Krzysztof Z. Łączkowski*, Katarzyna Jachowicz, Konrad Misiura, Anna Biernasiuk and Anna Malm

Synthesis and biological evaluation of novel 2-(1*H*-imidazol-2-ylmethylidene)hydrazinyl-1,3-thiazoles as potential antimicrobial agents

Abstract: Synthesis, characterization, and investigation of antimicrobial activities of nine novel imidazolylthiazoles are presented. Their structures were determined using ^{1}H NMR, ^{13}C NMR, and elemental analysis. Compounds **3e** and **3i** show very strong or strong bacteriostatic or bactericidal activity [minimal bactericidal concentration/minimal inhibitory concentration (MIC)=2–64] against *Staphylococcus* spp. (MIC=7.81–15.62 μg/mL), *Micrococcus luteus* ATCC 10240 (MIC=1.95–3.91 μg/mL), and *Bacillus* spp. (MIC=3.91–15.62 μg/mL). Compounds **3e** and **3i** also show the highest fungicidal effect (minimal fungicidal concentration/MIC=2–4) against reference strains of fungi belonging to *Candida* spp. with MIC ranging from 3.91 to 31.25 μg/mL.

Keywords: antibacterial drugs; antifungal drugs; *Candida* species; imidazole; thiazole.

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Introduction

Bacterial and fungal infections in patients with tumors, immunosuppression, immunodeficiency, and organ transplantation has increased dramatically in developing and developed countries [1]. The main reason for this is the widespread use of antibiotics and the consequent emergence of multi-drug-resistant microorganisms [2]. The search for new drugs that will replace those that have become less effective as a result of antimicrobial resistance is the mainstream research of many laboratories. In particular, in recent years, much attention has been given to the design and synthesis of five-membered antimicrobial heterocyclic compounds containing sulfur and nitrogen atoms [3]. This group of compounds includes imidazole and thiazole as important biological building blocks, which are present in histidine, related hormone histamine, nucleic acids, and bactericidal peptides, for example, microcin B17 [4]. Additionally, imidazole plays an important role in charge-transfer processes and as a hydrogen donor and acceptor in enzymes.

Various methods for the synthesis of imidazole derivatives with unique mechanism of action have huge potential for the preparation of a number of derivatives of various biological activities, such as antimicrobial [5–7], antitumor [8], and antiviral agents [9]. Also, a number of compounds with a thiazole scaffold acting as antimicrobial, antifungal, antiviral, antioxidant, analgesic, anti-inflammatory, anticonvulsant, neuroprotective, and antitumor drugs have been prepared [10–13].

Continuing our previous investigation on the effect of systematic structural modifications of the thiazole ring on antimicrobial [14–17] and antiproliferative activities [18], we decided to incorporate imidazole moiety into the thiazole ring to obtain strongly polar compounds with relatively high biological availability and having both antibacterial and antifungal properties.

Their antibacterial and antifungal activities against a panel of reference strains of 20 microorganisms were evaluated. The microorganisms came from American Type Culture Collection (ATCC), routinely used for the evaluation of antimicrobials.

***Corresponding author: Krzysztof Z. Łączkowski,** Faculty of Pharmacy, Department of Chemical Technology and Pharmaceuticals, Collegium Medicum, Nicolaus Copernicus University, Jurasza 2, 85-089 Bydgoszcz, Poland, e-mail: krzysztof.laczkowski@cm.umk.pl
Katarzyna Jachowicz and Konrad Misiura: Faculty of Pharmacy, Department of Chemical Technology and Pharmaceuticals, Collegium Medicum, Nicolaus Copernicus University, Jurasza 2, 85-089 Bydgoszcz, Poland
Anna Biernasiuk and Anna Malm: Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Medical University, Chodźki 1, 20-093 Lublin, Poland

