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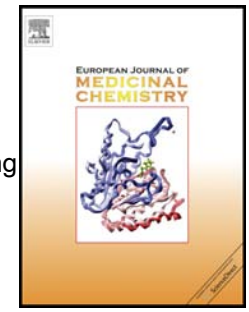

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Graphical Abstract

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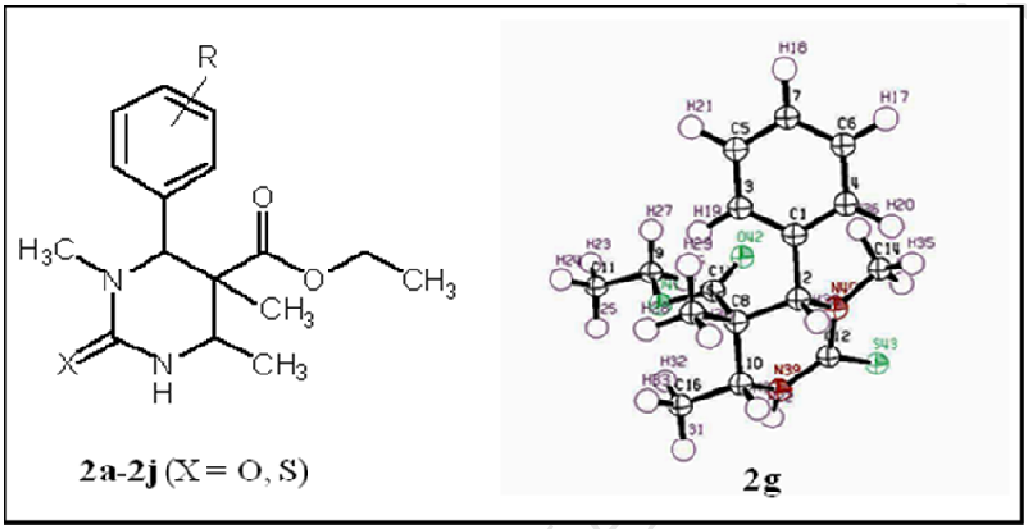

The novel N-1 and C-5 alkyl substituted derivatives of Pyrimidine were synthesized, characterised along with crystal structure determination and biological screening is reported.

Synthesis, Characterisation, Crystal Structure Determination and Biological Screening of Novel N-1 and C-5 Alkyl Substituted Scaffolds of Pyrimidine

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Abstract

The novel N-1 and C-5 alkyl substituted derivatives of pyrimidine were synthesized by using tetra butyl ammonium bromide (TBAB) as phase transfer catalyst at 20-25 °C with excellent productivity (85-95%). The new compounds were evaluated for their antibacterial activities by screening them against Gram + ve and Gram-ve bacterial strain: S. aureus ATCC 6538P, S. abony NCTC 6017: *E. coli* ATCC 8739, S. epidermidis ATCC 12228. Among all compounds evaluated the molecule **2c** and (**2g-j**) exhibit the most pronounced antibacterial activity against E. *coli*, *S. aureus* and *S. abony* with MICs value 25 µg/mL.

Keywords: Alkyl pyrimidines, antibacterial activity, crystal structure, characterisation.

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1. Introduction

Despite a numerous attempts to develop new structural prototype in the search of more effective antimicrobial, the pyrimidine skeleton still remains as one of the most versatile class of compounds against microbes and is of great importance to chemists as well as biologists as it is available in a large variety of naturally occurring compounds and also in clinically useful molecules [1-3]. Pyrimidines are an integral part of DNA and RNA and exhibit diverse pharmacological properties as effective bactéricides, fungicides, virocides and insecticides [4]. Certain pyrimidines and annulated pyrimidines derivatives are also known to display anticancer [5], antimalerial [6], antileishmanial [7] and antifilarial activities [8]. Due to resistance and safety concerns, antimicrobial like chloramphenicol is no longer a first-line agent for any infection in developed nations, although it is sometimes used topically for eye infections. In low-income countries, chloramphenicol is still widely used because it is inexpensive and readily available. The most serious adverse effect associated with chloramphenicol treatment is bone marrow toxicity, which may occur in two distinct forms: bone marrow suppression, which is a direct toxic effect of the drug and is usually reversible, and aplastic anemia which in general is fatal [18]. The increasing incidence of microbial infections over the world demand to search and synthesize a new class of antimicrobial compounds that has resulted in the development of resistance to these drugs with important implications for morbidity, mortality and health care cost in the current regimen [9-12]. Among the various pharmacophores responsible for the antimicrobial activities, the pyrimidine derivatives seem to be a viable lead structure. There fore pyrimidine and its derivatives have been extensively studied for their close association with life processess. Hence, we developed a new methodology that allows the rapid synthesis of N- & C-alkylpyrimidine derivatives using tetrabutyl ammonium bromide, a Phase-Transfer-Catalyst. Each of the alkylpyrimidine analogues **2a-j** prepared has been tested for their antimicrobial activities and the results are reported in comparison with Chloromphenicol and Streptomycin as standars drugs (**Figure 1**). As an extensive continuation of our study on the structure-antibacterial activity relationships in β-lactam derivatives and biologically active heterocycles [22, 27], we performed an investigation of compounds **2a-j** because they represent an attractive model for a theoretical and experimental study of the pharmacophore and their medical applications because of the large variability and combination in their substituent. We present here the results of our virtual screening investigation into possible alternative structures for these compounds. A comparison between experimental and theoretical

predictions of the antibacterial activity has enabled us to identify alternative combined pharmacophore sites structures.

2. Chemistry

Asymmetric C- & N-alkylation of differently substituted ethyl 1, 2, 3, 4-tetrahydro-6-methyl-2-oxo/thioxo-4-phenylpyrimidine-5-carboxylates **(1a-j)** were outlined in **scheme 1.** Substituted ethyl 1, 2, 3, 4-tetrahydro-4-methyl-2-oxo/thioxo-6-phenylpyrimidine-5-carboxylates **(1a-g)** required as the starting material were prepared according to literature procedure [13]. Substituted ethyl 1, 2, 3, 4-tetrahydro-4-methyl-2-oxo/thioxo-6-phenylpyrimidine-5-carboxylate **(1a-g)** when subjected to alkylation with methyl iodide in acetone in the presence of K_2CO_3 using TBAB yields corresponding Substituted ethyl hexahydro-1, 4, 5-trimethyl-2-oxo/thioxo-6-phenylpyrimidine-5-carboxylates **(2a-j).** The purity of the compounds was monitored by thin layer chromatography and the structures of all derivatives **(2a-j)** were supported and elucidated by IR, ^{1}HNMR, C^{13}NMR, and MASS spectral data.

