SYNTHESIS AND ANTIMICROBIAL ACTIVITY SCREENING OF 4, 6-DISUBSTITUTED AND 2,4,6-TRISUBSTITUTED THIENO[2,3-D] PYRIMIDINE ANALOGUES

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ABSTRACT : In an attempt to study the biological activities of 4, 6-disubstituted and 2,4,6-trisubstituted thieno[2,3-d] pyrimidine and series of thieno[2,3-d] pyrimidine analogues were synthesized and characterized by IR and ^{1}H NMR and Mass spectroscopy. All the targeted compounds were screened for their antibacterial activity against S.aureus, B.subtilis E.coli, P.aeruginosa and antifungal activity against Candida albicans and Candida krusei. Among synthesized compounds 5a showed potent antibacterial activity against all the strains but it was found to be more potent on S. aureus. Compound 5b and 5c shows more potent on E.coli on compare to other bacterial. Compound 5b shows more potent against C. albicans and compound 5c against C. krusei.

Key words: Antibacterial activity, Antifungal activity, Streptomycin and Clotrimazole.

I.INTRODUCTION

Antimicrobial agents are the natural or synthetic compounds which at certain concentrations inhibit the growth of or kill microorganisms completely. The term antimicrobials are collective for antiviral, antibacterial, antifungal and antiprotozoal. Due to the rapid development of microorganism's resistance to antimicrobial agents, it is necessary to discover new synthetic compound to help in the battle against pathogenic microorganisms.

Thienopyrimidines are a class of fused heterocycles which are common sources for thedevelopment of new potential therapeutic agents. There are three isomeric thienopyrimidines corresponding to the three possible types of annulation of thiophene to the pyrimidine ring:thieno[2,3-d]pyrimidine, thieno[3,4-d]pyrimidine, and thieno[3,2-d]pyrimidine.

Antiallergic,[1] antiatherosclerotic,[2] antibacterial,[3] antidepressive,[4] antidiabetic,[5] antihypertensive,[6] antihistaminic,[7] analgesic and anti-inflammatory,[8] antiviral[9] and spasmolytic[3] activities have been reported for certain thienopyrimidine derivatives.

The similarity between the physicochemical properties of benzene and thiophene is striking.

For example, the boiling point of benzene is 81.1°C and the one of thiophene is 84.4°C (at 760mm Hg) and therefore, thiophene and benzene are a well known example of bioisosterism. The change of a benzene moiety into a thiophene often results in superior pharmacodynamic, pharmacokinetic, or toxicological properties. For example, the thiophene analogue of piroxicam (a non-steroid anti-inflammatory agent used in arthritis patients) has the same biological activity, with the same mechanism of action as piroxicam, and even displayed a longer plasma half-life than piroxicam.[10] Thiophene isosteres of mianserin (a tetracyclic antidepressive agent) also act as serotonin receptor (5-HT) antagonists.[11]

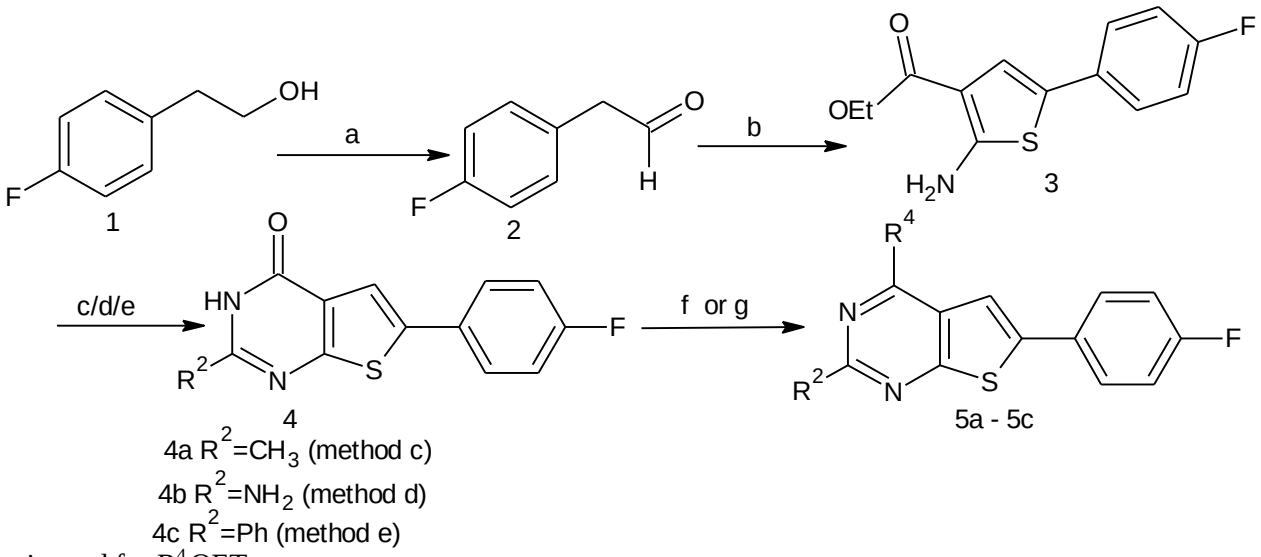
However, this study focuses on the synthesis and biological evaluation of the thienopyrimidines compounds as antibacterial and antifungal agents.

