Charles University in Prague
Faculty of Pharmacy in Hradec Králové
Department of Inorganic and Organic Chemistry

Synthesis and structure-antimycobacterial activity relationship study of dinitrobenzylsulfanyl heteroaromates

Ing. Galina Karabanovich

PhD THESIS

Hradec Králové

2014

Declaration

I declare that this thesis is my original work. All used literature and sources are listed in the list of references at the end of the thesis and are properly cited.

This work has not been used to gain equal or different degree.

Hradec Králové 2014 Galina Karabanovich

ACKNOWLEDGEMENTS

I would like to thank Prof. Alexandr Hrabálek, my supervisor, for the opportunity to study at Charles University, for his valuable advices and professional leadership.

I am very thankful to Jaroslav Roh, PhD, who devoted much time to discuss the results of my work with me, for his efficient advices and constant support throughout my study.

I thank Prof. Grigorii I. Koldobskii, my Russian supervisor for diploma thesis, Tatiana V. Artamonova and Prof. Yurii E. Zevatskii, for opportunity to continue my study at Charles University, for their useful advices and belief in my ability to successfully finish it.

I would like to thank Assoc. Prof. Věra Klimešová and Assoc. Prof. Kateřina Vávrová for their beneficial consultations, Assoc. Prof. Jiří Kuneš, who recorder the NMR spectra and Prof. Milan Pour, who taught me the interpretation of the NMR spectra.

My thanks are given to all my colleagues at the Department of Inorganic and Organic Chemistry for multilateral help in my work.

I am very thankful to Mukund Ghavre for thoroughly checking English grammar of the body of this thesis

I thank my family and friends for their patience and support.

Finally, I acknowledge the financial support from the Charles University (Project GAUK 55610/2010 - principal researcher), Czech science foundation (projects GAČR 14-08423S and 13-15008S) and Charles University Research Centre (UNCE 204019/304019/2012).

Content

List of publications…….. 9

Conference abstracts…… 10

List of abbreviations …… 11

1. Abstract…… 13

   Abstrakt…… 15

2. History of Tuberculosis……... 17

3. History of antiTB drug discovery and development of TB treatment……... 26

   3.1. Discovery and development of currently used antiTB drugs…… 26

   3.2. Current antiTB therapy and new requirements to antiTB candidates…… 41

   3.3. Highly effective antiTB candidates being in preclinical or clinical development……. 43

4. Mycobacterial cell wall…… 48

5. Background of current work…….. 54

6. Aim of the work……. 57

7. Description of obtained results……. 58

   7.1. Synthesis and structure – antimycobacterial activity relationships study of 1-substituted-5-alkylsulfanyl-1*H*-tetrazoles and their isosteric analogs……. 58

   7.2. Sulfanyl/selanyl tetrazole regioisomers: synthesis and structure - antimycobacterial activity relationships study…….. 70

   7.3. Tetrazole-free compounds containing 3,5-dinitrobenzylsulfanyl moiety: synthesis and antimycobacterial efficiency..……... 75

   7.4.Synthesis and structure - antimycobacterial activity relationships study of 2-alkyl-5-alkylsulfanyl-1,3,4-oxadiazoles and 2-alkyl-5-alkylsulfanyl-1,3,4-thiadiazoles…… 76

8. Hypothetical mechanism of action of compounds bearing 3,5-dinitrobenzylsulfanyl fragment……... 84

9. Conclusion…… 87

10. References……. 92

11. Attachments……. 103

11.1. Attachment I…… 103
11.2. Attachment II…… 117
11.3. Attachment III…….. 137
11.4. Attachment IV…….. 187

List of publications

This thesis is based on the following publications, which are referred in the text by the Roman numerals I – IV and are attached at the end of the thesis:

I. Karabanovich, G.; Roh, J.; Padělková, Z.; Novák, Z.; Vávrová, K.; Hrabálek, A. One-pot synthesis of 1-substituted-5-alkylselanyl-1*H*-tetrazoles from isoselenocyanates: unexpected formation of *N*-alkyl-*N*-arylcyanamides and (Z)-*Se*-alkyl-*N*-cyano-*N,N'*-diarylisoselenoureas. *Tetrahedron*. **2013**, 69, 8798-8808.

II. Karabanovich, G.; Roh, J.; Smutný, T.; Něměcek, J.; Vicherek, P.; Stolaříková, J.; Vejsová, M.; Dufková, I.; Vávrová, K.; Pávek, P.; Klimešová, V.; Hrabálek, A. 1-Substituted-5-[(3,5-dinitrobenzyl)sulfanyl]-1H-tetrazoles and their isosteric analogs: A new class of selective antitubercular agents active against drug-susceptible and multidrug-resistant mycobacteria. *Eur. J. Med. Chem*. **2014**, 82, 324-340.

III. Karabanovich, G.; Roh, J.; Soukup, O.; Pávková, I.; Pasdiorová, M.; Tambor, V.; Stolaříková, J.; Vejsová, M.; Vávrová, K.; Klimešová, V.; Hrabálek, A. Tetrazole Regioisomers in the Development of Nitro Group-Containing Antitubercular Agents. *Med. Chem. Commun*. **2014**. DOI: 10.1039/c4md00301b

IV. Hrabálek, A.; Roh, J.; Karabanovich, G.; Klimešová, V.; Němeček, J.; Pávek, P. Oxa- and thia-diazoles useful in the treatment of Tuberculosis. Patent WO2014/161516 A1.

Conference abstract

Karabanovich, G.; Roh, J.; Vávrová, K.; Stolaříková, J.; Klimešová, V.; Hrabálek, A. Structure-antimycobacterial activity relationship study of 1-substituted 5-(dinitrobenzylsulfanyl)-1*H*-tetrazoles. Book of Abstracts. Conference "Towards New Therapeutics for Diseases of the Developing World". Madrid, Spain. **2014**

Karabanovich, G.; Roh, J.; Vávrová, K.; Stolaříková, K.; Klimešová, V.; Hrabálek, A. Synthesis of heterocyclic compounds with high antimycobacterial activity. *Sborník abstraktů.* 42. Konference Syntéza a analýza léčiv. Velké Karlovice, CZ. **2013**

Karabanovich, G.; Roh, J.; Vávrová, K.; Stolaříková, J.; Klimešová, V.; Hrabálek, A. Derivatives of Nitrogen Heteroaromates as a Potential Antituberculotics. Book of Abstracts. Conference "Tuberculosis 2012: Biology, pathogenesis, invervention strategies". Paris, France, **2012**.

Karabanovich, G.; Roh, K.; Vávrová, K.; Stolaříková, J.; Klimešové, V.; Hrabalek, A. Synthesis of new antituberculotics derived from benzylsulfanylheteroaromates. *Chem. Listy*. **2012**, 106, S1037. EuChemMS Chemistry Congres, Prague, CZ, **2012**.

Karabanovich, G.; Roh, J.; Vávrová, K.; Hrabálek, A. Synthesis of potential antituberculotics based on nitrogen heterocycles. Book of Abstracts. 14. Austrian Chemistry Days. Linz, Rakousko, **2011**.

Karabanovich, G.; Roh, J.; Vávrová, K.; Hrabálek, A. Syntéza antimykobakteriálně aktivních 1-alkyl/aryl-5-alkylsulfanyl-1*H*-tetrazolů a jejich selenových analogů. *Sborník abstraktů.* 46. Pokroky v organické, bioorganické a farmaceutické chemii – "Liblice 2011". Lázně Bělohrad, CZ. **2011.**

Karabanovich, G.; Roh, J.; Vávrová, K.; Hrabálek, A. Potential antituberculotics based on 1-substituted 5-alkylsulfanyltetrazoles. *Sborník abstraktů.* 40. konference Syntéza a analýza léčiv, Brno, CZ, **2011**.

Karabanovich, G.; Roh, J.; Vávrová, K.; Hrabálek, A. Syntéza selenových analogů sirných antituberkulotik. *Zborník*, **2010**, 79. (39. Conference "Syntéza a analýza liečiv", Modra-Harmonia, Slovensko, **2010**.

Karabanovich, G.; Roh, J.; Vávrová, K.; Hrabálek, A. Syntéza selenových analogů antituberkuloticky účinných 1-R-5-alkylsulfanyl-1*H*-tetrazolů. *Chem. Listy*. **2010**, 104, 1091. 45. Pokroky v organické, bioorganické a farmaceutické chemii – "Liblice 2010". Lázně Bělohrad, CZ. **2010.**

List of abbreviations

ADME	Absorption, distribution, metabolism, and excretion. These process describe the disposition of a drug within an organism
AG	Arabinogalactan
antiTB	Antituberculosis
BlaC	*β*-lactamase
BTZ	Benzothiazinone
EMA CHMP	European Medicines Agency's Committee for Medicinal Products for Human Use
CNCTC	Czech National Collection of Type Cultures
DMF	*N*,*N*-Dimethylformamide
DOTS	Directly Observed Treatment Short course
EMB	Ethambutol
FAD	Flavin adenine dinucleotide
FDA	United State Food and Drug Administration
HIV	Human Immunodeficiency Virus
INH	Isoniazid
inhA	NADH-dependent enoyl-acyl carrier protein reductase
katG	catalase/peroxidase enzyme
mAGP	mycolyl-arabinogalactanpeptidoglycan complex
M.	*Mycobacterium*
MIC	Minimal inhibitory concentration
M.tb.	*Mycobacterium tuberculosis*
MDR-TB	Multidrug resistant TB
MTBC	*Mycobacterium tuberculosis* complex
NAD	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
PAS	*para*-Aminosalicylic acid
PG	Peptidoglycan
PZA	Pyrazinamide

RIF	Rifampicin
PZase	Pyrazinamidase/nicotinamidase
STR	Streptomycin
TAC	Thiacetazone
TB	Tuberculosis
TBAB	Tetrabutylammonium bromide
TDR-TB	Totally drug resistant TB
THF	Tetrahydrofuran
XDR-TB	Extensively drug-resistant TB
WHO	World Health Organization

1. Abstract

This thesis deals with the design and synthesis of novel heterocyclic compounds with high and selective antimycobacterial activity and the structure - antimycobacterial activity relationship study of these compounds.

The history of knowledge about Tuberculosis, drug/regimen development, a way to successfully understand the *Mycobacterium tuberculosis* (*M.tb.*) etiology and the search for suitable therapy for treatment of drug-susceptible and drug-resistant TB are described in chapters 2 and 3. The current situation of TB epidemic and efforts towards its solution are also included.

In chapter 4, the structure of mycobacterial cell wall is presented. It demonstrates the mycobacterial structural feature possessing an unusual, highly protective properties, which is essential for *M.tb*. growth and its survival in the host.

The synthesis, logical consistency of structural changes and the results of antimycobacterial evaluation of prepared compounds are described in chapter 7.

Within this work, four broad series of compounds were synthesized with following structural motifs: 1-alkyl/aryl-5-alkylsulfanyl-1*H*-tetrazoles, 2-alkyl-5-alkylsulfanyl-2*H*-tetrazoles, 2-alkyl/aryl-5-alkylsulfany-1,3,4-oxadiazoles and 2-alkyl/aryl-5-alkylsulfany-1,3,4-thiadiazoles (Fig.1).

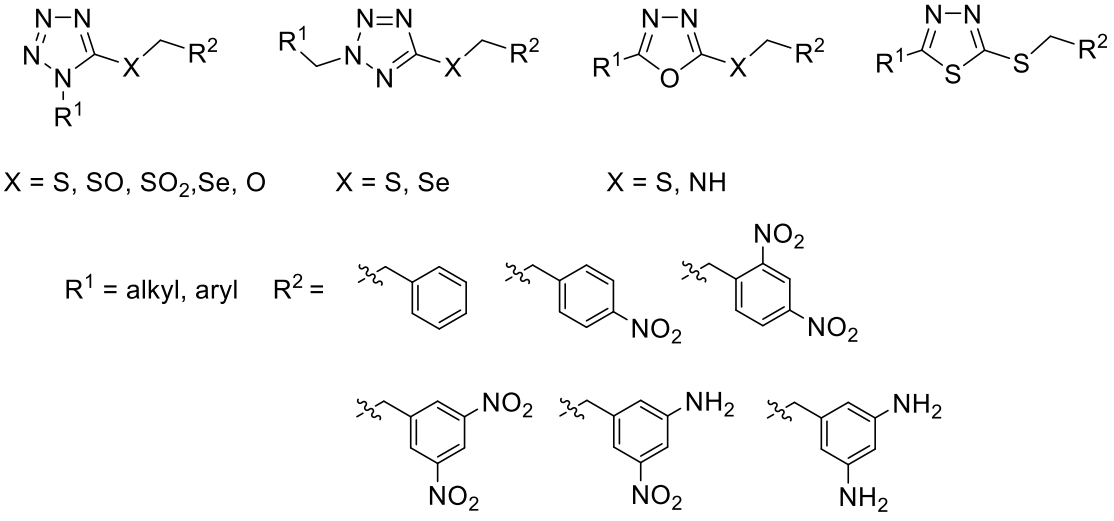


Figure 1. The general structure of compounds studied in this work.

The role of the individual structural fragments of these compounds in antimycobacterial activity was investigated. The influence of substituent R^1 on

heterocycle on compounds efficiency was studied. Additionally, the role of the number and position of nitro groups on benzyl moiety, reduction of dinitrobenzyl moiety, heteroatom linker and the heterocycle on antimycobacterial activity was also examined.

The certain regularities in relationships between the structures and antimycobacterial activity in all series of prepared compounds were described.

Two interesting, general lead structures were found in this work: 2-alkyl-5-[(3,5-dinitrobenzyl)sulfanyl]-2*H*-tetrazoles with minimal inhibitory concentration (MIC) 1 μM against *M.tb.* and 2-alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfanyl]-1,3,4-oxadiazole with MIC 0.03 μM against *M.tb.* These compounds were highly effective against both drug-susceptible and drug-resistant strains of *M.tb*. Moreover, such substances showed no antibacterial and antifungal effect and low cytotoxicity.

This work has become a basis for further research and led to the synthesis of series of compounds with promising antimycobacterial activity.

1. Abstrakt

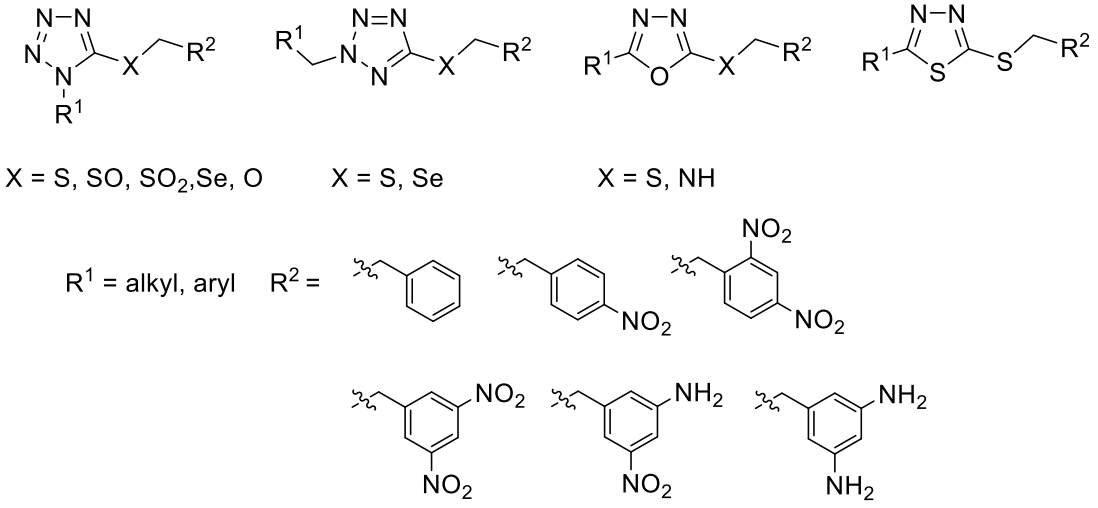
Cíle této práce spočívají v designu a syntéze nových heteroaromatických sloučenin vykazujících vysokou a selektivní antimykobakteriální aktivitu a ve studiu vztahů mezi strukturou těchto látek a jejich antimykobakteriální účinností.

V úvodu práce je shrnuta historie studia tuberkulózy (TB) a vývoje antituberkulotických léčiv, hledání vhodné terapie pro její léčbu a etiologie hlavního původce *Mycobacterium tuberculosis* (*M.tb*). Dále je v úvodu popsán současný stav epidemie TB a nové možnosti v její léčbě.

Další kapitola je věnována struktuře mykobakteriální buněčné stěny, důležitého a charakteristického strukturálního rysu mykobakterií, díky které jsou mykobakterie vysoce odolné vůči vnějším vlivům a která je nezbytná pro růst a přežití *M.tb*. v organismu hostitele.

Syntéza cílových látek, strategie prováděných strukturálních změn a výsledky antimykobakteriálních hodnocení připravených látek jsou popsány v kapitole 7.

V rámci této práce byly syntetizovány čtyři rozsáhlé sérii sloučenin s následujícími strukturními motivy: 1-alkyl/aryl-5-alkylsulfanyl-1*H*-tetrazoly, 2-alkyl-5-alkylsulfanyl-2*H*-tetrazoly, 2-alkyl/aryl-5-alkylsulfany-1,3,4-oxadiazoly and 2-alkyl/aryl-5-alkylsulfany-1,3,4-thiadiazoly (Obr. 1).



Obrázek 1. Obecné struktury sloučenin studovaných v této práci.

U těchto látek byl studován vliv jednotlivých strukturních fragmentů na antimykobakteriální aktivitu, především pak vliv substituentu R^1, vliv počtu a polohy

nitroskupin na benzylové skupině, spojovacího atomu X či redukce dinitrobenzylové skupiny.

Na základně těchto studií byly nalezeny dvě vysoce účinné struktury, 2-alkyl-5-[(3,5-dinitrobenzyl)sulfanyl]-2*H*-tetrazoly s minimální inhibiční koncentrací (MIC) 1 μM proti *M.tb*. a 2-alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfanyl]-1,3,4-oxadiazoly s MIC 0,03 μM proti *M.tb*. Tyto sloučeniny jsou vysoce aktivní proti citlivým i lékově rezistentním kmenů *M.tb*., nejsou antibakteriálně a antifungálně účinné a vykazují nízkou toxicitu proti lidským buňkám.

Tato práce se stala základem pro další výzkum a vedla k syntéze několika dalších sérií sloučenin s vysokou antimykobakteriální aktivitou.

2. History of Tuberculosis

The history of tuberculosis (TB) dates back hundreds of thousands of years. It is hypothesized that the genus *Mycobacterium* appeared more that 150 million years ago, however progenitor of *Mycobacterium tuberculosis* (*M.tb.*) developed only 3 million years ago infecting early hominids. (1) Evolution of tubercle bacilli led to appearance of various kinds of mycobacteria caused TB, which had a common African ancestor about 15,000-35,000 years ago and nowadays are combined in *M.tb.* complex (MTBC). MTBC includes *M.tb., M. africanum*, *M. canettii*, which are the human pathogens, and pathogens isolated mainly from animals: *M. bovis* (causative agent of TB commonly in cattle),[1] *M. microti* (cause of naturally acquired TB in voles), *M. pinnipedii* (primarily infects seals) and *M. caprae* (causes TB among goats and deers).

Modern strains of *M.tb.*, which arose during human migration out of Africa, are genetically different and also highly geographically structured. (2) Currently, the comparative genomic and molecular epidemiological tools allow to define the global population structure of *M.tb.*, reveal six main lineages, and 15 sublineages of *M.tb.* (Fig.2). Each of the six main lineages of *M.tb.* are associated with the specific geographical region, however, all of them are present in Africa. Such knowledge is useful, for example, in the case of vaccine application. It can be ineffective to certain *M.tb.* strains in certain geographic regions due to genetic variability of the pathogen population.

The earliest data about human infection by *M.tb.* were obtained from genetic studies of bone samples with lesion of a 9,000-year-old Neolithic human found in the eastern Mediterranean. (3; 4) Evidence of *M.tb.* infection was also found in the mummified tissues of ancient Egyptians (5) and Peruvians. Moreover, TB was described in Indian and Chinese written text 2000-3000 years ago. (1)

However, the first systematic description of TB (formerly known as Phthisis or Consumption) occurs in work of **Hippocrates** (c. 460 – c. 370 BC). In 'Book 1, Of the Epidemics' (6; 7) the Greek physician noted very accurate symptoms of the disease, which was later interpreted as TB: a general view of a patient, fever, chills, sweating, fatigue, exhaustion, loss of weight, cough, characteristic sputum and chest pain. He

[1] The principal cause of human TB is *M.tb.; M.bovis* has a wider host range and is the main cause of TB in other animal species. Humans can become infected by *M. bovis,* usually via milk, milk products or meat from an infected animal.

underlined that preferentially young adults suffered from phthisis, disease, which was usually fatal.

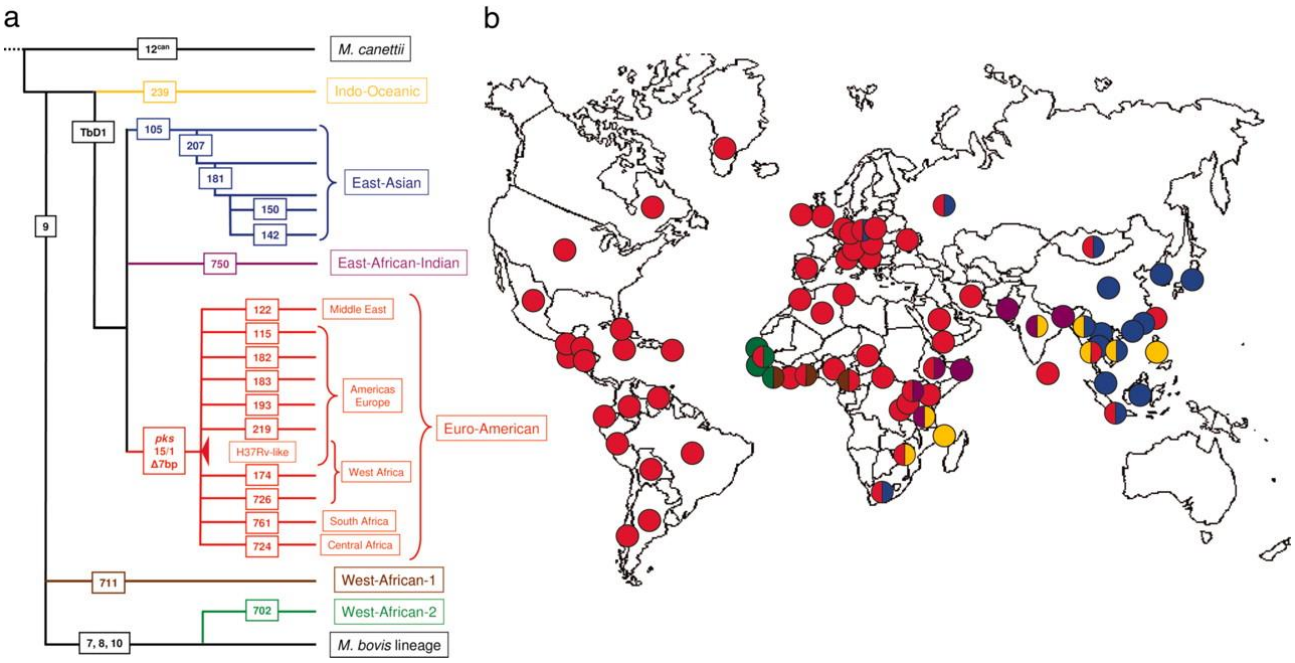


Figure 2. The global population structure and geographical distribution of *M.tb.* (2)
(**a**) Large sequence polymorphisms (LSPs) define a global phylogeny for *M.tb*. The names of the lineage-defining LSPs or regions of difference are shown in rectangles. The geographic regions associated with specific lineages are indicated. (**b**) The six main lineages of *M.tb*. are geographically structured. Each dot corresponds to 1 of 80 countries represented in the global strain collection. The colors of the dots relate to the six main lineages defined in Fig.2a and indicate the dominant lineage(s) in the respective countries.
© 2006 by The National Academy of Sciences of the USA

Hippocrates had no concepts concerning the causative agent (pathogen), he had no possibility to perform autopsies and had no knowledge about physiology and pathophysiology, and therefore he suggested that the phthisis was hereditary rather than being infectious in nature. While **Aristotle** (384 – 322 BC) adhered to another opinion about phthisis: he believed in contagious nature of the disease, noting that there was some pathogenic source in the air around such patients. (8; 9) Description of phthisis can also be found around the beginning of the Common Era in the works of other notable physicians such as **Aurelius Cornelius Celsus** (25 BC – 30 AD), (10) **Aretaeus of Cappadocia,** (11) the Greek physician of the 2nd century AD, and **Caelius Aurelianus**, the Roman physician of the 5th century AD, who continued to provide descriptions of the other symptoms, diagnosis and treatment of phthisis. (12)

The Roman physician **Claudius Galen** (c. 130 – c. 210 AD), the founder of experimental physiology, whom belongs the most comprehensive and long-lived doctrine about phthisis, had a similar view with Aristotle about contagious nature of phthisis and advised avoiding it. He considered that disease affected lungs and caused inflammation, which was accompanied by the formation of abscesses; the coughing patients excreted the contents of abscesses, resulting in the formation of the cavity, which was often increased in volume and constantly produced pus; thus lung tissue got destroyed, and the patients died from exhaustion and fever. Galen also stated that the symptoms of disease had to be explained by alterations in the body structure. (13)

The great contribution to further development of the doctrine about TB was made by the Persian physician **Avicenna** (980 – 1037) (Abu Ali Ibn Sina). Avicenna noted the influence of the environment on the development and course of TB, and also emphasized that the infection occurred from “bad” air and was spread by water and soil. The Persian physician considered phthisis contagious disease and believed in the possibility of patient recovery. (14)

However, there was the scant information about TB in the work of European physicians during the Middle Ages (5^{th} – 15^{th} centuries). The situation changed with the introduction of printing, when antique work of Hippocrates, Galen and other scientists was published and spread across the Europe, caused a new wave of interest in the research of phthisis.

In Renaissance period, the Italian physician **Girolamo Fracastoro** (1478 – 1553), the founder of theory of infection by microorganism, noted in his work ‘De contagion’ (‘On contagion’) (1546) that phthisis spread by an invisible “virus” and the disease came from an acquired lung ulcer, from which true pus was discharged. (8) In this work Fracastoro systematically described the major ways of disease transmission: by direct contact, through infectious items (fomites), which were in contact with pathogens, and on the distance. (9) From his work the term "phthisis" was established exclusively for pulmonary consumption.

The first pathoanatomical studies contributed to the understanding of the pathological changes in the lung were performed by **Andreas Vesalius** (1514 – 1564), the great anatomist of the Renaissance, author of one of the most influential books on human anatomy, ‘De humani corporis fabrica’ (‘On the Fabric of the Human Body’) (1543). The possibility to perform autopsies of patients died from phthisis and

enrichment of anatomy knowledge in the 16th - 17th centuries led to generation of exact pathological and anatomical description of the disease.

