

Three component solvent-free synthesis and fungicidal activity of substituted pyrimido [4,5-d] pyrimidine-2-(1h)-one

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ABSTRACT

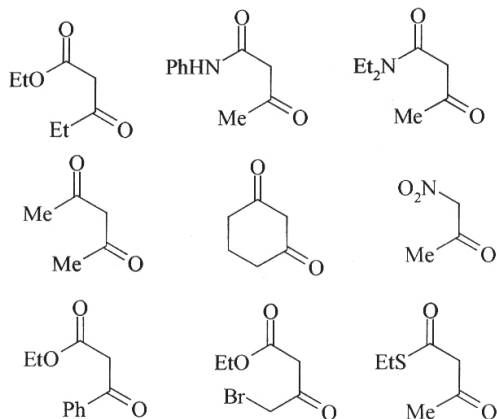
A novel three component condensation of aromatic aldehyde, urea or thiourea and barbituric acid or N,N-1,3-dimethylbarbituric acid was carried out using *L*-proline as an organo-catalyst under solvent-free microwave conditions. The resulting product, pyrimido [4,5-*d*] pyrimidine-2-(1H)-one was checked for the fungicide activity.

Keywords: Fungicidal activity, aromatic aldehyde.

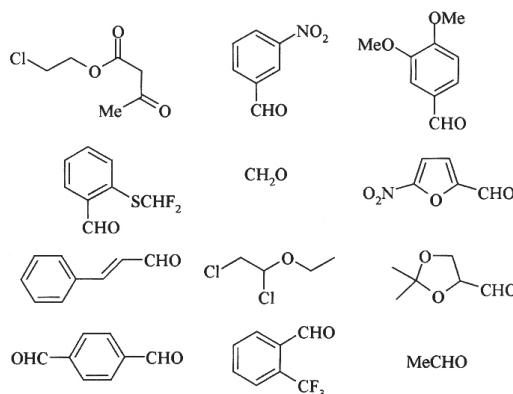
INTRODUCTION

Variations of building blocks in the classic version of Biginelli reaction:

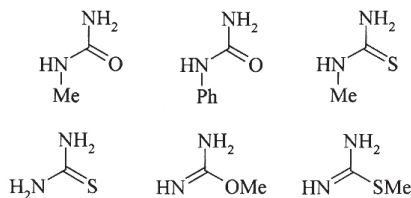
The great biological importance of DHPM nucleus has over the years prompted the development of various strategies for the synthesis of diversely functionalized dihydropyrimidine derivatives¹. Making the variations in the original three reacting components can modify the classical Biginelli reaction². The following building blocks have so far been documented as the variations of original Biginelli reaction.



Variations in 1,3-dicarbonyl components



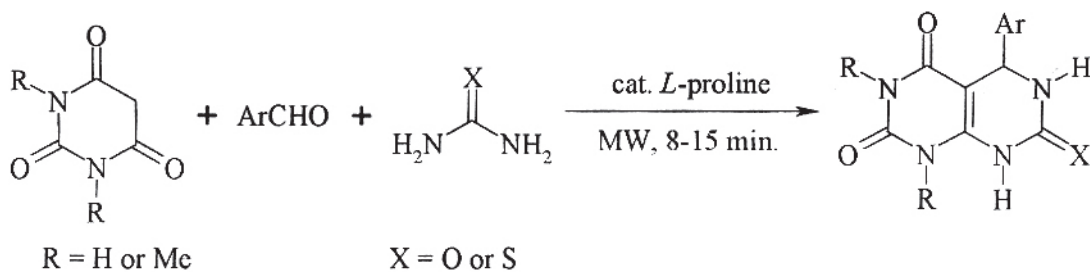
Variations in aldehyde components



Variations in urea or thiourea components

There are several improved protocols for the Biginelli reaction, including transition metal Lewis acid catalysis, solid phase synthesis and activation with certain additives, ionic liquids, heterogeneous catalysis, microwave irradiation and grinding techniques. All of the above improved procedures for the three component Biginelli reaction mainly utilize open chain β -dicarbonyl compounds (particularly, β -keto ester) as one of the substrates. The literature survey has revealed that there is

limited number of Biginelli reaction variations, which utilize cyclic 1,3-diketone to afford pyrimidine-fused heterocycles. Owing to the importance of the fused pyrimidine ring in natural products and drug chemistry, herein we wish to report one-pot three component condensation of barbituric acid, aldehyde and urea or thiourea using L-proline as an efficient organo-catalyst under solvent free microwave conditions (Scheme 1).



