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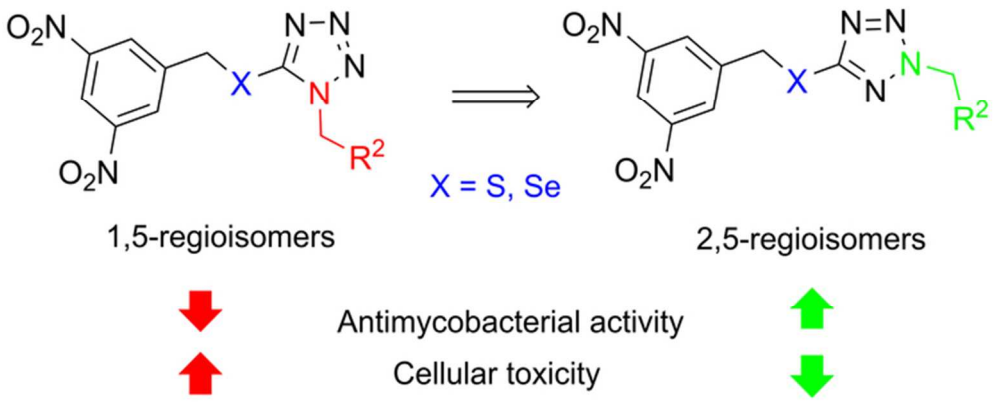

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ARTICLE

Tetrazole Regioisomers in the Development of Nitro Group-Containing Antitubercular Agents

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Tetrazole derivatives containing nitro substituents have been identified as promising antitubercular agents. In this study, the antitubercular potency, selectivity and toxicity of tetrazole 1,5- and 2,5-regioisomers were examined. We prepared a series of 1- and 2-alkyl-5-benzylsulfanyl-2*H*-tetrazoles and their selenium analogs with various nitro group substitutions. These 1,5- and 2,5-regioisomers were isolated and unambiguously identified using ^{1}H and/or ^{13}C NMR. Among the prepared compounds, 1- and 2-alkyl-5-[(3,5-dinitrobenzyl)sulfanyl]-2*H*-tetrazole derivatives and their selenium bioisosteres showed the highest antimycobacterial activity, with minimal inhibitory concentration (MIC) values of approximately 1 μM (0.37-0.46 μg/mL) against Mycobacterium tuberculosis CNCTC My 331/88. The 2-alkyl regioisomers exhibited consistently higher antimycobacterial activity and lower *in vitro* toxicity against a mammalian cell line compared to the 1-alkyl isomers. The antimycobacterial activity of the 2-alkyl regioisomers was less influenced by the type of alkyl substituent in contrast to 1-alkyl isomers. Furthermore, 3,5-dinitrobenzyl moiety *per se* is not the carrier of mutagenicity. These findings encourage the further optimization of the 2-alkyl chain to improve pharmacokinetic properties and toxicity of 2-alkyl-5-[(3,5-dinitrobenzyl)sulfanyl]-2*H*-tetrazole lead compounds.

Introduction

Tuberculosis (TB) is a widespread infectious disease and remains a serious global problem that takes millions lives each year.[1] The emergence and distribution of multi-drug resistant (MDR-TB) and extensively drug-resistant (XDR-TB) strains of *Mycobacterium tuberculosis (M.tb)* has become the biggest challenge in treatment with current anti-TB drugs. Current anti-TB drugs also suffer from low tolerability or adverse effects. Moreover, TB frequently occurs in HIV/AIDS patients who have a further reduced response to TB treatment.

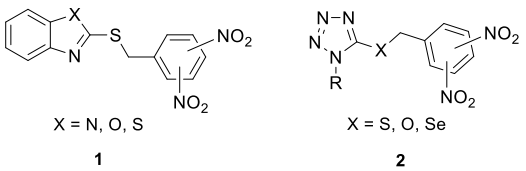

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Figure 1. Dinitrobenzyl-bearing benzazole and tetrazole derivatives with high and selective antimycobacterial activity.

This establishes the need for the discovery of new highly efficient antimycobacterial agents (for recent reviews, see refs 2, 3, 4 and 5). Recently, several nitro group-containing anti-TB agents were developed and two, nitroimidazole-based compounds PA-824[6] and OPC-67683 (delamanid),[7] are undergoing clinical trials.[8] Another promising group of nitro group-containing anti-TB agents are dinitrobenzamides[9] and benzothiazinones;[10] both of which are inhibitors of decaprenyl-phosphoribose epimerase (DprE1), an essential enzyme involved in arabinan biosynthesis.[11] The piperazinobenzothiazinone PBTZ 169, a benzothiazinone-derivative, is currently undergoing preclinical development.[12] In our previous work, we found that 2-(dinitrobenzylsulfanyl)benzazoles **1** exhibited high *in vitro* antimycobacterial activities with minimal inhibitory

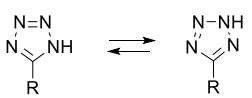

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Figure 2. 1*H*- and 2*H*- tautomers of 5-substituted tetrazoles.

