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Research Article

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Synthesis, Antimicrobial, Bactericidal and Anti-Biofilm Activities of novel Pyridyl Tetrazole Analogs

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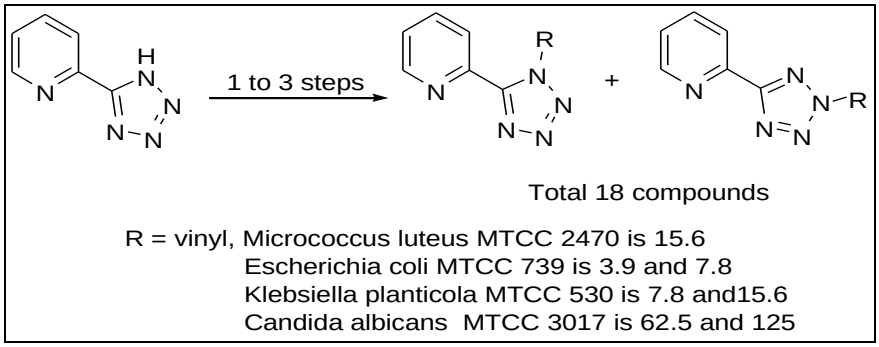
ABSTRACT

The antibacterial, bactericidal and anti-biofilm activity of pyridyl tetrazole derivatives were evaluated. In vitro antibacterial activity was investigated by microdilution method against both Gram-positive and Gram-negative bacterial strains. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) have been determined and found to be in the range of 3.9 to 15.6 µg ml^{-1}. It was that two test compounds 3D and 4D were more promising against Gram-negative bacteria with an MIC value as low as 3.9 µg ml−1 against E. coli MTCC 739. Anti-biofilm activity was investigated by crystal violet assay where M. luteus MTCC 2470, Escherichia coli MTCC 739 and Klebsiella planticola MTCC 530 were used as the test organisms. The half maximal biofilm inhibitor concentration (BIC$_{50}$) was determined and found to be in the range of 10.3 to 17.3 µg ml^{-1}.

GRAPHICAL ABSTRACT



Keywords: Antibacterial; Bactericidal; Biofilm; Pryidyl tetrazoles

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INTRODUCTION

Microorganisms attach to surfaces and develop biofilms. Biofilms are complex assemblages of microbial cells enclosed in a self synthesized polymeric matrix [1]. Biofilms may form on living or non-living surfaces and can be prevalent in natural, industrial and hospital settings like metals, plastics, mineral surfaces and living tissue in human host [2-9]. The CDC (Centers for Disease Control) estimates that over 65% of nosocomial (hospital-acquired)

infections are caused by biofilms. Bacteria growing in a biofilm are highly resistant to antibiotics, up to 1,000 times more resistant than the same bacteria not growing in a biofilm. Standard antibiotic therapy is often useless and the only recourse may be to remove the contaminated implant. Oral diseases, including dental caries and periodontal diseases, are commonly caused by a wide range of microorganisms associated with oral biofilm or dental plaque [10,11]. Biofilms are sources of diverse problems in food industry, medicine and everyday life. The presence of biofilms in food processing environments is a potential source of contamination that may lead to food spoilage and disease transmission [12,13]. Bacteria included in biofilm structure are generally more resistant to antimicrobial agents than planktonic cells [14,15]. A number of current researches on antibacterial and anti-biofilm activity of several compounds of natural and synthetic origin can be seen in the literatures. In this regard, many plant extracts have been showed to prevent biofilm formation and adherence [16,17].

In past, penicillin was discovered and used as strong antibacterial agent after which antibiotics have become critical to fight against infectious diseases caused by bacteria and other microbes. But widespread use of antibiotics has promoted the emergence of antibiotic-resistant pathogens, including multidrug resistant strains [18-20]. With the emergence of new microbial strains resistant to many conventional available antibiotics, there is growing interest in the discovery of new antibacterial agents to bate against pathogenic microorganism, especially the bacteria resistant to the current antibiotics. In an effort to develop potent synthetic compounds of pyridyl tetrazoles having diverse biological activities was undertaken and reported [21-23]. The present study has undertaken the study of anti-microbial and anti-biofilm activities of pyridyl tetrazoles.

