr>
<td>6s</td>
<td>29</td>
<td>31</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>26</td>
<td>24</td>
</tr>
</tbody>
</table>
Table 5. Minimum inhibitory concentration (µg/mL) of the synthesized compounds 6(a-s) against tested bacterial strains by macro broth dilution method

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Bacillus subtilis</th>
<th>Staphylococcus epidermidis</th>
<th>Xanthomonas campestris</th>
<th>Pseudomonas aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>6a</td>
<td>92</td>
<td>88</td>
<td>85</td>
<td>92</td>
</tr>
<tr>
<td>6b</td>
<td>59</td>
<td>56</td>
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<td>6c</td>
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<td><strong>59</strong></td>
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<td>6j</td>
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<td>104</td>
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<td>6k</td>
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<td>6q</td>
<td>82</td>
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<td>6r</td>
<td>48</td>
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<td>46</td>
<td>52</td>
</tr>
<tr>
<td>6s</td>
<td>41</td>
<td>39</td>
<td>42</td>
<td>54</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>64</td>
<td>76</td>
<td>88</td>
<td>66</td>
</tr>
</tbody>
</table>
2.3.7. Results and discussion

3-(2-(Substituted amino)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 6(a-s) were synthesized by coupling 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido(1,2-a)pyrimidin-4-one with different substituted aliphatic/ aromatic/heterocyclic amines. Literature has several instances that pyrimidone and its derivatives exert antimicrobial activities. Hence, all the novel synthesized compounds were screened for their antimicrobial efficacy against bacterial strains *Bacillus subtilis* (MTCC 121), *Staphylococcus epidermidis* (435), *Xanthomonas campestris* (7903) and *Pseudomonas aeruginosa* (MTCC 7908) by paper disk and micro dilution methods. Streptomycin sulphate was used as standard and the results are summarized in Table 4 and 5.

In general, the synthesized compounds showed potent, moderate and less activity against tested bacterial strains. Among the compounds 6(a-s), compounds 6b, 6c, 6f, 6i, 6o, 6r, 6s showed potent inhibitory activity against Gram-positive and Gram-negative bacteria. Among which compounds 6c and 6i showed two fold potent than the standard Streptomycin sulphate drug. From the earlier literature, we came to know that the piperazine analogues are known to have antimicrobial activity [42, 43]. Aromatic groups such as diphenyl methyl group attached to piperazine class of molecules by increasing lipophilicity as supported by our previous study [44]. From the results obtained, it reveals that the presence of piperazine heterocyclic moiety might be the reason for the potent inhibitory activity. Compounds 6c and 6i showed observable antibacterial activity at the lower concentration of 46 µg/mL and 45 µg/mL against *Pseudomonas aeruginosa* (MTCC 7908) and *Bacillus subtilis* (MTCC 121) respectively. Compounds 6a, 6d, 6e, 6j, 6k and 6q showed moderate inhibitory activity against tested bacterial strains. The remaining compounds 6g, 6h, 6l, 6n and 6p showed less inhibitory activity against all the tested strains.
From the results, we were able to draw some initial structure activity relationships. When we compare the antibacterial activity of the substituted heterocyclic amines with aliphatic and aromatic amines, heterocyclic amine moiety containing compounds $6(a-f), 6i, 6(o-s)$ showed potent inhibitory activity. Among the heterocyclic moiety containing derivatives, compounds $6b, 6c, 6f, 6i, 6o$ containing six membered heterocyclic ring showed potent inhibitory activity compared to the compounds $6a, 6d, 6e, 6p, 6q, 6r$ and $6s$ containing five membered heterocyclic ring. Among the compounds containing piperazine moiety, compound $6i$ with an aliphatic substitution showed better activity compared to other aromatic substituted piperazine moiety containing derivatives. When we compare the compounds with similar structure, compound $6c$ containing a free hydroxyl group showed good activity compared to the compound $6o$. When we compare the activity of benzyl amine ($6g$) and substituted benzyl amine ($6j$) containing derivatives, compound $6j$ containing 4-fluoro substitution on the phenyl ring of the benzylamino moiety showed more than two fold potent activity. Among the cyclic aliphatic amine substituted derivatives, compound $6h$ with a cyclopentyl moiety showed two fold potent activity compared to the compound $6n$ containing cyclohexyl ring.

Finally, irrespective of the presence of primary or secondary amines, almost all analogues of the series have shown potent activity than the standard drug streptomycin sulphate which could be due to the presence of various substituents.

From the above results and initial SAR studies, it is observed that compounds with substituted heterocyclic piperazine moiety showed better activity compared to other moiety containing derivatives.

2.3.8. Conclusion

In conclusion, a series of novel 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a] pyrimidin-4-one aliphatic/aromatic/heterocyclic amine derivatives were
synthesized with good yield. Few among the synthesized molecules showed good antibacterial activity. In particular, compounds 6c and 6i with piperazine moiety showed potent inhibitory activity.

2.4. General procedure for the synthesis of 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 8(a-g) and 9(a-f)

A solution of 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) (1.0 eq) in N,N-dimethylformamide was taken, potassium carbonate (3.0 eq) was added to the reaction mixture and stirred for 10 min, then appropriate sulfonyl chlorides (1.0 eq) or acid chlorides (1.0 eq) were added, the reaction mixture was allowed to stir at room temperature for 5-6 h. The progress of the reaction was monitored by TLC. Upon completion, the solvent was removed under reduced pressure and residue was taken in water and extracted with ethyl acetate. The organic layer was washed with 10% ammonium chloride solution and finally water wash was given to organic layer and dried over anhydrous sodium sulphate. The solvent was evaporated to get crude product which was purified by column chromatography over silica gel (60-120 mesh) using hexane: ethyl acetate (8:2) as eluent.

2.4.1. Synthesis of 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 8(a-g) and 9(a-f)

2-Methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 8(a-g) and 9(a-f) were prepared by the method summarized in Scheme 1. 2-Aminopyridine (1) and 3-acetyldihydrofuran-2(3H)-one (2) were refluxed in toluene in presence of catalytic amount of PTSA using Dean-Stark apparatus gave 3-(1-(pyridin-2-ylamino)ethylidene)dihydrofuran-2(3H)-one (3) in 80-85% yield. The structure of compound (3) was characterized by spectral and analytical methods. The compound (3) was cyclized to 3-(2-chloroethyl)-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one (4) having
3-chloroethyl side chain by refluxing in POCl₃. This cyclized compound (4) was characterized by IR, ¹H NMR and elemental analysis. Compound (4) undergoes hydrogenation in presence of Pd/C under H₂ atmosphere in methanol yielded 3- (2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (5). This was directly treated with piperazine and anhydrous potassium carbonate using isopropyl alcohol as a solvent at 80 °C to give the target key intermediate 2-methyl-3- (2- (piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7).

The nucleophillic substitution reactions of 2-methyl-3- (2- (piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) with different sulfonyl chlorides and acid chlorides were carried out in the presence of potassium carbonate and N, N-dimethyl formamide as solvent with good yield ranging from 65-80% with good purity. The products obtained were purified by column chromatography using hexane: ethyl acetate (8:2) as eluent. Synthesized molecules 8(a-g) and 9(a-f) were characterized by IR, ¹H NMR, and elemental analysis. The absence of N-H proton peak in synthesized derivatives 8(a-g) and 9(a-f) in IR and ¹H NMR spectra confirms our products. It is also confirmed by IR data, for sulfonamide series 8(a-g) which showed asymmetric stretching frequency of O=S=O in the range 1350-1370 cm⁻¹ and symmetric stretching frequency at 1270-1290 cm⁻¹ and similarly for carboxamide series 9(a-f), IR data showed stretching frequency of -C=O at 1630-1670 cm⁻¹. The chemical structures and yield of all the synthesized compounds are given in Table 6.
Reagent and reaction conditions: (i) K₂CO₃, piperazine, IPA, 80°C, 8h. (ii) sulphonyl chlorides, K₂CO₃, DMF, 4-5 h. (iii) acid chlorides, K₂CO₃, DMF, 4-5 h.
Table 6. Chemical structures and physical data of synthesized compounds 8(a-g) and 9(a-f)

