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Synthesis and Pharmacological Screening of Some Novel 2-arylhydrazino and 2-aryloxy- Pyrimido [2,1-b] Benzothiazole Derivatives

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Abstract: In the present study we have synthesized a series of 2-substituted-9-chloro-8-fluoropyrimido [2,1-b]benzothiazole-3-cyano-4(H)-ones. These series was synthesized by the reaction of 9-chloro-8-fluoropyrimido[2,1-b]benzothiazole-2-thiomethyl-3-cyano-4(H)-one with various arylamino in the presence of dimethyl formamide and anhydrous potassium carbonate. 9-chloro-8-fluoropyrimido [2,1-b]benzothiazole-2-thiomethyl-3-cyano-4(H)-ones were prepared by condensation of 2-amino-7-chloro-6-fluorobenzothiazole with ethyl-2-cyano-3,3-bismethylthio acrylate in the presence of dimethyl formamide and anhydrous potassium carbonate. All the synthesized compounds were screened for their anti-inflammatory activity *in-vitro* and *in-vivo*, antibacterial and antifungal activity. Selected compounds were also subjected for analgesic activity by acetic acid induced writhing method in albino mice.

Key words: Chloro-fluoro compounds · Pyrimidine · Biological activity · Antifungal and anti-inflammatory activity

INTRODUCTION

A literature survey reveals that very few references are available on the synthesis of pyrimido benzothiazole compounds [1-8] and continuation of our work on the synthesis of flouro-chloro-substituted benzothiazole. It is well known that the introduction of fluorine atom into an organic molecule causes dramatic changes in its biological profile, mainly due to high electronegativity of fluorine causes increase lipid solubility. For this purpose 2-amino 7-chloro- 6-fluoro benzothiazole is selected as 'synthon'. It is planned to synthesize some biologically and pharmacologically potent new pyrimido benzothiazole derivatives.

Experimental: The present work reported one pot synthesis of new pyrimido [2,1-b] benzothiazole-4-ones. The required 2-amino-7-chloro-6-fluorobenzothiazole was prepared from the 3-chloro-4-fluoroaniline on reaction with potassium thiocyanate in presence of bromine in acetic acid followed by the basification with ammonia adopting the standard procedure. Similarly, ethyl-2-cyano-3,3-bismethylthioacrylate was prepared by the condensation of ethyl cyanoacetate with carbon disulphide in sodium ethoxide/ ethanol, methylation of the sodium salt, followed by the condensation of resulting ethyl-2-cyano-3,3-Bismethylthioacrylate adopting a standard procedure.

Synthesis of 2-amino-6-fluoro-7-chloro benzothiazole (II)
General Procedure: To glacial acetic acid (20ml) cooled below room temperature were added 8gm (0.08mol) of potassium thiocyanate and 1.45gm (0.01 mol) of fluoro chloro aniline. The mixture was placed in freezing mixture of ice and salt and mechanically stirred, 1.6ml of bromine in 6ml of glacial acetic acid was added, from a dropping funnel at such a rate that the temperature never rose beyond room temperature. After all the bromine was added (105min), the solution was stirred for 2 hrs below room temperature and at room temperature for 10 hrs, it was then allowed to stand over night, during which period an orange precipitate settle at the bottom. Water (6ml) was added quickly and slurry was heated at 85°C on a steam bath and filter while hot. The orange residue was placed in a reaction flask and treated with 10ml of glacial acetic acid heated again to 85°C and filtered quickly. The combined filtrate was cooled and neutralized with concentrated ammonia solution to pH-6. A dark yellow

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precipitate was collected. Recrystallized from benzene, ethanol of (1:1) after treatment with animal charcoal gave yellow plates of 2-amino-6-fluoro-7-chloro-(1,3)-benzothiazole. After drying in a oven at 80°C, the dry product (1gm, 51.02%) melted at 210-212°C, was obtained.

