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Synthesis and Antimicrobial Activities of some Isoxazolo-[5′,4′ : 4,5]pyrimido[6,1-*c*]1,2,4-triazine Derivatives

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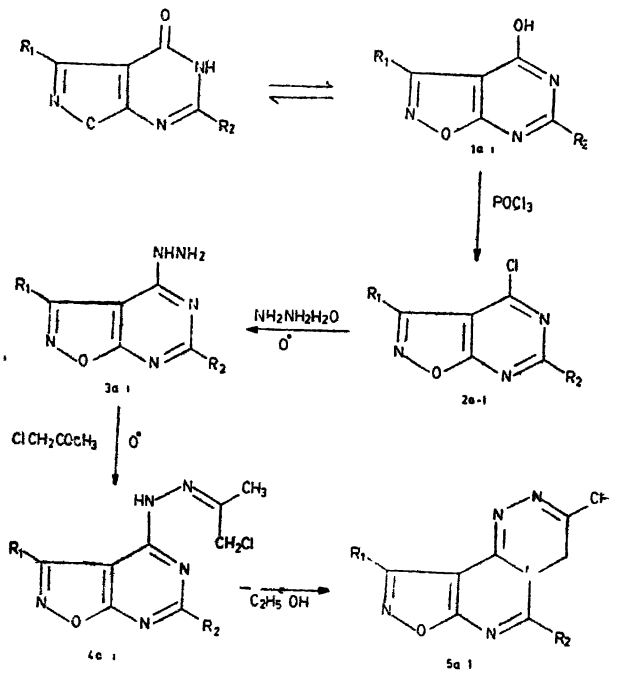
4-Hydrazino-3,6-disubstituted isoxazolo[5,4-*d*]pyrimidines (3a – i), when treated with 1-chloro-2-propanone yielded (3,6-disubstituted-isoxazolo[5,4-*d*]pyrimidine-4-yl)hydrazones of 1-chloro-2-propanone (4a – i), which on cyclisation produced the title compounds, viz 6,10-disubstituted-3-methyl-4*H*-isoxazolo[5′, 4′ : 4, 5]pyrimido[6,1 – *c*]1,2,4-triazines (5a – i). Structures of new compounds have been established by their spectral and elemental analyses. They have been subjected to *in vitro* antibacterial screening against five pathogenic strains and antifungal testing against three fungi; many compounds exhibited promising results

1,2,4-Triazine nucleus has been a seat of diverse biological activities[1] and 1,2,4-triazine derivatives exhibited various physiological activities[2]. On the commercial scene, they have great demand as effective herbicides[3]. Since a number of 3,5-disubstituted-1,2,4-triazines represent aza analogs of pyrimido nucleobase and many natural antibiotics are pyrimido[5,4-*e*]1,2,4-triazines, it prompted us to initiate research on the synthesis and biological activities of the title compounds wherein active 1,2,4-triazine moiety is fused to potent isoxazolo[5,4-*d*]pyrimidine nucleus[4].



1 – 5 a ; R_1 = 4-$CH_3C_6H_4$, R_2 = H
b ; R_1 = 4-$CH_3C_6H_4$, R_2 = CH_3
c ; R_1 = 4-$CH_3C_6H_4$, R_2 = C_2H_5
d ; R_1 = 4-ClC_6H_4, R_2 = H
e ; R_1 = 4-ClC_6H_4, R_2 = CH_3
f ; R_1 = 4-ClC_6H_4, R_2 = C_2H_5
g ; R_1 = C_6H_5, R_2 = H
h ; R_1 = C_6H_5, R_2 = CH_3
i ; R_1 = C_6H_5, R_2 = C_2H_5

