Synthesis and Bioevaluation of the Antimicrobial Features of Some New Thiazolyl-azoles

SMARANDA ONIGA[1], CATALIN ARANICIU[1]*, MARIANA PALAGE[1], CRISTINA STOICA[1], MARIANA CARMEN CHIFIRIUC[2,3], LUMINITA MARUTESCU[2,3]

[1] Iuliu Hatieganu University of Medicine and Pharmacy, Therapeutic Chemistry Department, 12 Ion Creanga Str., 400010, Cluj-Napoca, Romania

[2] University of Bucharest, Faculty of Biology, Microbiology Department,1-3 Portocalelor Lane, 60101 Bucharest, Romania

[3] Research Institute of the University of Bucharest (ICUB), 91-95 Splaiul Independentei, 050095, Bucharest, Romania

A new series of 4-methyl-2-(pyridin-3-yl)-thiazole-5-yl-azoles were synthesized using the key intermediate 4-methyl-2-(pyridin-3-yl)thiazole-5-carbohydrazide. The newly synthesized compounds were characterized by ^{1}HNMR, MS and elemental analysis. These molecules were screened for their antimicrobial activity against planktonic and adherent Gram-positive, Gram-negative bacteria and fungal strains.

Keywords: oxadiazoles, pyrazoles, triazoles, microbial growth inhibition, minimal inhibitory concentration, biofilm

Antibiotic resistance (AR) to traditional antibiotics remains an essential issue for the global public health, predicting the emergence of an "post-antibiotic" era, bringing major difficulties in treating bacterial infections [1], justifying the significant efforts that are being made in order to identify new therapeutic agents and strategies.

One of the ways by which bacteria and fungi develop phenotypic resistance is biofilm formation. Biofilms are defined as microbial communities of cells that are irreversibly attached to a substratum, to an interface, or to each other, and are embedded into a matrix of extracellular polymeric substances that they have produced. The biofilm phenotype can reduce antimicrobial susceptibility and increase tolerance up to 1000 - 4000 times, evidently decreasing the antimicrobial efficiency and leading to clinical therapeutic failures. The development of novel anti-biofilm strategies is therefore of major interest and currently constitutes an important field of investigation [2].

Among the pharmacophores responsible for antimicrobial activity, the thiazole nucleus is considered an important scaffold [3-7]. Taking into account the observed biological activities of thiazole compounds and as a part of our continuing research on the synthesis of new antimicrobial molecules [8-11] we synthesized and investigated the *in vitro* antimicrobial and antibiofilm activity of some novel structure hybrids incorporating the 2-(3-pyridine)-thiazole system, substituted with various heterocyclic ring systems (oxadiazoles, pyrazoles and triazoles).

Experimental part

Chemistry

Melting points were determined using open capillary tube method and are uncorrected. The purity of the synthesized compounds was verified by thin layer chromatography (TLC) and was carried out on pre-coated Silica Gel 60F254 sheets using heptan – ethyl-acetate 7:3 like developpant and UV absorption for visualization.

The ^{1}H NMR spectra were recorded at room temperature on a Bruker Avance NMR spectrometer operating at 500 MHz. Chemical shift values were reported relative to tetramethylsilane (TMS) as internal standard. GC-MS analyses were performed with an Agilent gas chromatograph 6890 equipped with an apolar Macherey Nagel Permabond SE 52 capillary column. Elemental analysis was registered with a Vario El CHNS instrument.

*Synthesis of ethyl 4-methyl-2-(pyridin-3-yl)-thiazole-5-carboxylate (**A**)*

Compound **A** was synthesized by refluxing a mixture of pyridine-4-carbothioamide (30 mmol) with ethyl 2-chloro-3-oxobutanoate (30 mmol) in absolute ethanol (30 mL) for 5 h. After cooling, the mixture was poured in cold water, neutralized with a sodium bicarbonate solution (10%) and the solid formed was filtered out, washed with water and recrystallized from water.

A: Yield 75%. m.p. 52-55^{0}C. ^{1}H NMR (DMSO-d6, δ, ppm) δ: 9.2 (s, 1H, pyridyl, C_2), 8.74 (d 1H, pyridyl C_4), 8.37 (d 1H, pyridyl, C_6), 7,58 (q 1H, pyridyl C_5), 4.87 (q, 2H, CH_2, OC_2H_5), 2.58 (s 3H, CH_3, thiazole C_4), 1.9 (t, 3H, CH_3, OC_2H_5); Anal. calcd. (%) for $C_{12}H_{12}N_2O_2S$ (248.30): C, 58.05; H, 4.87; N, 11.28; S, 12.91. Found: C, 58.20; H, 4.85; N, 11.3; S, 12.85. MS (EI, 70eV): m/z 248 (M+).