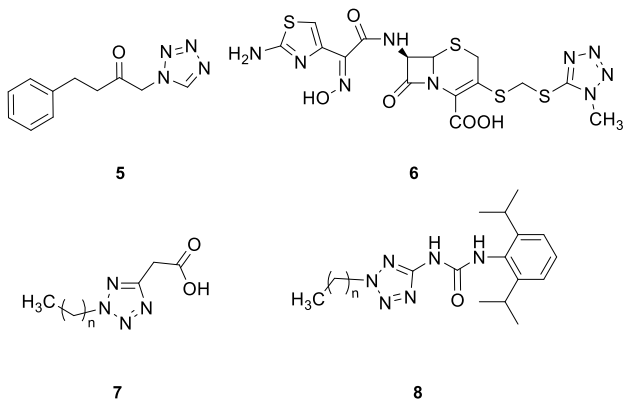


Figure 3. Examples of 1,5- and 2,5-disubstituted tetrazole-based compounds with regioisomer-dependent activity.

concentrations (MICs) from 2 to 8 μM against *M.tb* (Figure 1).[13-16] Hence, to continue this study, we prepared a series of 1-alkyl/aryl-5-(dinitrobenzylsulfanyl)-1*H*-tetrazoles **2** with 1-substituted tetrazole in place of the original benzazole moiety (Figure 1). The antimycobacterial activities of these compounds confirmed that the presence of two nitro groups on the benzylsulfanyl moiety is vital for increased antimycobacterial activity. 3,5-Dinitro substituted tetrazole derivatives of series **2** exhibited higher activities compared to the 2,4-dinitro analogs, with MIC values of 1 μM (0.36-0.44 μg/mL) against *M.tb*, i.e., values equivalent to the first-line anti-TB drug isoniazid, and 0.25-1 μM against six MDR *M.tb* strains and with no cross-resistance with common anti-TB drugs. Moreover, series of compounds **2** were highly selective for mycobacteria, because they exhibited no antibacterial or antifungal activity and low toxicity on selected mammalian cell lines. A structure-activity relationship study showed that the isosteric replacement of sulfur for the selenium or oxygen atom had no significant effect on the antimycobacterial activity of these compounds. In addition, twenty-three substituents at position 1 of the tetrazole

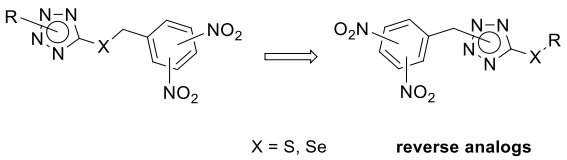
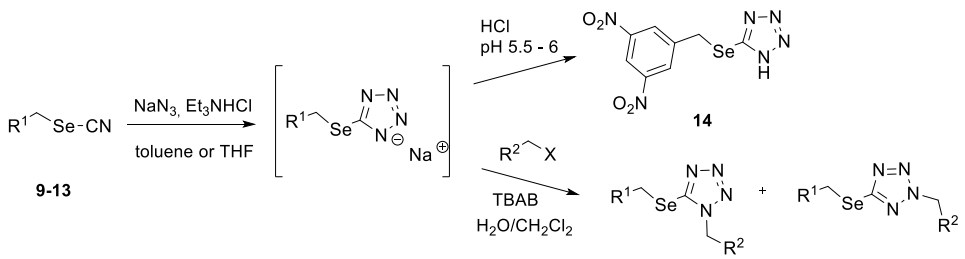


Figure 4. General structure of the studied compounds.

were studied, and these appeared to influence the antimycobacterial activity of the compound through their effects on its lipophilicity.[17]

As 5-substituted tetrazole forms 1*H*- and 2*H*-tautomers (**3** and **4**, Figure 2),[18-20] 1,5- and 2,5-disubstituted tetrazole isomers can be recognized. Several studies have indicated that the biological properties of 1,5- and 2,5-tetrazole regioisomers differ, particularly in their effective concentration levels.[21-23] 4-Phenyl-1-(1*H*-tetrazol-1-yl)-2-butanone (**5**, Figure 3), an inhibitor of heme oxygenase-1 (HO-1), displayed an IC_{50} value of 2.6 μM, while its 2*H*-tetrazol-2-yl analog exhibited greater than 3.5 times weaker inhibitory activity.[24] 1-Methyl derivatives of cephalosporin **6** displayed a two-fold increase in antibacterial activity against both Gram-positive and Gram-negative bacteria compared to the 2-methyl isomers.[25] Conversely, 2,5-disubstituted tetrazoles **7** were efficient Pseudomonas aeruginosa quorum-sensing inhibitors, whereas 1,5-disubstituted analogs had low inhibitory activity.[26] *N*-Aryl-*N'*-tetrazole-substituted ureas **8** containing a long carbon chain in position 2 of the tetrazole ring displayed 10-fold higher acyl-CoA:cholesterol *O*-acyltransferase inhibition compared to the 1-regioisomers (Figure 3).[27]

In this work, we focused on the tetrazole-based lead compounds **2** and studied how the position of substituent R on tetrazole influenced their biological properties, specifically, antimycobacterial activity. Moreover, their reverse analogs, with a dinitrobenzyl moiety on tetrazole and with substituent R on sulfur/selenium in position 5 of tetrazole,

