Accepted Manuscript

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PII: S0223-5234(16)30978-3

DOI: 10.1016/j.ejmech.2016.11.041

Reference: EJMECH 9074

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 11 October 2016

Revised Date: 18 November 2016

Accepted Date: 20 November 2016

Please cite this article as: G. Karabanovich, J. Němeček, L. Valášková, A. Carazo, K. Konečná, J. Stolaříková, A. Hrabálek, O. Pavliš, P. Pávek, K. Vávrová, J. Roh, V. Klimešová, *S*-substituted 3,5-dinitrophenyl 1,3,4-oxadiazole-2-thiols and tetrazole-5-thiols as highly efficient antitubercular agents, *European Journal of Medicinal Chemistry* (2016), doi: 10.1016/j.ejmech.2016.11.041.

S-Substituted 3,5-Dinitrophenyl 1,3,4-Oxadiazole-2-thiols and Tetrazole-5-thiols as Highly Efficient Antitubercular Agents

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ABSTRACT

Two new classes of antitubercular agents, namely 5-alkylsulfanyl-1-(3,5-dinitrophenyl)-1*H*-tetrazoles and 2-alkylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazoles, and their structure-activity relationships are described. These compounds possessed excellent activity against

Mycobacterium tuberculosis, including the clinically isolated multidrug (MDR) and extensively drug-resistant (XDR) strains, with no cross resistance with first or second-line anti-TB drugs. The minimum inhibitory concentration (MIC) values of the most promising compounds reached 0.03 μM. Furthermore, these compounds had a highly selective antimycobacterial effect because they were completely inactive against 4 gram positive and 4 gram negative bacteria and eight fungal strains and had low *in vitro* toxicity for four mammalian cell lines, including hepatic cell lines HepG2 and HuH7. Although the structure-activity relationship study showed that the presence of two nitro groups is highly beneficial for antimycobacterial activity, the analogues with a trifluoromethyl group instead of one of the nitro groups maintained a high antimycobacterial activity, which indicates the possibility for further structural optimization of this class of antitubercular agents.

Keywords

Tuberculosis
Antitubercular agent
Mycobacterium tuberculosis
Tetrazole
Oxadiazole
Structure-activity relationships

Introduction

Tuberculosis (TB) is an enormously widespread and dangerous infection disease. Despite the treatment success rates and progress in reducing the mortality rate since 1990, there were still an estimated 10.4 million incident cases of TB and 1.8 million deaths from TB in 2015 [1]. A particular problem is the development and increasing dissemination of multidrug-resistant forms

of TB (MDR-TB). In 2015, 3.9% of new TB cases and 21% of previously treated cases of TB were MDR-TB. Moreover, 9.5% of those cases involved the extensively drug-resistant strains of TB (XDR-TB), which have limited treatment options. Only 50% of patients with MDR-TB were successfully cured, whereas therapy of TB caused by the drug-susceptible strains of *Mycobacterium tuberculosis* (*M.tb.*) led to a 90% recovery rate. The low recovery rate of patients with MDR-TB is due to poor adherence to the treatment regimen because of its long duration, complexity and high cost and the adverse effects of the currently used anti-TB drugs when applied to a long TB therapy regimen. Furthermore, the wide spread of TB among HIV-positive patients worsens the TB epidemic situation and increases the death rates. The complicated diagnose of TB in HIV patients and drug-drug interactions between anti-TB drugs and antiretroviral drugs decreases the treatment success for both TB and HIV. All of the above mentioned problems highlight the necessity for the development of new, highly effective, anti-TB compounds.

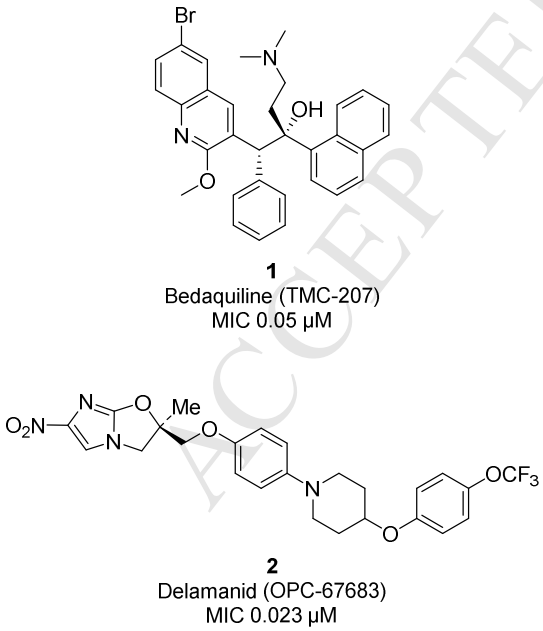


Figure 1. New anti-TB compounds for the treatment of MDR-TB.

In the last 40 years, only two novel anti-TB drugs have been approved for the treatment of pulmonary MDR-TB, bedaquiline (**1**) [2, 3] and delamanid (OPC-67683, **2**) [4] (Figure 1). Recently, several experimental anti-TB drugs, which have nitro group dependent antimycobacterial activities like delamanid, have been intensively studied. PA-824 (**3**) [5-7], TBA-354 (**4**) [8] and their parent compound, CGI-17341 (**5**) [9], are nitroimidazole-based compounds that are highly effective against drug-susceptible and drug-resistant TB forms and are active against both replicating and non-replicating mycobacteria (Figure 2A) [10]. Benzothiazinones BTZ043 (**6**) [11] and its piperazine derivative PBTZ-169 (**7**) [12] represent another class of nitro group containing compounds with excellent *in vitro* and *in vivo* antimycobacterial activity. These compounds act as irreversible inhibitors of decaprenylphosphoryl-*β*-D-ribose 2′-oxidase (DprE1), an essential enzyme in arabinan biosynthesis [13]. A structure–anti-TB activity study of BTZs showed that only one nitro-group is essential for the efficiency of these compounds, and derivatives with two nitro groups on the benzothiazinone core showed a lower potency [14]. Other nitro group-containing DprE1 inhibitors are also known: *N*-substituted 3,5-dinitrobenzamides (**8**) [15, 16], nitro group substituted quinoxaline VI-9376 (**9**) [17] or nitro group substituted triazole 377790 (**10**) (Figure 2B) [18].

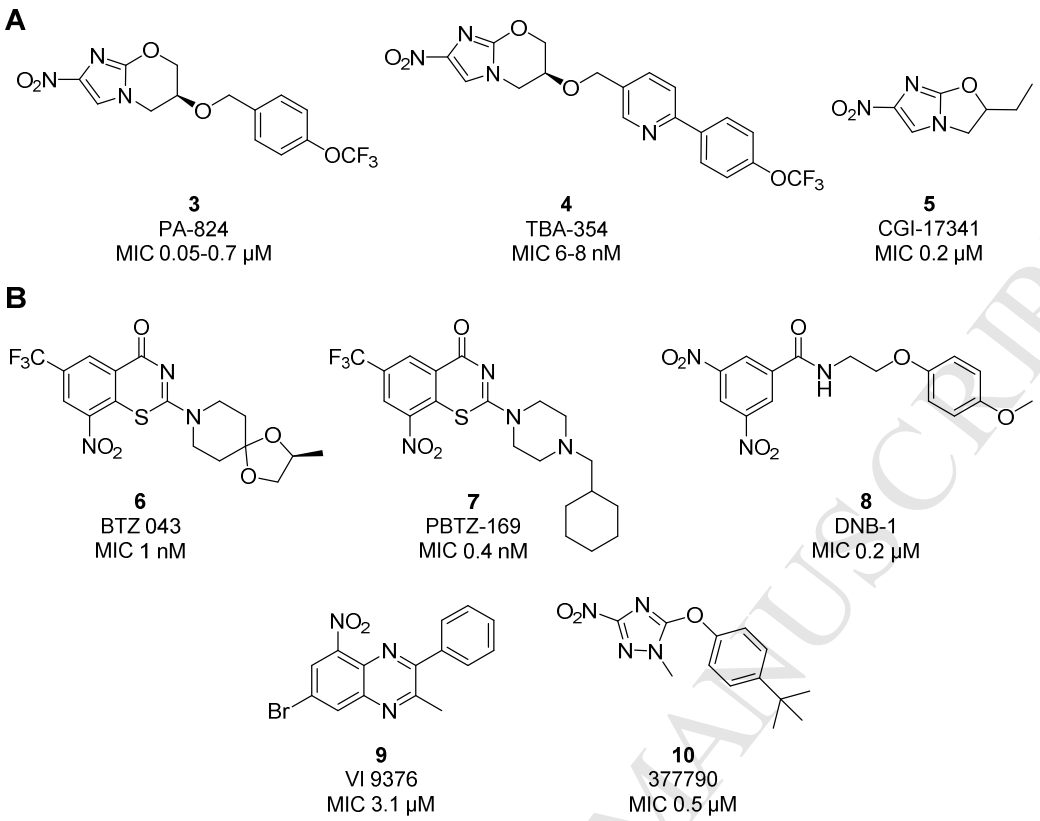


Figure 2. Experimental anti-TB drugs with nitro group dependent antimycobacterial activity: nitroimidazole-based compounds (**A**) and DprE1 inhibitors (**B**).

In our previous work, four classes of highly efficient nitro group containing anti-TB compounds were developed: 3,5-dinitrobenzylsulfanyl 1*H*- and 2*H*-tetrazoles (**11** and **12**, respectively) [19, 20], 1,3,4-oxadiazoles (**13**) and 1,3,4-thiadiazoles (**14**) (Figure 3) [21]. The majority of the compounds of series **11** - **14** possessed outstanding and highly selective antimycobacterial activity. They were highly effective against drug-susceptible *M.tb.*, multidrug-resistant clinically isolated strains of *M.tb.* and nonreplicating *M.tb.* They also displayed low cytotoxicity and mutagenicity. Despite the structural similarities with the DprE1 inhibitors, especially with the dinitrobenzamides (**8**), compounds **11** - **14** did not inhibit DprE1 [21]. The SAR study showed the necessity of both the 3,5-dinitrobenzylsulfanyl fragment and the tetrazole/oxadiazole heterocycle for high antimycobacterial efficiency. The compounds with a 3-

nitro-5-(trifluoromethyl)benzyl group instead of the 3,5-dinitrobenzyl group showed very low *in vitro* anti-TB activity [21].

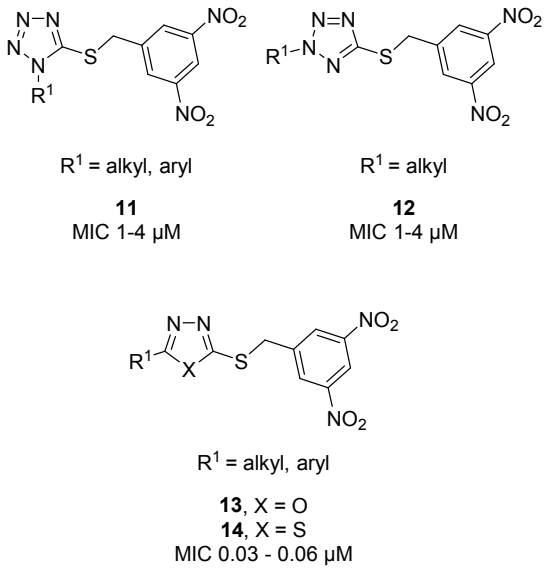
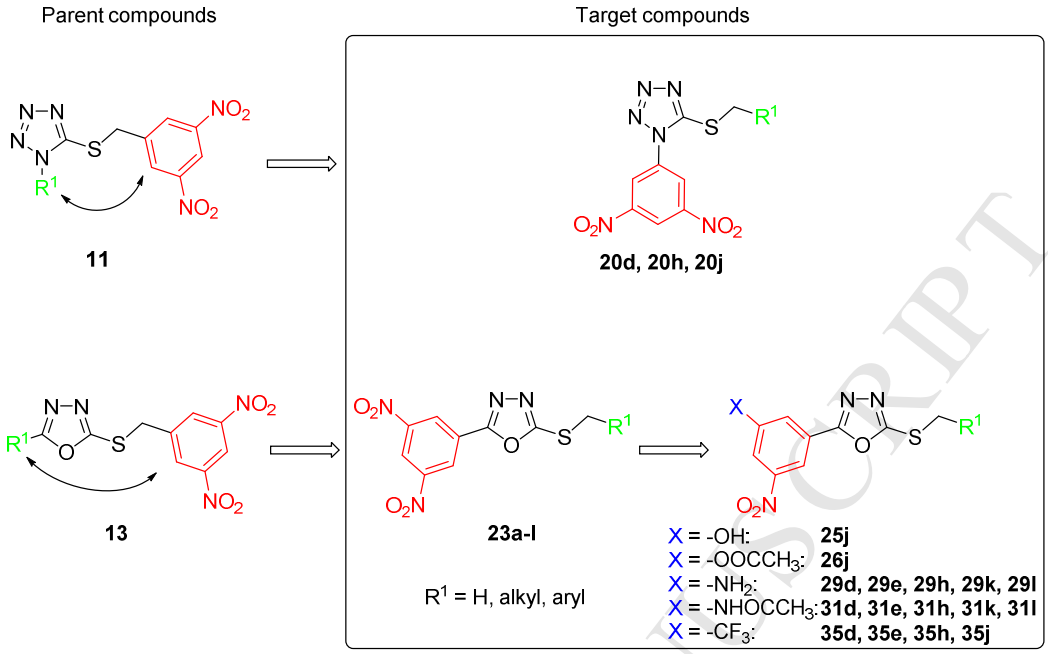


Figure 3. 3,5-Dinitrobenzylsulfanyl 1*H*- and 2*H*-tetrazoles (**11** and **12**), 1,3,4-oxadiazoles (**13**) and 1,3,4-thiadiazoles (**14**) as anti-TB lead structures.

As a continuation of our work, we were interested in how the direct attachment of a 3,5-dinitrophenyl group to the heterocycle would affect the antimycobacterial efficiency. Thus, the aim of this work was to synthesize a series of novel 5-alkylsulfanyl-1-(3,5-dinitrophenyl)-1*H*-tetrazoles (**20**) and 2-alkylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazoles (**23**) as the reverse analogues of the anti-TB lead compounds of series **11** and **13** and to investigate their structure-activity relationships. Furthermore, derivatives with hydroxy, acetoxy, amino and acetamido groups (**25**, **26**, **29** and **31**, respectively) instead of one of the nitro groups as well as derivatives with one trifluoromethyl group were prepared and studied (Table 1).

