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Antituberculosis activity of some 3,6-disubstituted 1,2,4,5-tetrazines

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Abstract

The increase in the incidence of tuberculosis, especially in the post-Covid period, associated with the presence of latent infections and multiple drug resistance, dictates the need to develop new antimicrobial drugs. Here we wish to report the synthesis of 3,6-disubstituted 1,2,4,5-tetrazine derivatives containing *N*-, *O*- and *C*-nucleophile residues, as well as pharmacophoric hydrazone and oligooxyethylene fragments, and their screening against the pathogen *Mycobacterium tuberculosis*. Substances with pronounced antimycobacterial activity *in vitro* were identified (MIC = 0.18–3.12 μg/ml). An acute toxicity *in vivo* and membranotropic properties towards biometals have been studied for the most active compounds.

Keywords

3,6-disubstituted 1,2,4,5-tetrazines
1,2,4,5-tetrazine podands
antimycobacterial activity
acute toxicity

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Key findings

- 1,2,4,5-Tetrazines carrying different *N*-, *O*- and *C*-nucleophile residues in 3 and 6 positions of the heterocycle were synthesized.
- Tetrazinyl amino and tetrazinyl hydrazone podands were synthesized.
- Antimycobacterial activity was determined in *in vitro* and *in vivo* experiments, promising inhibitors were found.

Supplementary materials

1. Introduction

3,6-Disubstituted 1,2,4,5-tetrazines are considered as a novel promising class of biologically active compounds. In particular, one can find in the literature data on their herbicidal [1], antifungal [2], antimalarial [3], antibacterial [4], anticoagulant and antiglycation action [5], they also have antitumor activity [6–8]. Several members of the biologically active 1,2,4,5-tetrazines [3, 4, 9] with different substituents and their minimum inhibitory concentrations (MICs) are shown in Figure 1. During the last decades, a special attention has been paid to the search for substances within the *s*-tetrazine derivatives of the above-mentioned class that exhibit antimycobacterial activity [9–12]. This is inspired by modern trends in practical medicine in connection with growth and widespread of multiple drug-resistant tuberculosis (MDR-TB), including the latest generation of drugs [13]. The first positive results of anti-tuberculosis activity of 1,2,4,5-tetrazines bearing amino acid ester substituents were published [9]. Some substances in this series suppress the growth of Mycobacterium tuberculosis in concentrations as high as 1.25–0.6 μg/mL, and inhibit in the 0.3–0.15 μg/mL range (Figure 1) [9].

Therefore, it was interesting to continue the search for effective tuberculostatic compounds within the novel representatives of this class of compounds. The present study describes the synthesis of 3,6disubstitued 1,2,4,5-tetrazines including those functionalized with podands in their reactions with *N*-, *O*-, and *C*-nucleophiles with the aim of estimating the tuberculostatic activity and acute toxicity of the synthesized derivatives.

2. Experimental

The starting compounds **1a** [14]; **1b**,**d**, **2e** [15]; **1c** [17]; **2a**,**k**, **7b** [16]; **3b**,**c** [5]; **4b** [18]; **2g**,**j**, **4a** [19]; **2b**,**k**, **7a-d**,**f** [20] have been described previously.

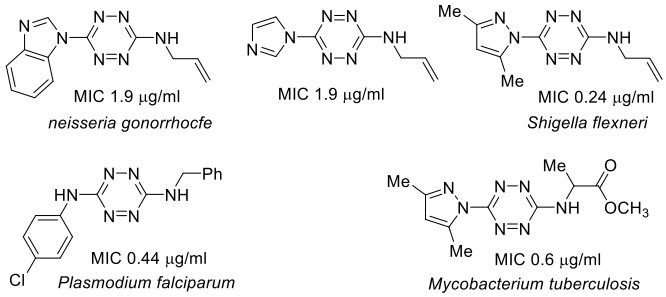

Figure 1 Examples of biologically active differently substituted 1,2,4,5-tetrazines.

^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance DRX-400» spectrometer (400 MHz) using DMSO-d_6 or $CDCl_3$ as solvents and Me4Si as an internal standard. Electrospray ionization mass spectra were recorded for positive ions on a qTOF maXis Impact HD ultra-high resolution mass spectrometer from Bruker Daltonik (USA) with a standard ionization source in the mass range 50–2300 Da by injection analysis for sample solutions in acetonitrile using a syringe pump (model No. 601553 kdScientific inc., USA); solution infusion rate of 240 μL/h) in the modified default method "Direct Infusion 100–1000". Calibration of the mass scale was external, according to the signals of a solution of lithium formate by enhanced quadratic or HPC method. All data were collected and processed using the Compass for oTof series 1.7 software package (oTOF Control 3.4; Bruker Compass Data Analysis 4.2). Elemental analysis data was obtained on Carlo Erba EA-1108 (Carlo Erba Instruments) and PE 2400 series II (Perkin Elmer) CHN automatic analyzers. IR spectra were recorded on a Perkin Elmer Spectrum One IR Fourier transform spectrometer using a diffuse reflectance sampling accessory (DRA). Melting points were measured on the instrument Boetius. The reaction control and the purity of the obtained compounds was carried out using Sorbfil TLC Plates on Aluminium Sheets with acetonitrile-hexane mixture (1:1) as the eluent.

2.1. General procedure of preparation of 2c,d,h,i,f,l, 3a, 5, 6a,b,c, 7e

Tetrazines **2c**,**d**,**h**,**i**,**l**, **6a**,**c** were obtained by suspending 0.11 mmol of the corresponding nucleophile in 25 mL of acetonitrile with 0.1 mmol of tetrazine **1** or **2d**, or **3a**. A reaction mixture was mixed for 2.0 hours. The precipitate was then filtered off, recrystallized from acetonitrile and air dried. The preparation methods for compounds **2f**, **3a**, **5**, **6b** and **7e** are given below, along with their physical and chemical properties.

2.1.1. 3-N-(*t*-butylamino)-6-(3,5-dimethylpyrazol-1-yl)-1,2,4,5-tetrazine 2c

Yield 230 mg, 93%; m.p. 127 °C. Calculated (%): C 53.42, H 6.93, N 39.65; $C_{11}H_{17}N_7$. Found (%): C 53.18, H 6.50, N 39.83. 1H NMR ($CDCl_3$) δ, ppm: 6.09 (*s*, 1 H, H-C(4)Pz), 5.85 (*br.s*, 1 H, NH), 2.57 and 2.35 (both *s*, 6 H, 2 CH_3 ,Pz), 1.56 (*s*, 9 H, C(CH3)3). ^{13}C NMR ($CDCl_3$) δ, ppm: 161.2, 157.2, 152.0, 141.8, 109.5, 52.6, 28.4, 13.7, 13.4 (Supplementary S1.1). IR (DRA, ν, cm^{-1}): 3280 (NH); 3044–3095 (C–H); 1557, 1448 (Pz), 1392, 1366 (–$C(CH_3)_3$). High-resolution mass spectrometry, found: *m/z* 248.1620 $[M + H]^+$. Calculated for $C_{11}H_{18}N_7^+$: 248.1618 $[M + H]^+$ (Supplementary S1.1).

