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Synthesis of novel isothiazolopyridines and their in vitro evaluation against *Mycobacterium* and *Propionibacterium acnes*

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ABSTRACT

In this paper we describe synthesis, structures and some physicochemical properties of **20** isothiazolopyridines **8**–**13** substituted differently into an isothiazole ring as well as their in vitro antibacterial assays against *Mycobacterium tuberculosis* H37Rv, *Mycobacterium fortuitum* PCM 672 and *Propionibacterium acnes* PCM 2400. Compound **13a** was found to be the most active derivative against *M. tuberculosis* H37Rv, demonstrating 100% growth inhibition of microorganisms in the primary screen (minimum inhibitory concentration [MIC] 6.25 μg/mL). Nineteen of the prepared compounds were evaluated against *M. fortuitum* PCM 672 and *P. acnes* PCM 2400 and only compounds **9** and **12d** exhibited excellent activity against individual strains of microorganisms with MIC_{90} <1 μg/mL. The inhibitory action of the remaining isothiazolopyridines towards the tested strains of the microorganism was low, absent, or a non-linear correlation prohibited accurate determination of MIC values. Unexpectedly, seven of the remaining isothiazolopyridines tested against *M. fortuitum* and *P. acnes* stimulated growth of the microorganisms in the range 10–50% or even more (**10b**) under experimental conditions.

1. Introduction

The treatment of mycobacterial infection has become an important problem due to emergence of multiple-drug-resistant microorganisms towards conventional therapeutic agents. Therefore there is a need to search for new antimycobacterial drugs with potent bioactivity and low toxicity. The new antimycobacterial agents are developed by modification of existing antimycobacterial drugs or by development of new classes of compounds. Among numerous new classes of compounds synthesized for this purpose, derivatives of bicyclic structures (benzoxazole, benzothiazole, benzisothiazole, quinolone, quinoxaline) have exhibited promising results.[1–9]

One of our research targets is synthesis of isothiazolo[5,4-*b*]pyridine derivatives for pharmaceutical purposes. We reported that several 4,6-dimethylisothazolo[5,4-*b*]pyridines, depending on substitution on the isothiazole ring, have biological activities including significant analgesic[10] or anorectic[11] effects. Other authors have also suggested antiaggregatory[12] and antiacne action[13] of derivatives of isothiazolo[5,4-*b*]pyridine. Additionally, derivatives of this system attracted our considerable interest as 7-azaanalogues of benzisothiazoles, which are cited for their promising activity against standard strains of *Mycobacterium tuberculosis* as well as against mycobacteria isolated from blood of infected patients (MIC 16–8 μg/mL).[3]

However, the mechanism of antimycobacterial action of benzisothiazoles remain unknown and there is poor knowledge about the structure–activity relationship which would be useful for rational projection of new bioactive compounds of this series.

Taking into account the interesting antimycobacterial activity of benzisothiazoles, a few of our 4,6-dimethylisothiazolo[5,4-*b*]pyridines possessing 4-aryl(benzyl)piperazinyl(piperidinyl) substructure linked to the N_2 or 3-O-atom of the isothiazolopyridine through different linkage (Fig. 1; **1** and **2**, respectively) and synthesized in connection with another project were additionally tested against *Mycobacterium tuberculosis* H37Rv.

By comparing the data concerning antimycobacterial potency against *M. tuberculosis* H37Rv within series **1** and **2** it was evident that insertion of the side chain in the N_2 position (series **1**) but not

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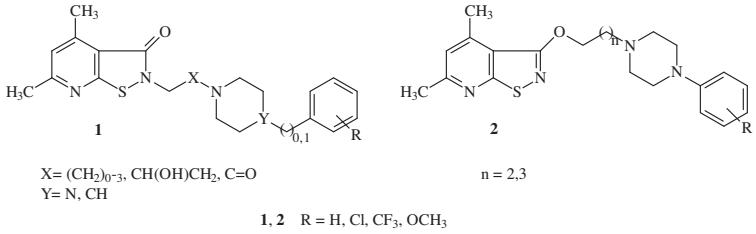


Figure 1. The general formula of isothiazolopyridines **1** and **2**.

in the 3-O position (series **2**), generates interesting activity against this strain of mycobacterium.[14,15] The most active compounds **1a–d** (Table 4) exhibited 100% inhibition of the mycobacteria after application at 6.25 μg/mL under preliminary testing.

The aim of this study was synthesis and evaluation under preliminary in vitro antibacterial screening of several classes of new isothiazolo[5,4-*b*]pyridines designed specially as potential antimycobacterial agents.

The new series of antimycobacterial isothiazolopyridines include derivatives with 4-nitrophenylpiperazine grouping in the structure of the side chain (**8**, **9**; Scheme 1), because such a substituent is characteristic for quinoxaline derivatives with significant antimycobacterial activity (MIC<1 μg/mL).[6]

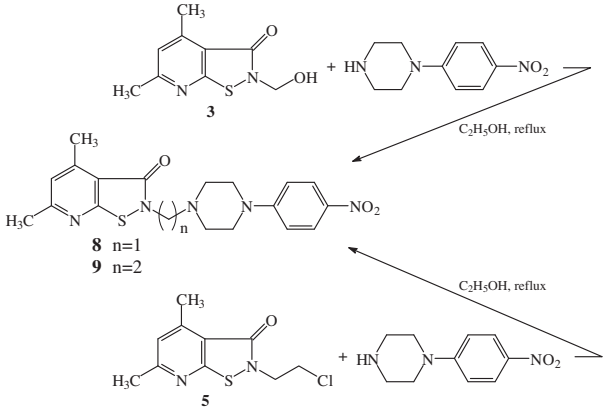
Pagani et al. revealed that carbamic esters of N_2-(2-hydroxyethyl)benzoisothiazole were interesting compounds for their promising in vitro antimycobacterial results (MIC 16–32 μg/mL).[4] In this context we prepared series of isomeric isothiazolopyridines of carbamic ester type **10** and **11** (Scheme 2).

As reported in the literature, N-alkylbenzylamines are compounds with specific action against mycobacteria.[16] On the other hand, our N_2-hydroxymethylisothiazolopyridine **3** (Fig. 2) showed 65% inhibition of *Mycobacterium tuberculosis* H37Rv.[15] A similar effect was exhibited by 2-hydroxymethylbenzoisothiazole **4** (Fig. 2).[5]

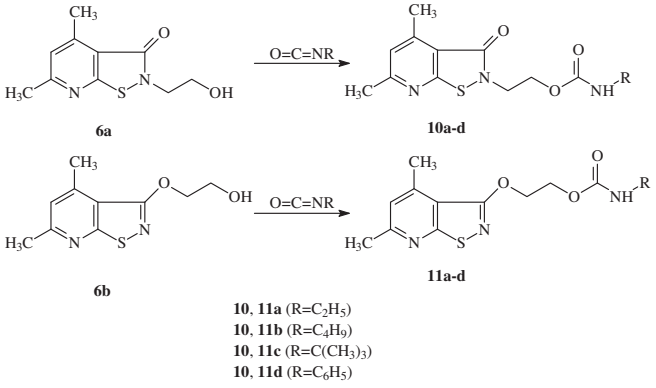
Based on these findings and combining benzylamines and hydroxymethylisothiazolopyridine **3** we prepared a series of hybrid compounds of specific Mannich base of type **12** as potential antimycobacterial agents (Scheme 3).

Additionally, to develop the structure–antimycobacterial relationship, we included in our investigation isomeric isothiazolopyridines **13a** and **13b** devoid of a base nitrogen atom within the structure of the side chain (Scheme 4).Compounds **8–13** were screened against *Mycobacterium*.