Synthesisofethyl-2-cyano-3,3-bismethylthioacrylate(III)
General Procedure: 6 gm of Na metal was dissolved in minimum quantity of ethanol cooled in ice bath and added 11.3ml of ethyl cyanoacetate. The mixture was treated drop wise with cooling and 0.1ml of carbon disulphide added drop-wise below 0°C and stirred for 1 hr, then the reaction mixture was allowed to stand for 1hr at room temperature and added 25.2 gm of dimethylsuphide drop by drop with stirring 10-15°C, the mixture was allowed to stand for 12 hrs at room temperature and poured into ice water. The solid obtained was filtered and washed with water.

Synthesis of 9-chloro-8-fluoropyrimido [2,1-b] benzothiazole-2-thiomethyl-3-cyano-4(H)-one(IV)
General Procedure: A mixture of 2-amino-6-fluoro-7-chloro benzothiazole (0.01mol) and ethyl -2-cyano3,3-bis methylthioacrylate (0.01mol) in 20-25ml of DMF and a pinch of anhydrous potassium carbonate was refluxed for 2-7 hrs. The reaction mixture was cooled to room temperature and poured into ice cold water. The separated solid product was filtered, washed with water and recrystallized from DMF: ethanol mixture.

Synthesis of 2-aryloxy-9-chloro-8-fluoropyrimido [2,1-b] benzothiazole-3-cyano-4(H)-ones(XB_{1-4})
General Procedure: Mixture of 9-chloro-8-fluoropyrimido [2,1-b]benzothiazole-2-thiomethyl-3-cyano-4-one (0.01mol) and different phenols (0.01mol) in 20-25ml of DMF and a pinch of anhydrous potassium carbonate was refluxed for 2-7 hrs. The reaction mixture was cooled to room temperature and poured in ice cold water. The separated solid product was filtered, washed with water and recrystallized from DMF: ethanol mixture to give a pure product.

Synthesis of 2-arylhydrazino-9-chloro-8-fluoropyrimido [2,1-b] benzothiazole-3-cyano-4(H)-ones (XC_{1-3})
General Procedure: Mixture of 9-chloro-8-fluoropyrimido [2,1-b]benzothiazole-2-thiomethyl-3-cyano-4-one (0.01mol) and different hydrazines (0.01mol) in 20-25ml of DMF and a pinch of anhydrous potassium carbonate was refluxed for 2-7 hrs. The reaction mixture was cooled to room temperature and poured into ice cold water. The separated solid product was filtered, washed with water and recrystallized from DMF: ethanol mixture to give a pure product.

Pharmacological Screening
Antibacterial Activity[9,11,12,13,15]
Cup Plate Method: The antibacterial activity of the synthesized compounds was studied systematically against four different strains of bacteria (gram-positive and gram-negative) by the agar diffusion method. All the test compounds were evaluated for antibacterial activity against *Staphylococcus aureus,Bacillus subtillis* (gram-positive), *Eschrichia coli, Pseudomonas* (gram-negative), by using the agar diffusion method of assay.

The organisms were subcultured using nutrient agar medium. The tubes containing sterilized medium were inoculated with respective bacterial strain. After incubation at 37±1°C for 24 hr. they were stored in a refrigerator. Thus stock cultures were maintained. Bacterial inoculum was prepared by transferring a loopful of stock culture to nutrient broth (100ml) in a clean and sterilized conical flask (250 ml). The flasks were incubated at 37±1°C for 18 hr. before the experimentation.

Solutions of the test compounds were prepared by dissolving 5 mg and 10 mg of each in dimethylformamide (10ml AR). A reference standard for gram-positive and gram-negative bacteria were made by dissolving accurately weighed quantity of Procaine Pencillin and Streptomycin, respectively in dimethylformamide solution, separately.