Results and discussion

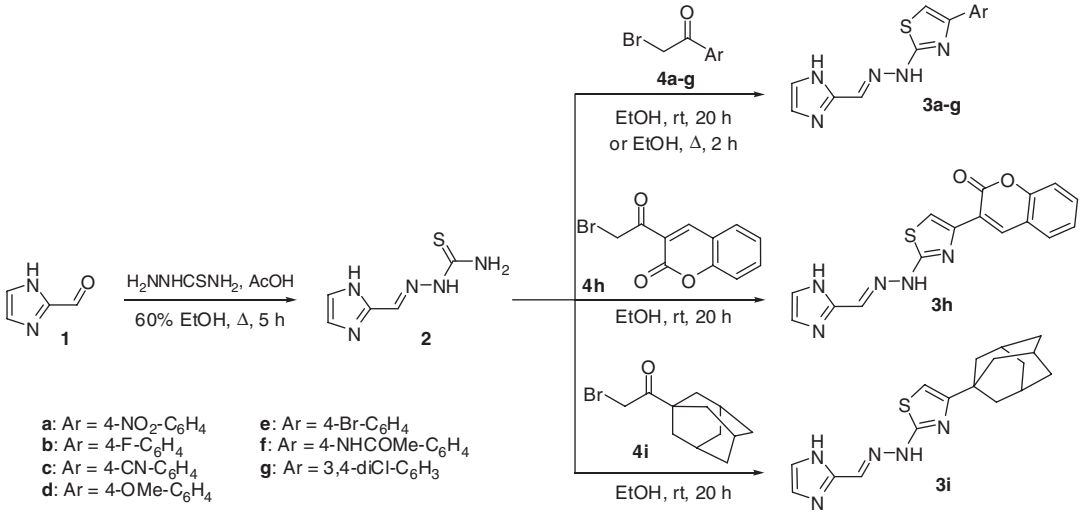
Chemistry

The desired thiazole compounds were prepared in two steps summarized in Scheme 1. In the first step, 1*H*-imidazole-2-carbaldehyde (**1**) was allowed to react with thiosemicarbazide in refluxing 60% ethyl alcohol solution to form 2-(1*H*-imidazol-2-ylmethylidene)hydrazinecarbothioamide (**2**) with 80% yield [19]. The carbothioamide **2** was further treated with various *para*-substituted bromoacetophenone or chloroacetophenones to form 2-(2-(1*H*-imidazol-2-ylmethylidene)hydrazinyl)-1,3-thiazole hybrid compounds **3a–3i** with good yield (25–72%) and high chemical purity. The structure of all compounds was confirmed by spectroscopic methods and elemental analysis. For example, the ^{1}H NMR spectrum of **2** shows typical three proton signals of NH_2 and NH groups at δ 8.30, 11.53, and 12.46, respectively. These three signals are from the exchange of hydrogen atom between the terminal NH_2 and the C=S groups. The ^{1}H NMR spectra of products **3a–3i** show a singlet at δ 6.40–7.71 due to the thiazole-5H proton and a singlet at δ 10.79–11.20, indicating the presence of hydrazide NH proton, which confirms the conversion of substrates into the expected products. All reactions were conducted at least twice and were found to be fully reproducible.

Antimicrobial activity *in vitro*

Data presented in Table 1 show that compounds **3e** and **3i** possess the widest spectrum of antibacterial activity and the highest activity against the tested strains of Gram-positive bacteria.

These two compounds exhibit very strong or strong bacteriostatic or bactericidal activity [minimal bactericidal concentration (MBC)/minimal inhibitory concentration (MIC)=2–64] against *Staphylococcus* spp. ATCC (MIC=7.81–15.62 μg/mL), *Micrococcus luteus* ATCC 10240 (MIC=1.95–3.91 μg/mL), and *Bacillus* spp. ATCC (MIC=3.91–15.62 μg/mL). The MBCs range from 7.81 to 500 μg/mL. The bacteria belonging to reference streptococci are also sensitive to compounds **3e** and **3i**, with MIC=7.81–125 μg/mL and MBC=125–500 μg/mL (MBC/MIC=2–16). Compound **3b** inhibits the growth of Gram-positive bacteria, with MIC=15.62–250 μg/mL and MBC=31.25–1000 μg/mL (MBC/MIC=1–8). The species *M. luteus* ATCC 10240 is especially sensitive to **3b** (MIC=15.62 μg/mL). Compound **3g** also shows very high bacteriostatic activity (MIC=7.81 μg/mL) against this microorganism and mild activity against *Staphylococcus aureus* ATCC 25923 (MIC=1000 μg/mL). The remaining compounds **3a**, **3d**, or **3h** show lower activity (MIC=500–1000 μg/mL) or no activity on the reference strains. The MBC of these compounds for the tested Gram-positive bacteria is >1000 μg/mL. Compound **3c** has no influence on the growth of Gram-positive bacteria. The results of our study indicate that the growth of Gram-negative bacteria is not inhibited by any of the compounds (Table 1). Compounds **3e** and **3i** show the highest bioactivity with fungicidal effect [minimal fungicidal concentration (MFC)/MIC=2–4] against reference strains of fungi belonging to *Candida* spp. (MIC=3.91–31.25 μg/mL and MFC=15.62–125 μg/mL). Compound **3e** exhibit very strong activity, especially against *Candida albicans* ATCC 10231 (MIC=3.91 μg/mL).