2.1.2. 3-N-(Aminoheptyl)-6-(3,5-dimethylpyrazol-1-yl)-1,2,4,5-tetrazine 2d

Orange crystals. Yield 202 mg, 70%; m.p. 48 °C. Calculated (%): C 58.11, H 8.01, N 33.88; $C_{14}H_{23}N_7$. Found (%): C 58.13, H 7.99, N 34.03. ^{1}H NMR ($CDCl_3$) δ, ppm: 6.09 (*c*, 1 H, H-C(4)^{Pz}), 5.93 (*br.s*, 1 H, NH), 3.63 (*k*, 2 H, –$NHCH_2$–, *J* = 7.0, 13 Hz), 2.57 and 2.35 (both *s*, 6 H, 2 CH_3), 1.71–1.76 (*m*, 2 H, –HN–$(CH_2)_5$–CH_2CH_3), 1.29–1.46 (*m*, 8 H, NH-$CH_2(CH_2)_4$-CH_2CH_3), 0.89 (*t*, 3 H, –NH–$(CH_2)_2$–CH_2CH_3, *J* = 7.0 Hz). ^{13}C NMR ($CDCl_3$) δ, ppm: 161.4, 157.7, 152.0, 141.9, 109.6, 41.7, 31.7, 29.1, 13.4; 28.9, 26.7, 22.54, 14.0, 13.7 (Supplementary-S1.2). IR (DRA, ν, cm^{-1}): 3248 (NH); 3060–3136 (C–H); 1567, 1459 (Pz), 1063, 1040, 970 (tetrazine). High-resolution mass spectrometry, found *m/z* 290.2090 $[M + H]^+$. Calculated for $C_{14}H_{23}N_7^+$: 290.2088 $[M + H]^+$ (Supplementary S1.2).

2.1.3. 6-(3,5-dimethylpyrazol-1-yl)-3-N-(2-Hydroxyphenylamino)- 1,2,4,5-tetrazine 2h

Dark-red crystals. Yield 256 mg, 94%; m.p. 218–218.5 °C. Calculated (%): C 55.12, H 4.59, N 34.63; $C_{13}H_{13}N_7O$. Found (%): C 55.23, H 4.50, N 34.43. ^{1}H NMR (DMSO-d_6) δ, ppm: 10.01 (*s*, 1 H, NH), 9.74 (*s*, 1 H, OH), 7.51 (*dd*, 1 H, Ar, J_1 = 1.6; J_2 = 7.8), 7.09–7.12 (*m*, 1 H, Ar), 6.95 (*dd*, 1 H, Ar, J_1 = 1.3; J_2 = 8.0), 6.86 (*td*, 1 H, Ar, J_1 = 1.4; J_2 = 11.4), 6.21 (*s*, 1 H, H-C(4)Pz), 2.42 and 2.38 (both s, 6 H, 2 CH3 at C(3)Pz and C(5)Pz). ^{13}C NMR (DMSO-d6) δ, ppm: 160.8, 157.1, 151.0, 150.3, 141.5, 126.6, 125.4, 124.3, 119.0, 116.0, 108.8, 13.3, 12.4 (Supplementary-S1.3). IR (DRA, ν, cm^{-1}): 3383 (OH); 3042–2938 (C_{ar}–H); 1575, 1557, 1410 (Pz); 1078, 1043, 926 (C=N, tetrazine). High-resolution mass spectrometry, found *m/z* 284.1254 $[M + H]^+$. Calculated for $C_{13}H_{14}N_7O^+$: 284.1254 $[M + H]^+$ (Supplementary S1.3).

2.1.4. 6-(3,5-Dimethylpyrazol-1-yl)-3-(Indol-3-yl-ethylamino)- 1,2,4,5-tetrazine 2i

Yield 307 mg, 92%; m.p. 197–198 °C. Calculated (%): C 61.09, H 5.67, N 33.64; $C_{17}H_{18}N_8$. Found (%): C 61.08, H 5.39, N 33.53. ^{1}H NMR (DMSO-d_6) δ, ppm: 10.85 (*s*, 1 H, NH), 8.92 (*t*, 1 H, NH–CH_2–, *J* = 5.8 Hz), 7.59 (*d*, 1 H, Ind, *J* = 7.8), 7.35 (*d*, 1 H, Ind, *J* = 8.1 Hz), 7.25 (*d*, 1 H, *J* = 2.2), 3.08 (*t*, 2 H, –CH_2–, *J* = 14.9 Hz), 3.75 (*k*, 2 H, –CH_2–), 7.05–7.09 and 6.98–7.01 (both *m*, 2 H, Ind), 6.18 (*s*, 1 H, H-

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C(4)^{Pz}), 2.26 and 2.35 (both *s*, 6 H, 2 CH_3). ^{13}C NMR (DMSO-d_6) δ, ppm: 12.1, 13.3, 24.3, 41.6, 108.3, 111.3, 11.4, 118.2, 118.3, 120.9, 123.0, 127.2, 136.2, 141.2, 149.9, 156.6, 161.3 (Supplementary-S1.4). IR (DRA, ν, cm^{-1}): 3336, 3257 (NH); 3074–3059 (C_{ar}–H); 2921–2850 (C_{alk}–H); 1593, 1489, 1420 (Pz); 1065, 1046, 969, 928 (tetrazine). High-resolution mass spectrometry, found *m/z* 335.1728 [M + H]$^+$. Calculated for $C_{17}H_{19}N_8^+$: 335.1727 [M + H]$^+$ (Supplementary S1.4).