<table>
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<th>Compound</th>
<th>R₁/R₂</th>
<th>Yield (%)</th>
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<td>75</td>
</tr>
<tr>
<td>8b</td>
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<td>8d</td>
<td>-</td>
<td>76</td>
</tr>
<tr>
<td>8e</td>
<td>-</td>
<td>69</td>
</tr>
<tr>
<td>8f</td>
<td>-CH₃</td>
<td>72</td>
</tr>
<tr>
<td>8g</td>
<td>-CH₃</td>
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</tr>
<tr>
<td>9e</td>
<td>NO₂/NO₂</td>
<td>80</td>
</tr>
<tr>
<td>9f</td>
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2.4.1.1. Synthesis of 3-(2-(4-(2,5-dichlorophenylsulfonyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (8a)

The product obtained was pale yellow oily from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), 2,5- dichlorobenzene sulfonyl chloride (0.09 g, 0.0361 mmol) and K$_2$CO$_3$ (0.15 g, 1.08 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 8.02 (s, 1H, Ar-H), 7.49 (d, 1H, Ar-H), 7.26 (d, 1H, Ar-H), 3.92 (t, 4H, (CH$_2$)$_2$), 3.75 (t, 4H, (CH$_2$)$_2$), 3.11 (t, 4H, (CH$_2$)$_2$), 2.93 (t, 4H, (CH$_2$)$_2$), 2.42 (t, 2H, -CH$_2$), 2.01 (t, 2H, -CH$_2$), 1.25 (s, 3H, -CH$_3$). IR (KBr, cm$^{-1}$): 1697, 1666, 1539, 1468, 1376, 1154. Anal. Calcd. For. C$_{21}$H$_{26}$Cl$_2$N$_4$O$_3$S (in %): C-51.32, H-6.56, N-11.40, S-6.52. Found C-51.30, H-6.52, N-11.43, S-6.54.

2.4.1.2. Synthesis of 3-(2-(4-(4-chlorophenylsulfonyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (8b)

The product obtained was pale yellow oily from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), 4-chlorobenzene sulfonyl chloride (0.08 g, 0.0361 mmol) and K$_2$CO$_3$ (0.15 g, 1.08 mmol). $^1$H NMR(DMSO-$d_6$, 400 MHz) $\delta$: 7.74 (dd, 2H, Ar-H), 7.35 (d, 2H, Ar-H), 4.01 (t, 4H, (CH$_2$)$_2$), 3.52 (t, 4H, (CH$_2$)$_2$), 2.95 (t, 4H, (CH$_2$)$_2$), 2.57 (t, 4H, (CH$_2$)$_2$), 2.16 (t, 2H, -CH$_2$), 2.01 (t, 2H, -CH$_2$), 1.25 (s, 3H, -CH$_3$). IR (KBr, cm$^{-1}$): 1681, 1540, 1502, 1460, 1378, 1148. Anal. Calcd. For. C$_{21}$H$_{27}$ClN$_4$O$_3$S (in %): C-55.93, H-6.03, N-12.42, S-7.12. Found C-55.89, H-6.01, N-12.45, S-7.10

2.4.1.3. Synthesis of 3-(2-(4-(2-nitrophenylsulfonyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (8c)

The product obtained was pale yellow oily from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), 2-nitrobenzene sulfonyl chloride (0.088 g, 0.0361 mmol) and K$_2$CO$_3$ (0.15 g, 1.08 mmol).
1H NMR (DMSO-\textit{d}_6, 400 MHz) δ: 7.97 (d, 1H, Ar-H), 7.72 (d, 2H, Ar-H), 7.69 (d, 1H, Ar-H), 3.93 (t, 4H, (CH\textsubscript{2})\textsubscript{2}), 3.81 (t, 4H, (CH\textsubscript{2})\textsubscript{2}), 3.01 (t, 4H, (CH\textsubscript{2})\textsubscript{2}), 2.82 (t, 4H, (CH\textsubscript{2})\textsubscript{2}), 2.22 (t, 2H, -CH\textsubscript{2}), 2.12 (t, 2H, -CH\textsubscript{2}), 1.25 (s, 3H, -CH\textsubscript{3}). IR (KBr, cm\textsuperscript{-1}): 1681, 1632, 1570, 1490, 1478, 1376. Anal. Calcd. For C\textsubscript{21}H\textsubscript{27}N\textsubscript{5}O\textsubscript{5}S (in %): C-54.65, H-5.90, N-15.17, S-6.95. Found C-54.68, H-5.93, N-15.15, S-6.98.

2.4.1.4. Synthesis of 3-(2-(4-(4-\textit{tert}-butylphenylsulfonyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4\textit{H}-pyrido[1,2-a]pyrimidin-4-one (8d)

The product obtained was pale yellow solid from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4\textit{H}-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), 4-\textit{tert}-butylbenzene sulfonyl chloride (0.092 g, 0.0361 mmol) and K\textsubscript{2}CO\textsubscript{3} (0.15 g, 1.08 mmol). 1H NMR (DMSO-\textit{d}_6, 400 MHz) δ: 7.65 (d, 2H, Ar-H), 7.52 (d, 2H, Ar-H), 3.89 (t, 4H, (CH\textsubscript{2})\textsubscript{2}), 3.74 (t, 4H, (CH\textsubscript{2})\textsubscript{2}), 2.98 (m, 4H, (CH\textsubscript{2})\textsubscript{2}), 2.89 (m, 4H, (CH\textsubscript{2})\textsubscript{2}), 2.29 (t, 2H, -CH\textsubscript{2}), 2.16 (t, 2H, -CH\textsubscript{2}), 1.70 (bs, 3H, -CH\textsubscript{3}), 1.34 (s, 9H, (CH\textsubscript{3})\textsubscript{3}). IR (KBr, cm\textsuperscript{-1}): 1747, 1647, 1539, 1462, 1399, 1348. Anal. Calcd. For. C\textsubscript{25}H\textsubscript{36}N\textsubscript{4}O\textsubscript{3}S (in %): C-63.53, H-7.68, N-11.85, S-6.78. Found C-63.55, H-7.69, N-11.88, S-6.80.

2.4.1.5. Synthesis of 3-(2-(4-(phenylsulfonyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4\textit{H}-pyrido[1,2-a]pyrimidin-4-one (8e)

The product obtained was pale yellow oily from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4\textit{H}-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), benzene sulfonyl chloride (0.07 g, 0.0361 mmol) and K\textsubscript{2}CO\textsubscript{3} (0.15 g, 1.08 mmol). 1H NMR (DMSO-\textit{d}_6, 400 MHz) δ: 8.01 (bs, 1H, Ar-H), 7.76 (dd, 2H, Ar-H), 7.62 (dd, 2H, Ar-H), 3.93 (t, 4H, (CH\textsubscript{2})\textsubscript{2}), 3.37 (t, 4H, (CH\textsubscript{2})\textsubscript{2}), 2.96 (t, 4H, (CH\textsubscript{2})\textsubscript{2}), 2.88 (t, 4H, (CH\textsubscript{2})\textsubscript{2}), 2.07 (t, 2H, -CH\textsubscript{2}), 2.03 (t, 2H, -CH\textsubscript{2}), 1.27 (s, 3H, -CH\textsubscript{3}). IR (KBr, cm\textsuperscript{-1}): 1694, 1665, 1462, 1414, 1377, 1350. Anal. Calcd. For. C\textsubscript{21}H\textsubscript{28}N\textsubscript{5}O\textsubscript{3}S (in %): C-60.55, H-6.78, N-13.45, S-7.70. Found C-60.58, H-6.80, N-1343, S-7.69.
2.4.1.6. Synthesis of 2-methyl-3-(2-(4-(methylsulfonyl)piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (8f)

The product obtained was pale yellow oily from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), methane sulfonyl chloride (0.041 g, 0.0361 mmol) and K$_2$CO$_3$ (0.15 g, 1.08 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 3.93 (t, 4H, (CH$_2$)$_2$), 3.37 (t, 4H, (CH$_2$)$_2$), 3.07 (t, 4H, (CH$_2$)$_2$), 2.92 (s, 3H, -CH$_3$), 2.88 (t, 4H, CH$_2$)$_2$, 2.06 (t, 2H, -CH$_2$), 2.03 (t, 2H, -CH$_2$), 1.28 (s, 3H, -CH$_3$).