Table 1. Nitro-substituted tetrazole and oxadiazole derivatives studied in this work

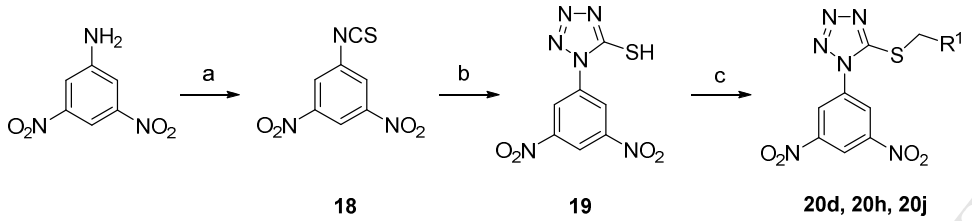


	R^1		R^1		R^1		R^1
a	H	**d**	Ph	**g**	3-ClPh	**j**	3,4-Cl_2Ph
b	CH_3CH_2	**e**	4-CH_3OPh	**h**	4-ClPh	**k**	4-BrPh
c	$CH_3(CH_2)_4$	**f**	2-ClPh	**i**	2,4-Cl_2Ph	**l**	4-NO_2Ph

Results and discussion

Chemistry. The synthesis of the 5-alkylsulfanyl-1-(3,5-dinitrophenyl)-1*H*-tetrazoles (**20d, 20h, 20j**), which are reverse analogues of the previously studied compounds of series **11**, is shown in Scheme 1. 3,5-Dinitrophenyl isothiocyanate (**18**) was prepared via the reaction of 3,5-dinitroaniline with thiophosgene in toluene and was converted into 1-(3,5-dinitrophenyl)-1*H*-tetrazole-5-thiol (**19**) via reaction with sodium azide in water at 80 °C. Alkylation of tetrazole **19** in acetonitrile in the presence of triethylamine gave the final products **20d**, **20h** and **20j** in 72 - 96% yields.

Scheme 1. Synthesis of 5-alkylsulfanyl-1-(3,5-dinitrophenyl)-1*H*-tetrazoles **20d, 20h** and **20j**.[a, b]



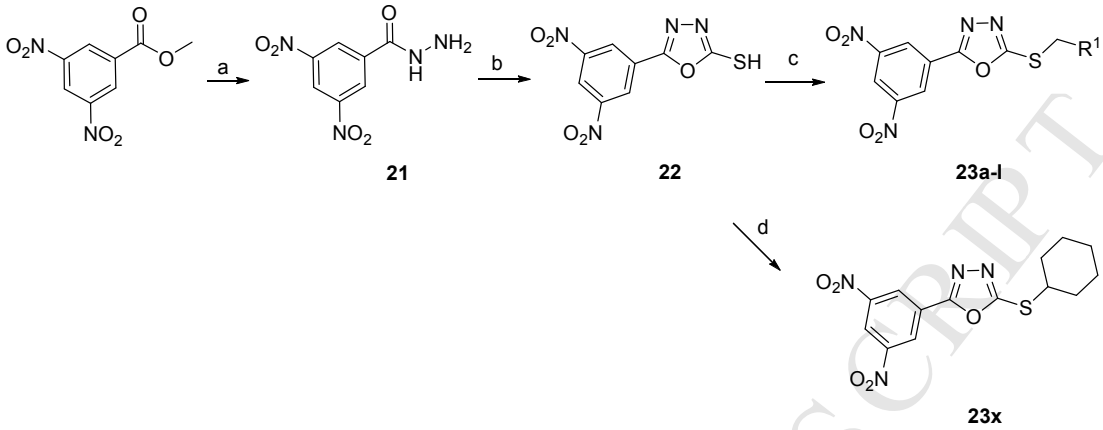
18 **19** **20d, 20h, 20j**

[a]Reagents and conditions: (a) $CSCl_2$, $PhCH_3$, 100 °C, 8 h, 51%; (b) NaN_3, H_2O, 80 °C, 5 h, 17%; (c) R^1CH_2X, Et_3N, CH_3CN, 2 h, 72 - 96%. [b] A list of R^1 groups can be found in Table 1.

2-Alkylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazoles (**23a - l, 23x**), which are reverse analogues of the previously studied compounds of series **13**, were synthesized as shown in Scheme 2. The slow addition of hydrazine hydrate to an ice cold suspension of methyl 3,5-dinitrobenzoate in ethanol resulted in the formation of the corresponding hydrazide **21**, which was further reacted with carbon disulfide and KOH in water at 90 °C. This reaction resulted in the formation of two main products, 5-(3,5-dinitrophenyl)-1,3,4-oxadiazole-2-thiol (**22**) in a 40% yield and 3-nitro-5-(5-sulfanyl-1,3,4-oxadiazol-2-yl)phenol (**24**) in a 32% yield.

The main product, 5-(3,5-dinitrophenyl)-1,3,4-oxadiazole-2-thiol (**22**), was converted into 2-alkylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazoles (**23a - l**) via the alkylation of oxadiazole-2-thiol **22** with various alkyl halides in acetonitrile in the presence of triethylamine (*Method A*) or under the conditions of phase-transfer catalysis in the presence of tetrabutylammonium bromide (TBAB) (*Method B*). The modified *Method A* was also used for the synthesis of 2-cyclohexylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazole (**23x**), which is the only example without the methylene linker between the sulfur atom and the substituent R^1 described in this work.

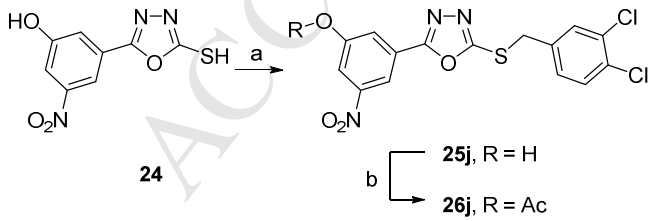
Scheme 2. Synthesis of 2-alkylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazoles **23a - l, 23x**.[a, b]



[a]Reagents and conditions: (a) 80% $NH_2NH_2.H_2O$, EtOH, 0 °C - rt, 4 h, 84%; (b) CS_2, KOH, H_2O, 90 °C, 10 h, 40%; (c) *Method A:* R^1CH_2X, Et_3N, CH_3CN, rt, overnight, 20 - 86%; *Method B:* R^1CH_2X, NaOH, TBAB, H_2O/CH_2Cl_2, rt, overnight, 52 - 68%; (d) $C_6H_{11}Br$, Et_3N, DMF, 80 °C, 20 h, 20%. [b] A list of R^1 groups can be found in Table 1.

3-Nitro-5-(5-sulfanyl-1,3,4-oxadiazol-2-yl)phenol (**24**) was alkylated using 3,4-dichlorobenzyl chloride to give the product **25j** in a 90% yield. In a further step, the hydroxy group of **25j** was acetylated to form compound **26j** (Scheme 3).

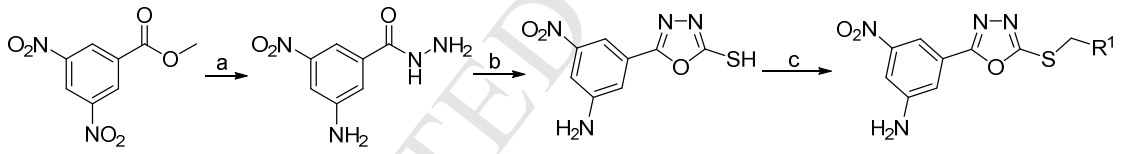
Scheme 3. Synthesis of 3-(5-((3,4-dichlorobenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitrophenol **25j** and its acetyl derivative **26j**.[a]



[a]Reagents and conditions: (a) 3,4-Cl_2PhCH_2Cl, Et_3N, CH_3CN, reflux, 1 h, 90%; (b) CH_3COCl, Et_3N, THF, 5 °C – reflux, 5 h, 52% or CH_3COCl, DIPEA, CH_3CN, rt, 48 h, 72%.

Furthermore, derivatives with one amino group instead of the original nitro group were prepared. In this case, methyl 3,5-dinitrobenzoate reacted with hydrazine hydrate in refluxing ethanol for 4 hours. The elevated temperature led to the formation of acyl hydrazide and the reduction of one nitro group. 3-Amino-5-nitrobenzohydrazide (**27**) was isolated in a 63% yield and was converted into the corresponding 1,3,4-oxadiazole-2-thiol (**28**) in an 82% yield. Alkylation of thiol **28** under phase-transfer catalysis conditions led to the formation of target 3-(5-alkylsulfanyl-1,3,4-oxadiazol-2-yl)-5-nitroanilines (**29d**, **29e**, **29h**, **29k**, **29l**) in high yields (51 - 91%) (Scheme 4). The alkylation of the amine group of compound **28** was not observed under these conditions.

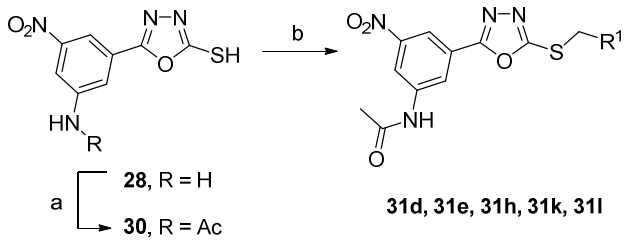
Scheme 4. Synthesis of 3-(5-alkylsulfanyl-1,3,4-oxadiazol-2-yl)-5-nitroanilines **29d**, **29e**, **29h**, **29k** and **29l**.[a, b]



[a]Reagents and conditions: (a) 80% $NH_2NH_2.H_2O$, EtOH, 4 h, reflux, 63%; (b) CS_2, KOH, EtOH, 8 h, reflux, 82%; (c) R^1CH_2X, NaOH, TBAB, H_2O/CH_2Cl_2, rt, overnight, 51 - 91%. [b] A list of R^1 groups can be found in Table 1.

The synthesis of the acetyl derivatives **31d, 31e, 31h, 31k** and **31l** is shown in Scheme 5. 5-(3-Amino-5-nitrophenyl)-1,3,4-oxadiazole-2-thiol (**28**) was acetylated with acetyl chloride in the presence of diisopropylethylamine (DIPEA) in acetonitrile. Alkylation of the resulting oxadiazole-2-thiol **30** in acetonitrile led to the formation of *N*-(3-(5-alkylsulfanyl-1,3,4-oxadiazol-2-yl)-5-nitrophenyl)acetamides (**31d, 31e, 31h, 31k, 31l**) in 52 - 92% yields.

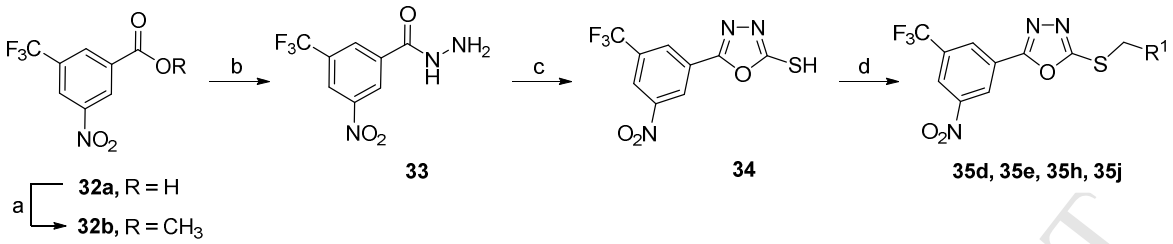
Scheme 5. Synthesis of *N*-(3-(5-alkylsulfanyl-1,3,4-oxadiazol-2-yl)-5-nitrophenyl)acetamides **31d, 31e, 31h, 31k** and **31l**.[a, b]



[a]Reagents and conditions: (a) CH_3COCl, DIPEA, CH_3CN, rt, 24 h, 81%; (b) R^1CH_2X, Et_3N, CH_3CN, reflux, 3 - 4 h, 52 - 92%. [b] A list of R^1 groups can be found in Table 1.

Trifluoromethyl group-containing analogues **35d**, **35e**, **35h** and **35j** were prepared as shown in Scheme 6. 3-Nitro-5-(trifluoromethyl)benzoic acid (**32a**) was prepared from commercially available 3-(trifluoromethyl)benzoic acid and was converted to methyl 3-nitro-5-(trifluoromethyl)benzoate (**32b**) via a standard acid-catalyzed esterification in boiling methanol. Hydrazinolysis of methyl 3-nitro-5-(trifluoromethyl)benzoate (**32b**) provided hydrazide **33**, which was then converted to 5-(3-nitro-5-(trifluoromethyl)phenyl)-1,3,4-oxadiazole-2-thiol (**34**) via a reaction with carbon disulfide in basic ethanol under reflux conditions. The target 2-(alkylsulfanyl)-5-(3-nitro-5-(trifluoromethyl)phenyl)-1,3,4-oxadiazoles (**35d**, **35e**, **35h** and **35j**) were prepared via alkylation of oxadiazol-2-thiol **34** under phase-transfer catalysis conditions.

Scheme 6. Synthesis of 2-(alkylsulfanyl)-5-(3-nitro-5-(trifluoromethyl)phenyl)-1,3,4-oxadiazoles **35d, 35e, 35h** and **35j**.[a, b]



[a]Reagents and conditions: (a) H_2SO_4, MeOH, 48 h, reflux, 87%; (b) $NH_2NH_2.H_2O$, EtOH, 48 h, reflux, 43%; (c) CS_2, KOH, EtOH, 48 h, reflux, 26%; (d) R^1CH_2X, NaOH, TBAB, H_2O/CH_2Cl_2, rt, 4 h, 16 - 54%. [b] A list of R^1 groups can be found in Table 1.

***In vitro* antimycobacterial activity.** The results of the *in vitro* antimycobacterial evaluation revealed very good activities for the 5-alkylsulfanyl-1-(3,5-dinitrophenyl)-1*H*-tetrazoles (**20d, 20h, 20j**) and outstanding activities for the 2-alkylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazoles (**23a - l, 23x**) against the drug-susceptible *M.tb*. My 331/88 strain, seven MDR/XDR strains of *M.tb.* and against the non-tuberculous strains *M. kansasii* My 235/80 and clinically isolated *M. kansasii* 6509/96 (Table 2, Table S1).

The direct attachment of the 3,5-dinitrophenyl moiety to the tetrazole nitrogen in the compounds of series **20** led to 2 - 4 times higher antimycobacterial efficiencies against *M.tb.* and both strains of *M. kansasii* compared to the activities of the parent compounds 1-alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles (**11**). The MIC values of the compounds of series **20** against *M.tb*. were in the range of 0.25 - 1 µM. The efficiency of the compounds of series **20** against *M. avium* My 330/88 also increased significantly, and the MIC values against *M. avium* were 4 - 32 µM, whereas the MIC values of the parent compounds **11** were in the range of 8 - 250 µM.

The antimycobacterial activities of the 1,3,4-oxadiazole reverse analogues **23a - l** and **23x** against *M.tb*. and against both *M. kansasii* strains were comparable or even better that those of the parent compounds **13** or the first-line anti-TB drugs. They had MIC values of 0.03 - 0.5 µM.