2.1.5. 3-(3,5-dimethylpyrazol-1-yl)-6-propargyloxy-1,2,4,5-tetrazine 2f

The 0.270 g (1.0 mmol) of 3,6-bis(3,5-dimethylpyrazol-1-yl)-1,2,4,5-tetrazine and 0.15 ml (3.0 mmol) of propargyl alcohol in 5 ml of toluene were refluxed for 0.5 hr until starting tetrazine disappeared (TLC control). The solvent was removed and the residue was dissolved in acetonitrile and concentrated. The product was purified by flash-chromatography on Silica (5/40, acetonitrile-benzene=1:1), R_f 0.8. Yield 90 mg, 33%, m. p. 57–59 °C. Calculated (%): C 52.17, H 4.38, N 36.51; $C_{10}H_{10}N_6O$. Found (%): C 52.05, H 4.38, N 36.61. ^{1}H NMR ($CDCl_3$) δ, ppm: 6.17 (*s*, 1 H, 4-CH, Pz), 5.31 (*s*, 2 H, –OCH_2–), 2.67 and 2.38 (both *s*, 6 H, 2CH_3, Pz), 2.60 (*s*, 1 H, CH) (Supplementary S1.5). IR (DRA, ν, cm^{-1}) 2920–2997 (C_{alk}–H); 3259, 2126 (CH); 1452, 751 (C=N); 1256, 1079 (C–O–C); 945, 921 (tetrazine). High-resolution mass spectrometry, Found: *m/z* 231.0989 [M + H]$^+$. Calculated for $C_{10}H_{11}N_6O^+$: 231.0989 [M + H]$^+$ (Supplementary S1.5).

2.1.6. *N′*-(6-(3,5-dimethylpyrazol-1-yl)-1,2,4,5-tetrazin-3-yl)hydrazide of 4-pyridinecarboxylic acid 2l

Yield 156 mg, 50%, m.p. 122–123 °C. Calculated (%): C 50.16, H 4.21, N 40.49; $C_{13}H_{13}N_9O$. Found (%): C 50.74, H 6.15, N 43.08. ^{1}H NMR (DMSO-d_6) δ, ppm: 2.23 (*s*, 3 H, 3-CH_3, Pz), 2.45 (*s*, 3 H, 5-CH_3, Pz), 6.23 (*s*, 1 H, 4-CH, Pz), 7.85 (*d*, 2 H, Py, *J* = 4.6 Hz), 8.82 (*d*, 2 H, Py, *J* = 4.0 Hz), 10.87, 11.28 (*br. s*, 2 H, 2 NH). ^{13}C NMR (DMSO-d_6) δ, ppm: 164.6, 162.4, 158.3, 150.8, 150.6, 141.8, 139.0, 121.4, 109.2, 13.3, 12.6 (Supplementary S1.6). IR (DRA, ν, cm^{-1}): 3203 (NH); 3039-2991 (C_{ar}–H); 1696 (C=O); 1484, 1426 (Pz); 1064, 1043, 956 (tetrazine); 1576 (Py). High-resolution mass spectrometry, Found: *m/z* 312.1318 [M + H]$^+$. Calculated for $C_{13}H_{14}N_9O^+$: 312.1316 [M + H]$^+$ (Supplementary S1.6).

2.1.7. 6-N-(1-Amino-4-hydroxybutyl)-3-(imidazol-1-yl)-1,2,4,5-tetrazine 3a

A mixture of 214 mg (1 mmol) of tetrazine **1b** and 0.1 ml of 1-aminobutanol-4 in 5 ml of acetonitrile in 5 minutes results in a formation of the product **3a**. Yield 172 mg, 73%; m.p. 157–158 °C. Calculated (%): C 45.95, H 5.57, N 41.60; $C_9H_{13}N_7O$. Found (%): C 46.07, H 5.72, N 41.70. ^{1}H NMR (DMSO-d_6) δ, ppm: 8.80 (*t*, 1 H, OH, *J* = 5.7 Hz), 8.52 and 7.93 and 7.22 (all *s*, 3 H, imidazole), 4.44 (*t*, 1 H, NH, *J* = 5.0 Hz), 1.70-1.63 and 1.55-1.48 (both *m*, 4 H, –NH–$(CH_2)_2$–CH_2CH_2OH), 3.47–3.42 (*m*, 4 H, –NH–$(CH_2)_2$-CH_2CH_2OH). ^{13}C NMR (DMSO-d_6) δ, ppm: 162.4 153.8, 134.5, 130.4, 116.0, 60.4, 40.6, 29.8, 24.9 (SupplementaryS1.7). IR (DRA, ν, cm^{-1}): 3139 (OH); 3251 (NH); 2938–2858 (C_{alk}–H); 1571 (C=C, C=N_{imid}); 1494, 962, 767 (C=N, C=C). High-resolution mass spectrometry, Found: *m/z* 236.1251 [M + H]$^+$. Calculated for $C_9H_{14}N_7O^+$: 236.1254 [M + H]$^+$ (Supplementary S1.7).

2.1.8. 1-(6-Mophoilin-4-yl-[1,2,4,5]-tetrazin-3-yl)-1H-benzotriazole 5

To a suspension of 144 mg (0.46 mmol) of tetrazine **1d** in 6 ml of acetonitrile a 5-fold excess of morpholine was dropwise added (0.5 ml). The reaction mixture was heated during for 1 hr. and a solution was formed. After cooling the precipitate was filtered off and recrystallized from acetonitrile. Yield 74 mg, 59%; m.p. 232–234 °C; R_f = 0.77 (CH_3CN:C_6H_6; 1:1). Calculated (%): C 50.70, H 4.25, N 39.42; $C_{12}H_{12}N_8O$. Found (%): C 50.63 H 4.15 N 39.59. 1H NMR ($CDCl_3$) δ, ppm: 8.33 and 8.22 (both *d*, 2 H, H-C(4), H-C(7), Ph, *J* = 10 and *J* = 10 Hz), 7.67 and 7.54 (both *t*, 2 H, H-C(5) and H-C(6), Ph, *J* = 9.3 and *J* = 9.3 Hz), 4.12 (*t*, 4 H, –CH_2–O–CH_2–, *J* = 5 Hz), 3.94–3.91 (*m*, 4 H, –CH_2–N–CH_2–). ^{13}C NMR ($CDCl_3$) δ, ppm: 161.2, 156.0, 146.5, 131.2, 129.6, 125.5, 120.5, 113.1, 66.4, 44.1 (Supplementary S1.8). IR (DRA, ν, cm^{-1}) 3096–3067 (C_{ar}–H); 2926–2873 (C_{alk}–H); 1702 (NH); 1541 (C=C, C=N_{pyr}); 1493, 978, 758 (C=N, C=C). High-resolution mass spectrometry, Found: *m/z* 285.1205 [M + H]$^+$. Calculated for $C_{12}H_{12}N_8O^+$: 285.1207 [M + H]$^+$ (Supplementary S1.8).