The nutrient agar medium was sterilized by autoclaving at 121°C (15 lb/sq. inch). The petri-plates, tubes and flasks plugged with cotton were sterilized in hot air-oven at 160°C, for an hour. Into each sterilized petri-plate (10cm diameter), about 30 ml each of molten nutrient bacteria (6 ml of inoculum to 300 ml of nutrient agar medium) was transferred, aseptically. The plates were left at room temperature to allow the solidification. In each plate, four cups of 6 mm diameter were made with a sterile borer. Then, 0.1 ml of the test solution was added to the cups, aseptically and labeled, accordingly. The plates were kept undisturbed for at least 2 hrs at room temperature to allow diffusion of the solution properly, into nutrient agar medium. After incubation of the plates at 37±1°C for 24 hr. the diameter of the zone of inhibition surrounding each of the cups was measured with the help of an 'antibiotic zone reader'. All the experiments were carried out in triplicate. Simultaneously, controls were maintained employing 0.1 ml of dimethylformamide to observe the solvent effects.

Minimal Inhibitory Concentration Method: The antibacterial activity of synthesized compounds were tested *in vitro* on strains of four microorganisms, *Escherichia coli, Bacillus subtilis, pseudomonas, Staphylococcus aureus*.

The antibacterial activity was evaluated by tube dilution method (turbidometric method). The turbidometric method depends upon the inhibition of growth of microbial culture in a uniform solution of antibacterial in a fluid medium that is favourable to its rapid growth in the absence of the antibacterial agent. In this method minimal inhibitory concentration (MIC) of the lowest concentration of an antibacterial agent that inhibits the growth of test organism can be detected.

The synthesized compounds were dissolved in DMF to prepare a stock solution of 1 mg/ml conc. with this stock solution different dilutions 800 µg to 5 µg/ml were prepared. The ciproflaxacin was also prepared in DMF to obtain a conc. of 800 µg/ml to 5 µg/ml.

Preparation of Double Strength Nutrient Media

Formula:

Peptone	1 gm.
Yeast	0.3 gm.
Sodium Chloride	0.5 gm.
Distill water	50 ml.

The solid ingredients were dissolved in water and pH adjusted to 7.4±0.2 and the media was sterilized by autoclaving at 15 lb/psi for 15 mins.

Preparation of Suspension of Micro Organism: Transferring the microorganism from culture to 5 ml of sterile normal saline (0.09%) solution made of each microorganism.

Determination of Minimal Inhibitory Concentration: The sterile test tube containing 1 ml of sterile media were added with 1 ml of different serially diluted test samples. To these tubes 0.1 ml of normal saline solution suspended with respective microorganism were inoculated and incubated at 37±2°C for 18 to 24 hrs. The growth in the tubes was observed visually for turbidity and inhibition was determined by lowest concentrations of sample that prevented the development of turbidity. The procedure was repeated to confirm the MIC.

Antifungal Activity [9]: All those compounds screened for antibacterial activity were also tested for their antifungal activity. The fungi employed for screening were: *Asperigillus flavus* and *Candida albicans*.

The test organisms were sub-cultured using potato dextrose agar medium. The tubes containing sterilized medium were inoculated with test fungi and after incubation at 25°C for 48 hr. they were stored 4° in a refrigerator.

The inoculum was prepared by taking a loopful of stock culture to about 100 ml of nutrient broth, in 250 ml clean ad sterilized conical flasks. The flasks were incubated at 25°C for 24 hr. before use.

The solutions of test substances were prepared by a similar procedure described under the antibacterial activity. A reference standard (0.5 mg and 1 mg/ml conc.) were prepared by dissolving 5 mg and 10 mg of Griseofulvin in 10ml of dimethylformamide to obtain a solutions of 50 µg/ml and 100 µg/ml concentration.

The potato-dextrose-agar medium was sterilized by autoclaving at 121°C (15 ib/sq. inch), for 15 minutes. The petri-plates, tubes and flasks plugged with cotton plugs were sterilized in hot air-oven at 150°C, for an hour. Into each sterilized petri-plate (10 cm diameter), about 30 ml each of molten potato dextrose-agar medium inoculated with respective fungus (6 ml of inoculum to 300 ml of potato-dextrose-agar medium) was transferred, aseptically. After solidification of the medium at room temperature four cups of 6mm diameter were made in each plate with a sterile borer. Accurately 0.1 ml (100 µg/ml conc.) of test solution was transferred to the cups, aseptically and labeled, accordingly. The reference standard 0.1 ml (50µg/ml conc., 100 µg/ml conc.) were also added to the cups in each plate. The plates were kept undisturbed for atleast two hours at room temperature to allow diffusion of the solution properly, into potato-dextrose-agar medium. Then the plates were incubated at 25°C for 48 hr. The diameter of the zone of inhibition was read with help of an 'antibiotic zone reader'. The experiments were performed in triplicate in order to minimize the errors.

