Article

N′-Substituted 4-Phenylpicolinohydrazonamides with Thiosemicarbazone Moiety as New Potential Antitubercular Agents: Synthesis, Structure and Evaluation of Antimicrobial Activity

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Abstract: Three new 4-phenylpicolin derivatives with a thiosemicarbazone structure were synthesized and evaluated for tuberculostatic activity. The compounds were obtained by the condensation of methyl 4-phenylpicolonimidate with the corresponding cycloalkylamino-1-carbothiohydrazides. The 1H NMR temperature spectra obtained showed proton lability at the nitrogen atom N2, and X-ray crystallography confirmed the zwitterionic structure of all products. ADME calculations indicate that the compounds can be tested as future drugs. All compounds were absorbed in the gastrointestinal tract. All compounds also showed very good tuberculostatic activity (MIC 3.1–12.5 µg/mL). Derivative 1b showed the best selectivity for M. tuberculosis compared to the other pathogenic species tested. The study has allowed the emergence of imine derivative 1b as a good structure for further optimization in the search for antitubercular drugs.

Keywords: picolinohydrazonamide; thiosemicarbazones; synthesis; temperature NMR; X-ray; ADME; tuberculostatic activity; antimicrobial activity

1. Introduction

Tuberculosis is one of the leading causes of death worldwide. It is an infectious disease declared a global public health emergency in 1993 by the WHO. Until the COVID-19 outbreak, tuberculosis was the leading infectious killer, surpassing HIV/AIDS [1,2]. It is a treatable disease, but its treatment regimen is complicated, as one should always use a combination of at least two drugs over a long period of time. This is done in order to increase the effectiveness of treatment as well as prevention of formation of resistant forms [3]. Mycobacteria can mutate on their own and develop resistance to the drugs used [4,5]. This happens as a result of improper treatment, e.g., inadequate duration of therapy, incorrectly selected medications, incorrect dose, or incomplete treatment cycle. In order to prevent this unfavorable phenomenon, the rules of pharmacotherapy should be scrupulously followed [6,7]. Due to the fact that tuberculosis is still one of the diseases leading to death and is a threat mainly due to the constantly developing chemotherapeutic and antibiotic resistant strains of Mycobacterium tuberculosis, new, effective anti-tuberculosis...
agents are being sought [8,9], especially given that the most effective anti-tuberculosis drug is still isoniazid, which was introduced into medical treatment in 1952 [10].

Previously, we described the synthesis and antimicrobial properties of the cycloalkylamine-1-thiosemicarbazone derivatives of picolinohydrazonamide DMK-15 and ACSc-5 (Figure 1) [11,12]. The first one, with two rings of secondary cyclic amines, pyrrolidine and morpholine, showed tuberculosis activity towards M. tuberculosis at the MIC (minimal inhibition concentration) level of 0.4 µg/mL with low cytotoxicity against the human dermal fibroblast last line HDF, with IC50 (half-maximal inhibitory concentration) of 36.18 µg/mL. The second one, with a methyl group in the C-6 position and a phenylpiperazine fragment at the end of the thiosemicarbazone substituent, showed considerable activity against the Staphylococcus aureus ATCC 25923 strain (MIC 15.6 µg/mL) and the Bacillus subtilis ATCC 6633 strain (MIC 7.8 µg/mL). These results show that the combination of a pyridine ring and a thiosemicarbazone moiety in one molecule can result in good antimicrobial activity without adversely affecting eukaryotic cells. Thus, further structural studies, both in solid and liquid states, seemed to be a crucial element in the search for the influence of tautomerism on the antimicrobial activity of thiosemicarbazone compounds.

Figure 1. Structures of picolinohydrazonamide DMK-15 and ACSc-5.

In this research, we describe the synthesis of three new 4-phenylpicoline derivatives with thiosemicarbazone structure. The interesting analytical results obtained for these products prompted us to study their structure thoroughly. We carried out 1H NMR temperature studies and X-ray crystallography. The compounds showed remarkable activity against both standard and resistant strains when tested with M. tuberculosis species. Then they were tested for the activity towards other species of pathogenic microorganisms. Compounds were also analyzed for their pharmacokinetic properties, drug similarity, and absorption.