2.1.9. 3-(5-Bromoindol-1-yl)-6-N-(heptylamino)-1,2,4,5-tetrazine 6a

Yield 290 mg, 75%; m.p. 147–149 °C. Calculated (%): C 52.45, H 5.44, N 21.59; $C_{17}H_{201}N_6Br$. Found (%): C 52.44, H 5.62, N 21.47. ^{1}H NMR ($CDCl_3$) δ, ppm: 8.42 (d, 1 H, indole, *J* = 8.9 Hz), 8.19 (*d*, 1 H, indole, *J* = 3.6 Hz), 7.78 (*d*, 1 H, indole, *J* = 1.9 Hz), 7.43 (*dd*, 1 H, indole, J_1 = 8.9, J_2 =1.9 Hz), 6.72 (*d*, 1 H, indole, *J* = 3.3 Hz), 5.70 (*t*, 1 H, –NH–, *J* = 5.5 Hz), 3.63 (*q*, 2 H, NH–CH_2–, *J* = 7.0, 13.0 Hz), 1.72–1.78 (*m*, 2 H, –CH_2CH_3), 1.30–1.46 (*m*, 8H, –$(CH_2)_4$–), 0.90 (*t*, 3 H, –CH_2CH_3, *J* = 6.9 Hz). ^{13}C NMR ($CDCl_3$) δ, ppm: 161.4, 157.3, 133.2, 132.5, 126.7, 125.4, 123.7, 116.7, 115.7, 106.9, 41.8, 31.7, 29.2, 28.9, 26.8, 22.6, 14.0 (Supplementary S1.9). IR (DRA, ν, cm^{-1}): 3246, 1572 (NH), 2931–2962 (C_{alk}–H); 1047, 951 (tetrazine), 1318 (C–N). High-resolution mass spectrometry, Found: *m/z* 389.1083 [M + H]$^+$. Calculated for $C_{17}H_{22}BrN_6^+$: 389.1084 [M + H]$^+$ (Supplementary S1.9).

2.1.10. 3,6-Di-propargyloxy-1,2,4,5-tetrazine 6b

The 214 mg (1 mmol) of 3,6-di-(imidazol-1-yl)-1,2,4,5-tetrazine was suspended in 3 ml of propargyl alcohol and heated for 3–5 min until starting material dissolved. The reaction stayed for 3 h until the starting tetrazine disappeared. The main product (R_f = 0.8) was collected by flash-chromatography (acetonitrile-benzene=1:1). Yield 125 mg, 66%; m.p. 99.5–102 °C. Calculated (%): C 50.53, H 3.18, N 29.47; $C_8H_6N_4O_2$. Found (%): C 50.47, H 3.18, N 29.26. ^{1}H NMR ($CDCl_3$) δ, ppm: 5.23 (*d*, 4 H, $CHCCH_2O$–, *J* = 2.0 Hz), 2.59

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(*s*, 2 H, $CHCH_2CO$–). ^{13}C NMR ($CDCl_3$) δ, ppm: 165.5, 76.6, 76.4, 57.0 (Supplementary S1.10). IR (DRA, ν, cm^{-1}) 2925–2963 (C_{alk}–H); 3259, 2126 (CH); 1454, 751 (C=N); 1255, 1079 (C–O–C); 945, 922 (tetrazine). High-resolution mass spectrometry, Found: m/z 191.0564 $[M + H]^+$. Calculated for $C_8H_7N_4O_2^+$: 191.0564 $[M + H]^+$ (Supplementary S1.10).

2.1.11. 6-(1-Amino-4-hydroxybutyl)-3-(5-chloropyridin-2-ylamino)-1,2,4,5-tetrazine 6c

Yield 236 mg, 80%; m.p. 221–222 °C. Calculated (%): C 44.67, H 4.74, N 33.16; $C_{11}H_{14}ClN_7O$. Found (%): C 44.50, H 4.77, N 33.25. ^1H NMR (DMSO-d_6) δ, ppm: 10.53 (*br.s*, 1 H, NH), 8.29 (*d*, 1 H, Py, *J* = 2.9 Hz), 8.06 (*t*, 1 H, –NH–, *J* = 5.6 Hz), 7.78–7.84 (*m*, 2 H, Py), 4.42 (*t*, 1 H, OH, *J* = 4.9 Hz), 3.37–3.39 and 3.42–3.45, and 1.60–1.65, and 1.48–1.54 (all *m*, 8 H, $(CH_2)_4$). ^{13}C NMR (DMSO-d_6) δ, ppm: 160.5, 157.7, 151.7, 146.1, 137.6, 122.6, 112.4, 60.4, 40.6, 29.9, 25.2 (Supplementary S1.11). IR (DRA, ν, cm^{-1}): 3249 (NH), 3053–3007 (C_{ar}–H); 2931–2870 (C_{alk}–H); 1702 (NH); 1574 (C=C, $C=N_{pyr}$); 1467, 960, 781 (C=N, C=C). High-resolution mass spectrometry, Found: *m/z* 296.1021 $[M + H]^+$. Calculated for $C_{11}H_{15}ClN_7O^+$: 296.1021 $[M + H]^+$ (Supplementary S1.11).

2.1.12. N-[6-(3,5-Dimethylpyrazol-1-yl)-1,2,4,5-tetrazin-3-yl]-N'-(3,4,5-trimethoxy-benzyliden)-hydrazine 7e

The product was obtained from 1.0 mmol of 3-hydrazino-6-(3,5-dimethylpyrazol-1-yl)-1,2,4,5-tetrazine **2a** and 1.0 mmol of 3,4,5-trimethoxybenzaldehyde according to known method [20]. Yield 366 mg, 95.5%; m.p. 190 °C. Calculated (%): C 53.12, H 5.24, N 29.15; $C_{17}H_{20}N_8O_3$. Found (%): C 52.85, H 5.11, N 28.92. ^1H NMR (DMSO-d_6) δ, ppm: 12.60 (*s*, 1 H, NH), 8.28 (*s*, 1 H, CH=N), 7.08 (*s*, 2 H, Ar), 6.23 (*s*, 1 H, 4Pz-CH), 3.86 (*s*, 6 H, 2*m*-OCH_3), 3.72 (*s*, 3 H, p-OCH_3), 2.47 (*s*, 3 H, 5Pz-CH_3), 2.25 (*s*, 3 H, 3Pz-CH3). ^{13}C NMR (DMSO-d_6) δ, ppm: 160.1, 157.8, 153.2, 150.5, 146.4, 141.6, 139.2, 129.6, 109.0, 104.1, 60.1, 55.9, 13.3, 12.5 (Supplementary S1.12). IR (DRA, ν, cm^{-1}): 3461 (NH); 3058 (C_{ar}–H); 2835, 1447 (Ar-OCH_3); 1615 (N=C); 1586 (N=N); 1329 (C–N); 1072, 1044, 949 (tetrazine). High-resolution mass spectrometry, Found: *m/z* 385.1729 $[M + H]^+$. Calculated for $C_{17}H_{21}N_8O_3^+$: 385.1731 $[M + H]^+$ (Supplementary S1.12).