Anti-Inflammatory Activity [3,6,16,19,20]

***In-vitro* Model:** Many invitro assays, each based on a specific biochemical or cellular mechanism have been developed for the initial screening of the anti-inflammatory compounds. A number of anti-inflammatory drugs are known to inhibit the denaturation of proteins as an invitro screening model for anti-inflammatory compounds.

The synthesized compounds are screened for anti-inflammatory activity by using inhibition of albumin denaturation technique. The standard drug and test compounds were dissolved in minimum amount of dimethyl formamide (DMF) and diluted with phosphate buffer (0.2 M, pH 7.4). Final concentration of DMF in

all solutions was less than 2.0%. Test solution (1 ml) containing different concentrations of drug was mixed with 1 ml of 1% mM albumin solution in phosphate buffer and incubated at 27°±1°C in BOD incubator for 15 min. Denaturation was induced by keeping the reaction mixture at 60°±1°C in water bath for 10 min. After cooling the turbidity was measured at 660 nm (UV-Visible Spectrophotometer SL-159, Elico India Ltd.). Percentage of inhibition of denaturation was calculated from control where no drug was added. Each experiment was done in triplicate and average was taken. The diclofenac sodium was used as standard drug.

$$\% \text{ of inhibition} = 100 \times ((Vc/Vt)-1)$$

Where, Vt and Vc are mean absorbance value of test group and control group.

Anti-inflammatory Activity (*In-vivo* Model) by Carrageenan Induced Paw Edema in Rat: Animals were divided into control, standard and different test groups comprising of five animals in each group. They were fasted overnight with free access to water before experiment. In all groups, acute inflammation was produced by sub-planter injection of 0.1ml of freshly prepared 1% suspension of carrageenan in the right hind paw of the rats and paw volume was measured plethysmometrically at 0 hr and 3hr after carrageenan injection. The test compounds (50mg/kg) was administered orally, standard group was treated with diclofenac (50mg/kg)orally 1 hrs before by the injection and control group received only vehicle. Mean difference in paw volume was measured statically by student t test (Dunnett). Mean difference in paw volume was measured and percentage inhibition was calculated by using formula

$$\% \text{ inhibition of edema} = (Vc-Vt / Vc) \times 100,$$

where Vt and Vc are the mean paw volume of test group and control group, respectively.

RESULTS AND DISCUSSION

All the compounds were characterized by running TLC, IR, NMR, MASS spectroscopy and elemental analysis. The melting points were determine by open capillary tube method and were uncorrected. The physicochemical parameters of all the synthesized compounds given in Table 1 and spectral analysis given in Table 2. The IR spectra of synthesized compounds were recorded on SHIMADZU FTIR-8400S spectrophotometer by using KBr pressed pellet technique. The IR spectrum of the compounds has shown characteristic absorption bands (in cm^{-1}) at 3310 (NH), 2217 (CN), 1650 (C=O), 1190(C-F) and 685 (C-CI). The proton NMR spectra are analyzed on Advance 300 MHz spectrometer The ^{1}HNMR spectrum of the compound (in DMSO-d6) has exhibited characteristic proton signal (in ä ppm) at 5.10 (s, 1H, NH-C_6H_5), 7.10-7.65 (m, 7H, Ar-H) and 8.16 (d, 1H, NH). The mass spectrum of the compound has recorded its molecular ion peak at m/z 385 (M^+) peak. This happens to be agreement with the mass number of assigned structure. The newly synthesized compounds have been screened for their pharmacological activities.