2. Materials and Methods

2.1. Chemistry

The reagents, solvents, and materials used were of analytical purity (Sigma-Aldrich-Merck KgaA, Darmstadt, Germany). The purity of the products was confirmed by thin-layer chromatography (TLC). In the analysis, silica gel plates were used, and a lamp emitting light with a wavelength of λ = 254 nm was used for detection. The melting points of the compounds obtained were measured once with a Stuart SMP30 (Stone, Staffordshire, UK). IR spectra for potassium bromide pellets of solids were recorded on a Satellite FT-IR spectrophotometer (Bruker, Madison, WI, USA). Standard 1H and 13C NMR spectra were performed in CDCl3 or DMSO-d6 using Varian Unity Plus (500 MHz) and Varian Gemini (200 MHz) instruments (Varian Medical Systems, Palo Alto, CA, USA). The 1H NMR spectra of 1a–c were acquired at various temperatures (233, 243, 253, 263, 273 and 300 K) using a Bruker Avance II Plus 16.4 T spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at an 1H frequency (700.16 MHz). The elemental composition of the products was determined on the basis of elemental analysis (% C, H, N), and the obtained results were consistent with the values calculated with a maximum deviation of 0.4%.
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Methyl 4-phenylpicolinimidate was synthesized according to method described by us elsewhere [13] and analytical data were consistent with those obtained by other authors [14].

2.1.1. General Procedure for the Synthesis of Hydrazonamides (1a-c)

Methyl 4-phenylpicolinimidate (0.36 g, 2 mmol) was dissolved in methanol (5 mL) and treated with DBU (0.35 mL, 2.3 mmol). The solution was refluxed for 30 min and then the appropriate cycloalkylamino-1-carbothiohydrazide (2 mmol) was added without cooling the mixture. The mixture was refluxed another 15 min, cooled in an ice bath and the precipitated products were filtered off and recrystallized from ethanol, giving yellow crystals. In the case of compound 1c, the ice was also added to the mixture.

4-Phenyl-N′-(pyrrolidine-1-carbonothioyl) Picolinohydrazonamide (1a)

From pyrrolidine-1-carbothiohydrazide (0.29 g), the compound 1a was obtained (0.23 g, 46%): m.p. 173–175 °C; IR: 3419, 3274 (v N-H), 3149, 3031, 2950, 2865 (v C-H), 1669, 1601 (v C=N), 1463, 1430 (v C=C), 1362, 1298, 1264, 1237 (v C-N), 763, 696 (v C-H) cm\(^{-1}\);

\( ^1H \) NMR (500 MHz, DMSO-\( d_6 \)): \( \delta \) 1.85–1.86 (m, 4H, 2CH\( _2 \)), 3.56–3.60 (m, 4H, 2CH\( _2 \)), 6.85 (br. s, 2H, NH\( _2 \) + D\( _2 \)O exchangeable), 7.55–7.61 (m, 4H, 3H Ph and 1H pyridine), 7.97–7.98 (m, 2H, Ph), 8.53 (s, 1H, pyridine), 8.81 (d, 1H, pyridine, J = 5 Hz), 12.62 (s, 1H, NH + D\( _2 \)O exchangeable); \( ^{13}C \) NMR (175 MHz): \( \delta \) 127.59 (2C), 129.76 (2C), 130.49, 136.60, 143.76, 145.31, 149.40, 150.94, 179.22 ppm; Anal. Calcd for C\( _{17}H_{16}N_2O \) (341.13): C, 62.74; H, 5.88; N, 21.54. Found: C, 62.95; H, 6.15; N, 21.19.

N′-(Morpholine-4-carbonothioyl)-4-phenylpicolinohydrazonamide (1b)