2.2. General procedure of preparation of Podands with amino-tetrazine fragments 2m,n

To a solution of 1 mmol of a corresponding aliphatic amine in 20 mL of acetonitrile 2.5 mmol of 3,6-bis-(3,5-dimethylpyrazol-1-yl)-1,2,4,5-tetrazine was added. The reaction was stirred for 1.0–1.5 h at 45–50 °C. After cooling the mixture was stayed for 10 h, and the precipitate formed was filtered off and recrystallized from ethanol. The compound **2n** was purified using flash chromatography using ethyl acetate as an eluent.

2.2.1. 1,5-Bis-(3,5-dimethylpyrazol-1-yl-sim-tetrazinylamino)-3-oxapentane 2m

Yield 420 mg, 93%, m.p. 160–162 °C. Calculated (%): C 47.78, H 5.35, N 43.34; $C_{18}H_{24}N_{14}O$. Found (%): C 47.79, H 5.30, N 43.30. ^1H NMR (DMSO-d_6) δ, ppm: 8.85 (*br.s*, 2 H, NH), 6.18 (*s*, 2 H, H_{Pyr}), 3.71–3.74 and 3.64–3.68 (both *m*, 8 H, NH–CH_2–CH_2), 2.38 (*s*, 6 H, CH_3), 2.21 (*s*, 6 H, CH_3). ^{13}C NMR (DMSO-d_6) δ, ppm: 161.32, 156.75, 149.93 141.26, 108.36, 68.02, 40.60, 13.27, 12.14 (Supplementary S1.13). IR (DRA, ν, cm^{-1}) 3256 (NH), 3145–3065 (C_{ar}–H); 2925–2810 (C_{alk}–H); 1702 (NH); 1594, 1568 (C=C, C=N); 1487, 969, 781 (C=N, C=C); 1125, 1022 (C_{ar} –O–C_{alk}). High-resolution mass spectrometry, found: m/z 453.2334 $[M + H]^+$. Calculated for $C_{18}H_{25}N_{14}O^+$: 453.2330 $[M + H]^+$ (Supplementary S1.13).

2.2.2. 1,8-Bis-(3,5-dimethylpyrazol-1-yl-sim-tetrazinylamino)-3,6-dioxaoctane 2n

Yield 422 mg, 85%, oil. Calculated (%): C 48.38, H 5.68, N 39.49; $C_{20}H_{28}N_{14}O_2$. Found (%): C 48.56, H 5.38, N 39.62. ^1H NMR (DMSO-d_6) δ, ppm: 8.81 (*t*, 2 H, *J* = 5.3 Hz, NH), 6.17 (*s*, 2 H, H_{Pyr}), 3.62–3.67 (*m*, 8 H, NH–CH_2–CH_2), 3.59 (*s*, 2 H, O–CH_2), 2.38 (*s*, 6 H, CH_3), 2.21 (*s*, 6 H, CH_3). ^{13}C NMR (DMSO-d_6) δ, ppm: 161.33, 156.74, 149.95, 141.26, 108.37, 69.62, 68.13, 40.52, 13.27, 12.14 (Supplementary S1.14). IR (DRA, ν, cm^{-1}) 3270 (NH), 3056 (C_{ar}-H); 2925–2871 (C_{alk}–H); 1702 (NH); 1571 (C=C, $C=N_{pyr}$); 1487, 969, 756 (C=N, C=C); 1125, 1042 (C_{ar}–O–C_{alk}). High-resolution mass spectrometry, found: *m/z* 497.2597 $[M + H]^+$. Calculated for $C_{20}H_{29}N_{14}O_2^+$: 497.2592 $[M + H]^+$ (Supplementary S1.14).

2.3. General procedure of preparation of Podands with hydrazone fragments 9a–c

To a solution of 1 mmol of formyl-podand in 25 ml of acetonitrile 2.2 mmol of a substituted hydrazine **2a** was added followed by addition of 2 drops of a conc. acetic acid. Reaction was brought to a boil, then left to cool with a constant stirring for 3 h and without stirring for 10 hours till the reaction ended. The formed precipitate was filtered off and recrystallized from DMF-ethanol mixture for the compounds **9a**,**b** or from ethanol for the compound **9c**.

2.3.1. 1,2-Bis-[2-(3,5-dimethylpyrazol-1-yl-sim-tetrazinylhydrazonomethylphenoxy)]-ethane 9a

Yield 623 mg, 90%, m.p. 220–221 °C. Calculated (%): C 55.48, H 5.24, N 32.35; $C_{30}H_{30}N_{16}O_2 \cdot C_2H_5OH$. Found (%): C 55.50, H 4.99, N 32.56. ^1H NMR (DMSO-d_6) δ, ppm: 12.5 (*br.s*, 2 H, NH), 8.77 (*s*, 2 H, N=CH), 7.96 (*dd*, 2 H, *J* = 7.8, 1.7 Hz, H_6(Ph)), 7.44–7.49 (*m*, 2 H, H_4(Ph)), 7.24 (*d*, 2 H, *J* = 7.8 Hz, H_3(Ph)), 7.08 (*t*, 2H, *J* = 7.8 Hz, H_5(Ph)), 6.2 (*s*, 2 H, H_{Pyr}), 4.52 (*s*, 4 H, CH_2), 2.42 (*s*, 6 H, CH_3), 2.22 (*s*, 6 H, CH_3). ^{13}C NMR (DMSO-d_6) δ, ppm: 160.01, 157.74, 156.82, 150.43, 142.17, 141.59, 131.45, 125.57, 122.69, 121.18, 113.06, 108.95, 67.25, 13.29, 12.49 (Supplementary S1.15). IR (DRA, ν, cm^{-1}) 3191 (NH), 3077–3033 (C_{ar}–H); 2927–2857 (C_{alk}–H); 1600 (C=C); 1563, 843 (C=C, C=N); 1480, 952, 796

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(C=N, C=C); 1244, 1041 (C_{ar}–O–C_{alk}). High-resolution mass spectrometry, found: *m/z* 647.2809 $[M + H]^+$. Calculated for $C_{30}H_{31}N_{16}O_2^+$: 647.2810 $[M + H]^+$ (Supplementary S1.15).