Table 1: Physicochemical Parameters of Synthesized Compound

SN	Comp Code	R	M.P.(°C)	Yield (%)	Mol. Formula	M.Wt
1	XB_1	2-Me	290	51	$C_{17}H_7ClFN_3O_3S$	387
2	XB_2	3-Me	310	47	$C_{17}H_7ClFN_3O_3S$	387
3	XB_3	4-NO_2	160	49	$C_{17}H_6ClFN_4O_4S$	416
4	XB_4	2-Cl	180	56	$C_{17}H_6C_{l2}FN_3O_2S$	406
5	XC_1	Ar(H)	112	57	$C_{17}H_9ClFN_5OS$	385
6	XC_2	Ar(2,4-NO_2)	142	62	$C_{11}H_5ClFN_5OS$	309
7	XC_3	H	183	60	$C_{17}H_7ClFN_7O_5S$	475

Table 2: Spectral data of the synthesized compounds

Comp No.	Spectral Analysis
XB_1	IR (KBr/cm^{-1})= C=N (2245), C=O (1690), O-Ar(1245), C-F (1160),C-Cl (715); elemental analysis(calculated/found)C%(52.66/52.20) H% (1.82/1.85) N% (10.84/10.90)
XB_2	IR (KBr/cm^{-1})=C=N (2220), C=O (1675), O-Ar (1252)C-F (1155), C-Cl (680); H^1 NMR ä/ppm in DMSO-D)= 6.93-7.60(m,6H, Ar-H); elemental analysis (calculated/found) C%(52.66/52.44) H%(1.82/1.58) **N%**(10.84/10.50)
XB_3	IR (KBr/cm^{-1})= C=N (2215), C=O (1680), O-Ar(1240), C-F (1190),C-Cl (710); NMR (ä/ppm in DMSO-D)= 6.88-8.09(m,6H, Ar-H)
XB_4	IR (KBr/cm^{-1})= C=N (2225), C=O (1670), O-Ar(1265), C-F (1175),C-Cl (693);
XC_1	IR (KBr/cm^{-1})= NH=NH (3310), C=N (2217), C=O (1650), C-F (1190),C-Cl (685); NMR ä/ppm in DMSO-D)= 5.0(s,1H, NH-C6H5), 7.10-7.68(m,7H,Ar-H), 8.16(d,1H,NH); MS:m/2 = 385(M^+) elemental analysis (calculated/found) C%(52.92/52.64) H%(2.35/2.58) N%(18.15/17.96)
XC_2	IR (KBr/cm^{-1})= NH=NH (3280), C=N (2215), C=O (1675), C-F (1174),C-Cl (680)
XC_3	IR (KBr/cm^{-1})= NH=NH (3330), C=N (2233), C=O (1660), C-F (1180),C-Cl (672); NMR ä/ppm in DMSO-D)= 5.1(s,2H,-NH2), 7.15-7.6(m,2H,Ar-H),7.98(s,1H,-NH)

Table 3: Antibacterial activity by Cup plate method

		Mean zone of inhibition in (mm)							
		B. subtillis (G+ve)		*P. seudomonas (G-ve)*		*Staphylococus aureus (+Ve)*		*Escherichia Coli (G-ve)*	
SN	Name of Compound	50 µg	100 µg	50 µg	100 µg	50 µg	100 µg	50 µg	100 µg
1	Procaine Pencillin	20	22	--	--	18	21	--	--
2	Streptomycin	--	--	19	21	--	--	20	22
3	XB_1	17	18	11	13	14	16	14	13
4	XB_2	11	12	10	12	16	17	7	9
5	XB_3	08	10	12	14	8	8	9	10
6	XB_4	15	17	13	14	7	9	11	12
7	XC_1	13	14	09	11	12	14	13	18
8	XC_2	12	13	15	16	11	12	14	15
9	XC_3	14	16	11	15	13	15	12	14

Table 4: Antibacterial activity by Minimal inhibitory concentration method

Sl. No.	Compound code	*Staphylococcus aureus(G +ve)*	*Bacillus subtillis (G +ve)*	*E.coli (G–ve)*	*Pseudomonas(G –ve)*
1	XB_1	100	400	200	400
2	XB_3	>800	200	400	50
3	XC_3	200	400	100	400

