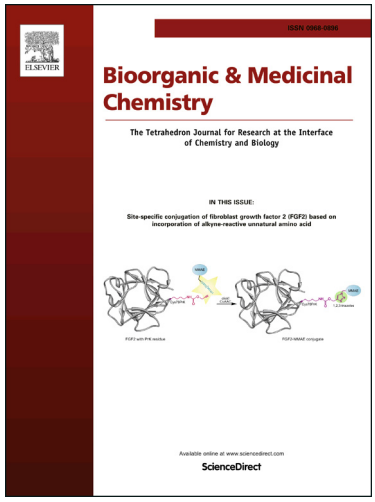
Accepted Manuscript



Development of Water-Soluble 3,5-Dinitrophenyl Tetrazole and Oxadiazole Antitubercular Agents

Jaroslav Roh, Galina Karabanovich, Hana Vl ková, Alejandro Carazo, Jan N me ek, Pavel Sychra, Lenka Valá šková, Oto Pavliš, Ji ina Stola íková, V ra Klimešová, Kate ina Vávrová, Petr Pávek, Alexandr Hrabálek

PII: S0968-0896(17)31113-6
DOI: http://dx.doi.org/10.1016/j.bmc.2017.08.010
Reference: BMC 13913

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 25 May 2017
Revised Date: 1 August 2017
Accepted Date: 7 August 2017

Please cite this article as: Roh, J., Karabanovich, G., ková, H.V., Carazo, A., me ek, J.N., Sychra, P., Valá šková, L., Pavliš, O., íková, J.i.S., Klimešová, V.r., Vávrová, K.i., Pávek, P., Hrabálek, A., Development of Water-Soluble 3,5-Dinitrophenyl Tetrazole and Oxadiazole Antitubercular Agents, *Bioorganic & Medicinal Chemistry* (2017), doi: http://dx.doi.org/10.1016/j.bmc.2017.08.010

Development of Water-Soluble 3,5-Dinitrophenyl Tetrazole and Oxadiazole Antitubercular Agents

Jaroslav Roh,[a,] Galina Karabanovich,[a] Hana Vlčková,[a] Alejandro Carazo,[a] Jan Němeček,[a] Pavel Sychra,[a] Lenka Valášková,[a] Oto Pavliš,[b] Jiřina Stolaříková,[c] Věra Klimešová,[a] Kateřina Vávrová,[a] Petr Pávek,[a] and Alexandr Hrabálek [a]*

[a] ` Ü-êäÉë=† åáî Éêëáíó Hradec Králové, Czech Republic

[b] Military Health Institute, Biological Defense Department, Techonin, Czech Republic

[c] Regional Institute of Public Health, Department of Bacteriology and Mycology, Partyzánské náměstí 7, 70200 Ostrava, Czech Republic

ABSTRACT

In this work, four series of tertiary amine-containing derivatives of 3,5-dinitrophenyl tetrazole and oxadiazole antitubercular agents were prepared, and their *in vitro* antimycobacterial effects were evaluated. We found that the studied compounds showed lipophilicity-dependent antimycobacterial activity. The *N*-benzylpiperazine derivatives, which had the highest lipophilicity among all of the series, showed the highest *in vitro* antimycobacterial activities against *Mycobacterium tuberculosis* CNCTC My 331/88 ($H_{37}Rv$), comparable to those of the

first-line drugs isoniazid and rifampicin. The presence of two tertiary amines in these *N*-benzylpiperazine derivatives enabled us to prepare water-soluble dihydrochloride salts, overcoming the serious drawback of previously described 3,5-dinitrophenyl tetrazole and oxadiazole lead compounds. The water-soluble 3,5-dinitrophenyl tetrazole and oxadiazole antitubercular agents described in this work are good candidates for further *in vitro* and *in vivo* pharmacokinetic and pharmacodynamic studies.

Keywords

Tuberculosis; Antitubercular agent; Mycobacterium tuberculosis; Structure-activity relationships; Lipophilicity; Solubility

Introduction

Tuberculosis (TB) is a widespread infectious disease caused by *Mycobacterium tuberculosis (M.tb.)* that took 1.8 million lives in 2015 and led to 10.4 million new cases. The major problem with TB treatment is the occurrence of drug-resistant strains of *M.tb.*[1] Multidrug-resistant (MDR) strains are resistant to at least two of the most powerful anti-TB drugs: isoniazid (INH) and rifampicin (RIF). Extensively drug-resistant (XDR) strains are resistant to all first-line anti-TB drugs and several second-line drugs, such as fluoroquinolones and injectable aminoglycosides.[2] The treatment of drug-resistant forms of TB usually lasts more than 20 months and has lower cure rates.[1] Thus, the development of new, efficient anti-TB drugs with different modes of actions is vital for success against TB. Recently, two new anti-TB drugs have been approved for treating pulmonary MDR-TB: nitroimidazol derivative delamanide[3] and diarylquinoline derivative bedaquiline.[4,5] Several experimental anti-TB compounds are in the preclinical and clinical phases of development, such as nitro group-containing nitroimidazoles PA-824[6] or TBA-

354[7] and the inhibitor of mycobacterial decaprenylphosphoryl-β-D-ribofuranose 2′-oxidase (DprE1) PBTZ-169 (Figure 1).[8-11]

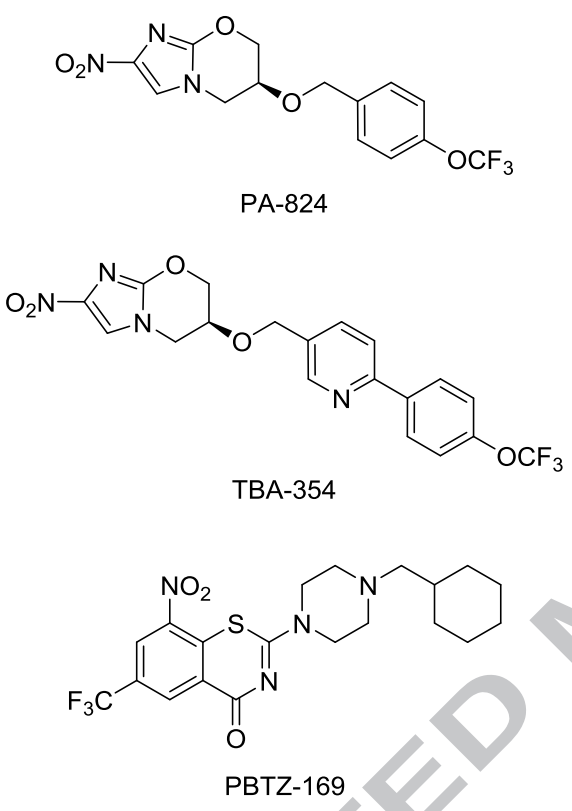


Figure 1. Structures of the nitro group-containing anti-TB agents nitroimidazoles PA-824 and TBA-354 and benzothiazinone PBTZ-169.

In our previous studies, we found that 1-substituted 5-(3,5-dinitrobenzylsulfanyl)tetrazoles (**1**) showed good *in vitro* antimycobacterial activity, in some cases comparable to that of the first-line anti-TB drug INH.[12] The structure-activity relationship study revealed the importance of both 3,5-dinitrobenzylsulfanyl and tetrazole moieties for high antimycobacterial effects and indicated that the lipophilicity of substituents on tetrazole can influence the compound's activity. Later, the 2,5-regioisomers, i.e., 2-substituted 5-(3,5-dinitrobenzylsulfanyl)tetrazoles (**2**), showed slightly better antimycobacterial effects than did the 1,5-regioisomers **1**[13] and became parent structures

for two new classes of potent antitubercular agents, 2-substituted 5-(3,5-dinitrobenzylsulfanyl)-1,3,4-oxadiazoles (**3**)[14] and 2-substituted 5-(3,5-dinitrophenyl)-2*H*-tetrazoles (**4**) (Figure 2). [15] 3,5-Dinitrobenzylsulfanyl oxadiazoles **3** showed outstanding *in vitro* antimycobacterial activity, with minimum inhibitory concentration (MIC) values reaching 0.03 μM against drug-susceptible and drug-resistant *M.tb.* strains. Furthermore, these compounds were highly effective against a non-replicating *M.tb.* strain and showed low cytotoxicity and genotoxicity. In contrast to other 3,5-dinitro-substituted antitubercular agents,[16] oxadiazoles **3** act in a DprE1-independent pathway.[14] Thus, 3,5-dinitrobenzylsulfanyl oxadiazoles **3** were chosen as one of the lead structures in this study (Figure 2).

The second lead structures of this study, 2-substituted 5-(3,5-dinitrophenyl)-2*H*-tetrazoles (**4**), are the analogues of tetrazoles **2** without the methylsulfanyl linker between 3,5-dinitrophenyl and tetrazole moieties.[15] These easily accessible compounds had submicromolar MICs against drug-susceptible and MDR/XDR strains of *M.tb.* and again showed low cytotoxicity in mammalian cell lines. Furthermore, these compounds are sulfur-free, which might be beneficial for their metabolic stability (Figure 2).