Franciscus Sylvius de la Boë (1614 – 1672), the Dutch physician and scientist, was the first who connected the revealed characteristic changes in the lungs in the form of little nodules (tubercles) observing at an autopsy with phthisis. In his work 'Opera Medica' (1679) Sylvius described the characteristic lesions in the lungs and other organs of patients as well as the progression of tubercles to the abscesses, the ulcers, and the empyema. (8) Sylvius believed in contagion of phthisis noting that the air expelled by consumptives, if drawn in by the nose and mouth by anyone, especially relatives, and particularly those of them who are of tender years, will produce phthisis. (15)

The first physician since Galen who envisaged a concept of the unity of TB through full description of disease symptomatology was **Richard Morton** (1637 – 1698). (9; 15) The English physician in his book 'Phthisiologia, seu exercitationes de Phthisi, tribus libris comprehensae' (1689) described the pathogenesis of TB cavitation, the changes in lung and highlighted the three stages of the development of disease process: initial inflammation giving way to tubercle formation, its progress to the ulcers and thus to phthisis. Morton considered that tubercles were always present in the lungs of patients with phthisis, although he mistakenly thought that tubercles were glandular degeneration. Morton regarded the phthisis as hereditary, although did not exclude the possibility of transmission by contact with patient.

At the same time, in 17th – 18th centuries, the research work of other notable European physicians on the etiology and pathophysiology of TB was published.

The Englishman **Thomas Willis** (1621 – 1675) in his 'Practice of Physick' (1684) described miliary chronic and fibrotic forms of TB. The Genevan physician **Jean-Jacques Manget** (1652 – 1742) explored the phenomenon of miliary TB and observed multiple small phthitic nodules in the lungs and other organs during autopsies. The English physician **Benjamin Marten** (1704 – 1782) considered that the phthisis was caused by small living organisms, which permeate into the body and produce the lesions and symptoms of phthisis. He also suggested that disease could be transmitted by inheritance as well as by contact with infected person. (16) In 1793 The Scottish physician and pathologist **Matthew Baillie** (1761 – 1823) described the caseous ("cheese-like") appearance of phthisic abscesses. Baillie considered that the essence of pulmonary TB was the appearance of tubercles, which were

morphological manifestation of TB and also the basis of all subsequent forms of the disease. (17) The French physician **Gaspard Laurent Bayle** (1774 – 1816) described the characteristic tubercle and its association with pulmonary and other forms of phthisis in his book 'Research of pulmonary tuberculosis' (1810). (18) He thought that the tubercles initiated the formation of the characteristic phthisis lesions and could exist before symptoms. He recognized that pulmonary TB may advance very slow or act as acute disease. (9) Physician performed a large number of autopsies of patients died from phthisis and combined the pathoanatomical study of cadavers with a detailed analysis of medicinal histories of patients. His work later gave the rationale for conducting clinical-pathological comparisons.

The further stage of development of study about TB is connected with name of French physician **René Théophile Hyacinthe Laennec** (1781 – 1826), well known as inventor of stethoscope (1816) (19). Based on auscultation and morphological results obtained by autopsy of patients died from phthisis, Laennec established the common origin, the etiological unity of the various forms of TB, pulmonary and extra-pulmonary (for example, in liver, intestines, meninges etc.). (1; 12) He wrote that the tuberculous process was a definite pathological change, whatever its site, and it occured in the two forms of tubercles and nodules, and diffused infiltration, both originating in a miliary nodule. (15) He described the appearance of tubercle in the lung in miliary form, progressing to caseous, their breakdown into pus and formation of cavities. However, Laennec considered that tubercles are the morbid growths and that the pulmonary phthisis is a constitutional disease and that it can never be developed from an acute or chronic pneumonia, or from bronchial haemorrhage; the every phthisis of the lung is owing to a new or morbid formation. (20) Laennec also did not believe in the curability of phthisis. (9)

To the beginning of 19th century TB reached the epidemic state and became known as the "Great white plague" and "The white death". However, to this time there was little information about pathogen of TB and there was no general opinion about etiology of phthisis. Physicians of this time debated the nature of TB - if the disease was infectious, hereditary, or a cancer. The question whether scrofula, tubercles, and phthisis were different diseases or different forms of the one disease also remained controversial.

The proof of infectiousness was a crucial stage in the study of TB. **Philipp Klencke** (1813 – 1881) and **Jean Antoine Villemin** (1827 – 1892) experimentally

demonstrated the transmissibility of TB. In 1843 the German physician **Klencke** successfully injected material from a tubercle to the rabbits, after which animals developed TB. Despite such evidence, Klencke believed that TB was a tumor disease. Later, the French military surgeon **Jean Antoine Villemin** proved that TB was actually an infectious disease, transmitted through contact from humans to animals and from one animal to another. He demonstrated that injections of blood or sputum of infected rabbits to others animals cause the development of tuberculous lesion. His experiments confirmed the unity of anatomically diverse processes that occur in TB. Moreover, Villemin hypothesized that the disease was caused by specific organism. (8)

Important contribution to the development of doctrine of TB was also made by **Carl von Rokitansky** (1804 – 1878) and **Jakob Henle** (1809 – 1885). The Viennese anatomist and pathologist **Carl von Rokitansky** performed many autopsies on TB patients exploring the anatomical changes of organs and tissues. In his book 'Pathological Anatomy' (1855) (21) he fully described the tubercle, its common forms, properties, formation and development, changes in surrounding textures, development of phthisis as well as its association with other diseases (typhus, bronchocele etc.). He also believed in infectious nature of TB. Due to his work the pathological anatomy became the foundation of pathology and scientific medicine. The German scientist, pathologist and anatomist **Jakob Henle** made contributions in elucidating the structure of both healthy and diseased tissues. He suggested that phthisis was contagious disease; he was a co-founder of the theory that microorganisms cause the infective diseases and noted that the specific causative agents had to induce only one disease.

Interestingly, the German doctor, anthropologist, pathologist, biologist, and politician **Rudolf Virchow** (1821 – 1902) held a view that scrofula, tubercles and phthisis were separate entities. Virchow presented a concept of the cell as a center of pathological changes and considered that there were no specific cells in diseased tissue - every morbid structure consisted of cells derived from pre-existent cells (22). However, he thought that tubercles, the smallest neoplasm, were formed from small round cells with little protoplasm; that the caseous material in tuberculosis-infected lungs were in fact the end product of degenerated cells; that almost all so-called miliary tubercles of the lungs were of bronchitis, peribronchitis, or pneumonic origin.

The major event which gave a start to modern understanding of etiology of TB and unambiguously confirmed the infectious nature of TB was the discovery of *M.tb.* made in 1882 by the German physician and scientist **Robert Koch** (1843 – 1912).

Before the discovery of *M.tb.*, Koch confirmed the infectious nature of anthrax caused by *Bacillus anthracis* (1876) and described the etiology of traumatic infectious diseases and the bacteria of six different kinds of surgical infection, with the pathological findings (1878). (18) In 1881, he developed the method of obtaining pure cultures of organisms by spreading liquid gelatin with meat infusion upon glass plates, forming a solid coagulum.

To find a causative agent of TB, Koch explored tissues of laboratory animals (guinea pigs), which were infected with tuberculous material obtained from other animals and died patients with pulmonary, extra-pulmonary TB as well as with scrofula cases. (23) His experiments demonstrated the development of the same disease in all cases and the presence of identical specific bacillus in tissues proved the single origin of various forms of TB.

A mycobacterial cell wall contains the large amounts of lipid substances, which causes its low permeability and give the bacteria the capability to resist to an action of chemicals and enzymes. Thus, it was difficult for a long time to distinguish pathogen of phthisis among other cell's organelles using standard procedures. To stain pathogens, Koch initially used methylene-blue, the common aniline dye, in water solution of caustic potash (KOH) with further addition of concentrated solution of vesuvin. In such condition only the previously blue-stained cell nuclei and detritus became brown, while the tubercle bacilli remained a blue. (24) However, later he used a medium of coagulated bovine serum developed by **John Tyndall** (1820 – 1893). It is important to note the contribution of the German physician and scientist **Paul Ehrlich** (1854 – 1915), an inventor of the first antisyphilitic agent Salvarsan, who improved the Koch's method of *M.tb*. staining by using heat and decolourising with acid. Moreover, two German doctors, the bacteriologist **Franz Ziehl** (1859 – 1926) and the pathologist **Friedrich Neelsen** (1854 – 1898) described acid-fast stain, in which carbol fuchsin (instead of aniline), acid alcohol, and methylene blue were used as reagents. In the case of the acid-fast stain mycobacteria became bright red.

Subsequently, Robert Koch discovered the causative agents of cholera (*Vibrio cholera*) and found the microorganisms responsible for Egyptian ophthalmia or

infectious conjunctivitis (1884) (18) as well as he made further important discoveries in bacteriology identifying the causative agents of typhoid fever, glanders and diphtheria. (8) The great Koch's another contribution to the development of bacteriology was the formulation of the criteria or postulates (1884), which established a causative relationship between a defined pathogen and a specific disease and were later published in a work "The Etiology of Tuberculosis". These postulates define that:

1. The pathogen (microorganism) causing the specific disease have to be found in infected tissues, in all cases of that disease;
2. The microorganism responsible for disease must be isolated and grown in pure culture;
3. The inoculation of a healthy organism by the cultured microorganism have to lead to the appearance of the same disease;
4. The microorganism must be re-isolated from the inoculated organism and identified as being identical to the original specific causative agent.

In 1882, the news about discovery and demonstration of pathogen of TB disease, which took away the hundreds of thousands of lives, rapidly spread around the World and produced the sensation, gave the hope that a new era for the study and control of disease had started and that drugs for the TB treatment will be found in the short time. All these hopes, all views were first of all turned to Robert Koch. And in 1890, Koch announced that he produced the glycerin extract of dead tubercle bacilli – tuberculin, remedy against TB. However, such statement was prematurely: it was detected that tuberculin was ineffective in fighting against this disease, although, it was defined later that it had a diagnostic potential and could be used to identify infected individuals. Injection of the tuberculin caused not only a local inflammatory response, but also the general intoxication with acute exacerbation of foci of TB infection.

In 1907, the Austrian scientist and pediatrician **Clemens von Pirquet** (1874 – 1929), who developed the study about allergy and allergen, used a small amount of diluted tuberculin intracutaneously and considered that the positive reaction in asymptomatic children reflected the latent TB. In 1908, the French physician **Charles Mantoux** (1877 – 1947) introduced the Mantoux skin test, also intradermally administered tuberculin, which became the standard method in determining previous exposure to TB. In the 1930's, the American biochemists **Florence B. Seibert** (1897

- 1991) and **Esmond R. Long** (1890 – 1979) improved diagnostic test through development of a tuberculin purified protein derivative (PPD), a precipitate of species-nonspecific molecules obtained from filtrates of sterilized, concentrated cultures, which produced less false negative results than previous tests and which is still being in use. (1)

While Robert Koch did not find the drug, which could cure TB, his contribution to bacteriology and development of doctrine about TB had the crucial role and is invaluable. He was awarded with the Nobel Prize in Physiology or Medicine in 1905 "for his investigations and discoveries in relation to tuberculosis."

In 1895, the German physicist **Wilhelm Konrad von Röntgen** (1845 – 1923) discovered X-ray, which proved crucial to the modern TB diagnosis and control. With Röntgen's technique, Röntgenograms, it became possible to look at internal organs in more detail and it was very effective in showing TB in the lung in its various stages. (25) In 1901, Röntgen was awarded with the Nobel Prize for this this importance discovery.

An establishment of antiTB vaccine was a further important stage in the fighting against TB. The French bacteriologists **Albert Calmette** (1863 – 1933) and **Camille Guerin** (1872 – 1961) developed a vaccine, which is still in use, especially, in countries with a high TB prevalence. Successive subculturing of the virulent strains of *M. bovis* on a medium containing ox bile led to formation of its non-virulent strain, which could not produce progressive tuberculous lesions and allowed to create live attenuated vaccine (1908), which was named as Bacillus Calmette-Guerin or BCG. By 1919, the scientists experimentally proved efficiency of created vaccine in animals and in 1921, the first vaccination was performed. Application of BCG to children led to a significant reduction of the most dangerous forms of TB, such as miliary TB and meningitis.

Discovery of antibiotics as sulphonamides (1930's) and penicillin (1940's) opened the new Era in the history of effective antimicrobial treatment. However, the new stage in TB history, stage of TB treatment, began with the development of streptomycin.

3. History of antiTB drug discovery and development of TB treatment

3.1. Discovery and development of currently used antiTB drugs

For centuries, the climatotherapy (sea voyage, walking in mountains, moving to somewhat cold climate or, in other period of history, to the gentle, protective and warm climate), the hygienic and dietetic treatment (mainly based on milk therapy), exercise (horseback riding, swinging etc.) and the herbal decoctions (for example, opium as in the syrup of meconium or diacodium) were the main tools in the TB treatment. Compounds such as arsenic, sulfur, chalk, iodine or potassium iodide, iron, the gases (carbon dioxide, nitrogen or hydrogen), calcium as well as tea and coffee were also recommended for the treatment. Moreover, bloodletting was often done as it was for many diseases. (1; 8; 12) However, treatment was directed towards the symptoms as cough, emaciation, fever etc.

Staying in the sanatorium as the approach of TB treatment became widespread in the middle of 19th century. Sanatorium treatment involved rest, a rich diet and carefully supervised exercise. Later, pulmonary collapse therapy (pneumothorax therapy) as well as the surgical treatment was introduced. (25)

Isolation of infectious consumptives was beneficial for public health. However, there was the urgent need for therapeutic medicaments. The first, but, unfortunately, unsuccessful attempt to develop the remedy for TB therapy was production of tuberculin by Robert Koch (see above). The discovered antibiotics in 1930's - 1940's were ineffective against *M.tb*. or too toxic to be used.

However, it was observed that metabolic products of saprophytic bacteria, such as *Staphylococci* and *Bacterium prodigiosum,* and *Aspergillus fumigatus,* inhibited growth of tubercle bacilli and decreased its virulence. (8) The breakthrough in drug investigation was brought by **Selman Waksman** (1888 – 1973) and his colleagues, who isolated streptomycin (STR) (Fig.3), the first antiTB drug, from *Streptomyces griseus* (1943). Waksman devoted a long time to study the soil fungi and bacteria and found the strong inhibitory effect of soil bacteria of actinomycete group on bacteria growth. Besides STR, Waksman discovered actinomycin and streptothricin, which were toxic for therapeutic application in animals and people. Furthermore, he isolated a number of other new antibiotics, including clavacin, grisein, neomycin,

fradicin, candicidin, and candidin. Selman Waksman was honored with the Nobel Prize in Physiology or Medicine 1952 for the discovery of the first antiTB drug. (1; 26)

Subsequently, in addition to STR, another aminoglycoside antiTB drug - kanamycin (isolated from *Streptomyces kanamyceticus* in 1957) and amikacin (semisynthetic compound derived from kanamycin; prepared in 1972) (Fig.3) were introduced in therapy and nowadays they are used as second-line medicaments in the treatment of MDR-TB.[2] (27) However, these compounds are not orally active, and have limited intracellular activity as well as possess nephrotoxicity, ototoxicity and other adverse effects.

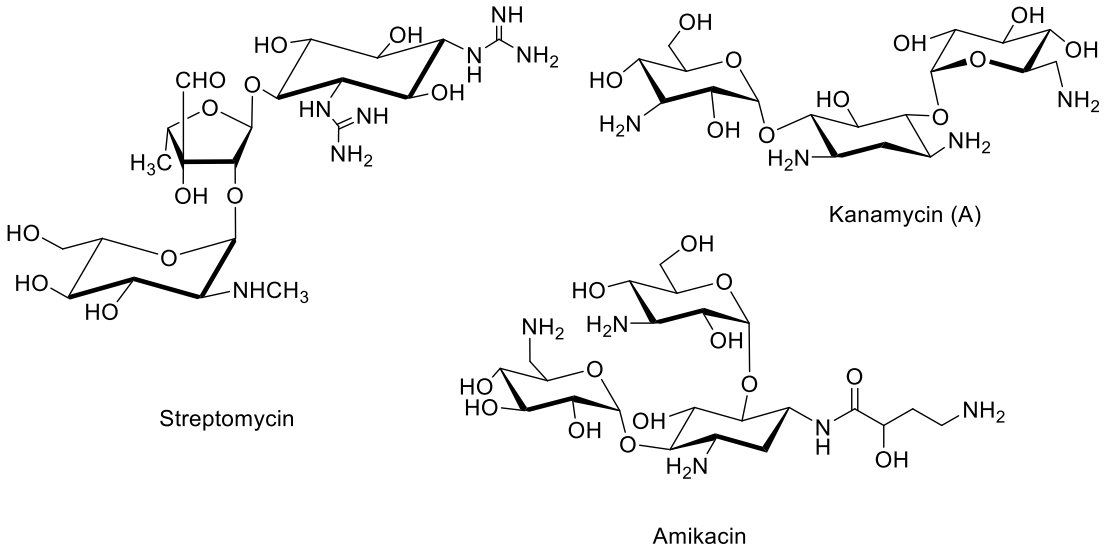


Figure 3. Aminoglycoside antiTB drugs: streptomycin (STR), kanamycin (A) and amikacin.

Aminoglycoside antiTB drugs act on *M.tb*. through the inhibition of its protein synthesis. These compounds penetrate mycobacterial cell wall by passive transport (via porin channels) to periplasmatic space, from which they are further transported across cytoplasmic membrane (by active transport) to cytosol, where they are accumulated. By binding to 30S ribosomal subunits, aminoglycosides prevent the formation of initiation complex and thus cause misreading of genetic code on mRNA. It subsequently leads to the formation of abnormal peptide chain and eventually erroneous protein. Despite the similarity in aminoglycoside structures and mode of action, these compounds have slight difference in binding to 30S ribosomal subunits.

[2] MDR-TB - Multidrug resistant TB caused by *M.tb.* bacilli resistant to at least INH and RIF.

Therefore cross-resistance between kanamycin and amikacin with STR is not frequent, that allows to use kanamycin and amikacin in current MDR-TB therapy.

Three years after STR discovery, this compound was widely administered to treat previously incurable patients with TB. Unfortunately, application of this drug in mono-therapy led to development of *M.tb*. resistant strains in short time; most patients treated in 1946 with STR developed STR-resistant strains. The results of a 5-year follow-up indicated that such mono-therapy had slight advantage compared with the ‘’bed rest’’ therapy. (28)

However, to this time *para*-aminosalicylic acid (PAS) (Fig.4) was synthesized by the Swedish chemist **Jörgen Lehmann** (1898 – 1989) and proved to be highly efficient. Its first application in 1944, just 3 months after its discovery, led to dramatic recovery of the patients. The drug proved to be better than STR as PAS was orally administrated and safer. In 1948, the clinical randomized trial at Britain's Medical Research Council, in which STR, PAS and combination STR/PAS were administered to TB patients, demonstrated that combined treatment with STR and PAS was superior to either drug alone and greatly reduced the appearance of STR resistance. (29) Unlike Waksman, Lehmann never received a Nobel Prize for his discoveries. Nowadays, PAS is used as a second-line anti-TB drug, although, with limitations due to its weak efficiency and low tolerability.

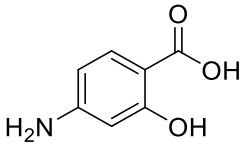


Figure 4. *para*-Aminosalicylic acid (PAS)

The mechanism of action of PAS is still under investigation. There are two suggested mechanisms. PAS possibly competes with *para*-aminobenzoic acid for dihydropteroate synthase, an essential enzyme in folate biosynthesis and thereby possess similar mode of action with sulfonamides. (30) On the other hand, it is supposed that PAS acts as an iron chelator and thus interferes with *M.tb*. iron intake. (31)

Since discovery of STR and PAS in 1940’s, the golden Era of antiTB drug development has started (Fig.5). (32)

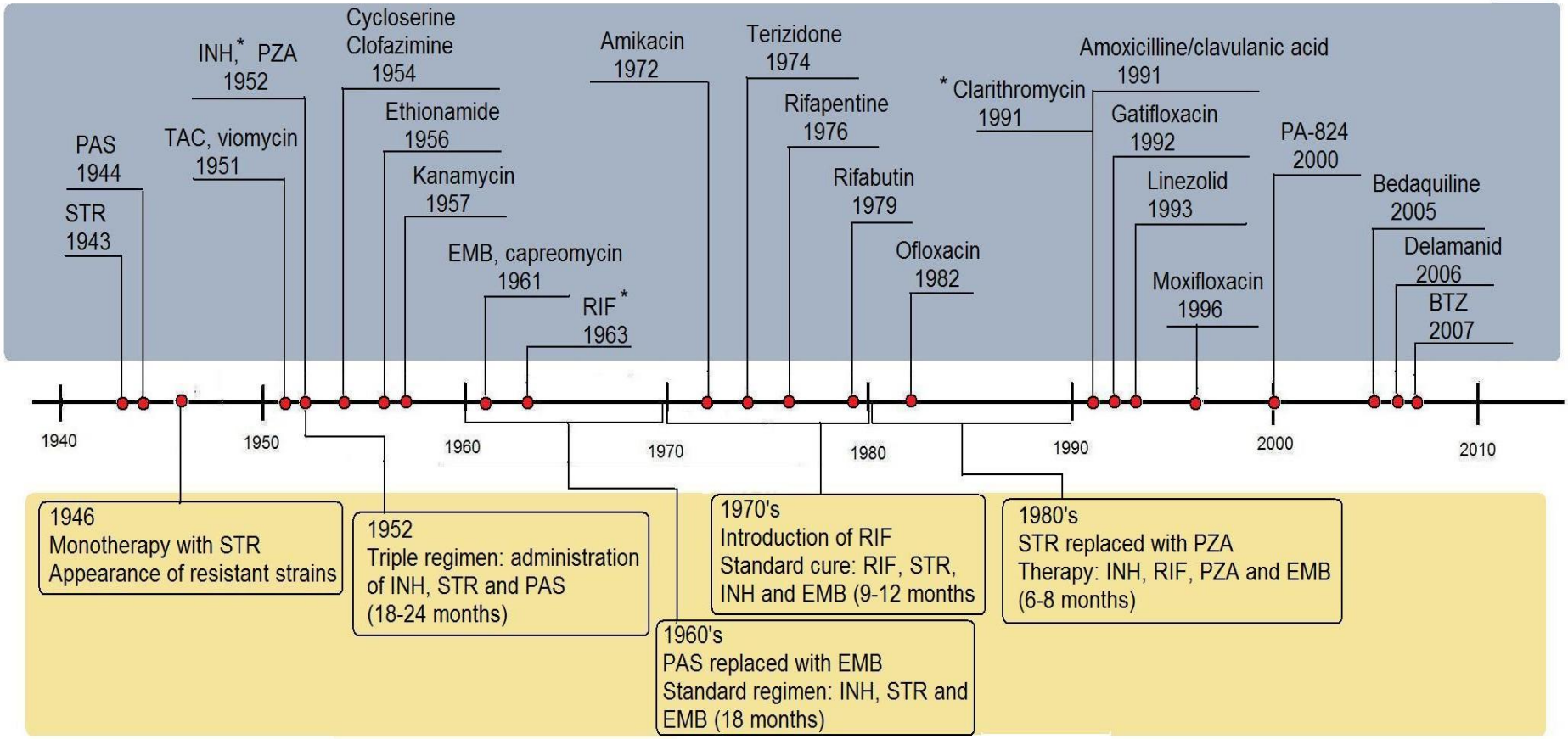


Figure 5. History of antiTB drug and treatment regimen development

Years of publication are presented; * - year of introduction to antiTB therapy

In 1951 Robert Behnisch, Fritz Mietzsch, Hans Schmidt, and **Gerhard Domagk** (1895 – 1964), the inventor of sulfonamide antibiotics, reported that thiacetazone (TAC) (Fig.6), thiosemicarbazones of the 4-acetaminobenzaldehyde, exhibited antiTB activity. Relationships between the structure of TAC and its antiTB activity was intensively studied and indirectly led to the discovery of isoniazid (INH) and pyrazinamide (PZA). Later, it was found that TAC was too toxic to be routine administrated, but in the end of 1940's clinical trials gave the erroneous results and since 1960's TAC began to be used in TB therapy. However it was employed mainly as alternative drug to expensive PAS and only in combination with INH or rifampicin (RIF) to prevent development of drug resistance. TAC had never been utilized itself as it has low efficiency against the mycobacteria. Moreover, nowadays there is no advantage of using TAC if ethambutol (EMB) is concluded in the regimen. TAC was recommended to be removed from the antiTB therapy due to adverse effects, especially in patients infected with human immunodeficiency virus (HIV). However, TAC is still being used in many countries in sub-Saharan Africa as it is cheap.

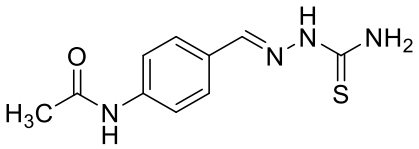


Figure 6. Thiacetazone (TAC).

Mechanism of action of TAC is still under investigation, but the cross-resistance of *M.tb*. strains to TAC, INH, ethionamide and prothioamide indicated the similar molecular target of these drugs. (31)

In 1912, **Hans Meyer** and **Josef Mally** synthesized INH (Fig.7), which later became the most successful and widely used antiTB drug and updated the TB therapy. The antiTB properties of INH were discovered only in 1951-1952 by Bayer, Hoffman la Roche and Squibb companies independently within the research directed to improvement of antiTB activity of TAC. In 1948, it was found that nicotinamide mildly inhibited the growth of *M.tb*. and therefore there were efforts to modify the structure of TAC through substitution of phenyl ring with pyridyl residue. However, INH, one of an intermediate, proved itself to be highly effective against *M.tb*. In 1952, INH was introduced in TB treatment and in the 1960's administration of STR, INH

and PAS for 18 months to 2 years became the standard treatment for pulmonary TB ("triple therapy"). Although such prolonged therapy was expensive and was difficult to adhere, this treatment effectively prevented acquired drug resistance.