3. Result and discussion

3.1 Spectral study

The structure of Ethyl hexahydro-1, 4, 5-trimethyl-6-phenyl-2-thioxo-Pyrimidin-5-carboxylate **2g** was supported by elemental analysis, IR, ^{1}HNMR, C^{13}NMR and MASS spectral data. IR absorption frequencies at 3195-3200 cm^{-1} was corresponding to an aromatic N-H stretching. Bands at 1715-1725 cm^{-1}, 1690-1710 cm^{-1} and 1085-1120 cm^{-1} corresponds to C=O group, C-C aromatic ring, >C=S respectively. Absorption band at 1145 cm^{-1} was corresponding to C-N-C stretching (>N-CH_3). In the ^{1}H NMR spectrum of compound **2g,** it showed the peaks at δ1.2 (t, 3H, Ar-C$\underline{H}_3$), δ 2.2 (s, 3H, COOCH$_2$C$\underline{H}_3$), δ 4.0 (q, 2H, COOC$\underline{H}_2$CH$_3$), δ 5.1 (s, 1H, Ar-H), δ 9.1 (br, s, 1H, N-H) and δ 7.0-7.5 (m, 5H, Ar-H). In the ^{13}CNMR spectrum of the compound **2g**, the signals belonging to the same groups were recorded at 13.72, 22.53, 62.13 and 102.83. The ^{1}H NMR & ^{13}C NMR spectra of compounds **(2a-j)** displayed additional signals into aromatic region, due to substituted aromatic ring placed at C-6 position.

The ^{1}H NMR spectra of compounds **(2a-j)** showed two more signals due to –CH_3 group. That is one singlet at 2.9 ppm which was integrating for 1N-C$\underline{H}_3$ obtained by the disappearance of the peak from 6.8 ppm of 1N-H and also a single peak at 2.5 ppm integrated for three protons which reflect the presence of methyl group attached to asymmetric carbon atom (5C-C$\underline{H}_3$). This data revealed the formation of N & C-alkylated product. The carbon signals for the same group at 58.90 recorded for the asymmetric carbon atom and also singlet at 36.0 corresponding to the alkyl group which attached to nitrogen atom leads credence to the formation of desired N-alkylated products. In addition, -OCH_3 group of compounds **2d** & **2i** resonated at 3.7 ppm integrating three protons as a single in the ^{1}H NMR spectrum, while this group was observed at 54.84 ppm in the ^{13}C NMR spectrum. Moreover, the signals derived from one –OH group in compounds **2e** & **2h** were recorded at 8.4 ppm integrating one proton. The compound **2f** showed singlet at 2.9 corresponding to the two methyl groups (CH_3-N-CH_3) integrating for six protons in ^{1}H NMR spectra.

3.2. Description of crystal structure

Yellow colour needle-like single crystals of **2g** suitable for X-ray diffraction were grown from methanol using the slow evaporation technique. Diffraction intensity data were collected with FR590 MACH3 single crystal diffractometer using Mo*kα* monochromatic radiation (λ = 0.7093cm^{-1}) at room temperature (299K). Crystal data collection and structure refinement data of Ethyl hexahydro-1, 4, 5-trimethyl-6-phenyl-2-thioxo-pyrimidin-5-carboxylate **2g** are listed in (**Table 1**). An integration type of absorption correction was applied to data sets. The structure was resolved by direct methods using the solution program SHELXS97 [7] in the WinGX package and refined by a full-matrix least-squares procedure on *F2* using SHELXS97. The additional data for the molecule **2g** are alternatively available from the Cambridge Crystallographic Data Centre as **CCDC 689147.** All non-hydrogen atoms were refined, first with isotropic and then with anisotropic parameters. Hydrogen atoms bonded to carbon were included using a riding model, starting from calculated positions. With the purpose of obtaining unambiguous evidence of the structures and determination of their conformations in crystal form, the X-ray structural analysis of these substances was carried out. Interestingly, with regards to the geometry of aromatic substituted alkyl pyrimidine ring, in particular of nodal atoms C2, C8, C10 and C40, the molecular structure shows a planar pyrimidine geometry and the phenyl ring corresponding to C2 being slightly twisted in respect to that moiety with the dihedral angle 109.51^{0}. Further by

comparing the length of the valence bond from nodal atoms C8 and C10, it is possible to note that the C8-C10 (1.51) bond is much larger than the double bond and is unindentical to aromatic benzene rings bonds. These data indicates that the presence of single bond is in the C8-C10 position. The methyl group (-C (8) H3 and –C (14) H3) are located similarly with regards to the plane of the pyrimidine skeleton. In the crystal, there is no intermolecular & intramolecular hydrogen bonding occurred. The final atomic coordinates for all atoms and a complete listing of bond distance and angles are tabulated (**Table 2 & 3**). An ORTEP drawing of the molecule **2g** with the atomic numbering scheme is shown in (**Figure 2**).

3.3. Antibacterial activity

In earlier reported work from our laboratory [14], it was found that imidazole-pyrimidine is associated with antimicrobial activities. Keeping this in view, new compounds (**2a-j**) were synthesized and tested in vitro against two Gram-positive bacteria namely *S. aureus* (ATCC 6538P), *S. abony* (NCTC 6017) and against two Gram-negative bacteria namely *E. coli* (ATCC 8739), *S. epidermidis* (ATCC 12228). The primary screening of the compounds for antibacterial activity was determined by measuring the diameter of growth inhibition summarized in **Table 4,** whereas the minimum inhibitory concentration (MIC) was given in **Table 5**. In general, all the newly synthesized compounds showed good antibacterial activity. Among these, **2c** and (**2g-j**) are the strong inhibitors of bacterial growth. The antibacterial activity of these compounds was also compared with two commercially available antibiotics namely chloromphenicol and streptomycin.