In our present investigation, a series of 4-chloro-3,6-disubstituted-isoxazolo[5,4-*d*]pyrimidines (2a – i) was synthesised from (un)substituted-benzaldehydes according to reported procedures[5]. These compounds were readily converted to 4-hydrazino-3,6-disubstituted-isoxazolo[5,4-*d*]pyrimidines (3a – i) by treating them with excess hydrazine hydrate at 0°. The 4-hydrazino derivatives (3a – i) on condensation with 1-chloro-2-propanone in tetrahydrofuran at 0° afforded the intermediates, (3,6-disubstituted-isoxazolo[5,4-*d*]pyrimidin-4-yl)hydrazones of 1-chloro-2-propanone (4a – i) which were isolated in pure state by column chromatography. Structures of 4a – i were confirmed by their satisfactory elemental analyses. The ir spectrum of 4d showed a sharp peak at 3 334 cm^{-1}, characteristic of NH stretching vibration and a very strong band at 1 667 cm^{-1} indicating the formation of C=N linkage as well as the presence of pyrimidine nucleus.

Further, the intermediates 4a – i were readily cyclised to 6,10-disubstituted-3-methyl-4*H*-isoxazolo[5′, 4′ : 4,5]pyrimido[6,1-*c*]1,2,4-triazines (5a – i) by refluxing them in absolute ethanol for 3–4 h. Structure of 5d was confirmed by its spectral and elemental analyses. In its ir spectrum 5d showed no absorption bands at 3 100 – 3 600 cm^{-1} indicating the absence of NH group which evidences the cyclisation. The uv spectrum of 5d exhibited a strong band at higher λ region, 382 nm confirming

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TABLE 1—ANALYTICAL AND PHYSICAL DATA OF COMPOUNDS 4a–i AND 5a–i

Compd. no.	R_1	R_2	Mol. formula (Mol. wt.)	M.p. °C	Yield %	Analysis % : Found/(Calcd.) C	H	N
4a	4-$CH_3C_6H_4$	H	$C_{15}H_{14}ClN_5O$ (315.60)	121–22d[a]	76	57.5 (57.1)	4.7 (4.4)	22.4 (22.2)
4b	4-$CH_3C_6H_4$	CH_3	$C_{16}H_{16}ClN_5O$ (329.61)	140–41d[a]	72	58.0 (58.3)	4.5 (4.9)	21.7 (21.2)
4c	4-$CH_3C_6H_4$	C_2H_5	$C_{17}H_{18}ClN_5O$ (343.62)	142–43d[a]	65	59 8 (59.4)	5.0 (5.2)	20.8 (20.4)
4d	4-ClC_6H_4	H	$C_{14}H_{11}Cl_2N_5O$ (336.05)	172[a]	80	50.6 (50.0)	3.7 (3.3)	20.3 (20.8)
4e	4-ClC_6H_4	CH_3	$C_{15}H_{13}Cl_2N_5O$ (350.05)	155–56d[b]	74	51.6 (51.5)	3.9 (3.7)	19.5 (20.0)
4f	4-ClC_6H_4	C_2H_5	$C_{16}H_{15}Cl_2N_5O$ (364.06)	168–69d[c]	68	52.5 (52.8)	4.4 (4.1)	19.0 (19.2)
4g	C_6H_5	H	$C_{14}H_{12}ClN_5O$ (301.59)	140–41[d]	74	55.3 (55.8)	3.7 (4.0)	23.6 (23.2)
4h	C_6H_5	CH_3	$C_{15}H_{14}ClN_5O$ (315.6)	132[a]	71	56.6 (57.1)	4.6 (4 4)	22.5 (22.2)
4i	C_6H_5	C_2H_5	$C_{16}H_{16}ClN_5O$ (329.61)	138–39[a]	78	58.7 (58.3)	4.5 (4.9)	21.5 (21.2)
5a	4-$CH_3C_6H_4$	H	$C_{15}H_{13}N_5O$ (279.15)	272–72d[e]	68	64.3 (64.5)	4.9 (4.7)	25.3 (25.1)
5b	4-$CH_3C_6H_4$	CH_3	$C_{16}H_{15}N_5O$ (293.16)	291–92d[f]	61	65.8 (65.5)	5.3 (5.1)	23 4 (23.9)
5c	4-$CH_3C_6H_4$	C_2H_5	$C_{17}H_{17}N_5O$ (307.17)	>300[f]	65	66.7 (66.5)	5.4 (5.5)	22.7 (22.8)
5d	4-ClC_6H_4	H	$C_{14}H_{10}ClN_5O$ (299.59)	287[e]	70	56.6 (56.1)	3.0 (3.3)	23.7 (23.4)
5e	4-ClC_6H_4	CH_3	$C_{15}H_{12}ClN_5O$ (313.6)	>300[e]	67	56.1 (56.4)	3.6 (3.8)	22.7 (22 3)
5f	4-ClC_6H_4	C_2H_5	$C_{16}H_{14}ClN_5O$ (327.61)	>300[f]	64	58.9 (58.7)	4.7 (4.3)	21.5 (21.4)
5g	C_6H_5	H	$C_{14}H_{11}N_5O$ (265.14)	285–86d[f]	62	63.6 (63.4)	4.4 (4.1)	26.0 (26.4)
5h	C_6H_5	CH_3	$C_{15}H_{13}N_5O$ (279.15)	282–83[g]	58	64.1 (64.5)	4.9 (4.7)	25.4 (25.0)
5i	C_6H_5	C_2H_5	$C_{16}H_{15}N_5O$ (293.16)	>300[h]	55	65.2 (65.5)	5.4 (5.1)	23.5 (23.9)