2.3.2. 1,5-Bis-[2-(3,5-dimethylpyrazol-1-yl-sim-tetrazinylhydrazonomethylphenoxy)]-3-oxapentane 9b

Yield 560 mg, 81%, m.p. 221–222 °C. Calculated (%): C 55.64, H 4.96, N 32.45; $C_{32}H_{34}N_{16}O_3$. Found (%): C 55.67, H 4.93, N 32.27. ^{1}H NMR (DMSO-*d*$_6$) δ, ppm: 12.57 (*br.s*, 2 H, NH), 8.74 (*s*, 2 H, N=CH), 7.92 (*dd*, 2 H, *J* = 7.6, 1.6 Hz, H_6(Ph)), 7.41–7.37 (*m*, 2 H, H_4(Ph)), 7.15 (*d*, 2 H, *J* = 7.8 Hz, H_3(Ph)), 7.03 (*t*, 2 H, *J* = 7.6 Hz, H_5(Ph)), 6.2 (*s*, 2 H, H_{Pyr}), 4.29 and 3.97 (*m*, 8 H, CH_2), 2.45 (*s*, 6 H, CH_3), 2.23 (*s*, 6 H, CH_3). ^{13}C NMR (DMSO-*d*$_6$) δ, ppm: 160.08, 157.80, 156.86, 150.48, 142.20, 141.63, 131.39, 125.51, 122.57, 120.99, 113.12, 108.98, 69.36, 68.27, 13.32, 12.51 (Supplementary S1.16). IR (DRA, ν, cm^{-1}) 3178 (NH), 3077–3033 (C_{ar}–H); 2950–2868 (C_{alk}–H); 1599 (C=C); 1572, 845 (C=C, C=N_{pyr}); 1471, 965, 757 (C=N, C=C); 1244, 1142, 1065 (C_{ar}–O–C_{alk}). High-resolution mass spectrometry, found: *m/z* 691.3078 $[M + H]^+$. Calculated for $C_{32}H_{35}N_{16}O_3^+$: 691.3073 $[M + H]^+$ (Supplementary S1.16).

2.3.3. 1,8-Bis-[2-(3,5-dimethylpyrazol-1-yl-sim-tetrazinylhydrazonomethylphenoxy)]-3,6-dioxaoctane 9c

Yield 609 mg, 78%, m.p. 184–186 °C. Calculated (%): C 55.37, H 5.68, N 28.70; $C_{34}H_{38}N_{16}O_4{\cdot}C_2H_5OH$. Found (%): C 55.67, H 5.67, N 28.82. ^{1}H NMR (DMSO-*d*$_6$) δ, ppm: 12.57 (*br.s*, 2 H, NH), 8.72 (*s*, 2 H, N=CH), 7.91 (*dd*, 2 H, *J* = 7.6, 1.6 Hz, H_6(Ph)), 7.36–7.39 (*m*, 2 H, H_4(Ph)), 7.10 (*d*, 2 H, *J* = 8.0 Hz, H_3(Ph)), 7.02 (*t*, 2 H, *J* = 7.6 Hz, H_5(Ph)), 6.23 (*s*, 2 H, H_{Pyr}), 4.21 and 3.84 (*m,* 8 H, CH_2), 3.71 (*s*, 4 H, CH_2), 2.45 (*s*, 6 H, CH_3), 2.24 (*s*, 6 H, CH_3). ^{13}C NMR (DMSO-*d*$_6$) δ, ppm: 160.08, 157.80, 156.84, 150.47, 142.19, 141.62, 131.36, 125.47, 122.52, 120.93, 113.04, 108.97, 70.18, 68.99, 68.05, 13.31, 12.51 (Supplementary S1.17). IR (DRA, ν, cm^{-1}) 3199 (NH), 3095–3038 (C_{ar}–H); 2950–2884 (C_{alk}–H); 1600 (C=C); 1568, 843 (C=C, C=N_{pyr}); 1477, 964, 746 (C=N, C=C); 1253, 1141, 1119, 1065 (C_{ar}–O–C_{alk}). High-resolution mass spectrometry, found: *m/z* 735.3333 $[M + H]^+$ (Supplementary-S1.17). Calculated for $C_{34}H_{39}N_{16}O_5^+$: 735.3335 $[M + H]^+$ (Supplementary S1.17).

2.4. Method for assessing tuberculostatic activity

The study of tuberculostatic activity was conducted using the vertical diffusion method with the laboratory strain *Mycobacterium tuberculosis* $H_{37}R_V$ in a dense nutrient medium "Novaya". The nutrient medium was poured into 5 ml tubes and tilted so that half of the bottom became free. The tilted medium in each tube was seeded with 0.1 ml of strain, diluted according to the standard turbidity 10 GKI units (Tarasevich State Research Institute for Standardization and Control of Biological Products), and placed in a tilted position in a thermostat for 24 h to allow mycobacteria to growth. In one day, the tubes were adjusted vertically, and then 0.3 ml of solution of studied substances in concentrations of 25.0, 12.5, 6.25, 3.12, 1.56, 0.75 μg/ml were added dropwise through the free wall of the vessel. The tubes were placed in a thermostat at 37 °C for 10 days of incubation. The estimation of the growth of mycobacteria tuberculosis was done with a standard method [21] where zones of growth retardation (over 10 mm) indicated the presence of tuberculostatic properties in the studied concentration of a substance. Each substance was tested in three parallel tubes at each concentration.

2.5. Acute toxicity study in mice

Acute toxicity, in accordance with the Guidelines for Experimental (Preclinical) Study of New Pharmacological Substances [21], was determined in outbred white mice (males) weighing 19.0±2.0 g, maintained on a standard diet and giving solutions of the test compounds in olive oil or purified water by gavage into the stomach (per os) under natural lighting conditions at room temperature. The animals were randomly assigned to experimental groups, with five selected animals in each group being observed for five days.

2.6. Study of membranotropic properties

A transport of cations of metals through the chloroform model membrane similar to publication [22] at 20±0.5 °C, at magnetic stirrer speed 100 rpm, during 6 hours and initial solutions concentrations of 10^{-4} mol/L both for ligand in chloroform and the metal picrate or amino acid in the first water phase was performed. A rate of metal cation transfer through the chloroform model membrane was monitored by measuring the picrate anion concentration in the second water phase using a spectrophotometer (Specord-UV-VIS, wave length 357 nm). A value for the metal cation transfer rate was calculated as an average as the arithmetic mean of the three measurements.

3. Results and Discussion

Continuing our studies in the search of novel tuberculostatic agents, we have obtained a wide range of 3,6-disubstituted *s*-tetrazines **2–6** (Scheme 1) using 3,6-bis-(azol-1-yl)-1,2,4,5-tetrazines **1a–d** as starting-compounds and a methodology of successive nucleophilic substitution of the azolyl groups in them with various *N*-, *O*- and *C*-nucleophiles. These include primary and secondary aliphatic and aromatic amines, acid hydrazides, propargyl alcohol, malononitrile and anhydro base of methylquinaldine, which form corresponding substitution products with yields ranging from 50 to 93%. Using polyoxyethylene diamines as nucleophiles the aminotetrazinyl podands **2m,n** were obtained in 85–90% yield. The compounds **2–6** are colored crystalline substances, readily soluble in chloroform, acetone, DMF, DMSO.