Scheme 1

Plants and animals possess healing properties that have been recorded for thousands of years in such documents as Dioscorides' "de Materia Medica" from 75 AD and the 3500-yr-old Papyrus Ebers³. During this four millennia and the research on the natural products although ensconced in more sophisticated drug-delivery systems, continue to help humankind, Modern examples include lovastatin, digitoxin, reserpine, morphine and cyclosporin A4. Moreover, plants⁵⁻⁷, marine organisms^{8,9} and microbes^{10,11} usually produce biologically active compounds in defense to predators and competition with neighbors¹². Thus, it seems logical that most of the drugs derived from natural sources have anticancer or anti-infective properties¹³. Just a few examples include vincristine, taxol and camptothecin (anticancer), quinine and artemisinin (antimalarial), tetracyclines, macrolides, and cephalosporins (antibacterial), and polyenes, echinocandins/pneumocandins, aureobasidins and sordarins (antifungals)^{10,14}.

Although Sir Fleming's discovery of penicillin¹⁵ (and more ancient findings) was somewhat serendipitous, and a little good fortune may still play a role in finding new drugs, antimicrobial drug discovery is more systematic and elaborate in the 21st century, but so are pathogenic

microbes. Fungal and bacterial infections persist as a threat to worldwide health and cost the world billions of dollars each year. Drug companies continue to focus on the development of antimicrobial drugs, especially with the increasing emergence of drug-resistant pathogens¹⁶.

In the current era, fungal infections have emerged as an important agricultural and clinical threat, with significant associated morbidity and mortality. Along with the emergence of fungal infections have come the development of antifungal resistance to existing antifungal agents and the development of agents directed at novel drug targets.

Biological activity can take many different forms and may be measured in many different ways. When the critical site and mechanism of action of a chemical are known, biological activity can be measured directly in terms of degree or % of inhibition *in vitro*. Moreover, such activity is measured indirectly through *in vitro* observations.

Therefore, attempts were made for screening these new compounds in the laboratory for their fungitoxicity. The present work deals with the study of antifungal activities of newly synthesised

compounds pyrimido[4,5-d]pyrimidine-2-1H-one against selected human and plant pathogens.

MATERIAL AND METHODS

Experimental Section:

Reaction vessels were flame-dried or oven dried. Barbituric acid, N,N-1,3-dimethyl barbituric acid and L-proline were purchased from Aldrich chemicals. Analytical TLC was performed on glass plates coated with silica gel. For the microwave irradiation experiments described below a conventional (unmodified) household microwave oven equipped with a turntable was used (Samsung, code number C 103 FL, 450 W) and operating at 2450 MHz.

Typical procedure:

Barbituric acid (5 mmol) or N,N-1,3-dimethyl barbituric acid, appropriate aromatic aldehyde (5 mmol), urea or thiourea (5 mmol) and L-proline (5 mol%) were mixed thoroughly in a mortar. The reaction mixture was then transferred to 100 mL conical flask and was then irradiated in a domestic microwave oven for 8-15 min. at 450 W. The progress of the reaction was monitored by TLC. After the completion of the reaction, it was poured into crushed ice. The resulting solid was filtered off and recrystallized from ethanol to afford pyrimido[4,5-d]pyrimidine-2-1H-one in reasonable purity.

Cultures of the *Alternaria brassicicola* (Ab) - leaf spot pathogen of spinach and related plants, *Helminthosporium tetramera* (Ht) - a seed pathogen of cereals were obtained from Botany Research Laboratory and Plant Disease Clinic, Science College, Nanded (M.S.). The fungal human pathogen cultures of *Candida albicans* (Ca) and *Epidermophyton floccosum* (Ef) were obtained from Department of Microbiology, Government Medical College, Aurangabad and Department of Pathology, SGGGS Medical College, Nanded.

The cultures were maintained on Malt Extract Agar (MEA) and subculturing was done after every fifteen days. In all experiments the cultures were incubated as $27 \pm 2^\circ\text{C}$ in the laboratory.