In 1984 and 1985, several patented *N*-(cyclo)alkyl, hydroxyalkyl, (un)substituted benzyl and carbamoylisothiazolo[5,4-*b*]pyridin-3-one compounds were claimed as useful agents against



Scheme 1. Synthetic route of compounds **8** and **9**.



Scheme 2. Synthetic route of compounds **10a–d** and **11a–d**.

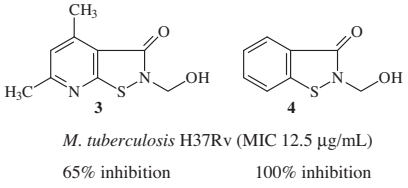
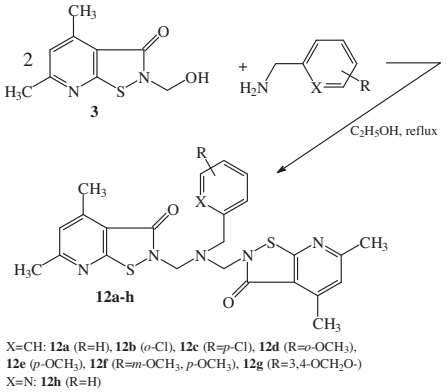
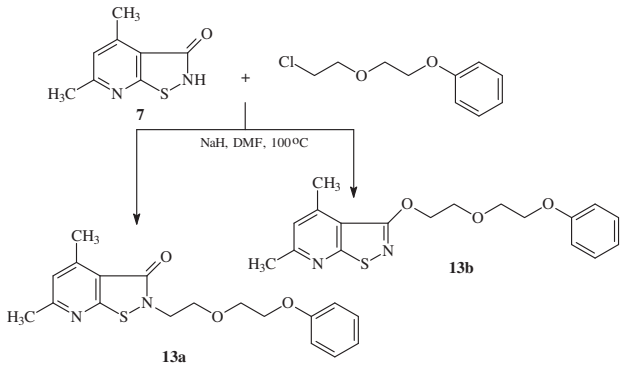


Figure 2. Antimycobacterial activity of 2-hydroxymethyl derivatives of isothiazolopyridine **3** and benzisothiazole **4**.



Scheme 3. Synthetic route of compounds **12a–h**.

Propionibacterium acnes,[13] which hydrolyzes sebum triglycerides to form the fatty acids responsible for inflammation in acne. Therefore all of our new isothiazolopyridines (except **11c**) were also tested against *Propionibacterium acnes* in order to complete their in vitro microbiological profile.



Scheme 4. Synthetic route of compounds **13a** and **13b**.

2. Chemistry

Different synthetic routes were used in preparation of the final isothiazolopyridines **8**–**13** (Schemes 1–4) with satisfactory yield.

In general, the new compounds were prepared starting from 4,6-dimethylisothiazolo[5,4-*b*]pyridin-3(2*H*)-one **7** (Scheme 4) or its hydroxy(chloro)alkyl derivatives **3**, **5**, **6** (Scheme 1–3). Intermediates **3**, **5**–**7** were described recently.[11,17–19]

Preparation of the 4-nitrophenylpiperazinylalkyl derivatives of isothiazolopyridine **8** and **9** involves alkylation of 4-nitrophenylpiperazine with corresponding intermediates **3** or **5**, respectively (Scheme 1).

The isomeric carbamates **10a**–**d** and **11a**–**d** were prepared after treatment of N_2- or 3-*O*-(2-hydroxyethyl)derivatives of isothiazolopyridine **6a** and **6b**, respectively, with appropriate alkyl and phenyl isocyanates (Scheme 2).

Alkylation of the commercially available benzylamines (un)substituted with different substituents in the *o*-, *m*- or *p*-positions (X = CH) or 2-aminomethylpyridine (X = N) with excess of 2-hydroxymethylisothiazolopyridine **3** led to formation of the specific Mannich bases of type **12** (Scheme 3).

Finally, alkylation of isothiazolopyridine **7** with 2-(2-phenoxy)ethoxyethyl chloride afforded the isomeric compounds **13a** and **13b** (Scheme 4).

Analytical purification of all new products **8**–**13** (20 compounds) was achieved by crystallization from the appropriate solvent. Final compounds were characterized by a sharp melting point, correct elemental (C, H, N) analyses, proton or carbon nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR) spectra, and in the case of **12d** by X-ray data.

The structures of the isomeric isothiazolopyridines **13a** and **13b** (Scheme 4) were assigned on the basis of ^{1}H NMR. In the N_2-substituted isomer **13a** the signal of the methylene protons adjacent to the N_2-nitrogen of the isothiazolopyridine was recorded ~3.8 ppm. The 3-O-substitution produced downfield shift of these protons (~4.7 ppm). The above spectral data agree with those previously reported by us for related isomeric N_2- and 3-*O*-hydroxyethyl substituted intermediates **6a** and **6b** (Scheme 2), respectively.

The ^{1}H NMR spectra of compounds **12**, independent of the type and position of R-substitution on the phenyl ring of the benzyl substructure (Scheme 3), showed a 2H singlet ~4.0 and 4H singlet ~5.0 ppm corresponding to the 2× NCH_2N methylene group and 1× arylmethylene group $ArCH_2N$. It may suggest a specific configuration of these compounds. Therefore the structure of the Mannich bases **12** were additionally determined by the X-ray diffraction method taking into account **12d** as a model compound (Section 2.1.).

Most of the compounds **8**–**13** were highly lipophilic substances and were characterized by $\log P_{calcd}$ 2.5–6.5 (Table 3). $\log P_{calcd}$ was calculated using the ChemPlus program from Hypercube, Inc., IBM PC version, implemented in the HyperChem program package.

The new synthesized compounds presented above, except for **8** and **9**, were tested in vitro for their activity against strains of *Mycobacterium tuberculosis* (18 compounds), and additionally all compounds **8**–**13**, except for **11c**, were evaluated against *Mycobacterium fortuitum* and *Propionibacterium acnes*.

2.1. Crystal structure of isothiazolopyridine 12d

The structure of compound **12d** was unambiguously confirmed by X-ray analysis (Fig. 3).

The geometry (bond lengths, angles and planarity) of the isothiazolopyridine rings in **12d** is very similar to that found in 4,6-dimethylisothiazolo[5,4-*b*]pyridine-3(2*H*)-one[20] and other related structures containing this ring.[21] The N atom of the tertiary amino group has pyramidal configuration with the sum of angles around it of 337.2° characteristic for sp^3 hybridization. The methylene bridges linking the isothiazolopyridine and phenyl rings with the central amino group have the *gauche–gauche–trans*, *gauche–trans–gauche* and *trans–gauche–trans* conformation in relation to the isothiazolopyridine rings A and B and phenyl ring C, respectively (Table 1) (see Fig. 4).

The dihedral angles between the mean planes of isothiazolopyridine and phenyl rings are: A/B = 55.74(4)°, A/C = 32.19(8)° and B/C = 87.72(9)°. The methoxy group, coplanar with the phenyl ring, is in *trans* conformation with respect to the methylene chain with the torsion angle C31–C32–O37–C38 of −178.1(3)°. The conformation of the molecule as a whole is stabilized by the C–H...X (X = O, N) intramolecular hydrogen bonds listed in Table 2.

The packing of molecules in the crystal structure of **12d** (Fig. 3) is governed by combination of a weak C12–H12C...O3A hydrogen bond linking the inversion-related molecules into dimers and a C11A–H11D...O3A hydrogen bond linking the dimers into molecular chains parallel to the *X* axis direction (Table 2).

Significant π–π interactions observed in the packing form the pairs of parallel isothiazole S1A...C8A rings and pairs of the parallel pyridine N7B...C6B rings belonging to inversion-related molecules with the centroid-to-centroid separations of 3.7348(15) and 3.9392(18) Å, respectively.

3. Microbiology and discussion

Structures of the compounds evaluated under the microbiological study are shown in Table 3.