One of the compounds with low level tuberculostatic activity (MIC: 12.5 μg/ml), namely 6-(3,5-dimethylpyrazol-1-

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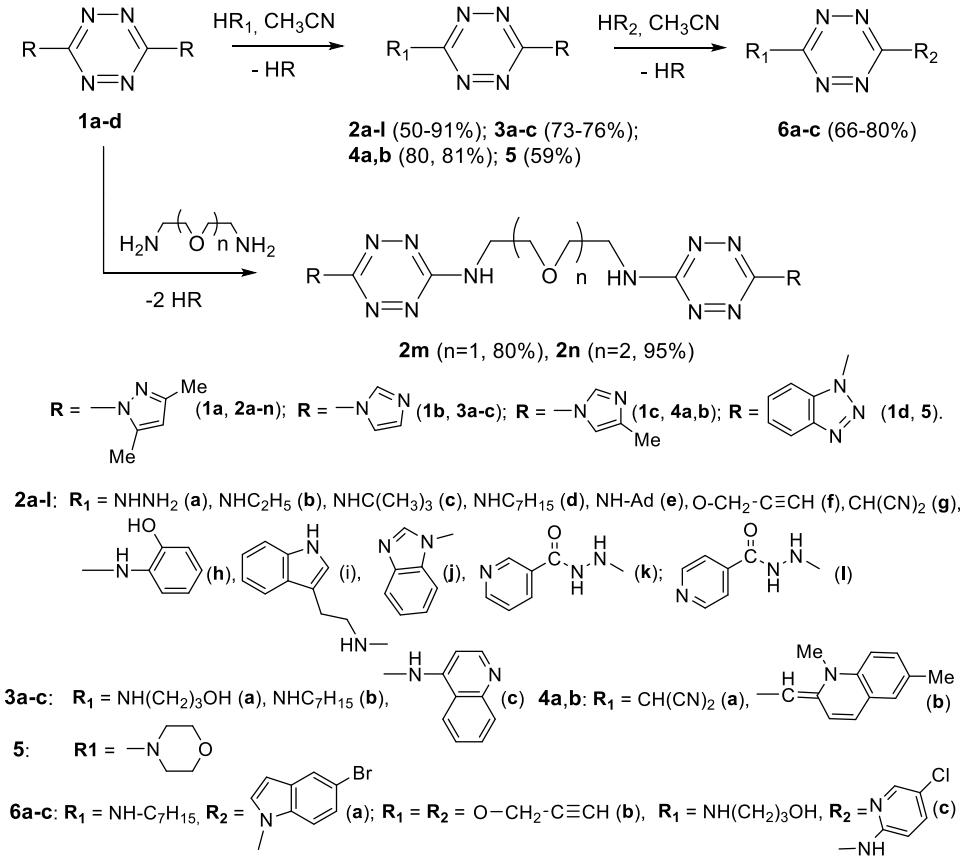
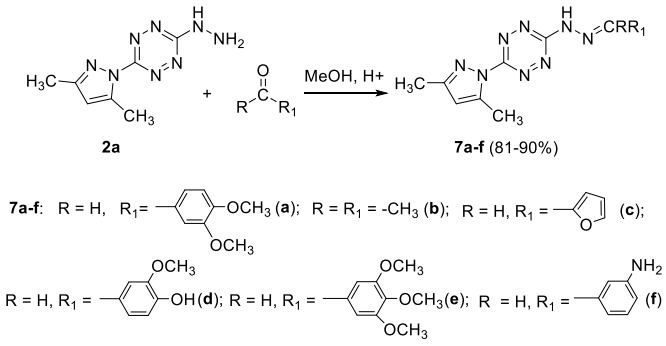
yl)-3-hydrazino-1,2,4.5-tetrazine (**2a**), was used in further modifications as a precursor for a series of hydrazones **7** (Scheme 2).

In similar reaction of formyl podands **8a–c** with hydrazinyl tetrazine **2a** corresponding tetrazinyl hydrazono-podands **9a–c** were obtained (Scheme 3).

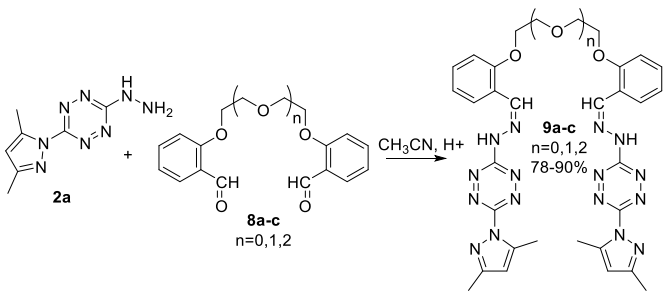
The starting substrates **1a,b,d** did not reveal themselves tuberculostatic activity, except for a slight activity of 3,6-bis-(3-methylimidazol-1-yl)-1,2,4,5-tetrazine (**1c**) with a MIC value of approximately 100 μg/ml.



Scheme 1 Synthesis of differently substituted 1,2,4,5-tetrazines.



Scheme 2 Synthesis of hydrazones **7**.



Scheme 3 Synthesis of podands **9a-c** with tetrazinyl hydrazone fragments.

Among the "mono" substituted series **2** with pyrazolyl group, the pronounced tuberculostatic activity was observed in only four compounds **2e**,**2i**,**2k**,**2l**, with moieties of adamantylamine, (indol-3-yl)ethylamine and hydrazides of nicotinic and iso-nicotinic acids with MIC values equal to 0.31, 3.12, 0.18, 0.75 μg/ml, respectively (Table 1). Furthermore, the activity of the nicotinic acid hydrazide derivative **2k** was four times higher than that of the isonicotinic acid hydrazide derivative **2l**, and comparable to that of a well-known drug for the treatment of tuberculosis, INH. The remaining compounds in this series, as well as the compounds **3–5,** exhibited low activity, varying within the MIC range of 12.5-100 μg/ml or more. As an exception, a tetrazine **3c** showed a MIC value of 0.75 μg/ml.

Table 1 Antituberculosis activity (strain $H_{37}R_v$) of compounds **2–9**.

