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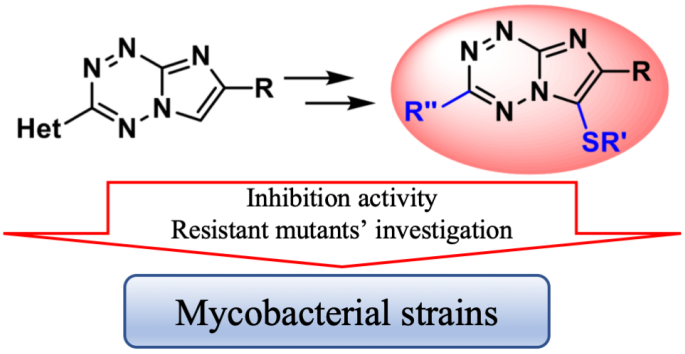

Synthesis and antimycobacterial activity of imidazo[1,2-*b*][1,2,4,5]tetrazines.

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Abstract.

Tuberculosis (TB) has recently become the leading killer among infectious diseases. Multidrug and extensively drug-resistant *Mycobacterium tuberculosis* strains urge the need to develop anti-TB drugs with a novel mechanism of action. We describe synthesis of 22 novel imidazo[1,2-*b*][1,2,4,5]tetrazine derivatives with different substituents at C(3) and C(6) positions, and their antimycobacterial activity *in vitro*. 8 compounds show activity as potential serine/threonine protein kinase (STPK) inhibitors in *M. smegmatis aphVIII+* test-system, which is characteristic for this class. 3 compounds out of 5 most active STPK inhibitors have a prominent minimal inhibitory concentration on *M. tuberculosis H37Rv* of 1 μg/ml. We were able to obtain *M. smegmatis mc2 155* mutants resistant to 4 compounds and show that they do not have cross resistance with other drugs, but have a common mechanism of resistance among these 4 imidazo[1,2-*b*][1,2,4,5]tetrazines. Compound 3h seems the most promising, combining a predicted STPK inhibitor activity, the lowest MIC on *M. tuberculosis* and a low frequency of drug resistant mutants' emergence.

Keywords: *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, imidazo[1,2-*b*][1,2,4,5]tetrazine, CH-functionalization, drug discovery, drug resistance, tuberculosis,.

1. Introduction

According to the World Health Organization, tuberculosis (TB) has recently become the deadliest among the infectious diseases, with about 10.4 million new TB cases and over 1.8 million deaths in 2016 [1]. The number of TB cases with multidrug resistance (MDR-TB, defined as TB resistant to rifampicin and isoniazid) is constantly growing, being a global threat for TB treatment and leading to the necessity of using more expensive and more toxic second-line drugs [1-3]. A custom case of MDR-TB is TB with extensive drug resistance (XDR-TB, defined as MDR-TB with resistance to the fluoroquinolones and second-line injectables), and recently cases of totally drug-resistance TB were also reported [4,5]. The emergence of *Mycobacterium tuberculosis* strains (the causative agent of TB) with MDR, XDR and TDR leads to an urgent need for novel anti-TB drugs with a novel mechanism of action.

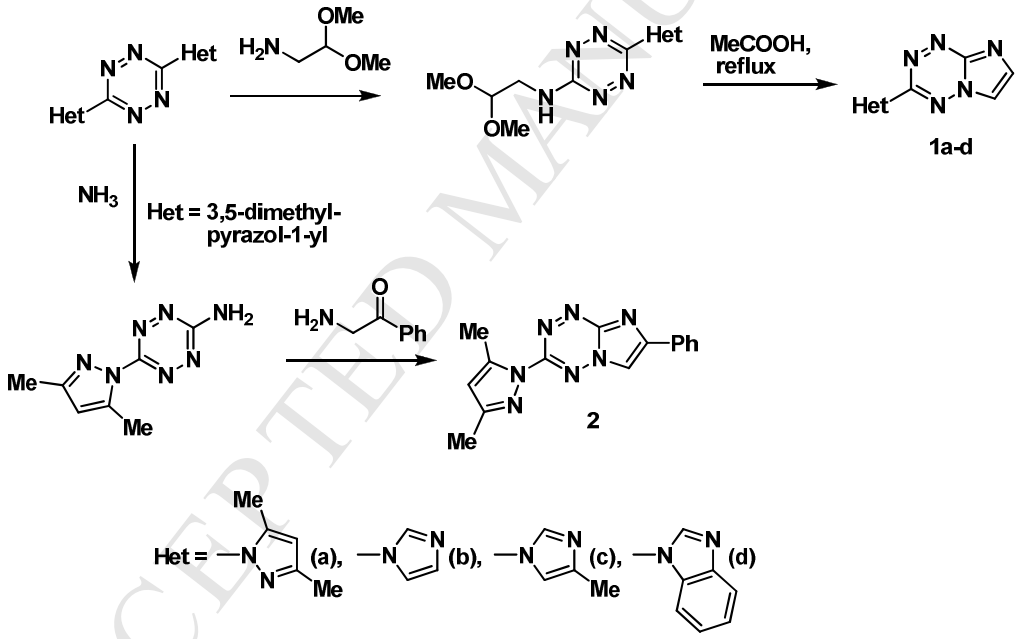
A promising way of search for new drugs, including anti-tuberculosis drugs, is the synthesis and bioscreening of polynitrogen heterocycles, in particular tetrazine derivatives. These compounds have an acceptor character and a large number of heteroatoms in the structure, which provides their high affinity to biotargets due to the possibility of valent and non-valent binding to electron-donor protein groups. Azole-annulated tetrazines are of particular interest for the study of biological activity, since they are polynitrogen-containing purine analogues. There are a number of publications showing the different biological activities of 3,6-disubstituted and azole-annulated 1,2,4,5-tetrazines. For example, antimicrobial activity of 1,4-dihydroimidazo[1,2-*b*][1,2,4,5]tetrazines [6], antibacterial and fungistatic action of aminesubstituted 1,2,4,5-tetrazines [7] and thiazolo[3,2-*b*][1,2,4,5]tetrazines [8], as well as antitumor activity of triazolo[4,3-*b*][1,2,4,5]tetrazines [9] and imidazo[1,2-*b*][1,2,4,5]tetrazines [10]. Important biomedical application of 1,2,4,5-tetrazines is bioorthogonal reactions of the [4+2] cycloaddition of 1,2,4,5-tetrazines and various dienophiles (inverse electron demand Diels–Alder reaction), for use in applications from protein labelling to cancer imaging or materials science [11,12]. Previously, tuberculostatic activity of imidazo[1,2-*b*]- and [1,2,4]triazolo[4,3-*b*]-[1,2,4,5]tetrazines [13], as well as 3-(3,5-dimethyl-1*H*-pyrazole-1-yl)-1,2,4,5-tetrazines containing in position C(6) fragments of amines [14,15], hydrazones [16] or amino acid esters [17] was detected and described. These examples show the expediency of finding new anti-tuberculosis drugs in a number of derivatives of 1,2,4,5-tetrazine, in particular azolo[1,2,4,5]tetrazines.

We have previously found that substituted imidazo[1,2-*b*][1,2,4,5]tetrazines bearing alkylthiol fragments at C(6) position of heterocyclic system can show the activity as serine/threonine protein kinase (STPKs) inhibitors in the *M. smegmatis aphVIII+* test-system [13].

This paper describes the synthesis of new imidazo[1,2-*b*][1,2,4,5]tetrazine derivatives with different substituents at C(3) and C(6) positions, screening of their activity as serine/threonine protein kinase (STPKs) inhibitors in the *M. smegmatis aphVIII+* test-system and investigation of antimycobacterial activity of leading compounds on *M. smegmatis* and *M. tuberculosis*, obtaining drug-resistant *M. smegmatis* mutants and their phenotypic characterization.