*Solvent of crystallisation : [a]benzene + petroleum ether, [b]benzene, [c]n-hexane, [d]petroleum ether, [e]DMF [f]aqueous DMF, [g]DMF + DMSO, [h]DMF + ethanol.

TABLE 2—ANTIMICROBIAL ACTIVITY DATA OF COMPOUNDS 4a–i AND 5a–i

Compd. no.*	Antibacterial activity: Zone of inhibition after 24 h**					Antifungal activity: % Transmission after 48 h***		
	E. coli	*P. vulgaris*	*P. aurigasa*	*K. pneumoniae*	*S. aureus*	*A. niger*	*C. albicans*	*A. flavus*
4a	++	++	+++	+	+	+	+	+
4b	++	++	+	+	++	++	+	–
4c	++	++	–	+	+	+	+	+
4d	+++	++	+	+++	+	++	+	+
4e	++	++	+++	++	+	++	+	+
4f	–	+	+++	–	+	+	+	–
4g	+++	++	++++	++	+	+	+	+
4h	++	++	+++	++	++	+	+	+
4i	++	+	++	+	+	++	+	+
5a	+	+++	–	+	++	++	+	+
5b	–	+	++	+	++	+++	++	+
5c	++	++	+	+	+	++	++	+
5d	+	+++	–	++	+	+++	+	++
5e	++	+	+++	++	+	++	+	+
5f	+	+	–	+	+	+++	+	+
5g	+	++	–	++	+	++	+	+
5h	+	+++	–	++	+	++	+	+
5i	+++	+	–	+	+	++	+	+
S-1	+	++	+	+	+			
S-2	+	++	+	–	+			
S-3						+++	+++	+++

*S-1 = Sulphanilamide, S-2 = phenol, S-3 = salicylic acid.

**Zone of inhibition (mm) : .14–16 (–) = no activity, (+) 17–19, (++) 20–22, (+++) 23–25, (++++) 26–28.

***% Transmission : (–) = 1–25 no activity, (+) 26-50, (++) 51–75, (+++) 76–100. Drug concentration = 200 μg. Solvent = DMF.

the cyclisation. The structure was further established by its ^{1}H nmr spectrum where CH_3 protons resonated as a sharp singlet at δ 2.55 and ring CH_2 protons appeared as another singlet at δ 4.65. The formation of **5d** was authentically established by its mass spectrum which showed a molecular ion (M^+) peak at m/z 300, agreeing with the calculated molecular weight of the compound. Spectral features of **3d**, **4d** and **5d** are given in the experimental part.