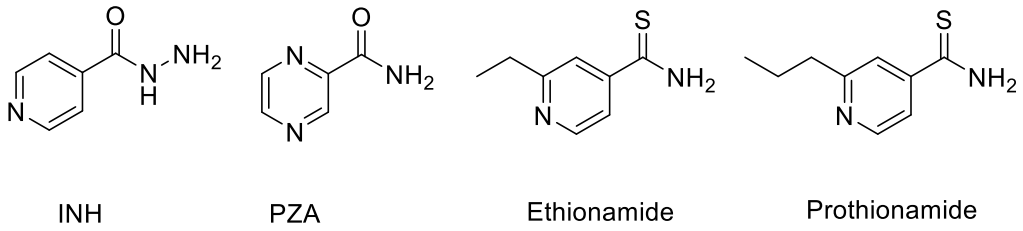


Figure 7. Isoniazid (INH), pyrazinamide (PZA), ethionamide and prothionamide

Mechanism of action of INH is inhibition of fatty acid biosynthesis. Fatty acids in *M.tb*. are represented by specific mycolic acids, the fatty acids with long chains, which are the major component of the mycobacterial cell wall. Mycolic acids as crucial part of the complex mycobacterial envelop (see more in section 4: Mycobacterial cell wall) are essential for the survival of *M.tb*. and protect against chemical injury, dehydration and provide low cell wall permeability to antibiotics. INH is a pro-drug and is activated by the catalase/peroxidase enzyme (katG); after penetration into *M.tb*. INH is oxidized by katG to isonicotinic acid, which gets accumulated in mycobacteria and interferes with nicotinic acid, which is as nicotinamide contained in NAD.[3] Thus activated by katG INH inhibits inhA, an NADH-dependent enoyl-acyl carrier protein reductase, which is involved in the mycolic acid biosynthesis.

The synthetic chemistry program, in which various analogs of nicotinamide were studied, led to the discovery of pyrazinamide (PZA) in 1952 (Fig.7) (Kaushner and coworkers of Lederle Laboratories), which was introduced into TB therapy in the early 1950's. In 1957, comparison of antiTB efficiency of four treatment regimens (INH/PZA; INH/STR; PAS/STR and INH/PAS) was performed and it was found that combination INH/PZA was the most effective and had the shortest time to sputum conversion[4]. (28) On the other hand, this regimen had the highest rates of toxicity.

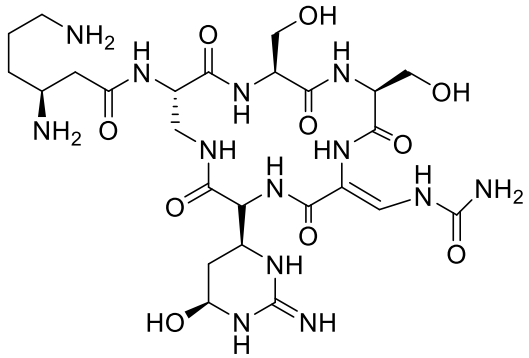
[3] Nicotinamide adenine dinucleotide (NADH) is necessary to synthesize adenosine triphosphate (ATP) via either glycolysis or via the mitochondrial respiratory chain

[4] Sputum culture conversion is a useful and appropriate interim indicator of treatment efficacy: conversion of sputum cultures from *M.tb.* positive to *M.tb*. negative is an important marker of treatment response and declining infectiousness

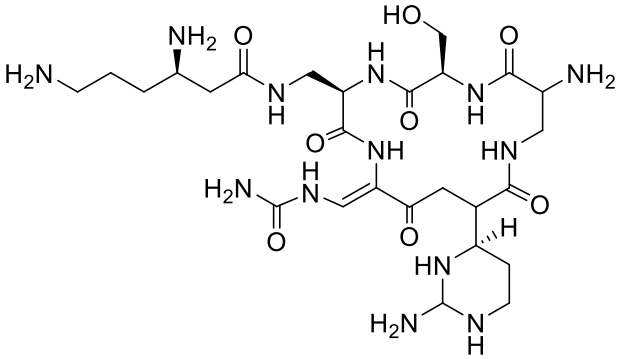
(33) Beside these results, PZA has been underestimated and was overshadowed by significantly higher efficiency of INH. However, subsequently the similar contribution of RIF and PZA in recovery of the patients treated by either STR/RIF or STR/PZA was demonstrated within the investigation of new antiTB therapeutic regimen (1972). In modern antiTB chemotherapy, PZA is used as part of a standard treatment regimen to treat drug-susceptible TB.

As in the case of PAS, mechanism of action of PZA is still not completely known. (34) It was defined that *in vitro* antiTB activity of PZA appears only under acidic pH. It is proposed that in the first step PZA is converted by bacterial pyrazinamidase/nicotinamidase (PZase) into pyrazinoic acid, and excreted by an efflux pump to pathogen's acidic environment. (35) Here, the pyrazinoate is protonated, reabsorbed and accumulated inside *M.tb*. Accumulation of pyrazinoic acid inside mycobacteria leads to disruption of bacterial membrane energetics, inhibition of membrane transport and thereby promotes the cellular death.

The exploration of nicotinamide activity and efforts on its improvement resulted in the discovery of two antiTB drugs - ethionamide and prothionamide (1956) (Fig.7). Currently these medicaments are utilized as second-line agents in the treatment of MDR and XDR-TB.[5] These antiTB agents are pro-drugs and are activated by different enzyme than INH (by ethA instead of katG), but the target of their action is similar (inhibition inhA). Therefore, cross-resistance between INH and ethionamide or prothionamide is expected, but not common.



viomycin



capreomycin

Figure 8. Polypeptide antituberculotics: viomycin and capreomycin.

[5] XDR-TD - extensively drug-resistant TB resistant to RIF, INH, plus any fluoroquinolone, and at least one of three injectable second-line drugs: amikacin, kanamycin, or capreomycin

In 1951, viomycin, one of the polypeptide antiTB drugs, which is produced by various *Streptomyces* species, was reported (Fig.8). (36) Viomycin as well as capreomycin, isolated from *Streptomyces capreolus* in 1961, belongs to the tuberactinomycin family[6] of antibiotics, which are among the most effective antibiotics against MDR-TB despite their toxicity and side effects. Polypeptide antiTB drugs have mechanism of action similar to aminoglycosides - they target the 30S subunit of ribosome and inhibit protein synthesis. However, polypeptides bind between 30S and 50S subunits. Therefore, cross-resistance between viomycin or capreomycin and kanamycin or amikacin is not usual.

Discovered in the early 1950's - 1952 (37) or 1954 (38) - the D-cycloserine (Fig.9) exhibited interesting properties. It inhibited *M.tb. in vitro*, but didn't exhibit antiTB activity *in vivo* either in guinea pigs or mice. On the other hand, it was effective in the TB therapy in humans. This difference was later explained by the presence of D-alanine (an antagonist to the antibiotic action of D-cycloserine) in the serum of guinea pigs and mice (humans do not have). D-Cycloserine can be isolated from *Streptomyces* species (*S. orchidaceus*, *S. garyphalus* and *S. lavendulae*) and nowadays is used as oral bacteriostatic second-line agent in the treatment regimens for MDR and XDR-TB. Its derivative, terizidone, (39) obtained by connection of two molecules of cycloserine through terephtalic dialdehyde, also belongs to second-line agents, although, its application is limited for TB cases when other medicaments can't be administrated.

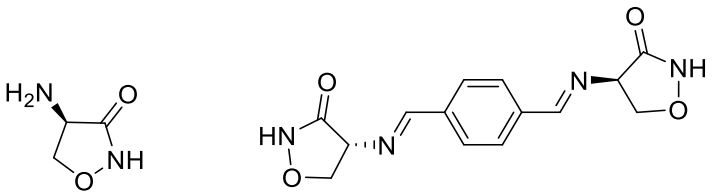


Figure 9. D-Cycloserine and terizidone

Due to structural similarity, D-cycloserine inhibits the D-alanine racemase and the D-alanine-D-alanine ligase, which are necessary for synthesis of correct UDP-muramyl-pentapeptide, the basic element of peptidoglycan. Peptidoglycan is the essential component of external mycobacterial envelope and inhibition of its biosynthesis leads to damage of bacterial cell wall.

[6] The tuberactinomycins are a group of cyclic peptide antibiotics, which are potent inhibitors of prokaryotic protein synthesis.

The further antiTB drug, ethambutol (EMB) was discovered in 1961 at Lederle Laboratories through extensive screening of library of compounds for antiTB activity. EMB proved to be more efficient than STR against susceptible strains and highly active against strain resistant to INH and STR (Fig.10). Owing bacteriostatic activity as PAS, EMB was more tolerable and therefore in 1960's PAS was replaced by EMB in standard "triple" treatment regimen. Moreover, EMB application led to reduction of the therapy duration to 18 months. (29) EMB continues to be used as part of a standard treatment regimen for TB cure till date. Similarly to other successful antiTB drugs EMB serves as lead structure, which scientists use to obtain new derivatives with improved properties. Such efforts led to the preparation of SQ109. This compound is more active than EMB both *in vitro* and *in vivo*, shows synergistic effects with many existing antiTB drugs and therefore is already in phase II of clinical development. (40)

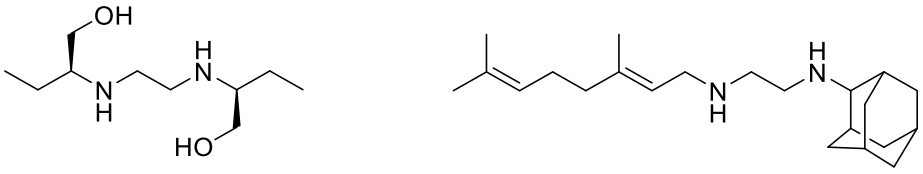


Figure 10. Ethambutol (EMB) and SQ-109.

EMB affects *M.tb*. through intervention into biosynthesis of its cell wall arabinogalactan by inhibition of mycobacterial arabinosyl transferase. (30) While the exact mechanism of action of EMB is still under investigation, its effect on cell wall plays significant role when EMB is administrated with other antiTB drugs. EMB improves the efficiency of other antibiotics (for example, clarithromycin, which is usually ineffective against *M.tb.*). Interestingly, SQ109 developed as EMB analog showed different mode of action. SQ109 targets MmpL3, a putative membrane protein transporter of trehalose monomycolate, precursor of threhalose dimycolate and cell wall mycolates. (40)

In the end of 1950's – early 1960's rifampicin (RIF), the most important antiTB drug for TB treatment up to now was discovered and later it improved the TB therapy significantly (Fig.11). In 1957, the broad-spectrum agents, rifamycins, were isolated from *Streptomyces mediterranei* in the Lepetit Pharmaceuticals research lab by scientific group of **Piero Sensi** (1920 – 2013) and **Maria Teresa Timbal** (1925 –

1969). The first substances were useless as therapeutic agents, because they had poor efficiency, bioavailability, low solubility and had short half-life. However, these properties were improved by the preparation of semisynthetic products. In 1959, the highly active and more tolerable compound, RIF, was obtained. Later, the semisynthetic analogs of RIF, rifapentine (1976) and rifabutin (1979), were prepared and introduced to therapeutic treatment. Rifapentine was approved for TB therapy in 1998. This derivate possesses higher activity, better pharmacokinetic profile and more than 5-fold longer halt-life than RIF and has the potential to shorten the duration of current TB therapy of latent and drug-susceptible TB. Rifabutin also has longer half-life than RIF and larger distribution volume. Moreover, this analog is recommended for HIV-co-infected patients as it affects CYP450 less than RIF and rifapentine. (40)

All derivatives of this class possess the unique mechanism of action. Rifamycins bind to *β*-subunit of bacterial DNA-dependent RNA-polymerase, prevent DNA transcription and thereby inhibit synthesis of bacterial mRNA, which has the crucial significance in the protein synthesis. Unfortunately, resistance to rifamycins occurs frequently.

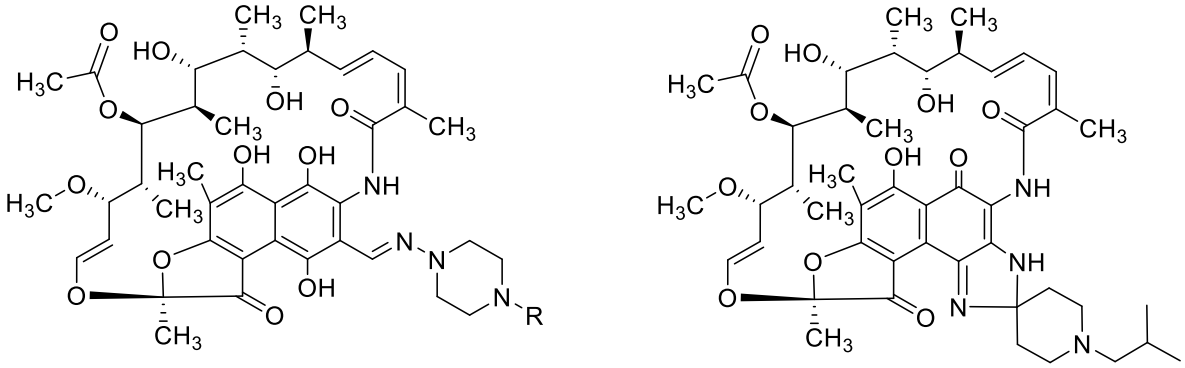


Figure 11. RIF, rifapentine and rifabutin.

Nevertheless, introduction of RIF in 1963 to therapeutic application resulted in the development of new regimens and allowed to shorten treatment duration to 6 months. First studies utilized the combination of STR, INH, RIF and EMB to show that such combined therapy leads to predictable cures in >95% in just 8-9 months. (28) After re-discovery PZA for TB therapy in early 1970's, the new course of TB

treatment was accepted. One of these regimens involved administration of STR, INH, RIF, and PZA combination for 2 months, and combination of INH and RIF for additional 4 months. This regimen proved to be highly effective and less toxic. Replacement of STR for EMB led to the similar regimen with EMB, INH, RIF and PZA for 2 months with an additional treatment for 4 months with RIF and INH. Further studies continued in many centers of east Africa, Zambia and Hong Kong demonstrated that INH, RIF and PZA were the basic elements of non-complicated TB cure and became the foundation of Directly Observed Treatment - Short course (DOTS). As RIF and PZA are expensive medicaments, alternative therapy was developed as administration of STR, INH, RIF and PZA for 2 months, followed by 6 months of TAC and INH. (41) Initially, this regimen took 8 months, but subsequently this therapy was reduced to 6 months. However, this drug combination can't be applied in patients with HIV as TAC worsens the adverse effect and promotes the occurrence of Stevens-Johnson syndrome.[7]

After the discovery of RIF more than 20 years had to pass for the development of new antiTB drugs. The antiTB activity of the fluoroquinolones (Fig.12) has been under investigation since the 1980's. Fluoroquinolones, antibiotics with a broad spectrum of antimicrobial activity, well oral bioavailability, with good tissue penetration and generally well tolerated, also proved to be highly effective against *M.tb*. Therefore fluoroquinolones of second generation – levofloxacin,[8] ofloxacin and ciprofloxacin are used in the modern treatment of MDR-TB. Fluoroquinolones exhibit slightly lower efficiency than RIF and INH, but resistance to them is rare.

Resently, investigations of fluoroquinolone antiTB drugs continue. Derivatives of third generation (moxifloxacin and gatifloxacin) exhibit higher activity against *M.tb*., are *in vitro* and *in vivo* more potent and are under clinical development. (42) These derivatives are studied for their potential use as first-line antituberculotics. Furthermore, new fluoroquinolone derivative DC-159a is in preclinical development and showed the highest activity among all fluoroquinolones against drug-susceptible and even quinolone-resistant MDR-TB strains. Nowadays, this class of antibiotics proved to be indispensable for the treatment of MRD-TB, for their mechanism of action. The target of fluoroquinolones is ATP-dependent DNA gyrase (topoisomerase

[7] Stevens-Johnson syndrome is a toxic epidermal necrolysis; an immune-mediated hypersensitivity of skin and mucous membranes; rare, potentially deadly disease that usually results from a drug reaction.
[8] Levofloxacin is the S- isomer, the active isomer, of the racemic mixture ofloxacin.

II), which catalyzes the supercoiling of DNA and thus provides DNA replication, transcription, and reparation.

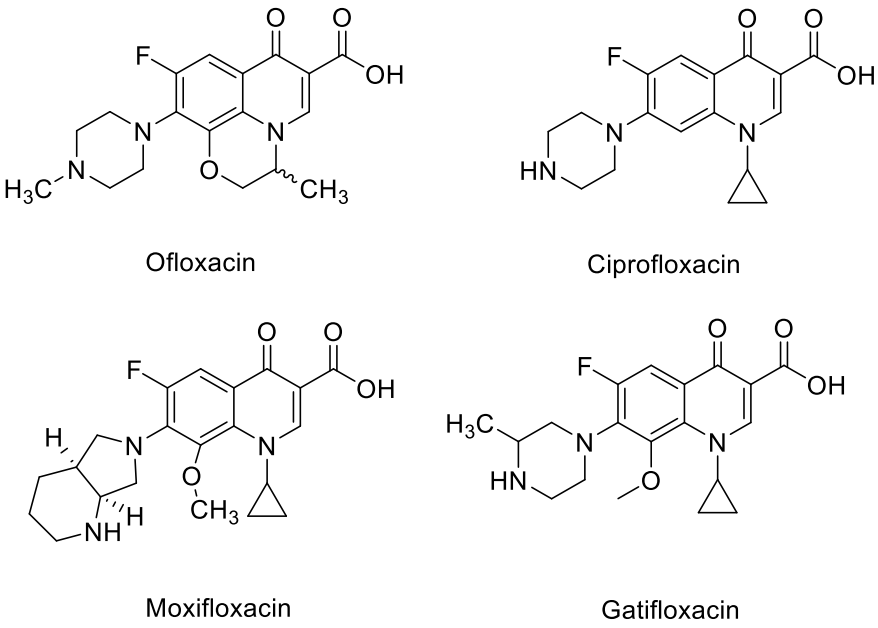


Figure 12. Fluoroquinolones.

Widely used approach for the development of new, highly efficient antiTB compounds is structure optimization or repurposition of known drugs. By this strategy, fluoroquinolones, rifamycins, *β*-lactams, oxazolidinones, riminophenazines and other substances were obtained and nowadays are being used in TB cure or they are in different stages of clinical trials.

The history of *β*-lactam antibiotics started with the discovery of penicillin made by Scottish scientist and Nobel laureate **Alexander Fleming** (1881 – 1955) in 1928. *β*-Lactams (Fig.13) are well-known antibiotics with high efficiency against Gram-positive and Gram-negative bacteria, but they were unsuccessful in TB therapy due to rapid enzymatic inactivation by *β*-lactamase (BlaC). Amoxicillin, semisynthetic derivative of 6-aminopenicillanic acid, was developed at Beecham group in the 1960's and became available for the treatment of various bacterial infections in 1972. Its combination with clavulanic acid (or clavulanate) (inhibitor of BlaC) was approved for use in the United States (U.S.) in 1984; and in 1991 it was suggested that amoxicillin/clavulanate might also be effective in the treatment of drug-resistant TB strains. (43; 44) Nowadays, combined application of amoxicillin with clavulanic acid is employed in the therapy of MDR-TB, only in those cases where other antiTB drugs can't be applied. Besides of amoxicillin, combination of imipenem, an intravenous *β*-

lactam antibiotic belonged to the carbapenem subgroup, with equal quantities of cilastatin, inhibitor of the human enzyme dehydropeptidase, which prevents antibiotic decay in the kidneys, is administrated in the case of MDR-TB. Additionally, meropenem, another derivative of carbapenem subgroup, in combination with clavulanic acid exhibits high efficiency and is considered as second-line agent. (45; 46)

The mechanism of action of *β*-lactams proceeds via inhibition of the transpeptidase that cross-links the peptide side chain making peptidoglycan, the backbone of the mycobacterial cell envelope.

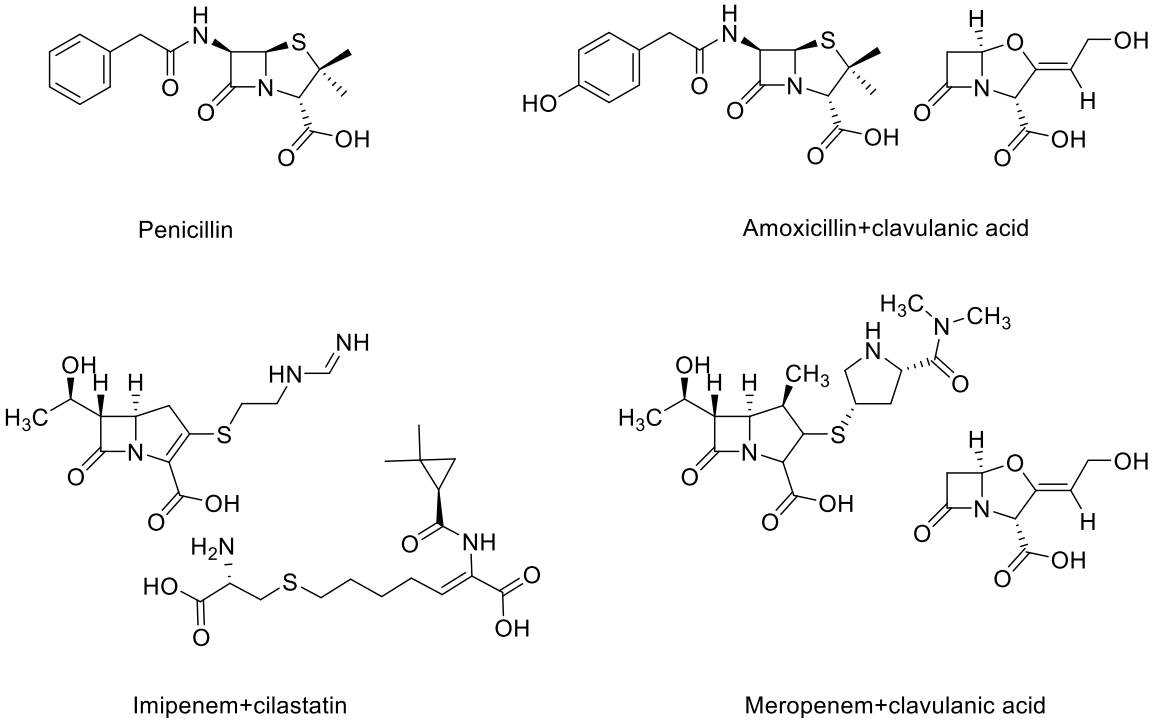


Figure 13. *β*-Lactam antibiotics.

Oxazolidinones is quite a new class of antituberculotics. Linezolid (Fig.14) was discovered in 1993 by **Steven J. Brickner** at Upjohn and was introduced for the treatment of serious Gram-positive infections, resistant to known antibiotics, in 2000. Since 2006, the World Health Organization (WHO) has considered linezolid for the treatment of MDR and XDR-TB despite the fact that its efficacy is unclear. (47) This agent exhibits high *in vitro* bacteriostatic activity against drug-susceptible and MDR-TB, although, possessed significant adverse effects. Therefore, linezolid is still under clinical trials; its administration in MDR-TB and XDR-TB cases is limited and

forbidden for HIV co-infected patients. Further investigations of oxazolidinones led to the development of two new derivatives, sutezolid and AZD-5847, which are also under clinical trials. New derivatives proved to be more effective and tolerable. (48) Linezolid acts as inhibitor of protein synthesis (as well as aminoglycosides and polypeptides), through a binding to 50S ribosomal subunit.

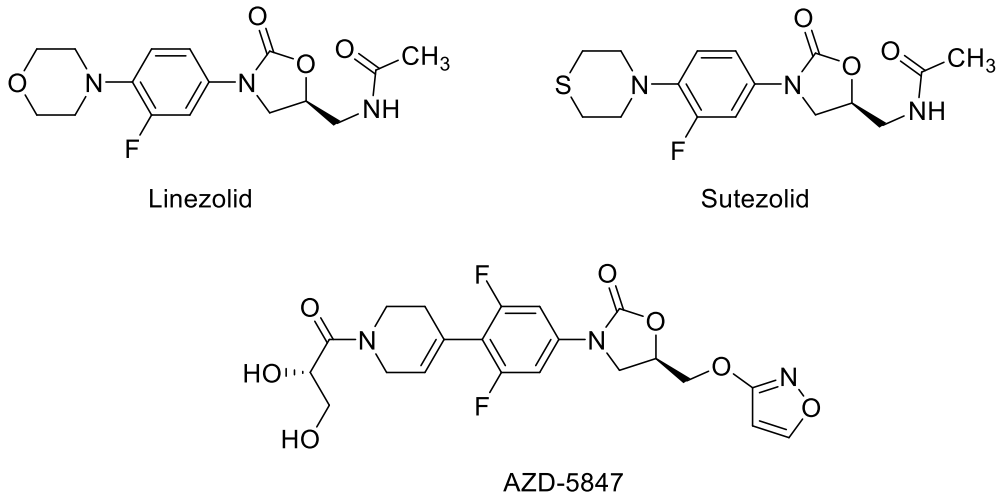
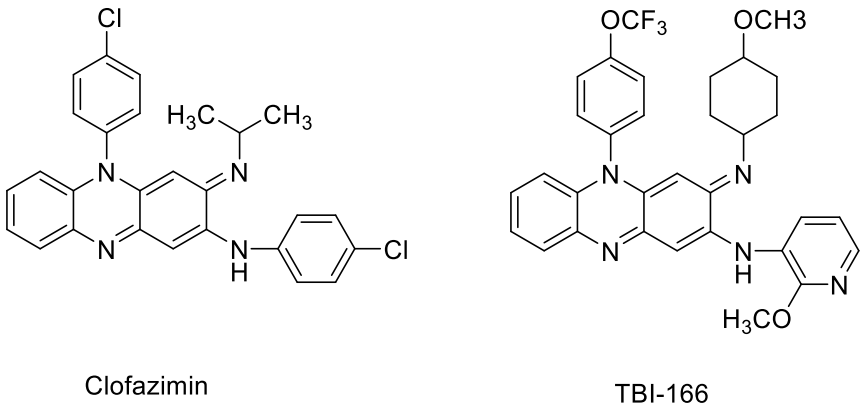


Figure 14. Oxazolidinones: linezolid, sutezolid, AZD-5847.

Other currently used medicament is clofazimine (Fig.15), a riminophenazine derivative, which was initially developed as medicament for the treatment of *M. Leprae* in 1960's, but later was repurposed for TB therapy. This compound showed high activity against dormant bacilli and MDR-TB, but accumulated in the tissues in excess and caused adverse effects. Therefore clofazimine is considered as second-line agent and is recommended by the WHO to treat MDR-TB, but as an agent for non-routine use. Current studies confirm its important role in the treatment regimen of XDR-TB, containing capreomcin, moxifloxacin, PAS, clarithromycin and EMB. (49) Optimization of clofazimine structure led to the derivative with safer profile and retained antiTB activity, TBI-166, which is in preclinical development.

Exact mechanism of action of clofazimine is still not defined. Its mode of action is difficult to study due to rare appearance of resistance. Postulated mechanisms of action include: inhibition of Ndn-2, and NADH-dehydrogenase in electron transport chain and energy production (36); bactericidal activity via reactive oxygen species (ROS) production following the reduction of clofazimine by mycobacterial NADH

quinone-oxidoreductase (50); intercalation with bacterial DNA and increasing levels of cellular phospholipase A2 (51); generalized membrane-disrupting effects. (31)



Clofazimin TBI-166

Figure 15. Riminophenazine: clofazimine and TBI-166.