Media:

The following media were used in this study

1. Sabouraud Agar

Dextrose	40gm
Peptone	10gm
Agar	20gm
Distilled water	1000ml
pH	5.5

2. Malt extract agar (MEA)

Dextrose	20gm
Peptone	1gm
Agar	20gm
Distilled water	1000ml
pH	5.5

Spore Germination

The spore suspension for germination studies was prepared by adding 5 mL of sterile distilled water to a heavily sporulating 8 day old MEA slant culture of fungus. The suspension was filtered through several layers of muslin cloth to reduce mycelial fragments. Spores were washed with sterile distilled water several times by centrifugation to remove nutrients from original medium. The spore finally obtained were diluted in water and adjusted to the desired concentration. The spore concentration was measured by Haemocytometer with Naubauer Counting Chamber¹⁷. Hanging drop slides were prepared from observation from the suspension and incubated in petriplates lined with moist blotters. Germination % was calculated by observing a minimum of 200 spores from different microscopic field.

Hanging Drop Technique¹⁸

One drop of a standard spore suspension was placed in the centre of the glass cover slip. Then it was kept inverted in the cavity of a cavity slide, due to which hanging position of the drop was maintained. The margin of cavity was lined with petroleum jelly in order to avoid the evaporation of the spore suspension. Then the slide was incubated for 1 hour under moist petridish at required temperature (27°C). Germination observations were made by observing the slides under microscope at 15 minutes time interval.

1 mg of the compound was dissolved in 1-2 drops of DMSO, and then volume was made to 1

ml with sterile distilled water. Thus by making the concentration of the solution as 1000 µg/ml. Solution without compound was used as control.

The spore germination in plant extract was compared with systemic fungicide Carbendazim. 1 gm. of technical grade Carbendazim obtained from BASFF, Mumbai was dissolved in 1000ml of sterile distilled water. This stock solution was used in spore germination studies.

1 ml of spore suspensions was mixed with 1 ml of compound solution. This mixture was subjected to observation by spore germination method. Relative humidity of the working place was determined and found to be 80% whereas the temperature was 27°C. Hence, experiments were performed to study the effect of the compounds on the spore germination. The spore germination with respect to time were recorded and subsequently the % inhibition of spore germination are given.

Antifungal activity was evaluated by well-diffusion method expressed by zone of inhibition mm in diameter for *Candida albicans*, *Epidermophyton floccosum*¹⁹.

The assay was carried out by using 1 ml of inoculum (1×10^6 colony forming units) prepared from an overnight culture for given test fungi. 1 ml of the resultant spore/cell suspension was poured in the petri plate and the plates were poured with respective medium to seed each prepared plate. The medium was allowed to solidify. Using a sterilized cork borer, wells of 5mm diameter were made in the solidified inoculated medium and the plate area uniformly. The wells were filled with 0.5ml of compound solution. Plates were then incubated aerobically at $37 \pm 2^\circ\text{C}$ for 72 h for fungi. Similarly, wells containing standard concentration of Amphotericin B were used to compare the antifungal property of the compound. 1gm of Amphotericin B (Hi-media, Mumbai) was dissolved separately in sterile distilled water and 0.5ml was used to fill the wells²⁰.

RESULTS AND DISCUSSION

In a typical experimental procedure, barbituric acid (3 mmol), aromatic aldehyde

(3 mmol) and thiourea (3 mmol) was intimately mixed in 25 mL beaker and L-proline (5 mol%) was added. This reaction mixture was subjected to intermittent microwave irradiation for the time period of 1 min interval in an unmodified domestic microwave oven at 450 W. This intermittent irradiation-mixing cycle was repeated for the total irradiation time as mentioned in Table 1, to afford the highly functionalized pyrimido[4,5-d]pyrimidine-2-1H-thione in excellent yields (79-92%). In all the cases, the products were purified by the recrystallization method from ethanol. After the microwave irradiation of every 1 min. interval, as glass thermometer was immediately immersed in the reaction mixture and the final reaction temperature under microwave activation was found to be less than 58°C. Based upon this observation, we carried out the solvent free reaction of barbituric acid (2 mmol), benzaldehyde (2 mmol), and thiourea (2 mmol) using L-proline (5 mol%) under conventional heating at 80-90°C for the time period of 2-3 hr. In this case the yield of 4m was significantly low (18-29%). Thus, microwave irradiation was found to be very efficient for the rapid synthesis of 4a-q in excellent yields. It is worth mentioning that in the absence of L-proline, the reaction does not proceed to form pyrimido[4,5-d]pyrimidine-2-1H-thione and only the condensation of barbituric acid and aromatic aldehyde was observed.