The comparison between the antimycobacterial activities of the tetrazole (**20**) and 1,3,4-oxadiazole derivatives (**23**) showed that the antimycobacterial activity of the reported compounds depended on the heterocycle involved. The 1,3,4-oxadiazole-based compounds of series **23** showed significantly higher activities than the tetrazole-containing substances of series **20**. This is the same result observed in our previous study. The 3,5-dinitrobenzylsulfanyl-1,3,4-oxadiazoles **13** also showed considerably higher antimycobacterial activities compared to the 3,5-dinitrobenzylsulfanyltetrazole analogues **11** and **12** [19-21]. The presence of 1,3,4-oxadiazole seems to be highly beneficial for the antimycobacterial activities of anti-TB agents based on the 3,5-dinitrobenzylsulfanyl moiety (**13**) and the 3,5-dinitrophenyl moiety (**23**).

The antimycobacterial activity of 2-alkylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazoles (**23a – l**, **23x**) was strongly influenced by the lipophilicity of the substituent R^1. This behavior was observed in our previous studies [19-21]. The significantly lower efficiency of 2-(3,5-dinitrophenyl)-5-methylsulfanyl-1,3,4-oxadiazole (**23a**) compared to other derivatives (**23b – l**, **23x**) can be explained by its lower lipophilicity. The loss of antimycobacterial activity in 3,5-dinitrobenzohydrazide (**21**) or 2-(3,5-dinitrophenyl)-1,3,4-oxadiazole-5-thiol (**22**) further supports this assumption.

The absence of antimycobacterial activity in the hydroxy analogue **25j**, its acetyl derivative **26j**, amino analogues **29d**, **29e**, **29h**, **29k**, **29l** and their acetyl derivatives **31d**, **31e**, **31h**, **31k**, **31l** confirmed our previous observation that both nitro groups are beneficial for a high antimycobacterial effect. However, in contrast to our previous studies, the compounds of series **35** with a trifluoromethyl group instead of one of the nitro groups also possessed antimycobacterial activities similar to the 3,5-dinitrophenyl derivatives of series **23** (Table 2, Table S1).

Table 2. *In vitro* antimycobacterial activities of the prepared compounds of series **20** - **23**, **25**, **26**, **29**, **31** and **35** expressed as MIC (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**
20d	0.5	4	2	2
20h	0.5	8	2	1
20j	0.25	8	1	0.5
21	62	125	125	125
22	62	62	125	125
23a	4	16	16	8
23b	0.25	8	2	2
23c	0.03	16	0.03	0.03
23x	0.03	16	0.03	0.03
23d	0.5	4	0.5	0.5
23e	0.03	4	0.125	0.25
23f	0.03	4	0.125	0.06
23g	0.03	4	0.06	0.06
23h	0.03	n.d.	0.06	0.03
23i	0.03	250	0.06	0.03
23j	0.03	16	0.03	0.03
23k	0.03	125	0.03	0.03
23l	0.5	2	0.5	0.5

25j	>250	>250	>250	>250
26j	1000	1000	1000	1000
29d	250	250	250	250
29e	125	250	250	250
29h	250	250	250	250
29k	125	125	125	125
29l	125	125	125	125
31d	>1000	>1000	>1000	>1000
31e	>1000	> 500	>1000	>1000
31h	250	>1000	>1000	>1000
31k	>500	>500	>500	>500
31l	>500	>500	>500	>500
35d	1	>250	4	4
35e	0.5	>250	4	2
35h	0.5	>250	1	1
35j	0.5	>250	1	1
INH	0.5	>250	>250	4

n.d. – not determined

Selected 5-alkylsulfanyl-1-(3,5-dinitrophenyl)-1*H*-tetrazoles (**20h**, **20j**), 2-alkylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazoles (**23e**, **23f**, **23g**, **23h**, **23j**, **23k**) and two trifluoromethyl analogues **35d** and **35j** were further tested against 7 multidrug-resistant strains of *M.tb*. (Table 3, Table S2). All of the compounds exhibited outstanding anti-TB activities with MIC values of 0.25 - 0.5 μM for the tetrazole derivatives **20h** and **20j** and 0.03 - 0.06 μM for the 1,3,4-

oxadiazole derivatives of series **23**, and no cross-resistance was observed with either the first or second-line anti-TB drugs. Trifluoromethyl analogues **35d** and **35j** had slightly lower but comparable activities to those of the dinitrophenyl derivatives of series **23**.

Table 3. *In vitro* antimycobacterial activities of selected compounds **20h**, **20j**, **23e**, **23f**, **23g**, **23h**, **23j**, **23k, 35d** and **35j** and common anti-TB drugs against MDR strains of *M. tb*. The results are expressed as MIC (μM) after 14 days of incubation.

	MDR *M. tuberculosis* strains						
	Praha 1	**Praha 4**	**Praha 131**	**9449/2007**	**234/2005**	**7357/1998**	**8666/2010**
20h	0.5	0.5	0.5	0.5	0.5	0.5	0.5
20j	0.25	0.25	0.25	0.25	0.25	0.25	0.25
23e	0.03	0.03	0.03	0.03	0.03	0.03	0.03
23f	0.06	0.03	0.06	0.06	0.06	0.06	0.06
23g	0.03	0.03	0.03	0.03	0.03	0.03	0.03
23h	0.03	0.03	0.03	0.03	0.03	0.03	0.03
23j	0.03	0.03	0.03	0.03	0.03	0.03	0.03
23k	0.03	0.03	0.03	0.03	0.03	0.03	0.03
35d	1	1	1	2	1	1	1
35j	0.5	0.25	0.5	0.5	0.5 / 0.5	0.5 / 0.5	0.5 / 0.5
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)
Rifampin	>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 the given antibiotic drug

R – Strain resistant to the given antibiotic drug

In vitro **antibacterial and antifungal activities.** To probe the selectivity of the inhibitory activities of the studied compounds towards mycobacteria, the effects of selected tetrazole-based compounds **20h** and **20j** and oxadiazole-based compounds **23e**, **23f**, **23h**, **23j** and **23k** against eight bacterial strains (4 gram positive cocci and 4 gram negative rods) and eight fungal strains (5 yeasts and 3 molds) were evaluated. The results showed that none of the studied compounds possessed antibacterial or antifungal activities, which indicated that the antimycobacterial effects of these compounds were highly selective (Table 4 and 5).

Table 4. *In vitro* antifungal activities of selected compounds expressed as MIC (μM).[a]

	Yeast strains										Mold strains					
	CA		**CT**		**CK**		**CG**		**TA**		**AF**		**AC**		**TM**	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	72 h	120h
20h	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
20j	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
23e	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
23f	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
23h	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
23j	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
23k	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125

FLU	0.82	-	1.6	-	105	-	26	-	210	-	>500	-	>500	-	105	-
AMB	0.54	-	0.54	-	1	-	0.54	-	0.27	-	0.54	-	1	-	0.54	-

[a]**CA** - *Candida albicans* ATCC 44859; **CT** - *Candida tropicalis* 156; **CK** - *Candida krusei* E28; **CG** - *Candida glabrata* 20/I; **TA** - *Trichosporon asahii* 1188; **AF** - *Aspergillus fumigatus* 231; **AC** - *Absidia corymbifera* 272; **TM** - *Trichophyton mentagrophytes* 445; **FLU** – Fluconazol; **AMB** – Amphotericin B.

Table 5. *In vitro* antibacterial activities of selected compounds expressed as MIC (μM).[a]

	Strains of gram positive cocci								Strains of gram negative rods							
	SA		**MRSA**		**SE**		**EF**		**EC**		**KP**		**KP-E**		**PA**	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
20h	125	125	125	125	500	500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
20j	62	62	125	125	250	250	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
23e	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
23f	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
23h	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
23j	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
23k	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
VAN	0.35	-	0.35	-	0.35	-	0.7	-	-	-	-	-	-	-	-	-
GEN	-	-	-	-	-	-	-	-	0.26	-	0.26	-	0.26	-	1	-

[a]**SA** - *Staphylococcus aureus* ATCC 6538; **MRSA** - methicillin resistant *Staphylococcus aureus* HK5996/08; **SE** - *Staphylococcus epidermidis* HK6966/08; **EF** - *Enterococcus* sp. HK14365/08; **EC** - *Escherichia coli* ATCC 8739; **KP** - *Klebsiella pneumoniae* HK11750/08; **KP-E** - extended spectrum β-lactamase producing *Klebsiella pneumoniae* HK14368/08; **PA** - *Pseudomonas aeruginosa* ATCC 9027; **VAN** – Vancomycin; **GEN** – Gentamicin.

***In vitro* effects of the studied compounds on mammalian cell proliferation/viability.** We tested the effects of the most promising compounds on mammalian cell viability using the HuH7 (human hepatocellular carcinoma), A431 (human epidermoid carcinoma), MDCK (Madin-Darby

canine kidney cells) and HepG2 (human hepatocellular carcinoma) cell lines (Table 6). 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 all the 3,5-dinitrophenyltetrazoles **20h** and **20j** and 3,5-dinitrophenyloxadiazoles **23e**, **23f**, **23g**, **23h**, **23j** and **23k** had negligible effects on the cell viability of these cell lines after 48 h of treatment. The selectivity indices, which were calculated as the ratio between the IC_{50} on the HepG2 cell lines and MIC against *M.tb.*, were higher than 60 for the tetrazole-based compounds **20h** and **20j** and higher than 1000 for the tested oxadiazole-based compounds **23e**, **23f**, **23g**, **23h**, **23j** and **23k** (Table 6).

Table 6. Viability determined via viability cell assays after 48 hours of treatment with compounds **20h**, **20j**, **23e**, **23f**, **23g**, **23h**, **23j** and **23k** for the Huh7, A431, MDCK and HepG2 cell lines and the calculated selectivity indices values. Vehicle-treated control viability was set to 100%. (CellTiter96® Assay)[a]

	HuH7		**A431**		**MDCK**		**HepG2**		**Selectivity index**
	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	IC_{50} (HepG2) / MIC (*M.tb.*)
20h	>30	115	>30	74	>30	85	>30	134	>60
20j	>30	137	>30	90	>30	84	>30	145	>120
23e	>30	118	>30	93	>30	119	>30	126	>1000
23f	>30	144	>30	91	>30	115	>30	129	>1000
23g	>30	158	>30	105	>30	105	>30	129	>1000
23h	>30	126	>30	100	>30	110	>30	126	>1000
23j	>30	153	>30	126	>30	91	>30	125	>1000
23k	>30	143	>30	99	>30	114	>30	113	>1000

[a]Standard deviations were < 10% of the mean

Conclusions

In this work we found that 5-alkylsulfanyl-1-(3,5-dinitrophenyl)-1*H*-tetrazoles (**20**) and 2-alkylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazoles (**23**) possessed excellent activity against replicating *M.tb.* strains, including the clinically isolated multidrug and extensively drug-resistant strains. The samples had MIC values as low as 0.03 µM, and they did not show any cross resistance with first or second-line anti-TB drugs. The studied compounds were designed as the reverse analogues of the previously studied 1-alkyl-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles (**11**) [19] and 2-alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfanyl]-1,3,4-oxadiazoles (**13**) [21]. Interestingly, the direct attachment of a 3,5-dinitrophenyl moiety to the tetrazole in compounds **20** or to the oxadiazole in compounds **23** did not lead to significant changes in the antimycobacterial activity compared to the previous lead compounds **11** and **13**. Furthermore, the compounds of series **20** and **23** had highly selective antimycobacterial effects because they were completely inactive against 4 gram positive and 4 gram negative bacterial strains and 8 fungal strains. The *in vitro* cytotoxicity experiments revealed that the IC_{50} values of the studied compounds against four mammalian cell lines, including hepatic cell lines HepG2 and HuH7, were higher than 30 µM. Thus, the selectivity indices of the most promising compounds **23e**, **23f**, **23g**, **23h**, **23j** and **23k** were higher than 1000. The structure-activity relationship study revealed that the presence of two nitro groups is highly beneficial for high antimycobacterial activity. The replacement of a nitro group for a hydroxy group in compound **25j** or the reduction of one nitro group to an amino group in series **29** compounds abolished the antimycobacterial activities, and acetylation of these hydroxy or amino groups did not restore the activity. However, in contrast to the previous oxadiazole lead compounds **13**, the replacement of one nitro

group in compounds **23** for another strong electron-withdrawing substituent, a trifluoromethyl group, did not diminish the activity. The antimycobacterial activities of the trifluoromethyl analogues **35** were only slightly lower than that of the original compounds **23**. The compounds studied in this work, in particular compounds of series **20**, **23** and **35**, proved to be highly active antimycobacterial agents with selective action and warrant further studies on their mechanisms of action and *in vitro/in vivo* assessments.

Experimental section

General. The structural identity of the prepared compounds was confirmed via ^{1}H-NMR and ^{13}C-NMR spectroscopy analyses. The purity of all the compounds reported was ≥95% as determined using elemental analysis. 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 using a Büchi B-545 apparatus (BUCHI Labortechnik AG, Flawil, Switzerland) and are uncorrected. The ^{1}H and ^{13}C NMR spectra were recorded using Varian Mercury Vx BB 300 or VNMR S500 NMR spectrometers (Varian, Palo Alto, CA, USA). Chemical shifts were reported as δ values in parts per million (ppm) and were indirectly referenced to tetramethylsilane (TMS) via the solvent signal. The elemental analysis was performed on an Automatic Microanalyser EA1110CE (Fisons Instruments S.p.A., Milano, Italy). HRMS (ESI+, ESI-) experiments were performed using the Q-Exactive Plus Mass Spectrometer (Thermo Scientific, Bremen, Germany) with direct infusion into the detector of the sample dissolved in acetonitrile:water, 1:1.

3,5-Dinitrophenyl isothiocyanate (**18**): Thiophosgene (2.51 g, 1.76 mL, 0.022 mol) was added dropwise to a solution of 3,5-dinitroaniline (2 g, 0.011 mol) in toluene (20 mL). The mixture was stirred under a nitrogen atmosphere at 100 °C for 8 hours. Upon completion, the reaction mixture was cooled to room temperature (rt) and neutralized with a saturated solution of K_2CO_3. The bilayer mixture was intensively stirred for 24 hours at rt. The organic phase was separated, dried with anhydrous sodium sulfate and evaporated under reduced pressure. The product was purified using column chromatography (Hexane/EtOAc, 10:1). Yield: 51% as an orange solid; mp 39 – 41 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.91 (t, *J* = 2.1 Hz, 1H), 8.38 – 8.33 (m, 2H), ^{13}C NMR (75 MHz, $CDCl_3$) δ 149.03, 143.45, 135.44, 125.63, 116.56. Anal. Calcd for $C_7H_3N_3O_4S$: C, 37.34; H, 1.34; N, 18.66; O, 28.42; S, 14.24. Found: C, 37.50; H, 1.17; N, 18.62; S, 14.36.