The third lead structures, *S*-substituted 5-(3,5-dinitrophenyl)oxadiazole-2-thiols (**5**), are the reverse analogues of oxadiazoles **3** (Figure 2).[17] As in the structure of tetrazoles **4**, the 3,5-dinitrophenyl group is attached directly to the heterocycle. 3,5-Dinitrophenyl oxadiazoles **5** showed similarly outstanding *in vitro* antimycobacterial activity as their parent 3,5-dinitrobenzylsulfanyl oxadiazoles **3**, with MIC values against drug-susceptible and MDR/XDR *M.tb.* strains as low as 0.03 μM. Furthermore, oxadiazoles **5** had highly selective antimycobacterial effects, because they showed low cytotoxicity against mammalian cell lines and had no antibacterial or antifungal activities.[17] However, the abovementioned lead structures suffered from very low aqueous solubility.

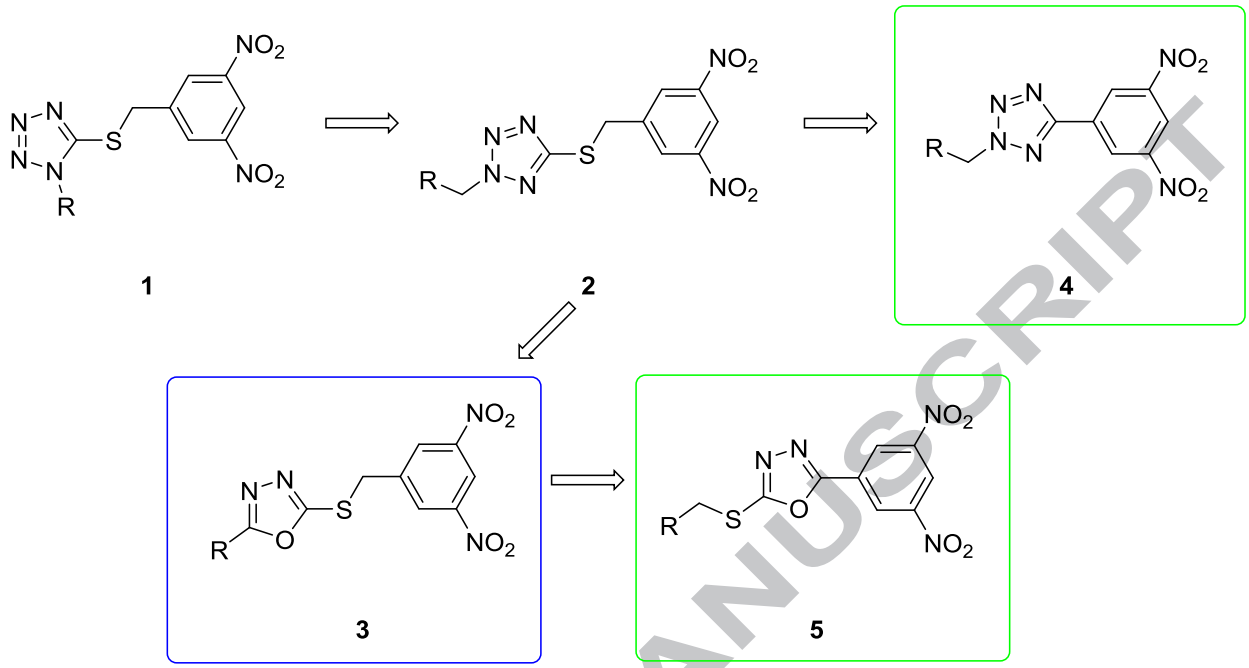


Figure 2. Antitubercular lead compounds used in this study: 3,5-dinitrobenzylsulfanyl oxadiazoles **3**[14] and 3,5-dinitrophenyl tetrazoles **4**[15] and oxadiazoles **5**.[17]

The aim of this study was to prepare derivatives of the lead structures **3**, **4** and **5** with increased aqueous solubility and to study their antimycobacterial activity. A way to improve the aqueous solubility is to introduce an acidic/basic moiety into the structure and to form a corresponding salt. As we previously demonstrated that acidic functionality has devastating effects on antimycobacterial activities of the abovementioned lead structures,[12] we decided to introduce a basic amine moiety into the structure. This approach has been widely used successfully to increase the aqueous solubility of water-insoluble drugs and bioactive substances.[11, 18-23] Therefore, derivatives **6a-d** and **7a-d** of the lead structure **3**, derivatives **8a-d** of the lead structure **4** and derivatives **9a-d** of the lead structure **5** with at least one tertiary amine in the substituent R were prepared, and their *in vitro* antimycobacterial activities were evaluated

(Figure 3). Compounds with highest *in vitro* antimycobacterial activity were converted to water-soluble hydrochloride salts, and their *in vitro* antitubercular activities were re-evaluated. Furthermore, the cytotoxicity of these water-soluble hydrochloride salts was assessed using four mammalian cell lines.

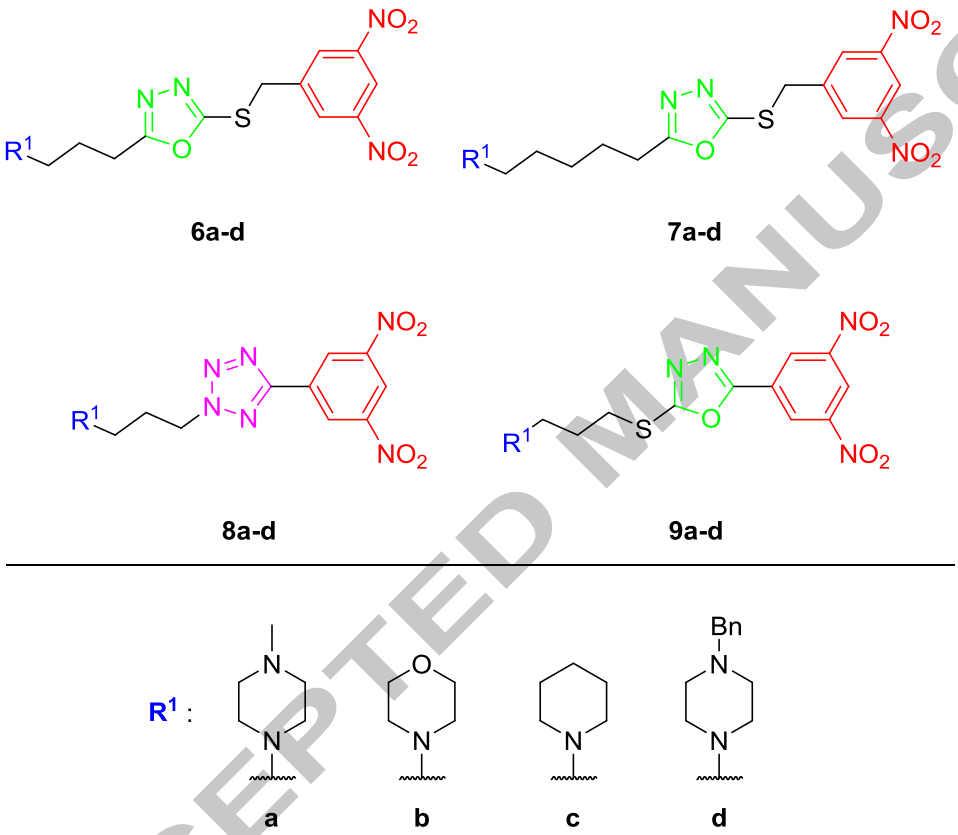
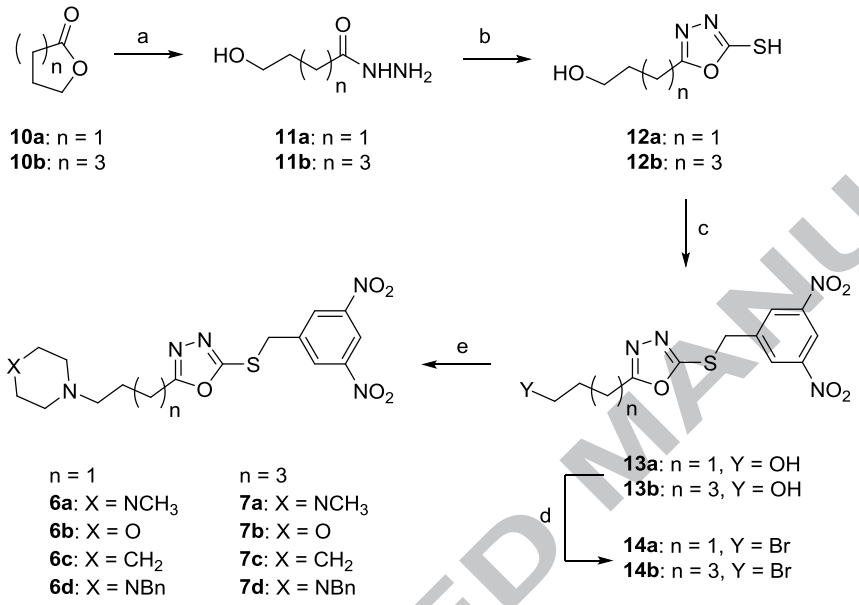


Figure 3. Tertiary amine-containing compounds of series **6**, **7**, **8** and **9** studied in this work.