*Synthesis of 4-methyl-2-(pyridin-3-yl)thiazole-5-carbohydrazide (**B**)*

A mixture of **A** (0.001 mol) and hydrazine hydrate (1 mL) was refluxed for 6 h in absolute ethanol (10 mL). The reaction mixture was cooled and the crystalline mass obtained was recrystallised from ethanol.

B: Yield 70%. m.p. 188-189,5 ^{0}C. ^{1}H NMR (DMSO-d6, δ, ppm) δ: 9.6 (s, 1H, NH), 9.2 (s, 1H, pyridyl, C_2), 8.74 (d 1H, pyridyl C_4), 8.37 (d 1H, pyridyl, C_6), 7,58 (q 1H, pyridyl C_5), 4.6 (s, 2H, NH_2), 2.8 (s 3H, CH_3, thiazole C_4); Anal. calcd. (%) for $C_{10}H_{10}N4OS$ (234.28): C, 51.27; H, 4.3; N, 23.91; S, 13.69. Found: C, 51.20; H, 4.28; N, 24.02; S, 13.58. MS (EI, 70eV): m/z 234 (M+).

*Synthesis of N-allyl-2-(4-methyl-2-(pyridin-3-yl)thiazole-5-carbonyl)hydrazinecarbothioamide (**B1**)*

A mixture of acid hydrazide **B** (0.001 mol) and allyl-isothiocyanate (0.001 mol) in absolute ethanol was refluxed for 3h. After cooling, the formed solid was filtered and recrystallised from ethanol.

B1: Yield 78%. m.p. 190-194 ^{0}C. ^{1}H NMR (DMSO-d6, δ, ppm) δ: 10.2 (s 1H, NH, -CO-NH), 9.5 (s 1H, NH, -NH-

* *email: araniciu.catalin@umfcluj.ro; Tel : 0740169952*

C=S), 9.2 (s 1H, pyridyl, C_2), 8.75 (d 1H, pyridyl, C_6), 8.4 (d 1H, pyridyl, C_4), 8.38 (s, 1H, NH –NH-CH_2-), 7,6 (q 1H, pyridyl, C_5), 5.85 (m 1H, CH, -CH=), 5.16 (d 1H, CH, =CH_2), 5.07 (d 1H CH, =CH_2), 4.13 (d 2H, CH_2, –NH-CH_2-), 2.63 (s 3H, CH_3, thiazole C_4); Anal. calcd. (%) for $C_{14}H_{15}N_5OS_2$ (333.43): C, 50.43; H, 4.53; N, 21.00; S, 19.23. Found: C, 50.50; H, 4.49; N, 21.08; S, 19.36. MS (EI, 70eV): m/z 333 (M+).

*Synthesis of 4-allyl-3-(4-methyl-2-(pyridin-3-yl)thiazol-5-yl)-1H-1,2,4-triazole-5(4H)-thione (**B2**)*

(0.001 mol) **B1** was refluxed 3h in a solution of potassium hydroxide (5%) in absolute ethanol (5 mL). After cooling, the solution was neutralized with a solution of hydrochloric acid 55 and the resulted solid was filtered and recrystallised from ethanol.

B2: Yield 70%. m.p. 215-219 ^{0}C. ^{1}H NMR (DMSO-d6, δ, ppm) δ: 14.31 (s 1H, NH, N_1-triazole), 9.19 (s 1H, pyridyl, C_2), 8.75 (d 1H, pyridyl, C_6), 8.6 (d 1H, pyridyl, C_4), 7,6 (q 1H, pyridyl, C_5), 5.86 (m 1H, CH, -CH=), 4.91 (d 1H, CH, =CH_2), 4.88 (d 1H CH, =CH_2), 4.66 (d 2H, CH_2, N_4-triazole), 2.48 (s 3H, CH_3, thiazole C_4). Anal. calcd. (%) for $C_{14}H_{13}N_5S_2$ (315.42): C, 53.31; H, 4.15; N, 22.20; S, 20.33. Found: C, 53.25; H, 4.18; N, 22.15; S, 20.41. MS (EI, 70eV): m/z 315 (M+).

*Synthesis of 2-methyl-5-(4-methyl-2-(pyridin-3-yl)thiazol-5-yl)-1,3,4-oxadiazole **(B3)***

A mixture of **B** (0.001 mol) and acetic anhydride (5 mL) was heated under reflux for 6 h. After cooling, the reaction mixture was poured into ice cold water. The resulted solid was filtered, washed with water, dried and recrystallised in ethanol [12,13].