Clarithromycin belongs to the group of antiTB drugs, which are being used, but are not routinely recommended for the treatment of MDR-TB (Fig.16). This representative of macrolide antibiotics was synthesized as analog of erythromycin by researchers at the Japanese company Taisho Pharmaceutical in the 1970's, and approved by the U.S. Food and Drug Administration (FDA) in 1991 for antimicrobial therapy. Clarithromycin alone displayed no antiTB activity, but showed *in vitro* synergistic activity in combination with other standard anti-TB drugs in MDR and XDR-TB treatment; such combinations make resistant TB strains susceptible to drugs. (52; 53)

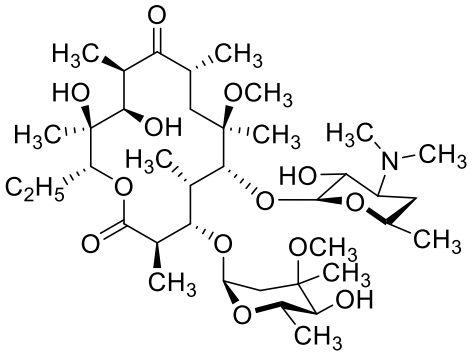


Figure 16. Macrolide clarithromycin

Clarithromycin inhibits the bacterial growth through inhibition of the proteins synthesis. This drug binds to the subunit 50S of the bacterial ribosome that is involved in the translation of mRNA and thus leads to the formation of incorrect peptides and proteins.

3.2. Current antiTB therapy and new requirements to antiTB candidates

Currently administrated antiTB drugs are classified into five groups.

First-line anti-TB drugs:

Group 1 oral drugs: isoniazid, rifampicin, pyrazinamide, ethambutol

Second-line anti-TB drugs:

Group 2: injectable aminoglycosides: streptomycin, kanamycin, amikacin; injectable polypeptides: capreomycin, viomycin.

Group 3: oral and injectable fluoroquinolones: ciprofloxacin, levofloxacin, ofloxacin, moxifloxacin, gatifloxacin.

Group 4: oral drugs: para-aminosalicylic acid, cycloserine, terizidone, ethionamide, prothionamide, thioacetazone, linezolid.

Third-line anti-TB drugs:

Group 5: linezolid, amoxicillin/clavulanic acid, imipenem/cilastatin, clarithromycin, clofazimine.

First-line drugs are recommended in a four-drug combination for the treatment of drug-susceptible TB within the Directly Observed Treatment - Short course (DOTS) since early 1990's, which involves application of all four drugs in the first two months followed by administration of RIF and INH during additional 4 months. It is suggested that initial period of therapy leads to the destruction of *M.tb*. in all growth stages, while further drugs application leads to the elimination of residual dormant bacilli and prevents development of MDR-TB strains. Rifapentine and rifabutin, conditionally, belong to this group due to their usage in place of RIF, e.g. in the treatment of HIV-positive TB patients.

Second-line agents are not used in the DOTS, because of their lower efficiency than the first-line drugs (e.g., PAS), toxic adverse effects (e.g., D-cycloserine) and unavailability in many developing countries (e.g., fluoroquinolones). The second line drugs are recommended to be used only to treat TB that is resistant to first-line drugs.

Third-line anti-TB drugs are substances with unclear efficacy, but showed some efficiency against *M.tb*. and thus are recommended for the treatment of MDR-TB, only if there is no alternative cure.

Unfortunately, second-line and third-line antiTB drugs are more expensive, toxic, and less effective than the first-line drugs. Treatment duration of MDR and XDR-TB is 18-24 months or longer.

Despite the significant treatment success rates and progress in reduction of mortality rate since 1990, TB remains enormously widespread and dangerous infectious disease. According to WHO, only in 2012, there were estimated 8.6 million cases of TB and 1.3 million deaths. (54) Development of MDR-, XDR- and totally drug resistant (TDR) TB strains remains the main problem of current TB cure. Interruption of antiTB therapy due to relative toxicity of antiTB drugs, poor adherence to the treatment regimen or drug interaction of antiTB agents with antiretroviral medicaments in HIV-positive patients cause development of drug-resistant strains. In 2012, 3.6% of new TB cases and 20% of previously treated cases of TB were MDR-TB. Almost 10% of them were represented by XDR-TB. Only 48% of patients with MDR-TB were successfully treated, while therapy of patients with the new cases of susceptible TB had 90% cure rate. Moreover, combination of TB with HIV leads to fast progress of both diseases and often is fatal.

Abovementioned problems underline the urgent need of further investigation of highly efficient antiTB drugs. New antiTB candidates have to fulfill the following requirements:

- activity against both replicating and non-replicating bacteria;
- activity against drug-susceptible and drug-resistant *M.tb*. strains;
- a novel mechanism of action allowing the treatment of MDR and XDR-TB;
- compatible or even synergistic effect with other antiTB agents;
- lack of drug-drug interactions with antiretroviral medicaments;
- reduction of antiTB treatment length and frequency of drug intake;
- minimization of side-effects and maintaining good tolerability.

Nowadays, the investigations of novel compounds fulfilling these requirements are being continued intensively (40; 55; 56). There are efforts directed to the optimization of properties of known, highly efficient substances or search for the compounds completely different from currently used antiTB drugs.

3.3. Highly effective antiTB candidates being in preclinical or clinical development

For the last 15 years, numerous compounds with high efficiency against susceptible TB, latent TB as well as against MDR and XDR-TB were obtained (57; 58), while only limited amount reached the clinical trials or approved for the TB treatment. Among such notable compounds are bedaquiline, nitroimidazole and benzothiazinone derivatives.

Bedaquiline (R207910, TMC207 or Sirturo) (Fig.17) is a diarylquinoline derivative, which was first reported in 2004 and currently is in the phase II of clinical development. However, in December 2012, FDA approved its use as an adjunct to existing regimens for the MDR-TB. (59) In December 2013, the European Medicines Agency's Committee for Medicinal Products for Human Use (CHMP) also recommended bedaquiline for its use as a part of the combination therapy for pulmonary MDR-TB in adult patients when there is no alternative effective regimen because of resistance to used antiTB drugs or their bad tolerability. (60)

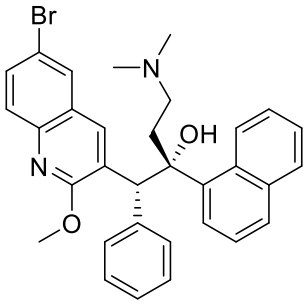


Figure 17. Bedaquiline (R207910, TMC207 or Sirturo)

Bedaquiline proved to be highly active both *in vitro* and *in vivo* active against drug-susceptible, MDR and XDR *M.tb*. strains as well as against both replicating and dormant *M.tb*. It possesses good oral bioavailability, high penetration into tissues such as lung and spleen, high tolerance and has no cross-resistance to current first-line drugs. (61; 62) Excellent activity of bedaquiline is explained by its unique mechanism of action, which completely differs from those that have been currently used antiTB drugs. It acts as an inhibitor of membrane-bound adenosine triphosphate (ATP) synthase, the proton pump of *M.tb*. and the essential enzyme in

the generation of energy for *M.tb*. Bedaquiline binds to subunit *c* of the proton pump of ATP synthase that leads to the conformational changes on ATP synthase, which disrupts normal proton flow; thus results in energy starvation of *M.tb*. and an imbalance in pH homeostasis. As human mitochondrial ATP synthase is 20,000-fold less sensitive to bedaquiline, it provides significant selectivity of its action on *M.tb.* (40) Furthermore, clinical trials demonstrated that bedaquiline is as effective as a combination of RIF, INH and PZA and more effective than RIF alone in mouse model. Combination of bedaquiline with RIF, INH and PZA results in negative cultures in mouse models after 2 months. (63)

Next important class of compounds with a high antiTB efficiency is nitroimidazoles. The parent compound of modern nitroimidazoles was azomycin, natural product isolated from *Streptomycete* in the 1950's. The first representative of its class with antiTB activity was metronidazole (Fig.18), which was synthesized by Rhone-Poulenc laboratories in the 1950's and in 1972, it was introduced for the treatment of Gram-positive and Gram-negative anaerobic bacteria and protozoa. In late 1980's metronidazole and some other nitro-containing compounds showed to be active against *M.tb*. (48) Although metronidazole displayed no activity in mice or guinea pig, its study is being continued. As this compound is effective only under hypoxic conditions, it is suggested that it can prevent reactivation of latent *M.tb*. and kill an anaerobic sub-population of *M.tb*. within patients with MDR-TB. (64)

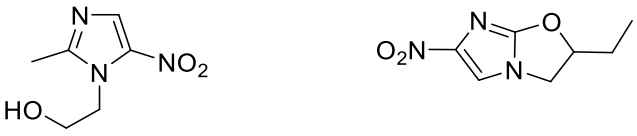


Figure 18. Nitroimidazole antiTB drugs: metronidazole and CGI-17341

Another derivative, CGI-17341, exhibited high antiTB activity against susceptible and drug-resistant strains of *M.tb.* and was active under both aerobic and anaerobic condition, but was not further developed because of its mutagenic properties. (65; 66)

Nitroimidazo-oxazine derivative, PA-824 (Fig.19), was announced as new drug candidate for the treatment of TB in 2000 (67) and since 2002 is being developed by the TB Alliance. Delamanid (OPC-67683), the nitroimidazo-oxazole derivative, was reported in 2005 (68); since 2006 is being investigated by Otsuka Pharmaceutical

Company. PA-824 (69; 70; 71) and more potent delamanid (72) proved to be highly *in vitro* and *in vivo* effective against drug-susceptible and drug-resistant TB forms and also proved to be active against both replicating and non-replicating bacteria.

Outstanding efficiency of these nitroimidazole derivatives is connected with its exceptional mode of action. It is proposed that these compounds are pro-drugs and are activated via bioreduction of nitro-group by deazaflavin-dependent enzymes. Formed metabolites (des-nitroimidazole) act as the inhibitors of cell wall biosynthesis (under aerobic condition) or as respiratory poison through nitric oxide release (under anaerobic condition).

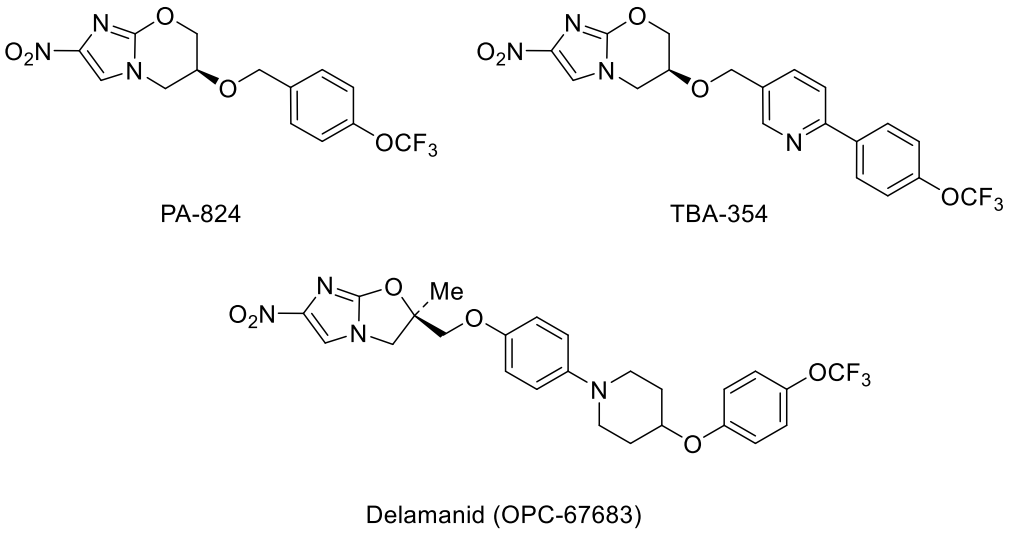
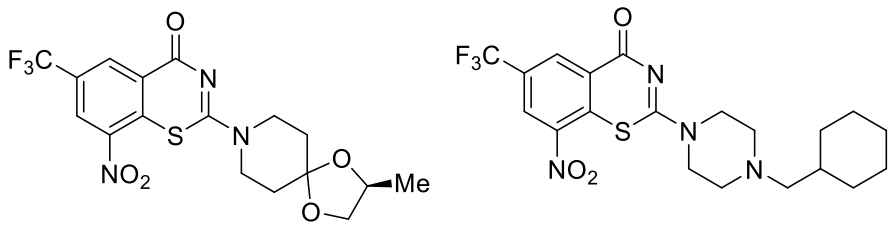


Figure 19. New nitroimidazoles: PA-824, delamanid (OPC-67683) and TBI-354

Both these nitroimidazoles have safe profiles, good bioavailability and tolerability. Moreover, delamanid is considered to be used for TB treatment of HIV-positive patients as it does not affect the activity of liver microsome enzymes (72). In November 2013, delamanid was approved by the CHMP for conditional use for MDR-TB therapy. PA-824 and delamanid are in final phases of clinical development, while TBA-354 (73) is in preclinical phase.

The new promising class of antiTB compounds, benzothiazinones, appeared in the middle 2000's (patent 2007). (74) The first derivative of this group, BTZ038, represented the racemic mixture of the two enantiomers BTZ043 (*S*) and BTZ044 (*R*), which exhibited the similar *in vitro* antiTB activity. (75) Nevertheless, further investigation of antiTB efficiency was performed with BTZ043 (Fig.20). This

compound proved to be highly effective in nanomolar concentration against both susceptible and drug-resistant (MDR and XDR-TB) *M.tb*. strains. Structure optimization of BTZ043 led to piperazine-containing benzothiazinone PBTZ-169 (76) with improved pharmacological properties and better affinity to cell target. This analog has three fold higher antiTB activity and ten fold lower toxicity than BTZ043. Moreover, PBTZ-169 possesses synergistic activity with bedaquiline and good tolerability with other antiTB drugs or drug candidates. Its combination with bedaquiline and PZA demonstrated better efficiency in mouse models of chronic TB than combination of INH, RIF and PZA. (76)



BTZ043 PBTZ-169

Figure 20. Benzothiazinones: BTZ043 and PBTZ169.

As in the case of bedaquiline and nitroimidazoles, phenomenal activity of benzothiazinones is explained by its new mechanism of action. These compounds similar to nitroimidazoles undergo the bioreduction of nitro group, but their active nitroso metabolites act as the inhibitors of decaprenyl-phosphoribose epimerase (DprE1), an essential enzyme in arabinan biosynthesis, which provides epimerization of decaprenylphosphoryl-*β*-D-ribose to decaprenylphosphoryl-*β*-D-arabinose, the only arabinose source for cell wall biosynthesis. Formed nitroso-metabolite reacts with Cys 287 of DprE1 irreversibly.

A few more DprE1 inhibitors currently known are: variously *N*-substituted 3,5-dinitrobenzamides, (77; 78) nitro-substituted quinoxaline VI-9376 (79) and nitro-substituted triazole 377790. (80)

Above mentioned compounds, which reached clinical or preclinical trials, are shown on figure 21. (47)

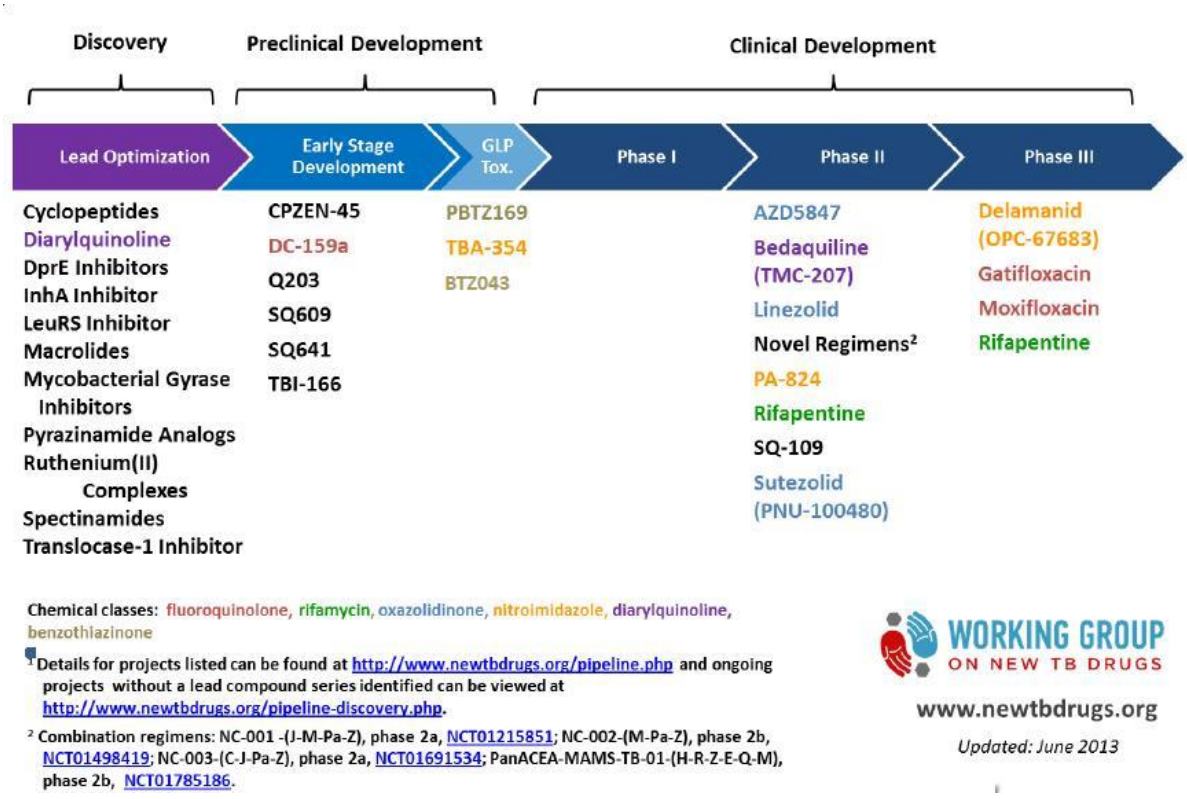


Figure 21. The development pipeline of new TB drugs, July 2013

Investigation of new highly efficient compounds against *M.tb*. is extensively being continued. But, despite these efforts, further research and drug development is arduous without the knowledge about the structure of mycobacterial cell wall, which is responsible for unique properties of *M.tb*. and without the knowledge about substances involving in its main biosynthetic pathways. (81; 82)

4. **Mycobacterial cell wall** (83; 84; 85; 86)

Mycobacterial cell wall (schematic structure - Fig.22) possesses an unusual, highly protective structure and is essential for *M.tb*. growth and survival in the infected host. These properties are provided by the large amounts of lipid substances in cell wall of *M.tb.*, making it hydrophobic, approximately 1000-fold less permeable for hydrophilic molecules than the cell wall of *Escherichia coli.* (61) Classified as acid-fast mycobacteria,[9] the virulence and slow growth of *M.tb*. have been also attributed to its unique cell wall structure.

The mycobacterial cell envelope contains three covalently linked macromolecules: peptidoglycan, arabinogalactan and mycolic acids forming mycolyl-arabinogalactanpeptidoglycan complex (mAGP); additionally there are free glycolipids.

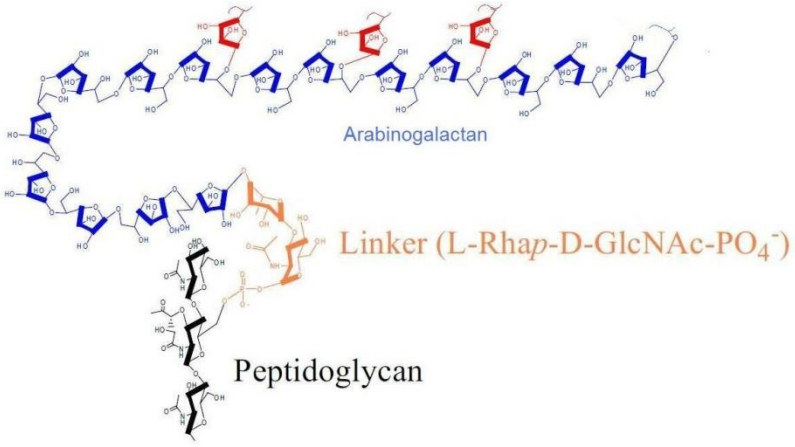


Figure 23. Attachment of arabinogalactan to peptidoglycan (87)
©2008 by American Society for Biochemistry and Molecular Biology

Peptidoglycan (PG), the backbone of the mycobacterial cell envelope, is located beyond the plasmatic membrane and is formed by alternating units of repeating *N*-acetyl-*β*-D-glucosaminyl-(1→4)-*N*-glycolylmuramic acid. The PG is cross-linked via an L-alanyl-D-*iso*glutaminyl-*meso*diaminopimelyl-D-alanine tetrapeptide attached to the muranic acid residues (with the diaminopimelic acid being further amidated). The

[9] Acid-fast bacteria is a bacteria, which has the special physical properties causing its resistant to decolorization by acids during staining procedures. Acid-fast organisms are difficult to characterize using standard microbiological techniques; the most common staining technique used to identify acid-fast bacteria is the Ziehl-Neelsen stain.

C6 of some of the muramic acid residues are bonded via phosphodiester bonds to C1 of *α*-D-*N*-acetylglucosamines, which in turn is (1→4) linked to *α*-L-Rhamnose (*α*-L-Rha*p*) residue. Forming bridge L-Rha*p*(13)-D-GlcNAc-1→P provides connection of PG with galactan of arabinogalactan (AG) (Fig.23).

AG is a polymer formed by covalently coupled D-galactofuranoses and D-arabinofuranoses, the polysaccharides, which are extremely rare in nature. D-galactofuranose polysaccharide is composed of approximately 30 *β*-D-galactofuranose (Gal*f*) units, which are linked through either C5 or C6 linearly: [*β*-D-Gal*f*(1→5)-*β*-D-Gal*f*-(1→6)]$_n$. The reducing residue of the galactan is linked to the PG through a phosphoryl-*N*-acetylglucosaminosyl-rhamnosyl linkage. The branched arabinan chains containing *α*-D-arabinofuranosyl (Ara*f*) are attached through the reducing end to C5 of the *β*-D-galactofuranose units. The arabinan domain is arranged with different *α*(1→5), *α*(1→3) and *β*(1→2) Ara*f* linkages.

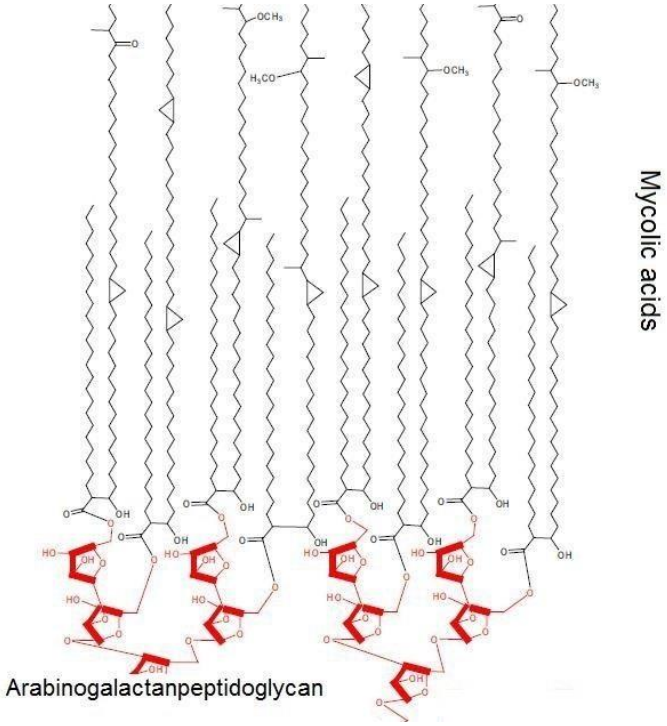


Figure 24. Attachment of mycolic acids to arabinogalactan (87)

©2008 by American Society for Biochemistry and Molecular Biology

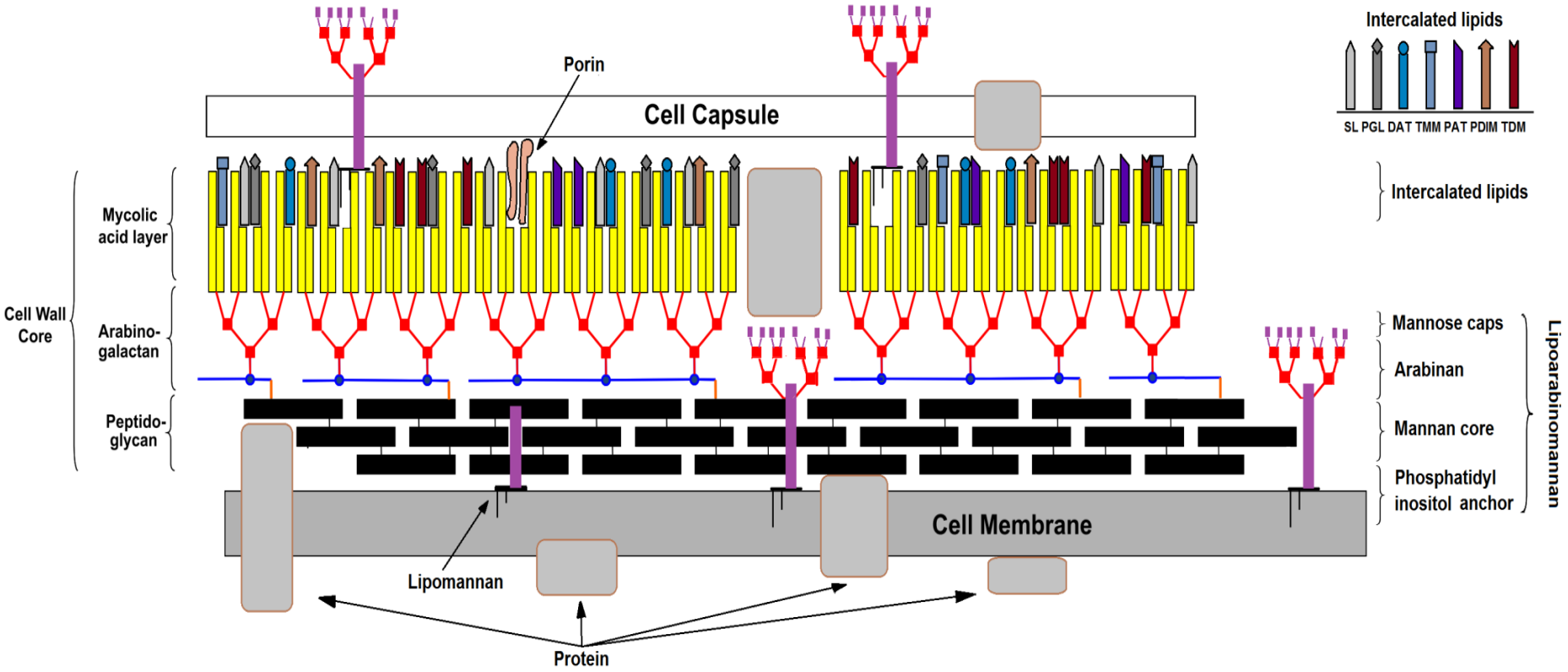


Figure 22. Schematic representation of the mycobacterial cell wall.