Initially all compounds, except **8** and **9**, were screened against *Mycobacterium tuberculosis* H37Rv strain at a single concentration of 6.25 μg/mL. Rifampicin, an antibiotic known for its potent activity against many strains of *M. tuberculosis*, was used as a reference drug. The microbiological data were provided by the GWL Hansen's Disease Center (Colorado State University) within the Tuberculosis Acquisition and Coordinating Facility (TAACF: NIH, NIAID Contract AI45264) screening program for the discovery of novel drugs for treatment of tuberculosis.

All the new compounds, except **11c**, were additionally tested for their in vitro action against *Mycobacterium fortuitum* PCM 672 as well for *Propionibacterium acnes* PCM 2400 obtained from the Polish Collection of Microorganisms (PCM). Isoniazid or erythromycin, respectively, were used as reference drugs.

The results of antibacterial studies of isothiazolopyridines **8**–**13** with the data for the control drugs are presented in Table 3.

To develop the preliminary S-A relationship, microbiologically tested compounds **8**–**13** were divided into four series (Table 3):

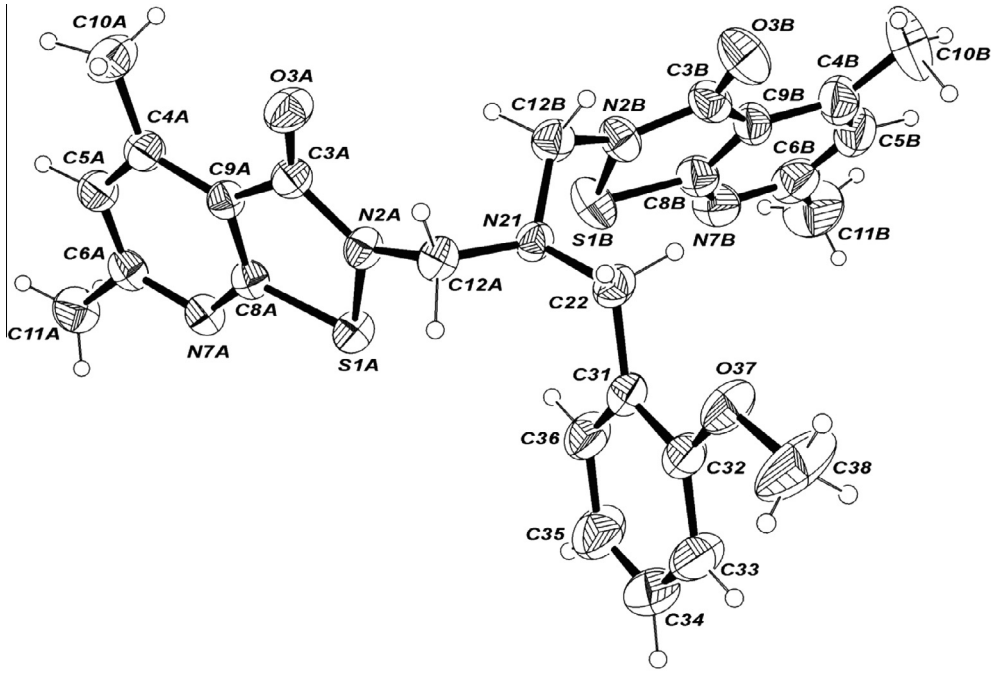


Figure 3. A view of the X-ray molecular structure of **12d** with the atomic labeling scheme (probability 50%).

Table 1
Selected torsion angles (°)

Torsion angle		Torsion angle	
S1A–N2A–C12A–N21	72.1(3)	S1B–N2B–C12B–N21	52.7(3)
N2A–C12A–N21–C12B	72.7(3)	N2B–C12B–N21–C12A	−153.6(2)
N2A–C12A–N21–C22	−159.4(2)	N2B–C12B–N21–C22	78.9(3)
C32–C31–C22–N21	−164.0(2)	C31–C32–O37–C38	−178.1(3)
C31–C22–N21–C12A	81.5(3)		
C31–C22–N21–C12B	−149.8(2)		

Table 2
Hydrogen-bond geometry (Å, °) for **12d**

D–H…A	D–H	H…A	D…A	D–H…A
C12A–H12C…O3A	0.97	2.46	2.850(3)	104
C10B–H10F…O3B	0.96	2.33	3.085(6)	135
C12B–H12F…O3B	0.97	2.51	2.879(4)	102
C36–H361…N21	0.93	2.50	2.843(4)	102
C11A–H11D…O3A$^{(i)}$	0.96	2.57	3.512(4)	166
C12A–H12C…O3A$^{(ii)}$	0.97	2.44	3.258(4)	142

Symmetry codes: (*i*) = 1 + *x*, *y*, *z*; (*ii*) = 1−x, 2−*y*, −*z*.

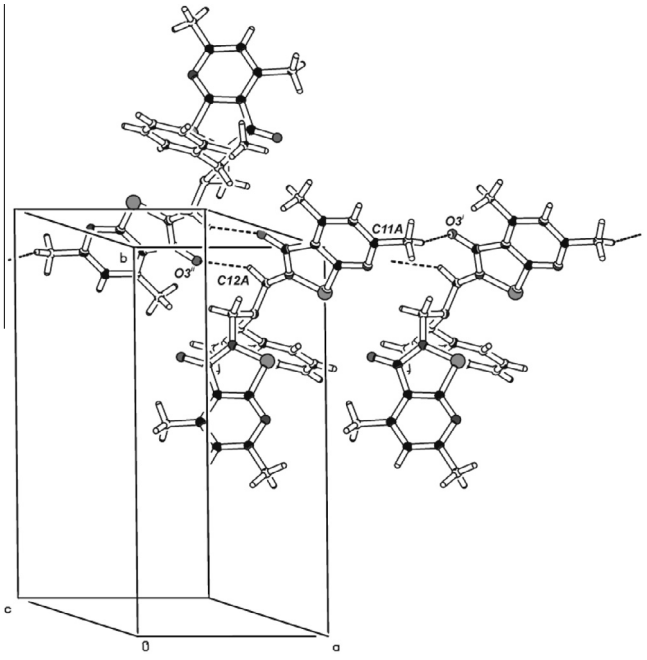


Figure 4. The molecular packing in crystal of **12d**. Dashed lines indicate intermolecular hydrogen bonds [symmetry codes: (*i*) = 1 + *x*, *y*, *z*; (*ii*) = 1−*x*, 2−*y*, −*z*)].

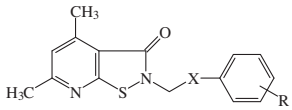
Series (I) N_2-substituted isothiazolopyridines **8**, **9**, **10d**, **13a** bearing in the structure of the side chain a terminal phenyl ring separated from the N_2-methyleneisothiazolopyridine fragment with a different spacer (*X*).
Series (II) N_2-substituted carbamates **10**, except for **10d**, which was included in series I.
Series (III) 3-O-substituted carbamates **11**. In this series 3-O-isomer **13b** was also included.
Series (IV) Specific Mannich bases of type **12**.

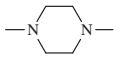
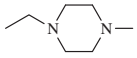
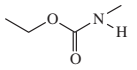
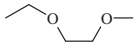
The data from Table 3 showed that carbamates **10a–c** (series II), **10d** (series I) and **11** (series III), which represent a group of compounds related to the antimycobacterial benzisothiazoles,[4] in contrast to their precursors, were completely inactive against *Mycobacterium tuberculosis* H37Rv.

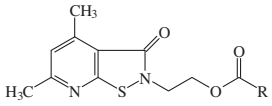
Similarly, lack of activity against this strain of mycobacterium was shown by highly lipophilic ($\log P_{calcd}$ ~5.5–6.6) Mannich bases **12** (Table 3, series IV), which combine corresponding benzylamines and two N_2-methyleneisothiazolopyridine moieties in a single molecule.