2.4.1.7. Synthesis of 2-methyl-3-(2-(4-tosylpiperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (8g)

The product obtained was pale yellow oily from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), 4-methylbenzene sulfonyl chloride (0.07 g, 0.0361 mmol) and K$_2$CO$_3$ (0.15 g, 1.08 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.52 (d, 2H, Ar-H), 7.38 (d, 2H, Ar-H), 3.89 (t, 4H, (CH$_2$)$_2$), 2.98 (m, 4H, (CH$_2$)$_2$), 2.89 (m, 4H, (CH$_2$)$_2$), 2.29 (t, 2H, -CH$_2$), 2.12 (s, 3H, -CH$_3$), 2.01 (t, 2H, -CH$_2$), 1.71 (bs, 3H, -CH$_3$). IR (KBr, cm$^{-1}$): 1685, 1620, 1535, 1498, 1388, 1374. Anal. Calcd. For C$_{22}$H$_{30}$N$_4$O$_3$S (in %): C-61.37, H-7.02, N-13.01, S-7.45. Found C-61.35, H-7.02, N-13.04, S-7.42.

2.4.1.8. Synthesis of 3-(2-(4-(2-fluorobenzoyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (9a)

The product obtained was pale yellow oily from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), 2-fluorobenzoyl chloride (0.06 g, 2.27 mmol) and K$_2$CO$_3$ (0.15 g, 1.08 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.41 (m, 2H, Ar-H), 7.13 (d, 1H, Ar-H), 3.91
(t, 4H, (CH₂)₂), 3.44 (t, 4H, (CH₂)₂), 3.01 (t, 4H, (CH₂)₂), 2.86 (t, 4H, (CH₂)₂), 2.30 (t, 2H, -CH₂), 2.01 (t, 2H, -CH₂), 1.87 (s, 3H, -CH₃). IR (KBr, cm⁻¹): 1746, 1665, 1536, 1462, 1191, 1011. Anal. Calcd. For C₂₂H₂₇F₄N₄O₂ (in %): C-66.31, H-6.83, N-14.06. Found C-66.29, H-6.85, N-14.05.

2.4.1.9. Synthesis of 3-(2-(4-(3-bromobenzoyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (9b)

The product obtained was pale yellow oily from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), 3-bromobenzoyl chloride (0.08 g, 2.27 mmol) and K₂CO₃ (0.15 g, 1.08 mmol). ¹H NMR (DMSO-d₆, 400 MHz) δ: 7.59 (s, 1H, Ar-H), 7.31 (d, 1H, Ar-H), 7.26 (t, 1H, Ar-H), 7.15 (d, 1H, Ar-H), 3.91 (t, 4H, (CH₂)₂), 3.42 (t, 4H, (CH₂)₂), 3.01 (t, 4H, (CH₂)₂), 2.88 (t, 4H, (CH₂)₂), 2.32 (t, 2H, CH₂), 2.01 (t, 2H, -CH₂), 1.87 (s, 3H, -CH₃). IR (KBr, cm⁻¹): 1735, 1664, 1597, 1462, 1093, 536. Anal. Calcd. For C₂₂H₂₇BrN₄O₂ (in %): C-57.52, H-5.92, N-12.20. Found C-57.50, H-5.94, N-12.22.

2.4.1.10. Synthesis of 3-(2-(4-(3-methoxybenzoyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (9c)

The product obtained was pale yellow oily from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), 3-methoxybenzoyl chloride (0.06 g, 2.27 mmol) and K₂CO₃ (0.15 g, 1.08 mmol). ¹H NMR (DMSO-d₆, 400 MHz) δ: 7.32 (bs, 1H, Ar-H), 6.95 (m, 3H, Ar-H), 3.83 (s, 3H, -OCH₃), 2.87 (t, 4H, (CH₂)₂), 2.73 (t, 4H, (CH₂)₂), 2.27 (t, 4H, (CH₂)₂), 2.16 (t, 4H, (CH₂)₂), 2.01 (t, 2H, -CH₂), 1.98 (t, 2H, -CH₂), 1.87 (s, 3H, -CH₃). IR (KBr, cm⁻¹): 1665, 1599, 1534, 1462, 1377, 1157. Anal. Calcd. For C₂₃H₃₀N₄O₃ (in %): C-67.29, H-7.37, N-13.65. Found C-67.27, H-7.40, N-13.68.
2.4.1.11. Synthesis of 3-(2-(4-(2,4-dichlorobenzoyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (9d)

The product obtained was pale yellow oily from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), 2,4-dichlorobenzoyl chloride (0.07 g, 2.27 mmol) and K$_2$CO$_3$ (0.15 g, 1.08 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.78 (d, 1H, Ar-H), 7.61 (d, 1H, Ar-H), 7.48 (d, 1H, Ar-H), 3.92 (t, 4H, (CH$_2$)$_2$), 3.41 (t, 4H, (CH$_2$)$_2$), 2.98 (t, 4H, (CH$_2$)$_2$), 2.88 (t, 4H, (CH$_2$)$_2$), 2.32 (t, 2H, -CH$_2$), 1.99 (t, 2H, -CH$_2$), 1.87 (s, 3H, -CH$_3$). IR (KBr, cm$^{-1}$): 1748, 1686, 1597, 1537, 1191, 722. Anal. Calcd. For C$_{22}$H$_{26}$Cl$_2$N$_4$O$_2$ (in %): C-58.80, H-5.80, N-12.47. Found C-58.81, H-5.78, N-12.49.

2.4.1.12. Synthesis of 3-(2-(4-(3,5-dinitrobenzoyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (9e)

The product obtained was red oily from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), 3,5-dinitrobenzoyl chloride (0.09 g, 2.27 mmol) and K$_2$CO$_3$ (0.15 g, 1.08 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 9.10 (s, 1H, Ar-H), 8.60 (s, 2H, Ar-H), 3.91 (t, 4H, (CH$_2$)$_2$), 3.74 (t, 4H, (CH$_2$)$_2$), 2.98 (t, 4H, (CH$_2$)$_2$), 2.83 (t, 4H, (CH$_2$)$_2$), 2.32 (t, 2H, -CH$_2$), 2.01 (t, 2H, -CH$_2$), 1.25 (s, 3H, -CH$_3$). IR (KBr, cm$^{-1}$): 1720, 1666, 1598, 1460, 1155, 1376. Anal. Calcd. For C$_{22}$H$_{26}$N$_6$O$_6$ (in %): C-56.16, H-5.57, N-17.86. Found C-56.18, H-5.60, N-17.90.

2.4.1.13. Synthesis of 3-(2-(4-(4-tert-butylbenzoyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (9f)

The product obtained was pale yellow oily from 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (7) (0.1 g, 0.361 mmol), 4-tert-butylbenzoyl chloride (0.07 g, 2.27 mmol) and K$_2$CO$_3$ (0.15 g, 1.08 mmol). $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$: 7.48 (d, 2H, Ar-H), 7.42 (s, 1H, Ar-H), 7.26 (s, 1H, Ar-H), 3.96 (t, 4H, (CH$_2$)$_2$), 3.75 (t, 4H, (CH$_2$)$_2$), 3.01 (t, 4H, (CH$_2$)$_2$), 2.89 (t, 4H, (CH$_2$)$_2$), 2.34
(t, 2H, -CH₂), 2.01 (t, 2H, -CH₂), 1.88 (s, 3H, -CH₃), 1.33 (s, 9H, (CH₃)₃). IR (KBr, cm⁻¹): 1700, 1667, 1540, 1462, 1156, 1379. Anal. Calcd. For C₂₆H₃₆N₄O₂ (in %): C-71.55, H-8.31, N-12.83. Found C-71.55, H-8.34, N-12.85.

2.4.2. Anti-microbial activity of 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 8(a-g) and 9(a-f)

Materials and methods of antimicrobial activity is discussed in Section 2.3.7.