The generality of the method was tested by making variations in all the three components. Thus, barbituric acid as well as N,N-1,3-dimethylbarbituric acid reacted with variously substituted aromatic aldehyde and urea or thiourea in the presence of L-proline under microwave irradiation to afford a range of diversely functionalized pyrimido [4,5-d]pyrimidine-2-1H-one in good to excellent yields. The results are shown in Table 1.

The structures of all pyrimido[4,5-d]pyrimidine-2-1H-thione (4a-q) were confirmed from the spectroscopic data and elemental analysis. The NMR spectra showed the absence of the methylene proton (δ 2.54 ppm) of the barbituric acid and the presence of a proton at δ 5.26-5.44 ppm. 5

Reaction Mechanismreaction as proposed by O.C. Kappe².

The mechanism of this reaction is assumed to be similar to the original Biginelli

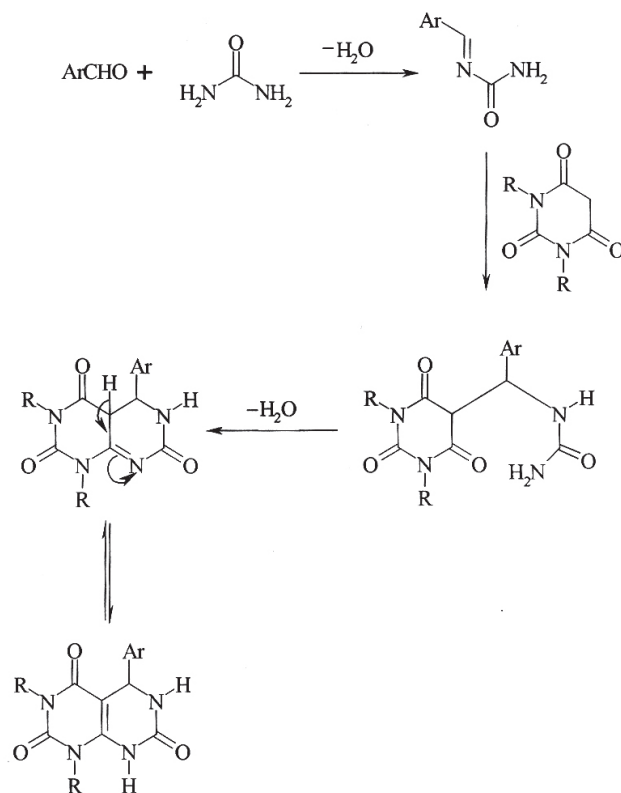
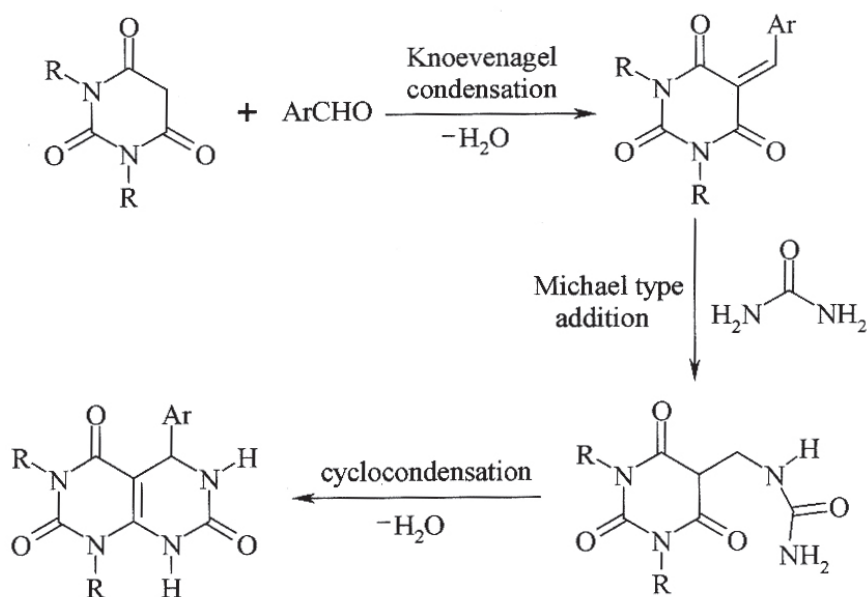
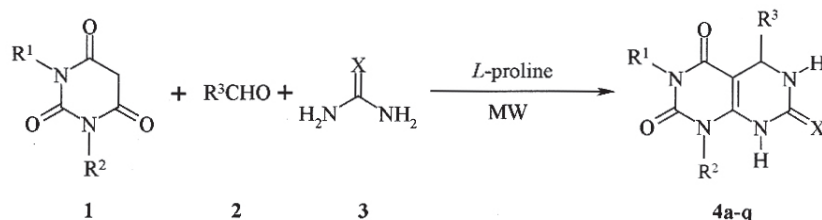
**Alternate mechanism**