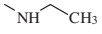
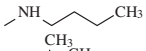
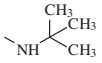
Significant action against *Mycobacterium tuberculosis* H37Rv (100% inhibition) was exhibited only by isothiazolopyridine **13a** (Table 3), devoid of a nitrogen atom within the structure of the side chain. At the same time, its 3-O-substituted isomer **13b** (Table 3, series III) was completely inactive under preliminary screening. The above results correspond with our recent observation within

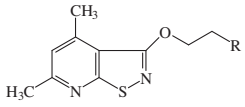
Table 3
In vitro antibacterial activity against Mycobacterium tuberculosis, Mycobacterium fortuitum, Propionibacterium acnes of isothiazolopyridines **8–13**

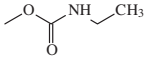
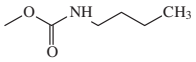
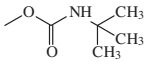
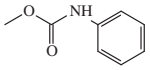
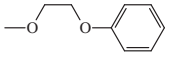


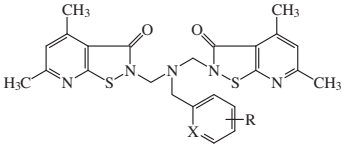
No.	X	R	*Mycobacterium tuberculosis*	*Mycobacterium fortuitum*		*Propionibacterium acnes*		Log P_{calcd}
			H37RV MIC 6.25 μg/mL % inhibition	MIC_{50} (μg/mL)	MIC_{90}	MIC_{50} (μg/mL)	MIC_{90}	
Series I								
8		*p*-NO_2	–	197	0	0	0	4.38
9		*p*-NO_2	–	0*	0	<1	<1	4.01
10d		H	12	0*	0	0	0	2.96
13a		H	100	nc	nc	nc	nc	2.98



	R					Log P_{calcd}
Series II						
10a		17	nc	nc	6.9	0	2.46
10b		0	125	0	0	0	2.63
10c		0	st	nc	nc	3.20	

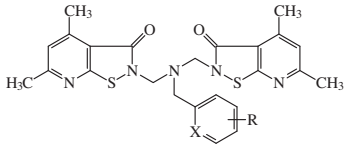


	R					Log P_{calcd}
Series III						
11a		6	st		184	0	1.58
11b		0	125	0	st		1.74
11c		16	–	–	–	–	2.31
11d		0	0	0	0	0	2.86
13b		0	nc	nc	0	0	2.69



	R	X						Log P_{calcd}
Series IV								
12a	H	CH	6	nc	nc		st	5.93
12b	*o*-Cl	CH	0	nc	nc	0	0	6.44
12c	*p*-Cl	CH	0	nc	nc	0	0	6.64
12d	*o*-OCH_3	CH	0	<1	<1	38	0	5.67
12e	*p*-OCH_3	CH	0	nc	nc		st	5.67
12f	*m*-OCH_3, *p*-OCH_3	CH	8	0*	0		st	5.42
12g	3,4-O$(CH_2)_2$O–	CH	0	nc	nc		st	5.61
12h	H	N	4	nc	nc	0*	0	5.01

Table 3 (*continued*)



R	X					LogP_{calcd}
Rifampicin		98 (125 μg/mL)				
Isoniazid			0.322 μg/mL	>250 μg/mL		
Erthromycin					<1 μg/mL	<1 μg/mL

—Compound not tested.
nc—At the lower concentration the preparation inhibited the growth of microorganisms, at the higher concentration this effect decreased and then increased again.
0—Not active up to 250 μg/mL
0*—Bacteriostatic effect against: *Mycobacterium fortuitum* – **9** (40–46% inh.), **10d** (42–50% inh.), **12f** (21–32% inh.), *Propionibacterim acnes* – **12h** (36–50% inh.).
st—Compound enhances bacterial replication: *Mycobacterium fortuitum* within range 10–50%: **10c** (max. effect was observed at concentration of 15.6–31.25 μg/mL), **11a** (0.97–250 μg/mL), *Propionibacterim acnes* within range 10–50%: **12a** (15.6 μg/mL), **12e**, **f** (0.97–250 μg/mL), **12g** (31.25–62.5 μg/mL); effect >50%: **11b** (3.9–15.6 μg/mL).

the series of isothiazolopyridines shown in Figure 1. For example, isothiazolopyridine **1a** (Table 4) exhibited 100% inhibition of mycobacteria under preliminary screening whereas its O-isomer (Fig. 1 and 2 (R = *m*-Cl, *n* = 2)) was a practically inactive compound.

Because the new isothiazolopyridines **10**–**12** and **13b** exhibited <20% inhibition in the preliminary screen against *M. tuberculosis* H37Rv at the assayed concentration of 6.25 μg/mL, these compounds were not further evaluated.

For compound **13a** causing >90% inhibition in the primary screen at concentration of 6.25 μg/mL a confirmatory advanced screening was performed against *M. tuberculosis* H37Rv in order to determine an actual MIC. In this investigation we also included recently synthesized and evaluated isothiazolopyridines **1a**–**d** (Fig. 1, Table 4), which under preliminary investigation exhibited significant action against *M. tuberculosis* H37Rv (100% inhibition, MIC 6.25 μg/mL).[14,15] Among these, compound **1b** resulted in the most inhibiting effect, with an MIC value of 1.56 μg/mL. For the remaining compounds MIC values were 6.25 μg/mL. So, the above results showed that none of the investigated compounds exhibited higher activity than that of rifampicin and the most interesting compound was ~12-fold less active than the reference drug, which showed an inhibition activity of 98% at a concentration of 0.125 μg/mL.

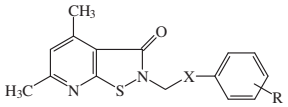
On the other hand, the significant inhibitory activity of **13a**, whose N_2-substituent structure represents a marked departure from those present in piperazine (piperidine) derivatives **1a**–**d** (Table 4), suggests that the nature of the central linkage *X* within series I (Table 3) does not play a relevant role in antimycobacterial activity.

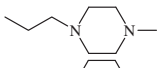
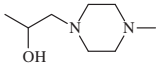
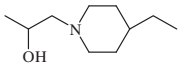
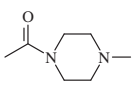
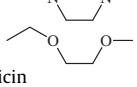
The balance of the therapeutic versus toxicological effects of bioactive agents is an important parameter when verifying their applicability as drugs. Therefore the most active isothiazolopyridines **1a**–**d** and **13a** were also tested in VERO cells for determination of cytotoxicity (IC_{50}) and the selectivity indexes (SI), defined as IC_{50}/MIC. All compounds demonstrate a degree of cytotoxicity ranging from 1.6 to 7 μg/mL and a low level of selectivity index SI = 0.25–2.69 (Table 4). The value of SI <10 indicates significant cytotoxicity, and these compounds were not considered to be evaluated further against *Mycobacterium tuberculosis*.

To determine whether the activity of isothiazolopyridines **1a**–**d** was exclusive to *Mycobacterium tuberculosis* H37Rv, compounds **1a** and **1b** were also tested against *Mycobacterium avium*, a naturally drug-resistant opportunistic pathogen, using clarithromycin as a standard. The tested compounds proved inactive after application at a concentration of 12.5 μg/mL, whereas clarithromycin exhibited inhibition of 98% of microorganisms at a concentration of 2 μg/mL.

