Med Chem Res (2012) 21:308–314
DOI 10.1007/s00044-010-9541-9

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and antimicrobial screening of *N*-(1-methyl-1*H*-pyrazole-4-carbonyl)-thiourea derivatives

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Received: 17 November 2009 / Accepted: 10 December 2010 / Published online: 25 December 2010

Abstract In the search of bioactive molecules in the class of functionally 4-substituted pyrazolic compounds, a series of *N*-(1-methyl-1*H*-pyrazole-4-carbonyl)-thiourea derivatives were prepared by addition of various substituted anilines to 1-methyl-1*H*-pyrazole-4-carbonyl isothiocyanate. The new thioureides and the intermediary compounds were characterized by spectroscopic data and elemental analyses and were evaluated for antibacterial and antifungal activities.

Keywords *N*-acyl-thiourea · 1-methyl-1*H*-pyrazole-4-carboxylic acid · Antibacterial

Introduction

In order to fight the overgrowing risk to appear antibiotics-resistant strains is essential to continue the development of new therapeutic solutions. For this purpose we joined in one single structure two important biologically active scaffolds, the pyrazole ring and thiourea. Pyrazole and its derivatives are an important class of compounds and attracted widespread attention because of their pharmacological properties, been reported to have a large spectrum of biological effects (antidepressant, anxiolytic, antiallergenic, antihypertensive, hypoglycemic, insecticidal, and fungicidal), currently, certain pyrazole derivatives being extensively employed as pharmaceuticals. The speciality literature mentions several pyrazole compounds with analgesic (Amir and Kumar, 2005), anti-inflammatory (Zelenin *et al.*, 1999), and antimicrobial activities (Akihiko *et al.*, 2005; Bildirici *et al.*, 2008; Goda *et al.*, 2003; Korgaokar *et al.*, 1996, Nauduri and Reddy, 1998, Susant *et al.*, 2007; Udupi *et al.*, 1998). Some pyrazole derivatives have the advantage of possesing all these three actions (Adnan *et al.*, 2005).

Literature survey reveals many *N*-acyl-thiourea derivatives with wide application as herbicides (Hackmann, 1960), insecticides (Aly *et al.*, 2007; Jirman and Resetova, 1986), parasiticidals (Müller *et al.*, 2009), antitumoral (Faidallah *et al.*, 2007), antimicrobials, and antifungals (Balotescu *et al.*, 2007). Giving attention to the possible synergetic antimicrobial effects of both thiourea and pyrayole moieties presence in an organic compound and following the promising model of various phenoxymethylbenzoic acids thioureides (Limban *et al.*, 2000, 2004, 2008a, b, c) we synthesized a series of *N*-(1-methyl-1*H*-pyrazole-4-carbonyl)-thiourea derivatives and tested their antimicrobial potential.

Results and discussion

The 1-methyl-1*H*-pyrazole-4-carboxylic acid (**1**) was converted into the 1-methyl-1*H*-pyrazole-4-carbonyl chloride (**2**) using thionyl chloride as chlorination reagent and 1,2-dichloroethane as solvent. The solvent and excess