Results and discussion

Chemistry. Tertiary amine-containing 2-substituted 5-(3,5-dinitrobenzylsulfanyl)-1,3,4-oxadiazoles **6a-d** and **7a-d** were prepared as shown in Scheme 1. Hydrazinolysis of the lactones **10a** and **10b** provided corresponding ω-hydroxyalkanehydrazides **11a** and **11b**, which were converted to 5-(ω-hydroxyalkyl)-1,3,4-oxadiazole-2-thiols **12a** and **12b**, respectively, by the

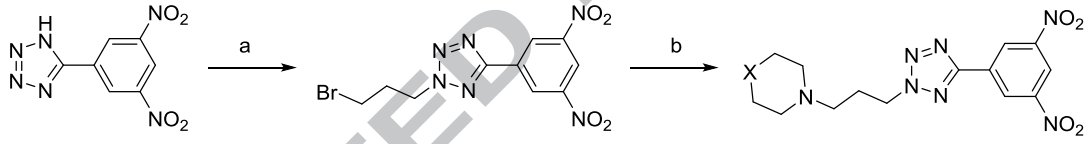
reaction with carbon disulfide under alkaline conditions. Oxadiazole-2-thiols **12a** and **12b** were then alkylated using 3,5-dinitrobenzyl chloride, and their hydroxy groups were converted to bromo groups and finally substituted with *N*-methylpiperazine, morpholine, piperidine or *N*-benzylpiperazine heterocycle (Scheme 1).



Scheme 1. Synthesis of tertiary amine-containing 2-subsituted 5-(3,5-dinitrobenzylsulfanyl)-1,3,4-oxadiazoles **6a-d** and **7a-d**. Reagents and conditions: (a) $NH_2NH_2.H_2O$, EtOH; rt, 24 h, 77% (**11a**); reflux, 70 h, 80% (**11b**); (b) CS_2, KOH, EtOH; rt, 48 h, 43% (**12a**); reflux, 24 h, 95% (**12b**); (c) 3,5-$(NO_2)_2$BnCl, Et_3N, MeCN, rt, 6 h, 82% (**13a**), 72% (**13b**) (d) PPh_3, NBS, rt, 3 h, 90% (**14a**), 71% (**14b**); (e) heterocyclic amine, MeCN; for conditions and yields, see Experimental section.

The 2-substituted 5-(3,5-dinitrophenyl)-2*H*-tetrazoles **8a-d** were prepared via two-step synthesis from 5-(3,5-dinitrophenyl)-1*H*-tetrazole (**15**, Scheme 2). In the first step, tetrazole **15**

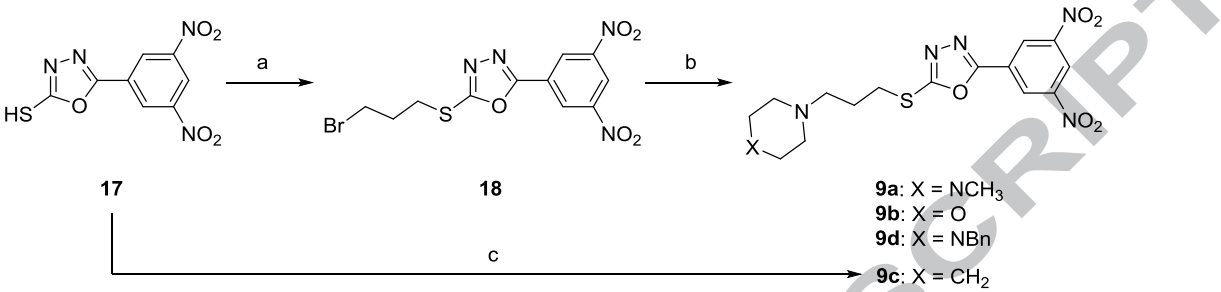
was alkylated using 1,3-dibromopropane, and the predominantly formed 2-regiosomer, 2-(3-bromopropyl)-5-(3,5-dinitrophenyl)-2*H*-tetrazole (**16**), was isolated. The chemical shift of the tetrazole carbon in the product **16** was above 160 ppm, which is typical for 2-substituted 5-aryltetrazoles.[15, 24] Furthermore, the correlation of the NCH_2 hydrogens (t, 4.96 ppm) in compound **16** with three nitrogen atoms was clearly visible in the ^{1}H-^{15}N gHMBC experiment, which would not be possible if the substituent was attached to position 1. Negligible correlation of the NCH_2 hydrogens with the aromatic hydrogens in 1D NOESY experiment further supports the presence of 2,5-regioisomer (see Supplementary material). The reaction of tetrazole **16** with *N*-methylpiperazine, morpholine, piperidine or *N*-benzylpiperazine gave the final products **8a**, **8b**, **8c** and **8d**, respectively (Scheme 2). 5-(3,5-Dinitrophenyl)-1*H*-tetrazole (**15**) was prepared as described previously.[15]



Scheme 2. Synthesis of tertiary amine-containing 2-substituted 5-(3,5-dinitrophenyl)-2*H*-tetrazoles **8a-d**. Reagents and conditions: (a) $Br(CH_2)_3Br$, Et_3N, MeCN, reflux, 5 h, 60%; (b) heterocyclic amine, MeCN; for conditions and yields, see Experimental section.

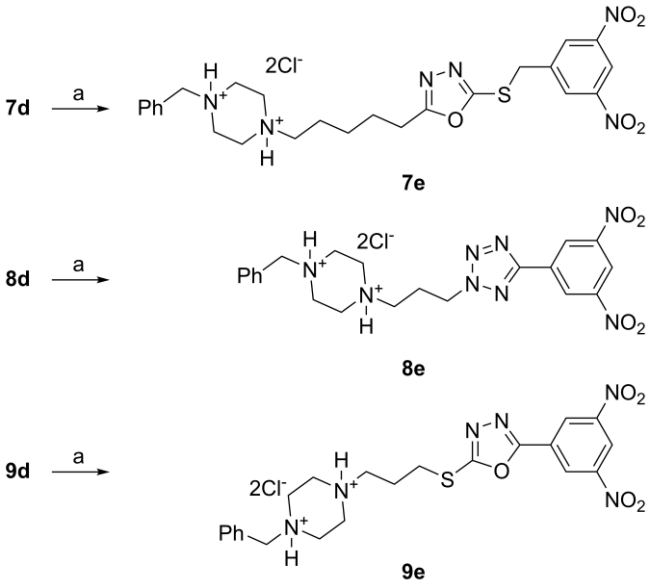
The synthesis of *S*-substituted 5-(3,5-dinitrophenyl)-1,3,4-oxadiazole-2-thiols **9a-d** started with the alkylation of 5-(3,5-dinitrophenyl)-1,3,4-oxadiazole-2-thiol **17**[17] with 1,3-dibromopropane (Scheme 3). The reaction of the resulting 2-(3-bromopropylsulfanyl)-5-(3,5-

dinitrophenyl)-1,3,4-oxadiazole **18** with *N*-methylpiperazine, morpholine, piperidine or *N*-benzylpiperazine heterocycles gave the final products **9a**, **9b**, **9c** and **9d**, respectively.



Scheme 3. Synthesis of tertiary amine-containing *S*-substituted 5-(3,5-dinitrophenyl)oxadiazole-2-thiols **9a-d**. Reagents and conditions: (a) $Br(CH_2)_3Br$, Et_3N, MeCN, 50 °C, 10 h, 61%; (b) heterocyclic amine, MeCN; for conditions and yields, see Experimental section; (c) 1-(3-chloropropyl)piperidine hydrochloride, Et_3N, MeCN, reflux, 12 h, 65%.

Finally, the three *N*-benzylpiperazine derivatives with the highest antimycobacterial activities, **7d**, **8d** and **9d**, were converted to water-soluble dihydrochloride salts **7e**, **8e** and **9e**, respectively, by dissolving the compounds in aq. hydrochloric acid and subsequent evaporation of all the volatiles (Scheme 4).



Scheme 4. Synthesis of dihydrochloride salts **7e**, **8e** and **9e** of *N*-benzylpiperazine derivatives **7d**, **8d** and **9d**. Reagents and conditions: (a) HCl, H_2O, 60 °C

The solubilities of dihydrochloride salts **7e**, **8e** and **9e** in pure water at room temperature were 17.1 mM (9 mg/mL), 20.9 mM (11 mg/mL) and 22.8 mM (12.5 mg/mL), respectively. As the solubility of these compounds was pH-dependent, we also measured the solubility in 0.05 M citrate buffer (pH 3), 0.1 M acetate buffer (pH 5) and phosphate-buffered saline (PBS, pH 7.4) using HPLC. The results are summarized in Table 1.