Some authors suggest that activity against the rapidly growing and less hazardous microorganism *Mycobacterium fortuitum* may be used as a measure of anti-*M. tuberculosis* action.[3] To verify this hypothesis, our new isothiazolopyridines **8**–**13** (except for **11c**) were also evaluated against this strain of microorganisms in vitro

Table 4
Antimycobacterial activity against *M. tuberculosis* H37Rv and cytotoxic activity of isothiazolopyridines **1a**–**d** and **13a**



No.	X	R	*Mycobacterium tuberculosis* H37RV % inhib.	MIC (μg/mL)	IC_{50} VERO cells (μg/mL)	Selectivity index SI IC_{50}/MIC
1a		*m*-Cl	100	6.25	3.3	0.53
1b		*m*-CF_3	100	1.56	4.2	2.69
1c		H	100	6.25	1.6	0.25
1d		H	100	6.25	7.0	1.12
13a		H	100	6.25	4.1	0.68
Rifampicin			98	0.125	68	

at concentrations of 0.97–250 μg/mL. The results of anti-*Mycobacterium fortuitum* activity studies are presented in Table 3. Initially the MIC_{50} values were determined. Isoniazid was used as a standard drug (MIC_{50} = 0.322 μg/mL). The data from Table 3 showed that only Mannich base **12d** significantly reduced growth of microorganisms and this effect was observed for concentration below 1 μg/mL, similar as for the reference drug. It should be noted that isothiazolopyridine **12d** was also active against *M. fortuitum* at MIC_{90} <1 μg/mL, and this compound will be the subject of further investigation to confirm the preliminary results.

The fact that very similar analogs **12** of isothiazolopyridine **12d** did not inhibit mycobacteria suggests that a specific interaction may exist between compound **12d** and some components specific for the *Mycobacterium fortuitum* strain.

The action of the remaining isothiazolopyridines against *Mycobacterium fortuitum* was generally poor, with MIC_{50} values of 125 μg/mL for carbamates **10b** and **11b** and 197 μg/mL for nitrophenylpiperazine derivative **8**. In this context it should be noted that isothiazolopyridines **9**, **10d** and **12f** were not active against *Mycobacterium fortuitum* at the MIC_{50} level; however, these compounds inhibited growth of 20–50% of microorganisms at all concentrations used. Thus, these isothiazolopyridines exhibited predominantly a bacteriostatic effect.

Taking into account the fact that the new isothiazolopyridines tested were inactive in general against *M. fortuitum* as well as against *M. tuberculosis* H37Rv, it is difficult to conclude whether the in vitro anti-*M. tuberculosis* H37Rv activity of our compounds can be related to their anti-*M. fortuitum* action.

The results of an initial in vitro microbiological evaluation of nineteen of our isothiazolopyridines against *Propionibacterium acnes* revealed that four of them (**9**, **10a**, **11a**, **12d**) were efficient antibacterial agents at the MIC_{50} level (Table 3). However, only **9** showed strong activity and produced >90% inhibition of microorganisms (MIC_{90} >1 μg/mL). Compound **9** also demonstrated higher activity at the concentration range of 1–0.25 μg/mL when compared to the reference drug (erythromycin).[22]

Studies are currently ongoing to explain the mode of unique action of **9** against *Propionibacterium acnes*. It should be noted that isothiazolopyridine **12h** inhibited 21–32% of microorganisms at all concentrations used. Thus, the compound has a bacteriostatic effect against *Propionibacterium acnes*.

The remaining isothiazolopyridines tested were inactive against *Mycobacterium fortuitum* and *Propionibacterium acnes* (0, Table 3), non-linear correlations prohibited accurate determination of MIC values (nc, Table 3) or unexpectedly **7** of them (carbamates **10c**, **11a**, **b** and Mannich bases **12a**, **e**, **f**, **g**) stimulated bacterial growth (st, Table 3). The compounds which stimulated growth of microorganisms were classified as high stimulating (stimulation >50%) and low stimulating (stimulation 10–50%) agents. However, it is unclear whether the low increase in bacterial replication ~10% is a consequence of enhancement of bacterial growth by the preparations or a lack of activity of the compounds to allow the natural growth of the microorganisms.

A low growth stimulation effect (10–50%) of the *Mycobacterium fortuitum* strain was shown by carbamates **10c** and **11a**. Compound **11a** stimulated at all concentrations used (0.97–250 μg/mL) whereas **10c** exhibited the best stimulation effect within the range of 15.6–31.25 μg/mL (Table 3).

The best stimulant of *Propionibacterium acnes* was carbamate **11b**, which exhibited the effect of stimulation >50% observed at a concentration of 3.9–15.6 μg/mL. Mannich bases **12a**, **e**, **f**, **g** have been found to stimulate growth of this strain of bacteria in the range of 10–50% at different concentrations (Table 3).

In this context of bacterial growth stimulation by compounds **11** and **12**, it should be noted that carbamates, like Mannich bases, are unstable substances and it cannot be ruled out that nitrogen-containing products of degradation of the side chain may be utilized by microorganisms to increase growth stimulation.

4. Conclusion

To investigate the influence of the introduction of various substituents in the isothiazolopyridine nucleus on antibacterial activity, twenty compounds **8**–**13** were synthesized and in vitro microbiologically evaluated, including activity against *Mycobacterium tuberculosis* H37Rv, *Mycobacterium fortuitum* PMC 672 and *Propionibacterium acnes* PCM 2400.

We found that the most active against *Mycobacterium tuberculosis* H37Rv, compound **13a** and isothiazolopyridines **1** (Table 4) recently investigated in this test, were characterized by MIC values within the range 1.56–6.25 μg/mL (Table 3). However, this activity was about 10–50 times lower than that observed for the reference drug rifampicin (0.125 μg/mL). Additionally, these compounds were characterized by a low value of the selectivity index (SI 0.25–2.69) and therefore they could not be considered for further evaluation. The investigation also proved rather low anti-*Mycobacterium fortuitum* activity of the tested compounds **8**–**13**, because only derivative **12d** produced 90% reduction of this microorganism at concentration <1 μg/mL. Only compound **9** exhibited significant activity against *Propionibacterium acnes* (MIC_{90} <1 μg/mL). The activity of **9** was better than that of erythromycin within the concentration range of 1–0.25 μg/mL. Unexpectedly, 7 of the 19 isothiazolopyridines tested against *M. fortuitum* and *P. acnes* stimulated growth of microorganisms in the range of 10–50% or even more (**11b**).

The promising isothiazolopyridines **9** and **12d** will be subjected to advanced biochemical investigation.

5. Experimental

5.1. Chemistry

5.1.1. Chemical experimental section

Melting points were determined with a Mel-Temp II apparatus (Laboratory Devices, USA) and were uncorrected. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 300 MHz spectrometer in $CDCl_3$ using tetramethylsilane (TMS) as an internal reference (chemical shift in δ ppm). The IR (KBr) spectra were recorded on a Specord-75 IR Spectrometer. Elemental C, H, N analyses were run on a Carlo Erba NA-1500 analyzer. The results were within ±0.4% of the values calculated for the corresponding formulae. Chromatographic separations were performed on a silica gel [Kieselgel 60 (70–230 mesh), Merck] column (CC). Progress of the reaction was monitored by TLC on silica gel plates with fluorescent indicator (Fluka) and visualized by UV light at 254 nm.

5.1.1.1. 2-[4-Nitrophenyl(piperazinyl)methyl]-4,6-dimethylisothiazolo[5,4-*b*]pyridin-3(2*H*)-one 8. To a stirred mixture of 2.4 mmol of 2-hydroxymethyl-4,6-dimethylisothiazolo[5,4-*b*]pyridin-3(2*H*)-one **3** in 20 mL of ethanol 2.4 mmol of 4-nitrophenylpiperazine was added. Next the mixture was stirred for 2 h at room temperature and then refluxed for 5 h. After cooling, the precipitated, crude product was filtered off and crystallized from methanol.

Mp: 201–203 °C, yield: 70%.

IR: 1650 (CO).

