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IN-VITRO ANTIOXIDANT SCREENING AND SYNTHESIS OF 3-CYANO-4-IMINO-2-(METHYLTHIO)-4*H*-PYRIMIDO[1,2-*a*]PYRAZINE AND ITS SUBSTITUTED DERIVATIVES

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ABSTRACT
2-amino pyrazine on condensation with bis(methylthio) methylene malanonitrile in presence of DMF and anhydrous K_2CO_3 as catalyst to afford 3-cyano-4-imino-2-(methylthio)-4*H*-pyrimido[1,2-*a*]pyrazine, which on further reaction with selected nucleophile like substituted aryl and heteryl amine, phenol, active methylene compound to afford 2-substituted derivatives**.** The synthesized compounds were screened for antioxidant potential against DPPH and OH radical scavenging assay.

KEYWORDS: 2-amino pyrazine, bis(methylthio)methylene malanonitrile, 3-cyano-4-imino-2-(methylthio)-4*H*-pyrimido[1,2-*a*]pyrazine.

INTRODUCTION
The class of fused heterocyclic compounds containing pyrimidine ring in their structure impart main role in biological activity, the pyrimido pyrimidine annulated uracils which correlate with purine-pteridine system. These fused pyrimido pyrimidine ring is found in marine obtained natural products like batzelladine[1], crambescidin alkaloid[2], also pyrimido pyrimidine and pyrimido pyridazine compounds exhibit anticancer[3], antibacterial activity[4,5], antifungal activity[6,7], anti-HIV activity[8], AKT-1 inhibitor[9], Further there are some reference available on synthesis of pyrimido pyrimdine and pyrimido pyridazine bearing compounds. Bazgir et al.[10] reported synthesis of 5,6-dihydro-1,3-dimethyl-5-phenyl pyrimido[4,5-*d*] pyrimidine-2,4,7(1*H*,3*H*,8*H*)-trione by refluxing a mixture of 6-amino-1,3-dimethyl uracil, benzaldehyde and urea in the presence of catalytic amount of acetic acid by microwave irradiation. Atkore et al.[11] reported synthesis of pyrimido[4,5-*d*]pyrimidine derivatives by three component reaction of aromatic aldehyde, urea or thiourea and barbituric acid using tetrabutyl ammonium bromide (TBAB) under solvent free condition by microwave irradiation. Mallette et al.[12] reported synthesis of 2,4-diamino pyrimido[4,5-*b*]pyrazine by cyclocondensation of 1,2-diketone with 2,4,5,6-tetra amino pyrimidine.

Recently our research group reported synthesis of diimino pyrimido pyrimidine by refluxing mixture of guanidine hydrochloride and bis (methylthio) methylene malononitrile and screened their antibacterial activity.[13] The biological activity of pyrimidine fused with pyrimidine and pyrimidine fused with pyridazines compounds and literature survey reveals that very few references are available on synthesis of pyrimido pyrazine. Which encourage us, plan to synthesize pyrimido pyrazine fused heterocyclic compounds as these compounds were not reported in literature survey.

MATERIALS AND METHODS
All the used chemical are of analytical grade, melting point were taken in electro thermal IA 9000 series digital melting point apparatus and were uncorrected. The progress of reaction were checked by thin layer chromatography, carried out on pre-coated sheets of silica-C plates of 0.25 mm thickness using UV chamber for detection. IR spectra were recorded in potassium bromide pallets on Shimadzu infrared spectrophotometer, ^{1}H NMR spectra were taken on Brukner avance spectrophotometer 400 MHz in DMSO-d_6 using tetramethyl silane as internal standard, mass spectra were recorded on FT-VC-7070 H mass spectrometer using the chemical ionisation technique at 70 eV. All the reactions were carried out under room condition.