Table 1. Solubilities (mM) of dihydrochloride salts **7e**, **8e** and **9e** in selected buffers.

	pH 3 **citrate buffer**	**pH 5** **acetate buffer**	**pH 7.4** **PBS**
7e	18.0 ± 0.4	9.0 ± 1.0	0.113 ± 0.007
8e	19.2 ± 0.6	11.6 ± 1.4	0.051 ± 0.005
9e	21.2 ± 0.4	14.2 ± 0.6	0.075 ± 0.005

***In vitro* antimycobacterial activity**

All tertiary amine-containing compounds of series **6**, **7**, **8** and **9** were evaluated for their *in vitro* antimycobacterial activity against *M.tb.* CNCTC My 331/88 ($H_{37}Rv$) and the non-tuberculous mycobacterial strains of *M. avium* CNCTC My 330/88, *M. kansasii* CNCTC My 235/80 and the clinically isolated *M. kansasii* 6509/96. The antimycobacterial activity of all compounds was evaluated after 14 days of incubation. The activities are expressed as minimum inhibitory concentrations (MICs). INH and RIF were used as standard anti-TB drugs (Table 2).

The antimycobacterial activities of all tertiary amine-containing compounds of series **6**, **7**, **8** and **9** were strongly influenced by their lipophilicity. *N*-Methylpiperazine derivatives **6a**, **7a**, **8a** and **9a** with very low lipophilicities (log P < 1) showed negligible antimycobacterial activity. The highest activities in all series showed the most lipophilic *N*-benzylpiperazine derivatives, i.e., compounds **6d**, **7d**, **8d** and **9d**. The MIC values of these compounds were 2, 1, 1 and 0.125 μM against the *M.tb.* strain, respectively, which are comparable to the activity of the first-line anti-TB drug INH. *N*-Benzylpiperazine-substituted 5-(3,5-dinitrophenyl)-1,3,4-oxadiazole **9d** showed the highest activity among all of the prepared compounds, with MIC values against all four tested mycobacterial strains better than those of INH and comparable with those of RIF.

Table 2. Calculated log P and antimycobacterial activities of compounds of series **6**, **7**, **8** and **9** expressed as MICs (μM) after 14 days of incubation.

	CLog P [a]	***M. tuberculosis*** **My 331/88**	***M. avium*** **My 330/88**	***M. kansasii*** **My 235/80**	***M. kansasii*** **6509/96**
6a	- 0.18	>32	1000	>32	>32
6b	0.75	8	500	8	8
6c	1.96	16	250	16	16

6d	3.03	2	32	2	2
7a	0.88	32	250	>32	>32
7b	1.81	8	125	8	8
7c	3.02	16	62.5	16	32
7d	4.09	**1**	16	2	2
8a	0.64	>32	250	32	>32
8b	1.55	16	500	16	16
8c	2.77	4	62.5	16	16
8d	3.84	**1**	8	2	**1**
9a	0.50	>32	125	16	> 32
9b	1.41	8	125	8	16
9c	2.62	8	62.5	32	16
9d	3.69	**0.125**	2	**1**	**0.5**
INH		**0.5**	**>250**	**>250**	**4**
RIF		**0.25**	**32**	**0.25**	**0.125**

[a] Calculated using ChemDraw Professional 15.0

The most active compounds **7d**, **8d** and **9d** were converted to water-soluble dihydrochloride salts **7e**, **8e** and **9e** and their *in vitro* antitubercular effects were re-evaluated. We found that the antimycobacterial activities of dihydrochloride salts remained excellent (Table 3). Moreover, dihydrochloride salts **7e**, **8e** and **9e** were highly efficient against clinically isolated MDR/XDR strains of *M.tb*. (Table 4). We can expect that in the biological system the equilibrium between (di)hydrochloride salt and the free-base forms of compounds of series **6**, **7**, **8** and **9** will occur according to pH in given compartments and, thus, that a lipophilic free-base form would be able to distribute itself through the biological system.

Table 3. Antimycobacterial activities of dihydrochlorides **7e**, **8e** and **9e** expressed as MICs (μM) after 14 days of incubation.

	M. tuberculosis **My 331/88**	*M. avium* **My 330/88**	*M. kansasii* **My 235/80**	*M. kansasii* **6509/96**
7e	2	32	2	2
8e	**1**	32	**1**	**1**
9e	**0.125**	2	2	**1**
INH	**0.5**	**>250**	**>250**	**4**
RIF	**0.25**	**32**	**0.25**	**0.125**

Table 4. Antimycobacterial activities of dihydrochlorides **7e**, **8e** and **9e** against seven clinically isolated MDR/XDR-TB strains expressed as MICs (μM) after 14 days of incubation.

	MDR/XDR *M. tuberculosis* strains						
	Praha 1	**Praha 4**	**Praha 131**	**9449/2007**	**234/2005**	**7357/1998**	**8666/2010**
7e	2	2	2	2	2	2	2
8e	**1**	**1**	**1**	**1**	**1**	**1**	**1**
9e	**0.125**	**0.25**	**0.125**	**0.125**	**0.125**	**0.25**	**0.125**
Streptomycin	16 (R)	>32 (R)	>32 (R)	>32 (R)	32 (R)	>32 (R)	>32 (R)
Isoniazid	16 (R)	16 (R)	16 (R)	64 (R)	16 (R)	16 (R)	32 (R)
Ethambutol	32 (R)	16 (R)	32 (R)	8 (S)	16 (R)	16 (R)	16 (R)
Rifampicin	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)
Ofloxacin	1 (S)	>16 (R)	16 (R)	2 (S)	0.5 (S)	8 (R)	8 (R)
Gentamicin	1 (S)	0.5 (S)	>8 (R)	1 (S)	0.25 (S)	1 (S)	2 (S)
Clofazimine	0.5 (R)	0.5 (R)	0.25 (S)	0.125 (S)	0.125 (S)	0.125 (S)	2 (R)

Amikacin	0.5 (S)	1 (S)	>32 (R)	0.5 (S)	0.5 (S)	1 (S)	2 (S)

S – strain susceptible to given anti-TB drug; R - strain resistant to given anti-TB drug

***In vitro* cell proliferation/viability assays**

To gain basic insight into the toxicity of the most active water-soluble derivatives **7e**, **8e** and **9e** in mammalian cell lines, their *in vitro* effects on Caco-2 (Caucasian colon adenocarcinoma), HepG2 (human hepatocellular carcinoma), A431 (human epidermoid carcinoma) and COS-1 (African green monkey kidney) cell lines were evaluated (Table 5). Because the values of IC_{50} were not reached for any compound, the data are presented as a relative viability at a concentration of 30 μM compared to the vehicle treated controls (100% viability). The results showed that the dihydrochloride salts of *N*-benzylpiperazine derivatives **7e**, **8e** and **9e** had negligible effects on cell viability after 48 h of treatment, thus demonstrating good selectivity toward mycobacterial cells.

Table 5. Viability determined by viability cell assay (CellTiter96® Assay) after 48 hours treatment with the dihydrochloride salts of *N*-benzylpiperazine derivatives **7e**, **8e** and **9e** in the Caco-2, HepG2, A431 and COS-1 cell lines. Vehicle-treated control viability was set to 100%.

	Caco-2		**HepG2**		**A431**		**COS-1**	
	IC_{50} (μM)	Viability at 30 μM	IC_{50} (μM)	Viability at 30 μM	IC_{50} (μM)	Viability at 30 μM	IC_{50} (μM)	Viability at 30 μM
7e	⩾30	105	⩾30	112	⩾30	86	⩾30	99
8e	⩾30	102	⩾30	118	⩾30	100	⩾30	100
9e	⩾30	121	⩾30	121	⩾30	93	⩾30	121

Conclusion

In this work, the tertiary amine-containing derivatives of previously described 3,5-dinitrophenyl tetrazole[15] and oxadiazole[14, 17] antitubercular lead compounds were prepared, and their antimycobacterial activities were studied. We found that all of the tertiary amine-containing compounds showed lipophilicity-dependent antimycobacterial activity. Compounds with low lipophilicity (log P < 1) practically lost their effects against mycobacteria. Among all of the series, the *N*-benzylpiperazine derivatives with the highest lipophilicities, i.e., **6d**, **7d**, **8d** and **9d**, showed the highest antimycobacterial activities. Their MIC values against *M.tb.* CNCTC My 331/88 ($H_{37}Rv$) were comparable to those of first-line anti-TB drugs INH and RIF, ranging from 0.125 to 2 μM. Dihydrochloride salts **7e**, **8e** and **9e** of these *N*-benzylpiperazine derivatives showed good activity against drug-susceptible and multidrug-resistant strains of *M.tb.* and had good aqueous solubility, thus overcoming the main drawback of previously described 3,5-dinitrophenyl tetrazole and oxadiazole lead compounds, which suffered from very low solubility in aqueous formulations.[14, 17] The water solubility of compounds **7e**, **8e** and **9e** ranged from 17.1 to 22.8 mM (9 - 12.5 mg/mL). Regarding the cytotoxicity, the dihydrochloride salts **7e**, **8e** and **9e** showed negligible effects on four mammalian cell lines at a concentration of 30 μM. The structure of *N*-benzylpiperazine derivatives can be further optimized, mainly in the linker between tertiary amine and heterocycle and on the benzyl moiety, to reach better selectivity and toxicity profiles.