^{1}H NMR: 2.60 (s, 3H, CH_3), 2.76 (s, 3H, CH_3), 2.81–2.97 (m, 4H, $CH_{2\text{-piperazine}}$), 3.10–3.25 (m, 4H, $CH_{2\text{-piperazine}}$), 4.70 (s, 2H, N–CH_2–N), 6.76 (s, 1H, $H_{\beta\text{-pyridine}}$), 6.90–6.99 (m, 2H, ArH), 8.05–8.20 (m, 2H, ArH).

5.1.1.2. 2-{2-[4-Nitrophenyl(piperazinyl)]ethyl}-4,6-dimethylisothiazolo[5,4-*b*]pyridin-3(2*H*)-one 9. To a stirred mixture of 1.2 mmol of 2-(2-chloro)ethyl-4,6-dimethylisothiazolo[5,4-*b*]pyridin-3(2*H*)-one **5** in 30 mL of ethanol 2.4 mmol of 4-nitrophenylpiperazine was added. Next the mixture was refluxed for 20 h. After cooling, the solvent was distilled off. The product was isolated from the resulting residue by column chromatography (ethyl acetate, R_f = 0.61).

Mp: 164–166 °C, yield: 37%.

IR: 1650 (CO).

^{1}H NMR: 2.62 (s, 3H, CH_3), 2.70–2.85 (m, 9H, CH_3 and $N(CH_2)_3$), 3.40–3.55 (m, 4H, $CH_{2\text{-piperazine}}$), 4.00–4.25 (m, 2H, $N_{isothiazole}$–CH_2), 6.76 (s, 1H, $H_{\beta\text{-pyridine}}$), 6.85–7.01 (m, 2H, ArH), 8.10–8.25 (m, 2H, ArH).

^{13}C NMR: 164.9, 163.7, 162.8, 154.7, 149.8, 138.8, 125.9, 122.5, 114.8, 112.9, 56.5, 52.4, 47.0, 40.2, 24.5, 17.5.

5.1.1.3. General procedure for preparation of the isomeric carbamates 10a–d and 11a–d. To a stirred mixture of a catalytic amount of 1,4-diazabicyclo[2.2.2]octane (DABCO) and 3.75 mmol of appropriate alkyl or phenyl isocyanate in 15 mL of dry xylene the solution of 2.5 mmol of 2-*N*- or 3-*O*-(2-hydroxyethyl) derivatives of isothiazolopyridine **6a** and **6b** in 10 mL of dry xylene was added. The reaction mixture was refluxed for 5 h. Next the solvent was distilled off and the resulting residue was chromatographed (except for **11b**). The obtained, crude product was crystallized from the appropriate solvent.

2-[2-(*N*-Ethylcarbamoiloxy)ethyl]-4,6-dimethylisotiazolo[5,4-*b*]pyridin-3(2*H*)-one 10a. Mp: 101–103 °C (*n*-hexane), yield: 54%.

CC (ethyl acetate, R_f = 0.56).

IR: 1660 (CO), 1720 (CO), 3340 (NH).

^{1}H NMR: 1.12 (m, 3H, CH_3CH_2), 2.59 (s, 3H, CH_3), 2.73 (s, 3H, CH_3), 3.16–3.25 (m, 2H, CH_3CH_2), 4.09 (t, J = 4.8 Hz, 2H, N_2–CH_2), 4.34 (t, J = 4.8 Hz, 2H, CH_2O), 4.86 (br s, 1H, NH), 6.93 (s, 1H, $H_{\beta\text{-pyridine}}$)

2-[2-(*N*-*n*-Buthylcarbamoiloxy)ethyl]-4,6-dimethylisothiazolo5,4-*b*pyridin-3(2*H*)-one 10b. Mp: 79–81 °C (cyclohexane), yield: 61.7%.

CC (ethyl acetate/chloroform 1:1, R_f = 0.54).

IR: 1670 (CO), 1735 (CO), 3420 (NH).

^{1}H NMR: 0.89 (t, J = 7.2 Hz, 3H, $CH_3CH_2CH_2CH_2$), 1.25–1.37 (m, 2H, $CH_3CH_2CH_2CH_2$), 1.40–1.50 (m, 2H, $CH_3CH_2CH_2CH_2$), 2.57 (s, 3H, CH_3), 2.71 (s, 3H, CH_3), 3.14–3.16 (m, 2H, $CH_3CH_2CH_2CH_2$), 4.07 (t, J = 5.1 Hz, 2H, N_2–CH_2), 4.32 (t, J = 5.1 Hz, 2H, CH_2O), 4.89 (br s, 1H, NH), 6.91 (s, 1H, $H_{\beta\text{-pyridine}}$).

2-[2-(*N*-*tert*-Buthylcarbamoiloxy)ethyl]-4,6-dimehtylisothiazolo5,4-*b*pyridin-3(2*H*)-one 10c. Mp: 106–107 °C (cyclohexane), yield: 18%.

CC (ethyl acetate/chloroform 1:1, R_f = 0.51).

IR: 1670 (CO), 1730 (CO), 3310 (NH).

^{1}H NMR: 1.31 (s, 9H, $C(CH_3)_3$), 2.60 (s, 3H, CH_3), 2.75 (s, 3H, CH_3), 4.07 (t, J = 5.2 Hz, 2H, N_2–CH_2), 4.32 (t, J = 5.1 Hz, 2H, CH_2O), 4.72 (br s, 1H, NH), 6.92 (s, 1H, $H_{\beta\text{-pyridine}}$).

2-[2-(*N*-Phenylcarbamoiloxy)ethyl]-4,6-dimethylizothiazolo[5,4-*b*]pyridin-3(2*H*)-one 10d. Mp: 135–136 °C (cyclohexane), yield: 61.3%.

CC (ethyl acetate/chloroform 1:1, R_f = 0.60).

IR: 1670 (CO), 1710 (CO), 3310 (NH).

^{1}H NMR: 2.59 (s, 3H, CH_3), 2.73 (s, 3H, CH_3), 4.15 (t, J = 5.4 Hz, 2H, N_2–CH_2), 4.45 (t, J = 5.4 Hz, 2H, CH_2O), 4.76 (br s, 1H, NH), 6.93 (s, 1H, $H_{\beta\text{-pyridine}}$), 7.03–7.08 (m, 2H, ArH), 7.29–7.39 (m, 3H, ArH).

^{13}C NMR: 164.5, 162.4, 152.8, 137.5, 129.1, 123.7, 122.7, 119.8, 118.9, 63.2, 42.7, 24.2, 17.5.

3-[2-(*N*-Ethylcarbamoiloxy)ethoxy]-4,6-dimethylisothiazolo5,4-*b*pyridine 11a. Mp: 125–127 °C (cyclohexane), yield: 33.8%.

CC (ethyl acetate/chloroform 1:1, R_f = 0.63).

IR: 1690 (CO), 3330 (NH).

^{1}H NMR: 1.12 (m, 3H, CH_3CH_2), 2.62 (s, 3H, CH_3), 2.66 (s, 3H, CH_3), 3.05–3.43 (m, 2H, CH_3CH_2), 4.53 (t, J = 4.8 Hz, 2H, CH_2O), 4.68 (t, J = 4.8 Hz, 2H, OCH_2), 4.94 (br s, 1H, NH), 6.93 (s, 1H, $H_{\beta\text{-pyridine}}$).

3-[2-(*N*-*n*-Butylcarbamoiloxy)ethoxy]-4,6-dimethylisothiazolo5,4-*b*pyridine 11b. Mp: 119–121 °C (cyclohexane), yield: 49.4%.

IR: 1690 (CO), 3320 (NH).