2. Chemistry

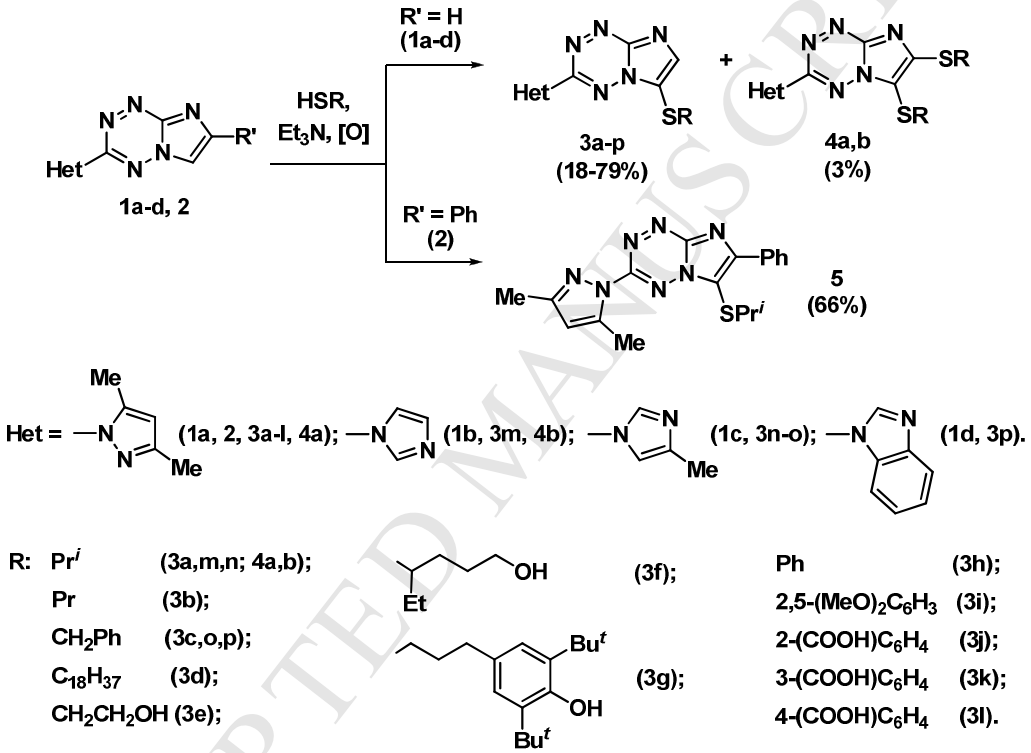
Synthesis of 3-(azol-1-yl)imidazotetrazines **1a-d** was carried out by the previously described method [18] by cyclocondensation of the corresponding 1,2,4,5-tetrazines containing 2,2-dimethoxyethylamine fragment obtained from the available 3,6-di(azol-1-yl)-1,2,4,5-tetrazines. To further evaluate the substituent's in C(7) position influence on antimycobacterial activity of imidazotetrazines we also synthesized 7-phenylimidazotetrazine **2** [19] by cyclization of 3-amino-6-(3,5-dimethylpyrazol-1-yl)-1,2,4,5-tetrazine with aminoacetophenone by boiling the reactants in ethanol (Scheme 1).



Scheme 1. Synthesis of 3-substituted and 3,7-disubstituted imidazo[1,2-*b*][1,2,4,5]tetrazines

Compounds **1a-d** and **2** were used as starting substances for modification of imidazotetrazine system in reactions with a wide range of S-nucleophiles (Scheme 2). Previously [20] in several examples, we have shown that S-nucleophile, in contrast to alcohols and amines, do not replace the leaving group in tetrazine cycle of azolotetrazines. Herewith in imidazo[1,2-*b*][1,2,4,5]tetrazines instead of the expected nucleophilic attack on the electrophilic atom C(3), an unusual replacement of the hydrogen atom in the annelated imidazole cycle is realized. In this paper, we show that the variation of the azolyl substituent in the tetrazine cycle, as well as the introduction of the aryl fragment to the C(7) position, has no significant effect on the direction

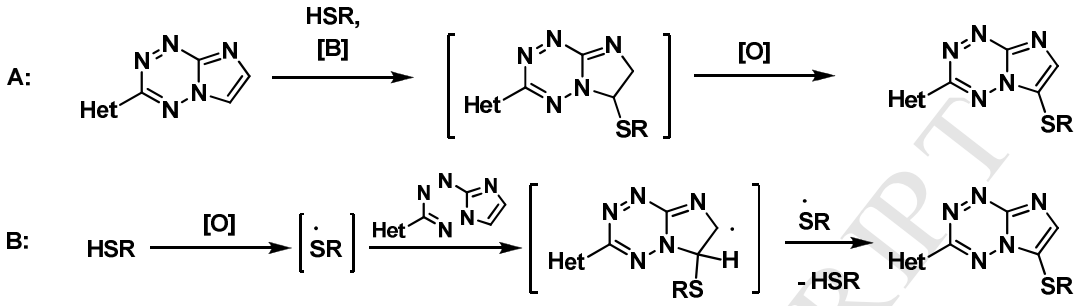
and yield of substitution reactions with mercaptans. The highest yields of 6-substituted imidazotetrazines **3** and **5** (50-75%) were achieved using alkylmercaptans, while in the reactions with thiophenol derivatives the yields of substitution products were reduced to 15-35%. In addition, it was observed that the small amounts of disubstituted derivatives **4** are formed along with the desired products **3** in the reactions of imidazotetrazines **1a-d** with alkylthiols. The structure of compounds **4** was confirmed by the ^{1}H NMR and the liquid chromatography mass spectrometry (LCMS) using products **4a,b**, which were isolated by column chromatography. In reactions with aromatic mercapto derivatives, the formation of disubstitution products was not observed.



Scheme 2. Synthesis of imidazo[1,2-*b*][1,2,4,5]tetrazines bearing alkylthiol fragments at C(6) position

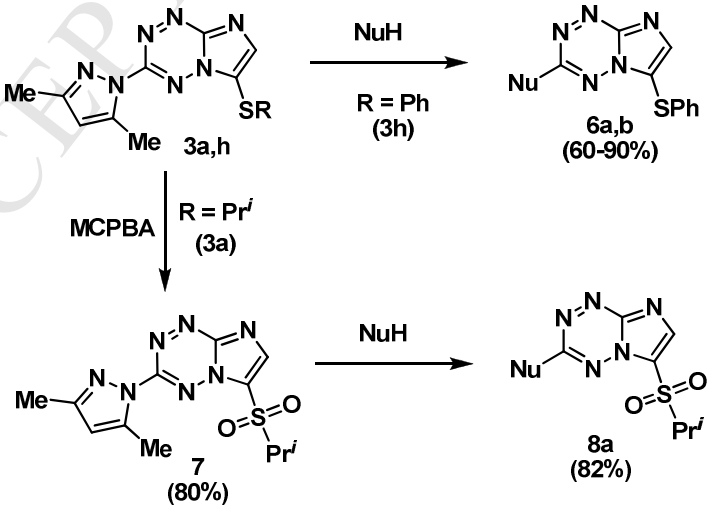
The interaction of imidazotetrazines with mercaptans was realized only in the presence of a base, as which triethylamine was used in this work. The use of K_2CO_3 also catalyzed these reactions, however, the yield of the target compounds was substantially reduced. When the reaction was carried out in an argon atmosphere without oxygen access, the substitution products also formed with a substantially lower yield. We can suppose two ways of passing the reaction of imidazotetrazines with mercaptans (Scheme 3). Route **A** includes the nucleophilic addition of a thiol to the C(6) atom in the imidazole ring, activated by the high-acceptor tetrazine core, and subsequent rapid oxidation of the formed adduct. It should be noted that the adduct was not possible to isolate or experimentally detect. Route **B** assumes a radical mechanism of the process

where the thiol radical produced by oxidation process replaces the hydrogen atom at C (6) in imidazotetrazine. In the case of both the **A** and **B** pathways, the acceptor molecules of the starting azolotetrazine may act as the oxidant and can be reduced to anion radicals or dihydro derivatives, which then oxidized back under the action of atmospheric oxygen.



Scheme 3. Possible ways of formation of 6-alkylthioimidazo[1,2-*b*][1,2,4,5]tetrazines

Using the example of the interaction of compound **3h** with heptylamine and ammonia (Scheme 4) the possibility of modification of 6-alkylthioimidazotetetrazines at the C (3) position with amine fragments has been shown. The corresponding nucleophilic substitution products of the 3,5-dimethylpyrazolyl group **6a,b** were formed under mild conditions with good yields. In addition, to further evaluation of the biological effect and the studying of reactivity of the 6-alkylthioimidazotetrazines modification products, the alkylthiol fragment in the compound **3a** was oxidized to the sulfonic group by 3-chloroperbenzoic acid. By the example of the reaction with heptylamine, it has been shown that the leaving group in the tetrazine ring of the sulfone **7** is easily substituted by the action of amines (Scheme 4).



Scheme 4. Modification of 6-alkylthioimidazo[1,2-*b*][1,2,4,5]tetrazines

3. Results and discussion

Mycobacterial serine-threonine protein kinases inhibition *in vitro* assay

The compounds **1b** and **3a** have been previously shown to be able to bind to the *M. tuberculosis* PknB adenine-binding socket by docking studies [13]. All synthesized imidazo[1,2-*b*][1,2,4,5]tetrazine derivatives were tested for the ability to inhibit mycobacterial eukaryotic type serine-threonine protein kinases (STPK). We used the *M. smegmatis aphVIII+* test-system, that was previously validated for mycobacterial STPK inhibitors screening, including those of *M. tuberculosis* [21-23]. This test-system implies the paper-disk method: the test-compound in subinhibitory concentration is applied on a paper-disk in combination with kanamycin and the diameter of the growth inhibition halo is compared to the one produced by kanamycin alone. Those compounds, that produce a larger growth inhibition halo in combination with kanamycin than kanamycin alone, are considered active STPK inhibitors. The results are presented in Table 1.

Table 1. Study of protein kinase inhibition activity of imidazo[1,2-*b*][1,2,4,5]tetrazines

Compound	*M. smegmatis* APHVIII+	
	Growth inhibition halo diameter, mm substance + kanamycin*	Subinhibitory concentration, nmol/disk
1b	12.8±0.4	50
3a	13.3±1.1	50
3c	13.7±0.5	100
3f	9.3±0.7	5
3h	11.5±0.7	5
3i	9.3±0.7	100
3m	13.8±0.4	25
3n	13.0±0.4	50
5	11.0±0.5	5
6a	12.0±1.0	50

*Growth inhibition halo produced by kanamycin alone (350 μg/disk) was 9.3±0.7 mm.