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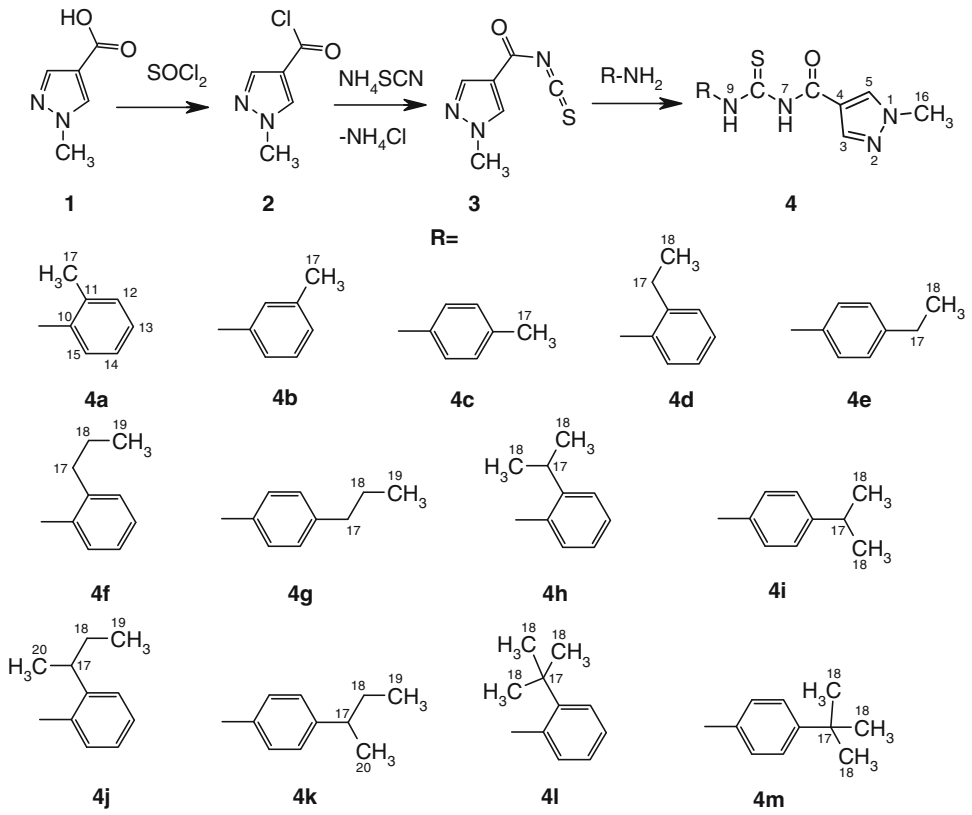
thionyl chloride were removed by reduced pressure distillation. For the next step the acyl chloride was used without additional purification. After solubilization in dry acetone, 1-methyl-1*H*-pyrazole-4-carbonyl chloride was treated with a solution of ammonium thiocyanate in acetone to afford 1-methyl-1*H*-pyrazole-4-carbonyl isothiocyanate (**3**). The resulting acyl isothiocyanate was not isolated from the mixture and was converted into the corresponding *N*-(1-methyl-1*H*-pyrazole-4-carbonyl)-*N*′-(aryl)-thioureas (**4a**–**m**) by adding various substituted anilines and refluxing for an hour in dry acetone. The synthesis process is described in the Scheme 1.

Following the aforementioned synthesis procedure, we obtained 13 new ***N***-(1-methyl-1*H*-pyrazole-4-carbonyl)-*N*′-(aryl)-thioureas, solid crystalline, white compounds that were characterized by NMR and IR spectra. The compounds purity was certified by elemental analyses, the results being within ±0.4 of the theoretical values. In the following section are presented the reaction yield, melting point, elemental composition and spectral data for each compound.

The qualitative screening of the susceptibility spectra of various microbial strains to the compounds **4a**–**m** was performed by adapted diffusion techniques. In Table 1 are presented the MICs for **4a**–**m** on the tested bacterial strains. The blank spaces indicate for MIC values over 1,000 μg/ml. All tested compounds generally exhibited low antimicrobial activity, excepting **4d** and **4f**. The low number of tested compounds unable us to perform a quantitative structure–activity relation study, but apparently the *ortho* substitution with a bulky group on the aryl moiety is somehow advantageous. This conclusion was obtained by comparing the activity of the isomer structures; the compound **4d** has a better antimicrobial effect then his *para* isomer, the compound **4e**. Similarly, the compound **4f** having a *ortho* propyl substituent is more active then the *para* isomer, **4g**. This relationship can be observed in a smaller degree to the compounds pairs **4h**–**i** and **4l**–**m**. One possible mechanism for the antimicrobial effect of the *N*-acyl-thioureas is their capacity to form complexes with various biologically important cations. The presence of a bulky alkyl near the thiourea group may hinder the formation of the inner hydrogen bond and thus facilitate the complexes formation.

The influence of the subinhibitory concentrations of the tested compounds on the expression of soluble enzymatic factors showed slight changes in the pattern of these virulence hallmarks. Comparing to the control only compounds **4d** and **4f** had a significant influence on the bacterian soluble enzymatic factors expression. In the presence of **4d** lecithinase production was inhibited in *Klebsiella pneumoniae*. Lecithinase is a bacterial toxin acting as an invasin by degrading membrane components and inserting pores into eucaryotic membranes promoting the bacterial invasion. Even though compound **4d** has a big