GENERAL PROCEDURE

Synthesis of 3-cyano-4-imino-2-(methylthio)-4*H*-pyrimido[1,2-*a*]pyrazine (3)

To a solution of 2-amino pyrazine **(1)** (0.95 g, 0.01mol) in 15 ml of DMF, bis (methylthio) methylene malanonitrile **(2)** (1.7 g, 0.01 mol) and 15 mg of anhydrous K_2CO_3 as catalyst was added and allowed to reflux for 3.5 hours. The process of reaction was monitored by TLC. After completion of reaction, the reaction mixture was cooled to room tempreture and poured into ice cold water containing crushed ice (100 ml). The separated solid mass was filtered washed with cold as well as hot water and recrystallized using absolute ethanol to afford as brown solid compound **(3)**

Synthesis of 3-cyano-4-imino-2-(substituted)-4*H*-pyrimido[1,2-*a*]pyrazine (4a-e), (5a-e), (6a-d) and (7a-d)

Refluxing the mixture of 3-cyano-4-imino-2-(methylthio)-4*H*-pyrimido[1,2-*a*] pyrazine (0.217 g, 0.001 mol) independently with various aromatic amines, substituted phenols, compounds containing active methylene group and heteryl amines (0.001 mol) in 15 ml of DMF and anhydrous K_2CO_3 (15 mg) for 3-4 hours. The process of reactions was monitored by TLC. The reaction mixture was cooled to room temperature and poured into ice cold water containing crushed ice (100 ml). The separated solid mass was filtered, washed with water and recrystallized from absolute ethanol to afford **(4a-e), (5a-e), (6a-d),** (**7a-d)** derivatives respectively.

3-Cyano-4-imino-2-(methylthio)-4*H*-pyrimido[1,2-*a*]pyrazine (3)

Brown solid, 72% yield, M.P. 197°C, IR (KBr) cm^{-1} 3332.36 (=NH stre), 2214.13 (CN stre), ^{1}H NMR (DMSOd_6, δ ppm) 2.639 (s, 3H, -SCH_3), 7.834, 8.847, 8.268-8.279 (s, 3H, Ar-H), 8.962 (s, 1H, =NH), ^{13}C NMR (DMSOd_6, δ ppm) 12.51, 115.23, 118.38, 133.18, 143.19, 152.06, 168.74, CI-MS (m/z) 218.2 (+ve mode), 216 (-Ve mode) (100%).

3-Cyano-4-imino-2-(4-nitro anilino)-4*H*-pyrimido[1,2-*a*]pyrazine (4e)

Brown solid, 79% yield, M.P. 196°C, IR (KBr) cm^{-1} 3352.75, 3228.67 (NH & =NH stre), 2214.73 (CN stre), ^{1}H NMR(DMSOd_6, δ ppm) 4.455 (s, 1H, -NH-), 7.595-8.351 (m,7H,-Ar-H), 8.826 (s, 1H, =NH), CI-MS (m/z) 308.3 (M +1).

3-Cyano-4-imino-2-(4-nitro phenoxy)-4*H*-pyrimido[1,2-*a*]pyrazine (5e)

Brown solid, 72% yield, M.P. 234-236°C, IR (KBr) cm^{-1} 3436.91, 3313.48 (=NH stre), 2210.27 (CN stre), ^{1}H NMR(DMSOd_6, δ ppm) 7.275-8.168 (m, 7H, -Ar-H), 8.736 (s, 1H =NH) MS-CI (m/z) 309.3 (M +1).

3-Cyano-4-imino-2-(α-malonyl)-4*H*-pyrimido[1,2-*a*]pyrazine (6d)

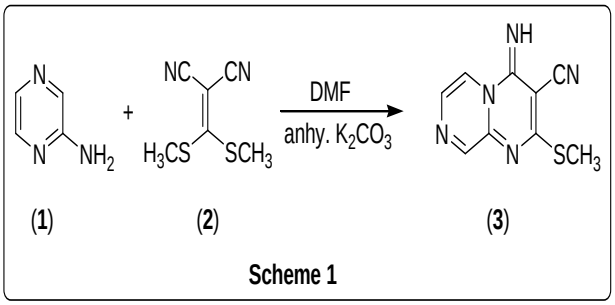
Brown solid, 76% yield, M.P. 232°C, IR (KBr) cm^{-1} 3151.47 (=NH stre), 2233.42 (CN stre), ^{1}H NMR (DMSOd_6, δ ppm) 4.158 (s, 1H, -CH-), 7.844-8.863 (m, 3H, Ar-H), 8.970 (s, 1H, =NH), MS-CI (m/z) 236 (M +1) (100%).