The study of biological activity of synthesized compounds in the *M. smegmatis aphVIII+* test-system has shown, that STPK inhibition activity increases (showing a higher increase in the growth inhibition halo diameter) with introduction of the alkyl(aryl)mercapto group into C(6) position of the imidazo[1,2-*b*][1,2,4,5]tetrazine system. The most promising STPK inhibitors were compounds bearing isopropylthiol (**3a**, **3m**, **3n**), benzylthiol (**3c**) and thiophenol (**3h**)

158 fragment in the C(6) position of the system. Modification of imidazotetrazine at C(3) position
159 revealed that the most active derivatives contain imidazolyl substituent (**1b**, **3n**, **3m**). However,
160 further study of such derivatives may be difficult due to their low solubility.
161 We were able to select 5 compounds among the most active ones with decent solubility (**1b, 3a,**
162 **3c, 3h** and **3n**) for further analysis.
163
164 **Antimycobacterial activity *in vitro***
165 We measured the MICs of the compounds in both liquid and solid media on different
166 mycobacterial strains. The results are presented in Table 2.
167 The activity of the imidazo[1,2-*b*][1,2,4,5]tetrazines on *M. smegmatis* was lower than on *M.*
168 *tuberculosis*, with **3h** and **3c** being the most active compounds both in weight-per-volume and
169 molar concentrations. The lower activity on *M. smegmatis* compared to *M. tuberculosis* was
170 expected and can be explained by a wider system of intrinsic antibiotic resistance in this
171 bacterium [24]. However, the active concentrations of the compounds allowed us to further use
172 the *M. smegmatis* model, providing faster results without the need to use the biosafety level 3
173 facility, for obtaining spontaneous resistant mutants and analyzing them.
174 **Table 2**. MICs of the imidazo[1,2-*b*][1,2,4,5]tetrazines on mycobacterial strains.

Compound	M_w	*M. tuberculosis H37Rv*		*M. smegmatis mc^2 155*			
		Liquid medium		Liquid medium		Solid medium	
		µg/ml	µM	µg/ml	µM	µg/ml	µM
3a	289,36	1	3.5	64	221.2	64	221.2
3h	323,38	1	3.1	32	99.0	20	61.8
1b	187,16	25	133.6	32	171.0	25	133.6
3n	275,33	25	90.8	64	232.4	25	90.8
3c	337,40	1	3.0	32	94.8	20	59.3

175
176

Obtaining spontaneous *M. smegmatis mc² 155* mutants resistant to imidazo[1,2-*b*][1,2,4,5]tetrazines

The acquired drug resistance may derive from mutations in genes encoding drug-targets, prodrug activators, efflux pumps, proteins that mediate cell-wall permeability, thus it is always determined genetically. The knowledge of the potential risk of drug-resistant mutants' emergence, as well as potential cross-resistance with other anti-tuberculosis agents is crucial in the development of drug-candidates for a complex treatment of tuberculosis and co-morbidities. Thus, we investigated the frequency of emergence of spontaneous *M. smegmatis mc² 155* mutants resistant to the studied compounds. We used the 3.5-4×times MIC concentrations of the imidazo[1,2-*b*][1,2,4,5]tetrazines to obtain spontaneous *M. smegmatis mc² 155* resistant mutants. The actual concentrations used and the frequency of drug-resistant mutants' emergence is shown in Table 3.

3c was the one compound, that generated most mutants at 3,75×MIC, with **3a** following it. As **3a** had the highest MIC, together with a high frequency of resistance emergence it makes this compound the least appealing as a possible anti-TB drug candidate. **3h** and **3n** had similar frequency of drug resistant mutants' emergence. The increase of **3h** compound concentration by 1×MIC (from 4× to 5×) lowered the frequency of spontaneous mutants emergence by 15,4 times, which is usual for bacteria: one mechanism of resistance provides one MIC value, and developing this mechanism might become less favorable when reaching this value or slightly overcoming it, while a higher increase in MIC might need a different mechanism of resistance, involving mutations in other genes, or even a combination of different mutations, leading to a more dramatic change in spontaneous resistant mutants [25]. Still a dispersion in the frequency by 10-100 times might may be observed when obtaining spontaneous mutants resistant to one concentration of the drug in different experiments on mycobacteria [26]. We could not obtain any spontaneous **1b** resistant mutants on 3-4×MIC. We suppose that due to the fact that this

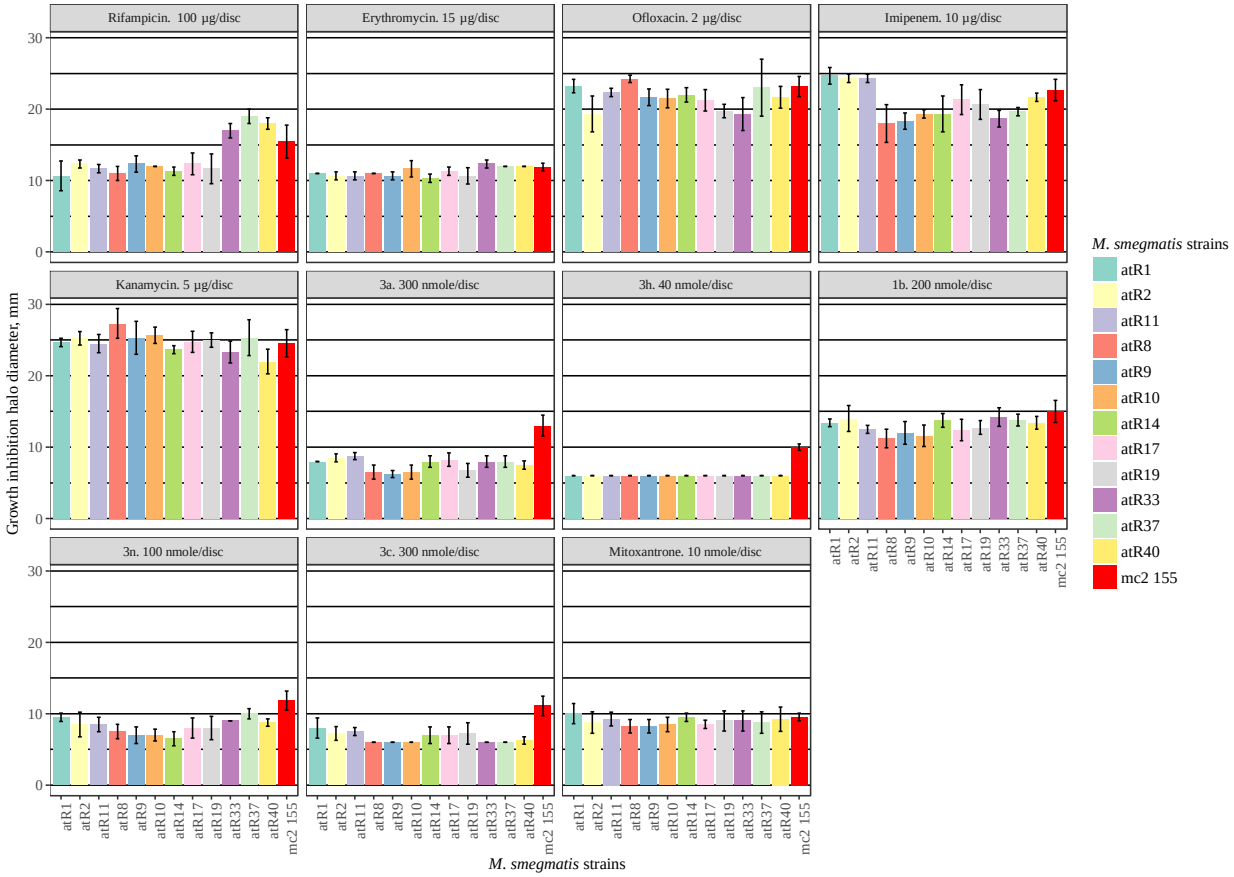
202 molecule has the smallest structure, it might be less specific than the others, and might have
203 more than one biotarget.
204 We selected 3 mutants resistant to each of the compounds for further analysis: **3a^{R}**
205 (*M. smegmatis* at^{R}8, at^{R}9, at^{R}10), **3h^{R}** (*M. smegmatis* at^{R}1, at^{R}2, at^{R}11), **3n^{R}** (*M. smegmatis*
206 at^{R}14, at^{R}17, at^{R}19) and **3c^{R}** (*M. smegmatis* at^{R}33, at^{R}37, at^{R}40).
207
208 **Drug resistant mutants' phenotypic characterization**
209 We confirmed the mutants' resistance to imidazo[1,2-*b*][1,2,4,5]tetrazines by determining the
210 MICs in liquid medium. Moreover, we discovered that the selected mutants had cross-resistance
211 among all the compounds, except for **1b**.
212 On **3a**, mutants 9, 14, 17, 19 had a MIC of 256 μg/ml (4× w.t. MIC), mutants 1, 8, 10, 11, 33, 37,
213 40 – 512 μg/ml (8× w.t. MIC) and mutant 2 – 1024 μg/ml (16× w.t. MIC).
214 On **3h** and **3c** all the mutants had a MIC higher than 64 μg/ml. The exact MIC could not be
215 determined in liquid medium due to the crystallization of the compounds at higher
216 concentrations.
217 On **3n**, mutants 8, 10 and 11 had a MIC of 256 μg/ml (4× w.t. MIC), while all the others – 128
218 μg/ml (2× w.t. MIC).
219 On **1b** all the mutants retained the w.t. MIC of 32 μg/ml.
220 We used paper-disc method to further analyze the mutants' susceptibility both to imidazo[1,2-
221 *b*][1,2,4,5]tetrazines and to antibiotics of different classes. This method allows a fast and
222 sensitive comparison of drug susceptibility among strains, though the results are less reliable
223 than MIC determination in liquid or solid medium. The results are shown on Picture 1.
224 We observed an increase in drug resistance that was significant according to our criterium of the
225 *M. smegmatis* at^{R}8, at^{R}10, at^{R}14, at^{R}1, at^{R}2 and at^{R}11 to rifampicin; *M. smegmatis* at^{R}8, at^{R}9,
226 at^{R}14, at^{R}1, at^{R}2 and at^{R}11 to erythromycin, *M. smegmatis* at^{R}40, at^{R}19 and at^{R}2 to ofloxacin,
227 *M. smegmatis* at^{R}33, at^{R}37, at^{R}9 and at^{R}10 to imipenem. All the mutants showed increased