Table 7. Inhibition zone (diameter mm) of the synthesized compounds against tested bacterial strains by paper disc diffusion method

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Gram-positive bacteria</th>
<th>Gram-negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacillus subtilis</td>
<td>Staphylococcus epidermidis</td>
</tr>
<tr>
<td>8a</td>
<td>37</td>
<td>39</td>
</tr>
<tr>
<td>8b</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>8c</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>8d</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>8e</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>8f</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>8g</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>9a</td>
<td>31</td>
<td>26</td>
</tr>
<tr>
<td>9b</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>9c</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>9d</td>
<td>39</td>
<td>36</td>
</tr>
<tr>
<td>9e</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td>9f</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>23</td>
<td>27</td>
</tr>
</tbody>
</table>
Table 8. Minimum inhibitory concentration of the synthesized compounds against tested bacterial strains by macro broth dilution method

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Gram-positive bacteria</th>
<th>Gram-negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Bacillus subtilis</em></td>
<td><em>Staphylococcus epidermidis</em></td>
</tr>
<tr>
<td>8a</td>
<td>55</td>
<td>58</td>
</tr>
<tr>
<td>8b</td>
<td>61</td>
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<tr>
<td>8c</td>
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<tr>
<td>8d</td>
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<td>113</td>
</tr>
<tr>
<td>8e</td>
<td>92</td>
<td>89</td>
</tr>
<tr>
<td>8f</td>
<td>120</td>
<td>117</td>
</tr>
<tr>
<td>8g</td>
<td>111</td>
<td>114</td>
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<tr>
<td>9a</td>
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</tr>
<tr>
<td>9b</td>
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<td>9d</td>
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<td>63</td>
</tr>
<tr>
<td>9f</td>
<td>131</td>
<td>124</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>100</td>
<td>96</td>
</tr>
</tbody>
</table>
2.4.3. Results and discussion

2-Methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 8(a-g) and 9(a-f) were synthesised and screened for their efficacy as antimicrobial agents against various pathogens *in vitro* by paper disc diffusion and micro dilution methods. Gentamycin was used as standard against both Gram-positive and Gram-negative bacteria. The results are shown in Table 7 and 8. Piperazine analouges are known to have antibacterial activity [45, 46]. Aromatic groups such as 6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one attached to piperazine help in improving antibacterial activity of piperazine class of molecules by increasing lipophobicity as supported by our previous study [47]. At the same time, heterocyclic sulphanamides and carboxamides are reported to shown antibacterial activity.

Therefore in the present work 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one sulphanamide and carboxamide derivatives were synthesized and screened for antibacterial activity. In general, the synthesized compounds showed significant, moderate and less inhibitory activity against pathogenic bacterial strains. Among sulfanamide derivatives 8(a-g), compound 8a and 8b showed significant inhibitory activity against Gram-positive *Bacillus subtilis* and *Staphylococcus epidermidis* (zone of inhibition 37-39 mm, and 20-26 mm respectively) and Gram-negative *Xanthomonas campestris*, and *Pseudomonas aeruginosa* (zone of inhibition 29-32 mm, and 29-31 mm respectively) bacteria. Compound 8c, and 8e showed moderate activity with the zone of inhibition in the range of (13-19 mm, and 17-23 mm) for Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus epidermidis* and (20-24 mm, and 15-19 mm) for Gram-negative *Xanthomonas campestris*, and *Pseudomonas aeruginosa* bacteria. Compound 8d, 8g, and 8f showed poor activity with the zone of inhibition in the range of (4-7 mm, 4-7 mm, and 5-8 mm respectively) for Gram-positive *Bacillus subtilis* and
Staphylococcus epidermidis bacteria, and (6-9 mm, 6-11 mm, and 13-15 mm) for Gram-negative Xanthomonas campestris, and Pseudomonas aeruginosa bacteria respectively. However, compound 8a and 8b showed observable antibacterial activity at the lower concentration of 55 µg/mL and 61 µg/mL respectively against Bacillus subtilis (MTCC 121). From the above results, it is observed that the presence of electron withdrawing groups on the phenyl ring of aryl sulphanamide moiety might be the reason for the significant inhibitory activity.

Among carboxamide series 9(a-f), compounds 9a, 9d and 9e showed significant inhibitory activity against Gram positive Bacillus subtilis and Staphylococcus epidermidis (zone of inhibition 26-31 mm, 36-39 mm, 23-26 mm respectively) bacteria, for Gram-negative Xanthomonas campestris, and Pseudomonas aeruginosa (zone of inhibition 25-39 mm, 20-28 mm, 16-20 mm respectively) bacteria. Significant inhibitory activities of the synthesized compounds are probably due to the presence of electronegative chlorine in 9d, electronegative fluorine in 9a and electron withdrawing nitro group in 9e. Compounds 9b, 9c and 9f showed low activity with the zone of inhibition in the range of 15-21 mm, 19-23 mm, and 5-12 mm, respectively for Gram-positive Bacillus subtilis and Staphylococcus epidermidis bacteria, and 9-19 mm, 12-14 mm, and 7-9 mm for Gram-negative Xanthomonas campestris, and Pseudomonas aeruginosa bacteria respectively. However, compound 9d showed observable antibacterial activity at the lower concentration of 47 µg/mL against Bacillus subtilis (MTCC 121).

2.4.4. Conclusion

In conclusion, a series of novel 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one sulfonamide and carboxamide derivatives were synthesized in good yield. Few among the synthesized molecules showed good
antibacterial activity. In particular, compounds 8a and 9d with dichloro substitution showed potent inhibitory activity.

2.5. Anti-angiogenic effects of 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 8(a-d) and 9(a-e) on Ehrlich Ascites Tumor [EAT] cells in-vivo

2.5.1. Introduction to angiogenesis

Angiogenesis, is the formation of new blood vessels from pre-existing vessels, is a complex process that normally occurs in adults only under specific conditions such as wound healing, inflammation and in menstrual cycle [48, 49]. Under normal conditions such as wound healing, the angiogenic process switches on and then off at the appropriate times indicating tight regulation of stimulatory and inhibitory factors [50]. Under certain pathological conditions, such as the growth of tumors, rheumatoid arthritis, psoriasis, and diabetic retinopathy, angiogenesis occurs in a less controlled manner [49-51]. Understanding angiogenesis and its unique characteristics in tumor growth has provided insights into a number of ways to interrupt the process. In the last decade research on antiangiogenic agents has exploded along with public interest in its potential [52]. We now have a clear understanding of the process of tumor angiogenesis, including key cytokines, differences between normal and immature tumor vasculature, and endogenous inhibitors, along with methods to quantify angiogenesis.

Angiogenesis is a complex process: In order for new vessels to develop, degradation of the basement membrane by proteolytic enzymes is necessary for tumor invasion, metastasis growth, and angiogenesis [53]. Subsequently, these new vessels maturate to form a vascular network [54, 55]. Strategies for regulating angiogenesis have been carried out mainly in molecular biology, such as the isolation and identification of the endogenous inhibitor [56]. As well as gene [57] and antibody therapy [58]. However, because of
bioavailability, biostability, and effectiveness, it is very important to discover the antiangiogenic small molecules that might be suitable for clinical therapies.

2.5.1.1. Angiogenesis as a therapeutic target in human tumors

Several attempts have been made to classify the therapeutic agents that interfere with the process of angiogenesis. This task, however, has revealed itself to be particularly arduous, because of the large number of complex and mainly unpredictable mechanisms involved in the angiogenic cascade. Because of the variability in experimental models designed to assess angiogenesis, and suboptimal reproducibility between pre-clinical and clinical effects, a classification system based on the efficacy of antiangiogenic agents as observed in pre-clinical studies does not seem adequate. In addition, although the majority of the angiogenic inhibitors have been specifically developed to interfere with angiogenesis, some therapeutic agents previously classified on the basis of other mechanisms of action (e.g. Bay 43-9006) [59] have been shown to possess antiangiogenic properties as an additional effect with antitumor property. For example, among anticancer agents, virtually all classes have been reported to have antiangiogenic activity, although significant variability exists across different compounds. An easily intuitive criterion for classifying a chemotherapeutic agent as antiangiogenic is based on the dose needed to reach endothelial cytotoxicity. In other words, if a drug shows its toxic effect on vessels at lower doses than those required to have a cytotoxic effect, it seems reasonable to consider it as antiangiogenic. Furthermore, it is important to emphasize that endothelial toxicity does not necessarily mean endothelial cell death. In fact, several agents may act by inhibiting endothelial cell proliferation, motility and invasiveness. Recently, as described by Pasquier et al., [60] a very low concentration of paclitaxel (0.1 nmol/L) causes an increase in the microtubule dynamics of living endothelial cells without significantly altering mitotic progression. Accordingly, they suggested that low doses of paclitaxel could be responsible for the
inhibition of angiogenesis through the impairment of microtubule interphase functions, which regulate cell polarization and migration. Similar observations of a non-cytotoxic inhibition of human endothelial cell growth have been reported with other drugs such as docetaxel [61] and topoisomerase-1 inhibitors [62]. A recently proposed classification of antiangiogenic agents is based on their targets and mechanism of action (Table 9) [63]. Three categories have been identified.