(all Minimal inhibitory concentration are in µ/ml)

Table 5: Antifungal activity[9,20]

		Mean zone of inhibition (in mm) (Activity index)			
		Candida albicans		*Aspergillus flavus*	
Sl. No	Name of the compounds	50 mg	100 mg	50 mg	100 mg
1	Griseofulvin	19	20	20	21
2	XB_1	14	16	8	09
3	XB_2	11	12	11	14
4	XB_3	07	09	12	15
5	XB_4	09	12	09	12
6	XC_1	12	13	13	14
7	XC_2	15	17	11	14
8	XC_3	07	09	14	16

Std: Griseofulvin, Mean zone of inhibition is including bore diameter, Bore diameter is 8 mm; Activity index = Test compound/Standard compound

Antibacterial Activity by Cup Plate Method: All the synthesized compounds were evaluated for antibacterial activity and the result are presented in Table 3. The test compounds have been found to active against both gram-positive and gram-negative organisms. Among these compounds sample No., XB_1, XB_4 has been found to superior in its antibacterial action against gram-positive bacteria. XC_2 has been found exhibit relatively significant inhibitory action against gram-negative bacteria.

Antibacterial Activity by MIC Method: Among the Synthesized compounds, some compounds were evaluated for antibacterial activity by MIC method and results are presented in Table 4. These compounds have been found to active against gram-positive and gram-negative organisms. Compounds XB_1 is found to sensitive and shown MIC activity at 100 µg/ml. *E.coli gram*(-ve) organisms responded very sensitively to compound XC_3. It has shown the MIC activity at 100µg/ml. Another gram (-ve) organisms *Pseudomonas* found to be very sensitive to compound XB_3 and shown MIC activity at 50µg/ml and 100µg/ml respectively.

Antifungal Activity: The same types of compounds were evaluated for their antifungal activity against *Candida Albicans* and *Aspergillus Flavus* at two different concentrations of 50µg/ml and 100 µg/ml. The results are presented in Table 5. All the compounds have been found to exhibit moderate to good antifungal activity against both the test fungi. They have been noted better zone of

Table 6: Anti-inflammatory activity (in-vitro model) [3,6,50,54,56]

SN	Name of the compounds	Absorbence value (Mean ± SE)	Inhibition of denaturation (in %)
1	Control	0.044±0.001	---
2	XB_1	0.061±0.001	38
3	XB_2	0.058±0.001	31
4	XB_3	0.056±0.002	27
5	XB_4	0.052±0.001	18
6	XC_1	0.054±0.002	22
7	XC_2	0.046±0.001	4
8	XC_3	0.049±0.003	11
9	Ibuprofen	0.080±0.002	81

Table 7: Anti-inflammatory activity (in-vivo model) by carrageenan induced paw edema in rat:

Sl No	Compound code	Dose (mg/kg)	Mean difference in paw volume ± SEM after 3 hrs.	Percentage of inhibition
01	Control	50	0.950±.122	----
02	Diclofenac sod. (Standard)	50	0.160±.010***	80
03	XB_1	50	0.410±.578**	56.84