Scheme 1 The synthesis procedure of the new compounds

Table 1 Antimicrobial activity (MIC values, μg/ml) of the new compounds **4a–m**

Tested strains	4a	4b	4c	4d	4e	4f	4g	4h	4i	4j	4k	4l	4m
Bacillus subtilis	–	1000	–	1,000	–	500	–	–	–	–	–	–	–
Enterococcus faecalis	–	–	–	–	–	250	–	–	–	–	–	–	–
Staphylococcus aureus	–	–	–	250	–	500	–	–	–	–	–	–	–
Pseudomonas aeruginosa	–	–	–	500	–	1,000	–	–	–	–	–	–	–
Acinetobacter baumannii	–	–	–	250	–	250	–	–	–	–	–	1,000	–
Klebsiella pneumoniae	–	1,000	–	1,000	–	500	–	1,000	–	–	–	–	–
Escherichia coli	–	–	–	–	–	1,000	–	–	–	–	–	–	–

MIC value on *Klebsiella pneumoniae* it can be useful by hindering the infection propagation. The compound **4f** inhibited the caseinase production in *Escherichia coli*, this proteolytic enzyme being known for its involvement in the animal tissues invasion. These compounds had no effect on enzymatic factors production in the other bacterial strains.

Additional studies are intended to understand the structure influence on the antimicrobial effect of these *N*-acylthioureas in order to prepare more active compounds.

Experimental section

General procedures

All starting materials and solvents were purchased from common comercial suppliers and used without purification, unless otherwise noted. The acetone was dried over potassium carbonate and distillated and the ammonium thiocyanate by heating at 100°C. Melting points are uncorrected and were measured in open capillary tubes on an Electrothermal 9100 apparatus. The elemental analyses were performed on a Perkin Elmer CHNS/O Analyser Series II 2400 apparatus. The IR spectra were recorded on a JASCO FT/IR-4200 spectrometer with ATR PRO450-S accessory and diamond crystal. The NMR spectra were recorded on a Varian Gemini 300BB instrument at room temperature, operating at 300 MHz for ^{1}H and 75.075 MHz for ^{13}C. The compounds were dissolved in DMSO-d_6 and the chemical shifts were recorded as δ values in parts per million (ppm) units downfield of tetrametylsilane used as internal standard. The coupling constants values are reported in hertz and the splitting patterns are abbreviated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; b, broad.

The synthesis of 1-methyl-1*H*-pyrazole-4-carbonyl chloride (**2**)

In a round-bottom flask equipped with condenser and drying tube is added a solution of 1-methyl-1*H*-pyrazole-4-carboxylic acid (12.61 g, 0.1 mol) in anhydrous 1,2-dichlorethane and thionyl chloride (14.5 ml, 0.2 mol). The mixture is refluxed for 3 h. The solvent and the excess thionyl chloride are removed by reduced pressure distillation. For the next step the acyl chloride is used in crude status.

The synthesis of 1-methyl-1*H*-pyrazole-4-carbonyl isothiocyanate (**3**)

The raw obtained 1-methyl-1*H*-pyrazole-4-carbonyl chloride (10 mmol) is dissolved in anhydrous acetone (30 ml) and added to a solution of ammonium thiocyanate (0.76 g, 10 mmol) in dry acetone. The reaction mixture is refluxed 1 h in a round-bottom flask with a condenser and drying tube.

General procedures for the synthesis of the new compounds (**4a–m**)

A solution of the suitable substituted aniline (10 mmol) dissolved in anhydrous acetone is added, while stirring, to the 1-methyl-1*H*-pyrazole-4-carbonyl isothiocyanate solution. The mixture is heated under reflux for 1 h and poured afterwards into 10 times its volume of cold water, when the *N*-(1-methyl-1*H*-pyrazole-4-carbonyl)-*N*′-(aryl)-thioureas (**4a–m**) precipitated as solids, or as slowly crystallizing oils that were recrystallized from the suitable solvent.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(2-methylphenyl)-thiourea (**4a**)

Prepared using the general procedure. Yield 70%, m.p. 133–134°C (isopropanol). ^{1}H-NMR: 12.44 (s, NH, H-9); 11.37 (s, NH, H-7); 8.60 (s, 1H, H-3); 8.22 (s, 1H, H-5); 7.60 (bd, 1H, H-15); 7.29 (td, 7.4, 2.4, 1H, H-14); 7.24 (dd, 7.4, 2.4, 1H, H-12); 7.20 (td, 7.4, 2.4, 1H, H-13); 3.90 (s, 3H, H-16); 2.24 (s, 3H, H-17). ^{13}C-NMR: 180.48 (C-8); 163.41 (C-6); 140.74 (C-5); 137.25 (C-10); 134.94 (C-3); 133.60 (C-11); 130.74 (C-15); 127.31 (CH); 126.91 (CH); 126.45 (CH); 115.88 (C-4); 39.41 (C-16); 18.00 (C-17).