i. Direct antiangiogenic drugs that act by targeting the endothelial cells and their functions involved in angiogenesis (proliferation, migration, formation of new vessels).

ii. Indirect antiangiogenic drugs that thwart the production of angiogenic factors by tumor and microenvironment cells, and/or interfere with extracellular processes.

iii. Mixed antiangiogenic drugs that may be able to interfere with both endothelial anti-tumor cells. Obviously, it is logical to hypothesize that changes in classification of antiangiogenic drugs will be parallel to improvement in our knowledge of angiogenesis and related mechanisms.
Table 9. Classification of anti-angiogenic agents

<table>
<thead>
<tr>
<th>Class</th>
<th>Target</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct antiangiogenic agents (e.g. angiostatin, endostatin, thalidomide)</td>
<td>Endothelial cells</td>
<td>Inhibition of endothelial cell functions (i.e. proliferation, migration, formation of new vessels)</td>
</tr>
<tr>
<td>Indirect antiangiogenic agents. (e.g. cetuximab, marimastat, gefitinib, erlotinib.)</td>
<td>Cancer cells, stromal cells, inflammatory cells</td>
<td>Interference with the production of angiogenic factors or with extracellular processes</td>
</tr>
<tr>
<td>Mixed antiangiogenic agents (e.g. bevacizumab, SU 11248, metronomic therapy)</td>
<td>Endothelial cells and cancer cells</td>
<td>Multiple mechanisms</td>
</tr>
</tbody>
</table>

iv. Several reports suggest that certain small molecules that can be taken orally will raise the endogenous expression of specific angiogenesis inhibitors or raise their plasma or serum level perhaps by alternative means, such as mobilization from matrix or platelets. For example, celecoxib can increase serum endostain [64]. Prednisolone and salazosulfapyridine can increase the endostain level in joint fluid [65]. Doxycycline [66] and rosiglitazone can increase expression of thrombospondin-1. A possible new pharmaceutical field could be developed around the future discovery of low molecular weight, orally available drugs that could increase endogenous angiogenesis inhibitors to protest against cancer as well as other angiogenesis depended diseases. This would help to
broaden antiangiogenic therapy of cancer. Endostatin is a paradigm of a broad spectrum endogenous antiangiogenic molecule.

2.5.1.2. Regulation of angiogenesis process

The regulation of angiogenesis both in physiologic and pathologic conditions is a complex, multistep process resulting from a dynamic balance between proangiogenic and antiangiogenic factors [67]. The mechanisms by which cancer cells stimulate pathological angiogenesis mimic those used by normal cells to foster physiological angiogenesis [68]. However, the tumor microvasculature is structurally, functionally and even genetically altered in comparison to that we observe in physiologic conditions [69, 70]. The ECs proliferate, migrate into the ECM toward the angiogenic stimulus, and form vessels. Finally, the association of pericytes and smooth muscle cells stabilizes the new vessels.

Angiogenesis is regulated by many cytokines including proangiogenic factors, such as VEGF, basic fibroblast growth factor (bFGF), placenta-like growth factor (PIGF), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), angiopoietin, angiogenin and interleukin (IL-8), a cascade of biologic events is switched on following the “cross-talk” between tumor cells and the local microenvironment. For example, homeostatic modifications such as those due to hypoxia, oxidative or mechanical stress may act as potent stimulators of tumor angiogenesis and induce the expression of multiple pro-angiogenic factors.
2.5.1.3. Vascular endothelial growth factor (VEGF) family and its receptors

Among the angiogenic regulators, vascular endothelial growth factor (VEGF) and its receptors (VEGFR) have been intensively studied in basic and clinical cancer research [71]. VEGF is a 45-kDa homodimer that belongs to a family of growth factors comprising six different glycoproteins: VEGF-A (commonly referred as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (P1GF). To date, at least six isoforms of VEGF-A, varying according to the number of amino acids in the protein chain (121, 145, 165, 183, 189 or 206 amino acids), have been identified. Recent findings suggest a tissue-specific expression of these VEGF variants with corresponding defined roles in vasculogenesis (i.e. the formation of a primitive network of vessels in embryonic development) and angiogenesis [72, 73]. VEGF has been shown to have a pluripotent effect in angiogenesis, stimulating both the motogenic (i.e., invasion and migration) activity of the endothelial cells and enhancing microvascular permeability [74-76]. In addition, although this issue is still controversial, a VEGF-mediated recruitment of endothelial precursor cells from the bone marrow has been hypothesized to be involved in tumor neovascularization [77].

2.5.1.4. Fibroblast growth factor (FGF) family

Unlike the VEGF family and despite their name, FGFs are strong mitogens for many cell types, not being restricted to vascular cells or fibroblasts. They are major growth and differentiation factors in embryonic development as well as in the adult playing a role in neuronal signaling, inflammatory process, hematopoiesis, angiogenesis, tumor growth and invasion. FGFs are small polypeptides of 155-268 amino acids, most of them are constitutively secreted, with a few family members remaining intracellular and a few FGFs lacking a signal sequence, still leaving the cell through an unknown mechanism, possibly involving a carrier protein [78]. The two most extensively studied members of
the FGF family, acidic FGF (a FGF, FGF1) and basic FGF (b FGF, FGF2) both adhere to this last group. The FGF family comprises 23 members to date [79, 80]. Matrix components, tumor promoters, and stress factors. This activation is attained after dual phosphorylation of the protein kinase ‘activating loop’ on threonine and tyrosine residues. Their specific upstream activators represent a set of dual specificity protein kinases, called M KK or MAPKKK. This cascade of three subsequent protein kinases, referred to as the ‘MAP kinase module’, is extremely well conserved from yeast to man, and has been the subject of intensive investigation [81]. The ERK signaling pathway, also known as the p42/p44 MAP kinase pathway, is a major determinant in the control of cell growth, cell differentiation and cell survival. This pathway, which operates down stream of Ras, is often up-regulated in human tumors and as such represents an attractive target for anticancer therapy [82, 83].

2.5.1.5. Transcriptional regulation of VEGF in tumor cells

The VEGF gene, which is located on human chromosome 6 and mouse chromosome 17, is expressed by a wide variety of normal and pathological cell types including tumor cells. Although most vascular endothelial cells do not express VEGF [84, 85]. Expression has been reported by EC under hypoxic conditions, hemangioma EC and some EC in culture [86, 87]. Given its importance during development, it is not surprising that VEGF is regulated at multiple levels—from transcriptional to post-translational. Transcriptional regulation of VEGF occurs via both the core promoter and through enhancers or repressors outside of the core promoter. The core promoter of VEGF does not contain typical transcriptional initiation recognition sequences, such as a TATA box, an initiator element, a downstream promoter element or a TFⅡB recognition element. Instead, the VEGF promoter is predicted to be controlled by Sp1 site that is 50 base pairs upstream of the transcription start site [88, 89]. Basal transcription of VEGF can be enhanced or
represented by the interaction of specific transcription factors with the VEGF promoter, either through or independent of the basal transcription machinery. Transcriptional regulation accounts for much of the up and down regulation of VEGF in tumors. There are many very well characterized transcription factors that can modulate VEGF expression. Some of them (e.g. HIF-1, AP-1, and Sp-1) are transcription factors, proteins that bind to the VEGF promoter to initiate and activate the transcription of a gene directly [90].