Table 1: *L*-Proline catalyzed multi-component synthesis of pyrimido[4,5-*d*]pyrimidien-2-1*H*-one under solvent-free conditions



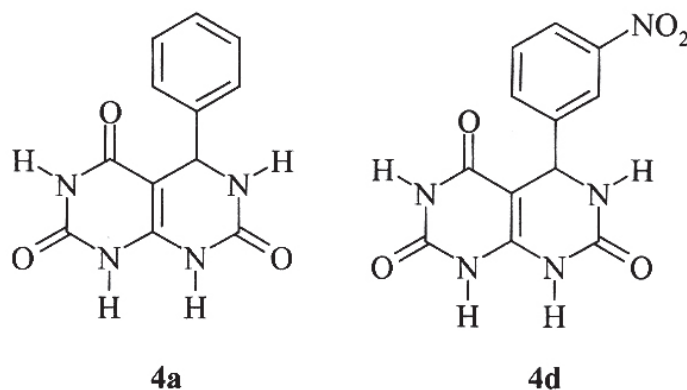
Entry	Product	R1	R2	R3	X	Reaction Time (min)	Yield ^a (%)
1	4a	H	H	C ₆ H ₅	O	12	87
2	4b	H	H	4-CH ₃ -C ₆ H ₄	O	14	84
3	4c	H	H	4-Cl-C ₆ H ₄	O	13	76
4	4d	H	H	3-NO ₂ -C ₆ H ₄	O	12	81
5	4e	CH ₃	CH ₃	C ₆ H ₅	O	13	79
6	4f	CH ₃	CH ₃	4-CH ₃ -C ₆ H ₄	O	17	83
7	4g	CH ₃	CH ₃	4-Cl-C ₆ H ₄	O	14	79
8	4h	CH ₃	CH ₃	3-NO ₂ -C ₆ H ₄	O	15	85
9	4i	CH ₃	CH ₃	3-CH ₃ O-4-HO-C ₆ H ₃	O	15	76
10	4j	CH ₃	CH ₃	C ₆ H ₅	S	13	82
11	4k	CH ₃	CH ₃	4-Cl-C ₆ H ₄	S	12	81
12	4l	CH ₃	CH ₃	4-CH ₃ -C ₆ H ₄	S	14	83
13	4m	H	H	C ₆ H ₅	S	12	78
14	4n	H	H	4-CH ₃ -C ₆ H ₄	S	14	77
15	4o	H	H	3-NO ₂ -C ₆ H ₄	S	13	84
16	4p	H	H	4-Cl-C ₆ H ₄	S	11	83
17	4q	H	H	4-CH ₃ -C ₆ H ₄	S	12	79

^a Isolated yields after recrystallization.

Spectral Analysis

IR Spectra were recorded on a Shimadzu FTIR-1710 spectrophotometer. The ¹H NMR spectra were recorded on 400 MHz instrument and the

chemical shifts were reported with TMS as an internal standard. The representative spectral analysis for few of the products is given below:



5,6-Dihydro-5-phenylpyrimido[4,5-d]pyrimidine-2,4,7(1H,3H,8H)-trione (4a)

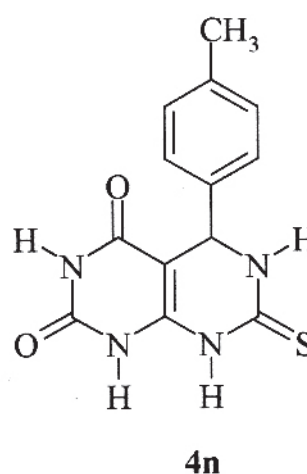
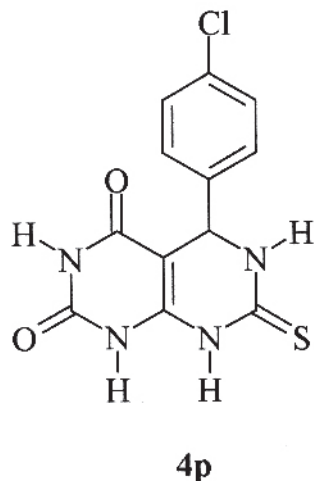
Yellow colored solid, M.p. 172-174°C.