It is clear from the results (**Table 4 and 5**) that the compounds (**2g-j**) showed maximum inhibition, which is even comparable to the commercially available toxic antibiotics bearing known and serious side effects [18]. It is to be mentioned that these compounds were found to be more potent against *E. coli* and *S. Aureus* (**Figure 3**), as compared to streptomycin where as **2j** showed excellent inhibitory activity against *E. coli, S. Aureus* and S. Abony as compared to chloromphenicol. We observed the activity of each compound at every concentration for 8 hr, 12 hrs and 24 hrs. It was evident that all the compounds showed significant activity at minimum concentration i.e. 25 µg/mL within 8hrs with the satisfactory zone of inhibition. As the time increases after 8 hrs, the activity of compounds gradually decreases and almost diminished about 12 hrs. *S. epidermidis* showed moderate sensitivity towards **2a** and **2b** while resistant to **(2c-j)**.

4. Conclusion

The alkyl substituted derivatives of Pyrimidine were synthesized by using tetra butyl ammonium bromide as phase transfer catalyst at 20-25 °C with excellent yield. The tested compounds are the Pyrimidine analogues substituted at different positions namely at N1, C4 and C5 in Pyrimidine subunit whereas at ortho and para positions in phenyl ring which is situated at C6 of Pyrimidine molecular frame **(2a-j)** were evaluated for their in vitro antimicrobial activity against the pathogenic bacteria. The preliminary structure-activity relationship (SAR) analysis suggested that the introduction of appropriate substituted phenyl ring into position 6 of thiopyrimidine ring enhanced antibacterial activities of these compounds.

5. Experimental

5.1. General and instrumental

Melting points were determined by open capillary method and are uncorrected. FTIR spectra were recorded on a Shimadzu FTIR spectrophotometer using KBr discs. ^{1}HNMR and ^{13}CNMR spectra were recorded from $CDCl_3$/ DMSO-d_6 solution on a Brucker Avance II 400 (400 MHz) NMR Spectrometer using TMS as an internal reference (chemical shift in δ ppm). Mass spectra were taken on Shimadzu gas chromatograph coupled with QP5050 Spectrometer at 1-1.5 ev. The purity of the compounds was checked on a silica gel-G plates and visualization was done using iodine/UV lamp.

5.2. *General Procedure for the synthesis of Substituted ethyl hexahydro-1, 4, 5-trimethyl-2-oxo/thioxo-6-phenylpyrimidine-5-carboxylate (2a-j)*

Tetra butyl ammonium bromide (9.23 g, 0.0272 mol) was added to the stirred solution of K_2CO_3 (6.9 g, 0.05 mol) in 30ml acetone. To this, was added a solution of β–ketoester (0.0277 mol) in 20 ml of acetone and the resulting solution was stirred for one hour followed by the addition of alkyl halide RX (0.034 mol) to the reaction mixture and it was further stirred at room temperature. The reaction was monitored by TLC. After the completion of the reaction, acetone was removed under vacuum; water was added to the residue and extracted twice with ether. Ether layer was dried over anhydrous Na_2SO_4, filtered, concentrated under vacuum and the crude product was then purified by column chromatography n-Hexane: ethyl acetate = 7:3).

*5.2.1. Ethyl hexahydro- 1, 4, 5- trimethyl-2-oxo-6-phenyl pyrimidine-5-carboxylate **(2a)***

Yield: 94%; m.p. 155 °C, IR (KBr, cm^{-1}) 3200, 1695, 1580, 1450, 1425, 1150; ^{1}HNMR ($CDCl_3$ + CCl_4, 400 MHz): 1.25 (t, 3H, *J* = 8), 2.36 (s, 3H), 2.55 (s, 3H), 2.68 (s, 1H), 2.98 (s, 1H), 2.99 (d, 3H), 3.65 (s, 1H), 4.10 (q, 2H, *J* = 4.75), 5.25 (s, 1H), 7.25-7.30 (m, 5H); ^{13}C-NMR (DMSO-d_6 , ppm): 12.58, 21.53, 56.90, 60.68, 101.83, 122.45, 140.43, 150.95, 161.20, 164.10. MASS (GCMS-EI(+)): m/z = 289 (M+1) . Elemental analyses Found (Calcl.): C: 65.96 (66.18); H: 7.50 (7.64); N: 9.70 (9.65).

*5.2.2. Ethyl 6-(4-chlorophenyl)-hexahydro-1, 4, 5-trimethyl-2-oxo pyrimidine-5-carboxylate **(2b)***

Yield: 92%; m.p. 180 °C , IR (KBr, cm^{-1}) 3190, 1720, 1690, 1580, 1450, 1419, 1135; ^{1}HNMR ($CDCl_3$ + CCl_4, 400MHz) 1.26 (t, 3H, *J* = 8), 2.37 (s, 3H), 2.56 (s, 3H), 2.69 (s, 1H), 2.99 (s, 1H), 3.01 (d, 3H), 3.67 (s, 1H), 4.12 (q, 2H, *J* = 4.75), 5.26 (s, 1H), 7.25-7.32 (m, 4H); ^{13}C-NMR (DMSO-d_6 , ppm): 12.60, 21.51, 56.91, 60.66, 101.85, 122.48, 140.44, 150.96, 161.22, 164.12. MASS (GCMS-EI(+)): m/z = 323 (M+1). Elemental analyses Found (Calcl.): C: 59.04 (59.17); H: 6.42 (6.52); N: 8.54 (8.62)

*5.2.3. Ethyl hexahydro- 1, 4, 5- trimethyl-6-(2-nitrophenyl)-2-oxo-pyrimidine-5-carboxylate (**2c**)*

Yield: 82%; m.p. 185°C , IR (KBr, cm^{-1}) 3195, 1715, 1710, 1690, 1580, 1435, 1419, 1120; ^{1}HNMR ($CDCl_3$ + CCl_4, 400MHz): 1.25 (t, 3H, *J* = 8), 2.36 (s, 3H), 2.55 (s, 3H), 2.68 (s, 1H), 2.97 (s, 1H), 2.99 (d, 3H), 3.64 (s, 1H), 4.09 (q, 2H, *J* = 4.75), 5.23 (s, 1H), 7.25-7.30 (m, 4H); ^{13}C-NMR (DMSO-d_6 , ppm): 12.62, 21.57, 56.97, 60.64, 101.89, 122.49, 140.47, 150.98, 161.25, 164.17. MASS (GCMS-EI(+)): m/z = 334 (M+1). Elemental analyses Found (Calcl.): C: 57.14 (57.30); H: 6.35 (6.31); N: 12.35 (12.35).