EXPERIMENTAL SECTION

Materials and methods

The solvents used in the synthesis of the pyridyl tetrazoles were distilled before use. All other chemicals were of AR grade and were used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Top Spin Instrument. Microbial Type Culture Collection (MTCC) is collected from CSIR-Institute of Microbial Technology, Chandigarh, India.

General procedure for the synthesis of 3 and 4

The pyridyl tetrazoles 3A-3G and 4A-4G (Table 1) were synthesized following our recently published procedures [22-24].

General procedure for the synthesis of 3H, 4H, 3I and 4I

To a solution of tetrazole 2 (1 g, 6.8 mmol) in DMF (15 ml) was added alkyliodide (6.8 mmol) followed by K_2CO_3 (10 mmol). The mixture was stirred for 16 h at 50 ^{o}C in a sealed tube, and then diluted with ethyl acetate (50 ml). The organic layer was washed successively water (40 ml $\times 3$) followed by brine solution (40 ml). The organic layer was dried over $MgSO_4$, filtered, and the solvent was evaporated under reduced pressure to afford a gummy brown liquid, which was purified by column chromatography using 10-17% EtOAc in hexane (V/V) to afford the alkylated tetrazoles.

3H: Off white solid M. P. 62 ^{o}C; ^{1}H-NMR**:** ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.74 (d, 1H, *J*=4.5 Hz), 8.36 (d, 1H, *J*=7.8 Hz), 7.91 (td, 1H, *J*=7.8, 1.5 Hz), 7.45 (m, 1H), 5.03 (q, 2H, *J*=14.4, 7.2 Hz), 1.59 (t, 3H, J=14.4, 7.2 Hz), 1.25 (s, 1H) ppm; ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 151.43, 149.56, 145.34, 137.76, 125.45, 124.86, 52.34, 22.65 ppm; MS: m/z 175 (M+1).

4H: Off white solid M. P. 66 ^{o}C; ^{1}H-NMR**:** ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.79 (d, 1H, *J*=3.8 Hz), 8.26 (d, 1H, *J*=7.5 Hz), 7.87 (t, 1H, J= 15.3, 7.5 Hz), 7.40 (t, 1H, J=11.1, 5.4 Hz), 4.77 (q, 2H, J=14.7, 7.5 Hz), 1.72 (t, 3H, J=14.7, 7.2 Hz) ppm; ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 164.34, 150.67, 147.34, 137.54, 124.37, 122.55, 57.44, 22.76 ppm; MS: m/z 175 (M+1).

3I: Off white solid M. P. 51 ^{o}C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.73 (d, 1H, J=4.8 Hz), 8.33 (d, 1H, J=7.8 Hz), 7.95-7.88 (m, 1H), 7.48-7.42 (m, 1H), 6.10-5.91 (m, 1H), 1.67 (d, 6H, J=6.9 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 151.25, 149.54, 145.45, 137.49, 125.26, 124.90, 52.70, 22.70 ppm; MS: m/z 189 (M+1).

4I: Off white solid M. P. 55 ^{o}C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.79 (d, 1H, J=4.5 Hz), 8.27 (d, 1H, J=7.8 Hz), 7.90-7.83 (m, 1H), 7.43-7.37 (m, 1H), 5.24-5.14 (m, 1H), 1.74 (d, 6H, J=6.6 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 164.55, 150.35, 147.17, 137.09, 124.71, 122.45, 57.07, 22.24 ppm; MS: m/z 189 (M+1).

Antimicrobial activity

The pathogenic reference strains were seeded on the surface of the media petri plates containing Muller–Hinton agar with 0.1 ml of previously prepared microbial suspensions individually containing 1.5×10^8 cfu ml^{-1} (equal to 0.5 McFarland). Wells of 6.0 mm diameter were prepared in the media plates using a cork borer, and the synthesized pyridyl-tetrazoles at a dose range of 250–0.9 µg per well were added in each well under sterile conditions in a laminar air flow chamber. A standard antibiotic solution of ciprofloxacin and miconazole at a dose range of 125–0.9 µg per well and the well containing methanol served as positive and negative controls, respectively. The plates were incubated for 24 h at 37 °C for bacteria and at 30 °C for Candida albicans. The well containing the least concentration showing the inhibition zone was considered as that with the minimum inhibitory concentration. All experiments were carried out in duplicates and values are represented as mean ± S.D.