Scheme 1: Synthesis of imidazolylthiazoles **3a–3i**.

Table 1: Antimicrobial activity data in MIC (μg/mL) and, in parentheses, as MBC or MFC (μg/mL) for imidazolylthiazoles **3a–3i**.

Species	3a	3b	3c	3d	3e	3f	3g	3h	3i	CIP/VA[a]/FLU[b]
S. aureus ATCC 6538	1000 (>1000)	125 (1000)	–	–	15.62 (500)	1000 (>1000)	–	–	15.62 (62.5)	0.244
S. aureus ATCC 25923	1000 (>1000)	250 (500)	–	–	15.62 (125)	1000 (>1000)	1000 (>1000)	–	15.62 (31.25)	0.488
S. epidermidis ATCC 12228	500 (>1000)	250 (1000)	–	–	15.62 (250)	1000 (>1000)	–	–	7.81 (125)	0.122
M. luteus ATCC 10240	1000 (>1000)	15.62 (62.5)	–	1000 (>1000)	1.95 (125)	1000 (>1000)	–	1000 (>1000)	3.91 (62.5)	0.976
B. subtilis ATCC 6633	1000 (>1000)	31.25 (31.25)	–	1000 (>1000)	3.91 (7.81)	–	–	1000 (>1000)	3.91 (7.81)	0.031
B. cereus ATCC 10876	1000 (>1000)	250 (1000)	–	–	15.62 (62.5)	–	–	–	15.62 (125)	0.061
Str. pneumoniae ATCC 49619	–	250 (500)	–	–	125 (500)	–	–	–	62.5 (500)	3.907[a]
Str. pyogenes ATCC 19615	–	125 (1000)	–	–	15.62 (250)	–	–	–	7.81 (125)	0.976[a]
Str. mutans ATCC 25175	–	125 (500)	–	–	62.5 (125)	–	–	–	62.5 (125)	0.488[a]
C. albicans ATCC 2091	1000 (>1000)	250 (250)	1000 (>1000)	1000 (>1000)	31.25 (125)	–	1000 (>1000)	–	31.25 (62.5)	0.245[b]
C. albicans ATCC 10231	250 (>1000)	125 (250)	1000 (>1000)	125 (500)	3.91 (15.62)	–	1000 (>1000)	–	31.25 (62.5)	0.976[b]
C. parapsilosis ATCC 22019	500 (>1000)	125 (250)	1000 (>1000)	250 (1000)	15.62 (31.25)	–	1000 (>1000)	–	15.62 (31.25)	1.953[b]

Standard antibiotics used as positive controls: [a]ciprofloxacin (CIP) or vancomycin (VA) for bacteria and [b]fluconazole (FLU) for fungi.

In the case of compounds **3a**, **3b**, and **3d**, the activity is good or mild with the fungicidal or fungistatic effect (MIC=125–1000 μg/mL and MFC=250–>1000 μg/mL) to *Candida* spp. ATCC strains. Compounds **3c** and **3g** show the lowest antifungal activity (MIC=1000 μg/mL and MFC >1000 μg/mL), whereas **3f** and **3h** have no activity to reference strains of yeasts (Table 1).

In summary, a structural combination of the imidazole and thiazole systems containing F, Br, and adamantanyl substituents have both antibacterial and antifungal activities, which is consistent with our earlier assumption. Previous studies have shown that the substitution with F and Br atoms can yield a good antifungal activity, which is consistent with current research [15]. However, the presence of an adamantanyl group makes the substituted compound also highly active, which is a surprising result because previous studies have indicated that the compounds with adamantanyl substituent are totally inactive.