*5.2.4. Ethyl hexahydro- 1, 4, 5- trimethyl-6-(4-methoxyphenyl)-2-oxo-pyrimidine-5-carboxylate (**2d**)*

Yield: 89%; m.p. 170 °C, IR (KBr, cm^{-1}) 3195, 1715, 1710, 1690, 1580, 1435, 1419, 1120; ^{1}HNMR ($CDCl_3$ + CCl_4, 400 MHz) 1.22 (t, 3H, *J* = 8), 2.34 (s, 3H), 2.50 (s, 3H), 2.61 (s, 1H), 2.97 (s, 1H), 2.98 (d, 3H), 3.65 (s, 1H), 3.85 (s, 3H), 4.05 (q, 2H, *J* = 4.75), 5.23 (s, 1H), 6.90-7.20 (m, 4H); ^{13}C-NMR (DMSO-d_6 , ppm): 12.68, 21.51, 56.91, 60.63, 101.85, 122.48, 140.44, 150.96, 161.24, 164.13. MASS (GCMS-EI(+)): m/z = 319 (M+1). Elemental analyses Found (Calcl.): C: 63.70 (63.73); H: 7.50 (7.55); N: 8.75 (8.74).

5.2.5. Ethyl hexahydro- 1, 4, 5- trimethyl-6-(4-hydroxyphenyl)--2-oxo-pyrimidine-5-carboxylate ***(2e)***

Yield: 90%; m.p. 115 °C, IR (KBr, cm^{-1}) 3180, 1720, 1680, 1580, 1450, 1419, 1140; ^{1}HNMR ($CDCl_3$ + CCl_4, 400 MHz) 1.19 (t, 3H, *J* = 8), 2.37 (s, 3H), 2.52 (s, 3H), 2.63 (s, 1H), 2.97 (s, 1H), 2.99 (d, 3H), 3.65 (s, 1H), 4.09 (q, 2H, *J* = 4.75), 5.23 (s, 1H), 6.90-7.20 (m, 4H), 8.45 (s, 1H); ^{13}C-NMR (DMSO-d_6 , ppm): 12.64, 21.51, 56.91, 60.66, 101.86, 122.48, 140.34, 150.96, 161.22, 164.16; MASS (GCMS-EI(+)): m/z = 305 (M+1). Elemental analyses Found (Calcl.): C: 62.96 (62.73); H: 7.30 (7.24); N: 9.05 (9.14).

5.2.6. Ethyl hexahydro- 1, 4, 5- trimethyl-6-(4-N',N dimethyl phenyl)-2-oxo-pyrimidine-5-carboxylate ***(2f)***

Yield: 84%; m.p. 235 °C, IR (KBr, cm^{-1}) 3195, 1715, 1710, 1690, 1575, 1425, 1415, 1120; ^{1}HNMR ($CDCl_3$ + CCl_4, 400MHz): 1.21 (t, 3H, *J* = 8), 2.33 (s, 3H), 2.51 (s, 3H), 2.62 (s, 1H), 2.96 (s, 1H), 2.99 (d, 3H), 3.05 (s, 6H), 3.65 (s, 1H), 3.85 (s, 3H), 4.07 (q, 2H, *J* = 4.75), 5.23 (s, 1H), 6.95-7.25 (m, 4H); ^{13}C-NMR (DMSO-d_6 , ppm): 12.61, 21.50, 56.92, 60.67, 101.86, 122.49, 140.45, 150.98, 161.23, 164.14; MASS (GCMS-EI(+)): m/z = 332 (M+1). Elemental analyses Found (Calcl.): C: 64.50 (64.84); H: 8.10 (8.16); N: 12.90 (12.65).

5.2.7. Ethyl hexahydro- 1, 4, 5- trimethyl-6-phenyl-2-thioxo-pyrimidine-5-carboxylate ***(2g)***

Yield: 96%; m.p. 105 °C, IR (KBr, cm^{-1}) 3195, 1715, 1710, 1690, 1580, 1445, 1425, 1145; ^{1}HNMR ($CDCl_3$ + CCl_4, 400MHz) 1.19 (t, 3H, *J* = 8), 2.34 (s, 3H), 2.50 (s, 3H), 2.61 (s, 1H), 2.96 (s, 1H), 2.99 (d, 3H), 3.61 (s, 1H), 4.05 (q, 2H, *J* = 4.75), 5.21 (s, 1H), 7.23-7.29 (m, 5H); ^{13}C-NMR (DMSO-d_6 , ppm): 13.58, 22.53, 58.90, 62.68, 102.83, 126.45, 141.43, 153.95, 162.20, 166.10. MASS (GCMS-EI(+)): m/z = 306 (M+1). Elemental analyses Found (Calcl.): C: 62.59 (62.7); H: 7.50 (7.24); N: 9.27 (9.16).

5.2.8. Ethyl hexahydro- 1, 4, 5- trimethyl-6-(4-hydroxyphenyl)-2-thioxo-pyrimidine-5-carboxylate ***(2h)***

Yield: 82%; m.p. 165°C , IR (KBr, cm^{-1}) 3180, 1720, 1680, 1580, 1450, 1419, 1140, ^{1}HNMR ($CDCl_3$ + CCl_4, 400MHz)) 1.18 (t, 3H, *J* = 8), 2.35 (s, 3H), 2.50 (s, 3H), 2.62 (s, 1H), 2.96 (s, 1H), 2.98 (d, 3H), 3.61 (s, 1H), 4.08 (q, 2H, *J* = 4.75), 5.22 (s, 1H), 6.90-7.20 (m, 4H), 8.41 (s, 1H). ^{13}C-NMR (DMSO-d_6 , ppm): 13.60, 22.55, 58.92, 62.69, 102.85, 126.48, 141.46, 153.96, 162.25, 166.12. MASS (GCMS-EI(+)): m/z = 322 (M+1). Elemental analyses Found (Calcl.): C: 59.62 (59.60); H: 6.96 (6.88); N: 8.35 (8.69).