3-Cyano-4-imino-2-(morpholino)-4*H*-pyrimido[1,2-*a*]pyrazine (7c)

Grey solid, 74% yield, M.P. 207°C, IR (KBr) cm^{-1} 3328.81 (=NH stre), 2214.13 (CN stre), ^{1}H NMR(DMSOd_6, δ ppm) 3.053 (t, 4H), 3.682 (t, 4H), 7.317 (s, 1H, Ar-H), 7.541 (s, 2H, Ar-H), 8.136 (s,1H,=NH), MS-CI (m/z) 256.1 (M^+).

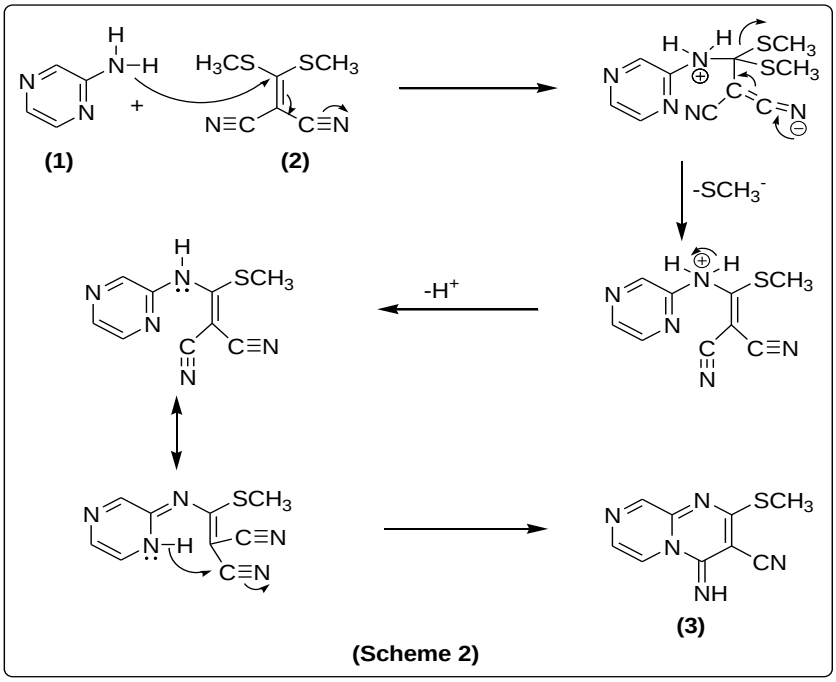
RESULTS AND DISCUSSION

The compound 3-Cyano-4-imino-2-(methylthio)-4*H*-pyrimido[1,2-*a*]pyrazine was synthesized by condensation of 2-amino pyrazine with bis(methylthio) methylene malanonitrile in presence of DMF and anhydrous K_2CO_3 as catalyst (scheme 1).



Scheme 1

The plausible mechanism of formation of compound (3) is given in scheme 2.