Compound	MIC, μg/ml	Compound	MIC, μg/ml
2a	12.5	4a	50.0
2b	12.5	4b	12.5
2c	>100	5	>100
2d	100	6a	>100
2e	**0.31**	6b	>100
2f	>100	6c	25.0

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2g	12.5	**7a**	**0.18**
2h	100	**7b**	**0.31**
2i	**3.12**	**7c**	**1.5**
2j	25.0	**7d**	**0.75**
2k	**0.18**	7e	100
2l	**0.75**	7f	100
2m	12.5	**9a**	**0.15**
2n	12.5	**9b**	**0.31**
3a	50.0	**9c**	**0.1**
3b	>100	*isoniazid*	0.15
3c	**0.75**		

The largest number of effective tuberculostatics was identified among hydrazone-containing tetrazines prepared from aromatic aldehydes. The most active compound in this series was derivative **7a** with two methoxy groups in the benzene ring (MIC: 0.18 μg/ml). Replacement of one methoxy group by a hydroxyl fragment (compound **7d**) resulted in a four-fold decrease in activity (MIC: 0.75 μg/ml). However, a catastrophic decrease in activity (MIC: 100 μg/ml) occurred when the second methoxy group was lost (compound **7f**). Conversely, the incorporation of a third methoxy group in the compound **7e** gave the same result as in the case of **7f** (MIC: 100 μg/mL).

Among the tetrazinyl podands (Scheme 3), the compounds **9a–c** were found to be more active, with MIC values ranging from 0.1 to 0.31 μg/ml (Table 2). These compounds had the hydrazonic fragment together with the tetrazine moiety. The absence of phenyl moieties in tetrazinyl podands **2m,n** (Scheme 1) resulted in a sharp loss of tuberculostatic activity up to 12.5 μg/ml as well as a loss of ion transport properties towards Cu(II) (Figure 2). It is known that one of the factors determining the survival of mycobacteria is their redox homeostasis, which depends on the intracellular concentration of transition metal ions, especially the divalent copper cations [23]. Model compound **2b** was not able to transport copper cations due to the absence of a spacer, which facilitates the "wrapping" of the ligand molecule around the metal cation, and, unlike tetrazine podands, also did not transfer potassium cations.

It is noteworthy that the tuberculostatic activity of compounds **9** depends on the length of the oligooxyethylene spacer. Thus, the most active compound in *in vitro* experiments was podand **9c** (MIC: 0.1 μg/ml) with four oxygen atoms in the spacer. In contrast, the podand **9b** with three oxygen atoms in the oligooxyethylene moiety had slightly reduced activity (MIC: 0.31 μg/mL).

The acute toxicity of some of the synthesized compounds was investigated, and the most active substances **2e**, **9a–c** with lipophilic groups as well as the water soluble compound **2i** with remarkable tuberculostatic activity (MIC: 3.2 μg/ml) were selected. The lowest toxicity (LD_{50} >5000 mg/kg) in the investigated series was exhibited by N-(2-(1H-indol-3-yl)ethyl-6-(3,5-dimethylpyrazol-1-yl)-1,2,4,5-tetrazin-3-amine **2i**. Replacement of the indolyl substituent with an adamantyl moiety resulted in a two-fold increase in acute toxicity while maintaining a high level of tuberculostatic activity (MIC: 0.31 μg/ml). A dependence of acute toxicity on the length of the oligooxyethylene spacers was observed in a series of tetrazine-containing podands was found (Table 2). The lowest acute toxicity was observed for podand **9a** (1500 mg/kg) with two oxygen atoms in the oxyethylene spacer, whereas podand **9b** with three oxygen atoms in the oxyethylene spacer showed a two-fold increase in acute toxicity (750 mg/kg).

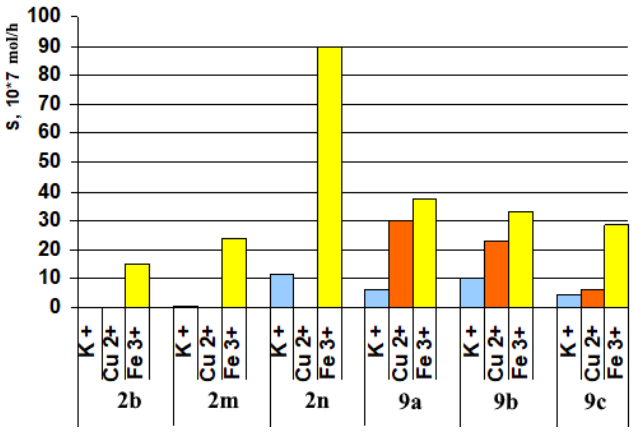

Figure 2 Transport properties of podands **2m**,**n** and **9a–c**.

Table 2 Acute toxicity and therapeutic indices of 3,6-disubstituted derivatives of 1,2,4,5-tetrazine.

Compound	LD_{50}[a], mg/kg (mice, per os)	Therapeutic index, TI (LD_{50}/MIC)
2e	>2500	>8064
2i	>5000	>1602
9a	1500	10000
9b	750	2419
9c	1250	12500
isoniazid	200	2000

[a]LD – lethal dose;

Consequently, 3,6-disubstituted derivatives of 1,2,4,5-tetrazine are significantly superior to the well-known drug isoniazid (150-200 mg/kg) in terms of acute toxicity (750–5000 mg/kg). Moreover, in terms of the therapeutic index, all the compounds tested, except **2i**, also surpass isoniazid; the best in this indicator is podand **9c** (TI = 12500).

4. Limitation

The strategy of stepwise nucleophilic substitution of easily leaving azole groups in the third and sixth positions of 1,2,4,5-tetrazine was used in the synthesis of a wide range of differently substituted tetrazines. However, the synthesis can be complicated by the formation of by-products, for example, through simultaneous substitution of both pyrazolyl groups by the reagent. Mild reaction conditions, selection of suitable dry solvents, and the use of solid carriers such as silicon or titanium oxides may be a solution to increasing the target product yields.

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5. Conclusions

Thus, the new 3,6-disubstituted derivatives of 1,2,4,5-tetrazine were synthesized, among which substances with antimycobacterial activity (MIC = 0.10–0.75 μg/ml) comparable to that of isoniazid, a well-known drug for the treatment of tuberculosis, and with acute toxicity tens of times lower than that were identified. Therefore, substituted 1,2,4,5-tetrazines are a promising class of heterocyclic systems for the development of effective tuberculostatic drugs with low-toxicity.

• Supplementary materials

NMR ^{1}H, ^{13}C and HRMS data presented as supplementary PDF file.

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• Conflict of interest

The authors declare no conflict of interest or any potential (ethical, financial) interests.

• Compliance with ethical standards

This article contains descriptions of studies using animals as subjects. Protocol ¾ dated May 25, 2023 (commission of Ural Phthisiopulmonology Research Institute).

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