II.METHOD AND METHODOLOGY

Present work

Synthesis of thieno[2,3-d]pyrimidine analogues

Within the isomeric thieno[3,2-d]pyrimidine series, a versatile starting material has beendescribed in literature.[45] 6-Bromo-4-chloro-thieno[3,2-d]pyrimidine(BOP) can beregioselectivity functionalized at positions 4 and 6. Palladium-catalyzed cross-coupling reactionsoccur exclusively at position 6, whereas the reaction with amines results in displacement of thechlorine at position 4. Therefore, it was envisioned that the corresponding 6-bromo-4-chloro-2-substituted-thieno[2,3-d]pyrimidine could similarly act as a versatile building block for the introduction of various substituent at position 4 and 6 to build up thieno[2,3-d]pyrimidinelibraries.



4a R^2=CH_3 (method c)

4b R^2=NH_2 (method d)

4c R^2=Ph (method e)

g is used for R^4 OET

a) PCC, CH2Cl2, rt; b) ethyl cyanoacetate, S, NEt3, DMF, 50°C to rt; c) RCN, 4 M HCl in dioxane, rt; d) chloroformamidine hydrochloride, dimethylsulfone, 130°C; e) PhCN, 4 M HCl in dioxane, rt then DMF, 100°C; f) BOP, DBU, amine, CH3CN, rt to 60°C; g) BOP, DBU, ethanol, CH3CN, rt to 60°C then ethanol, Na, reflux;

Figure: Reaction scheme for synthesis of new thieno[2,3-d]pyrimidine

Experimental part

General information

For all reactions, analytical grade solvents were used. All moisture-sensitive reactions were carried out in oven-dried glass-ware (135 °C). 1H and 13C NMR spectra were recorded with a Bruker Advance 300 (^{1}H NMR: 300 MHz, ^{13}C NMR: 75 MHz), using tetramethylsilane asinternal standard for ^{1}H NMR spectra and DMSO-*d6* (39.5 ppm) or CDCl3 (77.2 ppm) for ^{13}C NMR spectra. Abbreviations used are: s = singlet, d = doublet, t = triplet, q = quartet, m =multiplet, br s = broad signal. Coupling constants are expressed in Hertz. Mass spectra areobtained with a Finnigan LCQ advantage Max (ion trap) mass spectrophotometer from ThermoFinnigan, San Jose, CA, USA. Exact mass measurements are performed on a quadrupole time offlightmass spectrometer (Q-tof-2, Micromass, Manchester, UK) equipped with a standardelectrospray-ionization (ESI) interface. Samples were infused in *i*-PrOH/H2O (1:1) at 3 µl/min.

Melting points are determined on a Barnstead IA 9200 apparatus and are uncorrected. Pre-coated aluminum sheets (Fluka Silica gel/TLC-cards, 254 nm) were used for TLC. Column chromatography was performed on ICN silica gel 63-200, 60 Å.