To conclude, the dihydrochloride salts **7e**, **8e** and **9e** of the *N*-benzylpiperazine-containing compounds **7d**, **8d** and **9d** described in this work can be suitable candidates for further *in vitro* and, mainly, *in vivo* evaluation of their antitubercular activity.

Experimental section

General. The structural identities of the prepared compounds were confirmed by ^{1}H-NMR and ^{13}C-NMR spectroscopy and HPLC-HRMS experiments. All chemicals used for synthesis were obtained from Sigma-Aldrich (Schnelldorf, Germany) and were used as received. TLC was performed on Merck aluminum plates with silica gel 60 F_{254}. Merck Kieselgel 60 (0.040-0.063 mm) was used for column chromatography. Melting points were recorded with a Büchi B-545 apparatus (BUCHI Labortechnik AG, Flawil, Switzerland) and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded using a Varian Mercury Vx BB 300 or VNMR S500 NMR spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts were reported as δvalues in parts per million (ppm) and were indirectly referenced to tetramethylsilane (TMS) via the solvent signal. The elemental analysis was performed on an Automatic Microanalyzer EA1110CE (Fisons Instruments S.p.A., Milano, Italy). HPLC-HRMS (ESI) experiments were performed using the HRMS system Acquity UPLC I-class and a Synapt G2Si Q-TOF mass spectrometer (Waters, Milford, MA, USA). Atmospheric pressure chemical ionization (APCI) was performed using an Agilent 500 Ion Trap LC/MS (Agilent Technologies, Santa Clara, CA, USA).

General method for the synthesis of tertiary amine-containing 3,5-dinitrobenzylsulfanyl oxadiazoles 6a-d and 7a-d. *N*-Methylpiperazine, morpholine, piperidine (1.5 mmol) or *N*-benzylpiperazine (1 mmol) was added to a solution of 2-(3-bromopropyl)-5-(3,5-dinitrobenzylsulfanyl)-1,3,4-oxadiazole **14a** or 2-(5-bromopentyl)-5-(3,5-dinitrobenzylsulfanyl)-1,3,4-oxadiazole **14b** (0.5 mmol) in MeCN (10 mL). Upon completion, as determined by TLC, the solvent was evaporated, and the crude product was dissolved in EtOAc (20 mL) and washed with brine (2 × 20 mL). The organic phase was separated, dried over anhydrous Na_2SO_4 and evaporated. The product was purified using column chromatography.

*2-((3,5-Dinitrobenzyl)sulfanyl)-5-(3-(4-methylpiperazin-1-yl)propyl)-1,3,4-oxadiazole (**6a**):* The reaction was stirred at rt overnight. The product was purified using column chromatography

(mobile phase: $CHCl_3/CH_3OH$, 30:1). Yield: 57% yield as a brown oil. ^1H NMR (500 MHz, $CDCl_3$) δ8.96 (t, *J* = 2.1 Hz, 1H), 8.70 (d, *J* = 2.1 Hz, 2H), 4.60 (s, 2H), 2.85 (t, *J* = 7.5 Hz, 2H), 2.60-2.31 (m, 10H), 2.26 (s, 3H), 1.92 (p, *J* = 7.2 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ168.75, 161.77, 148.51, 141.02, 129.27, 118.28, 56.99, 55.06, 52.96, 45.97, 34.78, 23.51, 23.41. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{17}H_{23}N_6O_5S^+$: 423.1445; found: 423.1452.

*2-((3,5-Dinitrobenzyl)sulfanyl)-5-(3-(morpholine-4-yl)propyl)-1,3,4-oxadiazole (**6b**):* The reaction mixture was refluxed for 2 h. The product was purified using column chromatography (mobile phase: $CHCl_3/CH_3OH$, 25:1). Yield: 60% yield as a brown oil. ^1H NMR (500 MHz, $CDCl_3$) δ8.96 (t, *J* = 2.1 Hz, 1H), 8.80 – 8.53 (m, 2H), 4.60 (s, 2H), 3.56 (t, *J* = 4.7 Hz, 4H), 2.87 (t, *J* = 7.3 Hz, 2H), 2.48 – 2.26 (m, 6H), 1.93 (p, *J* = 7.0 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.75, 161.67, 148.51, 141.01, 129.26, 118.29, 66.77, 57.48, 53.40, 34.73, 23.49, 23.11. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{16}H_{20}N_5O_6S^+$: 410.1129; found: 410.1131.

*2-((3,5-Dinitrobenzyl)sulfanyl)-5-(3-(piperidin-1-yl)propyl)-1,3,4-oxadiazole (**6c**):* The reaction mixture was stirred at rt overnight. The product was purified using column chromatography (mobile phase: Hexan/EtOAc/Et_3N, 30:10:1). Yield: 74% as a brown oil. ^1H NMR (500 MHz, $CDCl_3$) δ8.96 (t, *J* = 2.1 Hz, 1H), 8.70 (d, *J* = 2.1 Hz, 2H), 4.60 (s, 2H), 2.85 (t, *J* = 7.4 Hz, 2H), 2.46 – 2.26 (m, 6H), 1.94 (p, *J* = 7.3 Hz, 2H), 1.56 – 1.48 (m, 4H), 1.44 – 1.35 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ168.79, 161.75, 148.49, 141.07, 129.29, 118.26, 57.81, 54.39, 34.77, 25.78, 24.25, 23.54, 23.46. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{17}H_{22}N_5O_5S^+$: 408.1336; found: 408.1331.

*2-(3-(4-Benzylpiperazin-1-yl)propyl)-5-((3,5-dinitrobenzyl)sulfanyl)-1,3,4-oxadiazole (**6d**):* The reaction mixture was stirred at rt for 48 h. The product was purified using column chromatography (mobile phase: EtOAc/Hexan/Et_3N, 70:10:2). Yield: 78% as a brown oil. ^1H NMR (500 MHz, $CDCl_3$) δ8.96 (t, *J* = 2.1 Hz, 1H), 8.70 (d, *J* = 2.1 Hz, 2H), 7.34 – 7.12 (m,

5H), 4.59 (s, 2H), 3.48 (s, 2H), 2.85 (t, *J* = 7.5 Hz, 2H), 2.51-2.37 (m, 10H), 1.98 – 1.87 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ168.74, 161.78, 148.50, 141.02, 137.99, 129.27, 129.13, 128.14, 126.98, 118.28, 62.99, 57.03, 53.00 (2C), 34.77, 23.51, 23.40. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{23}H_{26}N_6O_5S^+$: 499.1758; found: 499.1761.

*2-((3,5-Dinitrobenzyl)sulfanyl)-5-(5-(4-methylpiperazin-1-yl)pentyl)-1,3,4-oxadiazole (7**a**):* The reaction mixture was stirred at rt overnight. The product was purified using column chromatography (mobile phase: $CHCl_3/CH_3OH$, 40:1). Yield: 77% yield as a brown oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ8.96 (t, *J* = 2.1 Hz, 1H), 8.69 (d, *J* = 2.1 Hz, 2H), 4.59 (s, 2H), 2.80 (t, *J* = 7.6 Hz, 2H), 2.63 – 2.38 (m, 8H), 2.35 – 2.29 (m, 2H), 2.28 (s, 3H), 1.77 (p, *J* = 7.6 Hz, 2H), 1.58 – 1.48 (m, 2H), 1.42 - 1.35 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ168.70, 161.89, 148.53, 141.04, 129.30, 118.32, 58.22, 55.05, 53.14, 45.98, 34.82, 26.89, 26.34, 26.14, 25.32. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{19}H_{27}N_6O_5S^+$: 451.1758; found: 451.1759.

*2-((3,5-Dinitrobenzyl)sulfanyl)-5-(5-(morpholine-4-yl)pentyl)-1,3,4-oxadiazole (7**b**):* The reaction mixture was refluxed for 2 h. The product was purified using column chromatography (mobile phase: $CHCl_3/CH_3OH$, 25:1). Yield: 72% as a brown oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.96 (t, *J* = 2.1 Hz, 1H), 8.70 (d, *J* = 2.1 Hz, 2H), 4.60 (s, 2H), 3.73 – 3.66 (m, 4H), 2.81 (t, *J* = 7.6 Hz, 2H), 2.47 – 2.38 (m, 4H), 2.38 – 2.27 (m, 2H), 1.82 – 1.72 (m, 2H), 1.57 – 1.48 (m, 2H), 1.46 – 1.36 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ168.64, 161.88, 148.51, 141.01, 129.25, 118.29, 66.84, 58.55, 53.64, 34.80, 26.72, 26.07, 25.88, 25.28. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{18}H_{24}N_5O_6S^+$: 438.1442; found: 438.1445.