resistance to **3a**, **3h** and **3c**. All the mutants, except for *M. smegmatis* at^R37 and at^R2 showed significant increase in resistance to **3n**, though all of them showed increased resistance in liquid medium. *M. smegmatis* at^R8, at^R9, at^R10 and at^R17 showed increased resistance to **1b**, though they had the same MIC as the w.t. strain in liquid medium. *M. smegmatis* at^R10 and at^R17 showed increased resistance to mitoxantrone. Interestingly two mutants - *M. smegmatis* at^R37 and at^R40 showed increased susceptibility to rifampicin. No significant changes in susceptibility to kanamycin were observed.

Despite the fact that the observed differences in drug sensibility levels to rifampicin, erythromycin, ofloxacin and imipenem were significant according to our criterion, can't be considered as highly increased resistance, as compared to the resistance levels of the mutants to imidazo[1,2-*b*][1,2,4,5]tetrazines. Thus, we show that the selected compounds have a mechanism of resistance, different from the drugs of comparison, however a common mechanism leads to a resistant phenotype to 4 out of 5 compounds, that has to be elucidated by whole-genomic sequencing.

Table 3. Frequency of spontaneous drug resistant *M. smegmatis mc2 155* mutants' emergence.

Compound	Concentration		Drug resistance mutants' emergence frequency
	μg/ml	×MIC	
3a	256	4	$2.2*10^{-7}$
3h	80	4	$2*10^{-8}$
	100	5	$1.3*10^{-9}$
1b	75-100	3-4	– ($<10^{-9}$)
3n	100	4	$2.1*10^{-8}$
3c	75	3.75	$4*10^{-6}$



Picture 1. Growth inhibition halos, produced by various antibiotics and imidazo[1,2-*b*][1,2,4,5]tetrazines on w.t. and mutant *M. smegmatis* strains.

The X-axis represents different antibiotics and their concentrations per disc, the legend shows the *M. smegmatis* strains, the histograms represent the average values from triplicate measurements, while the error bars represent the standard deviation.

4. Experimental

Compound **1a-d**, **2**, **3h** have been described earlier [18-20].

All the reagents and solvents were purchased from commercial sources. Reactions were monitored by thin layer chromatography (TLC) on Sorbfil silica gel plates, visualization done by ultraviolet light. Column chromatography was performed on silica gel (0.040-0.063 mm, 230–400 mesh). The eluent for TLC and column chromatography was a benzene—acetonitrile (1:1) mixture. Preparative high-performance liquid chromatography was carried out on a device Agilent-1200, with CH_3CN-H_2O (1:1) as an eluent. 1H NMR (400, 500 MHz), 13C NMR (100,

125 MHz) spectra were recorded in $CDCl_3$ or DMSO-d6 solvents on Bruker Avance DRX-400 and Bruker Avance II 500 spectrometer using Me4Si as the internal standard. The chemical shifts are given in the δ scale in ppm. Spin multiplicities are given as s (singlet), br. s (broad singlet), d (doublet), dd (doublet of doublet), t (triplet), sept (septet), and m (multiplet). Coupling constants (J) are given in hertz. Elemental analysis was performed on a CHN PE 2400 Ser. II (Perkin—Elmer) analyzer. Mass spectra were recorded on Shimadzu LCMS-2010 in the electrospray ionization mode for solutions in MeOH. Melting points were determined using melting point apparatus Boetius.

4.1. Chemistry

6-Substituted 3-azolylimidazo[1,2-*b*][1,2,4,5]tetrazines 3a-p, 5.

To a solution of imidazo[1,2-*b*][1,2,4,5]tetrazine **1a-d**, **2** (1 mmol) in acetonitrile (5 mL), thiol (1.1 mmol) and triethylamine (101 mg, 1 mmol) were added. The reaction mixture was stirred 1 hour for compounds **3f-i,l**, 3h - **3o,p** 5-7h - **3a-c**, 24h - **3d,e,j,n**, 48h - **3k,m,5** at room temperature (TLC control). The solvent was concentrated. Compounds **3a-i,n-p, 5** were isolated by column chromatography (R_f = 0.43—0.92), **3m** were isolated by preparative HPLC (R_f = 0.68), **3j,k** were isolated by recrystallization from methanol, **3l** – from ethanol.

3-(3,5-Dimethyl-1*H*-pyrazol-1-yl)-6-(isopropylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (3a). Orange solid; yield: 60% (174 mg); mp 108–109 °C, R_f = 0.73. 1H NMR (400 MHz, DMSO-d6) δ: 1.30 (d, *J* = 6.7 Hz, 6H), 2.27 (s, 3H), 2.58 (s, 3H), 3.74 (sept, *J* = 6.7 Hz, 1H), 6.30 (s, 1H), 8.76 (s, 1H). 13C NMR (125 MHz, $CDCl_3$) δ: 13.8, 14.3, 23.6, 38.7, 111.1, 123.8, 143.2, 146.8, 146.9, 152.4, 153.4. Anal. calcd for $C_{12}H_{15}N_7S$: C, 49.81; H, 5.23; N, 33.88. Found: C, 49.78; H, 5.30; N, 33.58.

3-(3,5-Dimethyl-1*H*-pyrazol-1-yl)-6-(propylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (3b). Orange solid; yield: 51% (148 mg); mp 96–97 °C; R_f = 0.56. 1H NMR (400 MHz, $CDCl_3$) δ: 1.06 (t, *J* = 7.4 Hz, 3H), 1.71 (m, 2H), 2.40 (s, 3H), 2.71 (s, 3H), 3.12 (t, *J* = 7.2 Hz, 2H), 6.17 (s, 1H), 8.39 (s, 1H). 13C NMR (125 MHz, $CDCl_3$) δ: 13.1, 13.9, 14.4, 23.2, 34.8, 111.2, 125.1,

143.3, 145.3, 146.8, 152.4, 153.4. Anal. calcd for $C_{12}H_{15}N_7S$: C, 49.81; H, 5.23; N, 33.88. Found: C, 49.87; H, 5.23; N, 34.05.

6-(Benzylthio)-3-(3,5-dimethyl-1*H*-pyrazol-1-yl)imidazo[1,2-*b*][1,2,4,5]tetrazine (3c). Yellow solid; yield: 75% (253 mg); mp 139-140 °C; R_f = 0.85. 1H NMR (500 MHz, DMSO-d6) δ: 2.28 (s, 3H), 2.60 (s, 3H), 4.40 (s, 2H), 6.31 (s, 1H), 7.21-7.34 (m, 5H), 8.59 (s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 13.4, 36.0, 110.1, 122.7, 127.5, 128.5, 128.9, 136.9, 142.8, 146.3, 146.9, 151.5, 151.7. Anal. calcd for $C_{16}H_{15}N_7S$: C, 56.96; H, 4.48; N, 29.06. Found: C, 56.93; H, 4.65; N, 28.82.

3-(3,5-Dimethyl-1*H*-pyrazol-1-yl)-6-(octadecylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (3d). Yellow solid; yield: 67% (335 mg); mp 86-87 °C; R_f = 0.92. 1H NMR (400 MHz, $CDCl_3$) δ: 0.88 (t, *J* = 6.9 Hz, 3H), 1.25 (s, 25H), 1.38-1.48 (m, 2H), 1.62-1.72 (m, 2H), 2.39 (s, 3H), 2.71 (s, 3H), 3.12 (t, *J* = 7.3 Hz 2H), 6.17 (s, 1H), 8.38 (s, 1H). 13C NMR (125 MHz, $CDCl_3$) δ: 13.8, 14.1, 14.4, 22.7, 28.4, 29.0, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 32.9, 111.1, 125.3, 143.4, 145.3, 146.8, 152.3, 153.4. Anal. calcd for $C_{27}H_{45}N_7S$: C, 64.89; H, 9.08; N, 19.62. Found: C, 64.95; H, 9.02; N, 19.65.