5.2.9. Ethyl hexahydro-1, 4, 5-trimethyl-6-(4-methoxyphenyl)-2-thioxo-pyrimidine-5-carboxylate ***(2i)***

Yield: 79%; m.p. 120 °C , IR (KBr, cm^{-1}) 3195, 1715, 1705, 1695, 1580, 1435, 1419, 1120; ^{1}HNMR ($CDCl_3$ + CCl_4, 400MHz)) 1.20 (t, 3H, *J* = 8), 2.34 (s, 3H), 2.50 (s, 3H), 2.61 (s, 1H), 2.96 (s, 1H), 2.98 (d, 3H), 3.63 (s, 1H), 3.85 (s, 3H), 4.05 (q, 2H, *J* = 4.75), 5.23 (s, 1H), 6.90-7.22 (m, 4H); ^{13}C-NMR (DMSO-d_6 , ppm): 13.61, 22.54, 58.93, 62.68, 102.88, 126.46, 141.44, 153.97, 162.28, 166.15; MASS (GCMS-EI(+)): m/z = 336 (M+1). Elemental analyses Found (Calcl.): C: 60.55 (60.69); H: 7.25 (7.16); N: 8.08 (8.33).

5.2.10. Ethyl 6-(2-chlorophenyl)-hexahydro-1, 4, 5-trimethyl-2-thioxo pyrimidine-5-carboxylate ***(2j)***

Yield: 84%; m.p. 155 °C, IR (KBr, cm^{-1}) 1.17 (t, 3H, *J* = 8), 2.36 (s, 3H), 2.53 (s, 3H), 2.62 (s, 1H), 2.95 (s, 1H), 2.96 (d, 3H), 3.62 (s, 1H), 4.07 (q, 2H, *J* = 4.75), 5.22 (s, 1H), 6.91-7.25 (m, 54); ^{13}C-NMR (DMSO-d_6 , ppm): 13.54, 22.56, 58.98, 62.67, 102.85, 126.43, 141.42, 153.96, 162.22, 166.13; MASS (GCMS-EI(+)): m/z = 323 (M+1). Elemental analyses Found (Calcl.): C: 56.75 (56.38); H: 5.98 (6.21); N: 8.35 (8.22).

5.2 Biological assay

5.2.1 Medium

Medium used for the biological testing was nutrient agar media (NAM) of the following composition: peptone 10g; yeast extract 3g; sodium chloride 5g; nutrient agar 2% and final volume of medium was adjusted to 1000ml with sterile distilled water having pH 7.

5.2.2 In vitro antibacterial assay

Newly synthesized compounds (**2a-j**) were screened for their antibacterial activity against bacterial culture using agar well diffusion assay technique and minimum inhibitory concentration (MIC) method.

5.2.3 Primary Screening

The antibacterial activities of the newly synthesized compound were evaluated by agar well diffusion assay technique against two Gram-positive bacteria namely *S. aureus* (ATCC 6538P), *S. abony* (NCTC 6017) and against two Gram negative bacteria namely *E. coli* (ATCC 8739), *S. epidermidis* (ATCC 12228). The bacterial cultures were maintained on the

nutrient agar media by sub-culturing them on the fresh slants after every 4-6 weeks and incubating them at the appropriate temperature for 24h. All stock cultures were stored at 4^0C. For the evaluation of antimicrobial activity of the synthetic compounds, suspension of each test microorganism was prepared. Turbidity of each suspension was adjusted to 0.5 McFarland units by suspending the cultures in sterile distilled water. The size of final inoculums was adjusted to $5x10^7$ CFU/mL. A volume of 20 mL of agar media was poured into each Petri plate and plates were swabbed with broth cultures of the respective microorganism and kept for 15 min for adsorption to take place. Using a punch, ≈ 4mm diameter well was bored in the seeded agar plate and 100µL volume of each test compound reconstituted in DMSO was added into the wells. DMSO was used as control for all the test compounds. After holding the plates at room temperature for 2h to allow diffusion of the compounds in to the agar, the plates were incubated at 37 °C for 24h. Antibacterial activity was determined by measuring the inhibition zone diameter.

5.2.4 Minimum inhibitory concentration (MICs)

MIC is the lowest concentration of the antimicrobial agents that prevents the development of visible growth of microorganism after overnight incubation. MIC of chemically synthesized compounds against two Gram-positive bacteria namely *S. aureus* (ATCC 6538P), *S. abony* (NCTC 6017) and against two Gram negative bacteria namely *E. coli* (ATCC 8739), *S. epidermidis* (ATCC 12228) was determined by reported method [15]. Nutrient broth was adjusted to pH 7.0 used for the determination of MIC. The inoculum of the rest microorganism was prepared by using 16h old culture adjusted by reference to the 0.5 McFarland standards (10^8 cells/mL) [16]. These culture were further diluted up to 10 fold with nutrient broth to get inoculums size of $1.2x10^7$ CFU/mL. A positive control (containing inoculum but no compound) and a negative control (containing compound but no inoculums) were also prepared. A stock solution of 4 mg/mL of each compound was prepared in DMSO and the further appropriately diluted to get final concentration ranging from 250 to 0.03 µg/mL [17]. Then appropriately diluted test sample was added to each flask were mixed and incubated for 24-48 h at 37 °C. The test bacterial culture was spotted in a predefined pattern by aseptically transferring 5 µL of each bacterial culture on the surface of solidified agar-agar plates and incubated at 37 °C for 24h for determining the MIC value.

Acknowledgement

The authors deeply acknowledge Department of Microbiology, Sardar Patel Mahavidyalaya Chandrapur Maharashtra for antimicrobial study. We also thankful to Department of Pharmacy, Nagpur for IR spectral analysis; SAIF, Chandigarh for ^{1}HNMR, ^{13}CNMR spectral analysis; Pune for the Mass analysis and RSIC, Kolkata for single crystal X-ray diffraction data.

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[18] Note: The commercially available antibiotics have various side effects. In fact, Streptomycin cannot be given orally, but must be administered by regular intramuscular injections. Adverse effects of this medicine areototoxicity, nephrotoxicity, fetal auditory toxicity, and neuromuscular paralysis. On other hand, the most serious adverse effect associated with chloramphenicol treatment is bone marrow toxicity, and aplastic anemia, which isidiosyncratic but as it is both cheap and easy to manufacture it is frequently an antibiotic of choice in the Developing World. See for example: Rich, M.; Ritterhoff, R.; Hoffmann, R. (1950). "A fatal case of aplastic anemia following chloramphenicol (chloromycetin) therapy". Annals of Internal Medicine 33 (6): 1459–1467.

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FIGURE CAPTIONS:

Figure 1. Chemical structure of candidate drugs **2a-2j** and clinical toxic standard drugs [18].