Intercalated lipids: **SL** – sulpholipids; **TMM** - trehalose monomycolate; **TDM** – dimycolyltrehalose.; **DAT** – diacyl trehalose; **PAT** - penta-acyltrehaloses; **PGL** - phenolic glycolipids; **PDIM** - mycocerosate-based phthiocerol dimycocerosate.

The mycolic acids, 70-90 carbon atoms branched *α*-alkyl-β-hydroxy fatty acids, which consist of a saturated 20-26 carbon *α*-branch and a meromycolic acid moiety (a chain of up to 60 carbon atoms), are presented as tetramycolylpentaarabino-furanosyl clusters on the arabinan of mycolylarabinogalactan (mAG) (Fig.24).

Two types of mycolic acids are known: with oxygenated functional group such as ethoxy, methoxy, keto groups (methoxymycolates or ketomycolates) and without such groups (*α*-mycolates contain only cyclopropane rings as functional groups). The *α*-mycolates are the most abundant type found in *M.tb*. The mycolates esterified to the arabinan chain of AG form the inner cover of the mycobacterial outer membrane (MOM). Mycolylarabinogalactan peptidoglycan complex (mAGP) is the cell wall core (Fig.25).

Free glycolipids, important components of the mycobacterial cell wall, are present in *M.tb*. in high quantity. They are intercalated into the mAGP skeleton by interaction with the mycolyl moiety and form a pseudo bilayer that works as an additional hydrophobic barrier. Amongst these wall-associated lipids, dimycolyltrehalose (TDM), trehalose monomycolate (TMM), diacyl- (DAT) and penta-acyltrehalose (PAT), sulfolipids (SLs), phthiocerol dimycocerosate (PDIM), phenolic glycolipids (PGLs) can be found (Fig.22).

The lipoarabinomannan (LAM), is a glycolipid containing Ara*f* and mannopyranosyl residues. It passes through the MOM and the mAGP and is anchored in the cytoplasmic membrane through a phosphatidyl-*myo*-inositol diacylglycerol anchor. At the nonreducing end of LAM is a hexasaccharide moiety that lacks any mycolic acid elaboration. Mannose-capped lipoarabinomannam, the variant of LAM, as well as phosphatidylinositol mannosides (PIMs) and lipomannan (LP), which are also anchored into the cell membrane, appear to be involved in the host immune response.

The lipids and glycolipids on the outer surface of the mycobacterial cell envelope are responsible for the bacilli properties such as a strong resistance to the degradation by host enzymes, impermeability to toxic macromolecules, and inactivation of reactive oxygen and nitrogen derivatives.

As mycobacterial cell wall defines the unique survival properties of *M.tb.*, the compounds affecting its biosynthesis are successful antiTB. Ethionamide, INH, PZA prothionamide, TAC, PA-824 and delamanid act as inhibitors of fatty acids biosynthesis. D-cycloserine, EMB, amoxiciline, clofazimine, BTZ043 and PBTZ169

are arabinogalactan or peptidoglycan biosynthesis inhibitors. Kanamycin, amikacin, capreomycin, viomycin, clarithromycin, linezolid and STR inhibit protein synthesis (Fig. 26).

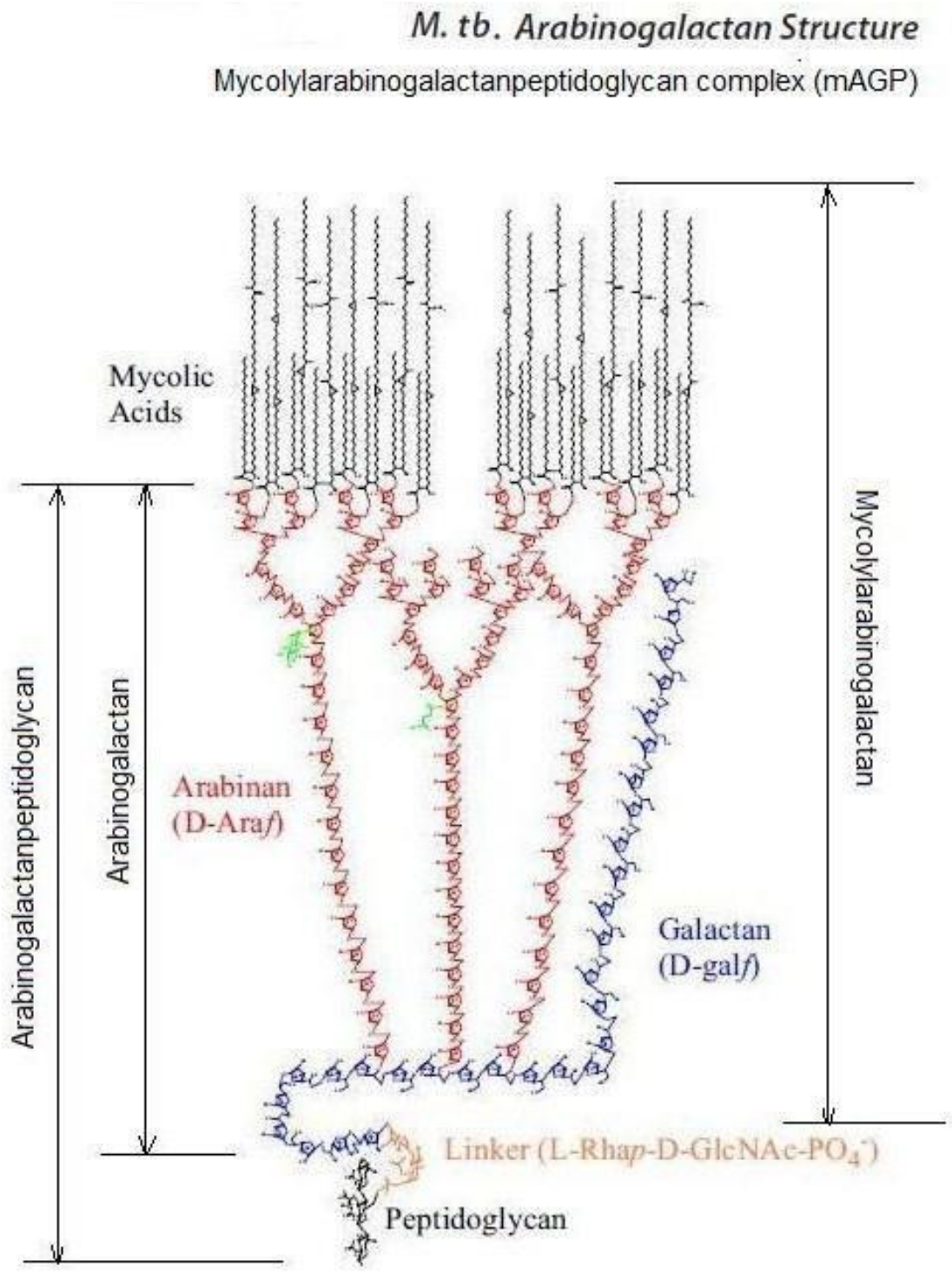


Figure 25. The structure of the mycolylarabinogalactan and its attachment to peptidoglycan (87). ©2008 by American Society for Biochemistry and Molecular Biology

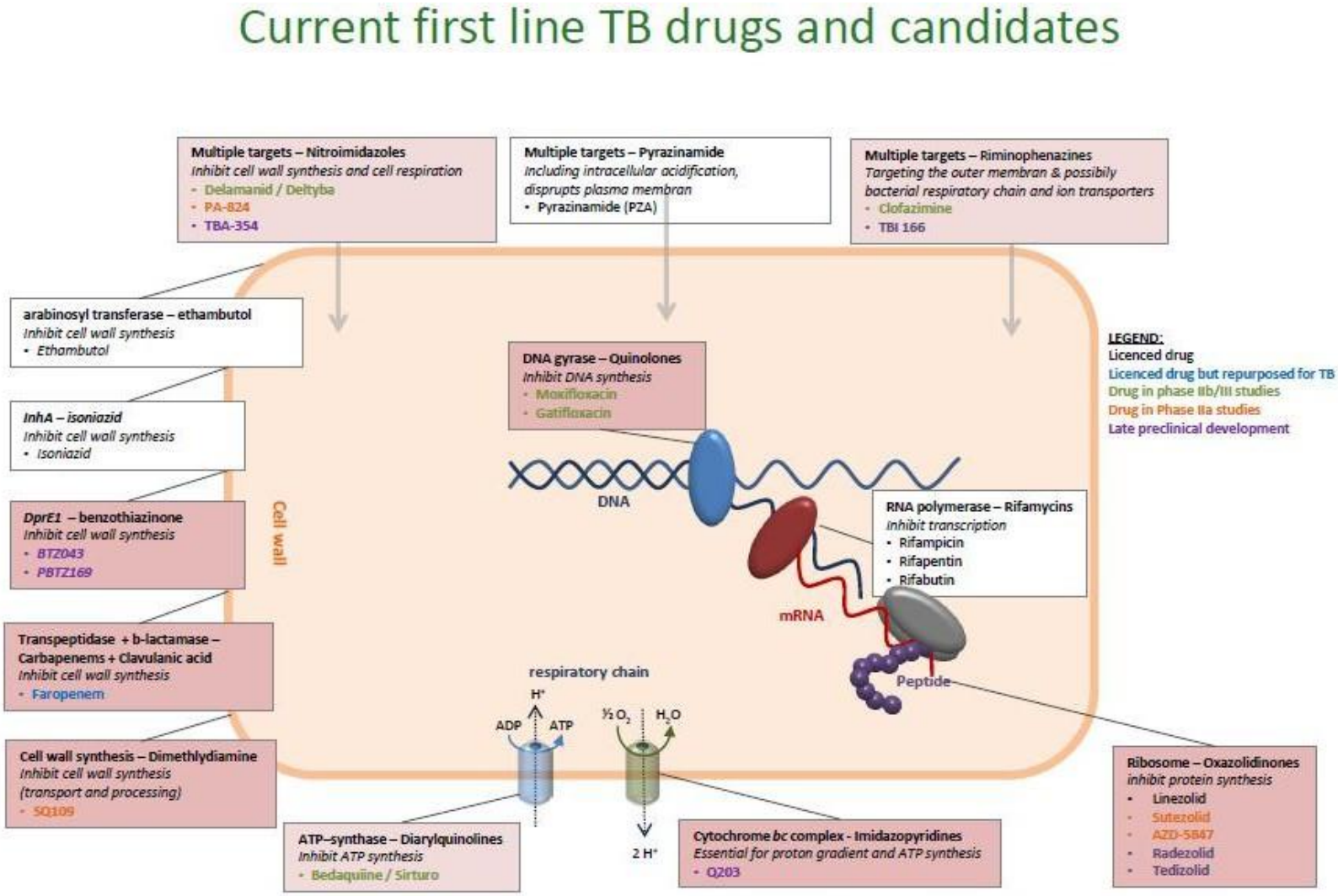
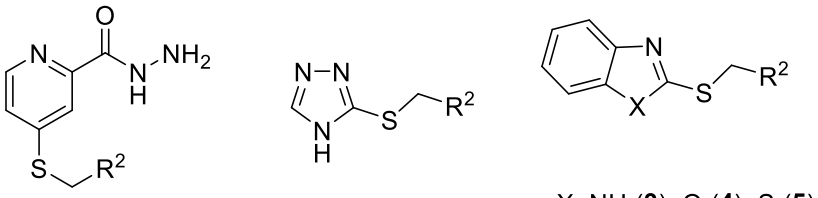


Figure 26. Mechanism of action of antiTB drugs and antiTB candidates (32).

5. Background of current work

Since the discovery of STR in 1943 and beginning of antiTB therapy the main problem in TB treatment was the development of resistant strains of *M.tb*. Application of triple therapy, combined application of STR, INH and PAS, and mainly DOTS - administration of INH, RIF, PZA and EMB - quite successfully solved this problem. But today, when MDR- and XDR-TB strains are spread disseminated, the new compounds with high antimycobacterial activity are urgently needed.

This work is aimed at finding the new substances with antimycobacterial activity identifying the fragment or fragments responsible for their efficiency and studying their structure - antimycobacterial activity relationships.



R^2 = 4-NO_2Ph: **1a-5a;** R^2 = 3,5-$(NO_2)_2$Ph: **1b-5b;** R^2 = 2,4-$(NO_2)_2$Ph: **2c-5c**

Figure 27. Previously studied heterocyclic aromates bearing benzylsulfanyl moiety

The basis of this research is previous work studying the antimycobacterial activity of various heteroaromates bearing differently substituted benzylsulfanyl fragments (Fig.27). The large series of 4-benzylsulfanylpyridine-2-carbohydrazides **1** (88), 3-benzylsulfanyl-1,2,4-triazoles **2** (89) as well as 2-benzylsulfanylbenzimidazole **3** (90; 91; 92), benzoxazole **4** (93) and benzothiazole **5** (94) derivatives were prepared and their efficiencies were evaluated.

Antimycobacterial activities of these compounds were evaluated against four mycobacterium strains: *M.tb.* My 331/88, the pathogen of TB, and non-tuberculous strains *M.avium* My 330/88, *M.kansasii* My 235/80, the strain obtained from CNCTC, and *M.kansasii* 6509/96, clinically isolated strain. These non-tuberculous mycobacteria are causative agents of severe disseminated infections. (95)

It was found that compounds bearing dinitrobenzylsulfanyl fragment generally displayed the highest antimycobacterial activity in each series and benzazole

derivatives **3**-**5** proved to be the most active among studied compounds. The antimycobacterial activities of benzazoles **3**-**5** were found to be high for all tested strains, however, these compounds didn't achieve antimycobacterial activity of INH against *M.tb*. and isolated *M.kansasii* 6509/96 (Table 1).

Table 1. Antimycobacterial activity of previously studied heterocyclic compounds **1**-**5**; expressed as MIC (μM).[10]

	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	
1a	4-NO_2Ph	8 / 8	62 / 62	8 / 8 / 16	8 / 16 / 16
1b	3,5-$(NO_2)_2$Ph	>62 / 125	>32 / >32	62 />62 />62	16 />32 />62
2a	4-NO_2Ph	1000 / 1000	500 / 500	250 / 1000 / 1000	250 / 1000 / 1000
2b	3,5-$(NO_2)_2$Ph	62 / 62	500 / 1000	32 / 62 / 125	62 / 62 / 125
2c	2,4-$(NO_2)_2$Ph	32 / 62	500 / 500	250 / 250 / 500	125 / 250 / 250
3a	4-NO_2Ph	62 / > 62	8 / 16	8 / 16 / 16	8 / 8 / 8
3b	3,5-$(NO_2)_2$Ph	4 / 4	8 / 8	4 / 8 / 8	4 / 8 / 8
3c	2,4-$(NO_2)_2$Ph	4 / 8	16 / 32	8 / 16 / 32	8 / 8 / 16
4a	4-NO_2Ph	>62 / >125	>32 / >62	32 / 32 / >62	32 / 32 / >32
4b	3,5-$(NO_2)_2$Ph	8 / 8	>16 / >32	2 / 4 / 4	4 / 8 / 8
4c	2,4-$(NO_2)_2$Ph	8 / 8	>16 / >32	4 / 4 / 4	4 / 8 / 8
5a	4-NO_2Ph	>32 / >125	32 / >62	32 / >62 / >250	32 / >32 / >125
5b	3,5-$(NO_2)_2$Ph	1 / 2	62 / >62	16 / 16 / >62	32 / 32 / >62
5c	2,4-$(NO_2)_2$Ph	2 / 4	>32 / >32	8 / 8 / 16	8 / 8 / 16
Isoniazid (INH)		0.5 / 1	250 / >250	>250 />250/>250	4 / 8 / 8

In several cases (denoted >), the MIC couldn't be determined due to the limited solubility of the compounds in the test medium.

Dinitrobenzylsulfanyl derivatives of benzimidazole (**3b, 3c**), benzoxazole (**4b, 4c**) and benzothiazole **5** (**5b**, **5c**) exhibited similar efficiency against non-tuberculous mycobacteria, but 2-[(dinitrobenzyl)sulfanyl]benzothiazoles **5** were more active

[10] MIC - Minimal inhibitory concentration, the lowest concentration of a substances at which mycobacterial growth inhibition occurred (the concentration that inhibited 99% of the mycobacterial population)

against *M.tb*. with the minimal inhibitory concentration (MIC) within 1-4 μM. These benzazoles also proved to be highly active against selected MDR strains of *M.tb.* (MIC within 1-4 μM) and ineffective against bacteria and fungi (yeasts or molds). Moreover, they displayed low antiproliferative effect and cytotoxicity.

Based on these data, it was supposed that the studied compounds **3**-**5** bearing dinitrobenzylsulfanyl fragment have to specifically influence the biosynthesis of characteristic mycobacterial cell wall that is present only in mycobacteria and are not in Gram-positive/Gram-negative bacteria or fungi. Comparable antitubercular efficiency of benzazoles against MDR and susceptible strains of *M.tb*. indicates that there is no cross-resistance with current antiTB drugs and therefore the mode of action might be new.

When summarizing the abovementioned results, several conclusions were made:

- dinitrobenzylsulfanyl group bound to various heterocycles is considered to be the fragment responsible for high antimycobacterial activity;
- position of two nitro groups has low influence on efficiency of active compounds **3**-**5**;
- heterocycle significantly affects the activity of evaluated compounds against all tested strains.

Compounds of series **3**-**5** with the highest antimycobacterial activity contain nitro groups in their structure, which are considered as potential carriers of toxicity and mutagenicity and thereby were often avoided in medicinal chemistry (96; 97). However, in the current clinical development there are substances, whose antimycobacterial activity is directly connected to the nitro-group in its structure. For example, delamanid (68; 72), PA-824 (67; 69; 70), TBA-354 (73) and benzothiazinones (75; 76) (BTZ043 and PBTZ169) (compounds have been described in detail above). All these compounds proved to be highly *in vitro* and *in vivo* antimycobacterial efficient as well as non-toxic, with safe profiles. Moreover, there were other nitro-containing compounds used as therapeutic medicaments, e.g., an antibiotic metronidazole. Nowadays, new biologically active compounds containing nitro group are being investigated. (96; 97; 98; 99)

Following abovementioned conclusions, our research is now focused on the investigation of new biologically active compounds containing nitro group.

6. Aim of the work

This work is focused on:

- synthesis of novel heterocyclic compounds with potential antimycobacterial activity; in particular bearing substituted benzylsulfanyl moiety.
- development and optimization of synthetic approaches.
- study the structure - antimycobacterial activity/toxicity/selectivity relationship of prepared compounds.

7. Description of obtained results:

In previous work (89; 90; 91; 92; 93; 94) the high and selective antimycobacterial activity of benzazoles **3**-**5** bearing dinitrobenzylsulfanyl fragment in position 2 was reported (MIC values 1-4 μM against drug-susceptible and drug-resistant strains of *M.tb.*; 2-62 μM against non-tuberculous mycobacteria). It was showed that the presence of two nitro groups on benzyl moiety and the benzazole heterocycle was necessary to reach high antimycobacterial activity.

Nowadays it is known that nitro group *per es* is not the carrier of mutagenicity and cytotoxicity. Nitro-containing compounds in preclinical and clinical development with safe profiles prove this observation.

This work is a continuation of research of novel potentially antimycobacterial active substances based on dinitrobenzylsulfanyl heteroaromates.

7.1. Synthesis and structure – antimycobacterial activity relationships study of 1-substituted-5-alkylsulfanyl-1*H*-tetrazoles and their isosteric analogs (I, II)

Tetrazole scaffold was chosen for the study of an influence of heterocycle on antimycobacterial activity. This nitrogen-containing heterocycle is synthetically accessible with ease and possesses valuable properties, which have widespread applications in various fields of industry and science, mainly in medicinal chemistry as lipophilic and metabolically stable surrogate for other functional groups. (100; 101) Additionally, tetrazole-5-thiols with general structure **6** are frequent fragments of biological active compounds (100; 102) with antiTB (103; 104), antibacterial (105), or antifungal activities (106).

Replacement of benzazole core of **3**-**5** with tetrazole cycle resulted in a few novel 1-substituted 5-(dinitrobenzyl)sulfanyl-1*H*-tetrazoles **7** and **8**, whose antimycobacterial efficiency was evaluated (Fig.28). It is important to note that the series of 1,5-disubstituted sulfanyl-1*H*-tetrazoles **6** were previously studied as potential antiTB agents, while such compounds exhibited only low to moderate efficiency. (103)

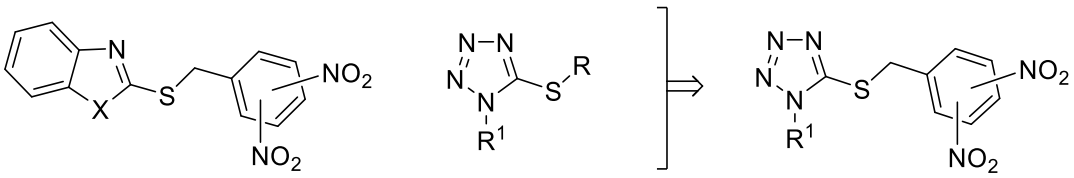
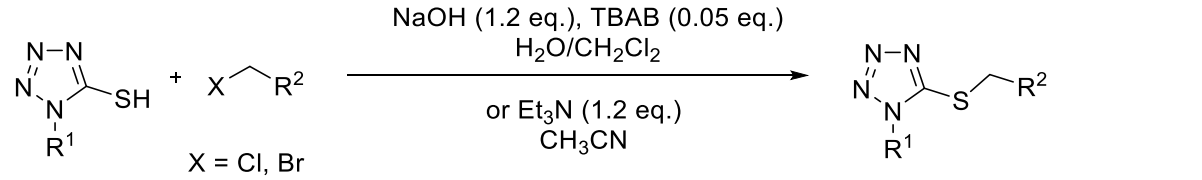


Figure 28. Previously studied compounds **3**-**5** and **6** and tetrazoles **7** and **8** designed in this study.

The preliminary results of antimycobacterial evaluation showed that tetrazoles **7** and **8** exhibited significant efficiency against all tested strains (*M.tb*. My 331/88, *M.avium* My 330/88, *M.kansasii* My 235/80 and *M.kansasii* 6509/96). Therefore, a large series of dinitrobenzyl derivatives along with analogs bearing 4-nitrosubstituted benzyl and unsubstituted benzyl moieties was prepared according to Scheme 1 and their structure - antimycobacterial activity relationships were studied (**II**).

Target compounds **7**-**10** were obtained according to well-known and efficient Williamson synthesis, which was carried out using tetrabutylammonium bromide (TBAB) as a phase-transfer catalyst. Another protocol used of triethylamine in acetonitrile. Both approaches provided high yields of benzylsulfanyltetrazoles (66-97%).



R^1 = alkyl, ary (Table 2)
R^2 = 3,5-$(NO_2)_2$Ph: **7a-7v**
R^2 = 2,4-$(NO_2)_2$Ph: **8e, 8g, 8j, 8s-8u**
R^2 = 4-NO_2Ph: **9u**
R^2 = Ph: **10u**

Scheme 1. Synthesis of 1-alkyl/aryl-5-alkylsulfanyl-1*H*-tetrazoles (**7**-**10**)

A number of various alkyl derivatives and aryl derivatives both with electron-withdrawing and donating groups were synthesized to explore the influence of substituent at position 1 of tetrazole ring on the efficiency of prepared compounds

(Table 2). Additionally, the effect of number and position of nitro groups on activity was studied.

It was determined that R^1-substituent affects the antimycobacterial activity of the compounds through determination of their physicochemical properties.

Lower activities of compounds with amino (**7a**) and methyl (**7b**) substituents can be related to their lower lipophilicity. The introduction of a carboxylic acid moiety to the molecule **7v** also led to a dramatic decrease of antimycobacterial activity. We believe that it is due to ionization of carboxylic group at physiological pH.[11] Esterification of carboxylic group restored the activity against *M.tb;* this corroborates our assumption.

Table 2. Substituent R^1 in the series of compounds **7**-**24.**

	R^1		**R^1**		**R^1**		**R^1**
a	$-NH_2$	**g**	Ph-	**m**	4-Et_3NPh-	**s**	2-NO_2Ph-
b	$-CH_3$	**h**	2-CH_3OPh-	**n**	4-BrPh-	**t**	3-NO_2Ph-
c	$-CH_2(CH_2)_4CH_3$	**i**	3-CH_3OPh-	**o**	4-ClPh-	**u**	4-NO_2Ph-
d	$-CH_2Ph$	**j**	4-CH_3OPh-	**p**	3,4-Cl_2Ph-	**v**	3-COOHPh-
e	$-C_6H_{11}$ (cyclohexyl)	**k**	3,4,5-$(CH_3O)_3$Ph-	**q**	3-FPh-	**w**	$-CH_2(CH_2)_2CH_3$
f	$-C_{10}H_{15}$ (1-adamantyl)	**l**	3,4-$(CH_3)_2$Ph-	**r**	4-FPh-	**x**	$-CH_2(CH_2)_6CH_3$

It was also found that alkyl and aryl derivatives of **7** or **8** exhibited similar antimycobacterial efficiency against all tested mycobacterium strains. Moreover, aryl derivatives exhibited analogous efficiency regardless of the electron-donating or electron-withdrawing substituents on the phenyl ring. However, position of substituent on phenyl ring affected the activity of target compounds; substances bearing 4-substituted phenyl, generally, displayed higher activities than the 2- or 3-substituted compounds. Activities of 2-, 3- and 4-nitrophenyl (**7s**, **7t**, **7u**), 2-, 3- and 4-methoxyphenyl (**7h**, **7i**, **7j**) as well as 3- and 4-fluorophenyl (**7q**, **7r**) derivatives are shown as examples. (Table 2, **II**)

Further, the importance of both nitro groups and their position for high antimycobacterial activity was confirmed. It was demonstrated that 5-[(3,5-dinitrobenzyl)sulfanyl]-1-(4-nitrophenyl)-1*H*-tetrazole (**7u**) and its 2,4-

[11] Physiological pH is 7.4

dinitrobenzylsylfanyl analog (**8u**) exhibited significantly higher efficiency that derivatives with 4-nitrosubstituted benzylsulfanyl (**9u**) or unsubstituted benzylsulfanyl (**10u**) moieties.