P<0.01,*P<0.001 when compare to control group

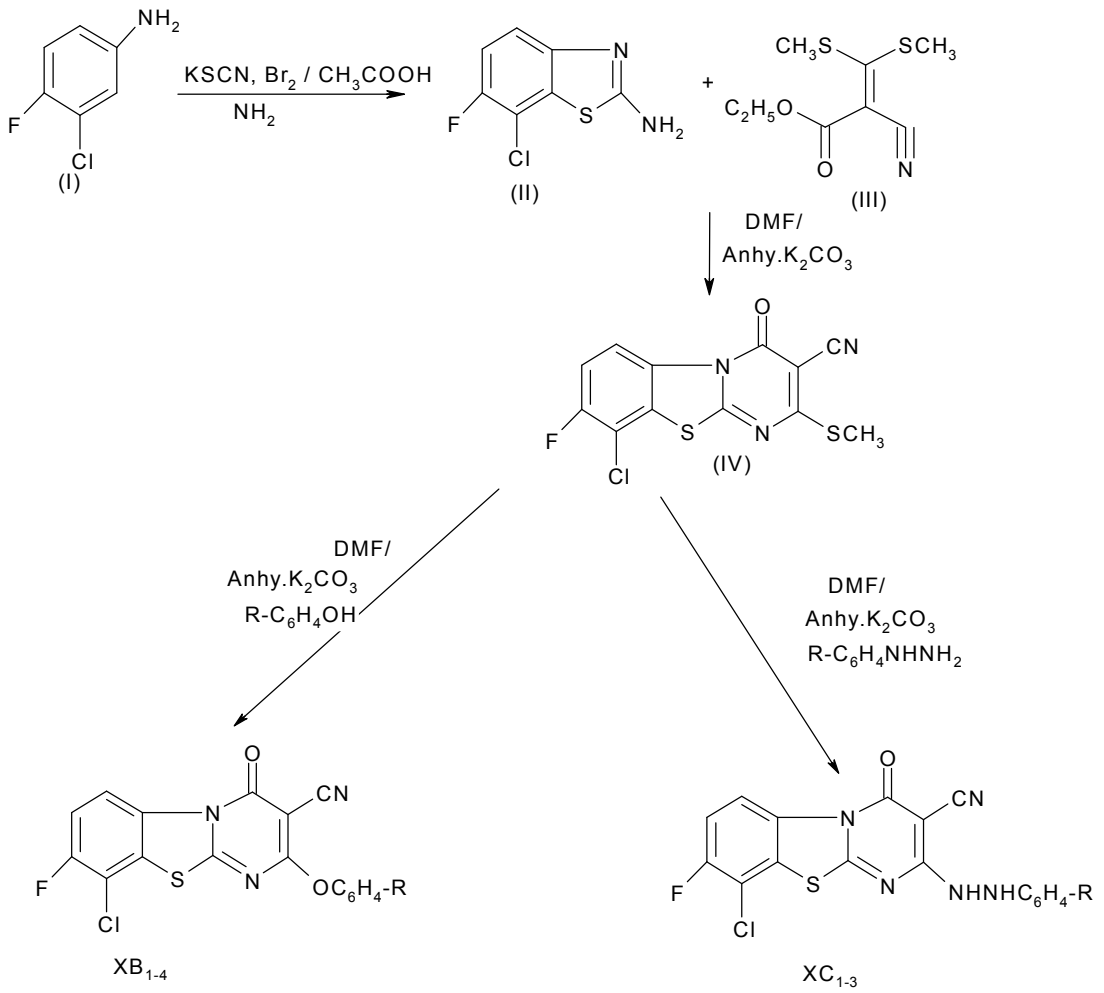

Fig. 1: Synthetic scheme for synthesis of 2-arylhydrazinoand 2-aryloxy- pyrimido [2,1-b] benzothiazole derivatives

inhibition at 100 µg/ml concentration than at 50µg/ml. Among the tested compound sample XB_1, XC_2 were found to be relatively better over the rest of the tested compounds.

Anti-inflammatory Activity

a) *In-vitro* Model: The results of the anti-inflammatory activity of test compounds presented in a Table 6 reveals that none of them could inhibit albumin denaturation significantly in comparison with standards drug, diclofinac sodium exhibited 81% inhibit albumin denaturation. However, some compounds could inhibit albumin denaturation considerably. The rest compounds tested were found to devoid of any inhibition of albumin denaturation.

b) *In-vivo* Model: On the bases of the results obtained in *in-vitro* anti-inflammatory screening, only one compound was screened *in-vivo*. Anti-inflammatory activities are carried out by carrageenan induced paw edema in rat. Results of activity are presented in Table 7. Tested compound XB_1(56.84%) show moderate anti-inflammatory activity as compare to standards compounds diclofenac sod. (80%).

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