IR (KBr, cm^{-1}): 3416, 3323, 3221, 2983, 1735, 1697, 1647, 1496, 1419, 1232, 758, 702 ^1H NMR (CDCl_3 , δ ppm): 5.49 (s, 1H, ArCH), 7.51-7.76 (m, 5H, ArH), 7.55 (s, 1H, NH), 10.16 (s, 1H, NH), 11.26 (s, 1H, NH), 11.63 (s, 1H, NH).**5,6-Dihydro-5-(3-nitrophenyl)pyrimido[4,5-d]pyrimidine-2,4,7(1H,3H,8H)-trione (4d)**

Yellow colored solid, M.p. 196-198°C.

IR (KBr, cm^{-1}): 3412, 3284, 3082, 2966, 1718, 1689, 1641, 1525, 1356, 808, 731. ^1H NMR (CDCl_3 , δ ppm): 5.43 (s, 1H, ArCH), 7.36 (t, $J=7.96$ Hz, 1H, ArH), 7.99 (d, $J=9.32$ Hz, 1H, ArH),8.09 (s, 1H, ArH), 8.15 (d, $J=8.2$ Hz, 1H, ArH), 9.17 (s, 1H, NH), 10.16 (s, 1H, NH), 11.35 (s, 1H, NH), 11.72 (s, 1H, NH).**5,6,7,8-Tetrahydro-5-(4-chlorophenyl)-7-thioxopyrimido[4,5-d]pyrimidine-2,4(1H,3H,8H)-dione (4p)**

Yellow colored solid, M.p. 182-184°C.

IR (KBr, cm^{-1}): 3213, 3090, 2850, 2360, 2332, 1755, 1703, 1603, 1775, 1442, 1342, 1288, 1091, 839, 792 ^1H NMR (DMSO, δ ppm): 5.26 (1H, s, ArCH), 7.28 (2H, d, $J=9.28$ Hz, ArH), 7.41 (2H, d, $J=8.6$ Hz, ArH), 7.61 (1H, s, NH), 8.37 (1H, s, NH), 11.31 (1H, s, NH), 11.19 (1H, s, NH).**5,6,7,8-Tetrahydro-7-thioxo-5-p-tolylpyrimido[4,5-d]pyrimidine-2,4(1H,3H)-dione (4n)**

Yellow colored solid, M.p. 165-167°C.

IR (KBr, cm^{-1}): 3219, 3088, 2847, 2360, 2343, 1762, 1760, 1710, 1687, 1577, 1556, 1442, 1398, 1315, 1296, 1219, 1192, 806, 794 ^1H NMR (DMSO, δ ppm): 2.42 (3H, s, ArCH₃), 5.29 (1H, s, ArCH), 7.25 (d, $J=7.92$ Hz, 2H, ArH), 7.52 (1H, s, NH), 8.13 (d, $J=7.98$ Hz, 2H, ArH), 8.42 (1H, s, NH), 10.91 (1H, s, NH), 11.05 (1H, s, NH).

2332, 1753, 1741, 1703, 1602, 1562, 1527, 1498, 1425, 1362, 1199, 829, 680

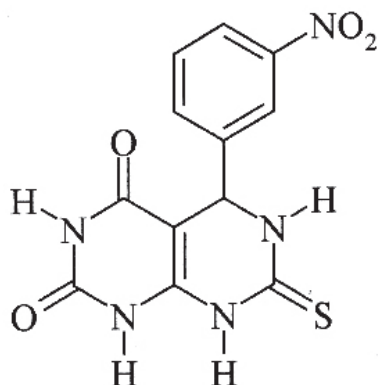
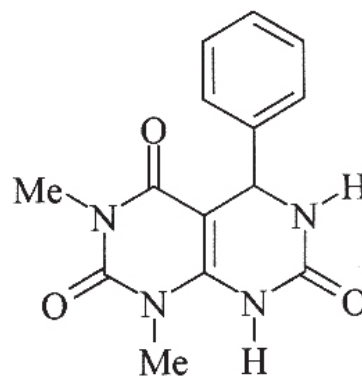
 ^1H NMR (DMSO, δ ppm): 5.44 (1H, s, ArCH), 7.56 (3H, m, ArH), 7.66 (d, $J=7.84$ Hz, 1H, ArH), 8.19 (1H, s, NH), 8.76 (1H, s, NH), 11.29 (1H, s, NH), 11.65 (1H, s, NH).**5,6,7,8-Tetrahydro-5-(3-nitrophenyl)-7-thioxopyrimido[4,5-d]pyrimidine-2,4(1H,3H)-dione (4o)**

Yellow colored solid, M.p. 175-177°C.