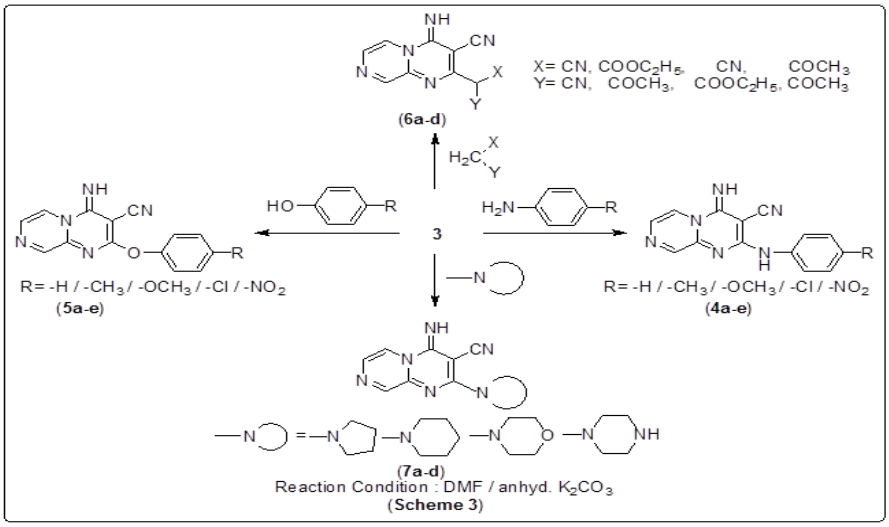


(Scheme 2)

The structure of the compound (3) was confirmed on the basis of analytic and spectral data. IR spectrum shows absorption frequency bands at 2214.13 cm^{-1} (-CN) and 3332.36 cm^{-1} (=NH) due to stretching vibrations in functional group region.

The ^{1}H NMR spectrum of the compound was recorded in $DMSOd_6$, shows singlet at δ2.639 ppm assignable to -SCH_3 group, singlet at δ7.834 ppm, δ8.847 ppm, δ8.268-8.279 ppm due to pyrazine ring protons and singlet at δ8.962 ppm due to =NH proton respectively. Mass spectrum exhibits molecular ion peak at m/z 218 in positive mode and m/z 216 (100% abundance) in negative mode which corresponds to its molecular weight and ^{13}C NMR spectrum of the compound gives seven peaks which are corresponding to carbon skeleton in the parent compound (3). Some specific carbon nuclei peaks observed at δ12.51, 115.23, 168.74 which are assignable to -SCH_3, -CN, C=NH carbon groups respectively.

The compound (4.35) have active methylthio group at 2-position which is easily replaceable; therefore, its susceptibility towards nucleophilic substitution reaction with different regents like aryl amines, substituted phenols, active methylene groups and heteryl amines has been investigated (scheme 3).



(Scheme 3)

Amino derivatives of (3) were prepared by reacting it independently with aniline, p-toulidine, p-anisidine, p-chloro aniline and p-nitro aniline in DMF and anhydrous K_2CO_3 as a catalyst to yield compounds (4a-e) (Scheme-3). The synthesized compounds exhibit strong absorption bands in the range of 2215-2206 cm^{-1} (-CN), 3360-3160 cm^{-1} (-NH, =NH) due to stretching vibration. Mass spectra showed the molecular ion peak which

corresponds to their respective molecular weight. ^{1}H NMR spectra showed absence of singlet at δ2.639 which indicates substitution of active methylthio group takes place.

Phenoxy derivatives of compound (3) were prepared by reacting it with independently with phenol, p-methyl phenol, p-methoxy phenol, p-chloro phenol and p-nitro phenol in DMF and anhydrous K_2CO_3 as catalyst, to yield compound (5a-e) (Scheme-3). All these compounds show strong absorption bands in IR spectrum within the range 2215-2205 cm^{-1} (-CN), 3440-3100 cm^{-1} (=NH) due to stretching vibration.

Different 3-cyano-4-imino-2-(substituted active methyleno)-4*H*-pyrimido[1,2-*a*]pyrazine (6a-*d*) were synthesized by condensing the parent compound (3) independently with acetyl acetone, ethyl cyano acetate, ethyl aceto acetate and malononitrile in DMF and anhydrous K_2CO_3 under reflux condition (Scheme-3). All these compounds shows absorption bands in the range 2235-2210 cm^{-1} (-CN) and 3160- 3050 cm^{-1} (=NH) due to stretching vibration. Mass spectra showed peaks which show their respective molecular weight. ^{1}H NMR spectrum show signals in the range δ4.1 ppm for -CH- and absence of signal δ2.639 ppm for -SCH_3, confirm replacement of -SCH_3 group by active methylene compounds.