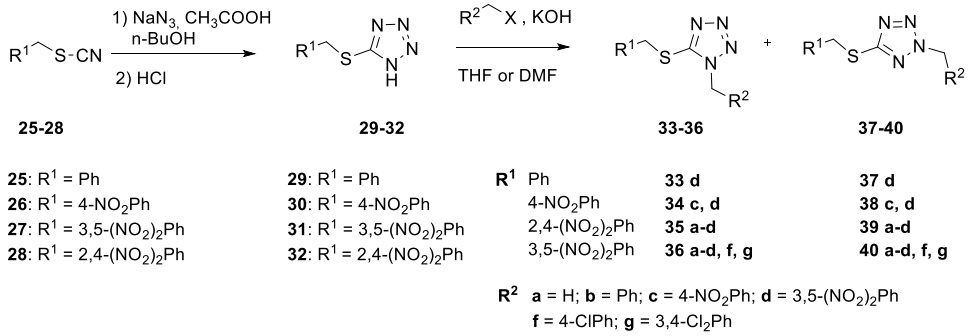


9: R^1 = Ph
10: R^1 = 4-NO_2Ph
11: R^1 = 2,4-$(NO_2)_2$Ph
12: R^1 = 3,5-$(NO_2)_2$Ph
13: R^1 = 4-MeOPh

R^1	**15**	**20**
Ph	**d, e**	**d, e**
4-NO_2Ph	**c-e**	**c-e**
2,4-$(NO_2)_2$Ph	**a-e**	**a-e**
3,5-$(NO_2)_2$Ph	**a-e**	**a-e**
4-MeOPh	**d, e**	**d, e**

R^2 **a** = H; **b** = Ph; **c** = 4-NO_2Ph; **d** = 3,5-$(NO_2)_2$Ph; **e** = 2,4-$(NO_2)_2$Ph

Scheme 1. Synthesis of 1-alkyl- and 2-alkyl-5-(alkylselanyl)-2*H*-tetrazoles.



Scheme 2. Synthesis of 1-alkyl and 2-alkyl-5-(alkylsulfanyl)-2*H*-tetrazoles.

were prepared to determine the optimal position of the crucial dinitrobenzyl fragment. We also examined the effect of isosteric replacement of sulfur for selenium on the biological properties of the target substances (Figure 4).

Results and discussion

Chemistry

Synthesis of 1-alkyl-5-(alkylselanyl)-1*H*-tetrazoles (**15-19**) and their 2-regioisomers (**20-24**) was conducted according to Scheme 1. The starting alkyl selenocyanates (**9-13**) were obtained by the reaction of the corresponding alkyl halides with a slight excess of potassium selenocyanate in THF or DMF. Subsequently, they were converted into 5-(alkylselanyl)-1*H*-tetrazolates by the reaction with sodium azide and triethylammonium chloride in toluene at an elevated temperature.[28, 29] For the preparation of 5-[(2,4-dinitrobenzyl)selanyl]-1*H*-tetrazolate, the reaction mixture was stirred in THF at room temperature overnight to avoid the precipitation of selenium, which was observed using the above-mentioned procedure in toluene. 5-[(3,5-Dinitrobenzyl)selanyl]-1*H*-tetrazolate was sensitive to acidification, and the corresponding tetrazole **14** could only be prepared in low yield; therefore, we alkylated selanyltetrazolates *in situ* under the conditions of phase-transfer catalysis in the presence of tetrabutylammonium bromide (TBAB). Expectedly, the alkylation of 5-(alkylselanyl)-1*H*-tetrazolates led to the formation of both regioisomers,[20] 1-alkyl-5-(alkylselanyl)-1*H*-tetrazoles (**15-19**) and 2-alkyl-5-(alkylselanyl)-2*H*-tetrazoles (**20-24**), in moderate yields. The 1- (**15-19**) and 2-isomers (**20-24**) of the target selanyltetrazoles were separated and purified by column chromatography. The ratios of 1- and 2-regioisomers ranged from 1:1 to 1:4.

Table 1. Selected ^{13}C and ^{1}H (in parentheses) NMR chemical shifts (δ, ppm) of 1-alkyl-5-(alkylselanyl)-1*H*-tetrazole (**15-19**) and 2-alkyl-5-(alkylselanyl)-2*H*-tetrazole (**20-24**) regioisomeric pairs.