Conclusions

An efficient and economic method for the synthesis of imidazolylthiazole hybrid molecules was developed. This hybrid scaffold could be promising in the discovery of new leading compounds having both antibacterial and antifungal properties. Among the derivatives, compounds **3e** and **3i** show strong activity against *Staphylococcus* spp. (MIC=7.81–15.62 μg/mL), *M. luteus* ATCC 10240 (MIC=1.95–3.91 μg/mL), and *Bacillus* spp. (MIC=3.91–15.62 μg/mL). Moreover, compounds **3e** and **3i** show the highest activity against reference strains of fungi belonging to *Candida* spp., with MIC ranging from 3.91 to 31.25 μg/mL. These results provide good starting templates for further structural optimization of this kind of derivatives.

Experimental

All synthetic experiments were carried out under air atmosphere. Reagents were generally the best-quality commercial-grade products and were used without further purification. ^{1}H NMR (700 MHz) and ^{13}C NMR (100 MHz) spectra were recorded in dimethyl sulfoxide (DMSO)-d_6 on a Bruker Avance III multinuclear instrument. Melting points were determined in open glass capillaries and are uncorrected. Analytical TLC was performed using Macherey-Nagel Polygram Sil G/UV$_{254}$ 0.2-mm plates. 1*H*-Imidazole-2-carbaldehyde, thiosemicarbazide, and appropriate chloroketones or bromoketones were commercial materials.

2-(1*H*-Imidazol-2-ylmethylidene)hydrazinecarbothioamide (2) [19] Thiosemicarbazide (0.47 g, 5.20 mmmol) was added to a stirred solution of 1*H*-imidazole-2-carbaldehyde (**1**, 0.50 g, 5.20 mmol) in 60% ethyl alcohol (25 mL). The mixture was stirred under reflux for 5 h and then at room temperature for 20 h. The resulting amorphous, slightly yellow precipitate of **2** was collected by filtration and washed with water; yield 0.70 g (80%); mp 224–226°C; ^{1}H NMR: δ 6.99 (s, 1H, CH), 7.28 (s, 1H, CH), 7.83 (s, 1H, CH), 8.06 (s, 1H, CH), 8.30 (s, 1H, NH), 11.53 (s, 1H, NH), 12.46 (s, 1H, NH).

2-((2*E*)-2-(1*H*-Imidazol-2-ylmethylidene)hydrazinyl)-4-(4-nitrophenyl)-1,3-thiazole (3a), a representative procedure Carbothioamide **2** (0.20 g, 1.18 mmol) was added to a stirred solution of 2-bromo-1-(4-nitrophenyl)ethanone (**4a**, 0.29 g, 1.18 mmol) in absolute ethyl alcohol (25 mL). The mixture was stirred at room temperature for 24 h. The separated precipitate was collected by filtration and suspended in water, and the mixture was neutralized with $NaHCO_3$ solution. The crude product was purified by silica gel column chromatography (230–400 mesh) using dichloromethane/methanol, 90:10, as an eluent (R_f=0.50) to afford the amorphous, deep yellow product **3a**; yield 0.33 g (70%) mp 239–240°C; ^{1}H NMR: δ 7.68–7.72 (m, 2H, 2CH), 7.85 (s, 1H, CH), 8.08 (d, 2H, 2CH, *J*=8.5 Hz), 8.26 (d, 2H, 2CH, *J*=8.5 Hz), 8.28 (s, 1H, CH), 13.10 (bs, 1H, NH), 14.40 (bs, 1H, NH); ^{13}C NMR: δ 111.0, 112.0, 124.6 (2C), 125.2, 126.9 (2C), 127.0, 140.5, 140.6, 146.8, 149.0, 167.6. Anal. Calcd for $C_{13}H_{10}N_6O_2S$: C, 49.67; H, 3.21; N, 26.74. Found: C, 49.65; H, 3.23; N, 26.78.

2-((2*E*)-2-(1*H*-Imidazol-2-ylmethylidene)hydrazinyl)-4-(4-fluorophenyl)-1,3-thiazole (3b) This compound was prepared from **2** and 2-bromo-1-(4-fluorophenyl)ethanone (**4b**) as described above; yield 0.33 g (89%); eluent – dichloromethane/methanol, 90:10, R_f=0.61; amorphous, deep yellow compound; mp 198°C (dec.); ^{1}H NMR: δ 7.22 (t, 2H, 2CH, *J*=8.8 Hz), 7.45 (s, 1H, CH), 7.72 (s, 1H, CH), 7.86 (q, 2H, 2CH, J_1=5.6 Hz, J_2=8.8 Hz), 8.04 (s, 1H, CH), 8.29 (s, 1H, CH), 13.00 (bs, 1H, NH), 14.40 (bs, 1H, NH); ^{13}C NMR: δ 106.0, 115.9, 120.9 (2C), 124.6, 128.0 (2C), 131.3, 140.6, 149.9, 161.0, 163.4, 167.2. Anal. Calcd for $C_{13}H_{10}FN_5S$: C, 54.34; H, 3.51; N, 24.38. Found: C, 54.31; H, 3.50; N, 24.40.