Likewise, the treatment of compound (3) with preferred heteryl amines like pyrolidine, piperidine morpholine and piperazine in DMF and K_2CO_3 to afford 3-cyano-4-imino-2-[pyrolidino/ piperidino/ morpholino/ piperazino]-4*H*-pyrimido[1,2-*a*]pyrazine (7a-d) (Scheme-3). All these compounds show IR absorption bands in the range 2215-2206 cm^{-1} (-CN) and 3350-3100cm^{-1} (=NH) due to stretching vibration. Mass spectrum showed ion peaks, which showed their respective molecular weights. ^{1}H NMR spectra are in agreement with the structure of the compounds.

The DPPH radical scavenging assay has been used for preliminary screening of the compound for antioxidant activity. The proton radical scavenging action is known as an important mechanism of antioxidant. The odd electron in the DPPH radical gives a strong absorption maximum at 517 nm and is purple in colour. The colour turns from purple to yellow when odd DPPH radical becomes paired with hydrogen from free radical scavenging antioxidant to form reduced DPPH. All tested compounds were compatible with DPPH radical scavenging activity, the tested compound **4a** (39.51 µg/ml) was found to be more DPPH radical scavenger, while minimum effect was found in compound **5a** (17.29 µg/ml), while remaining compounds have moderately radical scavenging potential having range of 20.91 to 31.62 µg/ml. Ascorbic acid (85.21µg g/ml) was used as reference compound.

The generation of OH radical in the cells due to decrease in antioxidant defence mechanism and excess accumulation of H_2O_2 as a result production of toxic OH radical through Fenton reaction. The tested compounds were screened for hydroxyl radical scavenging assay; all the tested compounds were potent with hydroxyl radical scavenging. The compound **7c** was shown maximum hydroxyl radicals scavenging potential (42.15 µg/ml), while compound **3** exhibit lowest OH radical scavenging potential (14.15µg/ml). The remaining tested compounds exhibit hydroxyl radical scavenging ability was considerable effective in the range of 18.98 to 40.58 µg/ml. Ascorbic acid (79.87µg/ml) was used as reference compound. The results of DPPH and OH radical scavenging assay are summarized in table 1.

1) DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay was carried out as per reported method.[14] In brief, 1 ml (1 mM) of the test sample is added to equal quantity of 0.1 mM solution of DPPH in ethanol. After 20 min of incubation at room temperature, the DPPH reduction was measured by reading the absorbance at 517 nm. Ascorbic acid (1 mM) was used as the reference compound.

2) OH radical scavenging assay

The OH radical scavenging activity was demonstrated with Fenton's reaction.[15] The reaction mixture contained, 60 µl of $FeCl_3$ (1 mM), 90 µl of 1-10 phenathroline (1 mM), 2.4 ml of phosphate buffer (0.2 M, pH 7.8), 150 µl of H_2O_2 (0.17 M) and 1.5 ml of individual compound (1 mM). The reaction was started by adding H_2O_2. After 5 min. incubation at room temperature, the absorbance was recorded at 560 nm. Ascorbic acid (1 mM) was used as a reference compound.

Table 1: The result of antioxidant potential of tested pyrimido[1,2-*a*]pyrazine derivatives.

Sr. No.	Compound	DPPH radical scavenging activity (%)	OH radical scavenging activity (%)
1	3	27.85	14.15
2	4a	39.51	23.17
3	4e	31.62	19.82
4	5a	17.29	32.51
5	5e	21.99	40.58
6	6d	26.19	18.98
7	7c	20.91	42.15
9	*Ascorbic Acid*	85.21	79.87

CONCLUSION

The present work provide convenient and simple method for preparation of pyrimido[1,2-*a*] pyrazine compounds using DMF as reaction solvent and anhydrous K_2CO_3 as catalyst. The result of antioxidant activity of tested compounds provide ready reference for further possible modification would act as good therapeutic drug which is supplement with pharmacological activity of pyrimido pyrazine compounds.

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