Determination of MBC

Bactericidal assay (NCCLS, 2000) was performed in sterile 2.0 ml microfuge tubes against a panel of pathogenic bacterial strains, including Micrococcus luteus MTCC 2470, Staphylococcus aureus MTCC 96, Staphylococcus aureus MLS-16 MTCC 2940, Bacillus subtilis MTCC 121, Escherichia coli MTCC 739, Pseudomonas aeruginosa MTCC 2453 and Klebsiella planticola MTCC 530 which were cultured overnight in Mueller Hinton broth. Serial dilutions of test compounds were prepared in Mueller Hinton broth with different concentrations ranging from 0 to 125 µg ml^{-1}. To the test compounds, 100 µl of overnight cultured bacterial suspensions were added to reach a final concentration of 1.5×10^8 cfu ml^{-1} (equal to 0.5 McFarland) and incubated at 37 °C for 24 h. After 24 h of incubation, the minimum bactericidal concentration (MBC) was determined by sampling 10 µl of suspension from the tubes onto Mueller Hinton agar plates and incubating for 24 h at 37 °C to observe the growth of test organisms. MBC is the lowest concentration of compound required to kill a particular bacterium. All the experiments were carried out in duplicates and values are represented as mean ± S.D.

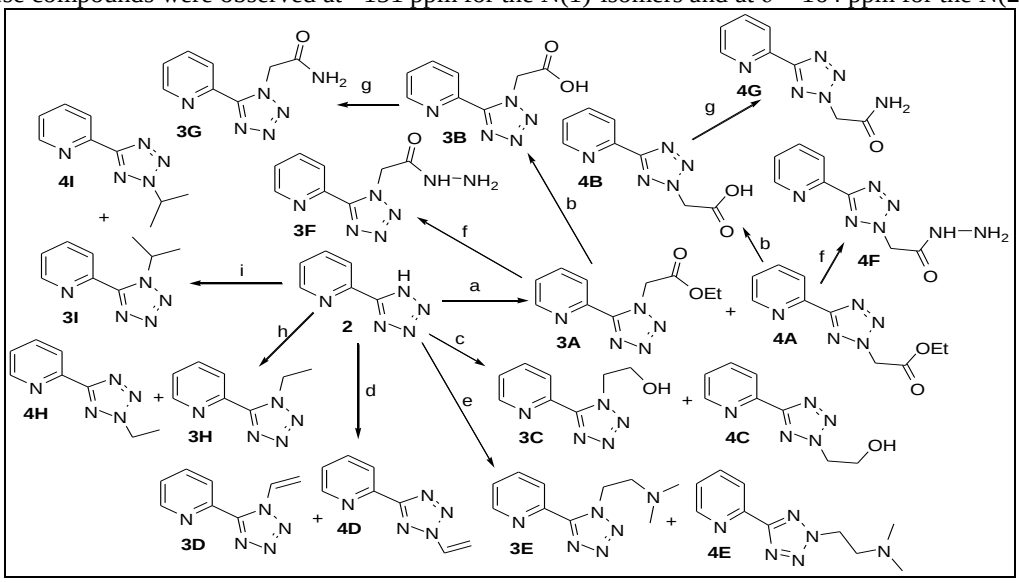
Biofilm inhibition crystal violet assay

The test compounds were screened in sterile 96 well poly- styrene microtiter plates using the modified biofilm inhibition assay, against a panel of pathogenic bacterial strains including Staphylococcus aureus MTCC 96, Staphylococcus aureus MLS-16 MTCC 2940 and Klebsiella planticola MTCC 530, which were cultured overnight in tryptone soy broth (supplemented with 0.5% glucose). The test compounds of predetermined concentrations ranging from 0 to 250 µg ml^{-1} were mixed with the bacterial suspensions having an initial inoculum concentration of 5×10^5 cfu ml^{-1}. Aliquots of 100 µl were distributed in each well and then incubated at 37 °C for 24 h under static conditions. The medium was then discarded and washed with phosphate buffered saline to remove the non-adherent bacteria. Each well of the microtiter plate was stained with 100 µl of 0.1% crystal violet solution followed by 30 min incubation at room temperature. Later the crystal violet solution from the plates was discarded, thoroughly washed with distilled water 3 to 4 times and air dried at room temperature. The crystal violet stained biofilm was solubilised in 95% ethanol (100 µl) and the absorbance was recorded at 540 nm using a TRIAD multi-mode reader (Dynex Technologies, Inc., Chantilly, VA, USA). Blank wells were employed as background check. The inhibition data were interpreted from the dose–response curves, where the IC_{50} value is defined as the concentration of inhibitor required to inhibit 50% of biofilm formation under the above assay conditions. All the experiments were carried out in triplicate and the values are indicated as mean ± S.D.