Figure 2. An ORTEP drawing of the Ethyl hexahydro-1, 4, 5-trimethyl-6-phenyl-2-thioxo-Pyrimidin-5-carboxylate **2g** showing the atomic numbering system.

TABLES:

Table 1. Crystal and experimental data for the title compound (**2g**).

Parameter	
Empirical formula	$C_{16}H_{22}N_2O_2S$
Formula weight (g mol–1)	306
Radiation	MoKα (λ = 0.70930)
Temperature	299 K
Crystal system	Triclinic
Space group	*P-1*
a (Å)	7.1270 (2)
b (Å)	9.7431 (2)
c (Å)	12.5031(3)
α (deg)	98.1693
β (deg)	103.0690
γ (deg)	107.0778
V($Å^3$)	68.203 (9)
Z	1
Dcalc (g/cm^{-3})	0.352
μ (mm^{-1})	0.7
F(000)	164
Crystal size (mm)	0.25 x 0.48 x 0.08
Temp (K)	299 (2)
θ range (deg)/ completeness (%)	2.9 to 27.00
R^b[I>2σ (I)]	0.0394
wR_2 [b](all data)	0.0828

Table 4. In vitro antibacterial activity of newly synthesized compounds by using well diffusion method.

Compd.	Concentration (µg/mL)	Diameter of Zone of Growth Inhibition			
		EC	***SA***	***S.Ab***	***SE***
2a	25	12	10	09	-
	100	13	10	10	14
2b	25	10	12	09	-
	100	14	16	10	13
2c	25	13	14	15	-
	100	18	28	24	-
2d	25	13	10	10	-
	100	14	12	13	-
2e	25	08	10	11	-
	100	10	12	14	-
2f	25	10	12	10	-
	100	14	16	22	-
2g	25	14	10	16	-
	100	21	13	18	-
2h	25	17	13	16	-
	100	20	15	20	-
2i	25	15	12	16	-
	100	22	16	15	-
2j	25	20	18	18	-
	100	20	23	28	-
Chlorom.	25	20	20	19	14
	100	24	28	32	35
Streptom.	25	14	12	25	-
	100	16	16	18	-

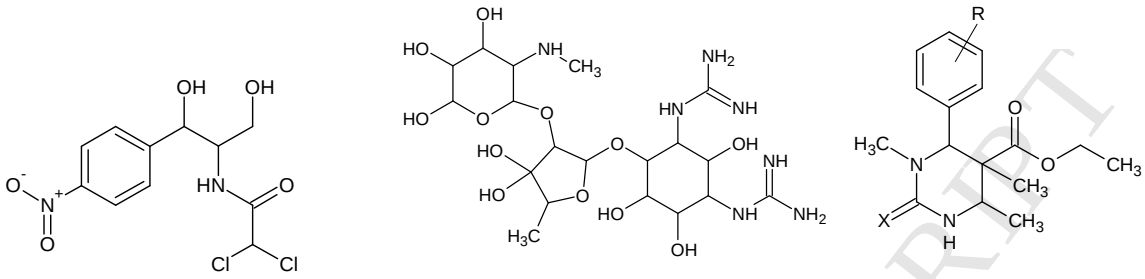
(-): No activity; E.C: *E. Coli*; S. A.: *S. Aureus*; S. Ab.: *S. Abony*; S. E.: *S. Epidermidis.* **Chlorom.**: Chloromphenicol; **Streptom.**: Streptomycin.

Table 5. MIC (µg/mL) of the compounds **2a-2j**.

Compd.	*E.C*	*S.A.*	*S.Ab*	*SE*
2a	68	125	125	68
2b	32	68	125	68
2c	32	08	16	>250
2d	68	68	68	>250
2e	125	68	68	>250
2f	68	32	16	>250
2g	16	68	32	>250
2h	32	32	32	>250
2i	16	32	32	>250
2j	16	16	08	>250
Chlorom.	16	08	04	04
Streptom.	32	32	32	>250

E.C: *E. Coli*; S. A.: *S. Aureus*; S. Ab.: *S. Abony*; S. E.: *S. Epidermidis.* Chlorom**.**: Chloromphenicol; Streptom. : Streptomycin.

FIGURES:



Chloromphenicol (**Chlorom)** Streptomycin **(Streptom.**) **2a-2j** (X = O, S)

Figure 1. Chemical structure of candidate drugs **2a-2j** and clinical toxic standard drugs [18].

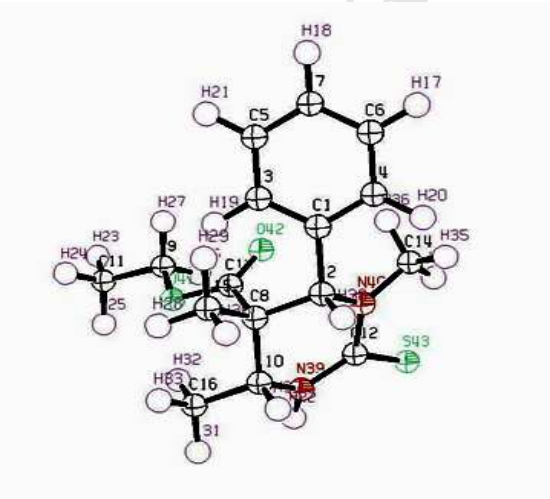
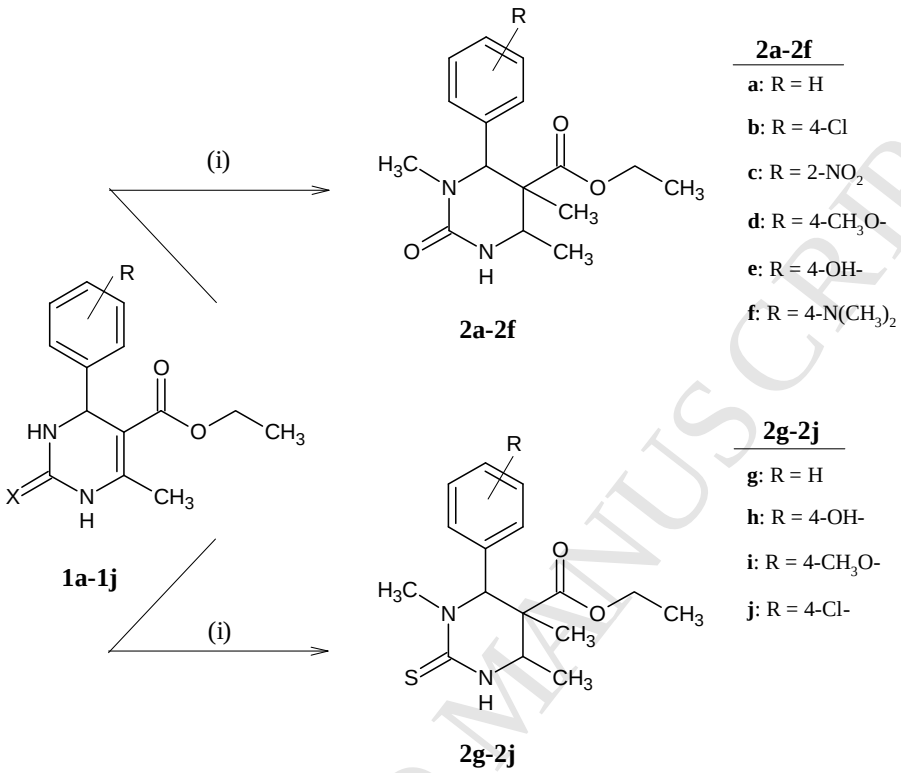