Table 3. *In vitro* antimycobacterial activities of selected compounds of series **7-24**; MIC (μM)

	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	
7e	1 / 1	16 / 32	4 / 4 / 4	2 / 2 / 4
7g	4 / 4	62 / 62	2 / 4 / 16	2 / 2 / 4
7j	2 / 4	16 / 32	1 / 4 / 4	1 / 1 / 2
7u	1 / 1	32 / 32	1 / 1 / 1	1 / 2 / 2
8e	8 / 16	16 / 32	4 / 8 / 16	16 / 62 / 62
8g	8 / 16	32 / 62	16 / 62 / 62	8 / 32 / 32
8j	8 / 8	62 / 62	32 / 62 / 62	8 / 16 / 32
8u	4 / 8	62 / 62	8 / 32 / 32	16 / 32 / 62
9u	250 / 250	250 / 250	250 / 250 / 250	250 / 250 / 250
10u	125 / 125	62 / 62	16 / 32 / 32	16 / 62 / 62
11j	125 / 125	1000 / 1000	250 / 500 / 500	250 / 500 / 500
12j	250 / 250	250 / 250	250 / 250 / 250	250 / 250 / 250
13g	62 / 62	125 / 125	62 / 250 / 250	125 / 250 / 250
14g	8 / 8	62 / 125	32 / 62 / 62	32 / 62 / 62
16e	1 / 2	32 / 32	1 / 1 / 2	2 / 4 / 4
16g	8 / 8	32 / 32	8 / 8 / 16	16 / 16 / 32
16j	1 / 1	32 / 32	1 / 2 / 2	2 / 4 / 8
16w*	2 / 2	125 / 125	4 / 8 / 8	4 / 8 / 16
16x*	2 / 2	>125 / >125	2 / 8 / 8	4 / 8 / 16
17e	4 / 8	32 / 32	4 / 16 / 16	16 / 62 / 62
18e	125 / 125	125 / 125	8 / 32 / 32	62 / 250 / 500
24j	2 / 2	16 / 32	4 / 8 / 16	4 / 16 / 16
24u	1 / 1	125 / 125	1 / 1 / 1	1 / 1 / 2
INH	0.5 / 1	250 / 250	250 / 250	2 / 4 / 4

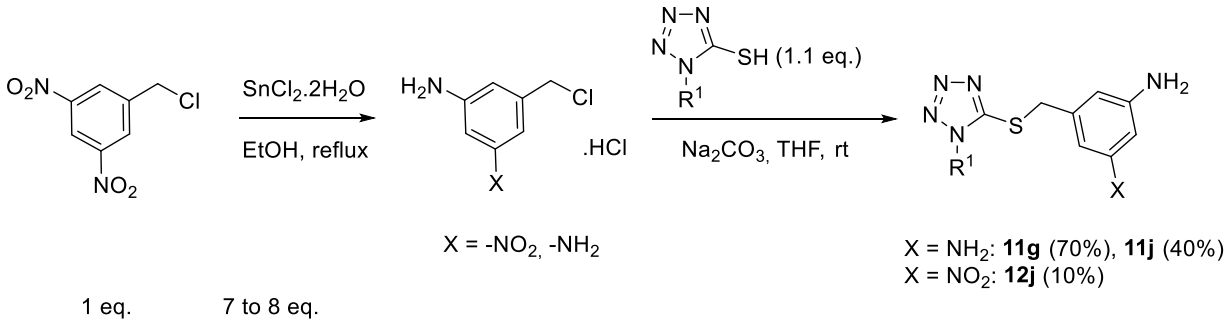
* - unpublished data

Moreover, position of two nitro groups has a considerable influence on efficiency of prepared substances; 3,5-dinitro derivatives **7** displayed significantly higher potency than 2,4-dinitro analogs **8** regardless of the substituent on tetrazole. This finding is in disagreement with the previous observation for dinitrobenzylsulfanyl benzazoles **3**-**5**, where 3,5-dinitro and 2,4-dinitro analogs showed similar antimycobacterial activity.

The most active 3,5-dinitrobenzylsulfanyl derivatives **7** exhibited comparable activity with INH against *M.tb*. and clinically isolated *M.kansasii* 6509/96 and proved to be highly effective against INH-resistant strains *M.avium* and *M.kansasii* My 235/80 (Table 3).

Loss of antimycobacterial activity of partially and fully reduced derivatives **11g**, **11j** and **12j** confirmed the importance of both nitro groups.

Synthesis of these derivatives was achieved by reduction of 3,5-dinitrobenzyl chloride with excess of tin(II) chloride dihydrate in ethanol followed by reaction of formed partially or fully reduced ammonium salts with 1-substituted-1*H*-tetrazole-5-thiol in THF in the presence of sodium carbonate (at pH 8-10).

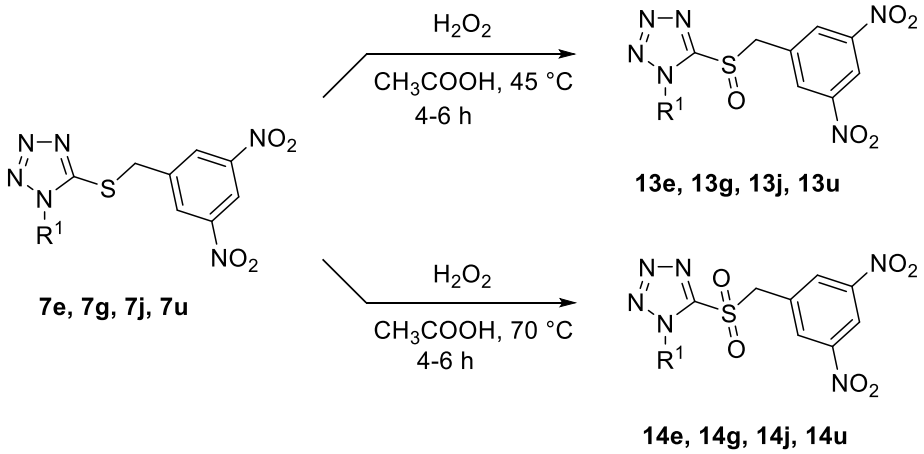


Scheme 2. Synthesis of partially and fully reduced derivatives **11g**, **11j** and **12j**.

As it was suggested that 1-alkyl/aryl-5-alkylsulfanyl-1*H*-tetrazoles might be metabolized *in vivo* to sulfoxides or sulfones, 1-substituted 5-[(3,5-dinitrobenzyl)sulfinyl]-1*H*-tetrazoles (**13e**, **13g**, **13j**, **13u**) and their sulfonyl analogs **14e**, **14g**, **14j** and **14u** were prepared. Hydrogen peroxide was used under mild condition to oxidase benzylsulfanyl derivatives **7** into corresponding sulfoxides or sulfones (Scheme 3). Full oxidation of selected sulfanyltetrazoles **7** led to corresponding sulfonyl derivatives **14** in moderate to high yields (62-91%). Sulfinyl derivatives **13** were prepared only in moderate yields (40-60%) as over-oxidation was

observed in these cases, despite of an incomplete consumption of starting material (**7**). (**II**)

It was found that both partial and full oxidation led to a significant decrease of antimycobacterial activity (Table 3).



Scheme 3. Synthesis of 1-alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfinyl]-1*H*-tetrazoles (**13**) and 1-alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfonyl]-1*H*-tetrazoles (**14**).

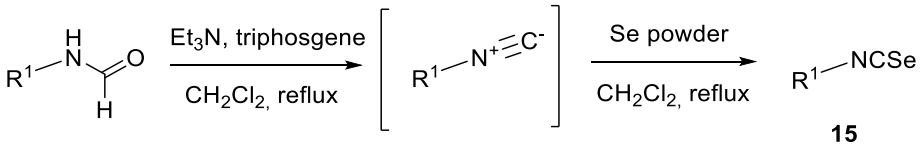
To explore the influence of heteroatom linkage between dinitrobenzyl moiety and tetrazole core on antimycobacterial activity, selenium and oxygen isosteric analogs of **7** were synthesized, i.e. 1-alkyl/aryl-5-[(3,5-dinitrobenzyl)selanyl]-1*H*-tetrazoles **15** and 1-aryl-5-[(3,5-dinitrobenzyl)oxy]-1*H*-tetrazoles **19**. (**I**, **II**) In a series of selenium and oxygen isosteric derivatives the influence of substituent R^1 in position 1 of tetrazole cycle on antimycobacterial activity was studied. Additionally, the crucial role of number and position of nitro groups on benzyl moiety was investigated in the series of selenium analogs bearing 3,5-dinitrobenzyl **15**, 2,4-dinitrobenzyl **16**, benzyl **17** and methyl **18** moieties.

Our interest in the selenium isosteric analogs was primarily motivated by the similarities in the chemistry of selenium and sulfur compounds. While 1-substituted 5-alkyl/arylsulfanyl-1*H*-tetrazoles have been widely explored, (**I**) their selenium analogs are scarcely described. Hence, we were keenly interested in an effect on antimycobacterial activity and cytotoxicity of target compounds upon the replacement of sulfur with selenium will affect both.

Selenium was considered as one of the most toxic elements until 1957, when Schwarz and Foltz described the relationship between selenium intake by food and

prevention of liver necrosis in rats. (107) Nowadays, it is well-known that selenium is a trace element, essential for normal human development, growth, metabolism, and function of the immune system. Moreover, in recent years attention has been paid to selenium-containing compounds due to their use as synthetic tools (108), and as promising biologically active substances exhibiting antifungal (109), antimycobacterial (110), antiviral (111), antitumor (112) or antihypertensive properties (113).

For the synthesis of 1-alkyl/aryl-5-alkylselanyl-1*H*-tetrazoles **16**-**19**, preparation of 1-substituted-1*H*-tetrazole-5-selenols was required, which was achieved by 1,3-dipolar cycloaddition of alkyl/arylisoselenocyanates **15** with sodium azide.

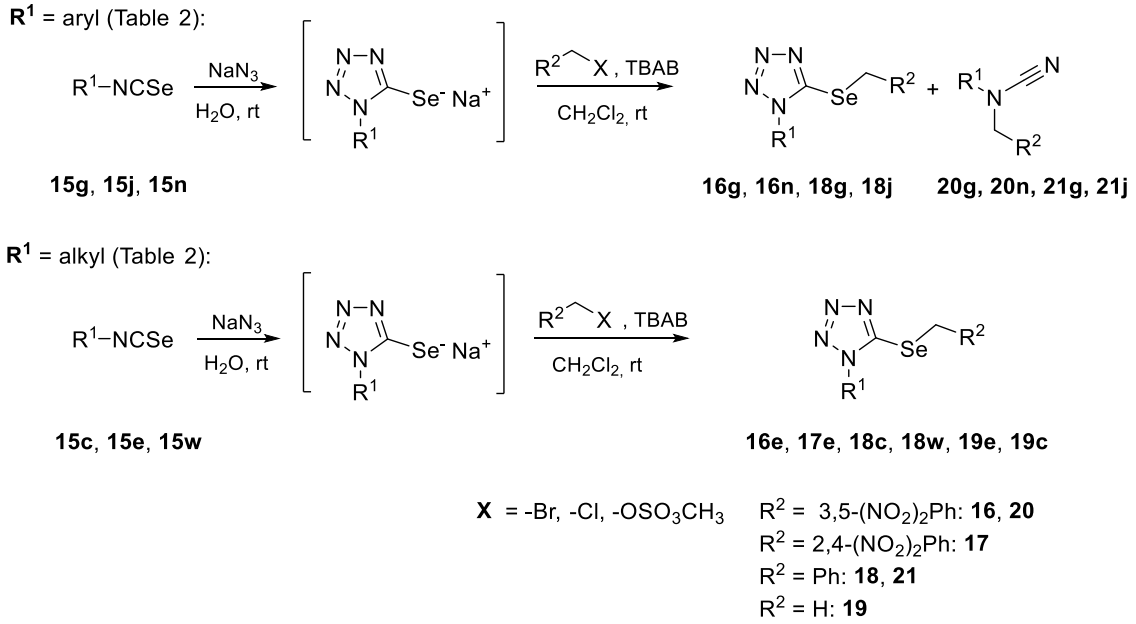


Scheme 4. Synthesis of alkyl/arylisoselenocyanates **15**.

The appropriate alkyl/arylisoselenocyanates were prepared from corresponding *N*-alkyl/arylformamides by well-described one-pot approach (114) proceeding through the formation of isonitriles, which were then reacted with selenium to form isoselenocyanates **15** (Scheme 4).

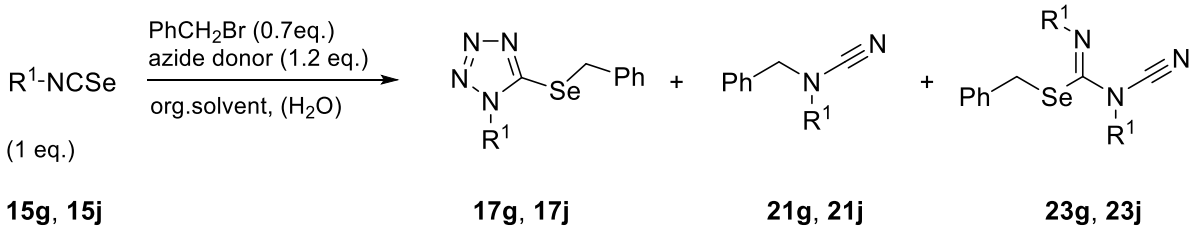
Many different approaches were explored to find suitable conditions for the preparation of desired 1-substituted 1*H*-tetrazole-5-selenols from the corresponding isoselenocyanates **15**. Unfortunately, all these reactions proceeded with precipitation of selenium powder. However, reaction of cyclohexylisoselenocyanate with sodium azide in water was successful and sodium 1-cyclohexyl-1*H*-tetrazole-5-selenolate was isolated as a mixture with residual sodium azide. Unfortunately acidification of sodium 1-cyclohexyl-1*H*-tetrazole-5-selenolate led to precipitation of red selenium at pH 5.5-6. (**I**) Therefore, it was decided to alkylate 1-substituted-1*H*-tetrazole-5-selenolates without isolation. Corresponding selenolates were synthesized via the reaction of isoselenocyanate with sodium azide in water, followed by alkylation with alkyl halides using phase-transfer catalyst (Scheme 5). Compounds **16e**, **16g**, **16n**, **17e**, **18c**, **18g**, **18j**, **18w**, **19c**, and **19e** were prepared by this method.

It was observed that the 1-alkyl-5-alkylselanyl-1*H*-tetrazoles were formed in poor to moderate yields (11-50%) as an only product, while aryl derivatives were formed in very low yields (5-14%) due to an unexpected formation of *N*-alkyl-*N*-arylcyanamides (**20**, **21**) as the main product.



Scheme 5. Synthesis of 1-alkyl/aryl-5-alkylselanyl-1*H*-tetrazoles **16**-**19.**

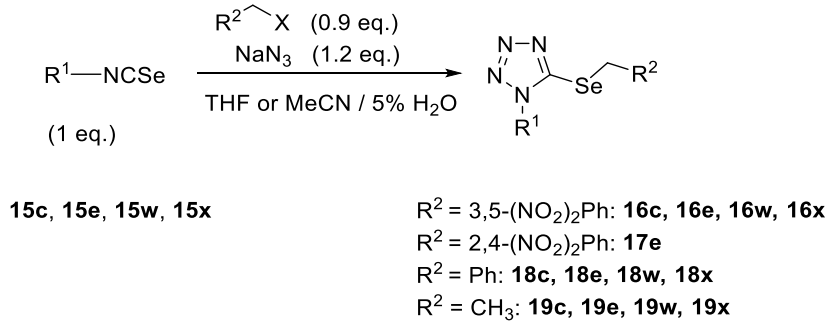
Encouraged by these results one-pot reactions were carried out in an attempt to alkylate tetrazole-5-selenolate immediately, before its decomposition. To find suitable conditions a number of experiments with phenylisoselenocyanate (**15g**) or 4-methoxyphenylisoselenocyanate (**15j**) as substrates and benzyl bromide as alkylating agent were carried out (Scheme 6). It was determined that almost in all cases reactions resulted in the formation of desired products; the 1-aryl-5-benzylselanyl-1*H*-tetrazoles (**18g**, **18j**), in low to moderate yields (4-45%) and *N*-aryl-*N*-benzylcyanamides (**20**, **21**) in low to moderate yields (10-71%). Furthermore, another unexpected side product was isolated and confirmed to be (*Z*)-*Se*-benzyl-*N*-cyano-*N*,*N*'-diarylisoselenourea (**23g**, **23j**) by NMR and X-ray crystallography experiments (Scheme 6). The highest yields for the compounds **18g** and **18j** were achieved when sodium azide in THF or CH_3CN with a small amount of water was employed. (**I**) Thus this method was chosen for the preparation of 1-alkyl/aryl-5-alkyl-1*H*-tetrazoles (**16**-**19**).



R^1 = Ph (**g**); 4-CH_3OPh (**j**)

Scheme 6. One-pot synthesis of 1-aryl-5-benzylselanyl-1*H*-tetrazoles (**17g**, **17j**).

Compounds **16g, 16j**, and **16n** were obtained in low yields (5-34%) by this approach; similarly to the previous protocol. Even utilization of 3,5-dinitrobenzyl iodide could not improve their yield significantly. Formation of *N*-aryl-*N*-(3,5-dinitrobenzyl)cyanamides and (*Z*)-*Se*-(3,5-dinitrobenzyl)-*N*-cyano-*N*,*N*'-diarylisoselenourea (**22g**, **22j**, **22n**) was also observed. None of the modifications of the reaction conditions or reactant ratios improved the yields of the target selanyltetrazoles **16**. In contrast to aryl analogues, 1-alkyl-5-alkylselanyl-1*H*-tetrazoles were obtained in good yields (30-84%) using analogous one-pot reactions (Scheme 7). Surprisingly, neither *N,N*-dialkylcyanamides nor *Se*-alkyl-*N*-cyano-*N,N'*-dialkylisoselenoureas were found.



Scheme 7. One-pot synthesis of 1-alkyl-5-alkylselanyl-1*H*-tetrazoles **16**-**19.**

Antimycobacterial activity of all dinitrobenzylselanyl derivatives **16** and **17** along with two benzyl analogs (**18e**, **18g**) were evaluated (**II**).

A conclusion was drawn that 1-alkyl- and 1-aryl-5-[(3,5-dinitrobenzyl)selanyl]-1*H*-tetrazoles **16** were comparably active against all tested strains. This observation was similar to that for sulfanyltetrazoles **7** and **8**. Thus, R^1-substituent analogously

influence the antimycobacterial activity of compounds **16** through determination of physicochemical properties.

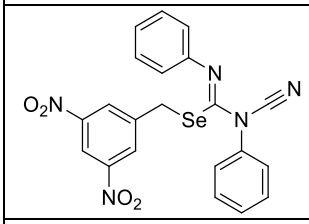
As in the previous series, 3,5-dinitro derivative 1-cyclohexyl-5-[(3,5-dinitrobenzyl)selanyl]-1*H*-tetrazole (**16e**) was more effective than its 2,4-dinitrobenzyl analog (**17e**). Derivatives with unsubstituted benzyl (**18e**) proved to be inactive (Table 3).

The most active 1-alkyl/aryl-[(3,5-dinitrobenzyl)selanyl]-1*H*-tetrazoles **16** exhibited comparable activity to INH against *M.tb*. and clinically isolated *M.kansasii* 6509/96 and proved to be highly effective against INH-resistant strains *M.avium* My 330/88 and *M.kansasii* My 235/80.

Surprisingly, presence of heteroatom (sulfur or selenium) has negligible effect on antimycobacterial activity when linkage between the dinitrobenzyl and the tetrazole moieties was considered. The efficiencies of sulfur and selenium isosteric analogs **7** and **16** were almost the same, regardless of the heteroatom involved.

(Z)-Se-(3,5-dinitrobenzyl)-*N*-cyano-*N,N'*-diphenylisoselenourea (**22g**), one of the side products, contains crucial 3,5-dinitrobenzylselanyl fragment attached to electron-deficient carbon atom. Therefore, its antimycobacterial efficiency was determined (Table 4). Interestingly, this compounds exhibited low potency against INH-susceptible *M.tb.* and INH-resistant *M.avium*, but was highly active against both strains of *M.kansasii*: INH-resistant strain My 235/80 and INH-susceptible clinically isolated strain 6509/96.

Table 4. Antimycobacterial activity of *(Z)-Se*-(3,5-dinitrobenzyl)-*N*-cyano-*N,N'*-diphenylisoselenourea (**22g**); MIC (μM)

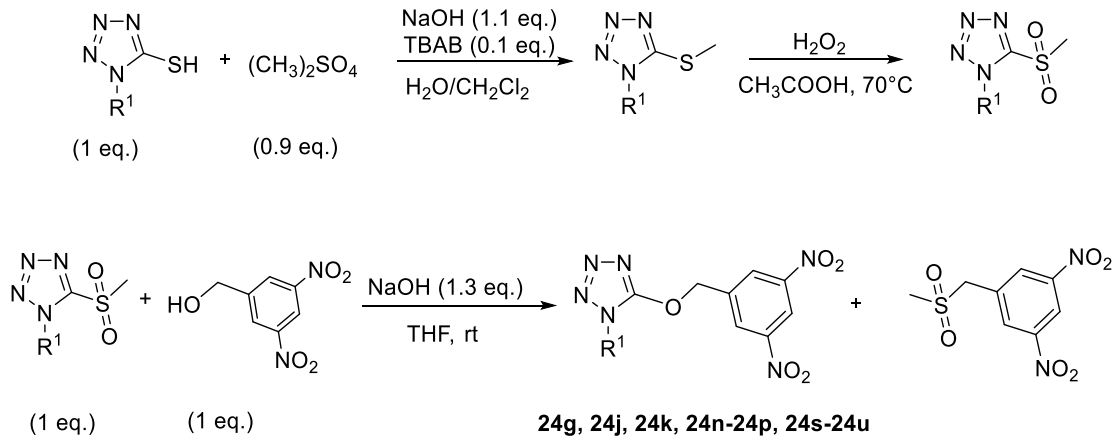
	M.tb. **My 331/88**	***M.avium*** **My 330/88**	***M.kansasii*** **235/80**	***M.kansasii*** **6509/96**
	14 / 21 days		7 / 14 / 21 days	
* 	>125 / >125	>125 / >125	1 / 4 / 4	2 / 4 / 8
INH	0.5 / 1	250 / 250	250 / 250	2 / 4 / 4

* - unpublished data

Oxygen, sulfur and selenium are the elements of group VIA and contain 6 electrons in valence shell, which has ns^2 np^4 configuration. Therefore, next step of

our research work was the replacement of the sulfur atom in 1-aryl-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles **7** with oxygen.

The synthesis of 1-aryl-5-[(3,5-dinitrobenzyl)oxy]-1*H*-tetrazoles **24** was carried out according to scheme 8. Methylation of tetrazole-5-thiols with dimethyl sulfate under phase-transfer catalytic conditions followed by oxidation with hydrogen peroxide resulted in formation of 1-aryl-5-(methylsulfonyl)-1*H*-tetrazoles in high yields (50-93%). In the next step, the corresponding methylsulfones underwent a nucleophilic substitution with 3,5-dinitrobenzyl alcohol in THF in the presence of KOH. Target 1-aryl-5-[(3,5-dinitrobenzyl)oxy]-1*H*-tetrazoles **24** were obtained in 23-55% yields, but none of the alkyl derivatives were obtained by this approach. Such low yields of 1-aryl-5-[(3,5-dinitrobenzyl)oxy]-1*H*-tetrazoles were due to the formation of a side product, (3,5-dinitrobenzyl)(methyl)sulfone in 30-40% yield.



Scheme 8. Synthesis of 1-aryl-5-[(3,5-dinitrobenzyl)oxy]-1*H*-tetrazoles **24**.

Formation of such byproduct via nucleophilic attack of the released methanesulfinate on benzylic position of the desired products **24** was not observed with such as methanol or benzylalcohol as substrates. (115)

Within structure – antimycobacterial activity relationships study, in a series of 1-aryl-5-[(3,5-dinitrobenzyl)oxy]-1*H*-tetrazoles **24** the same correlation between the substituent R^1 and antimycobacterial efficiency as in previous sulfanyl and selanyltetrazole series was found. Derivatives with electron-donating and electron-withdrawing substituents on phenyl ring displayed similar antimycobacterial activity. Position of substituent on phenyl ring slightly affected the compounds potency; 4-

nitrophenyl derivative **24u** was more effective than 2- or 3-nitrophenyl derivatives (**24s**, **24t**) (Tabl 2, **II**).

When the effect of heteroatom linkage between 3,5-dinitrobenzyl moiety and the tetrazole core was considered, identical conclusion as for the series of selenium isosteric analogs was drawn; type of linker heteroatom has negligible effect on the antiTB activity. Thus, all isosteric derivatives bearing 3,5-dinitrobenzyl fragment proved to be highly antimycobacterial active compounds exhibiting comparable efficiency with INH.

The compounds **7c-7e**, **7n-7p**, **7u**, **16c-16e**, **16j** and **24u,** which exhibited the high antiTB activities, were additionally tested against MDR strains of *M.tb*. 234/2005, 9449/2007, 8666/2010, Praha 1, Praha 4, and Praha 131. Compounds were highly efficient with MIC 0.25-2 μM against all tested strains. Moreover, there was no cross-resistance with either the first or second-line antiTB drugs. It indicates that the compounds of series **7**, **16** and **24** possessed different mechanism of action than the currently used antiTB drugs. Furthermore, these compounds have selective antimycobacterial efficiency because they showed no activity against selected bacteria and fungi. (**II**)

When the toxicity of prepared compounds was taken into consideration, the 3,5-dinitrobenzylsulfanyl derivatives **7d**, **7e**, **7j**, **7p**, **7t** and **7u** with promising antiTB activity showed limited effects on the cellular viability of four mammalian cell lines [HeLa (human cervix epithelioid carcinoma), HepG2 (human Caucasian hepatocyte carcinoma), HuH7 (human hepatocellular carcinoma) and MDCKII (Madin-Darby canine kidney cells)]. Conversely, compounds **8e**, **13e**, **16d**, **16e**, **16j** and **24p** showed some toxicity in at least one cell line, and **14e** and **17e** were toxic in at least three cell lines.

While summarizing the abovementioned results, several interim conclusions for compounds **7**-**24** can be drawn:

- substituent R^1 at position 1 of tetrazole cycle provides the suitable physicochemical properties to reach high antimycobacterial activity;
- the number and position of nitro groups on benzyl moiety has crucial significance for high antimycobacterial activity of target compounds - derivatives with 3,5-dinitrobenzyl moiety **7**, **16** and **24** showed the highest efficiency;

- type of heteroatom linkage between benzyl moiety and tetrazole core slightly affect the antimycobacterial efficiency;
- partial or full oxidation of sulfur atom of 1-alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles as well as partial or full reduction of dinitrobenzyl fragment lead to significant decrease of compounds potency.