1- / 2-isomers	R^1-CH_2Se	R^2-CH_2N	1-isomer		2-isomer	
			C_{tetr}	R^2-CH_2N^1	C_{tetr}	R^2-CH_2N^2
15d/20d	Ph	3,5-$(NO_2)_2$Ph	147.66	50.40 (5.87)	157.68	55.55 (6.31)
15e/20e		2,4-$(NO_2)_2$Ph	148.32	48.98 (6.03)	157.60	54.19 (6.48)
16c/21c	4-NO_2Ph	4-NO_2Ph	148.06	51.12 (5.74)	156.75	56.41 (6.10)
16d/21d		3,5-$(NO_2)_2$Ph	n.d.	50.47 (5.94)	157.08	55.62 (6.32)
16e/21e		2,4-$(NO_2)_2$Ph	148.10	49.13 (6.09)	156.91	54.26 (6.47)
17a/22a	2,4-$(NO_2)_2$Ph	H	147.14	34.67 (3.98)	155.86	40.09 (4.39)
17b/22b		Ph	n.d.	52.13 (5.57)	156.24	57.52 (5.90)
17c/22c		4-NO_2Ph	n.d.	51.21 (5.79)	156.73	56.47 (6.12)
17d/22d		3,5-$(NO_2)_2$Ph	147.79	50.59 (6.00)	157.07	55.70 (6.33)
17e/22e		2,4-$(NO_2)_2$Ph	n.d.	49.39 (6.14)	156.92	54.36 (6.49)
18a/23a	3,5-$(NO_2)_2$Ph	H	145.62	34.12 (3.94)	154.74	39.78 (4.35)
18b/23b		Ph	146.86	52.09 (5.57)	156.18	57.50 (5.87)
18c/23c		4-NO_2Ph	148.01	50.34 (5.79)	156.72	56.50 (6.10)
18d/23d		3,5-$(NO_2)_2$Ph	147.54	50.52 (5.99)	155.68	55.71 (6.32)
18e/23e		2,4-$(NO_2)_2$Ph	148.00	49.29 (6.12)	156.82	54.34 (6.47)
19d/24d	4-MeOPh	3,5-$(NO_2)_2$Ph	147.74	50.38 (5.87)	157.84	55.52 (6.31)
19e/24e		2,4-$(NO_2)_2$Ph	148.38	48.90 (6.02)	157.70	54.15 (6.47)

n.d. not determined because of a signal overlap or close proximity with the nitro group-bearing carbons

Table 2. Selected ^{13}C and ^{1}H (in parentheses) NMR chemical shifts (δ, ppm) of 1-alkyl-5-(alkylsulfanyl)-1*H*-tetrazole (**33-36**) and 2-alkyl-5-(alkylsulfanyl)-2*H*-tetrazole (**37-40**) regioisomeric pairs.

1- / 2 -isomers	R^1-CH_2S	R^2-CH_2N	1-isomer		2-isomer	
			C_{tetr}	R^2-CH_2N^1	C_{tetr}	R^2-CH_2N^2
33d/37d	Ph	3,5-$(NO_2)_2$Ph	154.65	49.80 (5.89)	165.02	55.61 (6.27)
34c/38c	4-NO_2Ph	4-NO_2Ph	154.10	50.54 (5.74)	164.06	56.45 (6.06)
34d/38d		3,5-$(NO_2)_2$Ph	154.31	49.92 (5.95)	164.37	55.69 (6.27)
35a/39a	2,4-$(NO_2)_2$Ph	H	153.77	34.77 (3.94)	162.96	40.14 (4.33)
35b/39b		Ph	153.72	51.51 (5.53)	163.35	57.57 (5.83)
35c/39c		4-NO_2Ph	154.15	50.61 (5.75)	163.83	56.53 (6.05)
35d/39d		3,5-$(NO_2)_2$Ph	154.38	49.94 (5.96)	163.28	54.90 (6.26)
36a/40a	3,5-$(NO_2)_2$Ph	H	152.33	35.37 (3.92)	162.02	39.82 (4.30)
36b/40b		Ph	153.06	50.51 (5.54)	163.34	57.54 (5.83)
36c/40c		4-NO_2Ph	153.91	50.61 (5.77)	163.83	56.51 (6.05)
36d/40d		3,5-$(NO_2)_2$Ph	153.30	49.11 (5.97)	163.25	54.92 (6.27)
36f/40f		4-ClPh	153.58	50.78 (5.57)	163.52	56.74 (5.85)
36g/40g		3,4-Cl_2Ph	152.15	50.00 (5.35)	163.70	56.13 (5.89)

The alkylation was preferentially directed to position 2, which is likely due to steric hindrance of the alkylselanyl substituent in position 5 of tetrazole. The 1,5- and 2,5-isomeric series were unambiguously identified according to the ^{1}H and ^{13}C NMR chemical shifts of the tetrazole carbon and the nitrogen-bound methylene group. These signals of 2-alkyl-5-(alkylselanyl)-2*H*-tetrazoles were observed downfield compared to the 1-isomers (Table 1). Correlations between methylene hydrogens in 1,5-isomer **19d** in 1D NOESY experiment and the absence of such correlations in 2,5-isomer **24d** confirmed their regioisomeric identitites (see Supplementary info). Furthermore, all 2-alkyl-5-(alkylselanyl)-2*H*-tetrazoles had lower retention (higher values of R_f) on silica gel compared to their 1-isomers, indicating that the 2-isomers were less polar. In contrast to the 1-alkyl-5-(alkylselanyl)-1*H*-tetrazole regioisomers,[30] a report of the 2-alkyl regioisomers has not yet been published. Nevertheless, our results are in agreement with previously published differences between NMR shifts of 1,5- and 2,5-disubstituted tetrazoles.[31, 32] The synthetic procedure for 1-alkyl-5-(alkylsulfanyl)-1*H*-tetrazoles (**33-36**) and 2-alkyl-5-(alkylsulfanyl)-2*H*-tetrazoles (**37-40**) is shown in Scheme 2. Unlike selenium analogs, 5-(alkylsulfanyl)-1*H*-tetrazoles (**29-32**) were isolated in moderate yields (50-68%). Alkylation of the tetrazoles (**29-32**) was performed in THF or DMF in the presence of KOH or under phase-transfer catalysis conditions (compounds **36a**, **36b**, **40a**, and **40b**). 1-Alkyl-5-(alkylsulfanyl)-1*H*-tetrazoles (**33-36**) and the respective 2-alkyl isomers (**37-40**) were separated by column chromatography and were obtained in ratios ranging from 1:1.6 to 1:4. The identification of the 1,5- and 2,5-isomers was performed using the ^{1}H and ^{13}C NMR chemical shifts of the tetrazole carbon and the methylene group on the tetrazole nitrogen.[31, 32] These results followed the same rules as in the above-mentioned selenium derivatives: the signals of 2-alkyl-5-(alkylsulfanyl)-2*H*-tetrazoles were observed downfield compared to the 1-isomers (Table 2), and the 2-isomers had lower retention (higher values of R_f) on silica compared to the 1-isomers. The regioisomeric identities were confirmed by 1D NOESY experiments of compounds **33d** and **37d**, which showed correlations between methylene hydrogens in 1,5-regioisomer **33d** only (see Supplementary info).