3-(3,5-Dimethyl-1*H*-pyrazol-1-yl)-6-(2-hydroxyethylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (3e). Orange solid; yield: 36% (105 mg); mp 134-135 °C; R_f = 0.43. 1H NMR (500 MHz, $CDCl_3$) δ: 2.36 (s, 3H), 2.75 (s, 3H), 3.28 (t, *J* = 5.8 Hz, 2H), 3.87 (t, *J* = 5.8 Hz, 2H), 6.16 (s, 1H), 8.44 (s, 1H). 13C NMR (125 MHz, $CDCl_3$) δ: 13.8; 14.7; 37.0, 61.6, 111.4, 123.8, 143.8, 147.0, 152.3, 153.8. Anal. calcd for $C_{11}H_{13}N_7OS$: C, 45.35; H, 4.50; N, 33.65. Found: C, 45.33; H, 4.45; N, 33.52.

3-(3,5-Dimethyl-1*H*-pyrazol-1-yl)-6-(6-hydroxyhexan-3-ylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (3f). Orange solid; yield: 70% (243 mg); mp 109-110 °C; R_f = 0.80. 1H NMR (400 MHz, $CDCl_3$) δ: 0.85 (t, *J* = 7.2 Hz, 3H), 1.33-1.43 (m, 1H), 1.48-1.69 (m, 5H), 2.38 (s, 3H), 2.79 (s, 3H), 3.51-3.59 (m, 1H), 3.95-4.02 (m, 1H), 4.36-4.44 (m, 1H), 5.32 (br. s, 1H), 6.18 (s, 1H), 8.46 (s, 1H). 13C NMR (125 MHz, $CDCl_3$) δ: 13.4, 13.8, 15.0, 20.0, 38.6, 39.0,

46.9, 58.4, 111.4, 123.8, 144.5, 147.1, 148.7, 152.5, 154.1. Anal. calcd for $C_{15}H_{21}N_7OS$: C, 51.85; H, 6.09; N, 28.22. Found: C, 51.40; H, 6.04; N, 27.94.

3-(3,5-Dimethyl-1*H*-pyrazol-1-yl)-6-(3-(4-hydroxy-3,5-di-tert-butylphenyl)propylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (3g). Orange solid; yield: 51% (500 mg); mp 111-112 °C; R_f = 0.90. 1H NMR (500 MHz, DMSO-d6) δ: 1.34 (s, 18H), 1.85 (m, 2H), 2.26 (s, 3H), 2.56 (s, 3H), 2.59 (m, 2H), 3.18 (m, 2H), 6.28 (s, 1H), 6.70 (s, 1H), 6.87 (s, 2H), 8.74 (s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 13.2, 13.4, 30.4, 31.6, 31.7, 33.7, 34.4, 123.7, 124.2, 131.8, 139.2, 142.6, 145.9, 147.0, 151.3, 151.7, 151.9. Anal. calcd for $C_{26}H_{35}N_7OS$: C, 63.26; H, 7.15; N, 19.86. Found: C, 63.38; H, 7.15; N, 19.66.

3-(3,5-Dimethyl-1*H*-pyrazol-1-yl)-6-(phenylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (3h). Yellow solid; yield 36% (117 mg); mp 139-140 °C; R_f = 0.80. 1H NMR (400 MHz, $CDCl_3$) δ: 2.38 (s, 3H), 2.50 (s, 3H), 6.13 (s, 1H), 7.34-7.38 (m, 3H), 7.40-7.44 (m, 2H), 8.29 (s, 1H). 13C NMR (125 MHz, $CDCl_3$) δ: 13.8, 14.1, 111.2, 123.9, 129.0, 129.3, 129.9, 131.6, 144.4, 146.0, 146.8, 152.2, 153.5. Anal. calcd for $C_{15}H_{13}N_7S$: C, 55.71; H, 4.05; N, 30.32. Found: C, 55.61; H, 3.76; N, 30.31.

6-(2,5-Dimethoxyphenylthio)-3-(3,5-dimethyl-1*H*-pyrazol-1-yl)imidazo[1,2-*b*][1,2,4,5]tetrazine (3i). Orange solid; yield 42% (161 mg); mp 105-106 °C; R_f = 0.74. 1H NMR (500 MHz, DMSO-d6) δ: 2.25 (s, 3H), 2.42 (s, 3H), 3.58 (s, 3H), 3.82 (s, 3H), 6.27 (s, 1H), 6.52 (d, J = 3.0 Hz, 1H), 6.84 (dd, J_1= 8.9 Hz, J_2 = 3.0 Hz, 1H), 7.03 (d, J = 8.9 Hz, 1H), 8.75 (s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 13.0, 13.4, 55.5, 56.6, 110.1, 112.6, 112.8, 115.3, 118.5, 120.6, 142.8, 147.5, 148.3, 150.3, 151.5, 151.8, 153.7. Anal. calcd for $C_{17}H_{17}N_7O_2S$: C, 53.25; H, 4.47; N, 25.57. Found: C, 53.26; H, 4.67; N, 25.46.

6-(2-Carboxyphenylthio)-3-(3,5-dimethyl-1*H*-pyrazol-1-yl)imidazo[1,2-*b*][1,2,4,5]tetrazine (3j). Orange solid; yield 25% (92 mg); mp 266-267 °C; R_f = 0.21. 1H NMR (500 MHz, DMSO-d6) δ: 2.23 (s, 3H), 2.27 (s, 3H), 6.23 (s, 1H), 6.76 (d, J = 7.6 Hz, 1H), 7.24-7.33 (m, 2H), 8.03 (dd, J_1 = 7.6 Hz, J_2 = 2.0 Hz, 1H), 8.89 (s, 1H), 13.59 (br. s, 1H). 13C NMR (125 MHz, DMSO-

d6) δ: 12.9, 13.3, 110.2, 118.7, 125.9, 127.0, 127.1, 131.4, 133.3, 137.0, 142.7, 147.9, 149.2, 151.6, 151.8, 167.5. Anal. calcd for $C_{16}H_{13}N_7O_2S$: C, 52.31; H, 3.57; N, 26.69. Found: C, 52.24; H, 3.52; N, 26.65.

6-(3-Carboxyphenylthio)-3-(3,5-dimethyl-1*H*-pyrazol-1-yl)imidazo[1,2-*b*][1,2,4,5]tetrazine (3k).

Yellow solid; yield 20 % (74 mg); mp 229-230 °C; R_f = 0.10. 1H NMR (400 MHz, DMSO-d6) δ: 2.24 (s, 3H), 2.30 (s, 3H), 6.25 (s, 1H), 7.31-7.35 (m, 1H), 7.61-7.67 (m, 1H), 7.78-7.83 (m, 1H), 7.91-7.96 (m, 1H), 8.90 (s, 1H), 13.06 (br. s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 13.0, 13.4, 110.2, 118.7, 125.9, 128.4, 129.2, 130.0, 132.1, 132.5, 133.0, 142.8, 147.4, 148.0, 151.6, 166.4. Anal. calcd for $C_{16}H_{13}N_7O_2S$: C, 52.31; H, 3.57; N, 26.69. Found: C, 52.37; H, 3.65; N, 26.54.

6-(4-Carboxyphenylthio)-3-(3,5-dimethyl-1*H*-pyrazol-1-yl)imidazo[1,2-*b*][1,2,4,5]tetrazine (3l). Yellow solid; yield 18 % (65 mg); mp 243-244 °C; R_f = 0.10. 1H NMR (400 MHz, DMSO-d6) δ: 2.24 (s, 3H), 2.30 (s, 3H), 6.25 (s, 1H), 7.33 (d, *J* = 8.5 Hz, 2H), 7.80 (d, *J* = 8.5 Hz, 2H), 8.90 (s, 1H), 13.02 (br. s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 12.9, 13.4, 110.3, 117.0, 126.9, 129.1, 130.2, 138.3, 142.8, 147.6, 148.5, 151.7, 151.8, 166.6. Anal. calcd for $C_{16}H_{13}N_7O_2S$: C, 52.31; H, 3.57; N, 26.69. Found: C, 52.37; H, 3.65; N, 26.54.

3-(1*H*-Imidazol-1-yl)-6-(isopropylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (3m). Orange solid; yield 34 % (89 mg); mp 139-140 °C; R_f = 0.68. 1H NMR (400 MHz, DMSO-d6) δ: 1.33 (d, *J* = 6.7 Hz, 6H), 3.88 (sept, *J* = 6.7 Hz, 1H), 7.27-7.32 (m, 1H), 8.04-8.08 (m, 1H), 8.76-8.79 (m, 1H), 8.80 (s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 23.5, 38.3, 116.7, 123.0, 131.2, 136.0, 147.8, 147.9, 149.2. Anal. calcd for $C_{10}H_{11}N_7S$: C, 45.96; H, 4.24; N, 37.52. Found: C, 46.02; H, 4.41; N, 37.53.