From morpholine-4-carbothiohydrazide (0.32 g), the compound 1b was obtained (0.55 g, 80%): m.p. 171–173 °C; IR: 3282 (v N-H), 3155, 3044, 2953, 2922, 2836 (v C-H), 1671 (v C=N), 1589, 1463, 1414 (v C=C), 1344, 1259, 1220 (v C-N), 1116 (v C-O), 1023 (\( \delta \) C-H), 892, 767 (v C-H) cm\(^{-1}\); \( ^1H \) NMR (200 MHz, CDCl\( _3 \)): \( \delta \) 3.74 (t, 4H, 2CH\( _2 \), J = 5 Hz), 3.98 (t, 4H, 2CH\( _2 \), J = 5Hz), 6.75 (br. s, 2H, NH\( _2 \) + D\( _2 \)O exchangeable), 7.51–7.68 (m, 6H, 5H Ph and 1H pyridine), 8.11 (s, 1H, pyridine), 8.70 (d, 1H, pyridine, J = 5 Hz) 12.53 (s, 1H, NH) ppm; \( ^{13}C \) NMR (175 MHz, DMSO-\( d_6 \)): \( \delta \) 47.19 (2C), 66.72 (2C), 119.21, 123.98, 127.60 (2C), 129.78 (2C), 130.53, 136.51, 145.18, 145.24, 149.45, 150.94, 179.22 ppm; Anal. Calcd for C\( _{17}H_{19}N_2OS \) (341.15): C, 59.80; H, 5.61; N, 20.51; Found: C, 59.44; H, 5.45; N, 20.15.

4-Phenyl-N′-(4-phenylpiperazine-1-carbonothioyl) Picolinohydrazonamide (1c)

From 4-phenylpiperazine-1-carbothiohydrazide (0.47 g), the compound 1c was obtained (0.37 g, 45%): m.p. 178–180 °C; IR: 3266 (v N-H), 3149, 3024, 2843 (v C-H), 1668, 1598 (v C=N), 1495, 1463, 1413 (v C=C), 1339, 1223 (v C-N), 1018 (\( \delta \) C-H), 763, 693 (v C-H) cm\(^{-1}\); \( ^1H \) NMR (500 MHz, DMSO-\( d_6 \)): \( \delta \) 3.13–3.15 (m, 4H, 2CH\( _2 \)), 4.04 (m, 4H, 2CH\( _2 \)), 6.81 (t, 1H, Ph, J = 7 Hz), 7.00 (d, 2H, Ph, J = 8 Hz), 7.24 (t, 2H, Ph, J = 7 Hz), 7.54–7.60 (m, 3H, Ph), 7.87 (br. s, 1H, NH + D\( _2 \)O exchangeable), 7.96–7.98 (m, 3H, 2H Ph and 1H pyridine), 8.57 (s, 1H, pyridine), 8.76–8.92 (m, 2H, 1H pyridine and 1H NH + D\( _2 \)O exchangeable), 12.69 (s, 1H, NH + D\( _2 \)O exchangeable) ppm; \( ^{13}C \) NMR (175 MHz): \( \delta \) 46.34 (2C), 49.04 (2C), 116.23 (2C), 119.23, 119.50, 123.95, 127.60 (2C), 129.42 (2C), 129.77 (2C), 130.50, 136.56, 145.15, 145.27, 149.47, 150.93, 151.70, 179.10 ppm; Anal. Calcd for C\( _{23}H_{24}N_6S \) (416.18): C, 66.32; H, 5.81; N, 20.18; Found: C, 66.28; H, 5.41; N, 19.86.

2.2. X-ray Study

X-ray diffraction experiments of the studied compounds were done on a diffractometer (Bruker SMART APEXII CCD) using CuKα radiation. Diffraction data were processed with SAINT ver. 8.34A, SADABS ver. 2014/4 (structure 1c with TWINABS ver. 2008/4) and XPREP ver. 2014/2. The structures were solved with the ShelXT (Version 2018/2) [15] and refined with ShelXL 2018/3 [16]. For visualization, ShelXle [17] and Olex2 [18] programs were used. Structure 1c was refined as a three-component twin with the following contributions: 0.88, 0.11, and 0.01. All H atoms (except those in NH and NH2 groups) were
geometrically optimized. All studied crystals were obtained by slow evaporation of solvent. The solvent used is a mixture of methanol and DMF (1:1). Programs publCIF [19] and Mercury [20] were used to prepare the manuscript. CCDC 2189843, 2189844, 2189856 contain the supplementary crystallographic data for this paper. The data were provided free of charge by the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures (accessed on 13 July 2022).

2.3. ADME

The compounds were analyzed for their pharmacokinetic properties, drug-likeness and absorption. An ADME analysis done with SwissADME service [21], a free web tool to evaluate pharmacokinetics, drug-likeness molecules and BOILED-Egg to predict gastrointestinal absorption and brain penetration of molecules [22].