In vitro antimycobacterial activity

In vitro antimycobacterial activities of the synthesized compounds were evaluated against *M.tb* CNCTC My 331/88 and non-tuberculous mycobacteria - *M. avium* CNCTC My 330/88, *M. kansasii* CNCTC My 235/80 and *M. kansasii* 6509/96. All strains were obtained from the Czech National Collection of Type Cultures (CNCTC), with the exception of *M. kansasii* 6509/96, which was a clinical isolate. The activities of the compounds were determined in Sula semisynthetic medium. Minimum inhibitory concentrations (MICs), i.e., the lowest concentration that inhibits the visible growth of mycobacteria, were determined after incubation at 37 °C for 7, 14, and 21 days for both strains of *M. kansasii* and after 14 and 21 days for *M.tb* and *M. avium*. The values of MIC are expressed in μM and are presented in Tables 3 and 4. Isoniazid (INH) was used as a prototype drug.

The antimycobacterial activities of the most potent compounds in the series of 1- and 2-isomers of selanyltetrazoles (**15-24**) and sulfanyltetrazoles (**33-40**) were 1 μM against *M.tb*, which is equivalent to the first-line anti-TB drug isoniazid (INH), and 1-2 μM against both INH-resistant and INH-susceptible *M. kansasii*. These results support our previous observations that 3,5-dinitrobenzylsulfanyl/selanyl derivatives had significantly higher activities than 2,4-dinitrobenzylsulfanyl/selanyl derivatives and both nitro groups are necessary for the high antimycobacterial efficiency of the studied compounds. Hence, the most active substances were 1-alkyl-5-[(3,5-dinitrobenzyl)selanyl]-2*H*-tetrazoles (**18a-e**), 2-alkyl-5-[(3,5-dinitrobenzyl)selanyl]-2*H*-tetrazoles (**23a-e**), 1-alkyl-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles (**36a-d, 36f, 36g**) and 2-alkyl-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles

Table 3. *In vitro* antimycobacterial activities of selanyltetrazoles **15-24** expressed as MIC (μM).