Experimental

Melting points were determined in open capillaries and are uncorrected. The ir spectra (nujol) were recorded on a Perkin-Elmer 1378 spectrophotometer, uv spectra (DMF) on a Hitachi 150-20 spectrophotometer, ^{1}H nmr spectra (DMSO-d_6) on a Varian T-60A spectrometer using TMS as internal reference, and mass spectra on a Finnigan 4121 GC spectrometer at 70 eV (low resolution).

4-Hydrazino-3,6-disubstituted isoxazolo[5,4-d] *pyrimidines* (**3a–i**): To a well stirred clear solution of freshly prepared 4-chloro-3,6-disubstituted-isoxazolo[5,4-*d*]pyrimidine (**2a–i**; 0.02 mol) in dioxan (90 ml), an excess of hydrazine hydrate (18 ml; 99%) was added dropwise under refrigeration with ice. The reaction mixture was worked out as per the reported procedure[6]; **3d** ν_{max} 3 175br ($NHNH_2$), 1 626s (pyrimidine), 1 587, 1 580 (CH=CH, Ar) and 725 cm^{-1} (CCl); λ_{max} 230 and 290 nm.

(*3,6-Disubstituted-isoxazolo*[5,4-d]*pyrimidin-4-yl*) *hydrazones of 1-chloro-2-propanone* (**4a–i**): A clear solution of 4-hydrazino-3,6-disubstituted-isoxazolo-[5,4-*d*]pyrimidine (0.01 mol) in dry tetrahydrofuran (200 ml) was treated with a solution of 1-chloro-2-propanone (0.012 mol, 1.15 g) in tetrahydrofuran dropwise with stirring at 0°. After stirring at 0° (20 ml) for 3 h, the resulting clear mixture was evaporated to dryness under reduced pressure. The residue was extracted with benzene repeatedly (twice or thrice) and the combined extract was treated with decolourising charcoal, filtered and concentrated under reduced pressure. It was treated with petroleum ether (b.p. 40–60; 100 ml) and allowed to stand to get yellow crystals of **4a–i**. In some cases, the benzene extract was chromatographed using neutral alumina to get pure compounds. Characterisation data of **4a–i** are summarised in Table 1; **4d** ν_{max} 3 334 (NH), 1 667 (C=N and pyrimidine), 1 626, 1 580 (CH=CH, Ar) and 775 cm^{-1} (CCl); λ_{max} 225, 256 and 305 nm.

*6,10-Disubstituted-3-methyl-4*H-*isoxazolo*[*5′, 4′ : 4,5*] *pyrimido* [*6, 1*-c] *1, 2, 4-triazines* (**5a–i**): A mixture of (3,6-disubstituted-isoxazolo[5, 4-*d*] pyrimidin-4-yl) hydrazone of 1-chloro-2-propanone (**4a–i**; 0.01 mol) and dry ethanol (100 ml) was refluxed for 3–4 h. The reaction mixture was concentrated to small bulk and cooled to get tan coloured solid. It was filtered, washed with ethanol and recrystallised from an appropriate solvent (Table 1); **5d** ν_{max} 1 653 (C=N and pyrimidine), 1 613, 1 587 (CH=CH, Ar) and 725 cm^{-1} (CCl); λ_{max} 382 nm; δ 2.55 (3H, s, CH_3), 4.65 (2H, s, CH_2), 7.3–7.9 (4H, m, Ar), 8.4 (1H, s, 6-H); m/z 300 (M^+), 285 (M-CH_3)$^+$, 272 (M-N_2)$^+$ and 264 (M-Cl)$^+$.