2-(4-Fluorophenyl) acetaldehyde (2)

To a stirred suspension of pyridinium chlorochromate (6.9 g, 21.4 mmol) in CH2Cl2 (100 ml) was added asolution of 2-(4-fluorophenyl) ethanol **1**(3.0 g, 21.4 mmol) in CH_2Cl_2 (10 ml). The resulting suspensionwas stirred for 2 hours at room temperature and was then diluted with ether. The resulting suspension wasfiltered through a pad of Celite and washed with ether. The solvents were removed under reduced pressureto yield the crude title compound **2**

Ethyl 2-amino-5-(4-fluorophenyl)thiophene-3-carboxylate (3)

Triethylamine (0.98 ml, 7.01 mmol) was added to a stirred suspension of ethyl cyanoacetate (2.79 ml, 13.7mmol) and sulfur (0.44 g, 13.7 mmol) in DMF (70 ml). A solution of 2-(4-fluorophenyl)acetaldehyde **2**(1.9 g, 13.7 mmol) in DMF (5 ml) was added dropwise over a period of 50 minutes, while the temperaturewas maintained at 50°C. The solution was cooled down to room temperature and stirred overnight. Thereaction was poured into water and the aqueous phase was extracted with diethylether. The organic layerwas separated and washed with water, brine and dried over Na_2SO_4. The solvents were evaporated and thecrude residue was purified by flash chromatography on silica gel (EtOAc/Hexane 1:15) to yield the titlecompound.

6-(4-Fluorophenyl)-2-methylthieno[2,3-d]pyrimidin-4(3*H*)-one (4a)

To a solution of ethyl 2-amino-5-(4-fluorophenyl)thiophene-3-carboxylate**3**(0.3 g, 1.13 mmol) andacetonitrile (0.56 ml, 11.3 mmol) in dioxane (4 ml) was added 4M HCl in dioxane (4 ml). The mixture wasstirred at room temperature overnight. The solvents were removed under reduced pressure. The residue wasdiluted with water and made alcaline with a saturated aqueous sodium bicarbonate solution. The precipitatewas filtered off, washed with

water and dried. The crude residue was purified by flash chromatography onsilica gel (CH_2Cl_2/MeOH 60:1) to yield the title compound.

2-Amino-6-(4-fluorophenyl)thieno[2,3-d]pyrimidin-4(3*H*)-one (4b)

A mixture of ethyl 2-amino-5-(4-fluorophenyl)thiophene-3-carboxylate**3**(0.3 g, 1.13 mmol),chloroformamidine hydrochloride (0.33 g, 2.83 mmol) and dimethylsulfone (0.53 g, 5.65 mmol) was heatedat 120-130°C for 30 minutes. After cooling down to room temperature, water (10 ml) was added andammonium hydroxide was used to neutralize the suspension. The solid was filtered off, washed with waterand dried. The crude residue was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 10:1) toyield the title compound.

6-(4-Fluorophenyl)-2-phenylthieno[2,3-d]pyrimidin-4(3*H*)-one (4c)

To a solution of ethyl 2-amino-5-(4-fluorophenyl)thiophene-3-carboxylate **3**(0.2 g, 0.75 mmol) andbenzonitrile (0.23 g, 2.26 mmol) in dioxane (4 ml) was added 4M HCl in dioxane (4 ml). The mixture wasstirred at room temperature overnight. The precipitate was filtered off, washed with diethyl ether and dried.The solid was redissolved in DMF and the mixture was heated at 100oC for 3 hours. The solvents wereremoved under reduced pressure. The residue was diluted with water and the solid was filtered off, washedwith water and dried. The crude residue was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH60:1) to yield the title compound.

Ethyl 6-(4-fluorophenyl)-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-2-carboxylate (4d)

Ethyl 2-amino-5-(4-fluorophenyl)thiophene-3-carboxylate (3) (0.98 ml, 7.01 mmol) was added to a stirred suspension of ethyl cyanoformate (2.79 ml, 13.7mmol) and sulfur (0.44 g, 13.7 mmol) in DMF (70 ml). A solution of 2-(4-fluorophenyl)acetaldehyde **2**(1.9 g, 13.7 mmol) in DMF (5 ml) was added dropwise over a period of 50 minutes, while the temperaturewas maintained at 50^oC. The solution was cooled down to room temperature and stirred overnight. Thereaction was poured into water and the aqueous phase was extracted with diethylether. The organic layerwas separated and washed with water, brine and dried over Na_2SO_4. The solvents were evaporated and thecrude residue was purified by flash chromatography on silica gel (EtOAc/Hexane 1:15) to yield the titlecompound.