4-(2-((2*E*)-2-(1*H*-Imidazol-2-ylmethylidene)hydrazinyl)-1,3-thiazol-4-yl)benzonitrile (3c) This compound was prepared from **2** and 4-(bromoacetyl)benzonitrile (**4c**) as described above; yield 0.18 g (48%); eluent – dichloromethane/methanol, 90:10, R_f=0.43; amorphous, slightly yellow compound; mp 223.8–228.5°C; ^{1}H NMR: δ 7.71–7.75 (m, 2H), 7.80 (s, 1H, CH), 7.90 (d, 2H, 2CH, *J*=8.6 Hz), 8.05 (d, 2H, 2CH, *J*=8.6 Hz), 8.07 (s, 1H, CH), 13.04 (bs, 1H, NH), 14.40 (bs, 1H, NH); ^{13}C NMR: δ 110.0, 110.4, 119.4, 121.0, 125.2, 126.6 (2C), 133.3 (2C), 138.8, 140.6, 149.4, 149.4, 167.5. Anal. Calcd for $C_{14}H_{10}N_6S$: C, 57.13; H, 3.42; N, 28.55. Found: C, 57.16; H, 3.40; N, 28.58.

2-((2*E*)-2-(1*H*-Imidazol-2-ylmethylidene)hydrazinyl)-4-(4-methoxyphenyl)-1,3-thiazole (3d) This compound was prepared from **2** and 2-bromo-1-(4-methoxyphenyl)ethanone (**4d**) as described above; yield 0.18 g (61%); eluent – dichloromethane/methanol, 90:10, R_f=0.23; amorphous, slightly yellow compound; mp 176°C dec; ^{1}H NMR: δ 3.78 (s, 3H, CH_3), 6.96 (d, 2H, 2CH, *J*=9.0 Hz), 7.08–7.19 (m, 3H, 3CH), 7.77 (d, 2H, 2CH, *J*=9.0 Hz), 7.93 (s, 1H, CH), 12.12 (bs, 1H, NH), 12.42 (bs, 1H, NH); ^{13}C NMR: δ 55.6, 102.2, 114.4 (2C), 124.6, 127.3 (2C), 127.5, 128.0, 133.4, 143.0, 150.8, 159.3, 168.1. Anal. Calcd for $C_{14}H_{13}N_5OS$: C, 56.17; H, 4.38; N, 23.40. Found: C, 56.14; H, 4.35; N, 23.44.

2-((2*E*)-2-(1*H*-Imidazol-2-ylmethylidene)hydrazinyl)-4-(4-bromophenyl)-1,3-thiazole (3e) This compound was prepared from **2** and 2,4′-dibromoacetophenone (**4e**) as described above; yield 0.17 g (70%); eluent – dichloromethane/methanol, 90:10, R_f=0.43; amorphous, yellow compound; mp 151°C dec; ^{1}H NMR: δ 7.24–7.31 (m, 2H, 2CH), 7.45 (s, 1H, CH), 7.60 (d, 2H, 2CH, *J*=8.5 Hz), 7.80 (d, 2H, 2CH, *J*=8.5 Hz), 7.96 (s, 1H, CH), 12.40 (bs, 1H, NH), 13.00 (bs, 1H, NH). ^{13}C NMR (DMSO-d_6, 100 MHz), δ (ppm): 105.7, 121.1, 123.8, 128.0 (2C), 131.7, 132.0 (2C), 132.7, 134.2, 142.4, 149.6, 168.1. Anal. Calcd for $C_{13}H_{10}BrN_5S$: C, 44.84; H, 2.89; N, 20.11. Found: C, 44.80; H, 2.86; N, 20.14.