FT-IR (v, cm^{-1}): 3047; 2987; 1675; 1608; 1585; 1517; 1459; 1335; 1242; 1209; 1158; 1095; 993; 895; 829; 784; 756; 736; 682; 651; 616; 558; 534; 469; 442. Anal. Calcd for $C_{13}H_{14}N_4OS$: C, 56.92; H, 5.14; N, 20.42; S, 11.69. Found: C, 56.75; H, 5.36; N, 20.70; S, 11.29.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(3-methylphenyl)-thiourea (**4b**)

Prepared using the general procedure. Yield 63%, m.p. 138–139°C (isopropanol). ^{1}H-NMR: 12.37 (s, NH, H-9); 11.31 (s, NH, H-7); 8.60 (s, 1H, H-3); 8.21 (s, 1H, H-5); 7.48 (m, 8.0, 2H, H-11, H-15); 7.29 (t, 8.0, 1H, H-14); 7.07 (d, 8.0, 1H, H-13); 3.90 (s, 3H, H-16); 2.32 (s, 3H, H-17). ^{13}C-NMR: 179.20 (C-8); 163.13 (C-6); 140.38 (C-5); 138.10 (C-10); 137.89 (C-12); 134.61 (C-3); 128.48 (C-14); 126.90 (C-13); 124.65 (C-11); 121.33 (C-15); 115.53 (C-4); 39.06 (C-16); 20.93 (C-17). FT-IR (v, cm^{-1}): 3673; 2987; 2153; 1746; 1655; 1606; 1546; 1489; 1399; 1347; 1308; 1256; 1225; 1148; 1093; 992; 884; 783; 739; 698; 681; 616; 544; 519; 448; 405. Anal. Calcd for $C_{13}H_{14}N_4OS$: C, 56.92; H, 5.14; N, 20.42; S, 11.69. Found: C, 56.81; H, 5.01; N, 20.63; S, 11.82.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(4-methylphenyl)-thiourea (**4c**)

Prepared using the general procedure. Yield 68%, m.p. 171–173°C (isopropanol). ^{1}H-NMR: 12.33 (s, NH, H-9); 11.30 (s, NH, H-7); 8.60 (s, 1H, H-3); 8.21 (s, 1H, H-5); 7.52 (d, 8.2, 2H, H-11, H-15); 7.21 (d, 8.2, 2H, H-12, H-14); 3.89 (s, 3H, H-16); 2.31 (s, 3H, H-17). ^{13}C-NMR: 179.24 (C-8); 163.10 (C-6); 140.36 (C-5); 135.58 (C-10); 135.43 (C-13); 134.58 (C-3); 129.09 (C-12, C-14); 124.23 (C-11, C-15); 115.55 (C-4); 39.05 (C-16); 20.60 (C-17). FT-IR (v, cm^{-1}): 2987; 2153; 1745; 1657; 1606; 1548; 1490; 1399; 1347; 1309; 1256; 1226; 1148; 1093; 992; 884; 783; 740; 698; 682; 616; 545; 520; 448; 406. Anal. Calcd for $C_{13}H_{14}N_4OS$: C, 56.92; H, 5.14; N, 20.42; S, 11.69. Found: C, 56.55; H, 5.20; N, 20.49; S, 11.91.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(2-ethylphenyl)-thiourea (**4d**)