IR (KBr, cm^{-1}): 3529, 3371, 3167, 3032, 2951, 2360,**5,6,7,8-Dihydro-1,3-dimethyl-5-phenylpyrimido[4,5-d]pyrimidine-2,4,7(1H,3H,8H)-trione (4e)**

Yellow colored solid, M.p. 108-110°C (Lit. 26 m.p.°C).

IR (KBr, cm^{-1}): 3416, 3323, 2983, 1697, 1647, 1496, 2332, 785, 702 ^1H NMR (CDCl_3 , δ ppm): 2.81 (s, 3H, CH₃), 2.97 (s, 3H, CH₃), 5.39 (s, 1H, ArCH), 6.31 (s, 1H, NH),

**4o****4e**

7.15-7.21 (m, 3H, ArH), 7.29 (d, J = 9.72 Hz, 2H, ArH), 7.60 (s, 1H, NH).

7.15(d, J = 8.48 Hz, 12H,ArH), 7.29(d, J = 8.48 Hz, 2H, ArH), 8.03 (s, 1H, NH).

5-(4-Chlorophenyl)-5,6-dihydro-1,3-dimethylpyrimido[4,5-d]pyrimidine-2,4,7(1H,3H,8H)-trione (4g)

Yellow colored solid, M.p. 148-150°C.

IR (KBr, cm^{-1}): 3209, 3074, 2553, 1693, 1489, 1381, 1222, 1089, 821, 678

^1H NMR (CDCl_3 , δ ppm): 2.82 (s, 3H, CH₃), 2.96 (s, 3H, CH₃), 5.34 (s, 1H, ArCH), 7.09 (s, 1H, NH),

5,6-Dihydro-1,3-dimethyl-5-(3-Nitrophenyl)pyrimido[4,5-d]pyrimidine-2,4,7(1H,3H,8H)-trione (4h)

Yellow colored solid, M.p. 161-163°C

^1H NMR (CDCl_3 , δ ppm): 2.80 (s, 3H, CH₃), 2.97 (s, 3H, CH₃), 5.56 (s, 1H, ArCH), 7.51 (s, 1H, NH), 7.51-7.61 (m, 2H, ArH), 7.89 (s, 1H, NH), 8.09, (s, 1H, ArH), 8.17 (d, J = 7.24 Hz, 1H, ArH).

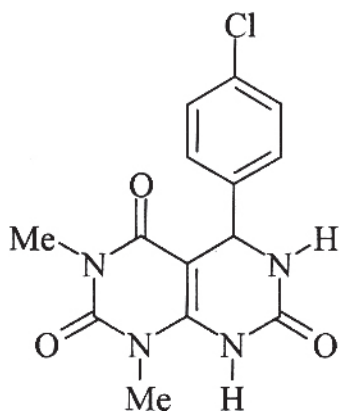
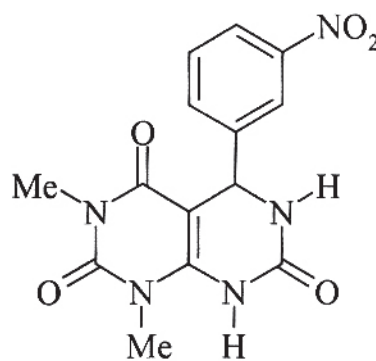
**4g****4h**

Table 2: Effect of *compounds* aqueous extracts on growth of human pathogenic fungi