RESULTS AND DISCUSSION

The syntheses of pyridyl tetrazoles was commenced from commercially available picolinonitrile. The tetrazoles 3A-3I and 4A-4I (Scheme-1) were synthesized following our recently published procedures [22-24]. The analytical data was in good agreement with the reported compounds. The regioisomers 3H and 4H were synthesized by reacting tetrazole 2 with ethyl iodide in dry DMF at 50 ^oC in a sealed tube. The alkylation at N(1) position afforded 3H and at N(2) afforded 4H. The compounds were assigned their structures by analysing their NMR spectra. The ^1H NMR spectra of four of the compounds showed four signals corresponding to pyridyl protons. The ^1H NMR spectrum of 3H showed a characteristic peak of $–CH_2$ of ethyl group at δ 5.0 as a quartet and the same peak in 4H is appeared at δ 4.8. The $-CH_3$ of ethyl group was observed at δ 1.5 as a triplet in 3H and the same peak in 4H was observed at δ 1.7. The regioisomers 3I and 4I were synthesized by reacting tetrazole 2 with isopropyl iodide in dry DMF at 50 ^oC in a sealed tube. The alkylation at N(1) position afforded 3I and at N(2) afforded 4I. The compounds were assigned their structures by analysing their NMR spectra. The ^1H NMR spectrum of 3I showed a characteristic peak of –CH of isopropyl group at δ 6.0 ppm and the $(CH_3)_2$ of isopropyl group is appeared as doublet at δ 1.6 ppm. The ^1H NMR spectrum of 4I showed a characteristic peak of –CH of isopropyl group at δ 5.1 ppm and the $(CH_3)_2$ of isopropyl

group is appeared as doublet at δ 1.7 ppm. The ^{13}C NMR chemical shift values for the tetrazole quaternary carbon of each of these compounds were observed at ~151 ppm for the N(1)-isomers and at δ ~ 164 ppm for the N(2)-isomers.



Scheme 1: Synthesis of Pyridyl tetrazoles. Reagents and conditions: (a) ethylbromo acetate, DMF, 70 ^{o}C, 8 h; (b) aq. NaOH (1N), MeOH, r.t., 6 h; (c) 2-chloroethane, K_2CO_3, MeCN, 80 ^{o}C, 24 h (d) 2-chloro-N,N-dimethylehylamine.HCl, K_2CO_3, DMF,70 ^{o}C 24 h (e) 2-chloro-N,N-dimethylehylamine.HCl, K_2CO_3, MeCN, reflux, 24 h (f) hydrazine hydrate, EtOH, 80 ^{o}C, 10 h. (g) BOC anhydride, pyridine, $(NH_4)_2CO_3$, dioxane, r.t., 8 h; (h) Iodoethane, K_2CO_3, DMF, 50 ^{o}C, sealed tube, 16 h; (i) 2-Iodopropane, K_2CO_3, DMF, 50 ^{o}C, sealed tube, 16 h

Antimicrobial activity

Total eighteen compounds 3A-3I and 4A-4I (scheme-1) were tested for antimicrobial activity against both Gram-positive and Gram-negative bacterial strains. The results illustrate that of 18 compounds, 2 compounds exhibited promising antimicrobial activity (Table 1) and bactericidal effects with minimum bactericidal concentration (MBC) values ranged between 3.9 and 15.6 µg ml^{-1}. Compound 3D and 4D found to be active against different Gram-positive bacterial strains with MIC values ranging between 3.9 and 15.6 µg ml^{-1}; however, it was more promising against Gram-negative bacteria with an MIC value as low as 3.9 µg ml^{-1} against E. coli MTCC 739. Compound 3D and 4D showed the lowest MIC value of 15.6 µg ml^{-1} against M. luteus MTCC 2470 and 7.8 µg ml^{-1} against K. planticola MTCC 530. Compound 3D was found to be active at 15.8 µg ml^{-1} and 4D at 7.8 µg ml^{-1} against *Klebsiella planticola* MTCC 530 and the same 3D, and 4D was shown promising activity against *Candida albicans* MTCC 3017 at 125 µg ml^{-1} and 62.5 µg ml^{-1} respectively.