*2-((3,5-Dinitrobenzyl)sulfanyl)-5-(5-(piperidin-1-yl)pentyl)-1,3,4-oxadiazole (7**c**):* The reaction mixture was stirred at rt overnight. The product was purified using column chromatography (mobile phase: Hexan/EtOAc/Et_3N, 30:10:1). Yield: 61% as a brown oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ8.96 (t, *J* = 2.1 Hz, 1H), 8.70 (d, *J* = 2.1 Hz, 2H), 4.59 (s, 2H), 2.96 –

2.68 (m, 2H), 2.35 (s, 4H), 2.30 – 2.22 (m, 2H), 1.77 (p, *J* = 7.6 Hz, 2H), 1.61 – 1.48 (m, 6H), 1.47 – 1.32 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ168.74, 161.83, 148.50, 141.02, 129.27, 118.29, 59.08, 54.60, 34.79, 27.01, 26.39, 26.16, 25.91, 25.30, 24.40. HRMS (ESI+): *m*/*z* $[M+H]^+$ calcd for $C_{19}H_{26}N_5O_5S^+$: 436.1649; found: 436.1656.

*2-(5-(4-Benzylpiperazin-1-yl)pentyl)-5-((3,5-dinitrobenzyl)sulfanyl)-1,3,4-oxadiazole (7**d**):* The reaction was stirred at rt for 48 h. The product was purified using column chromatography (mobile phase: EtOAc/Hexan/Et_3N, 70:10:2). Yield: 84% as a brown oil. ^{1}H NMR (500 MHz, Acetone-d_6) δ8.89-8.86 (m, 3H), 7.37 – 7.27 (m, 4H), 7.27 – 7.20 (m, 1H), 4.84 (s, 2H), 3.47 (s, 2H), 2.83 (t, *J* = 7.5 Hz, 2H), 2.51 – 2.32 (m, 8H), 2.28 (dd, *J* = 7.6, 6.5 Hz, 2H), 1.74 (p, *J* = 7.6 Hz, 2H), 1.58 – 1.44 (m, 2H), 1.45 – 1.34 (m, 2H). ^{13}C NMR (126 MHz, Acetone-d_6) δ169.31, 162.94, 149.33, 143.24, 139.71, 130.40, 129.63, 128.88, 127.60, 118.63, 63.47, 58.73, 54.05, 53.97, 35.19, 27.32, 27.02, 26.79, 25.64. HRMS (ESI+): *m*/*z* $[M+H]^+$ calcd for $C_{25}H_{31}N_6O_5S^+$: 527.2071; found: 527.2083.

*5-(3,5-Dinitrophenyl)-2-(3-(4-methylpiperazin-1-yl)propyl)-2H-tetrazole (**8a**):* Bromopropyltetrazole **16** (0.1 g, 0.28 mmol), potassium carbonate (0.039 g, 0.28 mmol) and *N*-methylpiperazine (0.040 mL, 0.36 mmol) were mixed in MeCN (10 mL) and the reaction mixture was refluxed for 5 h. Upon cooling, the solids were filtered off and the residue was evaporated under vacuum. The product was isolated using column chromatography (mobile phase: $CHCl_3$/MeOH, 17:1). Yield: 93% as a yellow oil. ^{1}H NMR (500 MHz, DMSO) δ9.05 (d, *J* = 2.1 Hz, 2H), 8.95 (t, *J* = 2.1 Hz, 1H), 4.84 (t, *J* = 6.9 Hz, 2H), 2.39 – 2.30 (m, 6H), 2.28 – 2.22 (m, 4H), 2.17 (p, *J* = 6.7 Hz, 2H), 2.11 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ161.06, 148.89, 129.79, 126.04, 119.89, 54.74, 54.37, 52.56, 51.89, 45.73, 25.92. HRMS (ESI+): *m*/*z* $[M+H]^+$ calcd for $C_{15}H_{21}N_8O_4^+$: 377.1680; found: 377.1682.

*5-(3,5-Dinitrophenyl)-2-(3-(morpholine-4-yl)propyl)-2H-tetrazole (**8b**):* Bromopropyltetrazole **16** (0.1 g, 0.28 mmol), potassium carbonate (0.039 g, 0.28 mmol) and morpholine (0.031 mL, 0.36 mmol) were mixed in MeCN (10 mL), and the reaction mixture was refluxed for 5 h. Upon cooling, the solids were filtered off and the residue was evaporated under vacuum. The product was isolated using column chromatography (mobile phase: EtOAc). Yield: 90% as a yellow oil. ^{1}H NMR (500 MHz, DMSO) δ9.06 (d, *J*= 2.1 Hz, 2H), 8.95 (t, *J* = 2.1 Hz, 1H), 4.86 (t, *J* = 6.9 Hz, 2H), 3.53 (t, *J* = 4.7 Hz, 4H), 2.41 (t, *J* = 6.7 Hz, 2H), 2.36 (t, *J* = 4.7 Hz, 4H), 2.24 – 2.17 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ160.87, 148.80, 129.84, 125.82, 119.45, 66.01, 54.61, 53.00, 51.66, 25.41. HRMS (ESI+): *m*/*z* $[M+H]^+$ calcd for $C_{14}H_{18}N_7O_5^+$: 364.1364; found: 364.1362.

*5-(3,5-Dinitrophenyl)-2-(3-(piperidin-1-yl)propyl)-2H-tetrazole (**8c**):* Bromopropyltetrazole **16** (100 mg, 0.28 mmol) and piperidine (0.055 mL, 0.56 mmol) were dissolved in MeCN (10 mL) and the reaction mixture was refluxed for 4 h. Upon completion, as determined by TLC, the solvent was evaporated under reduced pressure and the product was purified using column chromatography (mobile phase: $CHCl_3$/MeOH, 50:1). Yield: 78% as a dark yellow solid, mp 83-84 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ9.32 (d, *J* = 2.1 Hz, 2H), 9.13 (t, *J* = 2.1 Hz, 1H), 4.81 (t, *J* = 7.1 Hz, 2H), 2.45 – 2.35 (m, 6H), 2.32 – 2.23 (m, 2H), 1.61 – 1.52 (m, 4H), 1.47 – 1.40 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ161.47, 149.04, 131.23, 126.53, 119.63, 55.48, 54.54, 52.28, 26.71, 25.96, 24.32. HRMS (ESI+): *m*/*z* $[M+H]^+$ calcd for $C_{15}H_{20}N_7O_4^+$: 362.1571; found: 362.1581.

*2-(3-(4-Benzylpiperazin-1-yl)propyl)-5-(3,5-dinitrophenyl)-2H-tetrazole (**8d**):* Bromopropyltetrazole **16** (1.834 g, 5.14 mmol) and *N*-benzylpiperazine (1.810 g, 10.27 mmol) were dissolved in MeCN (100 mL), and the reaction mixture was refluxed for 13 h. Upon cooling, the solvent was evaporated under vacuum, and the residue was dissolved in EtOAc (80

mL) and washed with 10% aq. $KHCO_3$ (3 × 30 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated under vacuum. The product was isolated using column chromatography (mobile phase: $CHCl_3$/MeOH, 19:1). Yield: 76% as yellow oil. ^{1}H NMR (500 MHz, DMSO-d_6) δ9.04 (d, J = 2.1 Hz, 2H), 8.95 (t, J = 2.1 Hz, 1H), 7.31 – 7.19 (m, 5H), 4.83 (t, J = 6.8 Hz, 2H), 3.39 (s, 2H), 2.46 – 2.21 (m, 10H), 2.15 (p, J = 6.8 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ161.18, 148.99, 138.31, 129.90, 128.91, 128.24, 126.99, 126.16, 120.02, 62.23, 54.53, 52.81, 52.74, 52.01, 26.03. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{21}H_{25}N_8O_4^+$: 453.1993; found: 453.2003.

*2-(3,5-Dinitrophenyl)-5-((3-(4-methylpiperazin-1-yl)propyl)sulfanyl)-1,3,4-oxadiazole (**9a**):* Bromopropylsulfanyl oxadiazole **18** (200 mg, 0.514 mmol) and *N*-methylpiperazine (0.115 mL, 1.03 mmol) were dissolved in MeCN (15 mL), and the reaction mixture was refluxed for 4 h. Upon completion, as determined by TLC, the solvent was evaporated under reduced pressure and the product was purified using column chromatography (mobile phase: $CHCl_3$/MeOH, 19:1). Yield: 55% yield as a brownish oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ9.18 – 9.16 (m, 1H), 9.14 (d, J = 2.1 Hz, 2H), 3.43 (t, J = 7.1 Hz, 2H), 2.77 – 2.61 (m, 8H), 2.58 (t, J = 6.8 Hz, 2H), 2.43 (s, 3H), 2.08 (p, J = 6.9 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ167.25, 162.07, 149.09, 126.96, 126.08, 120.66, 56.14, 54.47, 51.88, 45.12, 30.69, 26.08. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{16}H_{21}N_6O_5S^+$: 409.1289; found: 409.1293.