Prepared using the general procedure. Yield 71%, m.p. 130–131°C (isopropanol). ^{1}H-NMR: 12.47 (s, NH, H-9); 11.40 (s, NH, H-7); 8.60 (s, 1H, H-3); 8.22 (s, 1H, H-5); 7.55–7.52 (m, 1H, H-15), 7.32–7.23 (m, 3H, H-12, H-13, H-14); 3.89 (s, 3H, H-16); 2.57 (q, 7.6, 2H, H-17); 1.14 (t, 7.6, 3H, H-18). ^{13}C-NMR: 180.80 (C-8); 163.34 (C-6); 140.47 (C-5); 139.04 (C-10); 136.30 (C-11); 134.70 (C-3); 128.84 (C-12); 127.44 (C-14); 127.36 (C-13); 126.15 (C-15); 115.58 (C-4); 39.12 (C-16); 24.21 (C-17); 14.51 (C-18). FT-IR (v, cm^{-1}): 3126; 2349; 1659; 1579; 1511; 1319; 1238; 1217; 1200; 1151; 990; 944; 879; 832; 755; 664; 612; 571; 530; 461. Anal. Calcd for $C_{14}H_{16}N_4OS$: C, 58.31; H, 5.59; N, 19.43; S, 11.12. Found: C, 58.07; H, 5.64; N, 19.32; S, 11.03.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(4-ethylphenyl)-thiourea (**4e**)

Prepared using the general procedure. Yield 76%, m.p. 155–156°C (isopropanol). ^{1}H-NMR: 12.72 (s, NH, H-9); 11.29 (s, NH, H-7); 8.59 (s, 1H, H-3); 8.21 (s, 1H, H-5); 7.54 (d, 8.5, 2H, H-11, H-15,); 7.24 (d, 8.5, 2H, H-12, H-14); 3.89 (s, 3H, H-16); 2.60 (q, 7.6, 2H, H-17); 1.18 (t, 7.6, 3H, H-18). ^{13}C-NMR: 179.28 (C-8); 163.17 (C-6); 141.95 (C-13); 140.42 (C-5); 135.67 (C-10); 134.62 (C-3); 127.98 (C-12, C-14); 124.33 (C-11, C-15); 115.60 (C-4); 39.10 (C-16); 27.79 (C-17); 15.60 (C-18). FT-IR (v, cm^{-1}): 2962; 2363; 2309; 2153; 1661; 1599; 1532; 1397; 1342; 1195; 1147; 1089; 996; 922; 872; 829; 810; 774; 753; 725; 671; 616; 543; 519; 458. Anal. Calcd for $C_{14}H_{16}N_4OS$: C, 58.31; H, 5.59; N, 19.43; S, 11.12. Found: C, 58.21; H, 5.71; N, 19.57; S, 11.10.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(2-*n*-proylphenyl)-thiourea (**4f**)

Prepared using the general procedure. Yield 71%, m.p. 118.5–120°C (isopropanol). ^{1}H-NMR: 12.49 (s, NH, H-9); 11.39 (s, NH, H-7); 8.60 (s, 1H, H-3); 8.23 (s, 1H, H-5); 7.58–7.55 (m, 1H, H-15); 7.30–7.22 (m, 3H, H-12, H-13, H-14); 3.89 (s, 3H, H-16); 2.51 (t, 7.6, 2H, H-17); 1.54 (sxt, 7.6, 2H, H-18); 0.87 (t, 7.6, 3H, H-19). ^{13}C-NMR: 180.74 (C-8); 163.35 (C-6); 140.44 (C-5); 137.37 (C-10); 136.45 (C-11); 134.73 (C-3); 129.73 (C-12); 127.47 (C-14); 127.14 (C-13); 126.17 (C-15); 115.55 (C-4); 39.10 (C-16); 33.11 (C-17); 23.07 (C-18); 13.83 (C-19). FT-IR (v, cm^{-1}): 3124; 2953; 2349; 2309; 2153; 1658; 1579; 1510; 1444; 1396; 1320; 1249; 1211; 1197; 1152; 1115; 1090; 1059; 989; 940; 874; 838; 812; 775; 756; 689; 657; 642; 610; 592; 553; 532; 467; 418. Anal. Calcd for $C_{15}H_{18}N_4OS$: C, 59.58; H, 6.00; N, 18.53; S, 10.60. Found: C, 59.64; H, 6.18; N, 18.43; S, 10.76.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(4-*n*-proylphenyl)-thiourea (**4g**)

Prepared using the general procedure. Yield 72%, m.p. 150–151°C (isopropanol). ^{1}H-NMR: 12.74 (s, NH, H-9); 11.28 (s, NH, H-7); 8.59 (s, 1H, H-3); 8.21 (s, 1H, H-5); 7.55 (d, 8.5, 2H, H-11, H-15); 7.21 (d, 8.5, 2H, H-12, H-14); 3.89 (s, 3H, H-16); 2.51 (t, 7.5, 2H, H-17); 1.58 (sxt, 7.5, 2H, H-18); 0.88 (t, 7.5, 3H, H-19). ^{13}C-NMR: 179.20