2.4. Tuberculostatic Activity Assay

The newly synthesized hydrazonamides were tested in vitro for their anti-tuberculosis activity against three strains of *M. tuberculosis*: H37Rv and two native strains isolated from patients of the National Institute of Tuberculosis and Lung Diseases in Warsaw, Poland. Strain Spec. 210 was resistant to clinically used anti-tuberculosis drugs: *p*-aminosalicylic acid (PAS), isoniazid (INH), ethambutol (ETB), and rifampicin (RMP). Spec. 192 showed full sensitivity. The tests were carried out using the classic test tube method described in detail earlier [23]. For the compound 1b, which showed the highest activity during the tests, the research was extended to include non-tuberculous strains *M. bovis*, *M. kanssasi*, *M. intracellulare*, and *M. scrafulaceum*. Each test was performed in triplicate. Representative data are presented.

2.5. In Vitro Antibacterial Activity Assay

Compounds 1a–c were screened for antibacterial and antifungal activities by microdilution broth method as described elsewhere [24]. For all compounds, minimal inhibition concentration (MIC), minimal bactericidal concentration (MBC), and minimal fungicidal concentration (MFC) were determined. The tested derivatives were evaluated for the panel of the reference microorganisms from American Type Culture Collection (ATCC), including Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Micrococcus luteus* ATCC 10240, *Bacillus cereus* ATCC 10876, *Bacillus subtilis* ATCC 6633, *Streptococcus pyogenes* ATCC 19615, *Streptococcus mutans* ATCC 25175, *Streptococcus pneumoniae* ATCC 49619), Gram negative bacteria (*Salmonella Typhimurium* ATCC14028, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 9027, *Proteus mirabilis* ATCC 12453), and fungi (*Candida parapsilosis* ATCC 22019, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019) (LGC Standards, Teddington, Middlesex, UK). Each experiment was performed in triplicate. Representative data are presented.

3. Results and Discussion

3.1. Chemistry

The initial compound 4-phenyl-2-cyanopyridine refluxed for 1 h with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in methanol gave methyl 4-phenylpicolinimidate with 85% yield (Figure 2) [13]. The addition of DBU is intended to prevent the substitution at position 4 of the pyridine ring. Received compound was refluxed with cycloalkylamino-1-carbothiohydrazides for 15 min to the hydrazonamides 1a–c. Cycloamino-1-carbothiohydrazides were obtained according to modified method described by Klayman et al. [25]. The substituents used were pyrrolidine, morpholine, and 4-phenylpiperazine, the reaction yield depended on the cyclic amine (45–80%).
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In 1H NMR experiments recorded for compounds 1a–c at various temperatures (Figures 3–5), it can be seen that the NH proton with a chemical shift of ~13.31 ppm at 300 K is very broadened and almost disappears in noise (Figure 6). As the temperature decreases, we get one sharp signal, which underwent an upfield shift (12.87 ppm). Changing the position of the labile proton at nitrogen atoms and the possibility of hydrogen bonding of this proton with the nitrogen atom in the aromatic ring changes the chemical environment of the NH₂ protons. The consequence of this is the magnetic inequality of protons in the amino group and the appearance of separate signals on the spectrum at 243 K, while at 300 K there is one average signal (~6.40 ppm). Both signals undergo exchange upon heavy water.

Figure 2. Synthesis of compounds 1a–c.

Figure 3. 1H NMR spectra of 1a as a function of temperature (700 MHz, CDCl₃).

Figure 4. 1H NMR spectra of 1b as a function of temperature (700 MHz, CDCl₃).

Figure 5. 1H NMR spectra of 1c as a function of temperature (700 MHz, CDCl₃).
Figure 3. $^1$H NMR spectra of 1a as a function of temperature (700 MHz, CDCl$_3$).

Figure 4. $^1$H NMR spectra of 1b as a function of temperature (700 MHz, CDCl$_3$).

Figure 5. $^1$H NMR spectra of 1c as a function of temperature (700 MHz, CDCl$_3$).