1-(3,5-Dinitrophenyl)-1H-tetrazole-5-thiol (**19**): The suspension of 3,5-dinitrophenyl isothiocyanate (**18**) (1.25 g, 5.55 mmol) and sodium azide (0.54 g, 8.33 mmol) in water (25 mL) was stirred at 80 °C for 5 hours. Upon completion, the reaction mixture was cooled down to rt, washed with EtOAc (2 × 25 mL) and then acidified with HCl to pH 1 - 2. The precipitated product was filtered, washed with water, dried and recrystallized from CH_3OH. Yield: 17% as a yellow solid; mp: 134 – 136 °C. ^{1}H NMR (300 MHz, DMSO) δ 9.33 (d, *J* = 2.1 Hz, 2H), 8.93 (t, *J* = 2.1 Hz, 1H). ^{13}C NMR (75 MHz, DMSO) δ 164.54, 148.26, 135.66, 124.15, 119.04. Anal. Calcd for $C_7H_4N_6O_4S$: C, 31.35; H, 1.50; N, 31.33; S, 11.95. Found: C, 31.33; H, 1.67; N, 31.29; S, 11.82.

General procedure for the synthesis of 5-alkylsulfanyl-1-(3,5-dinitrophenyl)-1*H*-tetrazoles (**20d**, **20h**, **20j**): An alkylating agent (0.51 mmol) was added to a solution of 1-(3,5-dinitrophenyl)-1*H*-tetrazole-5-thiol (**19**) (0.15 g, 0.56 mmol) and triethylamine (0.062 g, 0.61 mmol)) in CH_3CN (5 mL). The reaction mixture was refluxed for 2 hours. Upon completion, the

solvent was evaporated under reduced pressure. The crude product was dissolved in EtOAc (15 mL), washed with 5% aq. Na_2CO_3 (1 × 10 mL) and water (2 × 15 mL), and the organic layer was dried over anhydrous sodium sulfate and evaporated. The pure product was obtained using column chromatography (hexane/EtOAc, 10:1).

5-Benzylsulfanyl-1-(3,5-dinitrophenyl)-1H-tetrazole (**20d**): Benzyl bromide was used as an alkylating agent. Yield: 96% as a light yellow solid; mp 117 - 119 °C. ^{1}H NMR (500 MHz, acetone) δ 9.13 (td, *J* = 2.1, 0.6 Hz, 1H), 8.93 (dd, *J* = 2.0, 0.7 Hz, 2H), 7.46 – 7.41 (m, 2H), 7.34 – 7.25 (m, 3H), 4.67 (s, 2H). ^{13}C NMR (126 MHz, acetone) δ 155.41, 149.97, 136.86, 136.14, 130.05, 129.55, 128.87, 125.66, 120.59, 38.68. Anal. Calcd for $C_{14}H_{10}N_6O_4S$: C, 46.93; H, 2.81; N, 23.45; S, 8.95. Found: C, 47.31; H, 3.18; N, 23.59; S, 8.62.

5-[(4-Chlorobenzyl)sulfanyl]-1-(3,5-dinitrophenyl)-1H-tetrazole (**20h**): 4-Chlorobenzyl chloride was used as an alkylating agent. Yield: 82% as a light yellow solid; mp 100 - 102 °C. ^{1}H NMR (500 MHz, acetone) δ 9.13 (td, *J* = 2.1, 0.7 Hz, 1H), 8.96 (dd, *J* = 2.1, 0.7 Hz, 2H), 7.49 (d, *J* = 8.3 Hz, 2H), 7.34 (d, *J* = 8.3 Hz, 2H), 4.68 (s, 2H). ^{13}C NMR (126 MHz, acetone) δ 155.28, 149.98, 136.14, 134.23, 131.85, 129.52, 125.61, 120.60, 37.67. Anal. Calcd for $C_{14}H_9ClN_6O_4S$: C, 42.81; H, 2.31; N, 21.40; S, 8.16. Found: C, 42.46; H, 2.4; N, 21.68; S, 8.19.

5-[(3,4-Dichlorobenzyl)sulfanyl]-1-(3,5-dinitrophenyl)-1H-tetrazole (**20j**): 3,4-Dichlorobenzyl chloride was used as an alkylating agent. Yield: 72% as a light yellow solid; mp 97 - 99 °C. ^{1}H NMR (500 MHz, acetone) δ 9.14 (t, *J* = 2.1 Hz, 1H), 8.98 (d, *J* = 2.1 Hz, 2H), 7.73 (d, *J* = 2.1 Hz, 1H), 7.52 (d, *J* = 8.3 Hz, 1H), 7.48 (dd, *J* = 8.3, 2.1 Hz, 1H), 4.70 (s, 2H). ^{13}C NMR (126 MHz, acetone) δ 155.20, 150.00, 138.50, 136.10, 132.62, 132.18, 132.15, 131.51, 130.25, 125.62, 120.65, 36.94. Anal. Calcd for $C_{14}H_8Cl_2N_6O_4S$: C, 39.36; H, 1.89; N, 19.67; S, 7.50. Found: C, 39.52; H, 2.06; N, 19.87; S, 7.63.

3,5-Dinitrobenzohydrazide (**21**) [22]: Methyl 3,5-dinitrobenzoate was obtained in a 98% yield by refluxing 3,5-dinitrobenzoyl chloride in MeOH for 1 hour. Upon cooling, the precipitated product was filtered and washed with 1% NaOH. In the next step, hydrazine hydrate (80%, 0.94 g, 0.91 mL, 15 mmol) was added dropwise into the solution of methyl 3,5-dinitrobenzoate (1.13 g, 5 mmol) in EtOH (20 mL) at 0 °C. The reaction mixture was stirred for 4 hours at rt. Upon completion, the precipitated product was filtered and washed with EtOH (10 mL) and Et_2O (10 mL). The product was used without further purification. Yield: 84% as a yellowish solid; mp 153 - 154 °C (lit.[22] mp 155 - 157 °C). ^{1}H NMR (300 MHz, DMSO) δ 10.50 (s, 1H, N*H*), 9.00 (d, *J* = 2.1 Hz, 2H), 8.93 (t, *J* = 2.1 Hz, 1H), 4.74 (s, 2H, N*H*$_2$). ^{13}C NMR (75 MHz, DMSO) δ 161.50, 148.38, 136.09, 127.36, 120.84. Anal. Calcd for $C_7H_6N_4O_5$: C, 37.18; H, 2.67; N, 24.77. Found: C, 37.42; H, 2.68; N, 24.32.

5-(3,5-Dinitrophenyl)-1,3,4-oxadiazole-2-thiol (**22**) [23]: Carbon disulfide (0.7 g, 0.56 mL, 9.2 mmol) was added to a solution of 3,5-dinitrobenzohydrazide (**21**) (0.7 g, 3.1 mmol) and KOH (0.21 g, 3.75 mmol) in water (15 mL). The reaction mixture was heated at 90 °C for 10 hours. The product precipitated from the reaction mixture with some side products, and 1 M NaOH (3 mL) was added to the reaction mixture. The reaction mixture was stirred for 10 min and filtered. The aqueous solution was then acidified with HCl to pH 2 - 3 and extracted with EtOAc (2 × 20 mL). The organic layers were combined, washed with water (2 × 15 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure, and the product was purified using column chromatography (mobile phase: $CHCl_3$/hexane/CH_3COOH, 24:6:1, R*f* = 0.26). Yield: 40% as a yellow solid; mp 203 - 210 °C. ^{1}H NMR (300 MHz, acetone) δ 9.12 (t, *J* = 2.1 Hz, 1H), 9.02 (d, *J* = 2.1 Hz, 2H). ^{13}C NMR (75 MHz, acetone) δ 179.37, 158.54, 150.13,

127.02, 126.69, 121.91. Anal. Calcd for $C_8H_4N_4O_5S$: C, 35.83, H, 1.50; N, 20.89; S, 11.96. Found: C, 36.11; H, 1.90; N, 20.52; S, 11.63.

3-Nitro-5-(5-sulfanyl-1,3,4-oxadiazol-2-yl)phenol (**24**) was isolated as a by-product from a previous reaction (mobile phase: $CHCl_3$/hexane/CH_3COOH, 24:6:1, R*f* = 0.13). Yield: 32% as a yellow solid; mp 195 - 197 °C, ^{1}H NMR (500 MHz, DMSO) δ 14.82 (s, 1H), 7.62 (t, *J* = 1.8 Hz, 1H), 7.54 (t, *J* = 2.1 Hz, 1H), 7.41 (t, *J* = 1.9 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 177.64, 159.53, 151.10, 149.39, 124.31, 115.88, 110.06, 106.65. Anal. Calcd for $C_8H_5N_3O_4S$: C, 40.17; H, 2.11; N, 17.57; S, 13.4. Found: C, 40.63; H, 2.03; N, 17.32; S, 13.59.

General procedure for the synthesis of the 2-alkylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazoles (**23a - l**): *Method A:* An alkylating agent (0.51 mmol) was added to a solution of 5-(3,5-dinitrophenyl)-1,3,4-oxadiazole-2-thiol (**22**) (0.15 g, 0.56 mmol) and triethylamine (0.062 g, 0.61 mmol) in CH_3CN (7 mL). The reaction mixture was stirred overnight at rt. Upon reaction completion (as determined by TLC), the solvent was evaporated under reduced pressure. The crude product was dissolved in EtOAc (15 mL), and the organic solution was washed with 1% NaOH (15 mL) and water (2 × 15 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure, and the product was purified via crystallization or column chromatography.

Method B: The alkylating agent (0.51 mmol) and TBAB (0.025 mmol) solution in CH_2Cl_2 (5 mL) was added to the solution of 5-(3,5-dinitrophenyl)-1,3,4-oxadiazole-2-thiol (**22**) (0.15 g, 0.56 mmol) and NaOH (0.024 g, 0.61 mmol) in water (5 mL). The reaction mixture was gently stirred overnight at rt. Upon completion, the organic layer was separated, washed with water (2 × 7 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure, and the product was purified via crystallization or column chromatography.

5-(3,5-Dinitrophenyl)-2-methylsulfanyl-1,3,4-oxadiazole (**23a**): Dimethyl sulfate was used as an alkylating agent. The product was prepared via Method B and was purified using column chromatography (mobile phase: hexane/EtOAc, 3:1, R*f* = 0.41). Yield: 60% as a white solid; mp 124 - 127 °C. ^{1}H NMR (500 MHz, acetone) δ 9.10 (s, 3H), 2.86 (s, 3H). ^{13}C NMR (126 MHz, acetone) δ 167.82, 163.57, 150.13, 127.73, 126.90, 121.53, 14.72. Anal. Calcd for $C_9H_6N_4O_5S$: C, 38.30; H, 2.14; N, 19.85; S, 11.36. Found: C, 38.87; H, 2.35; N, 19.96; S, 11.22. The formation of 5-(3,5-dinitrophenyl)-3-methyl-1,3,4-oxadiazole-2(3*H*)-thione was also observed (mobile phase: hexane/EtOAc, 3:1, R *f* = 0.56) [24]. The compound was obtained in a 10% yield. ^{1}H NMR (500 MHz, acetone) δ 9.12 (t, *J* = 2.1 Hz, 1H), 9.01 (d, *J* = 2.1 Hz, 2H), 3.82 (s, 3H). ^{13}C NMR (126 MHz, acetone) δ 176.80, 155.52, 149.32, 125.88, 125.79, 36.13.

5-(3,5-Dinitrophenyl)-2-propylsulfanyl-1,3,4-oxadiazole (**23b**): Propyl bromide was used as an alkylating agent. The product was prepared via Method A and was purified using column chromatography (mobile phase: hexane/EtOAc, 6:1). Yield: 40% as a white solid; mp 70 - 72 °C. ^{1}H NMR (300 MHz, acetone) δ 9.10 (s, 3H), 3.39 (t, *J* = 7.2 Hz, 2H), 1.97 – 1.83 (m, 2H), 1.07 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, acetone) δ 167.20, 163.49, 150.10, 127.73, 126.91, 121.52, 34.97, 23.48, 13.20. Anal. Calcd for $C_{11}H_{10}N_4O_5S$: C, 42.58; H, 3.25; N, 18.06; S, 10.33. Found: C, 43.12; H, 3.35; N, 17.86; S, 10.58.

5-(3,5-Dinitrophenyl)-2-hexylsulfanyl-1,3,4-oxadiazole (**23c**): Hexyl bromide was used as an alkylating agent. The product was prepared via Method A and was purified using column chromatography (mobile phase: hexane/EtOAc, 7:1). Yield: 66% as a white solid; mp 72 - 75 °C. ^{1}H NMR (500 MHz, acetone) δ 9.12 (s, 3H), 3.47 – 3.38 (m, 2H), 1.96 – 1.83 (m, 2H), 1.57 – 1.47 (m, 4H), 1.37 – 1.35 (m, 2H), 0.93 – 0.88 (m, 3H). ^{13}C NMR (126 MHz, acetone) δ 176.98,

173.23, 159.86, 137.49, 136.64, 131.26, 42.87, 41.63, 39.20, 38.56, 32.86, 23.95. Anal. Calcd for $C_{14}H_{16}N_4O_5S$: C, 47.72; H, 4.58; N, 15.90; S, 9.10. Found: C, 48.1; H, 4.42; N, 15.53; S, 9.42.

2-Benzylsulfanyl-5-(3,5-Dinitrophenyl)-1,3,4-oxadiazole (**23d**): Benzyl bromide was used as an alkylating agent. The product was prepared via Method B and was purified using column chromatography (mobile phase: hexane/EtOAc, 3:1). Yield: 68% as a yellow solid; mp 155 - 157 °C (with decomposition). ^{1}H NMR (300 MHz, DMSO) δ 8.97 (t, *J* = 2.1 Hz, 1H), 8.94 (d, *J* = 2.1 Hz, 2H), 7.55 - 7.47 (m, 2H), 7.39 - 7.27 (m, 3H), 4.64 (s, 2H); ^{13}C NMR (75 MHz, DMSO) δ 165.41, 162.77, 148.88, 136.67, 129.29, 128.78, 128.02, 126.38, 125.92, 121.14, 36.08. Anal. Calcd for $C_{15}H_{10}N_4O_5S$: C, 50.28; H, 2.81; N, 15.64; S, 8.95. Found: C, 49.91, H, 2.74; N, 15.37; S, 9.12.

5-(3,5-Dinitrophenyl)-2-[(4-methoxybenzyl)sulfanyl]-1,3,4-oxadiazole (**23e**): 4-Methoxybenzyl chloride was used as an alkylating agent. The product was prepared via Method A and was purified by crystallization (EtOH/H_2O). Yield: 84% as a yellowish solid; mp 142 - 145 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.97 (t, *J* = 2.1 Hz, 1H), 8.94 (d, *J* = 2.1 Hz, 2H), 7.42 (d, *J* = 8.6 Hz, 2H), 6.90 (d, *J* = 8.6 Hz, 2H), 4.59 (s, 2H), 3.72 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 165.50, 162.71, 159.07, 148.87, 130.61, 128.27, 126.35, 125.92, 121.10, 114.15, 55.25, 35.82. Anal. Calcd for $C_{16}H_{12}N_4O_6S$: C, 49.48; H, 3.11; N, 14.43; S, 8.26. Found: C, 49.62: H, 3.28; N, 14.35; S, 8.64.