B3: Yield 70%. m.p. 207-210 ^{0}C. ^{1}H NMR (DMSO-d6, δ, ppm) δ: 9.17 (s 1H, pyridyl, C_2), 8.73 (d 1H, pyridyl, C_6), 8.6 (d 1H, pyridyl, C_4), 7,6 (q 1H, pyridyl, C_5), 2.78 (s 3H, CH_3, thiazole C_4), 2.61 (s 3H, CH_3, oxadiazole C_2); Anal. calcd. (%) for $C_{12}H_{10}N4OS$ (258.30): C, 55.80; H, 3.9; N, 21.69; S, 12.41. Found: C, 56.00; H, 3.85; N, 21.65; S, 12.44 MS (EI, 70eV): m/z 258 (M+).

*Synthesis of 2-ethyl-5-(4-methyl-2-(pyridin-3-yl)thiazol-5-yl)-1,3,4-oxadiazole **(B4)***

A mixture of **B** (0.001 mol) and propionic anhydride (5 mL) was heated under reflux for 6 h. After cooling, the reaction mixture was poured into ice cold water. The separated product was filtered, washed with water, dried and recrystallised from ethanol [12,13].

B4: Yield 74%. M.p. 196-198 ^{0}C. ^{1}H NMR (DMSO-d6, δ, ppm) δ: 9.22 (s 1H, pyridyl, C_2), 8.75 (d 1H, pyridyl, C_6), 8.62 (d 1H, pyridyl, C_4), 7,58 (q 1H, pyridyl, C_5), 2.98 (q 2H, CH_2 -C_2H_5 oxadiazole C_2) 2.76 (s 3H, CH_3, thiazole C_4), 1.34 (t 3H, CH_3, - C_2H_5 oxadiazole C_2); Anal. calcd. (%) for $C_{13}H_{12}N4OS$ (272.33): C, 57.34; H, 4.44; N, 20.57; S, 11.77. Found: C, 57.30; H, 4.34; N, 20.51; S, 11.82. MS (EI, 70eV): m/z 272 (M+).

*Synthesis of (3,5-dimethyl-1H-pyrazol-1-yl)(4-methyl-2-(pyridin-3-yl)thiazol-5-yl)methanone **(B5)***

0.001 mol acid hydrazide **B** was refluxed with 0.001 mol acetyl-acetone in absolute ethanol for 3 h. The resulted solid was filtered, washed with water and recrystallised from ethanol [14,15].

B5: Yield 70%. m.p. 209-212 ^{0}C. ^{1}H NMR (DMSO-d6, δ, ppm) δ: 9.22 (s 1H, pyridyl, C_2), 8.75 (d 1H, pyridyl, C_6), 8.4 (d 1H, pyridyl, C_4), 7,6 (q 1H, pyridyl, C_5), 6.34 (s 1H, pyrazole C_4) 2.82 (s 3H, CH_3, thiazole C_4), 2.59 (s 3H, CH_3, pyrazole C_5). 2.28 (s 3H, CH_3, pyrazole C_3). Anal. calcd. (%) for $C_{15}H_{14}N4OS$ (298.36): C, 60.38; H, 4.73; N, 18.78; S, 10.75. Found:. C, 60.25; H, 4.69; N, 18.58; S, 10.63 MS (EI, 70eV): m/z 298 (M+).

Biological Assays

The *in vitro* qualitative screening of the antimicrobial activity

The *in vitro* qualitative screening of the antimicrobial activity was carried out by an adapted agar disk diffusion technique using a bacterial suspension of 0.5 McFarland obtained from 24 h cultures. The antimicrobial activities of the newly synthesized compounds were determined against ATCC reference microbial strains: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27857, *Bacillus subtilis* ATCC 6683; Cantacuzino Institute reference microbial strain *Klebsiella pneumoniae* 13420, as well as clinical strains, recently isolated from different clinical specimens, encoded *Candida albicans* 393 and *Enterococcus faecium* E5.

The compounds were solubilised in dimethylsulfoxide to a final concentration of 10 mg/mL. A volume of 5µL of each tested compound solution was distributed directly on the solid medium previously seeded with the microbial inocula. The inoculated plates were incubated for 24 h at 37 °C. Antimicrobial activity was assessed by measuring the growth inhibition zones diameters expressed in mm [16, 17].