3.2. X-ray Study
Crystallographic data are presented in Table 1. All compounds crystallized in a monoclinic system. The compound 1c has two molecules in the asymmetric unit and was refined as a three-component twin, and the other two compounds have one molecule in the asymmetric unit (Figure 7). All compounds take the form of zwitterion. The compound 1a forms a chain of hydrogen bonds, and its conformation is stabilized by three intramolecular hydrogen bonds (Figure 8A). Infinite chains of hydrogen bonds strengthen the layered packing system (Figure 8B). Planar structure of molecules in compounds 1b and 1c are also intramolecular hydrogen bonds stabilizing the structure. In crystal structures of 1b and 1c, both hydrogen atoms of the NH$_2$ group form hydrogen bonds of the N-H S type, creating a three-dimensional structure. Both compounds form a similar packing (Figures 9 and 10).

Table 1. Crystal data, data collection, and refinement details.

| Compound | Crystal Data | Refinement Details |
|----------|--------------|--------------------|
| 1a       | C$_{17}$H$_{19}$N$_5$S |                  |
| 1b       | C$_{17}$H$_{19}$N$_5$OS |                |
| 1c       | 2(C$_{23}$H$_{24}$N$_6$S) |              |
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Table 1. Crystal data, data collection, and refinement details.

|      | 1a          | 1b          | 1c          |
|------|-------------|-------------|-------------|
| **Crystal Data** |             |             |             |
| Chemical formula | C_{17}H_{19}N_{5}S | C_{17}H_{19}N_{5}OS | 2(C_{23}H_{24}N_{6}S) |
| M_r  | 325.43      | 341.43      | 833.08      |
| Space group | P_{2}_1/c | P_{2}_1/c | P_{2}_1 |
| a, b, c (Å) | 13.3591 (4), 12.0691 (4), 9.9817 (3) | 5.9419 (4), 12.1487 (7), 22.9938 (13) | 6.2510 (3), 11.9035 (5), 27.8679 (12) |
| β (°) | 101.248 (2) | 96.549 (2) | 93.696 (2) |
| V (Å³) | 1578.46 (9) | 1649.01 (17) | 2069.30 (16) |
| Z    | 4           | 4           | 2           |
| μ (mm⁻¹) | 1.87       | 1.86        | 1.56        |
| Crystal size (mm) | 0.87 × 0.24 × 0.20 | 1.24 × 0.18 × 0.14 | 1.0 × 0.41 × 0.13 |

| **Data Collection** |             |             |             |
| No. of measured, independent and observed [I > 2σ(I)] reflections | 21324, 3119, 2979 | 17762, 3213, 3185 | 8030, 8030, 8024 |
| (sin θ/λ)max (Å⁻¹) | 0.618 | 0.618 | 0.618 |

| **Refinement** |             |             |             |
| R[F² > 2σ(F²)], wR(F²), S | 0.028, 0.076, 1.05 | 0.027, 0.073, 1.04 | 0.026, 0.074, 1.13 |
| No. of reflections | 3119 | 3213 | 8030 |
| No. of parameters | 220 | 230 | 568 |
| No. of restraints | 0 | 0 | 1 |
| Δ_max, Δ_min (e Å⁻³) | 0.22, −0.32 | 0.34, −0.18 | 0.20, −0.19 |
| Absolute structure | – | – | Flack x determined using 3129 quotients [(I+) − (I-)][(I+) + (I-)] [²⁶] |
| Absolute structure parameter | – | – | 0.046 (4) |
### Materials

**Space group**

- P2₁/c
- P2₁/c
- P2₁

| Parameter | Value 1 | Value 2 | Value 3 |
|-----------|---------|---------|---------|
| a (Å)     | 13.3591 (4) | 12.0691 (4) | 9.9817 (3) |
| b (Å)     | 5.9419 (4) | 12.1487 (7) | 22.9938 (13) |
| c (Å)     | 6.2510 (3) | 11.9035 (5) | 27.8679 (12) |
| β (°)     | 101.248 (2) | 96.549 (2) | 93.696 (2) |

**Volume (Å³)**

- 1578.46 (9)
- 1649.01 (17)
- 2069.30 (16)

**Z**

- 4
- 4
- 2

**µ (mm⁻¹)**

- 1.87
- 1.86
- 1.56

**Crystal size (mm)**

- 0.87 × 0.24 × 0.20
- 1.24 × 0.18 × 0.14
- 1.0 × 0.41 × 0.13

### Data Collection

- No. of measured, independent and observed [I > 2σ(I)] reflections
  - 21324, 3119, 2979
  - 17762, 3213, 3185
  - 8030, 8030, 8024

| (sinθ/λ)max (Å⁻¹) | Value 1 | Value 2 | Value 3 |
|--------------------|---------|---------|---------|
| 0.618 | 0.618 | 0.618 |