2-[(2-Chlorobenzyl)sulfanyl]-5-(3,5-dinitrophenyl)-1,3,4-oxadiazole (**23f**): 2-Chlorobenzyl chloride was used as an alkylating agent. The product was prepared via Method A and was purified by crystallization (EtOH/H_2O). Yield: 86% as a yellowish solid; mp 137 - 139 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.99 - 8.97 (m, 1H), 8.95 - 8.94 (m, 2H), 7.68 - 7.65 (m, 1H), 7.53 - 7.49 (m, 1H), 7.39 - 7.32 (m, 2H), 4.72 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 164.86, 162.98,

148.89, 133.93, 133.55, 131.89, 130.26, 129.84, 127.66, 126.42, 125.89, 121.20, 34.44. Anal. Calcd for $C_{15}H_9ClN_4O_5S$: C, 45.87; H, 2.31; N, 14.26; S, 8.16. Found: C, 46.06; H, 2.48; N, 14.28; S, 8.29.

2-[(3-Chlorobenzyl)sulfanyl]-5-(3,5-dinitrophenyl)-1,3,4-oxadiazole (**23g**): 3-Chlorobenzyl chloride was used as an alkylating agent. The product was prepared via Method A and was purified using column chromatography (mobile phase: hexane/EtOAc, 4:1). Yield: 74% as a yellowish solid; mp 112 - 114 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.97 (t, *J* = 2.1 Hz, 1H), 8.94 (d, *J* = 2.2 Hz, 2H), 7.62 – 7.60 (m, 1H), 7.50- 7.47 (m, 1H), 7.40 - 7.34 (m, 2H), 4.64 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 165.20, 162.87, 148.86, 139.47, 133.19, 130.56, 129.12, 127.99, 127.93, 126.38, 125.91, 121.15, 35.23. Anal. Calcd for $C_{15}H_9ClN_4O_5S$: C, 45.87; H, 2.31; N, 14.26; S, 8.16. Found: C, 46.41; H, 2.54; N, 13.94; S, 8.11.

2-[(4-Chlorobenzyl)sulfanyl]-5-(3,5-dinitrophenyl)-1,3,4-oxadiazole (**23h**): 4-Chlorobenzyl chloride was used as an alkylating agent. The product was prepared via Method A and was purified by crystallization (CH_3CN/H_2O). Yield: 79% as a light beige solid; mp 163 - 168 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.97 (t, *J* = 2.1 Hz, 1H), 8.93 (d, *J* = 2.1 Hz, 2H), 7.54 (d, *J* = 8.3 Hz, 2H), 7.41 (d, *J* = 8.3 Hz, 2H), 4.63 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 165.23, 162.83, 148.87, 136.00, 132.63, 131.17, 128.71, 126.38, 125.90, 121.14, 35.24. Anal. Calcd for $C_{15}H_9ClN_4O_5S$: C, 45.87; H, 2.31; N, 14.26; S, 8.16. Found: C, 46.56; H, 2.54; N, 14.53; S, 8.19.

2-[(2,4-Dichlorobenzyl)sulfanyl]-5-(3,5-dinitrophenyl)-1,3,4-oxadiazole (**23i**): 2,4-Dichlorobenzyl chloride was used as an alkylating agent. The product was prepared via Method A and was purified by crystallization ($EtOH/H_2O$). Yield: 63% as a white solid; mp 129 - 130 °C. ^{1}H NMR (500 MHz, acetone) δ 9.14 – 9.10 (m, 3H), 7.78 (d, *J* = 8.3 Hz, 1H), 7.60 (d, *J* = 2.2 Hz, 1H), 7.42 (dd, *J* = 8.3, 2.2 Hz, 1H), 4.77 (s, 2H). ^{13}C NMR (126 MHz, acetone) δ 166.00,

163.95, 150.12, 135.74, 135.26, 134.02, 133.75, 130.18, 128.39, 127.58, 127.02, 121.69, 34.55. Anal. Calcd for $C_{15}H_8Cl_2N_4O_5S$: C, 42.17; H, 1.89; C, 13.11; N, 7.50. Found: C, 41.8; H, 1.91; N, 12.79; S, 7.37.

2-[(3,4-Dichlorobenzyl)sulfanyl]-5-(3,5-dinitrophenyl)-1,3,4-oxadiazole (**23j**): 3,4-Dichlorobenzyl chloride was used as an alkylating agent. The product was prepared via Method A and was purified using column chromatography (mobile phase: hexane/EtOAc, 4:1). Yield: 85% as a white solid; mp 132 - 134 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.97 (t, *J* = 2.1 Hz, 1H), 8.93 (d, *J* = 2.1 Hz, 2H), 7.81 (d, *J* = 2.0 Hz, 1H), 7.61 (d, *J* = 8.3 Hz, 1H), 7.51 (dd, *J* = 8.3, 2.0 Hz, 1H), 4.63 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 165.09, 162.90, 148.86, 138.32, 131.28, 131.14, 130.83, 130.60, 129.66, 126.38, 125.91, 121.16, 34.61. Anal. Calcd for $C_{15}H_8Cl_2N_4O_5S$: C, 42.17; H, 1.89; N, 13.11; S, 7.50. Found: 42.71; H, 2.25; N, 12.86; S, 8.25.

2-[(4-Bromobenzyl)sulfanyl]-5-(3,5-dinitrophenyl)-1,3,4-oxadiazole (**23k**): 4-Bromobenzyl bromide was used as an alkylating agent. The product was prepared via Method A and was purified using column chromatography (mobile phase: hexane/EtOAc, 4:1). Yield: 74% as a white solid; mp 188 - 189 °C. ^{1}H NMR (300 MHz, DMSO) δ 8.97 (t, *J* = 2.1 Hz, 1H), 8.93 (d, *J* = 2.1 Hz, 2H), 7.55 (d, *J* = 8.5 Hz, 2H), 7.47 (d, *J* = 8.5 Hz, 2H), 4.62 (s, 2H). ^{13}C NMR (75 MHz, DMSO) δ 165.25, 162.87, 148.89, 136.47, 131.66, 131.54, 126.42, 125.90, 121.21, 121.18, 35.29. Anal. Calcd for $C_{15}H_9BrN_4O_5S$: C, 41.21; H, 2.07; N, 12.81; S, 7.33. Found: C, 41.15; H, 2.23; N, 12.82; S, 7.58.

5-(3,5-Dinitrophenyl)-2-[(4-nitrobenzyl)sulfanyl]-1,3,4-oxadiazole (**23l**): 4-Nitrobenzyl chloride was used as an alkylating agent. The product was prepared via Method B and was purified using column chromatography (mobile phase: hexane/EtOAc, 5:1). Yield: 52 % as a white solid; mp 187 - 188 °C. ^{1}H NMR (300 MHz, DMSO) δ 8.98 - 8.96 (m, 1H), 8.92 – 8.90 (m, 2H), 8.22 (d, *J*

= 9.0 Hz, 2H), 7.80 (d, *J* = 9.0 Hz, 2H), 4.76 (s, 2H). ^{13}C NMR (75 MHz, DMSO) δ 165.01, 162.94, 148.88, 147.11, 145.12, 130.61, 126.41, 125.87, 123.84, 121.19, 35.09. Anal. Calcd for $C_{15}H_9N_5O_7S$: C, 44.67; H, 2.25; N, 17.36; S, 7.95. Found: C, 44.7; H, 2.19; N, 17.2; S, 8.24.

2-Cyclohexylsulfanyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazole (**23x**): Cyclohexyl bromide was used as an alkylating agent. The product was prepared via Method A, but DMF was used as the solvent. The reaction mixture was heated at 80 °C for 20 hours, but the reaction was not complete. The product was purified using column chromatography (mobile phase: hexane/EtOAc, 10:1, R*f* = 0.31). Yield: 20% as a white solid; mp 89 - 90 °C. ^{1}H NMR (500 MHz, Acetone) δ 9.14 – 9.11 (m, 3H), 3.94 – 3.89 (m, 1H), 2.28 – 2.20 (m, 2H), 1.88 – 1.78 (m, 2H), 1.73 – 1.60 (m, 2H), 1.57 – 1.48 (m, 2H), 1.44 – 1.27 (m, 2H). ^{13}C NMR (126 MHz, Acetone) δ 166.41, 163.48, 150.10, 127.75, 126.95, 121.55, 47.64, 33.85, 26.36, 25.98. Anal. Calcd for $C_{14}H_{14}N_4O_5S$: C, 48.0; H, 4.03; N, 15.99; S, 9.15. Found: C, 48.86; H, 4.25; N, 15.41; S, 8.84.

Formation of 3-cyclohexyl-5-(3,5-dinitrophenyl)-1,3,4-oxadiazole-2(3*H*)-thione was also observed (mobile phase: hexane/EtOAc, 10:1, R*f* = 0.39). The compound was obtained in a 5% yield (yellow solid); mp 193 - 196 °C. ^{1}H NMR (500 MHz, acetone) δ 9.14 (t, *J* = 2.1 Hz, 1H), 9.06 (d, *J* = 2.1 Hz, 2H), 4.64 – 4.53 (m, 1H), 2.12 – 2.02 (m, 2H), 2.00 – 1.91 (m, 2H), 1.89 – 1.72 (m, 2H), 1.59 – 1.45 (m, 2H), 1.40 – 1.28 (m, 2H). ^{13}C NMR (126 MHz, acetone) δ 176.43, 156.76, 150.13, 126.95, 126.69, 121.90, 59.21, 30.99, 29.33, 25.67. Anal. Calcd for $C_{14}H_{14}N_4O_5S$: C, 48.0; H, 4.03; N, 15.99; S, 9.15. Found: C, 48.03; H, 4.41; N, 15.78; S, 9.3.

3-(5-((3,4-Dichlorobenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitrophenol (**25j**): 3,4-Dichlorobenzyl chloride (0.22 g, 1.12 mmol) was added to a solution of 3-(5-mercapto-1,3,4-oxadiazol-2-yl)-5-nitrophenol (**24**) (0.3 g, 1.25 mmol) and triethylamine (0.127 g, 0.175 mL,

1.25 mmol) in CH_3CN (10 mL). The reaction mixture was refluxed for 1 h. Upon cooling, the product precipitated from the reaction mixture. The product was filtered, washed with water, dried and recrystallized from EtOH/H_2O. Yield: 90% as a yellow solid; mp 179 - 181 °C. ^{1}H NMR (500 MHz, DMSO) δ 7.77 (d, *J* = 2.1 Hz, 1H), 7.69 (t, *J* = 1.8 Hz, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.55 (t, *J* = 2.2 Hz, 1H), 7.52 – 7.45 (m, 2H), 4.56 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 164.59, 163.68, 151.06, 149.41, 138.31, 131.23, 131.14, 130.83, 130.57, 129.60, 124.71, 116.22, 110.05, 107.12, 34.68. Anal. Calcd for $C_{15}H_9Cl_2N_3O_4S$: C, 45.24; H, 2.28; N, 10.55; S, 8.05. Found: C, 45.45; H, 2.50; N, 10.19; S, 8.33.

3-(5-((3,4-Dichlorobenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitrophenyl acetate (**26j**): Two methods were employed: 1. Triethylamine (0.053 g, 0.52 mmol) was added to a mixture of 3-(5-((3,4-dichlorobenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitrophenol (**25j**) (0.15 g, 0.38 mmol) in THF (7 mL). The mixture was cooled to 5 °C, and acetyl chloride (0.04 g, 0.51 mmol) was added. The reaction mixture was refluxed for 5 hours. The reaction mixture was then filtered and evaporated under reduced pressure. The product was purified using column chromatography (mobile phase: hexane/EtOAc, 3:1). Yield: 52%.

2. Acetyl chloride (0.071 g, 0.9 mmol) was added to a suspension of 3-(5-((3,4-dichlorobenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitrophenol (**25j**) (0.12 g, 0.3 mmol) and diisopropylethylamine (DIPEA) (0.117 g, 0.155 mL, 0.9 mmol) in CH_3CN (7 mL). The reaction mixture was stirred at rt for 48 h. The yellow suspension turned into a white suspension. Although the reaction was not complete, the solvent was evaporated under reduced pressure, and the crude product was dissolved in EtOAc (15 mL) and washed with water (2 × 15 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The product was purified using column chromatography (mobile phase: hexane/EtOAc, 5:2).

Yield: 72% as a white solid; mp 202 - 204 °C. ^{1}H NMR (500 MHz, acetone) δ 8.79 (t, *J* = 2.1 Hz, 1H), 8.63 (t, *J* = 1.8 Hz, 1H), 8.39 – 8.36 (m, 1H), 7.79 (d, *J* = 1.4 Hz, 1H), 7.58 – 7.54 (m, 2H), 4.64 (s, 2H), 2.19 (s, 3H). ^{13}C NMR (126 MHz, acetone) δ 169.91, 165.18, 150.09, 142.52, 138.93, 132.75, 132.21, 132.11, 131.61, 130.18, 126.33, 122.45, 116.72, 115.95, 35.76, 24.33. Anal. Calcd for $C_{17}H_{11}Cl_2N_3O_5S$: C, 46.38; H, 2.52; N, 9.54; S. 7.28. Found: C, 46.82; H, 2.71; N. 9.40; S, 7.05.

3-Amino-5-nitrobenzohydrazide (**27**): Methyl-3,5-dinitrobenzoate (1 g, 4.4 mmol) was dissolved in methanol (20 mL), and 80% hydrazine hydrate (1.07 mL, 22 mmol) was added. The reaction mixture was refluxed for 4 hours. The methanol was evaporated under reduced pressure, and the crude product was purified using column chromatography (mobile phase: $CHCl_3$/MeOH/Et_3N, 100:1:1). Yield: 63% as a yellow solid; mp 220 - 222 °C. ^{1}H NMR (300 MHz, DMSO) δ 9.91 (s, 1H), 7.74 – 7.71 (m, 1H), 7.48 – 7.45 (m, 1H), 7.38 (dd, *J* = 2.3, 1.4 Hz, 1H), 6.00 (s, 2H), 4.52 (s, 2H). ^{13}C NMR (75 MHz, DMSO) δ 164.73, 150.32, 148.87, 135.50, 118.63, 109.26, 108.16. Anal. Calcd for C, 42.86; H, 4.11; N, 28.56. Found C, 43.07; H, 4.00; N, 28.42.