*2-(3,5-Dinitrophenyl)-5-((3-(morpholine-4-yl)propyl)sulfanyl)-1,3,4-oxadiazole (**9b**):* Bromopropylsulfanyl oxadiazole **18** (200 mg, 0.514 mmol) and morpholine (0.089 mL, 1.03 mmol) were dissolved in MeCN (15 mL), and the reaction mixture was refluxed for 13 h. As a significant amount of the starting material **18** was detected in the reaction mixture, another portion of morpholine (0.089 mL, 1.03 mmol) was added. After an additional 6 hours of reflux, the solvent was evaporated under reduced pressure and the product was purified using column

chromatography (mobile phase: EtOAc). Yield: 58% as a yellow solid, mp 115-117 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ9.18 – 9.16 (m, 1H), 9.15 (d, *J* = 2.1 Hz, 2H), 3.75 – 3.70 (m, 4H), 3.45 (t, *J* = 7.1 Hz, 2H), 2.53 (t, *J* = 6.7 Hz, 2H), 2.50 – 2.45 (m, 4H), 2.08 (p, *J* = 6.8 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ167.35, 162.06, 149.12, 127.00, 126.08, 120.66, 66.92, 56.91, 53.60, 30.77, 25.88. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{15}H_{18}N_5O_6S^+$: 396.0972; found: 396.0975.

*2-(3,5-Dinitrophenyl)-5-((3-(piperidin-1-yl)propyl)sulfanyl)-1,3,4-oxadiazole (**9c**):* Bromopropylsulfanyl oxadiazole **18** (0.2 g, 0.75 mmol), 1-(3-chloropropyl)piperidine hydrochloride (0.148 g, 0.75 mmol) and Et_3N (0.15 g, 0.2 mL, 1.5 mmol) were dissolved in MeCN (10 mL), and the reaction mixture was refluxed for 12 h. Upon completion, the solvent was evaporated, and the residue was partitioned between EtOAc (20 mL) and 10% aq. K_2CO_3 (20 mL). The aqueous phase was then extracted with EtOAc (2 × 20 mL), the combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated. The product was purified using column chromatography (mobile phase: Hexane/EtOAc/Et_3N, 75:25:1). Yield: 65% as a yellow oil. ^{1}H NMR (500 MHz, Acetone-d_6) δ9.12 (s, 3H), 3.48 (t, *J* = 7.1 Hz, 2H), 2.50 (t, *J* = 6.6 Hz, 2H), 2.42 (br s, 4H), 2.09 – 2.04 (m, 2H), 1.60 – 1.54 (m, 4H), 1.48-1.40 (m, 2H). ^{13}C NMR (126 MHz, Acetone-d_6) δ167.55, 163.41, 150.10, 127.76, 126.87, 121.48, 57.73, 55.14, 31.43, 27.24, 26.68, 25.13. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{16}H_{20}N_5O_5S^+$: 394.1180; found: 394.1178.

*2-((3-(4-Benzylpiperazin-1-yl)propyl)sulfanyl)-5-(3,5-dinitrophenyl)-1,3,4-oxadiazole (**9d**):* Bromopropylsulfanyl oxadiazole **18** (0.277 g, 0.71 mmol) and *N*-benzylpiperazine (0.251 g, 1.42 mmol) were dissolved in MeCN (20 mL), and the reaction mixture was refluxed for 13 h. Upon completion, the solvent was evaporated, and the residue was dissolved in EtOAc (40 mL) and washed with 10% aq. $KHCO_3$ (3 × 30 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The product was purified using column chromatography (mobile phase: Hexane/EtOAc/Et_3N, 66:33:1). Yield: 81% as a yellowish oil. ^{1}H NMR (500

MHz, Acetone-d_6) δ9.13 – 9.11 (m, 3H), 7.34 – 7.29 (m, 4H), 7.25 – 7.21 (m, 1H), 3.50 – 3.45 (m, 4H), 2.54 – 2.38 (m, 10H), 2.09 – 2.03 (m, 2H). ^{13}C NMR (126 MHz, Acetone-d_6) δ167.49, 163.40, 150.08, 139.61, 129.62, 128.88, 127.74, 127.62, 126.86, 121.48, 63.42, 57.03, 53.93, 53.89, 31.35, 27.23. HRMS (ESI+): *m*/*z* $[M+H]^+$ calcd for $C_{22}H_{25}N_6O_5S^+$: 485.1602; found: 485.1613.

General method for the synthesis of dihydrochlorides 7e, **8e** and **9e**: *N*-Benzylpiperazine-containing derivative **7d**, **8d** or **9d** (0.1 g) was mixed with water (10 mL) and conc. HCl (3 equiv.). The reaction mixture was heated to 60 °C until a clear solution was obtained. Then, all of the volatiles were evaporated under reduced pressure, and the residue was dried under vacuum over P_2O_5 for 48 h. These reactions gave quantitative yields of products **7e**, **8e** and **9e**.

*2-(5-(4-Benzylpiperazin-1-yl)pentyl)-5-((3,5-dinitrobenzyl)sulfanyl)-1,3,4-oxadiazole dihydrochloride (**7e**):* Obtained as a yellowish solid, mp 201-202 °C. ^{1}H NMR (500 MHz, D_2O) δ 8.78 (t, *J* = 2.2 Hz, 1H), 8.55 (d, *J* = 2.1 Hz, 2H), 7.46 – 7.34 (s, 5H), 4.48 (s, 2H), 4.35 (s, 2H), 3.53 (br s, 8H), 3.18 – 3.09 (m, 2H), 2.72 (t, *J* = 7.4 Hz, 2H), 1.72 – 1.54 (m, 4H), 1.32 – 1.18 (m, 2H). ^{13}C NMR (126 MHz, D_2O) δ169.65, 163.64, 148.09, 141.16, 131.12, 130.53, 129.50, 129.37, 127.48, 118.27, 60.44, 56.50, 48.50, 48.03, 34.68, 24.71, 24.66, 24.24, 22.80. MS (APCI+): *m*/z (%) = 527.4 (100), 528.2 (25) $[M+H]^+$.

*2-(3-(4-Benzylpiperazin-1-yl)propyl)-5-(3,5-dinitrophenyl)-2H-tetrazole dihydrochloride (**8e**):* Obtained as a yellowish solid, mp 258-259 °C. ^{1}H NMR (500 MHz, D_2O) δ9.15 (d, *J* = 2.1 Hz, 2H), 9.10 (t, *J* = 2.1 Hz, 1H), 7.45 – 7.38 (m, 5H), 4.86 (t, *J* = 6.6 Hz, 2H), 4.35 (s, 2H), 3.53 (br s, 8H), 3.35 – 3.29 (m, 2H), 2.56 – 2.44 (m, 2H). ^{13}C NMR (126 MHz, D_2O) δ161.98, 148.80, 131.13, 130.53, 129.59, 129.39, 127.57, 127.08, 120.59, 60.47, 53.73, 50.53, 48.88, 48.30, 23.26. MS (APCI+): *m*/z (%) = 453.4 (100), 454.3 (20) $[M+H]^+$.

*2-((3-(4-Benzylpiperazin-1-yl)propyl)sulfanyl)-5-(3,5-dinitrophenyl)-1,3,4-oxadiazole dihydrochloride (**9e**):* Obtained as a yellowish solid, mp 246-248 °C. ^{1}H NMR (500 MHz, D_2O) δ 9.17 – 9.15 (m, 1H), 9.06 – 9.03 (m, 2H), 7.52 – 7.31 (m, 5H), 4.35 – 4.28 (m, 2H), 3.50 (s, 8H), 3.40 – 3.26 (m, 4H), 2.33 – 2.19 (m, 2H). ^{13}C NMR (126 MHz, D_2O) δ166.84, 163.33, 148.86, 131.09, 130.46, 130.42, 129.36, 126.85, 125.67, 121.71, 60.50, 55.08, 48.93, 48.34, 28.64, 23.69. MS (APCI+): *m*/z (%) = 485.4 (100), 486.1 (22) $[M+H]^+$.

***In vitro* antimycobacterial assay.** The *in vitro* antimycobacterial activity of the studied compounds was evaluated against *M.tb.* CNCTC My 331/88, *M. kansasii* CNCTC My 235/80 and *M. avium* CNCTC My 330/88 from the Czech National Collection of Type Cultures (CNCTC). Additionally, the *in vitro* antimycobacterial activity of the studied compounds was evaluated against the clinically isolated strain *M. kansasii* 6509/96 and clinically isolated MDR/XDR strains *M.tb*. 7357/1998, *M.tb.* 234/2005, *M.tb.* 9449/2007, *M.tb.* 8666/2010, *M.tb.* Praha 1, *M.tb.* Praha 4 and *M.tb.* Praha 131. Basic suspensions of the mycobacterial strains were prepared according to a 1.0 McFarland standard. From the basic suspension, subsequent dilutions of each strain were made: *M. tuberculosis,* 10^{-3}; *M. avium,* 10^{-5}; and *M. kansasii,* 10^{-4}. The appropriate dilutions of the strains were prepared, and 0.1 mL was added to each well of the microtiter plates containing the compounds. The activities of the compounds were determined via the micromethod for the determination of the minimum inhibitory concentration in Šula's semisynthetic medium (SEVAC, Prague). The compounds were dissolved in dimethyl sulfoxide and added to the medium at concentrations of 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06 and 0.03 μM for *M.tb.* and *M. kansasii* strains and at concentrations of 1000, 500, 250, 125, 64, 32, 16, 8, 4, 2, and 1 μM for the *M. avium* strain. The MICs, i.e., the lowest concentration of a substance at which mycobacterial growth inhibition occurred (the concentration that inhibited >99% of the

mycobacterial population), were determined after incubation at 37 °C for 14 days. Isoniazid (INH) and rifampicin (RIF) were used as the prototype drugs.