Ethyl 2-(6-(4-fluorophenyl)-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)acetate (4e)

Ethyl 2-amino-5-(4-fluorophenyl)thiophene-3-carboxylate (3) (0.98 ml, 7.01 mmol) was added to a stirred suspension of ethyl cyanoacetate (2.79 ml, 13.7mmol) and sulfur (0.44 g, 13.7 mmol) in DMF (70 ml). A solution of 2-(4-fluorophenyl)acetaldehyde(1.9 g, 13.7 mmol) in DMF (5 ml) was added dropwise over a period of 50 minutes, while the temperature was maintained at 50oC. The solution was cooled down to room temperature and stirred overnight. The
reaction was poured into water and the aqueous phase was extracted with diethylether. The organic layer was separated and washed with water, brine and dried over Na2SO4. The solvents were evaporated and the crude residue was purified by flash chromatography on silica gel (EtOAc/Hexane 1:15) to yield the title compound.

2-(4-Chlorophenoxy)-1-(4-(6-(4-fluorophenyl)-2-methylthieno[2,3-d]pyrimidin-4-yl)piperazin-1-yl)ethanone (5a)

To a solution of 6-(4-fluorophenyl)-2-methylthieno[2,3-d]pyrimidin-4(3*H*)-one **4a** (40 mg, 0.15 mmol)and BOP (88 mg, 0.20 mmol) in CH3CN (1 ml) was added DBU (34 µl, 0.23 mmol). After stirring for 10minutes at room temperature, 2-(4-chlorophenoxy)-1-(piperazin-1-yl)ethanone (59 mg, 0.23 mmol) wasadded. The reaction was stirred at room temperature overnight and then heated at 60oC for 4 hours. Thesolvents were removed under reduced pressure, and the crude residue was purified by flashchromatography on silica gel (CH_2Cl_2/MeOH 100:1) to yield the title compound.

Yield: (66 mg,) 86%; M.P.: 165^oC; IR: 2946.7 (C-H), 1647.3 (C=N) ; ^{1}HNMR: 8.70 (s, 1H, CH of imino), 7.79 (d, 2H, Ar-H), 7.21 (d, 2H, Ar-H), 3.22 (m, 2H, CH2), 2.89 (m, 2H, CH2), 2.43 (m, 2H, CH2), 1.57 (m, 3H, CH3) ; Mass: 327; M^+

1-(4-(2-Amino-6-(4-fluorophenyl) thieno[2,3-d]pyrimidin-4-yl)piperazin-1-yl)-2-(4-chloro phenoxy)ethanone (5b)

This compound was prepared from **4b**, according to the procedure for the synthesis ofcompound **5a**.To a solution of **2-Amino-6-(4-fluorophenyl) thieno[2,3-d] pyrimidin-4(3*H*)-one 4b**(40 mg, 0.15 mmol)and BOP (88 mg, 0.20 mmol) in CH3CN (1 ml) was added DBU (34 µl, 0.23 mmol). After stirring for 10minutes at room temperature, 2-(4-chlorophenoxy)-1-(piperazin-1-yl)ethanone (59 mg, 0.23 mmol) wasadded. The reaction was stirred at room temperature overnight and then heated at 60^oC for 4 hours. Thesolvents were removed under reduced pressure, and the crude residue was purified by flashchromatography on silica gel (CH_2Cl_2/MeOH 100:1) to yield the title compound.