***N*-(4-(2-((2*E*)-2-(1*H*-Imidazol-2-ylmethylidene)hydrazinyl)-1,3-thiazol-4-yl)phenyl)acetamide (3f)** This compound was prepared under reflux for 2 h from **2** and *N*-(4-(chloroacetyl)phenyl)acetamide (**4f**) as described above; yield 0.22 g (67%); eluent – dichloromethane/methanol, 80:20, R_f=0.71; amorphous, slightly orange compound; mp 196.1–199.4°C; ^{1}H NMR: δ 2.06 (s, 3H, CH_3), 7.04 (bs, 1H, CH), 7.16–7.25 (m, 2H, 2CH), 7.62 (d, 2H, 2CH, *J*=8.7 Hz), 7.77 (d, 2H, 2CH, *J*=8.7 Hz), 7.94 (s, 1H, CH), 10.00 (s, 1H, NH), 12.16 (bs, 1H, NH), 12.42 (bs, 1H, NH); ^{13}C NMR: δ 24.5, 103.0, 119.4 (2C), 126.4 (2C), 126.6, 130.0, 133.5, 139.2, 142.2, 143.0, 150.7, 168.0, 168.1. Anal. Calcd for $C_{15}H_{14}N_6OS$: C, 55.20; H, 4.32; N, 25.75. Found: C, 55.21; H, 4.30; N, 25.76.

2-((2*E*)-2-(1*H*-Imidazol-2-ylmethylidene)hydrazinyl)-4-(3,4-dichlorophenyl)-1,3-thiazole (3g) This compound was prepared from **2** and 2-bromo-3′,4′-dichloroacetophenone (**4g**) as described above; yield 0.20 g (59%); eluent – dichloromethane/methanol, 90:10, R_f=0.63; amorphous, slightly yellow compound; mp 233.8–238.3°C; ^{1}H NMR: δ 7.69 (d, 1H, CH, *J*=8.5 Hz), 7.72 (s, 1H, CH), 7.75 (s, 2H, CH), 7.84 (dd, 1H, CH, J_1=8.5 Hz, J_2=4.2 Hz), 8.07 (d, 1H, J=9.0 Hz), 8.08 (s, 1H, CH), 13.09 (bs, 1H, NH), 14.50 (bs, 1H, NH); ^{13}C NMR: δ 80.7, 109.5, 122.0, 126.0, 127.1, 128.6, 131.5, 132.5, 133.0, 136.2, 141.5, 149.5, 168.4. Anal. Calcd for $C_{13}H_9Cl_2N_5S$: C, 46.17; H, 2.68; N, 20.71. Found: C, 46.19; H, 2.71; N, 20.69.

3-(2-((2*E*)-2-(1*H*-Imidazol-2-ylmethylidene)hydrazinyl)-1,3-thiazol-4-yl)-2*H*-chromen-2-one (3h) This compound was prepared from **2** and 3-(2-bromoacetyl)-2*H*-chromen-2-one (**4h**) as described above; yield 0.21 g (62%); eluent – dichloromethane/methanol, 90:10, R_f=0.58; amorphous, slightly yellow compound; mp 230.0–233.5°C; ^{1}H NMR: δ 7.44 (t, 1H, CH, *J*=7.5 Hz), 7.48 (d, 1H, CH, *J*=8.0 Hz), 7.54–7.59 (m, 2H, 2CH), 7.78 (t, 1H, CH, *J*=7.5 Hz), 7.88 (s, 1H, CH), 7.98 (d, 1H, CH, *J*=8.0 Hz) 8.05 (s, 1H, CH), 8.53 (s, 1H, CH), 12.78 (bs, 1H, NH), 14.00 (bs, 1H, NH); ^{13}C NMR: δ 79.7, 112.5, 116.3, 119.5, 120.8, 122.0, 125.1, 127.5, 129.3, 132.2, 138.8, 141.2, 144.4, 152.8, 159.2, 167.0. Anal. Calcd for $C_{16}H_{11}N_5O_2S$: C, 56.96; H, 3.29; N, 20.76. Found: C, 56.99; H, 3.31; N, 20.77.