Cell proliferation/viability assay. The CellTiter 96® AQueousOne Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was performed to evaluate *in vitro* effects of compounds **7e**, **8e** and **9e** in the four mammalian cell lines. The method is based on the colorimetric method of MTS bioreduction into colored formazan via viable cells. The formazan production is thus proportional to the number of viable cells. All experiments were conducted according to the manufacturer's protocol. Briefly, the cells were treated with the test compounds or vehicle alone (DMSO) for 48 h in Opti-MEM, reduced serum medium (Life Technologies) to avoid potential binding of test compounds onto the serum proteins. At the end of the treatment, 20 μL of the MTS reagent was added directly to each culture well and further cultivated for 4 h. Finally, the absorbance of the converted formazan was recorded at 490 nm using a plate reader (BioTec Synergy 2, Winooski, VT, USA). In the toxic control, SDS (10% v/v) was added to the cells 45 min before the addition of the MTS reagent. The relative viability of the cells treated either by the vehicle (DMSO, 0.1%) or SDS was set to be 100% or 0%, respectively. The results were expressed as the relative cell viability at a 30 μM concentration. All experiments were performed in triplicates and repeated at least three times.

Solubility in Aqueous Media. Compound **7e, 8e** or **9e** (15 mg) was shaken in 0.05 M citrate buffer (pH 3, 1 mL), 0.1 M acetate buffer (pH 5, 1 mL) or PBS (pH 7.4, 5 mL) at rt for 2 h. The suspensions were decanted and the solutions were filtered through 0.22-μm PTFE filters. Clear solutions in citrate buffer and acetate buffer were diluted 500-times with water/acetonitrile 1:1 (v/v) prior to analysis. All samples were analyzed using a Shimadzu Prominence HPLC instrument (Shimadzu, Kyoto, Japan) consisting of LC-20AD pumps with a DGU-20A3

degasser, an SIL-20A HT autosampler, a CTO-20AC column oven, an SPD-M20A diode array detector and a CBM-20A communication module. The data were analyzed using LCsolutions 1.22 software. All experiments were repeated at least 4 times.

Compounds **7e**, **8e** and **9e** were analyzed using a Discovery® HS C18 150-4.6 mm column with 5-μm particles (Supelco Analytical, Sigma-Aldrich, Schnelldorf, Germany) at 30 °C. The mobile phase, composed of water/acetonitrile 1:1 (v/v) at a flow rate of 2.5 mL/min, was used. The samples were monitored at 235 nm for **7e** and 229 nm for **8e** and **9e**. The retention times of **7e, 8e** and **9e** were 6.1, 5.9 and 5.5 min, respectively. The calibration curves were linear in the range of 1 – 100 μM.

SUPPLEMENTARY MATERIAL

Experimental protocols and characterization data of compounds **11a**, **11b**, **12a**, **12b**, **13a**, **13b**, **14a**, **14b**, **16** and **18**. NMR a HRMS spectra of the final compounds.

AUTHOR INFORMATION

Corresponding Author

J. Roh. email: jaroslav.roh@faf.cuni.cz.

ACKNOWLEDGMENTS

This work was supported by the Charles University Grant Agency project 361215 and Charles University project SVV 260 401.

REFERENCES

1. World Health Organization, Global tuberculosis report 2015. http://www.who.int/tb.

2. Zumla A, Nahid P, Cole ST. Advances in the development of new tuberculosis drugs and treatment regimens. *Nat Rev Drug Discov.* 2013;12(5): 388-404.

3. Gler MT, Skripconoka V, Sanchez-Garavito E, et al. Delamanid for Multidrug-Resistant Pulmonary Tuberculosis. *N Engl J Med.* 2012;366(23): 2151-2160.

4. Diacon AH, Pym A, Grobusch M, et al. The Diarylquinoline TMC207 for Multidrug-Resistant Tuberculosis. *N Engl J Med.* 2009;360(23): 2397-2405.

5. Kakkar AK, Dahiya N. Bedaquiline for the treatment of resistant tuberculosis: Promises and pitfalls. *Tuberculosis.* 2014;94(4): 357-362.

6. Singh R, Manjunatha U, Boshoff HIM, et al. PA-824 Kills Nonreplicating Mycobacterium tuberculosis by Intracellular NO Release. *Science.* 2008;322(5906): 1392-1395.

7. Blaser A, Palmer BD, Sutherland HS, et al. Structure-Activity Relationships for Amide-, Carbamate-, And Urea-Linked Analogues of the Tuberculosis Drug (6S)-2-Nitro-6-{ 4-(trifluoromethoxy)benzyl oxy}-6,7-dihydro-5H-imidazo 2,1-b 1,3 oxazine (PA-824). *J Med Chem.* 2012;55(1): 312-326.

8. Makarov V, Manina G, Mikusova K, et al. Benzothiazinones Kill Mycobacterium tuberculosis by Blocking Arabinan Synthesis. *Science.* 2009;324(5928): 801-804.

9. Trefzer C, Skovierova H, Buroni S, et al. Benzothiazinones Are Suicide Inhibitors of Mycobacterial Decaprenylphosphoryl-beta-D-ribofuranose 2'-Oxidase DprE1. *J Am Chem Soc.* 2012;134(2): 912-915.

10. Mikusova K, Makarov V, Neres J. DprE1-from the Discovery to the Promising Tuberculosis Drug Target. *Curr Pharm Design.* 2014;20(27): 4379-4403.

11. Makarov V, Lechartier B, Zhang M, et al. Towards a new combination therapy for tuberculosis with next generation benzothiazinones. *EMBO Mol Med.* 2014;6(3): 372-383.

12. Karabanovich G, Roh J, Smutný T, et al. 1-Substituted-5-[(3,5-Dinitrobenzyl)sulfanyl]-1*H*-Tetrazoles and Their Isosteric Analogs: A New Class of Selective Antitubercular Agents Active against Drug-Susceptible and Multidrug-Resistant Mycobacteria. *Eur J Med Chem.* 2014;82: 324-340.

13. Karabanovich G, Roh J, Soukup O, et al. Tetrazole Regioisomers in the Development of Nitro Group-Containing Antitubercular Agents. *Med Chem Commun.* 2015;6(1): 174-181.

14. Karabanovich G, Zemanová J, Smutný T, et al. Development of 3,5-Dinitrobenzylsulfanyl-1,3,4-Oxadiazoles and Thiadiazoles as Selective Antitubercular Agents Active Against Replicating and Nonreplicating Mycobacterium tuberculosis. *J Med Chem.* 2016;59: 2362−2380.

15. Němeček J, Sychra P, Macháček M, et al. Structure-activity relationship studies on 3,5-dinitrophenyl tetrazoles as antitubercular agents. *Eur J Med Chem.* 2017;130: 419-432.

16. Christophe T, Jackson M, Jeon HK, et al. High Content Screening Identifies Decaprenyl-Phosphoribose 2' Epimerase as a Target for Intracellular Antimycobacterial Inhibitors. *PLoS Pathog.* 2009;5(10): e1000645.

17. Karabanovich G, Němeček J, Valášková L, et al. S-substituted 3,5-dinitrophenyl 1,3,4-oxadiazole-2-thiols and tetrazole-5-thiols as highly efficient antitubercular agents. *Eur J Med Chem.* 2017;126: 369-383.

18. Xu LB, Farthing AK, Dropinski JF, et al. Synthesis and antibacterial activity of novel water-soluble nocathiacin analogs. *Bioorg Med Chem Lett.* 2013;23(1): 366-369.

19. Ferlin MG, Marzano C, Dalla Via L, et al. New water soluble pyrroloquinoline derivatives as new potential anticancer agents. *Bioorg Med Chem.* 2005;13(15): 4733-4739.

20. Brunschweiger A, Koch P, Schlenk M, et al. 8-Substituted 1,3-dimethyltetrahydropyrazino 2,1-f purinediones: Water-soluble adenosine receptor antagonists and monoamine oxidase B inhibitors. *Bioorg Med Chem.* 2016;24(21): 5462-5480.

21. Luzzio MJ, Besterman JM, Emerson DL, et al. Synthesis and Antitumor-Activity of Novel Water-Soluble Derivatives of Camptothecin as Specific Inhibitors of Topoisomerase-I. *J Med Chem.* 1995;38(3): 395-401.

22. Kingsbury WD, Boehm JC, Jakas DR, et al. Synthesis of Water-Soluble (Aminoalkyl)Camptothecin Analogs - Inhibition of Topoisomerase-I and Antitumor-Activity. *J Med Chem.* 1991;34(1): 98-107.

23. MacNevin CJ, Atif F, Sayeed I, Stein DG, Liotta DC. Development and Screening of Water-Soluble Analogues of Progesterone and Allopregnanolone in Models of Brain Injury. *J Med Chem.* 2009;52(19): 6012-6023.

24. Trifonov RE, Ostrovskii VA. Protolytic equilibria in tetrazoles. *Russ J Org Chem.* 2006;42(11): 1585-1605.

Graphical abstract