The in vitro quantitative assay of the antimicrobial activity

The quantitative assay of the minimal inhibitory concentration (MIC, µg/mL) was based on liquid medium two-fold micro dilutions and performed in 96 multi-well plates. For this purpose, serial binary dilutions of the tested compounds were performed in a 200 µL volume of nutrient broth/YPG and each well was seeded with 20 µL microbial inocula of 0.5 McFarland density. The plates were incubated for 24 h at 37°C for bacterial strains, respectively for 48 h at 28°C for fungal strains. MICs were read as the last concentration of compound which inhibited the visible microbial growth [18].

Study of the influence of the tested compounds on the ability of microbial strains to colonize the inert substratum and to form biofilms

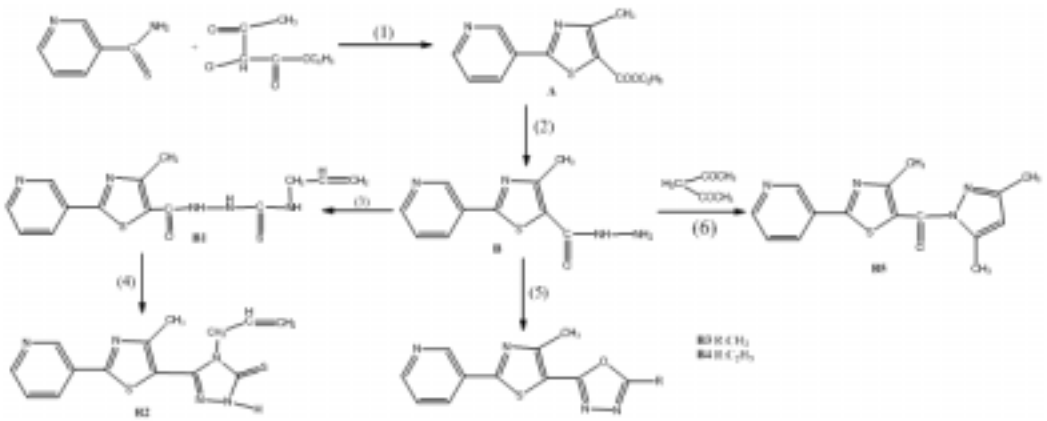
The anti-biofilm activity of the tested compounds was evaluated by the microtiter method. For this purpose, the microbial strains were grown in the presence of two-fold serial dilutions of the investigated compounds performed in liquid nutrient broth/YPG, distributed in 96-well plates and incubated for 24 h at 37°C for bacterial strains, respectively for 48 h at 28°C for the fungal strains. At the end of the incubation period, the plastic wells were emptied, washed two times with phosphate buffered saline (PBS), fixed with cold methanol 80% and stained with 1% violet crystal solution for 15 min. The stained biofilm formed on plastic wells was then resuspended in a 33% acetic acid solution. The last concentration that inhibited the development of microbial biofilm on the plastic wells, preventing the occurrence of a blue stained suspension, was considered the minimum biofilm eradication concentration (MBEC), expressed in ìg/mL [17, 18].

Results and discussions

Chemistry

All new compounds presented spectral data consistent with the proposed structure and microanalysis within 0.4% of the theoretical values.

Reagents and conditions: (1) ethanol, reflux; (2) NH_2NH_2, absolute ethanol, reflux 6h; (3) allylisothiocyanate, absolute



Scheme 1. The procedure for the synthesis of the 2(3-pyridyl)-thiazolyl-azoles

Table 1
QUALITATIVE SCREENING OF THE ANTIMICROBIAL ACTIVITY OF THE NEWLY SYNTHESIZED COMPOUNDS (DIAMETERS OF THE GROWTH INHIBITION ZONE ARE EXPRESSED IN mm)

Microbial strain / Compound	*B. subtilis* ATCC 6683	*E. faecium E5*	*S. aureus* ATCC 6538	*P.aeruginosa* ATCC 27857	*K. pneumoniae* IC 13420	*E. coli* ATCC 25922	*C. albicans* 393
B1	6	8	0	8	0	0	5
B2	6	7	0	0	0	0	5
B3	6	0	0	0	0	6	6
B4	6	0	0	6	0	0	6
B5	6	7	0	0	0	0	6
DMSO	0	0	0	0	0	0	0

Table 2
RESULTS OF THE QUANTITATIVE ASSAY (MIC VALUES EXPRESSED IN μg/mL)

Microbial strain / Compound	*B. subtilis* ATCC 6683	*E. faecium E5*	*S. aureus* ATCC 6538	*P.aeruginosa* ATCC 27857	*K. pneumoniae* IC 13420	*E. coli* ATCC 25922	*C. albicans* 393
B1	500	1000	-	1000	-	-	-
B2	500	1000	-	-	-	-	-
B3	500	-	-	-	-	1000	-
B4	500	-	-	1000	-	-	-
B5	> 1000	1000	-	-	-	-	-

ethanol, reflux 6h; (4) KOH 5%, absolute ethanol, reflux 6h; (5) acetic anhydride, reflux 6h; (6) propionic anhydride, reflux 6h; (7) absolute ethanol, reflux, 6h. >