		R^1	R^2	*M.tb* My 331/88	*M. avium* My 330/88	*M. kansasii* My 235/80	*M. kansasii* 6509/96
				14 / 21 days		7 / 14 / 21 days	
1,5-regioisomers	**15d**	Ph	3,5-$(NO_2)_2$Ph	8 / 8	32 / 32	8 / 16 / 32	16 / 32 / 32
	15e		2,4-$(NO_2)_2$Ph	1 / 2	16 / 32	32 / 62 / 62	16 / 62 / 125
	16c	4-NO_2Ph	4-NO_2Ph	62 / 125	250 / 250	62 / 125 / 125	32 / 62 / 62
	16d		3,5-$(NO_2)_2$Ph	8 / 8	250 / 250	16 / 32 / 32	32 / 32 / 62
	16e		2,4-$(NO_2)_2$Ph	2 / 4	16 / 32	32 / 62 / 62	32 / 62 / 62
	17a	2,4-$(NO_2)_2$Ph	H	32 / 32	250 / 250	62 / 125 / 125	62 / 125 / 250
	17b		Ph	8 / 16	250 / 250	16 / 32 / 125	16 / 62 / 125
	17c		4-NO_2Ph	16 / 16	62 / 62	16 / 32 / 32	32 / 62 / 62
	17d		3,5-$(NO_2)_2$Ph	16 / 16	250 / 250	16 / 32 / 62	32 / 62 / 62
	17e		2,4-$(NO_2)_2$Ph	8 / 16	32 / 32	32 / 62 / 62	32 / 62 / 62
	18a	3,5-$(NO_2)_2$Ph	H	8 / 8	500 / 500	8 / 32 / 32	16 / 32 / 32
	18b		Ph	1 / 2	32 / 32	2 / 4 / 4	4 / 8 / 8
	18c		4-NO_2Ph	2 / 4	125 / 125	4 / 16 / 32	4 / 8 / 16
	18d		3,5-$(NO_2)_2$Ph	4 / 8	125 / 125	16 / 62 / 62	16 / 62 / 62
	18e		2,4-$(NO_2)_2$Ph	4 / 8	125 / 125	8 / 32 / 62	4 / 16 / 32
	19d	4-CH_3OPh	3,5-$(NO_2)_2$Ph	16 / 16	125 / 125	16 / 32 / 62	16 / 32 / 62
	19e		2,4-$(NO_2)_2$Ph	4 / 8	125 / 125	16 / 32 / 62	4 / 16 /32
2,5-regioisomers	**20d**	Ph	3,5-$(NO_2)_2$Ph	8 / 8	16 / 16	8 / 16 / 32	16 / 32 / 32
	20e		2,4-$(NO_2)_2$Ph	1 / 1	4 / 8	8 / 16 / 16	8 / 16 / 32
	21c	4-NO_2Ph	4-NO_2Ph	62 / 62	250 / 250	32 / 125 / 125	62 / 125 / 125
	21d		3,5-$(NO_2)_2$Ph	32 / 32	250 / 250	32 / 62 / 62	32 / 62 / 62
	21e		2,4-$(NO_2)_2$Ph	2 / 2	8 / 16	1 / 2 / 2	2 / 4 / 8
	22a	2,4-$(NO_2)_2$Ph	H	16 / 16	62 / 62	32 / 125 / 125	16 / 62 / 125
	22b		Ph	16 / 16	62 / 62	16 / 16 / 32	32 / 32 / 62
	22c		4-NO_2Ph	32 / 32	125 / 125	16 / 32 / 32	32 / 62 / 62
	22d		3,5-$(NO_2)_2$Ph	16 / 16	250 / 250	8 / 16 / 32	16 / 32 / 62
	22e		2,4-$(NO_2)_2$Ph	2 / 4	16 / 32	16 / 32 / 62	32 / 62 / 62
	23a	3,5-$(NO_2)_2$Ph	H	1 / 2	250 / 250	2 / 4 / 8	2 / 4 / 4
	23b		Ph	1 / 1	16 / 32	1 / 1 / 1	1 / 1 / 2
	23c		4-NO_2Ph	1 / 1	125 / 125	1 / 2 / 4	2 / 4 / 4
	23d		3,5-$(NO_2)_2$Ph	1 / 1	125 / 125	2 / 4 / 8	2 / 4 / 8
	23e		2,4-$(NO_2)_2$Ph	2 / 2	125 / 125	4 / 8 / 16	2 / 8 /16
	24d	4-CH_3OPh	3,5-$(NO_2)_2$Ph	4 / 8	16 / 32	8 / 16 / 16	16 / 32 / 32
	24e		2,4-$(NO_2)_2$Ph	n.d.	n.d.	n.d.	n.d.
		INH		0.5 / 1	250 / 250	250 / 250 / 250	4 / 4 / 4

n.d. not determined

(**40a-d, 40f, 40g**). Considering the position of the alkyl substituent, the 2-isomer series (**23** and **40**) exhibited higher activity than the 1-isomer series (**18** and **36**). Interestingly, the antitubercular activities of the 2-isomers **23a-e**, **40a-d**, **40f** and **40g** reached MIC values of 1-2 μM regardless to the substituent on the tetrazole cycle. Conversely, the activities of the corresponding 1-isomers **18a-e**, **36a-d**, **36f** and **36g**, with MIC values ranging from 1 to 8 μM, appeared to be more influenced by the type of substituent on the tetrazole then their corresponding 2-isomers**.** The combination of a 3,5-dinitrobenzyl substituent on sulfur/selenium and a dinitrobenzyl substituent in position 1 of tetrazole (**18d, 18e, 36d**) decreased antimycobacterial activity compared to substances with a 3,5-dinitrobenzyl substituent on sulfur/selenium and a 1-benzyl (**18b, 36b**) or 1-(4-nitrobenzyl) (**18c, 36c**) on tetrazole. The MIC values of benzylselanyl/3,5-dinitrobenzyl and 3,5-dinitrobenzylselanyl/benzyl reverse analog pairs **15d**/**18b** and

Table 4. *In vitro* antimycobacterial activities of sulfanyltetrazoles **33-40** expressed as MIC (μM).