5-(3-Amino-5-nitrophenyl)-1,3,4-oxadiazole-2-thiol (**28**): Carbon disulfide (0.15 mL, 2.55 mmol) was added dropwise to a suspension of 3-amino-5-nitrobenzohydrazide (**27**) (0.5 g, 2.55 mmol) and KOH (0.143 g, 2.55 mmol) in ethanol (15 mL). The reaction mixture was refluxed for 8 hours and cooled to rt, and the solvent was evaporated under reduced pressure. The crude product was dissolved in water (50 mL), acidified with 2% HCl (5 mL) to pH = 1 and extracted with EtOAc (3 × 20 mL). The combined organic extracts were washed with water (2 × 15 mL), dried over anhydrous sodium sulfate and evaporated under reduced pressure. Yield: 82% as a yellow solid; mp 118 °C. ^{1}H NMR (300 MHz, DMSO) δ 7.62 – 7.59 (m, 1H), 7.53 (t, *J* = 2.1 Hz, 1H), 7.42 – 7.38 (m, 1H), 6.21 (s, 2H). ^{13}C NMR (75 MHz, DMSO) δ 177.62, 159.54, 151.09,

149.38, 124.30, 115.87, 110.06, 106.65. Anal. Calcd for $C_8H_6N_4O_3S$: C, 40.34; H, 2.54; N, 23.52; O, 20.15; S, 13.46. Found C, 39.13; H, 2.64; N, 22.62; S, 15.84.

3-(5-(Benzylsulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitroaniline (**29d**): Benzyl bromide (0.044 mL, 0.38 mmol) was added to a solution of 5-(3-amino-5-nitrophenyl)-1,3,4-oxadiazole-2-thiol (**28**) (0.1 g, 0.42 mmol) and triethylamine (0.025 mL, 0.42 mmol) in CH_3CN (7 mL). The reaction mixture was refluxed for 2 hours. Upon completion (as determined by TLC), the solvent was evaporated under reduced pressure. The crude product was dissolved in EtOAc (15 mL), and the organic solution was washed with 10% Na_2CO_3 (2 × 15 mL) and brine (1 × 15 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure, and the product was purified using column chromatography (mobile phase: hexane/EtOAc, 1:1). Yield: 51% as a yellow solid; mp 198 - 199 °C. ^{1}H NMR (500 MHz, DMSO) δ 7.70 (dd, *J* = 2.2, 1.4 Hz, 1H), 7.55 (t, *J* = 2.2 Hz, 1H), 7.51 (dd, *J* = 2.2, 1.4 Hz, 1H), 7.49 – 7.46 (m, 2H), 7.37 – 7.32 (m, 2H), 7.31 – 7.26 (m, 1H), 6.24 (s, 2H), 4.58 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 164.44, 163.99, 151.08, 149.43, 136.63, 129.23, 128.77, 127.99, 124.75, 116.20, 110.03, 107.12, 36.13. Anal. Calcd for $C_{15}H_{12}N_4O_3S$: C, 54.87; H, 3.68; N, 17.06; S, 9.76. Found C, 56.49; H, 4.04; N, 16.17; S, 9.55.

General procedure for the synthesis of 3-(5-alkylsulfanyl-1,3,4-oxadiazol-2-yl)-5-nitroanilines (**29e**, **29h**, **29k**, **29l**): A solution of the alkylating agent (0.57 mmol) and TBAB (0.057 mmol) in CH_2Cl_2 (5 mL) was added to a solution of 5-(3-amino-5-nitrophenyl)-1,3,4-oxadiazole-2-thiol (**28**) (0.15 g, 0.63 mmol) and NaOH (0.03 g, 0.76 mmol) in water (5 mL). The reaction mixture was gently stirred overnight at rt. Upon completion, the reaction mixture was diluted with CH_2Cl_2 (10 mL), and the organic layer was separated, washed with 10% Na_2CO_3 (2 × 15 mL) and brine (1 × 10 mL) and dried over anhydrous sodium sulfate. The solvent was

evaporated under reduced pressure, and the product was purified using crystallization or column chromatography.

3-(5-((4-Methoxybenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitroaniline (**29e**): 4-Methoxybenzyl chloride was used as an alkylating agent. The product was purified using column chromatography (mobile phase: hexane/EtOAc, 2:1). Yield: 91% as a yellow solid; mp 158 - 160 °C. ^{1}H NMR (300 MHz, DMSO) δ 7.71 (dd, *J* = 2.2, 1.4 Hz, 1H), 7.58 – 7.54 (m, 1H), 7.51 (dd, *J* = 2.2, 1.4 Hz, 1H), 7.39 (d, *J* = 8.7 Hz, 2H), 6.89 (d, *J* = 8.7 Hz, 2H), 6.25 (s, 2H), 4.53 (s, 2H), 3.72 (s, 3H). ^{13}C NMR (75 MHz, DMSO) δ 164.39, 164.09, 159.05, 151.09, 149.43, 130.58, 128.26, 124.78, 116.19, 114.18, 110.01, 107.11, 55.26, 35.84. Anal. Calcd for $C_{16}H_{14}N_4O_4S$: C, 53.61; H, 3.94; N, 15.63; S, 8.95. Found C, 53.61; H, 3.93; N, 15.37; S 8.87.

3-(5-((4-Chlorobenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitroaniline (**29h**): 4-Chlorobenzyl chloride was used as an alkylating agent. The product precipitated from the reaction mixture. It was filtered, washed with hexane (2 × 10 mL), and water (1 × 10 mL). Yield: 56% as a yellow solid; mp 203 - 204 °C. ^{1}H NMR (300 MHz, DMSO) δ 7.69 (s, 1H), 7.59 – 7.46 (m, 4H), 7.40 (m, 2H), 6.25 (s, 2H), 4.57 (s, 2H). ^{13}C NMR (75 MHz, DMSO) δ 164.52, 163.84, 151.08, 149.41, 135.98, 132.61, 131.13, 128.72, 124.73, 116.19, 110.04, 107.13, 35.27. Anal. Calcd for $C_{15}H_{11}ClN_4O_3S$: C, 49.66; H, 3.06; N, 15.44; S, 8.84. Found C, 49.89; H, 3.14; N, 15.21; S, 9.09.

3-(5-((4-Bromobenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitroaniline (**29k**): 4-Bromobenzyl bromide was used as an alkylating agent. The product partially precipitated from the reaction mixture. The precipitated product was filtered and washed with hexane (1 × 10 mL) and water (1 × 10 mL). The filtrate was extracted with $CHCl_3$ (2 × 15 mL), and the organic extracts were dried over anhydrous sodium sulfate and evaporated. An additional portion of the product was purified using column chromatography (mobile phase: hexane/EtOAc, 2:1). Yield: 85% as a yellow solid;

mp 209 - 211 °C. ^{1}H NMR (300 MHz, DMSO) δ 7.69 (t, *J* = 1.8 Hz, 1H), 7.59 – 7.48 (m, 4H), 7.47 – 7.39 (m, 2H), 6.25 (s, 2H), 4.55 (s, 2H). ^{13}C NMR (75 MHz, DMSO) δ 164.53, 163.84, 151.09, 149.42, 136.42, 131.66, 131.46, 124.74, 121.17, 116.20, 110.05, 107.14, 35.33. Anal. Calcd for $C_{15}H_{11}BrN_4O_3S$: C, 44.24; H, 2.72; N, 13.76; S, 7.87. Found C, 44.20; H, 2.84; N, 13.69; S, 7.97.

3-Nitro-5-(5-((4-nitrobenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)aniline (**29l**): 4-Nitrobenzyl chloride was used as an alkylating agent. The product partially precipitated from the reaction mixture. The precipitated product was filtered and washed with hexane (2 × 10 mL) and water (1 × 10 mL). The filtrate was extracted with $CHCl_3$ (2 × 15 mL), and the organic extracts were dried over anhydrous sodium sulfate and evaporated. An additional portion of the product was purified using column chromatography (mobile phase: hexane/EtOAc, 2:1). Yield: 71% as a yellow solid; mp 200 - 201 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.21 (d, *J* = 8.7 Hz, 2H), 7.77 (d, *J* = 8.7 Hz, 2H), 7.67 – 7.64 (m, 1H), 7.55 (t, *J* = 2.2 Hz, 1H), 7.50 – 7.48 (m, 1H), 6.24 (s, 2H), 4.70 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 164.60, 163.57, 151.06, 149.39, 147.06, 145.07, 130.51, 124.67, 123.80, 116.17, 110.05, 107.12, 35.14. Anal. Calcd for $C_{15}H_{11}N_5O_5S$: C, 48.26; H, 2.97; N, 18.76; S, 8.59. Found C, 48.28; H, 2.69; N, 18.38; S, 8.4.

N-(3-(5-sulfanyl-1,3,4-oxadiazol-2-yl)-5-nitrophenyl)acetamide (**30**): Acetyl chloride (1.41 g, 1.28 mL, 18 mmol) was added to a solution of 5-(3-amino-5-nitrophenyl)-1,3,4-oxadiazole-2-thiol (**28**) (1.5 g, 6.3 mmol) and diisopropylethylamine (DIPEA) (2.27 g, 3.14 mL, 18 mmol) in anhydrous CH_3CN (20 mL). The reaction mixture was stirred for 24 hours at rt. The solvent was evaporated under reduced pressure, and the crude product was dissolved in EtOAc (75 mL) and washed with 10% Na_2CO_3 (3 × 20 mL). The organic phase was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The product was purified using column

chromatography (hexane/EtOAc/CH_3COOH, 66:33:1). Yield: 81% as a yellow solid; mp 252 - 253 °C (with decomposition). ^{1}H NMR (500 MHz, DMSO) δ 14.90 (s, 1H), 10.63 (s, 1H), 8.64 (t, *J* = 2.1 Hz, 1H), 8.44 – 8.42 (m, 1H), 8.13 (t, *J* = 1.8 Hz, 1H), 2.12 (s, 3H).^{13}C NMR (126 MHz, DMSO) δ 177.57, 169.58, 158.91, 148.64, 141.44, 124.40, 120.97, 115.62, 114.39, 24.28. Anal. Calcd for $C_{10}H_8N_4O_4S$: C, 42.86; H, 2.88; N, 19.99; O, 22.84; S, 11.44. Found C, 43.02; H, 2.96; N, 19.36; S, 11.88.

General procedure for the synthesis of *N*-(3-(5-alkylsulfanyl-1,3,4-oxadiazol-2-yl)-5-nitrophenyl)acetamides (31d, 31e, 31h, 31k and 31l): An alkylating agent (0.32 mmol) was added to a solution of *N*-(3-(5-sulfanyl-1,3,4-oxadiazol-2-yl)-5-nitrophenyl)acetamide (**30**) (0.1 g, 0.36 mmol) and triethylamine (0.054 mL, 0.4 mmol) in CH_3CN (7 mL). The reaction mixture refluxed for 3 - 4 h. Upon completion (as determined by TLC), the solvent was evaporated under reduced pressure. The crude product was dissolved in EtOAc (15 mL), and the organic solution was washed with 10% Na_2CO_3 (2 × 15 mL) and brine (1 × 15 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure, and the product was purified using column chromatography.

N-(3-(5-(benzylsulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitrophenyl)acetamide (**31d**): Benzyl bromide was used as an alkylating agent. The reaction mixture was refluxed for 4 hours. The product was purified using column chromatography (mobile phase: hexane/EtOAc/CH_3COOH, 60:40:1). Yield: 52% as a yellow solid; mp 192 - 193 °C. ^{1}H NMR (300 MHz, DMSO) δ 10.65 (s, 1H), 8.67 (t, *J* = 2.1 Hz, 1H), 8.51 (t, *J* = 1.8 Hz, 1H), 8.22 (t, *J* = 1.8 Hz, 1H), 7.53 – 7.46 (m, 2H), 7.39 – 7.25 (m, 3H), 4.60 (s, 2H), 2.11 (s, 3H). ^{13}C NMR (75 MHz, DMSO) δ 169.60, 164.53, 163.85, 148.70, 141.46, 136.61, 129.28, 128.80, 128.02, 124.82, 121.37, 115.62, 114.87, 36.12,

24.30. Anal. Calcd for $C_{17}H_{14}N_4O_4S$: C, 55.13; H, 3.81; N, 15.13; S, 8.66. Found C, 55.27; H, 4.08; N, 14.89; S, 8.5.

N-(3-(5-((4-methoxybenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitrophenyl)acetamide (**31e**): 4-Methoxybenzyl chloride was used as an alkylating agent. The reaction mixture was refluxed for 4 hours. The product was purified using column chromatography (mobil phase: hexane/EtOAc/CH_3COOH, 60:40:1). Yield: 92% as a yellow solid; mp 189 - 192 °C. ^{1}H NMR (500 MHz, DMSO) δ 10.64 (s, 1H), 8.67 (t, *J* = 2.1 Hz, 1H), 8.51 (t, *J* = 1.8 Hz, 1H), 8.23 (t, *J* = 1.8 Hz, 1H), 7.41 (d, *J* = 8.5 Hz, 2H), 6.90 (d, *J* = 8.5 Hz, 2H), 4.54 (s, 2H), 3.72 (s, 3H), 2.11 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 169.56, 164.59, 163.78, 159.05, 148.68, 141.43, 130.57, 128.19, 124.81, 121.35, 115.58, 114.82, 114.17, 55.23, 35.85, 24.26. Anal. Calcd for $C_{18}H_{16}N_4O_5S$: C, 53.99; H, 4.03; N, 13.99; S, 8.01. Found C, 53.75; H, 3.96; N, 14.17; S, 9.72.

N-(3-(5-((4-chlorobenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitrophenyl)acetamide (**31h**): 4-Chlorobenzyl chloride was used as an alkylating agent. The reaction mixture was refluxed for 4 hours. The product was purified using column chromatography (mobile phase: hexane/EtOAc/CH_3COOH, 60:40:1). Yield 64 % as a yellow solid; mp 201 - 203 °C. ^{1}H NMR (500 MHz, DMSO) δ 10.66 (s, 1H), 8.68 (t, *J* = 2.1 Hz, 1H), 8.53 (t, *J* = 1.7 Hz, 1H), 8.24 – 8.23 (m, 1H), 7.53 (d, *J* = 8.4 Hz, 2H), 7.41 (d, *J* = 8.4 Hz, 2H), 4.59 (s, 2H), 2.12 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 169.59, 164.35, 163.93, 148.71, 141.44, 135.96, 132.60, 131.14, 128.71, 124.82, 121.42, 115.67, 114.91, 35.27, 24.28. Anal. Calcd for $C_{17}H_{13}ClN_4O_4S$: C, 50.44; H, 3.24; N, 13.84; S, 7.92. Found C, 50.66; H, 3.48; N, 13.57; S, 8.01.