(C-8); 163.18 (C-6); 140.42 (C-5); 140.33 (C-10); 135.70 (C-13); 134.60 (C-3); 128.53 (C-12, C-14); 124.16 (C-11, C-15); 115.55 (C-4); 39.10 (C-16); 36.84 (C-17); 24.09 (C-18); 13.69 (C-19). FT-IR (v, cm^{-1}): 2960; 2348; 1664; 1595; 1530; 1397; 1347; 1306; 1240; 1204; 1147; 1091; 994; 973; 945; 875; 827; 794; 743; 663; 617; 545; 519. Anal. Calcd for $C_{15}H_{18}N_4OS$: C, 59.58; H, 6.00; N, 18.53; S, 10.60. Found: C, 59.56; H, 5.99; N, 18.80; S, 10.77.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(2-*i*-proylphenyl)-thiourea (**4h**)

Prepared using the general procedure. Yield 65%, m.p. 177.5–179°C (isopropanol). ^{1}H-NMR: 12.41 (s, NH, H-9); 11.40 (s, NH, H-7); 8.60 (s, 1H, H-3); 8.22 (s, 1H, H-5); 7.41 (dd, 7.5, 1.8, 1H, H-12); 7.38 (dd, 7.5, 1.8, 1H, H-15); 7.30 (td, 7.5, 1.8, 1H, H-13); 7.23 (td, 7.5, 1.8, 1H, H-14); 3.89 (s, 3H, H-16); 3.04 (septet, 6.7, 1H, H-17); 1.18 (d, 6.7, 6H, H-18). ^{13}C-NMR: 181.26 (C-8); 163.33 (C-6); 143.74 (C-10); 140.46 (C-5); 135.64 (C-11); 134.69 (C-3); 127.96 (C-12); 127.71 (C-13); 125.98 (C-14); 125.86 (C-15); 115.58 (C-4); 39.12 (C-16); 27.99 (C-17); 23.07 (C-18). FT-IR (v, cm^{-1}): 3181; 2968; 1670; 1552; 1508; 1398; 1363; 1315; 1243; 1217; 1200; 1156; 1097; 1063; 1028; 995; 977; 937; 866; 834; 780; 762; 720; 689; 664; 643; 620; 582; 557; 532; 469; 439; 419. Anal. Calcd for $C_{15}H_{18}N_4OS$: C, 59.58; H, 6.00; N, 18.53; S, 10.60. Found: C, 59.44; H, 6.07; N, 18.68; S, 10.76.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(4-*i*-proylphenyl)-thiourea (**4i**)

Prepared using the general procedure. Yield 69%, m.p. 127–128°C (isopropanol). ^{1}H-NMR: 12.10 (bs, NH, H-9); 11.29 (s, NH, H-7); 8.59 (s, 1H, H-3); 8.21 (s, 1H, H-5); 7.55 (d, 8.4, 2H, H-11, H-15); 7.26 (d, 8.4, 2H, H-12, H-14); 3.89 (s, 3H, H-16); 2.89 (septet, 6.9, 1H, H-17); 1.19 (d, 6.9, 6H, H-18). ^{13}C-NMR: 179.47 (C-8); 163.39 (C-6); 146.76 (C-13); 140.65 (C-5); 135.95 (C-10); 134.84 (C-3); 126.74 (C-12, C-14); 124.54 (C-11, C-15); 115.84 (C-4); 39.34 (C-16); 33.33 (C-17); 24.12 (C-18). FT-IR (v, cm^{-1}): 3286; 3110; 3034; 2952; 1662; 1596; 1530; 1511; 1398; 1343; 1295; 1246; 1206; 1146; 1090; 1063; 1016; 994; 945; 918; 873; 830; 811; 761; 728; 664; 617; 548; 523; 480; 433. Anal. Calcd for $C_{15}H_{18}N_4OS$: C, 59.58; H, 6.00; N, 18.53; S, 10.60. Found: C, 59.87; H, 5.80; N, 18.70; S, 10.71.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(2-*s*-butylphenyl)-thiourea (**4j**)