^{1}H NMR: 0.91 (t, J = 7.2 Hz, 3H, $CH_3CH_2CH_2CH_2$), 1.01–1.12 (m, 2H, $CH_3CH_2CH_2CH_2$), 1.24–1.48 (m, 2H, $CH_3CH_2CH_2CH_2$), 2.63 (s, 3H, CH_3), 2.64 (s, 3H, CH_3), 3.09–3.17 (m, 2H, $CH_3CH_2CH_2CH_2$), 4.46 (t, J = 5.2 Hz, 2H, CH_2O), 4.66 (t, J = 5.2 Hz, 2H, OCH_2), 4.92 (br s, 1H, NH), 6.92 (s, 1H, $H_{\beta\text{-pyridine}}$).

3-[2-(*N*-*tert*-Buthylcarbamoiloxy)ethoxy]-4,6-dimethylisothiazolo5,4-*b*pyridine 11c. Mp: 109–111 °C (*n*-heptane), yield: 30.5%.

CC (ethyl acetate/chloroform 1:1, R_f = 0.55).

IR: 1730 (CO), 3260 (NH).

^{1}H NMR: 1.31 (s, 9H, $C(CH_3)_3$), 2.62 (s, 3H, CH_3), 2.65 (s, 3H, CH_3), 4.42–4.52 (m, 2H, CH_2O), 4.64–4.72 (m, 3H, OCH_2 and NH), 6.92 (s, 1H, $H_{\beta\text{-pyridine}}$).

3-[2-(*N*-Phenylcarbamoiloxy)ethoxy]-4,6-dimethylisothiazolo5,4-*b*pyridine 11d. Mp: 144–146 °C (cyclohexane), yield: 32.8%.

CC (ethyl acetate/chloroform 1:1, R_f = 0.65).

IR: 1740 (CO), 3230 (NH).

^{1}H NMR: 2.61 (s, 6H, $2CH_3$), 4.64-4.67 (m, 4H, OCH_2CH_2O), 4.99 (br s, 1H, NH), 6.93 (s, 1H, $H_{\beta\text{-pyridine}}$), 7.05–7.37 (m, 5H, ArH).

^{13}C NMR: 160.3, 152.2, 136.8, 128.1, 122.8, 121.0, 118.0, 65.7, 62.1, 24.1, 18.4.

5.1.1.4. General procedure for preparation of benzylamine derivatives of isothiazolo5,4-bpyridine 12. To a stirred mixture of 10 mmol 2-hydroxymethyl-4,6-dimethylisothiazolo[5,4-*b*]pyridin-3(2*H*)-one **3** in 20 mL of ethanol 5 mmol of an appropriate benzylamine was added. Next the mixture was refluxed for 2 h. After cooling, the precipitated, crude product (**12a–c**, **12e**, **12g**) was filtered off and crystallized from the appropriate solvent. In the case of compounds **12d** and **12h–f** the ethanol solution was evaporated and the oil residue was cleaned by crystallization with charcoal from appropriate solvent.

***N*,*N*-Bis(4,6-dimethyl-3-oxo-2,3-dihydroisothiazolo5,4-*b*]pyridin-2-ylmethyl)-*N*-benzylamine 12a.** Mp: 192–194 °C (ethanol), yield: 36.7%.

IR: 1670 (CO).

^{1}H NMR: 2.59 (s, 6H, $2CH_3$), 2.72 (s, 6H, $2CH_3$), 3.97 (s, 2H, NCH_2), 4.94 (s, 4H, $2N_2$–CH_2), 6.92 (s, 2H, $2H_{\beta\text{-pyridine}}$), 7.22–7.52 (m, 5H, ArH).

***N*,*N*-Bis(4,6-dimethyl-3-oxo-2,3-dihydroisothiazolo5,4-*b*]pyridin-2-ylmethyl)-*N*-2-chlorobenzylamine 12b.** Mp: 190–192 °C (ethanol), yield: 45.6%.

IR: 1670 (CO).

^{1}H NMR: 2.59 (s, 6H, $2CH_3$), 2.72 (s, 6H, $2CH_3$), 4.14 (s, 2H, NCH_2), 5.00 (s, 4H, $2N_2$–CH_2), 6.90 (s, 2H, $2H_{\beta\text{-pyridine}}$), 7.15–7.36 (m, 4H, ArH).

***N,N*-Bis(4,6-dimethyl-3-oxo-2,3-dihydroisothiazolo5,4-*b*]pyridin-2-ylmethyl)-*N*-4-chlorobenzylamine 12c.** Mp: 162–164 °C (ethanol), yield: 38%.

IR: 1670 (CO).

^{1}H NMR: 2.60 (s, 6H, $2CH_3$), 2.71 (s, 6H, $2CH_3$), 3.94 (s, 2H, NCH_2), 4.93 (s, 4H, $2N_2$–CH_2), 6.93 (s, 2H, $2H_{\beta\text{-pyridine}}$), 7.14–7.30 (m, 4H, ArH).

***N,N*-Bis(4,6-dimethyl-3-oxo-2,3-dihydroisothiazolo5,4-*b*]pyridin-2-ylmethyl)-*N*-2-methoxybenzylamine 12d.** Mp: 178–180 °C (cyclohexane), yield: 28.7%.

IR: 1660 (CO).

^{1}H NMR: 2.57 (s, 6H, $2CH_3$), 2.70 (s, 6H, $2CH_3$), 3.80 (s, 3H, OCH_3), 4.06 (s, 2H, NCH_2), 4.97 (s, 4H, $2N_2$–CH_2), 6.89 (s, 2H, $2H_{\beta\text{-pyridine}}$), 7.21–7.51 (m, 4H, ArH).

^{13}C NMR: 165.2, 163.4, 162.8, 157.8, 150.0, 130.7, 128.9, 125.2, 122.4, 120.5, 115.3, 110.4, 61.0, 55.2, 48.7, 24.5, 17.5.

***N,N*-Bis(4,6-dimethyl-3-oxo-2,3-dihydroisothiazolo5,4-*b*]pyridin-2-ylmethyl)-*N*-4-methoxybenzylamine 12e.** Mp: 152–154 °C (ethanol), yield: 46%.

IR: 1670 (CO).

^{1}H NMR: 2.59 (s, 6H, $2CH_3$), 2.72 (s, 6H, $2CH_3$), 3.78 (s, 3H, OCH_3), 3.94 (s, 2H, NCH_2), 4.93 (s, 4H, $2N_2$–CH_2), 6.88 (s, 2H, $2H_{\beta\text{-pyridine}}$), 7.22–7.49 (4H, ArH).

***N,N*-Bis(4,6-dimethyl-3-oxo-2,3-dihydroisothiazolo5,4-*b*]pyridin-2-ylmethyl)-*N*-2-(3,4-dimethoxyphenylo)methylamine 12f.** Mp: 157–159 °C (acetone), yield: 30%.

IR: 1670 (CO).

^{1}H NMR: 2.59 (s, 6H, $2CH_3$), 2.72 (s, 6H, $2CH_3$), 2.81–3.10 (m, 4H, NCH_2CH_2), 3.79 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 4.97 (s, 4H, $2N_2$–CH_2), 6.70–6.74 (m, 3H, 3ArH), 6.94 (s, 2H, $2H_{\beta\text{-pyridine}}$).

***N,N*-Bis(4,6-dimethyl-3-oxo-2,3-dihydroisothiazolo5,4-*b*]pyridin-2-ylmethyl)-*N*-3,4-methylenodioxybenzylamine 12g.** Mp: 179–181 °C (ethanol), yield: 48.5%.

IR: 1650 (CO).

^{1}H NMR: 2.59 (s, 6H, $2CH_3$), 2.71 (s, 6H, $2CH_3$), 3.84 (s, 2H, NCH_2), 4.93 (s, 4H, $2N_2$–CH_2), 5.91 (s, 2H, OCH_2O) 6.66–7.31 (m, 5H, $2H_{\beta\text{-pyridine}}$ and 3ArH).