### Refinement

- R(F² > 2σ(F²)), wR(F²), S
  - 0.028, 0.076, 1.05
  - 0.027, 0.073, 1.04
  - 0.026, 0.074, 1.13

| No. of reflections | Value 1 | Value 2 | Value 3 |
|--------------------|---------|---------|---------|
| No. of parameters | 220 | 230 | 568 |
| No. of restraints | 0 | 0 | 1 |
| Δmax, Δmin (e Å⁻³) | 0.22, -0.32 | 0.34, -0.18 | 0.20, -0.19 |

### Absolute structure

- Flack x determined using 3129 quotients [(I+) − (I⁻)] / [(I+) + (I⁻)] [26]
- Absolute structure parameter – – 0.046 (4)

**Figure 7.** The molecular structures of compounds (a–c), with atom-labeling schemes. Displacement ellipsoids are drawn at the 50% probability level, and H atoms are shown as small spheres of arbitrary radii. Drawings were prepared with Mercury software.

**Figure 8.** Cont.
Figure 8. (A) Intermolecular hydrogen bonds in 1a. (B) The packing of molecules in 1a (a, b, c—unit cell).

Figure 9. (A) Intermolecular hydrogen bonds in 1b. (B) The packing of molecules in 1b (a, b, c—unit cell).
Figure 9. (A) Intermolecular hydrogen bonds in 1b. (B) The packing of molecules in 1b (a, b, c—unit cell).

Figure 10. (A) Intermolecular hydrogen bonds in 1c. (B) The packing of molecules in 1c (a, b, c—unit cell).

The superposition of the molecules (Figure 11) indicates that the zwitterion form stabilizes the stretched form, and all compounds assume a similar conformation.

Figure 11. Superimposition of molecules for all determined structures; 1a—red, 1b—green, 1c—blue/turquoise. H atoms were omitted for clarity.

3.3. ADME

Bioavailability radars for all studied compounds were made (Figure 12). For drug-like properties, the compounds were found to have a good bioavailability score (0.55). The compounds 1a and 1b meets with the rules of Lipinski [27], Ghose [28], Egan [29], Veber [30] and Muegge [31]. Compound 1c does not fulfill Ghose’s rules. All compounds are good
drug candidates. In the BOILED-Egg diagram (Figure 13), all compounds are absorbed in the gastrointestinal tract, which may make it an effective drug. No compounds permeate the blood–brain barrier.

Figure 12. Bioavailability radar for (a–c).

Figure 13. BOILED-Egg diagram for all compounds (lipophilicity (WLOGP) and polarity (tPSA), human intestinal absorption (white area) and blood–brain barrier permeation (yellow area).
3.4. Tuberculostatic Activity

The obtained compounds were tested for in vitro tuberculostatic activity against *M. tuberculosis*. The MIC values were defined as the minimum concentration inhibiting the growth of the tuberculosis strains tested relative to the control without the test compound. INH and PZA were used as reference drugs. The tested derivatives showed diversified activity (Table 2). However, two of the obtained compounds showed better activity than not only the reference PZA, which showed activity against the standard H37Rv strain and susceptible strain Spec. 192 at the level of MIC value 25 µg/mL and against the resistance strain Spec. 210 over 400 µg/mL, but also INH (MICs respectively 12.5 µg/mL and 25 µg/mL). Two derivatives (1a and 1b) showed high activity toward all strain types (MIC 3.1–12.5 µg/mL). The presence of hydrophilic cyclic amine (pyrrolidine or morpholine) increased the activity, while the presence of highly lipophilic phenylpiperazine moiety (1c) significantly decreased the antitubercular potency (MIC 50 µg/mL). It is noteworthy that mostly the MIC values against the resistant strain were at the same level as against the standard strain.

Table 2. In vitro tuberculostatic activity of compounds 1a–c 1,2,3.

| Compd. | H37Rv 2 [µg/mL] | Spec. 192 | Spec. 210 |
|--------|-----------------|-----------|-----------|
| 1a     | 6.2             | 12.5      | 6.2       |
| 1b     | 3.1             | 3.1       | 3.1       |
| 1c     | 50              | 50        | 50        |
| INH 3  | 12.5            | 12.5      | 25        |
| PZA 3  | 25              | 25        | >400      |

The values obtained for the most potent compounds are marked in bold. 1 Minimal inhibiting concentration has been determined by the classical test-tube method of successive dilution. 2 *M. tuberculosis* H37Rv, Spec. 192, Spec. 210. 3 INH isoniazid; PZA pyrazinamide.