Bactericidal activity

The compounds were evaluated for bactericidal activity (Table 2) and exhibited good to promising activity against all the tested bacterial strains. All the compounds showed a bactericidal effect on different strains with MBC values ranging from 3.9 to 15.6 µg ml^{-1}. Compounds 3D and 4D were compared to ciprofloxacin with an MBC value of 0.9 µg ml−1 against M. luteus MTCC 2470 and promising against K. planticola MTCC 530 and E. coli MTCC 739.

Anti-biofilm activity

The Compounds 3D and 4D exhibiting antibacterial and bactericidal properties were also checked for bacterial biofilm inhibition (Table 3). They were found to inhibit biofilm formation with IC50 values ranging between 10.3 and 17.3 µg ml−1. Compound 3D and 4D were found to be potent as compared to Ciprofloxacin against M. luteus MTCC 2470, Escherichia coli MTCC 739 and Klebsiella planticola MTCC 530.

Table 1: Antimicrobial activity of the compounds against several standard strains

Test Compounds	Minimum inhibitory concentration (µg/ml)							
	Micrococcus luteus **MTCC 2470**	***Staphylococcus aureus*** **MTCC 96**	***Staphylococcus aureus*** **MLS-16 MTCC 2940**	***Bacillus subtilis*** **MTCC 121**	***Escherichia coli*** **MTCC 739**	***Pseudomonas aeruginosa*** **MTCC 2453**	***Klebsiella planticola*** **MTCC 530**	***Candida albicans*** **MTCC 3017**
3A	>250	>250	>250	>250	>250	>250	>250	>250
4A	>250	>250	>250	>250	>250	>250	>250	>250
3B	>250	>250	>250	>250	>250	>250	>250	>250
4B	>250	>250	>250	>250	>250	>250	>250	>250
3C	>250	>250	>250	>250	>250	>250	>250	>250
4C	>250	>250	>250	>250	>250	>250	>250	>250
3D	15.6	>250	>250	>250	7.8	>250	15.6	125
4D	15.6	>250	>250	>250	3.9	>250	7.8	62.5
3E	>250	>250	>250	>250	>250	>250	>250	>250
4E	>250	>250	>250	>250	>250	>250	>250	>250
3F	>250	>250	>250	>250	>250	>250	>250	>250
4F	>250	>250	>250	>250	>250	>250	>250	>250
3G	>250	>250	>250	>250	>250	>250	>250	>250
4G	>250	>250	>250	>250	>250	>250	>250	>250
3H	>250	>250	>250	>250	>250	>250	>250	>250
4H	>250	>250	>250	>250	>250	>250	>250	>250
3I	>250	>250	>250	>250	>250	>250	>250	>250
4I	>250	>250	>250	>250	>250	>250	>250	>250
Ciprofloxacin (Standard)	1.8	0.9	1.8	0.9	0.9	0.9	1.8	-[a]
Miconazole (Standard)	-	-	-	-	-	-	-	7.8

Table 2: MBC values of compounds against several bacterial strains

Test Compounds	Minimum bactericidal concentration (µg/ml)			
	Micrococcus luteus **MTCC 2470**	***Escherichia coli*** **MTCC 739**	***Klebsiella planticola*** **MTCC 530**	***Candida albicans*** **MTCC 3017**
3D	15.6	7.8	15.6	125
4D	15.6	3.9	7.8	62.5
Ciprofloxacin (Standard)	1.8	0.9	1.8	-[a]
Miconazole (Standard)	-	-	-	7.8

Table 3: Anti-biofilm activity of compounds against different strains

Test Compound	IC_{50} values in (µg/mL)		
	Klebsiella planticola **MTCC 530**	***Micrococcus luteus*** **MTCC 2470**	***Escherichia coli*** **MTCC 739**
3D	10.3 ± 0.43	18.23 ±0.36-	3D
4D	12.5 ± 0.36	17.233 ±0.29	4D
Ciprofloxacin (Standard control)	0.8 ± 0.11	0.9 ± 0.12	Ciprofloxacin (Standard control)

CONCLUSION

All the synthesized compounds were tested for the antibacterial, bactericidal and anti-biofilm avtivities. It was found that two compounds namely 3D and 4D are promising compared to the other tested compounds.

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