2.5.1.6. Hypoxia and Hypoxia inducible factor-1

Tissue hypoxia causes a deficiency in the supply of oxygen, and a demand for more oxygen in the tissue. In most malignant tumors, unlimited proliferation induces oxygen deficiency, leading to tissue hypoxia; nevertheless, the tumors continue to grow and invade because of their adaptation to hypoxia through angiogenesis, hyperemia, and glycolysis in the tumor [91]. Hypoxia-inducible factor-1 (HIF-1) is a ubiquitously and constitutively expressed heterodimeric transcription factor composed of $\alpha$ and $\beta$ subunits. HIF-1$\alpha$ is involved in the hypoxic response [92, 93]. Under normoxic conditions, HIF-1$\alpha$ is unstable due to degradation by von-Hippel-Lindau tumor suppressor protein [94]. In hypoxia, it separates from von-Hippel-Lindau protein and becomes stable and dimerized with HIF-1$\alpha$, and binds to the hypoxia-response element in the target genes-vascular endothelial growth factor (VEGF), [95] and erythropoietin (EPO), [96, 97] and glucose transporters and glycolytic enzymes. These substances are activated to promote angiogenesis, erythropoiesis and glycolysis, respectively [98]. Therefore, malignant tumors are able to survive and proliferate under hypoxic conditions. VEGF was originally recognized as a vascular permeability factor (VPF) that promoted the influx of serum albumin, leading to ascites in tumor implanted guinea pigs [99]. VPF increases the permeability of capillaries, postcapillary venules, and muscular venules by causing fenestration of the endothelium of these vessels [100]. Moreover, VEGF acts as a specific
mitogen for endothelial cells, [101, 102] induces proliferation of endothelial cells to form vessels [103] and activates chemoattractants for both endothelial cells and non-endothelial cells, monocytes and macrophages [104, 105]. In malignant tumors, VEGF/VPF mRNA is expressed at the boundary of the necrotic area of glioblastoma tumors indicating that hypoxia stimulates the production of VEGF.

VEGF has been reported to be expressed in ovarian [106] cancers.

It has been shown that inhibition of hypoxia inducible factor-1α (HIF-1α) decreases vascular endothelial growth factor (VEGF) secretion and tumor growth in malignant gliomas [107]. It is therefore possible that constitutive up-regulation or increased inducibility of HIF-1α may be associated with increased VEGF induction and tumor growth.

2.5.1.7. Transcription factor Ap-1

Transcription factor AP-1 (activator protein-1) binds to TRE (TPA response element), which is located in the promoter region of many genes responsible for the proliferation and tumor progression of cells. Hypoxia highly induces the binding ability of Ap-1 to DNA as well as the transcriptional activation of genes [108, 109]. The whole complex of factors (HIF-1, AP-1) contributes to activation and expression of the VEGF gene in hypoxic conditions. However, transcription factor AP-1 is not necessary in the induction of expression of the VEGF gene [110]. The functional role of AP-1 was investigated in hypoxia-induced expression of VEGF by using dexamethasone, where as hypoxia-induced VEGF expression was not inhibited by dexamethasone. It was also proved that intracellular calcium (Ca$^{2+}$) is required for the expression of hypoxia-inducible genes [111]. However, in contrast to hypoxia, the elevation of intracellular Ca$^{2+}$ neither induces the HIF-1α protein nor stimulates HIF-1-dependent transcription. On the contrary, it increase levels of c-Jun protein, causing its phosphorylation.
During hypoxia an increase in intracellular Ca\(^{2+}\) activates a HIF-1-independent signaling pathway that involves AP-1-dependent transcription, and the cooperation between the HIF-1 and AP-1 pathways allows fine regulation of gene expression during hypoxia.

2.5.1.8. Transcription factor Sp-1

The transcription factor Sp1 plays a significant role in the constitutive and induced expression of a variety of genes and therefore contributes to the process of tumorigenesis or the promotion of VEGF gene transcription by interacting directly and specifically with the protein kinase C zeta (PKC zeta) isoform [112]. The study of human pancreatic cancer cells revealed an elevated study-state level of VEGF mRNA due to enhanced VEGF gene transcription and increased constitutive VEGF promoter activity that was preceded by the activation of transcription factor Sp1[113]. Therefore it is clear that a constitutive Sp1 activation is essential for the differential over expression of VEGF, which in turn plays an important role in angiogenesis and the progression of cancer. It was shown that altered expression of transcription factor Sp1 critically impacts the angiogenic phenotype of human gastric cancer. The expression of Sp1 also precedes the increased synthesis of bFGF, PDGF and VEGF during the healing of duodenal ulcers [114]. It was shown that Sp1 contributes to accelerated healing without any changes in HCl secretion. In the same way, genetic therapy using the VEGF and the angiopoietin genes contributes to a similar healing of gastric ulcers [115].

2.5.1.9. Transcription factor NF-kB

The transcription factor NF-kB is one of the key regulators of the genes involved in the immune/inflammatory response as well as in survival from apoptosis. NF-kB is an inducible transcription factor made up of homo and heterodimers of p50, p65 RelA), p52, RelB, and c-rel subunits that interacts with a family of inhibitory kB proteins, of which 1-kB is the best characterized [116]. In most cell types, these proteins sequester NF-kB in
the cytoplasm by masking its nuclear localization sequence. Antigen stimulation in T-cells triggers a signaling pathway that results in phosphorylation, ubiquitination, and subsequent degradation of IκB proteins, resulting in the translocation of NF-κB from the cytoplasm of the nucleus [116]. Complex is a heterodimer containing p50 and p65, and in addition to the control of NF-κB activity exerted at the nuclear translocation level, there is increasing evidence for another complex level of regulation that is mediated by post-translational modifications of both subunits [117-119]. It was shown that NF-κB is also involved in the upregulation of VEGF and its activity in breast cancer cell lines (MDA-MB-231) was associated with the high expression of VEGF mRNA [120]. Advanced glycation end products (AGE), which occur during diabetes, increase the transcriptional activity of NF-κB and therefore upregulate mRNA levels of VEGF [121]. Cervistation (a hydroxymethylglutaryl CoA reductase inhibitor) completely abolishes this process and might therefore be a promising therapy for patients with proliferative diabetic retinopathy [121].

2.5.1.10. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are degradative enzymes that play an important role in all aspects of tumor progression by enhancing tumor-induced angiogenesis and destroying local tissue architecture and basement membranes to allow tumor invasion and metastasis. Efficient breakdown of the ECM surrounding invasive cancer islands involves interplay between tumor cells, stromal cells, and inflammatory cells, all of which express a distinct set of MMPs. Besides the classical role of MMPs in degradation of ECM, MMPs may also indirectly influence the tumor microenvironment through the release of growth factors, cryptic sites or angiogenic factors, or through the generation of matrix fragments that inhibit tumor cell proliferation, migration, and angiogenesis, MMP-2 (type IV collagenas/
gelatinase A) and MMP-9 (gelatinase B), in particular, are responsible for tumor angiogenesis [122].

2.5.1.11. Tumor antiangiogenic therapy

In the field of cancer therapy, the majority of traditional chemotherapeutic agents target dividing cells. However, large doses of drugs also display a significant toxicity in proliferating normal cells. Therefore, a more selective targeting is necessary for the treatment of cancer. It is widely accepted that most solid tumors cannot grow without blood supply. Similarly, the formation of metastasis is dependent on neovascularization of the primary tumor. As a consequence, inhibition of angiogenesis represents an alternative strategy to block tumor growth and metastasis. Anti-angiogenic therapy is one of the most promising strategies to inhibit tumor growth and metastatic spread [123, 124]. Angiogenesis is promoted by various factors, most of which are cytokines. These angiogenic factors are produced in tumor cells and surrounding stromal cells, bind to their receptors on endothelial cells nearby, and transduce angiogenic signals. Thus stimulated endothelial cells acquire the abilities of proliferation, motility, and invasion to form vascular construction. Therefore if these angiogenic signals are blocked, angiogenesis should inhibited and tumor growth suppressed indirectly. A recently proposed classification of antiangiogenic agents is based on their targets and mechanism of action [125]. Three categories have been identified.