6-(Isopropylthio)-3-(4-methyl-1*H*-imidazol-1-yl)imidazo[1,2-*b*][1,2,4,5]tetrazine (3n). Orange solid; yield 53 % (146 mg); mp 157-158 °C; R_f = 0.50. 1H NMR (400 MHz, $CDCl_3$) δ: 1.40 (d, *J* = 6.8 Hz, 6H), 2.36 (s, 3H), 3.77 (sept, *J* = 6.8 Hz, 1H), 7.66 (s, 1H), 8.43 (s, 1H), 8.69

(s, 1H). 13C NMR (125 MHz, $CDCl_3$) δ: 13.2, 23.7, 38.9, 112.5, 125.2, 135.2, 140.2, 147.5, 147.7, 149.3. Anal. calcd for $C_{11}H_{13}N_7S$: C, 47.98; H, 4.76; N, 35.61. Found: 47.76; H, 4.76; N, 35.56.

6-(Benzylthio)-3-(4-methyl-1*H*-imidazol-1-yl)imidazo[1,2-*b*][1,2,4,5]tetrazine (3o). Yellow solid; yield: 43 % (139 mg); mp 80-81 °C; R_f = 0.52. 1H NMR (400 MHz, DMSO-d6) δ: 2.25 (s, 3H), 4.45 (s, 2H), 7.22-7.34 (m, 5H), 7.75 (s, 1H), 8.63 (s, 1H), 8.65 (s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 13.5, 35.8, 112.5, 123.3, 127.5, 128.5, 128.9, 135.4, 136.9, 140.1, 146.7, 147.6, 149.0. Anal. calcd for $C_{15}H_{13}N_7S$: C, 55.71; H, 4.05; N, 30.32. Found: C, 55.74; H, 4.07; N, 30.26.

6-(Benzylthio)-3-(1*H*-indazol-1-yl)imidazo[1,2-*b*][1,2,4,5]tetrazine (3p). Yellow solid; yield: 79 % (284 mg); mp 165-167 °C; R_f = 0.86. 1H NMR (400 MHz, DMSO-d6) δ: 4.47 (s, 2H), 7.19-7.30 (m, 3H), 7.31-7.37 (m, 2H), 7.43-7.49 (m, 1H), 7.68-7.76 (m, 1H), 7.99-8.04 (m, 1H), 8.56-8.61 (m, 2H), 8.71 (s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 36.0, 114.4, 121.8, 122.7, 123.8, 126.1, 127.5, 128.5, 128.9, 129.0, 137.0, 138.8, 140.4, 146.1, 147.2, 152.5. Anal. calcd for $C_{18}H_{13}N_7S$ (FW =): C, 60.15; H, 3.65; N, 27.28 %. Found: C, 60.22; H, 3.50; N, 27.15 %.

3-(3,5-Dimethyl-1*H*-pyrazol-1-yl)-6,7-bis(isopropylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (4a). Orange solid; R_f = 0.79. Спектр 1H NMR (400 MHz, $CDCl_3$) δ: 1.32 (d, *J* = 6.8 Hz, 6H), 1.56 (d, *J* = 6.8 Hz, 6H), 2.39 (s, 3H), 2.67 (s, 3H), 3.84 (sept, *J* = 6.8 Hz, 1H), 4.32 (sept, *J* = 6.8 Hz, 1H), 6.22 (s, 1H). MS (ESI) m/z (%) = 364.15 (100) $[M + H]^+$ (calcd for $C_{15}H_{22}N_7S_2^+$, 364.14).

3-(1H-Imidazol-1-yl)-6,7-bis(isopropylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (4b). Orange solid; yield 3 % (10 mg); mp = 104–106 °C; R_f = 0.74. Спектр 1H NMR (400 MHz, DMSO-d6) δ: 1.28 (d, *J* = 6.7 Hz, 6H), 1.51 (d, *J* = 6.8 Hz, 6H), 3.82 (sept, *J* = 6.7 Hz, 1H), 4.32 (sept, *J* = 6.8 Hz, 1H), 7.27 (s, 1H), 8.03 (s, 1H), 8.71 (s, 1H). MS (ESI) m/z (%) = 336.05 (100) $[M + H]^+$ (calcd for $C_{13}H_{18}N_7S_2^+$, 336.11).

3-(3,5-Dimethyl-1*H*-pyrazol-1-yl)-6-(isopropylthio)-7-phenylimidazo[1,2-*b*][1,2,4,5]tetrazine (5). Orange solid; yield: 66% (241 mg); mp 150–151 °C, R_f = 0.90. 1H NMR (400 MHz, DMSO-d6) δ: 1.22 (d, *J* = 6.7 Hz, 6H), 2.29 (s, 3H), 2.61 (s, 3H), 3.75 (sept, *J* = 6.7 Hz, 1H), 6.32 (s, 1H), 7.56-7.66 (m, 3H), 8.41-8.46 (m, 2H), 8.76 (s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 13.2, 13.4, 23.2, 110.0, 118.3, 128.8, 128.9, 130.6, 131.8, 142.6, 146.5, 151.3, 151.8, 155.2. Anal. calcd for $C_{18}H_{19}N_7S$: C, 59.16; H, 5.24; N, 26.83. Found: C, 59.08; H, 5.12; N, 26.57.

3-(Heptylamino)-6-(phenylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (6a). To a solution of 1 mmol (323 mg) 3-(3,5-dimethyl-1*H*-pyrazol-1-yl)-6-(phenylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (**3h**) in 1.5 ml of acetonitrile was added 1.5 ml of heptylamine, stirred for 2 hours, then the reaction solution was evaporated, acetic acid was added to the residue, the product was precipitated with water, and filtered. Yellow solid; yield: 60 % (206 mg); mp 89–90 °C, R_f = 0.92. 1H NMR (400 MHz, DMSO-d6) δ: 0.85 (t, *J* = 7.0 Hz, 3H), 1.17-1.28 (m, 8H), 1.42-1.52 (m, 2H), 3.04-3.19 (m, 2H), 7.20-7.33 (m, 5H), 8.23 (s, 1H), 8.40 (br. s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 13.9, 22.0, 26.3, 27.5, 28.3, 31.2, 40.7, 116.4, 127.0, 128.3, 129.3, 132.5, 142.6, 146.1, 157.3. Anal. calcd for $C_{17}H_{22}N_6S$: C, 59.62; H, 6.48; N, 24.54. Found: C, 59.66; H, 6.69; N, 24.44.

3-(Amino)-6-(phenylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (6b). A solution of 1 mmol (323 mg) of 3-(3,5-dimethyl-1*H*-pyrazol-1-yl)-6-(phenylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (**3h**) in dimethylformamide, ammonia was flushed for 15 minutes, the reaction was carried out in a microwave oven for 10 minutes at a temperature of 150 ° C, the solvent was evaporated, the product was precipitated from acetonitrile with water, filtered and washed with hexane. Orange solid; yield: 90 % (220 mg); mp 204–205 °C, R_f = 0.78. 1H NMR (400 MHz, DMSO-d6) δ: 7.12-7.18 (m, 2H), 7.20-7.25 (m, 1H), 7.26-7.32 (m, 2H), 7.69 (br. s, 2H) 8.26 (s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 115.3, 126.7, 127.1, 129.5, 133.4, 143.9, 146.5, 158.5. Anal. calcd for $C_{10}H_8N_6S$: C, 49.17; H, 3.30; N, 34.40. Found: C, 49.35; H, 3.18; N, 34.19.

3-(3,5-Dimethyl-1*H*-pyrazol-1-yl)-6-(isopropylsulfonyl)imidazo[1,2-*b*][1,2,4,5]tetrazine (7). To a solution of 1 mmol (289 mg) 3-(3,5-dimethyl-1*H*-pyrazol-1-yl)-6-(isopropylthio)imidazo[1,2-*b*][1,2,4,5]tetrazine (**3a**) in acetonitrile was added 3.5 mmol (604 mg) of 3-chloroperbenzoic acid, stirred for 2 hours, the solvent was evaporated, the residue was washed with diethyl ether and filtered. Orange solid; yield: 80% (258 mg); mp 205–206 °C, R_f = 0.85. 1H NMR (500 MHz, DMSO-d6) δ: 1.29 (d, *J* = 6.9 Hz, 6H), 2.29 (s, 3H), 2.58 (s, 3H), 3.63 (sept, *J* = 6.9 Hz, 1H), 6.35 (s, 1H), 8.95 (s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 13.3, 13.4, 14.6, 55.3, 110.7, 121.6, 143.1, 145.0, 146.7, 151.3, 152.3. Anal. calcd for $C_{12}H_{15}N_7O_2S$: C, 44.85; H, 4.70; N, 30.51. Found: C, 44.77; H, 4.88; N, 30.51.