7.2. Sulfanyl/selanyl tetrazole regioisomers: Synthesis and structure - antimycobacterial activity relationships study. (III)

As 5-substituted tetrazole has 1*H*- and 2*H*-tautomers (Fig.29) and 1,5-disubstituted and 2,5-disubstituted tetrazole isomers can be recognized, it was conspicuous to investigate the influence of the position of substituent R^1 on tetrazole cycle on the biological properties of sulfanyltetrazoles, in particular their antimycobacterial activity. Therefore, the series of 1-alkyl-5-alkylsulfanyl-1*H*-tetrazoles (**27**-**30**) and corresponding 2*H*-regioisomers (**31**-**34**) with a various substituted benzyls at positions 1 or 2 and at position 5 on sulfanyltetrazole core were synthesized. Continuing the study on influence of isosteric replacement of linker heteroatom on antimycobacterial activity, a series of 1-alkyl-5-alkylselanyl-1*H*-tetrazoles (**36**-**40**) along with corresponding 2*H*-regioisomers (**41**-**45**) was also prepared. (**III**)

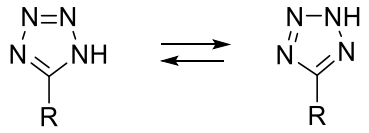
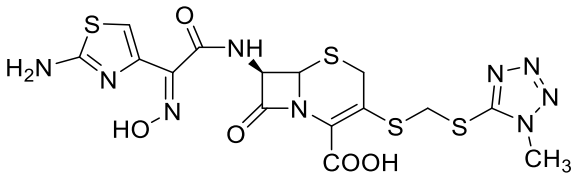


Figure 29. 1*H*- and 2*H*- tautomers of 5-substituted tetrazoles

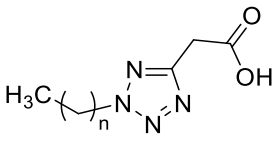
Our suggestion about the difference in antimycobacterial efficiency of 1*H*- and 2*H*-regioisomers was based on previous publications where the difference of the biological properties of the 1,5- and 2,5-regioisomers was demonstrated, in particular in the effective concentration levels. (**III**) For example, 7*β*-[(*Z*)-2-(2-aminothiazol-4-yl)-2-hydroxyiminoacetamido)cephalosporin derivatives with 1-methyltetrazol-5-yl fragment in the side chain **25** displayed more than twice times higher antibacterial

activity against both Gram-positive and Gram-negative bacteria than its 2-methyl isomer (Fig.30). On the other hand, 2-(2-alkyl-2*H*-tetrazol-5-yl)acetic acid **26** proved to be efficient *Pseudomonas aeruginosa* quorum-sensing inhibitor, whereas 1,5-disubstituted analogs had only low inhibitory activity.

At this point of research, the focus was on the exploration of the optimal position of the crucial dinitrobenzyl fragment on sulfanyltetrazole or selanyltetrazole scaffold for antimycobacterial activity.



25

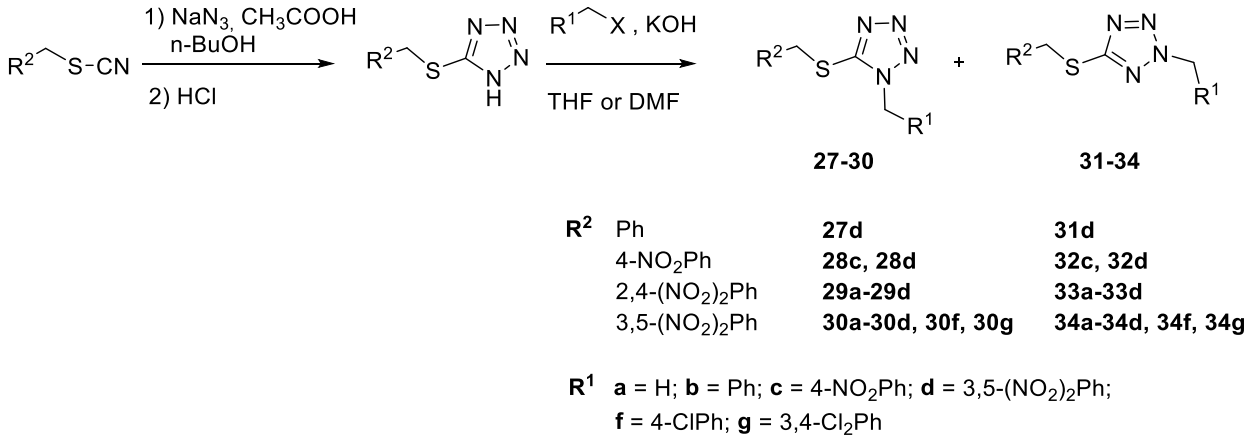


26

Figure 30. Tetrazole-based compounds with the different effective concentrations of their 1,5- and 2,5-tetrazole regioisomers.

The importance of number and position of nitro groups on benzyl fragment was widely explored in detail. Within this study, benzyl, 4-nitrobenzyl, 3,5-dinitrobenzyl, 2,4-dinitrobenzyl as well as 4-methoxybenzylsulfanyl-1*H*-tetrazole and selanyl-1*H*-tetrazolates were prepared and further alkylated to methyl (**a**), benzyl (**b**), 4-nitrobenzyl (**c**), 3,5-dinitrobenzyl (**d**), 2,4-dinitrobenzyl (**e**), 4-chlorbenzyl (**f**) or 3,4-dichlorbenzyl (**g**) analogs.

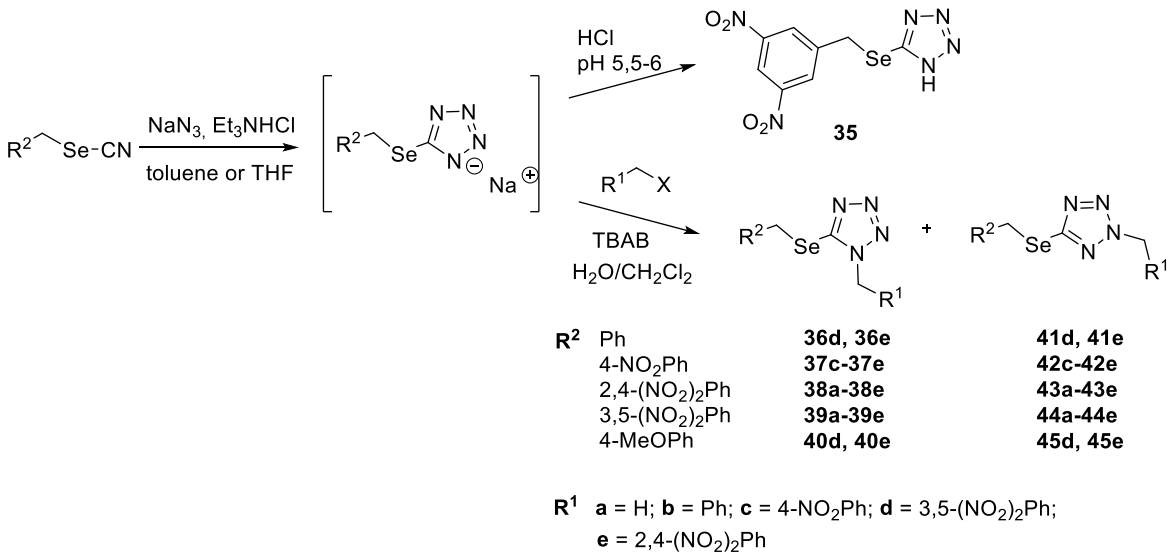
1-Alkyl-5-alkylsulfanyl-1*H*-tetrazoles (**27**-**30**) and 2-alkyl-5-alkylsulfanyl-2*H*-tetrazoles (**31**-**34**) were synthesized from corresponding R^2 alkyl halides. Alkyl thiocyanates were prepared by the reaction of corresponding alkyl halide with potassium thiocyanate in high yields (75-96%) and converted into 5-alkylsulfanyl-1*H*-tetrazoles via reaction with sodium azide and acetic acid in n-butanol with moderate yields (50-68%) (Scheme 9). Alkylation of tetrazoles was performed in THF or DMF in the presence of KOH or under phase-transfer catalytic conditions leading to the formation of both 1- and 2- regioisomers, in the ratios ranging from 1:1.6 to 1:4, which were separated by column chromatography.



Scheme 9. Synthesis of 1-alkyl- (**27**-**30**) and 2-alkyl-5-alkylsulfanyl-2*H*-tetrazoles (**31**-**34**).

Unlike easy and successful approach to target 1-alkyl-5-alkylsulfanyl-1*H*-tetrazoles and 2*H*-tetrazoles, the synthesis of 1-alkyl-5-alkylselanyl-1*H*-tetrazoles (**36**-**40**) and 2-alkyl-5-alkylselanyl-2*H*-tetrazoles (**41**-**45**) was complicated due to high sensitivity of selanyl-1*H*-tetrazolate towards acidification and low yields of corresponding tetrazole **35** (Scheme 10). Therefore, alkylation of selanyltetrazolates obtained from corresponding alkyl selenocyanates was performed *in situ* under conditions of phase transfer catalysis, which resulted in the formation of both 1- and 2-isomer regioisomers in ratios ranging from 1:1 to 1:4. (**III**) Alkylation of sulfanyl-1*H*-tetrazoles and selanyl-1*H*-tetrazolates was preferentially directed to position 2, which is likely to happen due to steric hindrance of the alkylsulfanyl or alkylselanyl substituent at position 5 of tetrazole.

The 1,5- and 2,5-isomeric series of alkylsulfanyl and alkylselanyl tetrazoles were unambiguously identified by ^{1}H and ^{13}C NMR and 1D NOESY experiments [**III**, Supplementary info]. Signals of the tetrazole carbon and the nitrogen-bound methylene group of 2-alkyl-5-(alkylselanyl)-2*H*-tetrazoles were always observed downfield as compared to the 1-isomers.



Scheme 10. Synthesis of 1-alkyl (**35**-**40**) and 2-alkyl-5-alkylselanyl-2*H*-tetrazoles (**41**-**45**).

1-Alkyl- (**27**-**30**) and 2-alkyl-5-alkylsulfanyl-2*H*-tetrazoles (**31**-**34**) together with their selenium analogs (**36-45**) were evaluated for their antimycobacterial activities. Antimycobacterial activities of selected derivatives are presented in table 5.

Table 5. *In vitro* antimycobacterial activities of selected compounds of series **27**-**45**; MIC (μM).

	isomer	R^1	R^2	***M.tb.* My 331/88**	***M.avium* My 330/88**	***M.kansasii* 235/80**	***M.kansasii* 6509/96**
				14 / 21 days		7 / 14 / 21 days	
27d	1	3,5-$(NO_2)_2$Ph	Ph	8 / 8	125 / 125	32 / 62 / 62	16 / 32 / 32
29b		Ph	2,4-$(NO_2)_2$Ph	8 / 8	250 / 250	8 / 32 / 62	16 / 32 / 62
30b		Ph	3,5-$(NO_2)_2$Ph	1 / 2	250 / 250	2 / 8 / 8	2 / 4 / 4
31d	2	3,5-$(NO_2)_2$Ph	Ph	16 / 16	125 / 125	16 / 32 / 32	16 / 32 / 32
33b		Ph	2,4-$(NO_2)_2$Ph	4 / 4	32 / 62	16 / 32 / 62	8 / 16 / 32
34b		Ph	3,5-$(NO_2)_2$Ph	1 / 1	62 / 125	8 / 16 / 16	4 / 4 / 8
36d	1	3,5-$(NO_2)_2$Ph	Ph	8 / 8	32 / 32	8 / 16 / 32	16 / 32 / 32
39b		Ph	3,5-$(NO_2)_2$Ph	1 / 2	32 / 32	2 / 4 / 4	4 / 8 / 8
43b	2	Ph	2,4-$(NO_2)_2$Ph	16 / 16	62 / 62	16 / 16 / 32	32 / 32 / 62
44b		Ph	3,5-$(NO_2)_2$Ph	1 / 1	16 / 32	1 / 1 / 1	1 / 1 / 2
INH				0.5 / 1	250 / 250	250 / 250	2 / 4 / 4

It was identified that the 1-alkyl-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles (**30a-30d**, **30f**, **30g**), 2-alkyl-5-[(3,5-dinitrobenzyl)sulfanyl]-2*H*-tetrazoles (**34a-34d**, **34f**, **34g**), 1-alkyl-5-[(3,5-dinitrobenzyl)selanyl]-1*H*-tetrazoles (**39a-39e**) and 2-alkyl-5-[(3,5-dinitrobenzyl)selanyl]-2*H*-tetrazoles (**44a-44e**) exhibited the highest antimycobacterial activity and reached 1 μM against *M.tb*. and 1-2 μM against both INH-resistant and INH-susceptible *M.kansasii*.

An influence of number and position of nitro groups on benzylsulfanyl/selanyl moiety was confirmed; 3,5-dinitrobenzylsulfanyl/selanyl derivatives possessed significantly higher activities than 2,4-dinitrobenzylsulfanyl/selanyl derivatives and both nitro groups were necessary for the high antimycobacterial efficiency of the studied compounds.

Moreover, it was demonstrated that the position of the 3,5-dinitrobenzyl substituent on sulfur/selenium is beneficial. The 3,5-dinitrobenzyl substitution of tetrazole moiety led to a significant decrease in the antimycobacterial activity. With regard to position of the alkyl substituent on nitrogen atoms of tetrazole cycle, the 2-isomer series (**34** and **44**) exhibited higher activity (MIC 1-2 μM) than the 1-isomer series (**30** and **39**) (MIC 1-8 μM). It was also found that the antiTB activities of the 2-isomers **34a-34d**, **34f**, **34g** and **44a-44e** were not affected by the substituent on the tetrazole while the activities of the corresponding 1-isomers **30a-30d**, **30f**, **30g** and **39a-39e** appeared to be slightly influenced by the type of substituent.

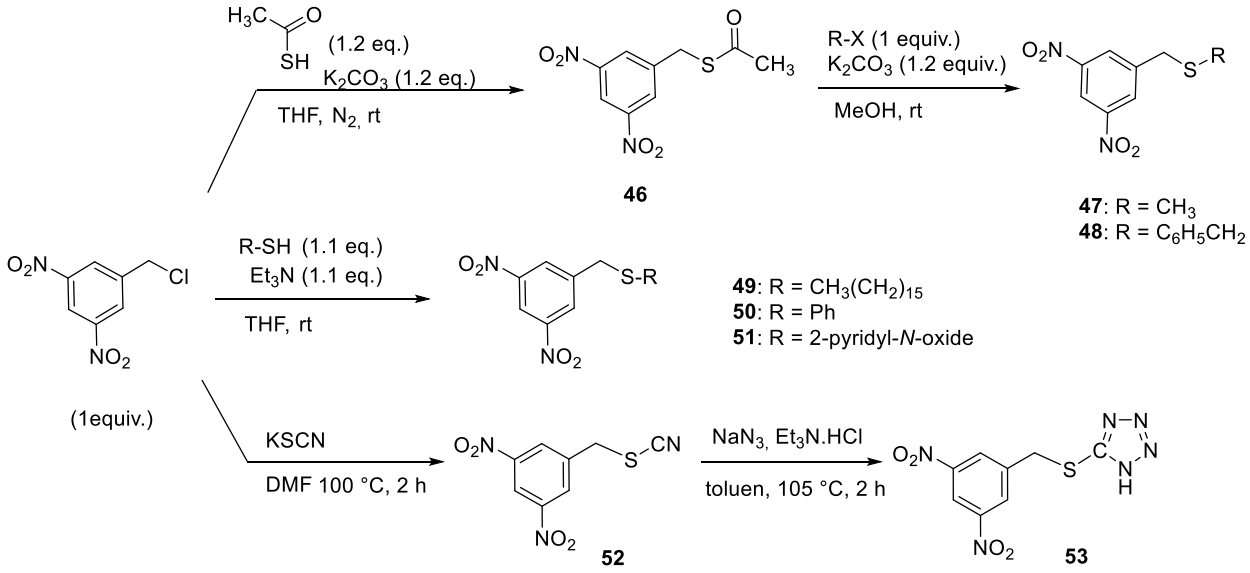
The antimycobacterial selectivity of the compounds **30b**, **34b**, **34d**, **39b** and **44b** with promising antimycobacterial activity was supported by the MIC values against 8 bacterial and 8 fungal strains. All compounds showed no antibacterial and no antifungal activity.

To further probe the selectivity of the studied compounds, the effects of selected substances on the viability of the Chinese hamster ovary (CHO-K1) cell line was evaluated. The resulting IC_{50} values indicated that there was no significant difference in the toxicity between sulfur or selenium derivatives. However, position of nitro group highly affected toxicity; the 2,4-dinitro derivatives were highly toxic regardless of their substitution (IC_{50} values of 8.5-22 μM). The toxicities of the 1-isomers were either similar or higher than the 2-isomers. Interestingly, the most toxic compound was 1-(3,5-Dinitrobenzyl)-5-[(3,5-dinitrobenzyl)selanyl]-1*H*-tetrazole **44d** containing four nitro groups in its structure with an IC_{50} of 6.5 μM, however, there is no clear correlation between toxicity and the number of nitro groups presented.

Moreover, 3,5-dinitrobenzyl moiety could not be connected with frame shift or base-exchange mutagenicity. (**III**)

7.3. Tetrazole-free compounds containing 3,5-dinitrobenzylsulfanyl moiety: synthesis and antimycobacterial efficiency (II)

To probe the importance of the heterocycle moiety for high antimycobacterial activity of 1-alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazole **7,** compounds **46**-**53** were designed and prepared with 3,5-dinitrobenzylsulfanyl moiety attached to selected alkyl and aryl groups (Scheme 11) instead of tetrazole. *S*-(3,5-Dinitrobenzyl) ethanethioate **46** was prepared followed by one-pot deacetylation together with Williamson reaction resulted in the formation of sulfides **47** and **48**. Alkylation of alkyl/arylthiols by 3,5-dinitrobenzyl chloride in THF in the presence of triethylamine led to the formation of sulfides **49**, **50** and **51**. 3,5-Dinitrobenzyl thiocyanate **52** was obtained by the reaction of 3,5-dinitrobenzyl chloride with excess of potassium thiocyanate in DMF and further was converted into corresponding 1*H*-tetrazole **53** by the [1+3] dipolar cycloaddition with sodium azide, in the presence of triethylammonium chloride in good yield (68%).



Scheme 11. Synthesis of compounds **46**-**53** containing 3,5-dinitrobenzylsulfanyl fragment

Overall, low antimycobacterial activity of tetrazole-free compounds indicates that tetrazole is also an important structural feature needed for high activity. Nonetheless, comparably high efficiency of derivatives **46** and **52** (Table 6) demonstrates that the attachment of 3,5-dinitrobenzylsulfanyl moiety to the electron-withdrawing group is beneficial for antimycobacterial efficiency. The absence of any antimycobacterial activity of acidic 1H-tetrazole **53** is likely connected with the ability of unsubstituted tetrazole ring to ionize at physiological pH (similarly with **7v**).

Table 6. *In vitro* antimycobacterial activities of selected tetrazole-free compounds; MIC (μM).

	M.tb. **My 331/88**	***M.avium*** **My 330/88**	***M.kansasii*** **235/80**	***M.kansasii*** **6509/96**
	14 / 21 days		7 / 14 / 21 days	
7u	1 / 1	32 / 32	1 / 1 / 1	1 / 2 / 2
46	8 / 16	62 / 125	16 / 32 / 125	62 / 125 / 500
52	4 / 4	32 / 62	2 / 8 / 16	4 / 8 / 16
INH	0.5 / 1	250 / 250	250 / 250	2 / 4 / 4

7.4. Synthesis and structure - antimycobacterial activity relationships study of 2-alkyl-5-alkylsulfanyl-1,3,4-oxadiazoles and 2-alkyl-5-alkylsulfanyl-1,3,4-thiadiazoles. (IV)

In extension to our search tetrazole core was replaced for 1,3,4-oxadiazole and 1,3,4-thiadiazole scaffolds. These five member heterocycles have attracted heightened interest due to their use as a bioisosteric replacement of carboxylic acids, esters or carboxamides like tetrazoles, and importantly, due to the broad spectrum of biological activities of their derivatives. Compounds based on 1,3,4-oxadiazoles and 1,3,4-thiadiazoles exhibited antibacterial, anti-inflammatory, antitumor and antiretroviral activities (116; 117) as well as noteworthy antiTB effect. (118; 119; 120; 121).

Derivatives of 4-(5-substituted-1,3,4-oxadiazol-2-yl)pyridine **54** bearing long, highly lipophilic pentadecyl and heptadecyl chains in position 5 showed 0.35 μM and

0.65 μM activities, respectively against *M.tb*. H37Rv and were highly efficient against drug-resistant strain CIBIN 112 (Fig.31). (122) Interestingly 4-[5-(3,5-dinitrophenyl)-1,3,4-oxadiazol-2-yl]pyridine showed weak antiTB activity. 2-{[5-(4-Methoxyphenyl)-1,3,4-oxadiazol-2-yl]sulfanyl}-*N*-phenethylacetamide **55** is another example of highly efficient derivative of 1,3,4-oxadiazole-5-thiol possessing IC_{90} value of 0.14 μg/mL. (123; 124; 125)

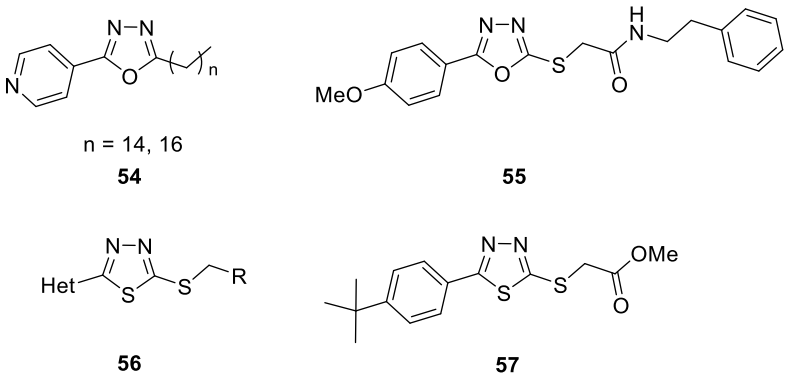
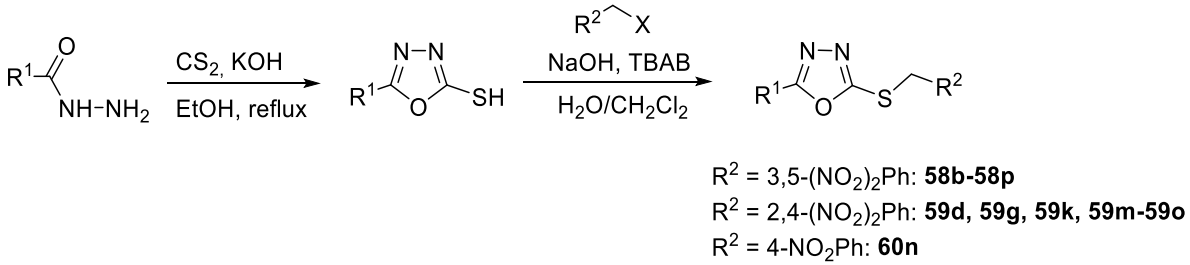


Figure 31. Structures of 1,3,4-oxadiazole and 1,3,4-thiadiazole derivatives with high antimycobacterial activities.

5-Alkylsulfanyl-1,3,4-thiadiazoles **56** with heteroaromatic substituents (5-nitro-2-thienyl, 5-nitro-2-furyl or 1-methyl-5-nitro-2-imidazolyl) at position 2 inhibited mycobacterial growth at ≥0.39 μg/mL. (126) Methyl {2-[(5-(4-*t*-butylphenyl]-1,3,4-thiadaizol-2-yl)thio} acetate **57** also presented high antimycobacterial activity with IC_{90} 0.2 μg/mL.

Therefore, a series of novel 2-alkyl/aryl-5-benzylsulfanyl-1,3,4-oxadiazoles **58**-**60** and 1,3,4-thiadiazoles **61**-**63** was synthesized. The influence of R^1 substituent at position 2 (Table 7) as well as the number and position of both nitro groups on benzyl fragment on the antimycobacterial activity was studied. Furthermore, derivatives with one or both reduced nitro groups (**64** and **65**, respectively) were prepared and their activities were compared with 3,5-dinitro analogs **58**.

Synthesis of 2-alkyl/aryl-5-benzylsulfanyl-1,3,4-oxadiazoles (**58b-58p**, **59d**, **59g**, **59k**, **59m-59o**, **60n**) was carried out according to a published method, (127) in four steps, starting from corresponding hydrazides (Scheme 7, Table 7).

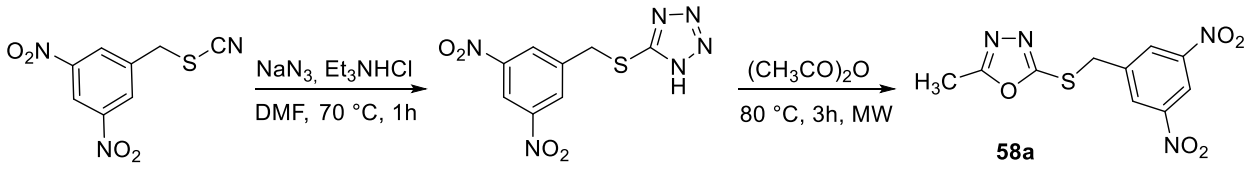


Scheme 12. Synthesis 2-alkyl/aryl-5-benzyl-1,3,4-oxadiazoles **58-60**.

Table 7. R^1 substituents of compounds **58-65.**

a	CH_3	**e**	2-ClPh	**i**	4-CH_3Ph	**m**	3-NO_2Ph
b	$CH_3(CH_2)_9$	**f**	3-ClPh	**j**	3-CH_3OPh	**n**	4-NO_2Ph
c	C_6H_{11}	**g**	4-ClPh	**k**	4-CH_3OPh	**o**	Pyridine-4-yl
d	Ph	**h**	2,4-Cl_2Ph	**l**	3,5-$(CH_3O)_2$Ph	**p**	3,5-$(CH_3)_2$Ph

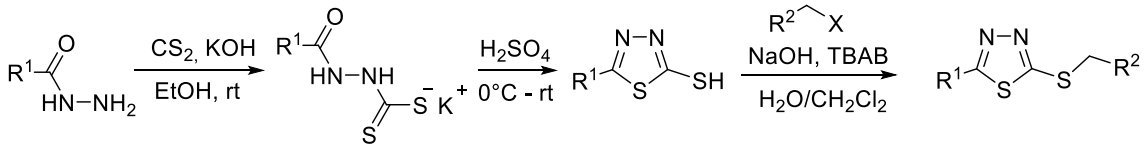
Alkyl/arylhydrazides were converted into 2-substituted-1,3,4-oxadiazole-5-thiols by the reaction with carbon disulfide and KOH in ethanol, which were then alkylated with alkyl halide. The target 2-alkyl/aryl-5-alkylsulfanyl-1,3,4-oxadiazoles **58**-**60** were obtained in high yields (45-96%). The reaction of 5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazole with acetic anhydride afforded 2-[(3,5-dinitrobenzyl)sulfanyl]-5-methyl-1,3,4-oxadiazole (**58a**) (Scheme 13). (128; 129)



Scheme 13. Synthesis of 2-[(3,5-dinitrobenzyl)sulfanyl]-5-methyl-1,3,4-oxadiazole (**58a**).