Figure 2. An ORTEP drawing of the Ethyl hexahydro-1,4,5-trimethyl-6-phenyl-2-thioxo-Pyrimidin-5-carboxylate **2g** showing the atomic numbering system.

SCHEMES:



Scheme 1. Synthesis of new tested compounds **2a-2j**. Conditions (i): CH_3I/ TBAB (catalyst)/ K_2CO_3/ Acetone (solvent)/ 20-25 °C/ 12 h.

HIGHLIGHTS

- The N-1 and C-5 alkyl substituted derivatives of Pyrimidine were synthesized.
- Crystal structure of a compound was determined.
- The new compounds were evaluated for their antibacterial activities.
- SAR of these compounds is described.

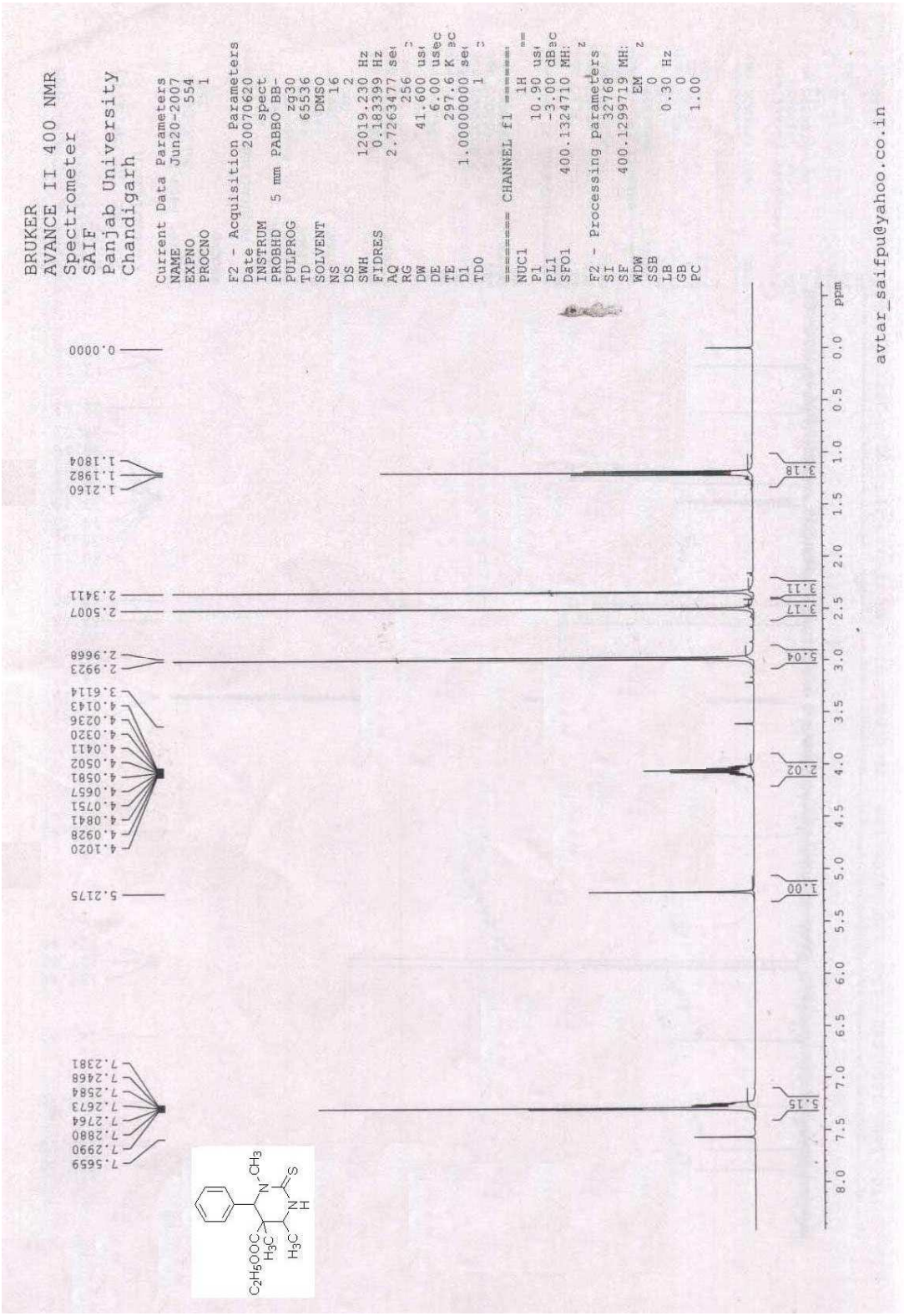
SUPPLEMENTARY DATA

Table 2. Selected bond lengths (in angstrom) of Ethyl hexahydro-1,4,5-trimethyl-6-phenyl-2-thioxopyrimidine-5-carboxylate (**2g**).