N-(3-(5-((4-bromobenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)-5-nitrophenyl)acetamide (**31k**): 4-Bromobenzyl bromide was used as an alkylating agent. The reaction mixture was refluxed for 3 hours. The product was purified using column chromatography (mobile phase:

hexane/EtOAc/CH_3COOH, 60:40:1). Yield: 60% as a yellow solid; mp 208 - 209 °C. ^{1}H NMR (500 MHz, DMSO) δ 10.63 (s, 1H), 8.65 (t, *J* = 2.1 Hz, 1H), 8.50 (t, *J* = 1.7 Hz, 1H), 8.21 – 8.20 (m, 1H), 7.53 (d, *J* = 8.5 Hz, 2H), 7.46 (d, *J* = 8.5 Hz, 2H), 4.56 (s, 2H), 2.11 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 169.55, 164.33, 163.89, 148.65, 141.42, 136.35, 131.63, 131.45, 124.76, 121.34, 121.16, 115.59, 114.83, 35.33, 24.26. Anal. Calcd for $C_{17}H_{13}BrN_4O_4S$: C, 45.45; H, 2.92; N, 12.47; S, 7.14. Found C, 45.74; H, 3.08; N, 12.26; S, 7.27.

N-(3-nitro-5-(5-((4-nitrobenzyl)sulfanyl)-1,3,4-oxadiazol-2-yl)phenyl)acetamide (**31l**): 4-Nitrobenzyl chloride was used as an alkylating agent. The reaction mixture was refluxed for 3 hours. The product was purified using column chromatography (mobile phase: hexane/EtOAc/CH_3COOH, 60:40:1). Yield: 63% as a yellow solid; mp 177 - 178 °C. ^{1}H NMR (500 MHz, DMSO) δ 10.63 (s, 1H), 8.62 (t, *J* = 2.1 Hz, 1H), 8.49 (t, *J* = 1.7 Hz, 1H), 8.20 (d, *J* = 8.8 Hz, 2H), 8.17 (dd, *J* = 2.2, 1.5 Hz, 1H), 7.78 (d, *J* = 8.8 Hz, 2H), 4.71 (s, 2H), 2.10 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 169.60, 164.12, 164.03, 148.65, 147.09, 145.09, 141.44, 130.57, 124.75, 123.84, 121.38, 115.66, 114.87, 35.17, 24.27. Anal. Calcd for $C_{17}H_{13}N_5O_6S$: C, 49.16; H, 3.15; N, 16.86; S, 7.72. Found C, 49.45; H, 3.32; N, 16.54; S, 8.07.

3-Nitro-5-(trifluoromethyl)benzoic acid (**32a**) [21]: Fuming nitric acid (5.5 mL) was added dropwise into a mixture of 3-trifluoromethylbenzoic acid (5 g, 0.026 mol) and concentrated sulphuric acid (23 mL) under vigorous stirring. The reaction mixture was stirred for 3 hours at rt and then poured on ice. The crude product was filtered, dissolved in EtOAc (30 mL) and washed with water (2 × 25 mL) and brine (1 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. Yield: 87% as a light yellow solid; mp 128 - 130 °C. ^{1}H NMR (500 MHz, acetone) δ 9.00 (t, *J* = 1.8 Hz, 1H), 8.78 (t, *J* = 1.9 Hz, 1H), 8.66 (t, *J* =

1.7 Hz, 1H). ^{13}C NMR (126 MHz, acetone) δ 164.52, 149.77, 134.72, 132.76 (q, *J* = 34.2 Hz), 132.37 (q, *J* = 3.6 Hz), 128.46, 125.20 (q, *J* = 3.9 Hz), 123.72 (q, *J* = 272.4 Hz).

Methyl 3-nitro-5-(trifluoromethyl)benzoate (**32b**) [25]: 3-Nitro-5-trifluoromethylbenzoic acid (**32a**) (1 g, 4.25 mmol) was dissolved in methanol (15 mL), and 5 drops of concentrated sulfuric acid were added. The mixture was refluxed for 48 h. The solvents were then evaporated under reduced pressure, and the residue was dissolved in EtOAc (20 mL) and washed with 5% Na_2CO_3 (3 × 20 mL) and brine (1 × 20 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. Methyl 3-nitro-5-trifluoromethylbenzoate was used without further purification. Yield: 87% as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.05 (t, *J* = 1.8 Hz, 1H), 8.68 (t, *J* = 1.8 Hz, 1H), 8.63 (s, 1H), 4.05 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.62, 148.47, 133.16, 132.83 (q, *J* = 34.7 Hz), 131.87 (q, *J* = 3.5 Hz), 127.50, 124.42 (q, *J* = 3.8 Hz), 122.37 (q, *J* = 273.2 Hz), 53.26.

3-Nitro-5-(trifluoromethyl)benzohydrazide (**33**) [26]: Hydrazine hydrate (2 mL, 20.06 mmol) was added into a solution of methyl 3-nitro-5-(trifluoromethyl)benzoate (**32b**) (1 g, 4.01 mmol) in ethanol (20 mL). The mixture was refluxed for 2 hours. The solvent was evaporated, and the residue was dissolved in $CHCl_3$ (20 mL) and washed with water (3 × 20 mL) and brine (1 × 20 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The product was purified using column chromatography (mobile phase: $CHCl_3$/MeOH, 20:1). Yield: 43% as a yellowish solid; mp 120 - 122 °C. ^{1}H NMR (500 MHz, acetone) δ 9.94 (s, 1H), 8.96 (s, 1H), 8.66 (s, 1H), 8.63 (s, 1H). ^{13}C NMR (126 MHz, CD_3OD) δ 165.45, 150.03, 137.44, 133.36 (q, *J* = 34.3 Hz), 130.72 (q, *J* = 3.6 Hz), 126.67, 124.20 (q, *J* = 272.3 Hz), 123.99 (q, *J* = 3.9 Hz).

5-(3-Nitro-5-(trifluoromethyl)phenyl)-1,3,4-oxadiazole-2-thiol (**34**): Carbon disulfide (0.353 mL, 5.85 mmol) was added to a mixture of 3-nitro-5-(trifluoromethyl)benzohydrazide (**33**) (486 mg, 1.95 mmol) and KOH (110 mg, 1.95 mmol) in EtOH (20 mL). The reaction mixture was refluxed for 48 hours. Upon completion, the reaction mixture was filtered, and the filtrate was evaporated and extracted with 5% Na_2CO_3 (3 × 25 mL). The aqueous extracts were combined and acidified with HCl to pH = 1. The precipitate was filtered, dried and purified using column chromatography (mobile phase: hexane/EtOAc/CH_3COOH, 70:10:1). Yield: 26% as a yellow solid; mp: 179 - 181 °C. ^{1}H NMR (500 MHz, acetone) δ 13.59 (s, 1H), 8.94 (t, *J* = 1.9 Hz, 1H), 8.76 (t, *J* = 2.1 Hz, 1H), 8.63 (d, *J* = 1.8 Hz, 1H). ^{13}C NMR (126 MHz, acetone) δ 179.36, 158.93, 150.10, 133.49 (q, *J* = 34.6 Hz), 129.07 (q, *J* = 3.7 Hz), 127.01, 125.20, 124.09 (q, *J* = 3.8 Hz), 123.53 (q, *J* = 272.5 Hz).

General procedure for the synthesis of 2-(alkylsulfanyl)-5-(3-nitro-5-(trifluoromethyl)phenyl)-1,3,4-oxadiazoles (35d, 35e, 35h and 35j): 5-(3-Nitro-5-(trifluoromethyl)phenyl)-1,3,4-oxadiazole-2-thiol (**34**) (75 mg, 0.26 mmol) and NaOH (10 mg, 0.26 mmol) were dissolved in water (20 mL). A solution of the alkylating agent (0.26 mmol) and tetrabutylammonium bromide (0.026 mmol) in CH_2Cl_2 (20 mL) was carefully added to the reaction mixture. The resulting two phase system was gently stirred for 4 h. The organic layer was then separated, washed with 5% Na_2CO_3 (3 × 25 mL), dried over anhydrous Na_2SO_4 and evaporated. The product was purified using column chromatography (mobile phase: hexane/EtOAc, 6:1).

2-(Benzylsulfanyl)-5-(3-nitro-5-(trifluoromethyl)phenyl)-1,3,4-oxadiazole (**35d**): Benzyl bromide was used as an alkylating agent. Yield: 54%; mp 94 – 96 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.99 (t, *J* = 1.8 Hz, 1H), 8.63 (t, *J* = 1.9 Hz, 1H), 8.58 (d, *J* = 1.7 Hz, 1H), 7.51 – 7.47

(m, 2H), 7.40 – 7.31 (m, 3H), 4.58 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.02, 162.75, 148.83, 135.05, 133.58 (q, *J* = 34.8 Hz), 129.13, 128.87, 128.59 (q, *J* = 3.6 Hz), 128.31, 126.45, 124.28, 122.89 (q, *J* = 3.7 Hz), 122.22 (q, *J* = 273.3 Hz), 36.92. HRMS (ESI+): *m*/*z* $[M + H]^+$ calcd for $C_{16}H_{11}F_3N_3O_3S$: 382.04677; found: 382.04639.

2-((4-Methoxybenzyl)sulfanyl)-5-(3-nitro-5-(trifluoromethyl)phenyl)-1,3,4-oxadiazole (**35e**): 4-Methoxybenzyl chloride was used as an alkylating agent. Yield: 16%; mp 93 – 95 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.00 (t, *J* = 1.8 Hz, 1H), 8.65 – 8.62 (m, 1H), 8.60 – 8.57 (m, 1H), 7.41 (d, *J* = 8.6 Hz, 2H), 6.89 (d, *J* = 8.6 Hz, 2H), 4.55 (s, 2H), 3.81 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.18, 162.70, 159.58, 148.86, 133.60 (q, *J* = 34.9 Hz), 130.43, 128.58 (q, *J* = 3.5 Hz), 126.89, 126.52, 124.28, 122.86 (q, *J* = 3.8 Hz), 122.24 (q, *J* = 273.3 Hz), 114.26, 55.28, 36.66. HRMS (ESI+): *m*/*z* $[M + H]^+$ calcd for $C_{17}H_{13}F_3N_3O_4S$: 412.05734; found: 412.05713.

2-((4-Chlorobenzyl)sulfanyl)-5-(3-nitro-5-(trifluoromethyl)phenyl)-1,3,4-oxadiazole (**35h**): 4-Chlorobenzyl chloride was used as an alkylating agent. Yield: 45%; mp 112 – 114 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.99 (t, *J* = 1.8 Hz, 1H), 8.63 (t, *J* = 1.9 Hz, 1H), 8.59 – 8.57 (m, 1H), 7.43 (d, *J* = 8.5 Hz, 2H), 7.33 (d, *J* = 8.5 Hz, 2H), 4.54 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.69, 162.90, 148.88, 134.30, 133.76, 133.64 (q, *J* = 34.9 Hz), 130.53, 129.05, 128.61 (q, *J* = 3.5 Hz), 126.41, 124.31, 122.98 (q, *J* = 3.8 Hz), 122.24 (q, *J* = 273.4 Hz), 36.12. HRMS (ESI+): *m*/*z* $[M + H]^+$ calcd for $C_{16}H_{10}ClF_3N_3O_3S$: 416.00780; found: 416.00739.

2-((3,4-Dichlorobenzyl)sulfanyl)-5-(3-nitro-5-(trifluoromethyl)phenyl)-1,3,4-oxadiazole (**35j**):

3,4-Dichlorobenzyl chloride was used as an alkylating agent. Yield: 48%; mp 92 – 94 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.00 (t, *J* = 1.8 Hz, 1H), 8.64 (t, *J* = 1.9 Hz, 1H), 8.59 (t, *J* = 1.6 Hz, 1H), 7.61 (d, *J* = 2.1 Hz, 1H), 7.43 (d, *J* = 8.2 Hz, 1H), 7.35 (dd, *J* = 8.2, 2.1 Hz, 1H), 4.52 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.36, 163.03, 148.87, 135.54, 133.67 (q, *J* = 35.0 Hz),

132.92, 132.59, 131.05, 130.76, 128.65 (q, J = 3.6 Hz), 128.55, 126.33, 124.34, 123.06 (q, J = 3.8 Hz), 122.22 (q, J = 273.6 Hz), 35.49. HRMS (ESI+): m/z $[M + H]^+$ calcd for $C_{16}H_9Cl_2F_3N_3O_3S$: 449.96883; found: 449.96869.

***In vitro* antimycobacterial assay.** The *in vitro* antimycobacterial activity of the prepared compounds was evaluated against the mycobacterial strains *Mycobacterium tuberculosis* 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); clinically isolated *M. kansasii* 6509/96; and the multidrug-resistant strains *M. tuberculosis* 7357/1998, *M. tuberculosis* 234/2005, *M. tuberculosis* 9449/2007, *M. tuberculosis* 8666/2010, *M. tuberculosis* Praha 1, *M. tuberculosis* Praha 4 and *M. tuberculosis* 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 sample 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 1000, 500, 250, 125, 62, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06 and 0.03 μmol/L. 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 7/14/21 days for both strains of *M. kansasii* and after 14/21 days for the *M. tuberculosis* and *M. avium* strains. Isoniazid (INH) was used as a prototype drug.

***In vitro* antibacterial and antifungal assays.** For the assessment of *in vitro* antibacterial and antifungal activities of the synthesized substances, the broth microdilution method was used. This method was performed according to CLSI/EUCAST (Clinical&Laboratory Standard Institute/European Committee on Antimicrobial Susceptibility Testing) instructions with slight modifications. The set of tested fungi included 5 yeasts and yeast-like organisms (*Candida albicans* ATCC 44859 (CA), *Candida tropicalis* 156 (CT), *Candida krusei* E28 (CK), *Candida glabrata* 20/I (CG) and *Trichosporon asahii* 1188 (TA)) and 3 molds (*Aspergillus fumigatus* 231 (AF), *Absidia corymbifera* 272 (AC) and *Trichophyton mentagrophytes* 445 (TM)). The procedure was performed with two-fold dilutions of the studied substances in RPMI 1640 cultivation medium buffered at pH 7.0 with 0.165 mol/L of 3-morpholinopropane-1-sulfonic acid. The compounds were dissolved in DMSO, and the final concentrations of the substances ranged from 500 to 0.488 μM (because of solubility limits the final concentrations of some of the substances ranged from 250 or 125 to 0.488 μM). Positive controls (drug-free cultivation medium inoculated with the tested fungi) and negative controls (drug-free cultivation medium) were included. The MIC values were determined after 24 and 48 h of static incubation at 35 °C. For *T. mentagrophytes*, the final MICs were determined after 72 and 120 h of incubation. Fluconazole and amphotericin B were used as prototype drugs.

The set of the tested bacteria included 4 strains of gram positive cocci (*Staphylococcus aureus* ATCC 6538 (SA), methicillin resistant *Staphylococcus aureus* H 5996/08 (MRSA), *Staphylococcus epidermidis* H 6966/08 (SE) and *Enterococcus* sp. J 14365/08 (EF)) and 4 strains of gram negative rods (*Escherichia coli* ATCC 8739 (EC), *Klebsiella pneumoniae* D 11750/08 (KP), *Klebsiella pneumoniae* (a producer of extended-spectrum beta-lactamases) (ESBL) J 14368/08 (KP-E) and *Pseudomonas aeruginosa* ATCC 9027 (PA)). The concentration range was

the same as that used for the aforementioned fungi. The Mueller Hinton broth was used as the cultivation medium for the antibacterial susceptibility testing. The MIC values were determined after 24 and 48 h of static incubation at 35 °C. Vancomycin was used as a prototype drug for gram positive cocci, and gentamicin was used as a prototype drug for gram negative rods.