3-(Heptylamino)-6-(isopropylsulfonyl)imidazo[1,2-*b*][1,2,4,5]tetrazine (8a). To a solution of 1 mmol (321 mg) of 3-(3,5-dimethyl-1*H*-pyrazol-1-yl)-6-(isopropylsulfonyl)imidazo[1,2-*b*][1,2,4,5]tetrazine (**7**) in acetonitrile 10 mmol of heptylamine were added, after 10 minutes the solvent was evaporated, the residue was washed with diethyl ether and filtered**.** Yellow solid; yield: 82% (279 mg); mp 98-99 °C, R_f = 0.87. 1H NMR (500 MHz, DMSO-d6) δ: 0.86 (t, *J* = 7.0 Hz, 3H), 1.27 (d, *J* = 6.9 Hz, 6H), 1.28-1.40 (m, 8H), 1.63 (m, 2H), 3.24 (s, 2H), 3.64 (sept, *J* = 7.0 Hz, 1H), 8.30 (s, 1H), 8.80 (br. s, 1H). 13C NMR (125 MHz, DMSO-d6) δ: 13.9, 14.6, 22.0, 26.4, 27.5, 28.4, 31.2, 40.9, 54.5, 120.3, 139.8, 145.3, 157.1. Anal. calcd for $C_{14}H_{24}N_6O_2S$: C, 49.39; H, 7.11; N, 24.69. Found: C, 49.36; H, 7.18; N, 24.61.

4.2. Antimycobacterial activity testing

Bacterial strains and growth conditions

M. smegmatis mc^2 155 strain was used in the study. Lemco-Tw broth (5 g/l Lemco Powder, Oxoid, UK; 5 g/l NaCl, 5 g/l bacto peptone, Oxoid, UK, 0.05 % v/v Tween-80) and Middlebrook 7H9 medium (Himedia, India) supplemented with OADC (Himedia, India), 0.1% Tween-80 (v/v) and 0.4% glycerol (v/v) were used as liquid media, while the M290 Soyabean

Casein Digest Agar (Himedia, India) was used as solid medium. Cultures in liquid medium were incubated in the Multitron incubator shaker (Infors HT, Switzerland) at 37 °C and 250 rpm.

Minimal inhibitory concentrations determination on M. tuberculosis

M. tuberculosis H37Rv strain was used in the study. MICs were determined in liquid medium on the automated Bactec MGIT 960 system (Becton–Dickinson, USA) as described in [22].

Minimal inhibitory concentrations determination on M. smegmatis

Minimal inhibitory concentrations (MICs) of the studied compounds on *M. smegmatis* were determined in liquid and on solid media.

For the MIC determination in liquid medium, *M. smegmatis* was cultured overnight in Lemco-Tw, then diluted in the proportion of 1:200 in fresh Lemco-Tw broth. 196 µl of the diluted culture were poured in the Greiner CELLSTAR® 96 well flat-bottom plates for suspension cultures (Sigma-Aldrich, USA) and 4 µl of serial two-fold dilutions of the tested compounds in DMSO were added to the wells to final concentrations of 0.5 to 1024 µg/ml for **3a**, **1b** and **3n**, and 0.5 to 64 µg/ml, for **3c** and **3h**, as they were not soluble in higher concentrations. 64 µg/ml concentrations of **3c** and **3h** were also supplemented with 4% DMSO (v/v), that did not affect the growth of mycobacteria. The plates were incubated at 37°C and 250 rpm for 48 hours. The MIC was determined as the lowest concentration of the compound with no visible bacterial growth.

For the determination of MICs on solid medium, M290 Petri dishes were prepared with serial two-fold dilutions of the tested compounds, ranging from 8 to 64 µg/ml for **3h**, **1b** and **3c**, and from 8 to 128 µg/ml for **3a** and **3n**, additional Petri dishes with 20 and 25 µg/ml of the tested compound were prepared in some cases. *M. smegmatis* was grown in Lemco-Tw broth up to OD_{600}=1,8 (approximately 3×10^8 colony forming units per milliliter, CFU/ml). Serial ten-fold dilutions of the culture were prepared and 3 µl of each dilution were poured on the plates. The

MIC for each compound was determined as the minimal concentration, inhibiting the growth of 99% of CFU.

Drug resistant M. smegmatis mutants' isolation

Serial ten-fold dilutions of *M. smegmatis mc^2 155* containg 10^7-10^9 were plated on Petri dishes with M290 medium, supplemented with 3.5-4×times MIC of the tested compounds. The Petri dishes were incubated at 30°C for 5-7 days, until the colonies were visible [27]. The number of colonies was counted to calculate the frequency of drug resistance emergence. Twelve colonies were plated on new Petri dishes with the same concentration of the compound, afterwards those that have shown growth were plated on compound-free M290 medium and then plated on M290 medium supplemented with the compound again to confirm drug resistance [28]. Three mutants with confirmed resistance to each of the corresponding compound were randomly selected for further analysis.

Drug susceptibility testing with the paper disc method

Mycobacterial cultures were grown in Middlebrook 7H9 broth to mid-exponential phase (OD600 = 1.2). Afterwards the cultures were diluted in the proportion of 1:9:10 (culture:water:M290 medium) and 5 ml were poured as the top layer on Petri dishes with agarized M290 medium. The plates were allowed to dry for at least 30 min, and Sensi-Discs with antibiotics (erythromycin, ofloxacin, imipenem were plated as Sensi-Discs, while rifampicin, kanamycin, mitoxantrone and the imidazo[1,2-*b*][1,2,4,5]tetrazines were impregnated on sterile paper discs and also plated. The plates were incubated for 2–3 days at 37°C, until the bacterial lawn was fully grown. Growth inhibition halos were measured to the nearest 1 mm. The experiments were carried out as triplicates, the average diameter and standard deviation (SD) were calculated. Those differences that had no intersection of the SDs with the control (*M. smegmatis mc^2 155*) were considered significant.

Conclusions

The method of chemical functionalization of imidazo[1,2-*b*][1,2,4,5]tetrazines at C(6) position in the reactions with alkyl and aryl mercaptanes has been developed. The possibility of further modification of obtained products via nucleophilic substitution with amines or oxidation reaction has been shown. These methods allowed to obtain the wide range of novel imidazo[1,2-*b*][1,2,4,5]tetrazine derivatives with different substituents at C(3) and C(6) positions.

Our testing in the *M. smegmatis aphVIII+* test-system [23] showed that 8 out of 28 synthesized compounds (**1b**, **3a**, **3c**, **3h**, **3m**, **3n**, **5** and **6a**) are active mycobacterial STPK inhibitors. 5 compounds selected for further analysis (**1b**, **3a**, **3c**, **3h**, and **3n**) showed antimycobacterial activity on both *M. tuberculosis* H37rv and *M. smegmatis mc2 155*, with 3 of them (**3a**, **3h** and **3c**) in a concentration as low as 1 μg/ml. MICs on *M. smegmatis* were higher, due to a wider system of intrinsic antibiotics resistance [24], however this was not an obstacle to obtain resistant mutants to 4 out of 5 compounds (**3a**, **3c**, **3h** and **3n**). The investigation of the *M. smegmatis* mutants' drug resistance phenotype revealed that the 4 imidazo[1,2-*b*][1,2,4,5]tetrazine have a common resistance mechanism, though it differs from those of the tested antibiotics.

The compound **3h** seems to be the most promising compound among the studied imidazo[1,2-*b*][1,2,4,5]tetrazines, due to its relatively high antimycobacterial activity on *M. tuberculosis* H37Rv (1 μg/ml), as well as a relatively low frequency of drug resistance mutants' emergence ($2.0*10^{-8}$ at 4×MIC and $1.3*10^{-9}$ at 5×MIC).

Though the alleged target of the selected imidazo[1,2-*b*][1,2,4,5]tetrazines are mycobacterial STPKs, as was shown in previous docking experiments [13] and in this study on the *M. smegmatis* APHVIII+ test-system [23], the exact biotarget of these compounds, as well as the mechanism of resistance are yet to be established by whole-genomic sequencing and "reverse-genetics" approach, which is a usual practice in the "drug-to-target" pathway of drug development [29].

Conflict of interests

The authors have no competing financial interests to declare.

Author contributions

Maslov D. A. and Korotina A. V. contributed equally to this work. Charushin V. N., Rusinov G. L. and Danilenko V. N. organized the collaboration. Maslov D. A. planned the biological experiments. Maslov D.A., Shur K. V., Vatlin A. A. and Bekker O.B. carried out the in vitro experiments. Koritna A. V., Tolshchina S. G., Ishmetova R. I. and Ignatenko N. K. synthesized the compounds. Maslov D. A., Korotina A. V., Tolshchina S. G. and Bekker O. B. drafted the manuscript. All authors read and approved the manuscript.

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References

[1] World Health Organization, Global Tuberculosis Report 2017, Geneva, 2017.

[2] N.R. Gandhi, P. Nunn, K. Dheda, H.S. Schaaf, M. Zignol, D. van Soolingen, et al., Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis, The Lancet. 375 (2010) 1830–1843. doi:10.1016/S0140-6736(10)60410-2.

[3] J.A. Caminero, G. Sotgiu, A. Zumla, G.B. Migliori, Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis, Lancet Infect Dis. 10 (2010) 621–629. doi:10.1016/S1473-3099(10)70139-0.