2-((2*E*)-2-(1*H*-Imidazol-2-ylmethylidene)hydrazinyl)-4-(adamant-1-yl)-1,3-thiazole (3i) This compound was prepared from **2** and 1-adamantyl bromomethyl ketone (**4i**) as described above; yield 0.25 g (76%); eluent – dichloromethane/methanol, 90:10, R_f=0.34; amorphous, slightly yellow compound; mp 173°C (dec); ^{1}H NMR: δ 1.65–1.75 (m, 6H, $3CH_2$), 1.84–1.88 (m, 6H, $3CH_2$), 1.99–2.03 (m, 3H, 3CH), 6.39 (s, 1H, CH), 7.29 (s, 2H, 2CH), 7.88 (s, 1H, CH), 12.30 (bs, 1H, NH), 13.40 (bs, 1H, NH); ^{13}C NMR: δ 29.4 (3C), 37.4, 37.8 (3C), 42.9 (3C), 57.5, 80.7, 101.8, 124.2, 130.9, 143.3, 168.7. Anal. Calcd for $C_{17}H_{21}N_5S$: C, 62.36; H, 6.46; N, 21.39. Found: C, 62.40; H, 6.43; N, 21.42.

Antimicrobial *in vitro* assays

The examined compounds were screened *in vitro* for antibacterial and antifungal activities using the broth microdilution method according to European Committee on Antimicrobial Susceptibility Testing [20] and Clinical and Laboratory Standards Institute guidelines [21].

Reference strains of microorganisms from ATCC were used including Gram-positive bacteria (*S. aureus* ATCC 6538, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *Streptococcus pyogenes* ATCC 19615, *Str. pneumoniae* ATCC 49619, *Str. mutans* ATCC 25175, *Bacillus subtilis* ATCC 6633, *B. cereus* ATCC 10876, *M. luteus* ATCC 10240), Gram-negative bacteria (*E. coli* ATCC 3521, *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 12453, *Bordetella bronchiseptica* ATCC 4617, *Sal. typhimurium* ATCC 14028, *Pse. aeruginosa* ATCC 9027), and fungi belonging to yeasts (*C. albicans* ATCC 2091, *C. albicans* ATCC 10231, *C. parapsilosis* ATCC 22019).

The microbial cultures were subcultured on nutrient agar or Sabouraud agar at 35°C for 18–24 h or at 30°C for 24–48 h for bacteria and fungi, respectively. The surface of Mueller-Hinton agar or Mueller-Hinton agar with 5% sheep blood (for bacteria) and Roswell Park Memorial Institute (RPMI, developed for the culture of human normal and neoplastic cells *in vitro*) 1640 with 3-(*N*-morpholino) propanesulfonic acid (MOPS) (for fungi) were inoculated with suspensions of bacterial or fungal species. Microbial suspensions were prepared in sterile 0.85% NaCl with an optical density of 0.5 McFarland standard scale and approximately 1.5×10^8 colony-forming units (CFU)/mL for bacteria and 0.5 McFarland standard scale and approximately 5×10^5 CFU/mL for fungi.

Subsequently MIC, defined as the lowest concentration of the samples showing complete bacterial or fungal growth inhibition, was examined by the microdilution broth method using their twofold dilutions from 1000 to 0.488 μg/mL in Mueller-Hinton broth or Mueller-Hinton broth with 5% sheep blood (for bacteria) and RPMI 1640 broth with MOPS (for fungi) prepared in microtiter plates. Next, 2 μL of each bacterial or fungal suspension with an optical density of 0.5 McFarland standard was added to each well containing 200 μL broth with various concentrations of the examined compounds. The microplates were incubated at suitable conditions (37°C or 30°C, 24 h for bacteria or fungi, respectively), and then MIC was assessed spectrophotometrically. Appropriate growth and sterile controls were carried out. The medium with no tested substances was used as control.

The MBC and MFC are described as the lowest concentration of the compounds that is required to kill a particular bacterial or fungal species. The MBC/MIC or MFC/MIC ratios were calculated to determine bactericidal/fungicidal (MBC/MIC≤4, MFC/MIC≤4) or bacteriostatic/fungistatic (MBC/MIC>4, MFC/MIC>4) effect of the tested compounds.

MBC/MFC was determined by removing 20 μL of the culture using for MIC determinations from each well and spotting onto appropriate agar medium. After incubation (37°C or 30°C, 24 h for bacteria or fungi, respectively), the lowest compounds concentrations with no visible growth observed was assessed as a bactericidal/fungicidal concentration. All experiments were performed three times, and bioactivities were defined as in reference [22].

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