A. Direct antiangiogenic drugs that act by targeting the endothelial cells and their functions involved in angiogenesis (proliferation, migration, formation of new vessels). B. Indirect anti-angiogenic drugs that inhibit the production of angiogenic factors by tumor and microenvironment cells, and/or interfere with extracellular processes. C. Mixed anti-angiogenic drugs that may be able to interfere with both endothelial and tumor cells.
2.5.1.12. Inhibitors of angiogenic factors and their receptors

A growing number of angiogenic factors have been shown to regulate tumor angiogenesis. Among them, vascular endothelial growth factor (VEGF) and fibroblast growth factor (bFGF, or FGF-2) are considered the main effectors, although other factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and platelet derived-endothelial cell growth factor thymidine phosphorylase (PD-ECGF/TP) are currently the subject of much attention as potential targets. Compounds that affect the production of angiogenic factors, their binding to receptors on endothelial cells, and receptor signaling are already in clinical studies. These inhibitors range from small molecules selected from chemical libraries, to monoclonal antibodies, cytokines and a ribozyme. Other compounds in development include inhibitors of the VEGF receptor 2 (VEGFR-2, also known as Flk-1/KDR), such as ZD6474 and AG13925, antagonists of the FGF-2 receptor, such as PD173074, and inhibitors of both factors, such as the porphyrin analogs [126]. Inhibitors of angiogenic factors, VEGF in particular, not only prevent endothelial-cell survival mechanisms [127]. Apart from VEGF and FGF-2 receptors, other potential targets for blocking angiogenesis include the EGF receptor (inhibitor, ZD1839), the PDGF receptor (inhibitor, ST1571) and PD-ECGF/TP [128].

2.5.1.13. Drugs targeting integrins and adhesion molecules

Integrins are the main family of cell receptors for the extracellular matrix. Compounds targeting integrins, in particular $\alpha v \beta 3$, and affecting endothelial cell matrix adhesion and survival [129] are in clinical trials. They include anti-integrin antibodies; cyclic peptides based on the integrin recognition sequence Arg-Gly-Asp (in single-letter amino acid code RGD), and compounds derived from peptidomimetic libraries. Newer integrin receptor antagonists include inhibitors of $\alpha v \beta 3$ and $\alpha v \beta 5$ (e.g. SD983 and SCH221153 [130] and of $\alpha 2\beta 1$ integrin (e.g. ER-68203-00). The relevance of $\beta 1$ integrins as targets for
antiangiogenic therapy is supported by the recent finding that endostatin binds to $\alpha_5\beta_1$ integrin. Cell-cell adhesion molecules, such as cadherins, can also be targets for antiangiogenic therapies [131].

2.5.1.14. Endogeneous inhibitors of angiogenesis

Endostatin (a fragment of type VIII collagen) and angiostatin (a fragment of plasminogen), now entering clinical trials, are the best-known examples of the large family of endogeneous inhibitors of angiogenesis [132]. Other such inhibitors are a fragment of prolactin, platelet factor-4 [133], thrombospondin TSP-1 and TSP-2, pigment epithelium derived factor (PEDF) [134], and fragments of fibronectin [135] and of type IV collagen [136, 137]. Interestingly, in many cases only a fragment of the molecule is antiangiogenic and the entire protein has no activity. Prolactin and TSP-1 are particular cases; fragments of both molecules are anti-angiogenic, but the whole molecule (prolactin) or different fragments (TSP-1) are able to actually stimulate angiogenesis [138,139]. Peptides and recombinant fragments corresponding to the antiangiogenic sites can be exploited as antiangiogenic agents.

2.5.1.15. Inhibitors of matrix degradation

Encouraging pre-clinical results have brought inhibitors of matrix metalloproteinases (MMPs), enzymes involved in matrix degradation, to clinical trials. MMP inhibitors include a large number of molecules, most of them synthetic compounds, directed against the active site of these enzymes. The clinical results have so far been rather discouraging and in some cases, studies have been halted because of nonadvantageous or even determental effects of the treatments [140]. Attempts to analyze these initial results have brought to light several considerations. First, that MMPs not only degrade the extracellular matrix but also activate cell-surface molecules and receptors, generate active fragments from matrix
components, and release growth factors stored in the matrix [141, 142]. It is, therefore, not surprising that modulation of MMP activity can have for reaching effects. Second, MMPs are bidirectional regulators of morphogenic processes. Low levels of MMPs promote the formation of tubular structures in a three dimensional matrix, but an excess of MMPs prevents tubulogenesis and promotes cell invasion [143-145]. Paradoxically, inhibition of MMPs can lead to stabilization of the formed tubular structures, by preventing MMP-mediated reabsorption [145]. MMP inhibitors will, therefore, oppositely affect different stages of the angiogenesis process. Third, more studies are needed to investigate the host response to pronged treatment with protease inhibitors. Finally, the lack of surrogate markers and end points had hindered the correct design of clinical trials and the identification of the active dose. The clinical experience with MMP inhibitors and recent preclinical findings indicate that alternative approaches to MMP inhibition may be more successful. Changing the target from the soluble MMPs targeted so far (in particular MMP-2 and MMP-9) is one approach. Preclinical studies indicate that membrane-type (MT-1)-MMP may be a more appropriate target [146,147].

2.6. Biology: In-vivo anti-cancer and angio-inhibitory effects of synthetic 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 8(a-d) and 9(a-e).

2.6.1. Compounds

Synthesized pyrimidinone derivatives 8(a-d) and 9(a-e) were used as compounds for the experiments. The compounds were weighed and dissolved in 0.1% DMSO to get required concentrations. The compound treatments were initiated on the day 7 of tumor transplantation on the advanced stage of tumor when the cells enter into exponential growth period.
2.6.1.1. Animals and tumor model

Inbred Swiss albino mice, 6-8 weeks old, weighing 25±5 g of either sex, were used for the experiments. They were Inbred and maintained in the Animal House, Department of Studies in Zoology, Manasagangotri, Mysore, India. Ehrlich ascites tumor was grown in adult Swiss albino mice intraperitoneally (ip). Cell viability was tested by trypan blue exclusion assay. Experimental animals were prepared by injecting 5×10⁶ viable tumor cells into the intraperitoneal cavity of Swiss mice. Tumor growth was followed by recording the animal weights. EAT cells begin their exponential growth phase from the 7th day after tumor cell injection and the animal succumbs to the ascites tumor burden on day 16-20 after injection.

2.6.1.2. Animal survival

After 7 days of tumor cell injection, the animals were divided into groups of 10 each and were treated as follows: Control: 0.2 mL of 0.1% DMSO was given on day 7, 9 and 11 of tumor transplantation. Compound treated groups the compounds 8(a-d) and 9(a-e) were given to different groups of tumor bearing mice. The compound 100 mg/kg body wt was injected intraperitoneally (ip) into the mice using 26 gauge needle on day 7, 9, and 11 of tumor transplantation. All the mice were weighed on the day of tumor inoculation and at weekly intervals. Animal survival was recorded up to 40 days. The tumor response was assessed on the basis of median survival time (MST) and percent increase in life span (% ILS). MST and % ILS was calculated from the mortality data within the observation period. Enhancements of life span by 25% are more over that of the control was considered as effective anti-tumor response [148].

2.6.1.3. Tumor growth inhibition and antiangiogenesis

Ehrlich ascites tumor-bearing mice were divided into groups of five each and treated as follows. Group 1 (control) 0.2 mL of 0.1% DMSO on days 7, 9, and 11 after tumor
transplantation. Group 2 (compounds 8(a-d) and 9(a-e) -treated groups): at a dosage of 100 mg/kg body weight was injected i.p. on days 7, 9, and 11 after tumor cell inoculation. The tumor growth response was assessed by measuring the cell number and ascites volume. On day 12, the control and compound 8(a-d) and 9(a-e) treated tumor-bearing mice were sacrificed, an incision was made in the abdominal region and EAT cells along with the ascites fluid were harvested into a beaker containing 2 mL saline and centrifuged at 3000 rpm for 10 min at 4°C, subtracting the volume of saline added previously from the volume of the supernatant gave the volume of ascites fluid. After harvesting the EAT cells, the cells were resuspended in 0.9% saline and counted using a haemocytometer.

2.6.1.4. Cytological changes in EAT cells
The cells were collected from the control and treated compounds 8(a-d) and 9(a-e) tumor-bearing mice on day 12 after tumor transplantation and were studied for the changes in the morphology of EAT cells.