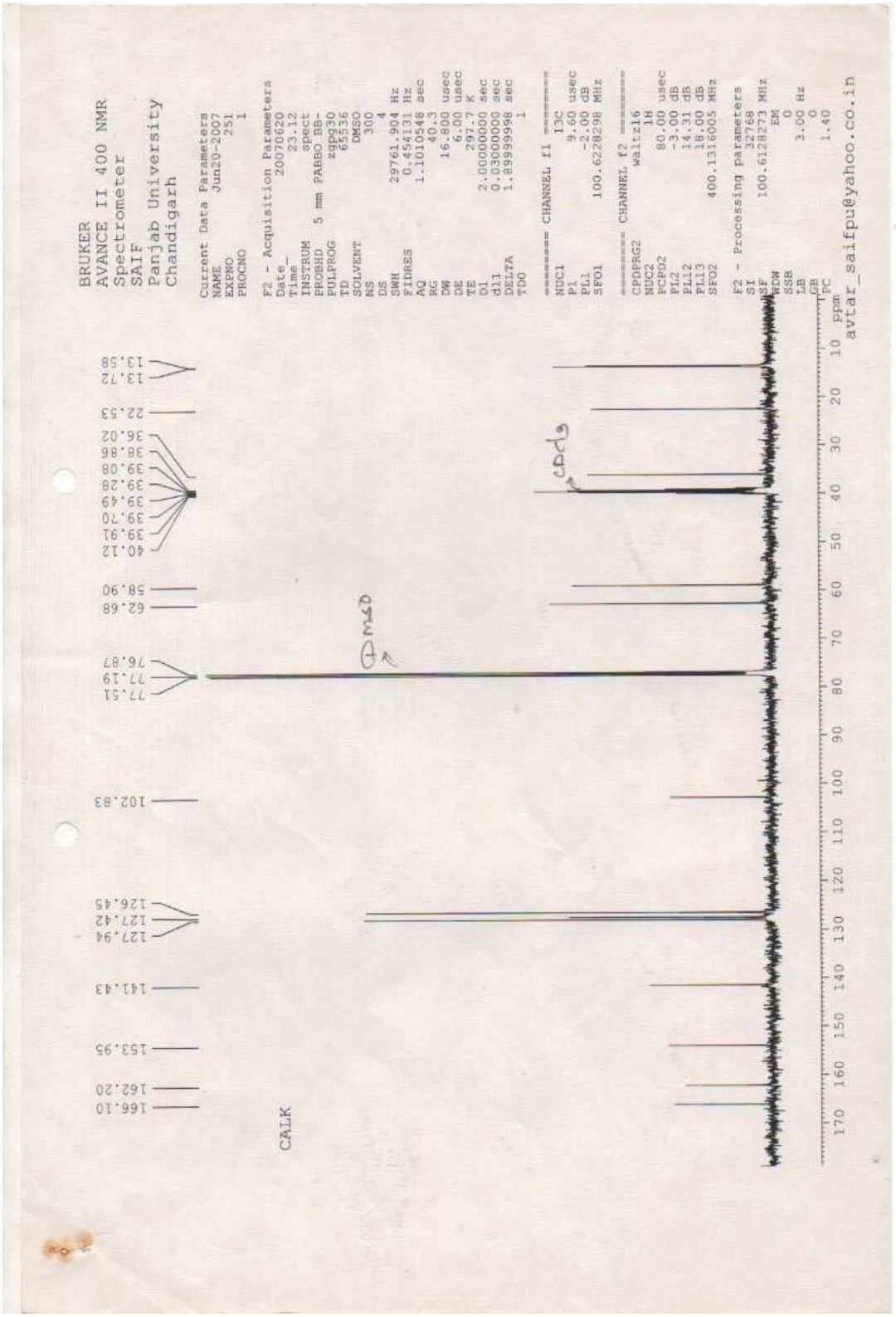
A- B	Distance	A- B	Distance	A- B	Distance
C1- C4	1.386	C15- H33	1.122	H30- C16	1.122
C2- H38	1.123	C16- H30	1.122	H31- C15	1.122
C3- H19	1.122	H17- C6	1.122	H32- C15	1.122
C4- H20	1.122	H18- C7	1.122	H33- C15	1.122
C5- C7	1.387	H19- C3	1.122	H34- C14	1.122
C6- H17	1.122	H20- C4	1.122	H35- C14	1.122
C7- C6	1.386	H21- C5	1.121	H36- C14	1.122
C8- C10	1.54	H22- N39	1.028	H37- C10	1.122
C9- C14	1.541	H23- C11	1.123	H38- C2	1.123
C10- N39	1.445	H24- C11	1.122	N39- C10	1.445
C11- H25	1.121	H25- C11	1.121	N40- C2	1.446
C12- O41	1.213	H26- C9	1.122	O41- C12	1.213
C12- N40	1.446	H27- C9	1121	O42- C13	1.41
C13- O43	1.212	H28- C16	1.122	O42- C9	1.411
C14- H35	1.122	H29- C16	1.122	O43- C13	1.212

Table 3. Selected bond angles (in degrees) of Ethyl hexahydro-1,4,5-trimethyl-6-phenyl-2-thioxopyrimidine-5-carboxylate (**2g**).

A- B- C	Angle	A- B- C	Angle	A- B- C	Angle
C4- C1- C3	120.01	C10- C8- C2	109.49	O42- C13- C8	119.98
C3- C1- C2	119.99	C13- C8- C2	109.51	H36- C14- H35	109.32
H38- C2- N40	109.31	C2- C8- C14	109.49	H35- C14- H34	109.51
N40- C2- C1	109.52	H27- C9- H26	109.36	H34- C14- C8	109.48
C1- C2- C8	109.49	H26- C9- O42	109.47	H31- C15- H32	109.52
H19- C3- C5	120.02	O42- C9- C11	109.46	H32- C15- H33	109.35
C5- C3- C1	119.98	H37- C10- N39	109.49	H33- C15- C10	109.49
H20- C4- C1	119.99	N39- C10- C8	109.52	H29- C16- H28	109.52
C1- C4- C6	120.01	C8- C10- C15	109.5	H30- C16- N40	109.43
H21- C5- C3	120.02	H25- C11- H24	109.35	H28- C16- N40	109.5
C3- C5- C7	119.99	H24- C11- H23	109.46	H22- N39- C10	122.65
H17- C6- C7	120	H23- C11- C9	109.52	C10- N39- C12	117.53
C7- C6- C4	120.01	O41- C12- N39	127.58	C2- N40- C12	120.01
H18- C7- C6	119.98	N39- C12- N40	119.64	C12- N40- C16	120.01
C6- C7- C5	120.01	O43- C13- O42	120.03	C13- O42- C9	109.46



[1]H-NMR Spectrum of compound 2g



^{13}C-NMR Spectrum of compound 2g