Antibacterial activity: Compounds **4a–i** and **5a–i** were subjected to *in vitro* antibacterial testing against five pathogenic microorganisms, viz. *Escherichia coli*, *Proteus vulgaris*, *Proteus aurigasa*, *Klebsiella pneumoniae* (all gram-negative) and *Staphylococcus aureus* (gram-positive) using paper-disc diffusion method[7]. In the method, the sterilised nutrient agar (25 ml, pH 7.2) while hot, was poured into the sterilised petri dishes and allowed to cool to room temperature. The agar plates were inoculated with 18–24 h old test culture by spreading uniformly with sterile swabs. Sterilised discs (10 mm) were impregnated with 0.1 ml (concentration 200 μg) of drug solution (prepared by dissolving 10 mg of the compound in 5 ml of DMF) and were placed in the petri dishes. The dishes were then incubated for 24 h at 37° and the zone of inhibition was measured. In control, the filter paper discs saturated with pure DMF were used simultaneously. Sulphanilamide and phenol were used as standards. The results are subjectively graded and are presented in Table 2.

Antifungal activity: Compounds **4a–i** and **5a–i** were screened for antifungal activity against three fungi, viz. *Aspergillus niger*, *Candida albicans* and *Aspergillus flavus* using turbidity method[8]. 0.1 ml of the test compound (200 μg in DMF) in 5 ml of sterilised fungi medium was treated with 3–4 drops of 48 h old culture in a sterilised test tube. The tubes were shaken well and incubated for 48 h at 37°. The extent of inhibition was determined by measuring the decrease in turbidity in terms of percentage of transmission at 660 mμ. Salicylic acid (5%) was used as standard and DMF as solvent control. The results are subjectively graded and summarised in Table 2.

Results and Discussion

From the results of antibacterial screening of the compounds it can be concluded that most of the hydrazones (**4a–i**) showed significant activity against *P. aurigasa* and *E. coli*. Amongst them, compounds **4a**, **4e–h** and **4d**, **4g** exhibited marked activity against *P. aurigasa* and *E. coli*, respectively. It is observed that a majority of the compounds displayed moderate activity against *P. vulgaris* and *S. aureus*. Against *K. pneumoniae*, **4d** showed maximum activity whereas other compounds exhibited significant activity. In general, it can be concluded that compounds **4a**, **4c**, **4d**, **4e**, **4g** and **4h** are highly potent antibacterial agents. From the structure-activity relationship point of view, it could by seen that substitution at position-3 by 4-$CH_3C_6H_4$, 4-ClC_6H_4 or C_6H_5 did not appear to alter the

activity noticeably, except in a few cases ; however, unsubstitution at position-6 of isoxazolo-[5,4-*d*]pyrimidine enhanced the activity considerably.

Cyclisation of **4a–i** to **5a–i** seems to decrease the antibacterial activity in most of the cases. But no change in activity has been observed against *S. aureus*. A majority of the compounds exhibited appreciable activity against *P. vulgaris* and *E. coli*, out of which **5a**, **5d**, **5h** and **5i** were found to be very active against *P. vulgaris* and *E. coli*, respectively. Against *K. pneumoniae*, the compounds exhibited moderate activity compared to that of standards. In general, **5e** was found to be active antibacterial agent. It may be concluded that substitution by 4-$CH_3C_6H_4$, 4-ClC_6H_4 and C_6H_5 at position-5 did not affect the activity considerably, but unsubstitution at position-6 enhanced the activity to a little extent.

From the results of antifungal screening, it is clear that almost all the hydrazones (**4a–i**) showed nearly same extent of activity, but they are less active compared to standard salicylic acid. It is interesting to note that upon cyclisation **5a–i** showed increased antifungal activity. They exhibited better activity against *A. niger* when compared to their activity against *C. albicans* and *A. flavus*. In general, **5b**, **5d** and **5f** were found to be effective antifungal agents.

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