		R^1	R^2	***M.tb* My 331/88**	***M. avium* My 330/88**	***M. kansasii* My 235/80**	***M. kansasii* 6509/96**
				14 / 21 days		7 / 14 / 21 days	
1,5-regioisomers	**33d**	Ph	3,5-$(NO_2)_2$Ph	8 / 8	125 / 125	32 / 62 / 62	16 / 32 / 32
	34c	4-NO_2Ph	4-NO_2Ph	500 / 500	250 / 250	250 / 250 / 250	250 / 250 / 250
	34d		3,5-$(NO_2)_2$Ph	8 / 8	250 / 250	4 / 8 / 16	16 / 16 / 32
	35a	2,4-$(NO_2)_2$Ph	H	16 / 16	125 / 500	125 / 500 / 500	125 / 500 / 500
	35b		Ph	8 / 8	250 / 250	8 / 32 / 62	16 / 32 / 62
	35c		4-NO_2Ph	4 / 4	62 / 62	32 / 62 / 62	32 / 62 / 62
	35d		3,5-$(NO_2)_2$Ph	4 / 4	16 / 32	32 / 62 / 62	16 / 32 / 62
	36a	3,5-$(NO_2)_2$Ph	H	4 / 8	500 / 1000	4 / 16 / 32	16 / 32 / 32
	36b		Ph	1 / 2	250 / 250	2 / 8 / 8	2 / 4 / 4
	36c		4-NO_2Ph	2 / 4	250 / 250	1 / 4 / 4	2 / 8 / 16
	36d		3,5-$(NO_2)_2$Ph	4 / 8	250 / 250	4 / 8 / 8	8 / 16 / 32
	36f		4-ClPh	2 / 4	125 / 125	2 / 2 / 4	4 / 8 / 8
	36g		3,4-Cl_2Ph	2 / 2	125 / 125	2 / 4 / 4	2 / 4 / 8
2,5-regioisomers	**37d**	Ph	3,5-$(NO_2)_2$Ph	16 / 16	125 / 125	16 / 32 / 32	16 / 32 / 32
	38c	4-NO_2Ph	4-NO_2Ph	250 / 250	250 / 250	250 / 250 / 250	250 / 250 / 250
	38d		3,5-$(NO_2)_2$Ph	8 / 8	250 / 250	4 / 8 / 16	16 / 16 / 32
	39a	2,4-$(NO_2)_2$Ph	H	4 / 8	125 / 125	32 / 125 / 125	32 / 125 / 125
	39b		Ph	4 / 4	32 / 62	16 / 32 / 62	8 / 16 / 32
	39c		4-NO_2Ph	n.d.	n.d.	n.d.	n.d.
	39d		3,5-$(NO_2)_2$Ph	4 / 4	250 / 250	4 /16 / 32	8 /16 / 16
	40a	3,5-$(NO_2)_2$Ph	H	2 / 4	125 / 250	4 / 8 / 8	8 / 16 / 16
	40b		Ph	1 / 1	62 / 125	8 / 16 / 16	4 / 4 / 8
	40c		4-NO_2Ph	1 / 2	250 / 250	2 / 2 / 4	2 / 8 / 8
	40d		3,5-$(NO_2)_2$Ph	1 / 2	250 / 250	1 / 2 / 2	2 / 4 / 8
	40f		4-Cl	1 / 2	16 / 16	4 / 8 / 16	16 / 16 / 32
	40g		3,4-Cl_2Ph	1 / 1	125 / 125	8 / 16 / 32	16 / 32 / 32
		INH		0.5 / 1	250 / 250	250 / 250 / 250	4 / 4 / 4

n.d. not determined

20d/23b and the respective sulfanyl reverse analog pairs **33d/36b** and **37d/40b** showed that the position of the 3,5-dinitrobenzyl substituent on sulfur/selenium is beneficial, while the 3,5-dinitrobenzyl substituted tetrazole moiety was generally unfavorable for antimycobacterial activity. Isomers **15e** and **20e** bearing a 2,4-dinitrobenzyl moiety on tetrazole were exceptions and exhibited surprisingly high antimycobacterial activity. However, this observation is likely connected with the high non-selective toxicity of 2,4-dinitrobenzyl derivatives (see below).

Nitro group-containing anti-TB agents display various mechanisms of antimycobacterial action such as inhibition of DprE1 (dinitrobenzamides, benzothiazinones),[11] inhibition of mycolic acid biosynthesis or NO poisoning of cytochrome *c* oxidase (PA-824, delamanid).[33] Although the most potent compounds **23a-e**, **40a-d**, **40f** and **40g** contain the 3,5-dinitrophenyl fragment as the DprE1 inhibiting dinitrobenzamides, their actual mechanism of action remains to be elucidated.

***In vitro* antibacterial and antifungal activity.**

The selectivities of the antimycobacterial effect of compounds **18b**, **23b**, **36b**, **40b** and **40d**, which exhibited promising antimycobacterial activity, were evaluated by determining the MIC values against 8 bacterial and 8 fungal strains. All compounds showed no antibacterial and no antifungal activity (Table S1 and S2, see Supplementary info).

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

To further probe the selectivity of the studied compounds, their effects on the viability of mammalian cells were evaluated. We were also interested in how the type and position of the substituents on tetrazole or on the sulfur/selenium atom and the presence of either sulfur or selenium in position 5 of tetrazole would influence the overall toxicity of the studied compounds. Therefore, the effects of five 1,5- and 2,5-regioisomeric pairs of the selenium derivatives, **15d**/**20d**, **17b**/**22b**, **18b**/**23b**, **18c**/**23c** and **18d**/**23d**, five pairs of the sulfur regioisomers, **33d**/**37d**, **36b**/**40b**, **36c**/**40c**, **36f**/**40f** and **36g**/**40g**, and compounds **39b** and

40d on the viability of the Chinese hamster ovary (CHO-K1) cell line were evaluated (Table 7).

The resulting IC_{50} values indicated that there is no significant difference in the toxicity between sulfur or selenium derivatives. The 2,4-dinitro derivatives (**17b**, **22b** and **39b**) were highly toxic regardless of their substitution (IC_{50} values of 8.5 – 22 μM). The toxicities of the 1-isomers were either similar to or higher than the 2-isomers; this is observed in the IC_{50} values of the isomeric pairs **18b**/**23b**, **18c**/**23c**, **18d**/**23d**, **36c**/**40c** and **36f**/**40f**. The most toxic compound, **18d**, with an IC_{50} of 6.5 μM has four nitro groups in its structure; however, there is no clear correlation between toxicity and the number of nitro groups present. Interestingly, introduction of a chlorine atom to the molecule decreased its toxicity (as in **36b** and **36f** or **40b** and **40f**); however, this effect did not show a clear dependence on the number of chlorines, as observed in compounds **36g** and **40 g**.