The synthesis of the target compounds is outlined in scheme 1. The core intermediary B was prepared in two steps, starting from 3-pyridil-carbo-thioamide. The presence of the carbohydrazide group in compound **B** makes it a useful intermediate in the synthesis of several heterocyclic compounds such as 1,3,4-oxadiazole, pyrazole and 1,2,4-triazole. The type of the heterocycle depends on the reagents and reaction conditions used.

Compounds **B1** and **B2** were synthesized by the reaction of hydrazide **B** with allyl-isothiocyanate in refluxing ethanol and the intramolecular cyclization of N-allyl-2-(4-methyl-2-(pyridin-4-yl)thiazole-5-carbonyl)hydrazine-carbothioamide **B1** formed with 5% KOH in ethanol.

The direct cyclization of the hydrazide **B** with acetic anhydride, propionic anhydride or acetyl-acetone gave the corresponding methyl-oxadiazole **B3**, ethyl-oxadiazole **B4** and dimethyle-pyrazole derivative **B5**.

The ^{1}H NMR spectra revealed characteristic signals for CH_3 and C_2H_5 groups (oxadiazole nucleus) for compounds **B3** and **B4,** while the spectrum of compound **B5** presented characteristic singlet signals at 2.23, 2.59 ppm (3H), (methyl groups in the 3 and 5 positions of the pyrazole nucleus) and at 6.35 ppm (1H), (CH in the 4 position of the pyrazole nucleus). Assignment of the **B2** structure is supported by the ^{1}HNMR spectrum which showed a singlet signal at 14.31 ppm (NH 4, triazole ring) and the corresponding signals for all the protons from the allyl group.

Biological evaluation

The qualitative screening of the susceptibility spectra of the tested compounds revealed a moderate antimicrobial activity and a diverse antimicrobial spectrum for different conpounds (table 1). The highest values of the growth inhibition diameters were recorded for derivative **B1** against *E. faecium* and *P. aeroginosa*, followed by **B2** and **B5** against *E. faecium.* All compounds seem to be effective against the *B. Subtillis* strain.

Subsequently, quantitative assays were performed for the compounds that proved to be active on different microbial strains in the qualitative screening assay. The results of the quantitative assay revealed relatively high MIC values against the tested strains (500 - >1000 μg/mL) (table 2). The best activity of the tested compounds was observed, similar to the qualitative screening, against the *B. subtillis* strain.

The compounds were further tested in order to establish their efficiency against the adherence of cells grown in biofilms developed on plastic wells, taking into account that, in many cases, the sub-inhibitory concentrations of the antimicrobial substances could interfere with the expression of different virulence features, such as adhesins or toxins [19].

Compound \ Microbial strain	*B. subtilis* ATCC 6683	*E. faecium* E5	*S. aureus* ATCC 6538	*P. aeruginosa* ATCC 27857	*K. pneumoniae* IC 13420	*E. coli* ATCC 25922	*C. albicans* 393
B1	1000	500	-	1000	-	-	-
B2	500	1000	-	-	-	-	-
B3	1000	-	-	-	-	250	-
B4	500	-	-	1000	-	-	-
B5	> 1000	1000	-	-	-	-	-

Table 3
RESULTS OF THE MICROTITER ASSAY OF THE INFLUENCE OF THE TESTED COMPOUNDS ON THE ADHERENCE ABILITY AND BIOFILM DEVELOPMENT ON THE INERT SUBSTRATUM. MBECs ARE EXPRESSED IN μg/mL

The results revealed that some of the tested compounds exhibited a better anti-biofilm activity than the antimicrobial one (table 3). This is the case of the compound **B3** which exhibited a good anti-biofilm activity against *E. coli*.

Conclusions

A novel series of 4-methyl-2-(pyridin-4-yl)-thiazole-5-yl-azoles were synthesized. All the physicochemical and spectral data of the compounds were in agreement with the proposed structures. The newly synthesized compounds were initially screened for their anti- microbial activity with promising results. Following further quantitative assays the molecules have shown high MIC and MBEC values. The most susceptible strain in planktonic state to the tested compounds was *B. subtillis*, while derivative **B3** proved to be the most efficient in inhibiting *E. coli* adherence and biofilm development on the inert substratum.

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