[4] M. Klopper, R.M. Warren, C. Hayes, N.C. Gey van Pittius, E.M. Streicher, B. Müller, et al., Emergence and Spread of Extensively and Totally Drug-Resistant Tuberculosis, South Africa, Emerging Infect. Dis. 19 (2013) 449–455. doi:10.3201/eid1903.120246.

[5] A.A. Velayati, P. Farnia, M.R. Masjedi, The totally drug resistant tuberculosis (TDR-TB), Int J Clin Exp Med. 6 (2013) 307–309.

[6] A.S. Shawali, N.M. Tawfik, Novel facile synthesis of imidazo[1,2-*b*]-[1,2,4,5]tetrazines with potential antimicrobial activity, Arch. Pharm. Res. 32 (2009) 975–982. doi:10.1007/s12272-009-1701-4.

[7] R.I. Ishmetova, N.K. Ignatenko, I.A. Belyaninova, N.A. Gerasimova, N.P. Evstigneeva, N.V. Zil'berberg, et al., Selective antibacterial agents, represented by 3-(azol-1-yl)-6-amino-substituted 1,2,4,5-tetrazines, Patent RU 2642882, 2018.

[8] J. Mohan, Shikha, Heterocyclic systems containing bridgehead nitrogen atom. Facile synthesis and biological activity of spiro[cycloheptane-1,7'(8'H)-[6H]pyrazolo[3',4':4,5]thiazolo[3,2-*b*]-*s*-tetrazines], Ind. J. Heterocycl. Chem. 15 (2005) 187–188.

[9] F. Xu, Z.-Z. Yang, J.-R. Jiang, W.-G. Pan, X.-L. Yang, J.-Y. Wu, et al., Synthesis, antitumor evaluation and molecular docking studies of [1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazine derivatives, Bioorg. Med. Chem. Lett. 26 (2016) 3042–3047. doi:10.1016/j.bmcl.2016.05.007.

[10] O.B. Bekker, V.N. Danilenko, N.K. Ignatenko, R.I. Ishmetova, G.L. Rusinov, S.G. Tolshchina, et al., 6-substituted 3-azolylimidazo[1,2-*b*][1,2,4,5]tetrazines showing anticancer activity, Patent RU 2527258, 2014.

[11] B.L. Oliveira, Z. Guo, G.J.L. Bernardes, Inverse electron demand Diels-Alder reactions in chemical biology, Chem Soc Rev. 46 (2017) 4895–4950. doi:10.1039/c7cs00184c.

[12] H. Wu, N.K. Devaraj, Advances in Tetrazine Bioorthogonal Chemistry Driven by the Synthesis of Novel Tetrazines and Dienophiles, Acc. Chem. Res. 51 (2018) 1249–1259. doi:10.1021/acs.accounts.8b00062.

[13] O.B. Bekker, V.N. Danilenko, R.I. Ishmetova, D.A. Maslov, G.L. Rusinov, S.G. Tolshchina, et al., Substituted azolo[1,2,4,5]tetrazines - inhibitors of antibacterial serine-threonine protein kinases, Patent RU 2462466, 2012.

[14] R.I. Ishmetova, N.K. Ignatenko, I.N. Ganebnykh, S.G. Tolschina, A.V. Korotina, M.A. Kravchenko, et al., Synthesis and tuberculostatic activity of amine-substituted 1,2,4,5-tetrazines and pyridazines, Russ Chem Bull. 63 (2014) 1423–1430. doi:10.1007/s11172-014-0613-8.

[15] G.L. Rusinov, O.V. Fedorova, R.I. Ishmetova, I.N. Ganebnykh, I.G. Ovchinnikova, M.A. Kravchenko, et al., Selective anti-tuberculosis agents, representing 3-aminosubstituted 6-(3,5-dimethylpyrazol-1-yl)-1,2,4,5-tetrazines, Patent RU 2519218, 2014.

[16] G.L. Rusinov, O.V. Fedorova, R.I. Ishmetova, I.N. Ganebnykh, I.G. Ovchinnikova, M.A. Kravchenko, et al., Selective antituberculosis agent representing 3-hydrazono-6-(3,5-dimethylpyrazol-1-yl)- 1,2,4,5-tetrazine and method for preparing it, Patent RU 2479311, 2013.

[17] G.L. Rusinov, N.I. Latosh, R.I. Ishmetova, M.A. Kravchenko, I.N. Ganebnykh, V.A. Sokolov, et al., Synthesis and Tuberculostatic Activity of Some Substituted Amino Acid Methyl Esters with Sym-Tetrazine Moieties, Pharm Chem J. 39 (2005) 8–10.

[18] R.I. Ishmetova, N.K. Ignatenko, I.A. Belyaninova, S.G. Tolshchina, A.V. Korotina, P.A. Slepukhin, et al., Synthesis and antifungal activity of 3-substituted imidazo[1,2-*b*][1,2,4,5]tetrazines, Russ Chem Bull. 64 (2015) 2100–2105. doi:10.1007/s11172-015-1124-y.

[19] I.N. Ganebnykh, R.I. Ishmetova, S.G. Tolshchina, A.V. Korotina, D.S. Filatov, P.A. Slepukhin, et al., Synthesis and reactions of 7-phenylimidazo[1,2-*b*][1,2,4,5]tetrazines with nucleophiles, Russ Chem Bull. 67 (2018) 1716–1723. doi:10.1007/s11172-018-2281-6.

[20] S.G. Tolshchina, R.I. Ishmetova, N.K. Ignatenko, A.V. Korotina, I.N. Ganebnykh, V.A. Ol'shevskaya, et al., Reactions of 1,2,4,5-tetrazines with S-nucleophiles, Russ Chem Bull. 60 (2011) 985–991. doi:10.1007/s11172-011-0155-2.

591 [21] O.B. Bekker, V.N. Danilenko, D.A. Maslov, Test system of *Mycobacterium smegmatis*
592 *aphVIII+* for screening of inhibitors of serine-threonine protein kinases of eukaryotic type,
593 Patent RU 2566998, 2015.
594 [22] O.B. Bekker, D.N. Sokolov, O.A. Luzina, N.I. Komarova, Y.V. Gatilov, S.N.
595 Andreevskaya, et al., Synthesis and activity of (+)-usnic acid and (−)-usnic acid derivatives
596 containing 1,3-thiazole cycle against *Mycobacterium tuberculosis*, Med Chem Res. (2015) 1–13.
597 doi:10.1007/s00044-015-1348-2.
598 [23] D.A. Maslov, O.B. Bekker, A.G. Alekseeva, L.M. Kniazeva, D.A. Mavletova, I.I.
599 Afanasyev, et al., Aminopyridine- and aminopyrimidine-based serine/threonine protein kinase
600 inhibitors are drug candidates for treating drug-resistant tuberculosis, Bulletin of RSMU. (2017)
601 38–43. doi: 10.24075/brsmu.2017-01-04.
602 [24] J. Burian, S. Ramón-García, G. Sweet, A. Gómez-Velasco, Y. Av-Gay, C.J. Thompson,
603 The mycobacterial transcriptional regulator *whiB7* gene links redox homeostasis and intrinsic
604 antibiotic resistance, J. Biol. Chem. 287 (2012) 299–310. doi:10.1074/jbc.M111.302588.
605 [25] J.L. Martinez, F. Baquero, Mutation frequencies and antibiotic resistance, Antimicrob.
606 Agents Chemother. 44 (2000) 1771–1777. doi:10.1128/aac.44.7.1771-1777.2000.
607 [26] M. McGrath, N.C. Gey van Pittius, P.D. van Helden, R.M. Warren, D.F. Warner,
608 Mutation rate and the emergence of drug resistance in *Mycobacterium tuberculosis*, J
609 Antimicrob Chemother. 69 (2014) 292–302. doi:10.1093/jac/dkt364.
610 [27] K. Andries, P. Verhasselt, J. Guillemont, H.W.H. Göhlmann, J.-M. Neefs, H. Winkler, et
611 al., A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*, Science.
612 307 (2005) 223–227. doi:10.1126/science.1106753.
613 [28] T. O'Malley, E. Melief, Isolation and Characterization of Compound-Resistant Isolates of
614 *Mycobacterium tuberculosis*, in: Mycobacteria Protocols, Springer New York, New York, NY,
615 2015: pp. 317–328. doi:10.1007/978-1-4939-2450-9_19.

616 [29] G.D. Coxon, C.B. Cooper, S.H. Gillespie, T.D. McHugh, Strategies and challenges
617 involved in the discovery of new chemical entities during early-stage tuberculosis drug
618 discovery, J. Infect. Dis. 205 Suppl 2 (2012) S258–64. doi:10.1093/infdis/jis191.

Highlights

- New imidazo[1,2-b][1,2,4,5]tetrazines with substituted 3 and 6 positions synthesized
- 6-(alkylthio)imidazo[1,2-*b*][1,2,4,5]tetrazines obtained by CH-functionalization
- The compounds' reactivity assessed by oxidation and interaction with *N*-nucleophiles
- This class exhibits antibacterial activity on *M. tuberculosis* as STPK inhibitors
- Drug resistant mutants do not show cross-resistance with several other drugs