Prepared using the general procedure. Yield 66%, m.p. 153–154°C (isopropanol). ^{1}H-NMR: 12.4 (bs, NH, H-9); 11.4 (s, NH, H-7); 8.60 (s, 1H, H-3); 8.22 (s, 1H, H-5); 7.42 (bd, 7.5, 1H, H-15); 7.35–7.20 (m, 3H, H-12, H-13, H-14); 3.90 (s, 3H, H-16); 2.9 (sxt, 6.9, 2H, H-17,); 1.55 (q, 6.9, 2H, H-18); 1.16 (d, 6.9, 3H, H-20); 0.75 (t, 6.9, 3H, H-19). ^{13}C-NMR: 181.46 (C-8); 163.65 (C-6); 142.79 (C-10); 140.69 (C-5); 136.30 (C-11); 134.95 (C-3); 128.18 (CH); 127.90 (CH); 126.57 (CH); 126.15 (CH); 115.79 (C-4); 39.16 (C-16); 35.12 (C-17); 30.19 (C-18); 21.22 (C-20); 12.37 (C-19). FT-IR (v, cm^{-1}): 3129; 2956; 1670; 1510; 1319; 1220; 1201; 1149; 1093; 1056; 995; 878; 830; 758; 712; 688; 655; 610; 537; 461. Anal. Calcd for $C_{16}H_{20}N_4OS$: C, 60.73; H, 6.37; N, 17.71; S, 10.13. Found: C, 60.44; H, 6.76; N, 17.46; S, 9.86.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(4-*s*-butylphenyl)-thiourea (**4k**)

Prepared using the general procedure. Yield 70%, m.p. 151–152°C (isopropanol). ^{1}H-NMR: 12.4 (bs, NH, H-9); 11.4 (s, NH, H-7); 8.53 (s, 1H, H-3); 8.15 (s, 1H, H-5); 7.58 (d, 8.5, 2H, H-12, H-14), 7.21 (d, 8.5, 2H, H-11, H-15), 3.90 (s, 3H, H-16); 2.60 (sxt, 7.1, 2H, H-17,); 1.55 (q, 7.1, 2H, H-18); 1.19 (d, 6.9, 3H, H-20); 0.77 (t, 7.1, 3H, H-19). ^{13}C-NMR: 183.61 (C-8); 163.64 (C-6); 144.74 (C-10); 140.32 (C-5); 136.07 (C-11); 134.38 (C-3); 126.24 (CH); 126.07 (CH); 110.28 (C-4); 38.96 (C-16); 31.26 (C-18); 30.53 (C-17); 21.71 (C-20); 12.11 (C-19). FT-IR (v, cm^{-1}): 2986; 2901; 1661; 1587; 1516; 1452; 1393; 1315; 1251; 1201; 1147; 1066; 877; 825; 757; 672; 613; 547. Anal. Calcd for $C_{16}H_{20}N_4OS$: C, 60.73; H, 6.37; N, 17.71; S, 10.13. Found: C, 60.46; H, 6.71; N, 17.55; S, 9.81.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(2-*t*-butylphenyl)-thiourea (**4l**)

Prepared using the general procedure. Yield 66%, m.p. 146–147.5°C (isopropanol). ^{1}H-NMR: 12.30 (s, NH, H-9); 11.46 (s, NH, H-7); 8.62 (s, 1H, H-3); 8.24 (s, 1H, H-5); 7.44 (dd, 7.0, 2.6, 1H, H-15); 7.25–7.35 (m, 3H, H-12, H-13, H-14); 3.89 (s, 3H, H-16); 1.33 (s, 9H, H-18). ^{13}C-NMR: 181.29 (C-8); 163.59 (C-6); 145.03 (C-10); 140.50 (C-5); 136.32 (C-11); 134.82 (C-3); 131.18 (C-14); 127.57 (CH); 126.73 (CH); 126.38 (CH); 115.48 (C-4); 39.12 (C-16); 34.57 (C-17); 30.50 (C-18). FT-IR (v, cm^{-1}): 3125; 2972; 2901; 1657; 1517; 1443; 1396; 1315; 1232; 1214; 1200; 1149; 1066; 990; 935; 877; 846; 825; 763; 745; 683; 616; 547; 485; 438. Anal. Calcd for $C_{16}H_{20}N_4OS$: C, 60.73; H, 6.37; N, 17.71; S, 10.13. Found: C, 60.90; H, 6.33; N, 17.74; S, 10.35.