Yield: of 55%,; MP 128^oC; IR: 2917.9 (C-H), 1651.4 (C=N) ; ^{1}H MNR: 8.30 (s, 1H, CH of imino), 7.61 (m, 2H, Ar-H), 7.52 (m, 3H, Ar-H), 2.96 (m, 2H, CH2), 2.40 (m, 2H, CH2), 2.25 (m, 3H, CH3), 1.72 (m, 2H, CH2), 1.59

(m, 4H, CH2) ; Mass: 321; M+

2-(4-Chlorophenoxy)-1-(4-(6-(4-fluorophenyl)-2-phenylthieno[2,3-d]pyrimidin-4-yl)piperazin-1-yl)ethanone (5c)

This compound was prepared from **4c** according to the procedure for the synthesis ofcompound **5a**. To a solution of **6-(4-Fluorophenyl)-2-phenylthieno[2,3-d] pyrimidin-4(3*H*)-one (4c)** (40 mg, 0.15 mmol)and BOP (88 mg, 0.20 mmol) in CH3CN (1 ml) was added DBU (34 µl, 0.23 mmol). After stirring for 10minutes at room temperature, 2-(4-chlorophenoxy)-1-(piperazin-1-yl) ethanone (59 mg, 0.23 mmol) wasadded. The reaction was stirred at room temperature overnight and then heated at 60°C for 4 hours. Thesolvents were removed under reduced pressure, and the crude residue was purified by flashchromatography on silica gel (CH_2Cl_2/MeOH 100:1) to yield the title compound.

Yield:77%,; MP: 220-221oC; IR: 2950.6 (C-H), 1623.3 (C=N) ; ^{1}H NMR: 8.36 (s, 1H, CH of imino), 7.61 (m, 2H, Ar-H), 7.51 (m, 2H, Ar-H), 3.31 (m, 2H, -CH2), 3.09 (s, 6H, 2CH3), 2.81 (m, 2H, CH2), 2.12 (m, 3H, CH3), 1.71 (m, 2H, CH2), 1.68 (s, 4H, 2CH2) ; Mass: 364; M+

Table 1: Synthesized thieno[2,3-d] pyrimidines

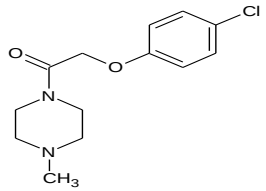
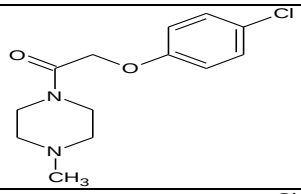
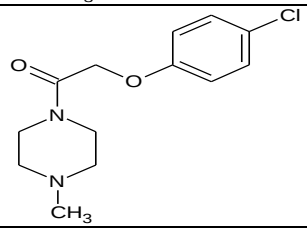
Compound	R^2	R^4
5a	CH_3	
5b	NH_2	
5c	C_6H_5	

Table 2: Physical characterization of synthesized compound (5a - 5e)

Sl. No	Compound	Molecular formula	Melting point	% yield	Colour	R_f value
1	5a	$C_{25}H_{23}ClFN_4O_2S$	165°C	86%	white solid	0.85
2	**5b**	$C_{24}H_{22}ClFN_5O_2S$	128°C	55%	white solid	0.88
3	**5c**	$C_{30}H_{25}ClFN_4O_2S$	220-221°C	77%	white solid	0.84
4	**5d**	$C_{27}H_{25}ClFN_4O_4S$	192-193°C	89%,	white solid	0.86
5	**5e**	$C_{28}H_{27}ClFN_4O_4S$	114-115°C	58%	white solid	0.82

Antimicrobial activity

The experimental methods that were employed for the evaluation of title compounds for antibacterial, antifungal have been described here under.

Anti-bacterial activity

The titled compounds were evaluated for antibacterial activity as per the reported methods[12-14]. The antibacterial activity of synthesized compounds was carried out against two gram positive bacteria viz., S.aureus, B.subtilis and two gram negative bacteria viz., E.coli and P.aeruginosa by using disc diffusion method. Streptomycin was used as standard to compare the results of antibacterial activity.

Preparation of test solution

The entire test compounds equivalent to concentration of 1000µg/ml and 500µg/ml were prepared by dissolving in Dimethylsulphoxide (DMSO).

Preparation of standard solution

Weight equivalent to concentration of 100µg/ml was prepared by dissolving streptomycin in DMSO.