The extended non-tuberculous assays have revealed that compound 1b shows weaker inhibition of non-tuberculotic mycobacteria (Table 3). MIC for *M. kansasii*, *M. intracellulare* and *M. scrofulaceum* was in the range of 6.2–12.5 µg/mL. The activity against *M. bovis* was merely at the same level as for tuberculous mycobacteria (3.1 µg/mL).

Table 3. In vitro activity of compound 1b towards various Mycobacterium strains.

| Mycobacterium Strain | MIC [µg/mL] |
|----------------------|-------------|
| *M. tuberculosis* H37Rv | 3.1         |
| *M. bovis*           | 3.1         |
| *M. kansasii*         | 12.5        |
| *M. intracellulare*   | 6.2         |
| *M. scrofulaceum*     | 12.5        |

3.5. Antimicrobial Activity

Extensive microbiological tests were conducted for all the newly synthesized compounds (1a–c). The antibacterial and antifungal activities of tested compounds are presented in Table 4. Vancomycin (Van), ciprofloxacin (Cip) and nystatin (Nys) were used as the standard drugs. Markedly, the tested compounds showed mostly bacteriostatic activity (MBC/MIC ratio > 4). The minimal inhibition concentration (MIC) values for Gram-positive reference bacteria, indicated very strong (MIC < 10 mg/L) anti-staphylococcal (*S. aureus*, *S. epidermidis*), anti-micrococcal (*M. luteus*) and good (MIC 25–125 mg/L) anti-streptococcal (*S. pyogenes*, *S. pneumoniae*, *S. mutans*) activity of tested compounds [32]. Good bioactivity was also observed against Gram-negative bacteria (*S. typhimurium*, *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*) with MIC of 25–125 mg/L. Compound 1a revealed the best antibacterial efficiency against Gram-positive bacteria. The most sensitive to tested com-
pounds were staphylococcal strains. Antifungal bioactivity (C. albicans, C. parapsilosis) of tested compounds was determined as very strong (1a) and strong (1b, 1c).

Table 4. In vitro antimicrobial activity of compounds 1a–c against reference bacterial and fungal strains.

| Gram-Positive Bacteria | MIC | MBC | MBC/MIC Ratio | MIC | MBC | MBC/MIC Ratio | MIC |
|------------------------|-----|-----|---------------|-----|-----|---------------|-----|
| S. aureus ATCC 25923   | 1.95| 15.6| 8             | 1.95| 1.95| 1             | 0.98|
| S. aureus ATCC 43300   | 3.9 | 125 | 32            | 1.95| 250 | 128           | 0.49|
| S. aureus ATCC 6538    | 3.9 | 125 | 32            | 1.95| 15.6| 8             | 0.49|
| S. epidermidis ATCC 12228 | 1.95| 125 | 32            | 0.98| 31.3| 16            | 0.49|
| M. luteus ATCC 10240   | 0.49| 15.6| 32            | 1.95| 1.95| 1             | 0.06|
| B. cereus ATCC 10876   | 3.9 | 31.3| 8             | 7.8 | >1000| >128         | 31.3|
| B. subtilis ATCC 6633  | 3.9 | 62.5| 16            | 0.98| 31.3| 2             | 0.24|
| S. pyogenes ATCC 19615 | 7.8 | 15.6| 2             | 15.6| 125 | 2             | 0.24|
| S. mutans ATCC 25175   | 3.9 | 15.6| 4             | 31.3| >1000| >32          | 62.5|
| S. pneumoniae ATCC 49619 | 7.8 | 31.3| 1             | 31.3| 125 | 1             | 0.24|
| Gram-Negative Bacteria | Cip |     |               |     |      |               |     |
| E. coli ATCC 25922     | 1000| >1000| Nd           | 125 | >1000| >8           | 125 |
| S. typhimurium ATCC 14028 | 250 | >1000| >4           | 125 | >1000| >8           | 125 |
| K. pneumoniae ATCC 13883 | >1000| >1000| Nd          | 500 | >1000| >8           | 125 |
| P. mirabilis ATCC 12453 | 125 | >1000| >8           | 31.3| >1000| >32          | 62.5|
| P. aeruginosa ATCC 9027 | 500 | >1000| Nd          | 31.3| >1000| >32          | 62.5|
| Yeasts                 |     | MIC | MFC          | MIC | MFC |               | MIC |
| C. albicans ATCC 102231 | 15.6| 62.5| 4            | 15.6| 62.5| 2             | 7.8 |
| C. parapsilosis ATCC 22019 | 62.5| 62.5| 1            | 15.6| 125 | 8             | 7.8 |