2.6.1.5. Ethidium bromide/acridine orange staining
The harvested EAT cells were smeared on clean glass slide and fixed in methanol and acetic acid and stained with Wright’s giemsa stain.

Nuclear staining was performed according to the method describe by Srinivas et al., [149]. In brief EAT cells of control and compounds 8(a-d) to 9(a-e) treated mice were smeared on a clean glass slide, fixed with methanol/acetic acid (3:1), and air dried in a humidified chamber. The cells were hydrated with phosphate-buffered saline (PBS) and stained with a mixture of ethidium bromide/acridine orange (1:1), (10 mg/mL) solutions. The cells were immediately washed with PBS thrice and viewed under Leitz- DIAPLAN fluroscence microscope [150].
2.6.1.6. Antiangiogenic effects of the compounds on mouse peritoneum

The peritoneum of the mice were cut open on day 12 after tumor cell inoculation and the inner lining of the peritoneal activity were examined for angiogenesis in both control and 8(a-d) to 9(a-e) treated tumor-bearing mice and photographed [151].

2.6.1.7. Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA). Values of p<0.05 were considered significant.

![Graph showing body weight change](image)

**Fig 1.** Change of body weight in compounds treated mice group and control results expressed as body weight of mouse in gram.
**Fig 2.** Effect (*in vivo*) of compounds 8(a-d) and 9(a-e) on proliferation of EAT cells and formation ascites volume.

**Fig 3.** Effect of 8(a-d) and 9(a-e) on cell number of EAT bearing mice. The treatments showed difference in cell number from the control. The error bars represent standard deviation of the mean.
Fig 4. Supression of angiogenesis *in vivo* by 8(a-d) and 9(a-e) decreased blood vessels was observed in treated groups compared to control.

Fig 5. Changes in the morphology of EAT cells after treatment with synthetic compounds 8(a-d) and 9(a-e) acridine orange:ethidium bromide staining. The apoptotic bodies and condensed nuclei are evident in the compound treated acridine orange staining with EAT cells.
2.7. Results and discussion

Table 10. Effect of 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 8(a-d) and 9(a-e) on survival of mice bearing Ehrlich Ascites Tumor

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>No of Animals</th>
<th>Schedule Days</th>
<th>MST Days</th>
<th>ILS (%)</th>
<th>Avg. Wt Changes.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.1% DMSO)</td>
<td>5</td>
<td>7,9,11</td>
<td>16</td>
<td>---</td>
<td>8.62 ± 0.29</td>
</tr>
<tr>
<td>8a</td>
<td>5</td>
<td>7,9,11</td>
<td>18</td>
<td>12.5</td>
<td>-1.87 ± 0.13</td>
</tr>
<tr>
<td>8b</td>
<td>5</td>
<td>7,9,11</td>
<td>20</td>
<td>25.00</td>
<td>-2.02± 0.25</td>
</tr>
<tr>
<td>8c</td>
<td>5</td>
<td>7,9,11</td>
<td>22</td>
<td>37.5</td>
<td>-3.67 ± 0.33</td>
</tr>
<tr>
<td>8d</td>
<td>5</td>
<td>7,9,11</td>
<td>32</td>
<td>100</td>
<td>-4.60 ± 0.40</td>
</tr>
<tr>
<td>9a</td>
<td>5</td>
<td>7,9,11</td>
<td>31</td>
<td>93.75</td>
<td>-4.59 ± 0.39</td>
</tr>
<tr>
<td>9b</td>
<td>5</td>
<td>7,9,11</td>
<td>19</td>
<td>16.75</td>
<td>- 2.13 ± 0.72</td>
</tr>
<tr>
<td>9c</td>
<td>5</td>
<td>7,9,11</td>
<td>18</td>
<td>12.5</td>
<td>-1.6 ± 0.40</td>
</tr>
<tr>
<td>9d</td>
<td>5</td>
<td>7,9,11</td>
<td>21</td>
<td>31.25</td>
<td>-3.47 ± 52</td>
</tr>
<tr>
<td>9e</td>
<td>5</td>
<td>7,9,11</td>
<td>17</td>
<td>6.25</td>
<td>-1.55±0.45</td>
</tr>
</tbody>
</table>
2.7.1. Growth inhibition of Ehrlich Ascites Tumor \textit{in vivo}

The data on decreasing body weight are presented in Table 10. In the vehicle injected controls the tumor grew progressively and body weight increased gradually, the treatment of compounds produced significant decrease in the body weight. Intraperitoneal administration of three doses of synthesized compounds 8(a-d) and 9(a-e) on days 7, 9, and 11 after tumor transplantation showed effective anti-tumor response and resulted in decrease in the body weights of treated animals. Compounds 8d and 9a showed higher tumor inhibitory compared to other compounds in the series. The body weight of controlled treated mice increased gradually, the treated group exhibited reduction in body weights due to deceased tumor burden and showed no other toxic side effects. The results demonstrated that the compounds were effective in suppressing the proliferation of tumor cells.

2.7.1.2. Ascites volume and cell number

The inhibitory effects of 8(a-d) and 9(a-e) derivatives on EAT cells \textit{in vivo} was evaluated in terms of volume of ascites and total number of cells in mice treated with vehicle or compounds. From the results obtained the mean cell number and ascites volume in control animals was found to be 7.25±25 mL, and 1700.2±2 mL x 10^6 cells/mouse respectively (Fig 4 and 5). Among the treated compounds 8(a-d) and 9(a-e), the best response was obtained with the compounds 8d and 9a. Compound 8d decreases the mean ascites volume to 2.8±55 mL with corresponding reduction of mean cell number to 800.4±4x10^6 cells/mouse. Compound 9a showed the mean ascites volume to 2.5±5 mL, with corresponding reduction of mean cell number to 750.6±6 x 10^6 cells/mouse. The compounds 8d and 9a showed mean ascites volume of 2.8±55 mL, and 2.5±5 mL, which is more than 60% less than the control. This indicates the compounds were efficient in delaying tumor-growth as they show significant decrease in mean number of cell number.
and ascites volume, the remaining compounds were ineffective in prolonging the life span of EAT bearing mice.

2.7.1.3. Inhibition of tumor induced neovascularization by synthesized 2-methyl-3-(2-(piperazin-1-yl)ethyl)-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one derivatives 8(a-d) and 9(a-e)

A greater number of blood vessels were observed in peritoneum of EAT bearing vehicle treated control mice. The peritoneum wall tissue of 8(a-d) and 9(a-e) treated tumor bearing mice exhibited significant decrease in blood vessel formation compared to the control. From the results obtained (Fig 1) treatment of synthesized compounds 8d and 9a to EAT bearing mice. Significantly decreased peritoneal angiogenesis suggesting the inhibiton of the secretion of ascites fluid and their by preventing the formation of new blood vessels.

2.7.1.4. Changes in morphology of EAT cells

The inhibitory effects of synthesized compounds may be due to induction of apoptosis. The EAT cells were stained with nuclear stain (ethidium bromide/acridine orange) and the slides were observed under microscope and photographed. The apoptotic bodies and nuclear condensation are evident in compound treated groups.

2.8. Conclusion

From our studies it is clear that the compounds 8d and 9a as anti angiogenic effect as shown by peritoneal angiogenesis assay and reduction in the EAT cell number, ascities volume, changes in morphology of EAT cells, and body weight of the animals in vivo. The above study shed light toward the identification of new anti-angiogenic molecules to the cancer therapy. Further research to know the mechanism of inhibition and modifications of the compounds 8d and 9a to improve their potency is currently under progress in our laboratory.
2.9. $^1$H NMR Spectra of 3-(2-(4-(4-chlorophenylsulfonyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (8b)
$^1$H NMR Spectra of 3-(2-(4-(4-tert-butylphenylsulfonyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one (8d)
$^1$H NMR Spectra of 3-(2-(4-(3,5-dinitrobenzoyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-$4H$-pyrido[1,2-$a$]pyrimidin-4-one (9e)
$^{1}$H NMR Spectra of 3-(2-(4-(4-tert-butylbenzoyl)piperazin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydro-4H-pyrdo[1,2-a]pyrimidin-4-one (9f)
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