***N,N*-Bis(4,6-dimethyl-3-oxo-2,3-dihydroisothiazolo5,4-*b*]pyridin-2-ylmethyl)-*N*-2-pyridylmethylamine 12h.** Mp: 181–183 °C (cyclohexane/toluene 1:2), yield: 26.4%.

IR: 1660 (CO).

^{1}H NMR: 2.59 (s, 6H, $2CH_3$), 2.72 (s, 6H, $2CH_3$), 4.14 (s, 2H, NCH_2), 5.05 (s, 4H, $2N_2$–CH_2), 7.03 (s, 2H, $2H_{\beta\text{-pyridine}}$), 7.16–7.89 (m, 3H, $2H_\beta + H_{\gamma\text{-pyridine}}$), 8.67–8.77 (m, 1H, $H_{\alpha\text{-pyridine}}$).

5.1.1.5. 2-[2-(2-Phenoxyethoxy)ethyl]-4,6-dimethylisothiazolo[5,4-b]pyridin-3(2H)-one 13a and 3-[2-(2-phenoxyethyl)ethoxy]-4,6-dimethylisothiazolo[5,4-b]pyridine 13b. To a solution of sodium ethoxide prepared from 0.1 mol of sodium and 100 mL of anhydrous ethanol 0.1 mol of phenol and 0.2 mol of bis(2-chloroethyl)ether were added. The reaction mixture was refluxed 12 h and then filtered. The solvent was removed and the oily residue was distilled under reduced pressure. The 136 °C/5 mm Hg fraction was collected to give 6 g of oily 2-(2-phenoxy)ethoxyethyl chloride. 0.02 mol of 2-(2-phenoxy)ethoxyethyl chloride were added to the stirred mixture of 0.01 mol of 4,6-dimethylisothiazolo[5,4-*b*]pyridin-3(2*H*)-one **7** and 0.01 mol of sodium hydride (~60% suspension in mineral oil) in 50 mL of anhydrous dimethylformamide (DMF). The reaction mixture was heated at 100 °C for 10 h and the solvent was distilled off. The isomers **13a** and **13b** were isolated by column chromatography (toluene/ethyl acetate – 10:1). Evaporation fraction of $R_f = 0.53$ afforded 1.15 g of O-isomer **13b** mp: 67–69 °C (*n*-hexane). Evaporation fraction of $R_f = 0.28$ gave 0.44 g of N-isomer **13a** mp: 59–61 °C.

Compound **13a** ^{1}H NMR: 2.58 (s, 3H, CH_3), 2.72 (s, 3H, CH_3), 3.79–3.9 (4H, $2 \times CH_2$), 4.02–4.19 (m, 4H, $2 \times CH_2$), 6.83–6.99 (m, 4H, 3ArH and $H_{\beta\text{-pyridine}}$), 7.16–7.35 (m, 2H, 2ArH).

Compound **13b** ^{1}H NMR: 2.62 s (3H, CH_3), 2.64 s (3H, CH_3), 3.92–4.15 m (6H, $OCH_2CH_2OCH_2$), 4.63–4.69 m (2H, 3-OCH_2), 6.84–6.93 m (4H, 3ArH and $H_{\beta\text{-pyridine}}$), 7.18–7.35 (m, 2H, 2ArH).

5.2. Crystallography

5.2.1. X-ray structure determinations of 12d

X-ray data of **12d** were collected on the Bruker SMART APEX II CCD diffractometer; crystal sizes 0.21 × 0.13 × 0.04 mm, MoKα ($\lambda = 0.71073$ Å) radiation, ω scans, absorption correction: multi-scan SADABS,[23] $T_{min}/T_{max} = 0.9509/0.9904$. The structure was solved by direct methods using SIR92[24] and refined by full-matrix least-squares with SHELXL97.[25] The H atoms were positioned geometrically and treated as riding on their parent C atoms with C–H distances of 0.93 Å (aromatic), 0.97 Å (CH_2) and 0.96 Å (CH_3). All H atoms were refined with isotropic displacement parameters taken as 1.5 times those of the respective parent atoms. All calculations were performed using WINGX version 1.64.05 package.[26] CCDC-859050 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre (CCDC), 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 (0) 1223 336 033; email: deposit@ccdc.cam.ac.uk].

5.2.1.1. Crystal data of 12d. $C_{26}H_{27}N_5O_3S_2$, $M = 521.65$, monoclinic, space group $P2_1/c$, $a = 8.2284(3)$, $b = 16.8045(6)$, $c = 20.1143(6)$ Å, $\beta = 110.743(1)°$, $V = 2601.00(15)$ Å^3, $Z = 4$, $d_{calcd} = 1.332$ Mg m^{-3}, $F(000) = 1096$, μ(Mo Kα) = 0.242 mm^{-1}, $T = 293$ K, 21949 measured reflections (θ range 1.62–19.58°), 2282 unique reflections ($R_{int} = 0.026$), final $R = 0.029$, $wR = 0.078$, $S = 1.068$ for 2013 reflections with $I > 2\sigma(I)$.

5.3. Pharmacology

5.3.1. In vitro evaluation of antimycobacterial activity against *M. tuberculosis* H37Rv

Primary screening was conducted at 6.25 μg/mL against *M. tuberculosis* H37Rv in BACTEC 12B medium using the BACTEC 460 radiometric system.[27] Compounds causing <90% inhibition in the primary screen (MIC >6.25 μg/mL) were not considered for further evaluation. Compounds demonstrating at least 90% inhibition in the primary screen were re-tested by serial dilution beginning at the concentration of 6.25 μg/mL to determine the actual minimum inhibitory concentration (MIC). The MIC is defined as the lowest concentration inhibiting 99% of the inoculum.

Also, compounds were screened by serial dilution to assess toxicity to a VERO cell line (IC_{50}), beginning at 10 × MIC if sample solubility in culture media was permitted. The selectivity index (SI) is defined as the ratio of the measured IC_{50} in VERO cells to the MIC described above.

5.3.2. In vitro evaluation of antibacterial activity against *Mycobacterium fortuitum*

(PCM 672), *Staphylococcus aureus* (PCM 2602) and *Propionibacterium acnes* (PCM 2400)

5.3.2.1. Bacterial strains and growing conditions. The strains of *Mycobacterium fortuitum* (PCM 672) and *Staphylococcus aureus* (PCM 2602) and *Propionibacterium acnes* (PCM 2400) (obtained from the Polish Collection of Microorganisms (PCM) of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences) were used throughout the study. Bacteria were cultivated on liquid 79 culture medium (for *M. fortuitum*), Luria–Bertani (LB) medium (for *S. aureus*) at 37 °C for 24 h under aerobic conditions. Then bacterial cells were diluted with the same media, respectively, to obtain suspension of about 2×10^5 cfu/mL of each strain.

5.3.2.2. Antibacterial susceptibility test. The antibacterial activities of synthesized compounds were determined against bacterial strains by the microplate Alamar Blue assay according to Ahmed et al.[28] Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO) 1 mg/mL and were diluted with appropriate media in the range 0.03–1000 μg/mL on a cell culture microtitration plate. To the wells containing 100 μL of drug compound, aliquots of 100 μL of the diluted suspension of the strain were added. The control wells consisting of either bacteria only or medium only and those containing different drug concentrations (100 μL) were inoculated with 100 μL of the diluted bacterial cells. Plates were incubated at 37 °C for 48 h and after that 20 μL of Alamar Blue (10× diluted) and 12.5 μL of 20% Tween 80 solutions were added to the wells and incubation was continued at 37 °C for 2 h. Fluorescence was measured using Victor apparatus (Wallac, Perkin Elmer). The experiment was repeated two or three times. The minimal inhibitory concentration (MIC) was defined as the lowest drug concentration which prevented a color change from blue to pink, inhibiting the bacterial growth by $\geqslant$90%. The means and standard error values were determined using the program STATISTICA.

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