S. No.	Compounds	Zone of inhibition (mm)					
		<i>C. albicans</i>			<i>E. floccosum</i>		
		Test Solution	Standard	Control	Test Solution	Standard	Control
1	4a	17	14	0	15	12	0
2	4b	17	14	0	16	12	0
3	4c	16	14	0	16	12	0
4	4e	12	14	0	11	12	0
5	4f	13	14	0	12	12	0
6	4g	14	14	0	13	12	0
7	4h	13	14	0	12	12	0
8	4i	17	14	0	15	12	0
9	4j	15	14	0	13	12	0
10	4k	12	14	0	10	12	0
11	4l	16	14	0	14	12	0
12	4n	17	14	0	15	12	0
13	4o	13	14	0	14	12	0
14	4p	14	14	0	15	12	0
15	4q	18	14	0	17	12	0

Standard – Amphotericin B, Control – blank

Table 3: Effect of *compounds* on spore germination of plant pathogenic fungi

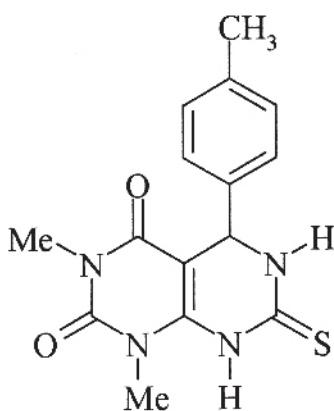
S. No.	Compounds	% inhibition of spore germination 9at 180 min)					
		<i>A. brassicicola</i>			<i>H. tetramera</i>		
		Test Solution	Standard	Control	Test Solution	Standard	Control
1	4a	100	100	0	100	100	0
2	4b	100	100	0	100	100	0
3	4c	100	100	0	100	100	0
4	4e	94	100	0	91	100	0
5	4f	97	100	0	95	100	0
6	4g	99	100	0	97	100	0
7	4h	97	100	0	96	100	0
8	4i	100	100	0	100	100	0
9	4j	100	100	0	100	100	0
10	4k	95	100	0	94	100	0
11	4l	100	100	0	100	100	0
12	4n	100	100	0	100	100	0
13	4o	95	100	0	94	100	0
14	4p	896	100	0	93	100	0
15	4q	100	100	0	100	100	0

Standard - Carbendazim, Control - blank

5,6,7,8-Tetrahydro-1,3-dimethyl-7-thioxo-5-p-tolylpyrimido[4,5-d]pyrimidine-2,4(1H,3H,8H)-dione (4I)

Yellow colored solid, M.p. 137-139°C (Lit.26 M.p.°C). IR (KBr, cm⁻¹): 3408, 3157, 3057, 2941, 1691, 1610, 1496, 1205, 1136, 1057, 815, 762

¹HNMR (CDCl₃, δ ppm): 2.28 (s, 3H, CH₃), 2.77 (s, 1H, NH), 2.94 (s, 1H, NH), 3.21 (s, 3H, CH₃), 3.29 (s, 3H, CH₃), 5.24(s, 1H, ArCH), 7.00, (d, J = 8.04 Hz, 2H, ArH), 7.09 (d, J = 7.6 Hz, 2H, ArH).

**4I****Antifungal activity**

Fifteen compounds were assessed for their antifungal activities against selected plant and human fungal pathogens. It is clear from the results that all the compounds exhibited antifungal activities. However, there was slight variation in their toxicity against the test pathogenic fungi. It may be attributed to different structures and functional group attached to the basic nucleus. However the functional group and their position imparts toxic

effect against fungi. From these results, inference is drawn that the presence of hydroxyl groups imparts maximum toxicity against pathogens. Beside this, the presence of methoxy groups in the ring also enhances their toxicity.

It is clear that, these compounds possess antifungal properties and the use of these compounds further need an elaborate study. This involves the study of effect on the host and pathogen system both in plant and animal models²¹. So, many of the pathogens used have been reported as resistant against common antibiotics and fungicides. However, it is clear from the present results/reports that preliminary studies showed their good inhibitory properties, therefore further studies is needed in order to have these compounds as fungicides,.

CONCLUSION

Multi-Component synthesis of pyrimido [4,5-d]pyrimidine-2-1H-one is associated with following important merits:

- Novel synthesis of diversely functionalized bicyclic pyrimidine derivatives.
- The products pyrimidines possess good fungicidal activities.
- Short reaction time.
- Elimination of solvent i.e. solvent-free protocol.
- High yields.
- Purification of the product simply by recrystallization.

In conclusion, we have developed a new, rapid and simple multicomponent cyclocondensation protocol for the synthesis of biologically active pyrimido[4,5-d]pyrimidine-2-1H-one in high yields.

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