Measurement of zone of inhibition

The inoculum of test organism was spreaded on the prepared agar plates by using a glass spreader under laminar airflow cabinet. Sterile discs were soaked in the desired test solution for about 15 mins and the discs were placed on the agar plates appropriately. Then the plates were kept undisturbed for at least 2h at room temperature to allow proper diffusion of the test and standard solution into the nutrient agar medium, later plates were incubated at 37±1ºC for 24h. Measurement of the diameter of zone of inhibition, simultaneously controls was maintained employing DMSO to observe the solvent effects. The results of antibacterial activity expressed in terms of zone of inhibition along with standard deviation are presented in tables 3.

Antifungal activity

All the synthesiszed compounds were evaluated for antifungal activity. The fungi selected for this screening were Candida albicans and Candida krusei. The anti-fungal activity was done by employing filter paper strip method in Sabouraud dextrose and czapexs dox agar medium[12-14]. The test compounds were screened for antifungal activity using agar medium. Result is given in table no. 3.

Statistical analysis: All the tests were done in triplicate and expressed as mean and standard deviation.

Table 3: Antibacterial and Anti-fungal activity of new thieno[2,3-d] pyrimidine derivatives

Compound		Diameter of zone of inhibition(mm) Mean# ± SD$					
		Antibacterial activity				Antifungal activity	
		S. aureus	B. subtilis	P. aeruginosa	E. coli	C. albicans	C. krusei
5a	500µg	16±0.5	15±0.5	16±0.5	16±0.5	13±0.6	12±0.3
	1000µg	20±0.6	19±0.4	19±0.2	19±0.5	15±0.7	15±0.5
5b	500µg	13±0.3	12±0.6	13±1.9	14±0.6	12±0.6	12±0.8
	1000µg	16±0.9	17±0.7	16±0.8	18±0.8	16±0.7	15±0.7
5c	500µg	13±1.7	13±0.6	14±0.3	13±0.3	13±0.9	14±1.0
	1000µg	16±1.5	17±0.4	17±0.5	19±0.6	15±0.9	16±1.2
5d	500µg	11±0.5	14±0.5	15±0.5	15±0.5	12±0.6	13±0.3
5e	1000µg	18±0.6	17±0.4	18±0.2	18±0.5	15±0.7	14±0.5
Streptomycin	100 µg	23±0.4	24±0.3	23±0.4	24±0.1	-	-
Clotrimazole	100 µg	-	-	-	-	22±0.3	23±0.5

#Mean values of Zone of inhibition of individual experiment in triplicate (the diameter of the sterile disc (6mm) included), $ standard deviation; NZI = No zone of inhibition (zone diameter ≤ 6mm).

III.RESULT AND DISCUSSION

The synthesized compounds were after synthesis and purification taken for spectral analysis like IR, ^{1}H NMR and Mass spectroscopy. The spectrl data for each compound was given in experimental part of material and method section. Compound 5d had more % of yield.

All three synthesized compounds (thieno pyrimidines) have been evaluated for *in vitro* antibacterial activity using disc diffusion method. Streptomycin was taken as standard to compare antibacterial potency of test compounds on four strains of bacteria in which two were gram positive and two were gram negative. The diameter of zone of inhibition of each test compound was measured at 500µg/ml and 1000µg/ml concentration. However, in the present discussion only the diameter of the zone of inhibition (Mean±SD) of test compounds at 1000µg concentration is considered for comparison.

Among synthesized compounds 5a showed potent antibacterial activity against all the strains but it was found to be more potent on **S. aureus**. Compound 5b and 5c shows more potent on E.coli on compare to other bacterial.

All the three compounds of thieno pyrimidines have been subjected to *in vitro* antifungal activity against two fungal strains, two from *Candida* species. The results thus obtained are compared with standard antifungal agent Clotrimazole. The diameter of zone of inhibition of each test compound was measured at 500µg/ml and 1000 µg/ml concentration. However, in the present discussion only the diameter of the zone of inhibition (Mean ± SD) of test compounds at 1000 µg concentration is considered for comparison.

Among the synthesized compound 5b shows more potent against **C. albicans** and compound c against **C. krusei.**

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