In vitro* cell proliferation/viability assay (MTS assay).** ***Cell lines and culture conditions: The MDCK cells (Madin-Darby canine kidney cells), A431 (human epidermoid carcinoma) and HepG2 (human hepatocellular carcinoma) cell lines were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) non-essential amino acids. The HuH7 cells (human hepatocellular carcinoma) were maintained in DMEM with 10% (v/v) fetal bovine serum (FBS).

The cells were seeded into 96-well cultivation plates (20 x 10^3 cells per well) 24 h before the treatment. Then, the cells were exposed to the test compounds for 48 h at a concentration of 30 μM. Stock solutions of the test compounds were prepared in DMSO at a concentration of 30 mM (the final concentration of DMSO in the culture did not exceed 0.1% v/v).

Cell proliferation/viability assay: The CellTiter 96®AQ_{ueous}One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was performed to evaluate the toxicity of the test compounds *in vitro* in the four mammalian cell lines. The method is based on the colorimetric method of MTS tetrazolium ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] 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 the 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 minutes 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.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was supported by the Czech Science Foundation project 14-08423S and Charles University Grant Agency project 361215. L.V. thanks Charles University project SVV 260 291.

ABBREVIATIONS

CNCTC, Czech National Collection of Type Cultures; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DprE1, Decaprenylphosphoryl-*β*-D□ribose 2′□oxidase; INH, isoniazid; MDR, multidrug-resistant; MIC, minimum inhibitory concentration; MTS, [3-

(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; R*f*, retention factor; SDS, sodium dodecyl sulfate; TB, tuberculosis; TBAB, tetrabutylammonium bromide; THF, tetrahydrofuran; TLC, thin layer chromatography; XDR, extensively drug-resistant.

SUPPLEMENTARY DATA

Table S1, Table S2, copies of NMR spectra

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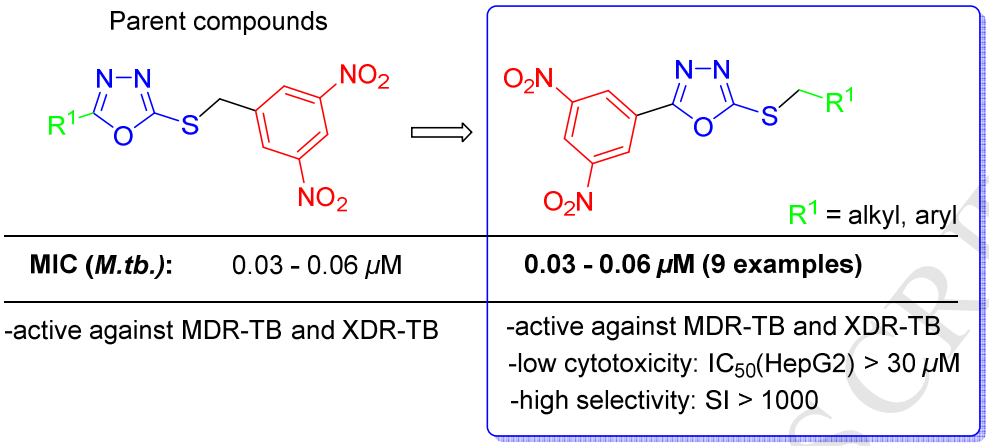
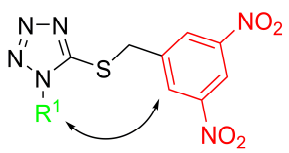
Highlights

- 3,5-Dinitrophenyl tetrazoles and oxadiazoles with high antimycobacterial activity
- MIC values of 0.03 - 0.06 µM against *Mycobacterium tuberculosis*
- Activity against MDR/XDR strains and no cross-resistance with common anti-TB drugs
- Selective antitubercular effect and low *in vitro* toxicity to mammalian cell lines
- Defined structure-activity relationships

Graphical Abstract



Parent compounds



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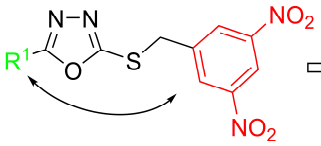

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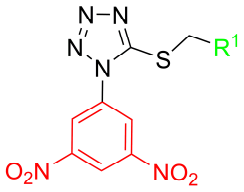
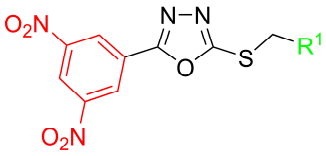
Target compounds

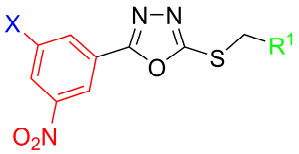


20d, 20h, 20j

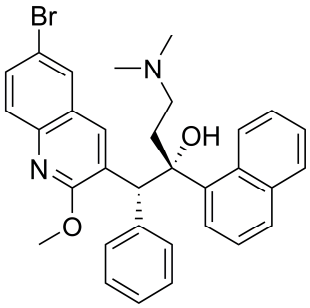


23a-l

R^1 = H, alkyl, aryl



X = -OH: **25j**
X = -$OOCCH_3$: **26j**
X = -NH_2: **29d, 29e, 29h, 29k, 29l**
X = -$NHOCCH_3$: **31d, 31e, 31h, 31k, 31l**
X = -CF_3: **35d, 35e, 35h, 35j**



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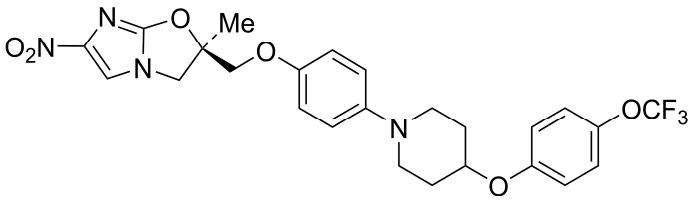
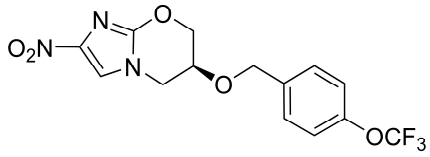
Bedaquiline (TMC-207)
MIC 0.05 μM



2
Delamanid (OPC-67683)
MIC 0.023 μM

A



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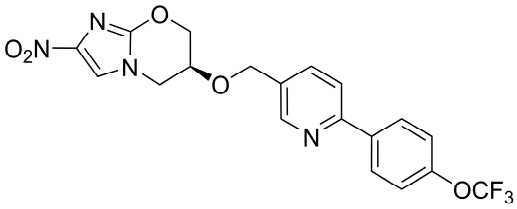
PA-824
MIC 0.05-0.7 μM



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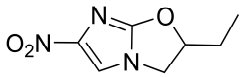
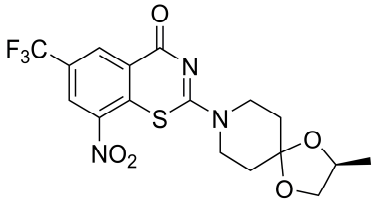
TBA-354
MIC 6-8 nM

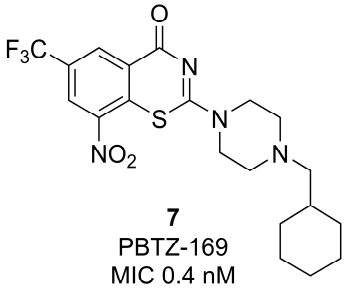


5
CGI-17341
MIC 0.2 μM

B



6
BTZ 043
MIC 1 nM



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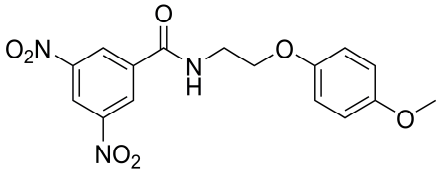
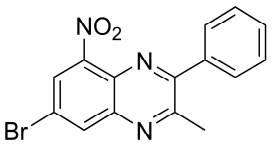
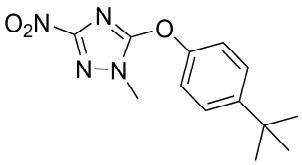
PBTZ-169
MIC 0.4 nM



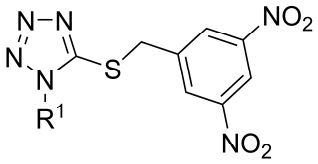
8
DNB-1
MIC 0.2 μM



9
VI 9376
MIC 3.1 μM

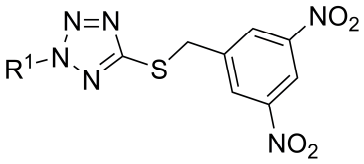


10
377790
MIC 0.5 μM



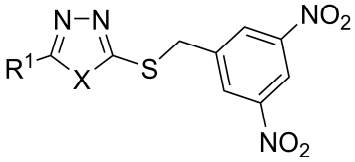
R^1 = alkyl, aryl

11
MIC 1-4 μM



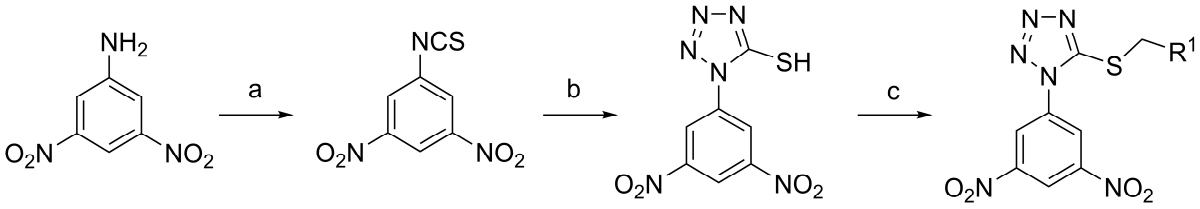
R^1 = alkyl

12
MIC 1-4 μM

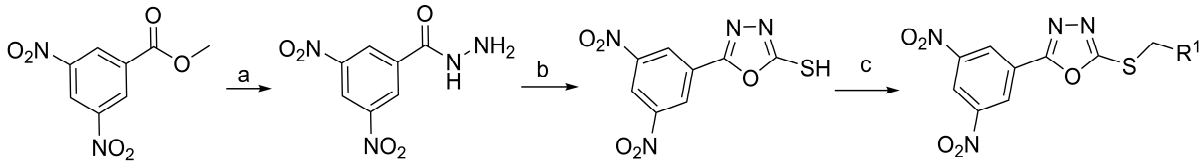


R^1 = alkyl, aryl

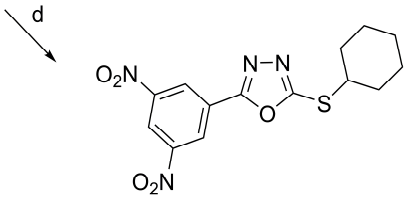
13, X = O
14, X = S
MIC 0.03 - 0.06 μM



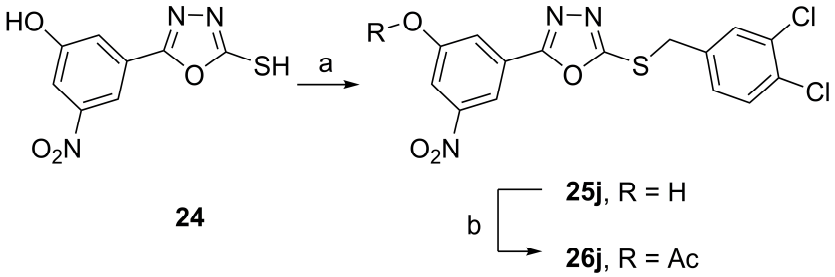
18 **19** **20d, 20h, 20j**

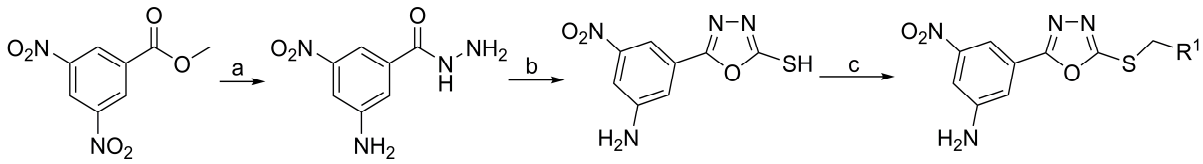


21 **22** **23a-l**

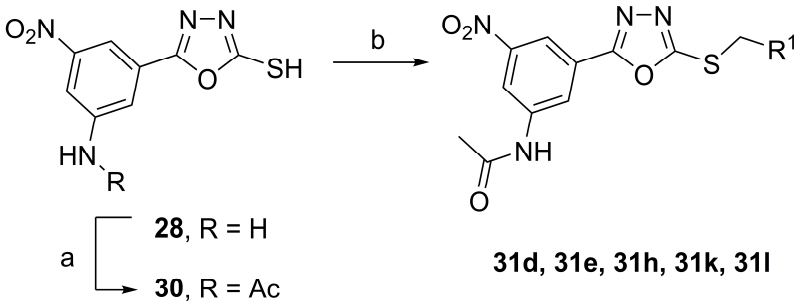


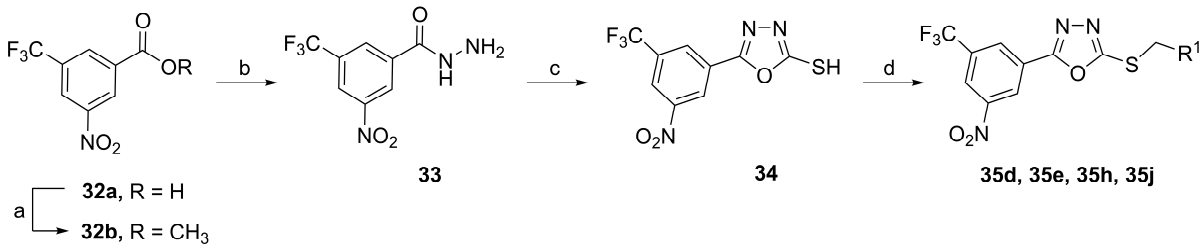
23x





27 **28** **29d, 29e, 29h, 29k, 29l**





Highlights

- 3,5-Dinitrophenyl tetrazoles and oxadiazoles with high antimycobacterial activity
- MIC values of 0.03 - 0.06 µM against *Mycobacterium tuberculosis*
- Activity against MDR/XDR strains and no cross-resistance with common anti-TB drugs
- Selective antitubercular effect and low *in vitro* toxicity to mammalian cell lines
- Defined structure-activity relationships