N-[(1-methyl-1*H*-pyrazole-4-yl)carbonyl]-*N*′-(4-*t*-butylphenyl)-thiourea (**4m**)

Prepared using the general procedure. Yield 70%, m.p. 152–153°C (isopropanol). ^{1}H-NMR: 12.74 (s, NH, H-9);

11.30 (s, NH, H-7); 8.60 (s, 1H, H-3); 8.21 (s, 1H, H-5); 7.58 (d, 8.5, 2H, H-12, H-14); 7.42 (d, 8.5, 2H, H-11, H-15); 3.89 (s, 3H, H-16); 1.29 (s, 9H, H-18). ^{13}C-NMR: 178.99 (C-8); 163.08 (C-6); 148.70 (C-13); 140.40 (C-5); 135.40 (C-10); 134.59 (C-3); 125.40 (C-11, C-15); 123.84 (C-12, C-14); 115.57 (C-4); 39.34 (C-16); 34.31 (C-17); 31.12 (C-18). FT-IR (ν, cm^{-1}): 3235; 2954; 2197; 1660; 1586; 1515; 1390; 1363; 1318; 1251; 1201; 1144; 1093; 994; 969; 875; 823; 753; 673; 614; 545. Anal. Calcd for $C_{16}H_{20}N_4OS$: C, 60.73; H, 6.37; N, 17.71; S, 10.13. Found: C, 60.40; H, 6.60; N, 17.94; S, 10.24.

Qualitative and quantitative screening of the antimicrobial properties

The synthesized compounds **4a–m** were evaluated for their in vitro antibacterial activity against pathogenic bacteria. The susceptibility spectra of various microbial strains to the compounds was performed by adapted diffusion techniques (paper filter disks impregnated with the tested compound, agar wells filled with the tested compound solution and distribution of the tested compound solution directly on the solid medium previously seeded with the bacterial inoculums). The quantitative assay of the minimal inhibitory concentration (MIC, μg/ml) was based on liquid medium two-fold microdilutions, the compounds being solubilized in DMSO (Dimethylsulfoxide) starting from a concentration of 1,000 to 1.95 μg/ml. The compounds were tested against a microbial inoculum of $\sim 1.5 \times 10^8$ CFU/cm^3, corresponding to 0.5 McFarland density. The MIC was considered to be the lowest concentration that completely inhibited growth on agar plates. The tested microorganisms were represented by Gram-positive (*Bacillus subtilis* 10.10.2006, *Enterococcus faecalis* 13773, *Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa* 5541, *Acinetobacter baumannii* 15896, *Klebsiella pneumoniae* 126/2007 and *Escherichia coli* 637/2007) reference or recently isolated strains. All bacterial strains were provided by Cantacuzino Institute, Bucharest.

Investigation of the influence of compounds **4a–m** on the expression of soluble virulence factors

The tested microbial strains were cultivated for 24 h in liquid medium in the presence of subinhibitory concentrations of the tested compounds. For the investigation of lipase production, the treated strains were spotted on Tween 80 agar at a final concentration of 1% and the plates were incubated at 37°C for 7 days. An opaque zone around the spot was registered as positive reaction. The amylase production was tested on 10% starch supplemented agar medium. The strains were stubbed and incubated at 24 h at 37°C, starch hydrolysis was registered by the presence of a clear area around the culture spot. For the lecithinase production assay, the cultures were spotted into 2.5% egg yolk agar and incubated at 37°C for 7 days. An opaque zone around the spot indicated the lecithinase production. The protease (caseinase/gelatinase) activity was determined using as substrate 15% soluble casein agar and 17.5% gelatin agar. The strains were spotted and after incubation 24 h at 37°C, a precipitation zone surrounding the bacteria growth indicated the enzyme production. The DNase production was studied on DNA supplemented medium. The strains were spotted and after incubation 24 h at 37°C, a drop of 1 M solution of hydrochloric acid was added upon the spotted cultures; a clear zone around the culture was interpreted as a positive reaction. The esculin hydrolysis test (that indicates the production of esculethol, a bacterial siderophore used for the iron acquisition and subsequent expression of other soluble virulence factors, especially toxins) was performed using bacterial strains incubated at 37°C for 24h in esculin and ferric citrate medium. A dark coloration indicates a positive result.

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