1 MIC—minimal inhibitory concentration, MBC—minimal bactericidal concentration, MFC—minimal fungicidal concentration. 2 CIP ciprofloxacin, VAN vancomycin, Nys nystatin. 3 Nd not detected.

4. Conclusions

In conclusion, three new 4-phenylpyridine thiosemicarbazone derivatives were successfully synthesized from methyl 4-phenylpicolinimidate and cycloalkylaminio-1-carbothiohydrazides. Spectroscopic analysis and structural crystallography allowed us to identify the labile proton at the nitrogen atom N2. In the crystalline state, these take the extended form of zwitterions. The planar conformation is stabilized by intramolecular hydrogen bonds. The packing of molecules in space is influenced by hydrogen bonds of the N-H S type. ADME calculations indicate that the compounds can be tested as future drugs. All compounds are absorbed in the gastrointestinal tract. This is a significant advantage, especially for potential anti-tuberculosis drugs, due to long-term therapy and patient comfort. Studies of tuberculostatic activity showed significantly high activity of the newly obtained derivatives. Extended tests for compound 1b against non-tuberculous species of the Mycobacterium genus showed a broad spectrum of activity of this compound. At the same time, this compound showed the best selectivity of all the products obtained, having a lower effect on other species of pathogenic microorganisms. The most important finding, therefore, is that—due to its high tuberculostatic potential—compound 1b may be a good starting structure for further research into new antimicrobial drugs.

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Abbreviations

- a, b, c (Å): unit cell parameters in angstroms
- ACSc-5: (Z)-6-methyl-N′-(4-phenylpiperazine-1-carbonothioyl) picolinohydrazoneamide
- ADME: absorption, distribution, metabolism, and excretion
- AIDS: acquired immune deficiency syndrome
- ATCC: American Type Culture Collection
- CCDC: Cambridge Crystallographic Data Centre
- Cip: ciprofloxacin
- COVID-19: Coronavirus Disease 2019
- DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene
- DMK-15: (Z)-N′-(morpholine-4-carbonothioyl)-4-(pyrrolidin-1-yl)picolinohydrazoneamide
- DMSO-d6: isotopologue of dimethyl sulfoxide
- ETB: ethambutol
- F2: structure factor squared
- FLEX: flexibility
- FT-IR: Fourier-transform infrared spectroscopy
- H37Rv: standard *M. tuberculosis* strain
- HDF: human dermal fibroblast line
- HIV: human immunodeficiency virus
- IC50: half-maximal inhibitory concentration
- INH: isoniazid
- INSATU: insaturation
- INSOLU: insolubility
- IR: infrared radiation
- *M. tuberculosis*: *Mycobacterium tuberculosis*
- MBC: minimal bactericidal concentration
- MFC: minimal fungicidal concentration
- MIC: minimal inhibition concentration
- *M*<sub>r</sub>: molecular mass
- NMR: nuclear magnetic resonance
- Nys: nystatin
- PAS: *p*-aminosalicylic acid
- POLAR: polarity
- PZA: pyrazinamide
- R: index based on structure factors describing compatibility of the model with X-ray diffraction data
- RMP: rifampicin
- S: goodness of fit of the refined X-ray structure
- Spec. 192: strain fully sensitive to the administrated tuberculostatic drugs
- Spec. 210: strain isolated from tuberculosis patients resistant to PAS, INH, ETB, and RMP
- tPSA: polarity
- UV: ultraviolet
- V (Å<sup>3</sup>): volume of a unit cell in cube angstroms
- Van: vancomycin
- WHO: World Health Organization
- WLOGP: lipophilicity
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