Table 7. Viability of CHO-K1 cells (IC_{50} expressed in μM ± SEM) determined by proliferation/viability cell assays after a 24-h treatment with test compounds.

1-isomer	IC_{50}	2-isomer	IC_{50}
15d	85 ± 21	**20d**	118 ± 19
17b	8.5 ± 0.2	**22b**	22 ± 2
18b	71 ± 34	**23b**	136 ± 6
18c	27 ± 14	**23c**	205 ± 29
18d	6.5 ± 0.2	**23d**	40 ± 9
33d	115 ± 18	**37d**	148 ± 11
35b	n.d.	**39b**	21 ± 1.4
36b	59 ± 11	**40b**	41 ± 6
36c	163 ± 40	**40c**	243 ± 6
36d	n.d.	**40d**	133 ± 5
36f	94 ± 16	**40f**	301 ± 9
36g	140 ± 9	**40g**	150 ± 34

n.d. not determined

Ames Fluctuation test

The mutagenic activity of regioisomeric pairs **18b**/**23b**, **36b**/**40b**, **36c**/**40c** and **36f**/**40f** was detected using the 96-well micro-plate version of the *Salmonella typhimurium* Ames Test. At 50 μM we found highly variable potencies of the tested compounds to induce reverse mutation on the *S. typhimurium* strain TA98 and no mutagenicity on the strain TA100, with no apparent structure-mutagenicity relationships. Importantly, compound **36b** did not induce any mutations in both strains. This indicates that 3,5-dinitrobenzyl moiety is not generally connected with frame shift or base-exchange mutagenicity (see Supplementary info).

Conclusions

In this study, a series of nitro group-containing regioisomeric 1-alkyl- and 2-alkyl-5-(alkylsulfanyl)-2*H*-tetrazoles and their selenium bioisosteres were prepared and characterized. All 1-alkyl and 2-alkyl regioisomers were isolated and unambiguously identified by the ^{1}H and ^{13}C NMR chemical shifts of the methylene group adjacent to the tetrazole nitrogen and the ^{13}C NMR chemical shift of the tetrazole carbon. The regioisomeric identities were confirmed by NOESY experiments.

Antimycobacterial evaluation indicated that 1-alkyl- (**36a-d**, **36f**, **36g**) and 2-alkyl-5-[(3,5-dinitrobenzyl)sulfanyl]-2*H*-tetrazoles (**40a-d**, **40f**, **40g**) and their selenium analogs (**18a-e**, **23a-e**) exhibited promising *in vitro* antimycobacterial activity against *M.tb* CNCTC My 331/88, with MIC values as low as 1 μM. These derivatives also showed high activities against non-tuberculous *M. kansasii* 6509/96 and INH-resistant *M. kansasii* CNCTC My 235/80, with MIC values similar or slightly lower than that of *M.tb*. Furthermore, the antimycobacterial effects of these compounds were found to be highly specific, because they showed no antibacterial or antifungal activity and low cytotoxicity in a mammalian cell line. Interestingly, no differences in these biological properties between sulfur and selenium bioisosteres were found. We also found that 3,5-dinitrobenzyl moiety *per se* is not the carrier of mutagenicity.

The structure-activity relationship study showed that the position of the 3,5-dinitrobenzyl substituent on the sulfur/selenium atom in position 5 of tetrazole is beneficial, as the reverse analogs, i.e., 1- or 2-(3,5-dinitrobenzyl)tetrazole derivatives, exhibited significantly lower antimycobacterial activities. Derivatives bearing a 2,4-dinitrobenzyl moiety generally exhibited lower antimycobacterial activities and higher *in vitro* cytotoxicity compared to the 3,5-dinitrobenzyl derivatives. Considering the position of the alkyl substituent on tetrazole, 2-alkyl-5-[(3,5-dinitrobenzyl)sulfanyl]-2*H*-tetrazoles and their selenium analogs showed higher antimycobacterial activity against *M.tb* and lower cytotoxicity compared to the 1-alkyl isomers. Consequently, 2-alkyl-5-[(3,5-dinitrobenzyl)sulfanyl]-2*H*-tetrazoles (**40**) are new lead antimycobacterial compounds because they are superior to 1-alkyl derivatives in their antimycobacterial effect and exhibit lower cytotoxicity. Moreover, the antimycobacterial activity of the 1-alkyl isomers was more influenced by the type of alkyl substituent than were the 2-alkyl isomers. Thus, variation of the 2-alkyl substituent may further optimize the ADME properties and toxicity of these compounds while maintaining the antimycobacterial efficiency.

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Notes and references

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† Electronic supplementary information (ESI) available: Details of chemical synthesis and characterization of all the reported compounds. Details of *in vitro* antimycobacterial, antibacterial, antifungal, cell

proliferation/viability assays and AMES Fluctuation test. Tables S1 and S2. See DOI: 10.1039/b000000x/

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