2-Aryl-5-benzylsulfanyl-1,3,4-thiadiazoles were synthesized according to a protocol depicted in scheme 14. Reaction of the substituted benzohydrazides with carbon disulfide and KOH in ethanol at room temperature resulted in formation of corresponding potassium 2-aroylhydrazine-1-carbodithioate, which were then

cyclized to the corresponding 2-aryl-1,3,4-thiadiazole-5-thiols in low to moderate yields (35-76%) using concentrated sulfuric acid. Alkylation of 2-aryl-1,3,4-thiadiazole-5-thiols under the conditions of phase-transfer catalysis led to the formation of target 2-aryl-5-benzylsulfanyl-1,3,4-thiadiazoles **61a**, **61d-61g**, **61i-61k**, **61m-61p**, **62d**, **62g**, **62n** and **63n** in good yields (48-87%).



R^1 = table 7
R^2 = 3,5-$(NO_2)_2$Ph: **61a, 61d-61g, 61i-61k, 61m-61p**
R^2 = 2,4-$(NO_2)_2$Ph: **62d, 62g, 62n**
R^2 = 4-NO_2Ph: **63n**

Scheme 14. Synthesis 2-aryl-5-benzyl-1,3,4-thiadiazoles **61-63**.

Antimycobacterial activity of prepared compounds **58**-**63** against four mycobacterial strains was assessed. 3,5-Dinitrobenzyl derivatives of 1,3,4-oxadiazole **58** and 1,3,4-thiadiazole **61** displayed excellent antimycobacterial activity against all tested strains (MIC values as low as 0.03 μM; 0.01 μg.mL^{-1}). Even 2,4-dinitrobenzyl derivatives **59** and **62** exhibited significant antimycobacterial efficiency (MIC 0.5 - 2 μM against *M.tb.*). (**IV**)

The importance of two nitro groups and their 3,5-position on benzylsulfanyl fragment for high antimycobacterial activity was unambiguously demonstrated in both series of 1,3,4-oxadiazoles and 1,3,4-thiadiazoles (Table 8). Substances **58** and **61** bearing 3,5-dinitrobenzylsulfanyl moiety proved to be considerably more active than first-line antiTB drug INH and 2,4-dinitrobenzyl derivatives **59** and **62**.

2,4-Dinitrobenzyl derivatives of 1,3,4-oxadiazoles **59** and 1,3,4-thiadiazoles **62** showed comparable antimycobacterial activity with above described 1-alkyl/aryl-5-(3,5-dinitrobenzylsulfanyl)-1*H*-tetrazoles **7,** and **30** and their isosteric derivatives. As in previous cases, compounds **59** and **62** containing 2,4-dinitrobenzyl fragment showed to be better antimycobacterial agents than 4-nitrobenzyl derivatives **60** and **63**.

Table 8. *In vitro* antimycobacterial activities of selected compounds of series **58**-**61** expressed in MIC (μM).

	M.tb. **My 331/88**	***M. avium*** **My 330/88**	***M. kansasii*** **My 235/80**	***M. kansasii*** **6509/96**
58c	0.03 / 0.03	n.d.	0.06 / 0.125 / 0.25	0.06 / 0.25 / 0.25
58d	0.06 / 0.06	16 / 32	0.5 / 1 / 1	0.5 / 1 / 2
58g	0.125 / 0.125	>125 / >125	0.125 / 0.25 / 0.25	0.125 / 0.25 / 0.5
58k	0.125 / 0.125	16 / 32	0.125 / 0.25 / 0.25	0.125 / 0.25 / 0.5
58n	0.125 / 0.125	>125 / >125	0.25 / 0.25 / 0.5	0.25 / 0.5 / 1
59k	0.5 / 1	4 / 4	2 / 8 / 16	2 / 8 / 16
59m	16 / 16	32 / 32	8 / 16 / 32	16 / 16 / 32
59n	2 / 2	>125 / >125	2 / 4 / 8	2 / 4 / 8
60n	250 / 250	250 / 250	250 / 250 / 250	62 / 62 / 62
61d	0.06 / 0.125	8 / 16	0.25 / 0.5 / 1	0.25 / 0.5 / 1
61k	0.125 / 0.125	8 / 16	0.25 / 1 / 1	0.25 / 1 / 1
61n	0.125 / 0.125	>16 / >16	0.25 / 0.5 / 1	0.25 / 0.5 / 1
62d	8 / 16	8 / 32	1 / 4 / 4	1 / 2 / 4
62n	16 / 32	250 / 250	1 / 4 / 8	1 / 4 / 8
63n	62 / 62	250 / 250	250 / 250 / 250	62 / 62 / 62
64n	125 / 125	250 / 250	250 / 250 / 250	250 / 250 / 250
65n	250 / 250	250 / 250	250 / 250 / 250	250 / 250 / 250
66*	1 / 1	4 / 8	1 / 2 / 4	1 / 1 / 2
INH	**0.5 / 1**	**>250 / >250**	**>250 / >250 / >250**	**2 / 4 / 4**

* - unpublished data
n.d. - not determined

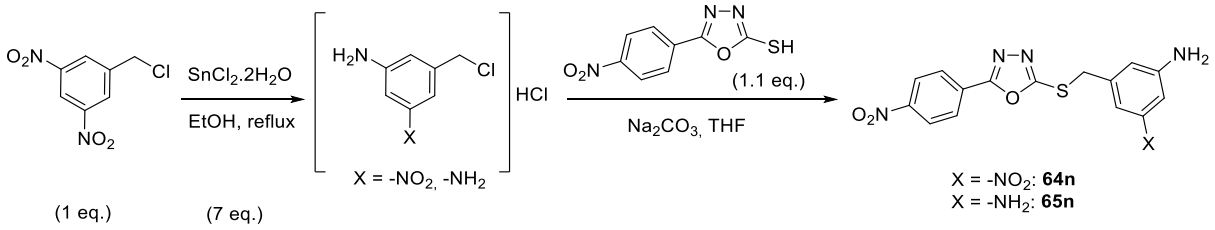
Effect of the substituent at position 2 of 2-alkyl/aryl-5-benzylsulfanyl-1,3,4-oxadiazoles **58**, **59** and 2-aryl-5-benzylsulfanyl-1,3,4-thiadiazoles **61** on antimycobacterial activity is identical as in the case of abovementioned compounds of series **7**, **8**, **16**, and **24**. Lower activities of 2-[(3,5-dinitrobenzyl)sulfanyl]-5-methyl-

1,3,4-oxadiazole (**58a**) and its thiadiazole analog (**61a**) are likely associated with its lower lipophilicity.

It was also observed that the antimycobacterial efficiency of prepared compounds **58**, **59** and **61** is not influenced by type of substituent on phenyl ring. Compounds with electron-donating and withdrawing substituents exhibited similar potency.

The loss of antimycobacterial activity after partial or full reduction of dinitrobenzyl fragment (**64n**, **65n**, table 8) is a direct proof of high significance of both nitro groups on benzylsulfanyl moiety. Derivatives with one or two amino groups were synthesized according to above described method for tetrazole derivatives **11** and **12** (Scheme 15).

Earlier it was concluded that replacement of sulfur with selenium or oxygen in linker between the 3,5-dinitrobenzyl and the tetrazole moieties only slightly affected the antimycobacterial activity of prepared compounds (Table 3, 5, and 8).

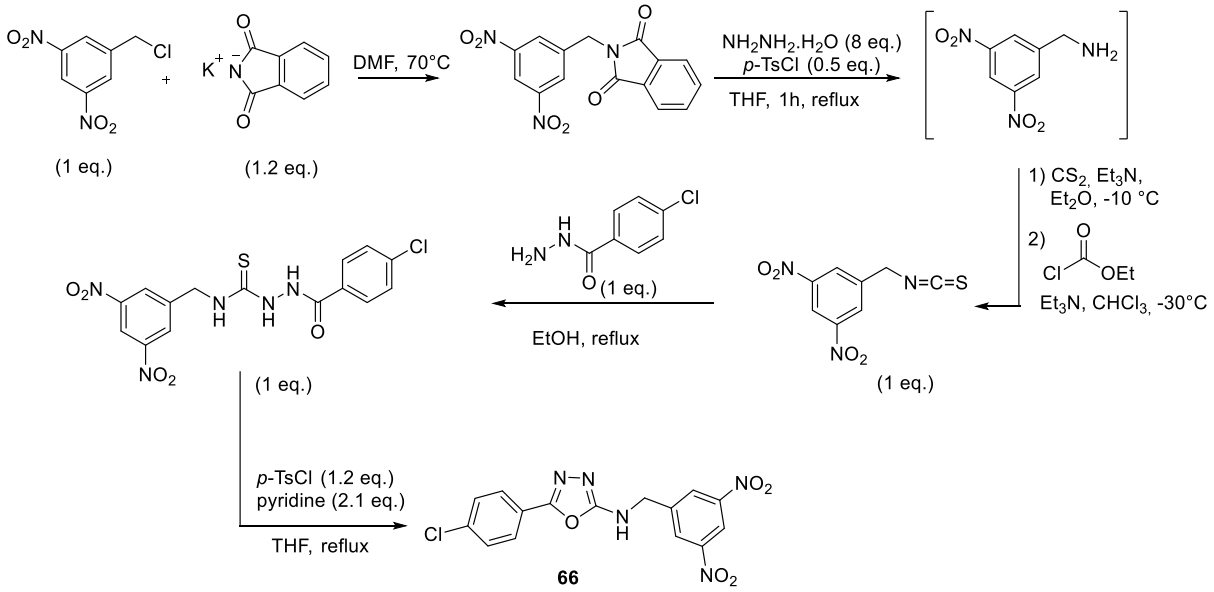


Scheme 15. Synthesis of partially and fully reduced derivatives **64n** and **65n**.

In order to explore the effect of nitrogen as a heteroatom in linker, the sulfur atom of 2-(4-chlorophenyl)-5-[(3,5-dinitrobenzyl)sulfanyl]-1,3,4-oxadiazole **58g** was replaced with amine group. *N*-[2-(4-Chlorophenyl)-1,3,4-oxadiazol-5-yl]-*N*-(3,5-dinitrobenzyl)amine **66** was synthesized according to method shown in scheme 16 and tested for antimycobacterial activity (Table 8).

The starting material, 3,5-dinitrobenzyl amine was prepared by Gabriel synthesis via the reaction of 3,5-dinitrobenzyl chloride with a slight excess of potassium phthalimide in DMF followed with the cleavage of *N*-(3,5-dinitrobenzyl)phthalimide by hydrazine monohydrate in the presence of *p*-tosyl chloride in THF. It resulted in the formation of desired amine in 32% yield over two reaction steps. By well-known approaches (130) 3,5-dinitrobenzyl amine was

converted into isothiocyanate in 15% yield (over two steps), which was reacted with 4-chlorobenzohydrazide. The resultant 1-(4-chlorobenzoyl)-4-(3,5-dinitrobenzyl)thiosemicarbazide, in final step, was cyclized to target *N*-[2-(4-chlorphenyl)-1,3,4-oxadiazol-5-yl]-*N*-(3,5-dinitrobenzyl) amine **66** by a treatment with *p*-tosyl chloride and pyridine in THF at reflux. Target compound was obtained in high yield (68%).



Scheme 16. Synthesis of *N*-[2-(4-chlorophenyl)-1,3,4-oxadiazol-5-yl]-*N*-(3,5-dinitrobenzyl)amine **66**

A significant decrease of antimycobacterial activity of **66** against *M.tb*. and both strains of *M.kansasii* was observed in comparison with sulfur derivative **58g.** Although, its efficiency remained high and comparable with the antimycobacerial activity of 2-alkyl/aryl-5-[2,4-dinitrobenzyl)sulfanyl]-1,3,4-oxadiazoles **59** and previously prepared heterocyclic compounds bearing 3,5-dinitrobenzyl fragment (**7**, **16**, **24**, **30**, **34**, **39**, **44**).

Selected derivatives of 1,3,4-oxadiazoles and 1,3,4-thiadiazoles bearing 3,5-dinitrobenzyl group (**58d, 58k, 58n, 61k, 61n**) were tested against clinically isolated MDR strains of *M.tb*. (234/2005; 9449/2007; 8666/2010; Prague 1; Prague 4; Prague 131). These compounds displayed outstanding activities against all six strains with MIC values 0.03 - 0.25 μM. Finally, it is proved that prepared compounds of series

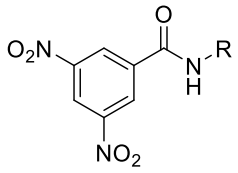
58, **59**, **61** and **62** are selective antimycobacterial agents as they were antibacterially and antifungally inactive and showed low toxicities against human cell lines.

Outstanding selective antimycobacterial activity of 2-alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfanyl]-1,3,4-oxadiazoles **58** and 2-aryl-5-[(3,5-dinitrobenzyl)sulfanyl]-1,3,4-thiadiazoles **61** against drug-susceptible and drug-resistant strains of *M.tb.* enable to postulate that these substances possess new mechanism of action than currently used antiTB drugs.

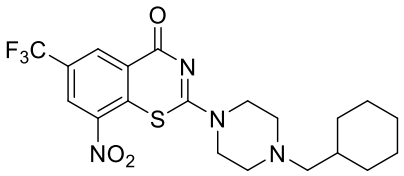
8. Hypothetical mechanism of action of compounds bearing 3,5-dinitrobenzylsulfanyl fragment

Compounds with highest antimycobacterial activity prepared in this work contain 3,5-dinitrobenzyl fragment (**7**, **16**, **24**, **30**, **34**, **39**, **44**, **58**, **61**). Initially it was supposed that these compounds could exhibit the same mode of action as 3,5-dinitrobenzamides due to presence of two nitro groups in the same position on phenyl ring.

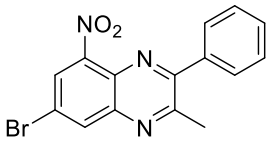
Mechanism of action of variously *N*-substituted 3,5-dinitrobenzamides (77; 78) (Fig.32) lies in the inhibition of cell wall biosynthesis by blocking DprE1.



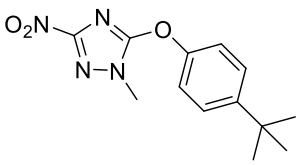
R= -CH_2CH_2O(4-MeOPh): **DNB-1** MIC 0.2 μM
R= 4-BrPh: MIC 20 μM



PBTZ-169
MIC 0.4 nM



VI 9376
MIC 3.1 μM



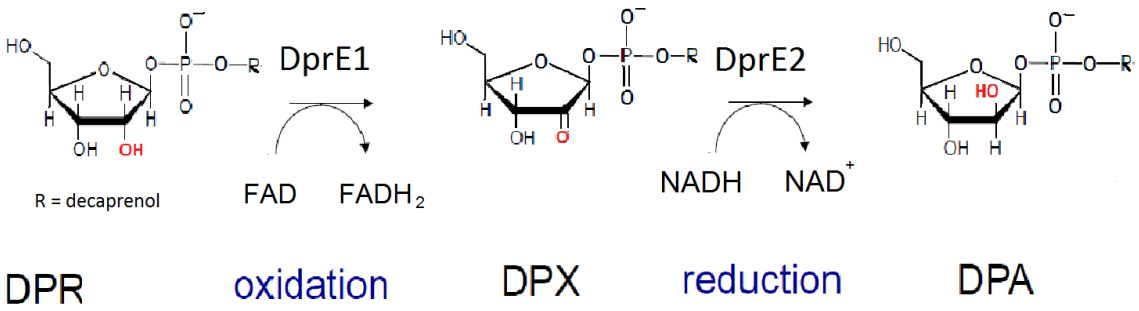
377790
MIC 0.5 μM

Scheme 32. DprE1 inhibitors

DprE1 is an essential enzyme in arabinan biosynthesis and is involved in epimerization of decaprenylphosphoryl-*β*-D-ribose (DPR) to decaprenylphosphoryl-*β*-D-arabinose (DPA), which is the only arabinose source for cell wall biosynthesis. Nitro-substituted quinoxaline VI-9376 (79) and nitro-substituted triazole 377790 (80) also act as DprE1 inhibitors.

The most effective DprE1 inhibitor being in preclinical development is PBTZ-169, piperazine-containing benzothiazinone (Fig.32). It is known that benzothiazinones are pro-drugs, which undergo the bioreduction of nitro group and

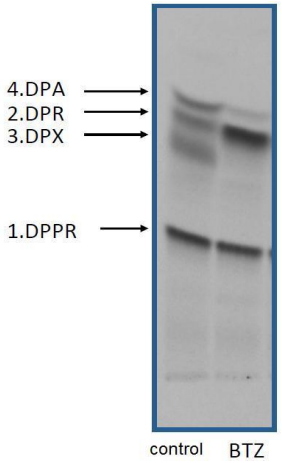
formed active nitroso metabolites covalently bind to Cys 287 of DprE1. Thus benzothiazinones inhibit DprE1 irreversibly.



DPR - decaprenylphosphoryl-*β*-D-ribose; DPX - decaprenylphosphoryl-D-2′-keto-erythro-pentofuranose; DPA - decaprenylphosphoryl-*β*-D-arabinose; FAD - flavin adenine dinucleotide; NAD – nikotinamid adenindinukleotid

Figure 33. Epimerization of DPR to DPA via keto intermediate DPX.

Derivatives of this class are highly *in vitro* and *in vivo* active in nanomolar concentration against both susceptible and drug-resistant (MDR and XDR-TB) *M.tb*. strains. Mechanism of action of BTZ derivatives was established using mycobacterial membranes *in vitro* (Fig. 33, 34). Both DprE1 and DprE2 are needed for the epimerization of DPR to DPA. Process proceeds via formation of intermediate, decaprenylphosphoryl-D-2′-keto-erythro-pentofuranose (DPX). DprE1 oxidases DPR to DPX through a flavin adenine dinucleotide (FAD)-dependent mechanism, DprE2 reduces the DPX to DPA (75; 130) in the presence of nicotinamide adenine dinucleotide (NAD) as a cofactor.



Briefly, mixture of cell wall proteins, radiolabel 5-phosphoribose diphosphate (DPPR) and BTZ was stirred and then worked out by extraction of lipids with organic solvent. Lipids analysis by thin layer chromatography was performed. It was observed that keto intermediate DPX and DPA were not formed in the presence of BTZ. It indicated that BTZ inhibits only DprE1.

Figure 34. Identification of cellular target of BTZ

DprE1 inhibitory activity of three compounds (**7u**, **46i** and **49g**), which were prepared in this work and displayed the highest antimycobacterial activity, along with compounds **3** (Fig.34), were evaluated.

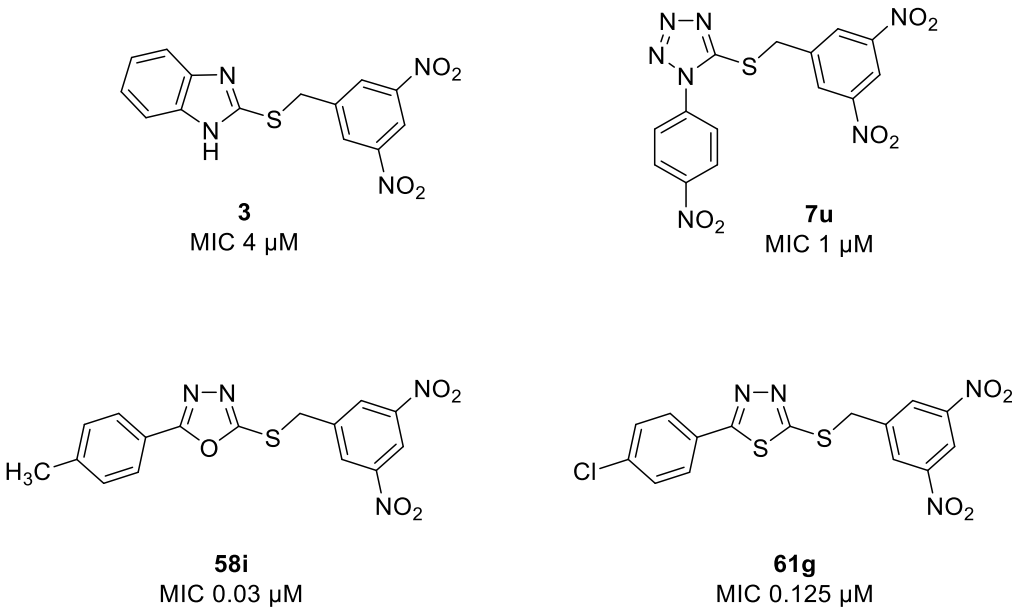


Figure 34. Compounds bearing 3,5-dinitrobenzylsulfanyl moiety, which were evaluated for mechanism of action

Experiments performed by group of prof. Mikušová[12] identified that all these compounds do not inhibit epimerization of DPR to DPA. The specific cellular target of compounds **3**, **7u**, **58i** and **61g** remains to be elucidated.

[12] Department of Biochemistry, Faculty of Natural Sciences, Comenius University, Mlynská dolina, 84215 Bratislava, Slovakia.

9. Conclusion

Tuberculosis (TB) – is a dangerous infectious disease caused by *Mycobacterium tuberculosis* (*M.tb.*), which is carried by airborne droplets and affects predominantly the lungs. Nowadays, the antiTB therapy (DOTS) provides successful treatment of disease caused by susceptible *M.tb*. strains with 90% cure rate. However, dissemination of multi-drug resistant (MDR-TB) and extensively drug resistant (XDR-TB) strains remains the major difficulty in the current TB treatment as the options of their therapy are limited; second-line antiTB are low tolerable, highly toxic and low efficient. Number of patients infected by drug-resistant strains of *M.tb*. is increasing every year. Global dissemination of TB remains enormous and this situation has not been changed the past ten years: annually there were around 9 million of new TB cases and approx. 1.5 million of deaths.

Aim of this work was to find the new heteroaromatic compounds with high and selective antimycobacterial activity and to study their structure - antimycobacterial activity relationships.

This work was based on previous observation that benzazoles bearing dinitrobenzylsulfanyl fragment (Fig.35) exhibited promising antimycobacterial efficiency with MIC against *M.tb*. in the range 1-8 μM, whereas dinitrobenzyl moiety was considered as a main fragment responsible for the high activity of studied compounds. Therefore, the benzazole core was replaced with the different heterocycles and the antimycobacterial activity was studied. Tetrazole, 1,3,4-oxadiazole and 1,3,4-thiadiazole scaffolds were chosen because compounds containing such heterocycles were shown to display broad spectrum of biological properties. (100; 116; 117)

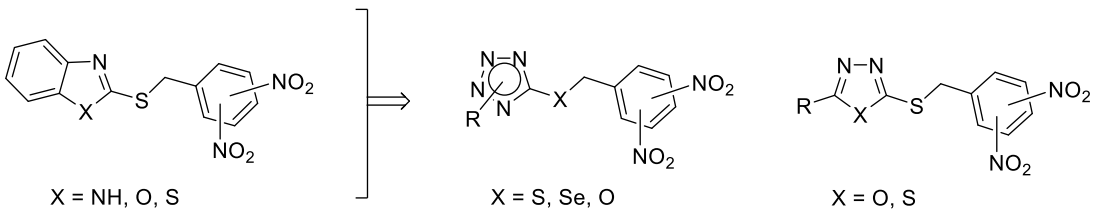


Figure 35. Previously studied compounds and target compounds prepared in this study

In this work, a series of 1-alkyl/aryl-5-alkylsulfanyl-1*H*-tetrazoles, 2-alkyl-5-alkylsulfanyl-2*H*-tetrazoles, 2-alkyl/aryl-5-alkylsulfanyl-1,3,4-oxadiazoles and 1,3,4-thiadiazoles were prepared and the role of an individual structural fragments of these compounds on antimycobacterial activity was investigated (Fig. 36, 37, 38).

All compounds prepared in this work were evaluated against *M.tb*. My 331/88 and non-tuberculosis strains *M.avium* My 330/88, *M.kansasii* My 235/80 and clinically isolated *M.kansasii* 6509/96.

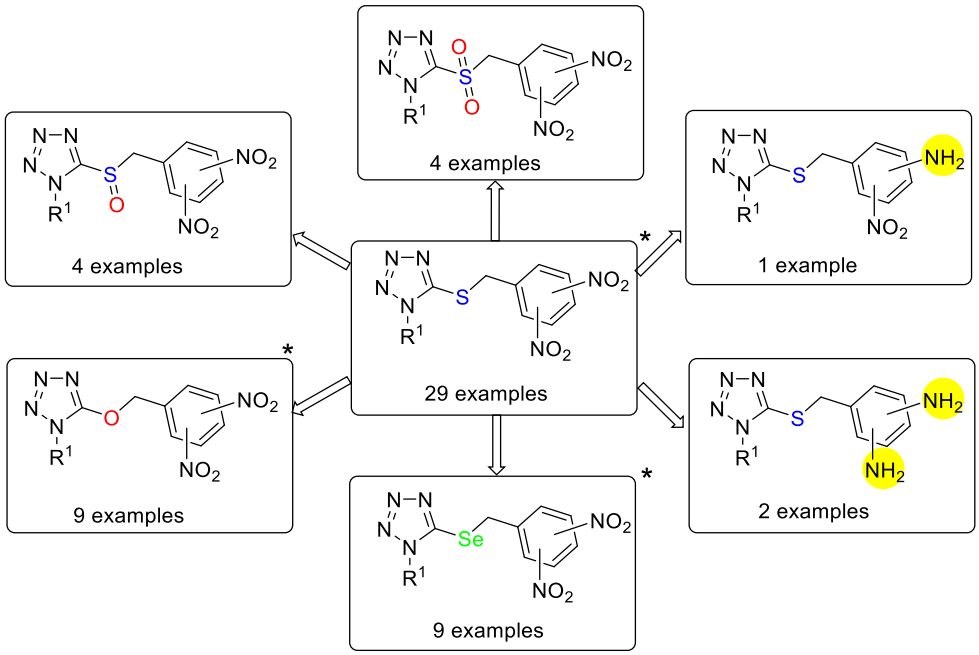


Figure 36. Structure – antimycobacterial activity relationships study of 1-substituted-5-alkyl/arylsulfanyl-1*H*-tetrazoles and their isosteric analogs. (**I, II**)

A few trends were observed in relationships between the structures and antimycobacterial activity of prepared compounds, which are as follows:

1. The role of various alkyl/aryl substituents R^1 at positions 1 or 2 of tetrazole core, at position 2 of 1,3,4-oxadiazole or 1,3,4-thiadiazole cycle in compounds efficiency consists in the determination of their physicochemical properties. Generally, more lipophilic compounds displayed higher activity. Derivatives, which were able to ionize at physiological pH showed no antimycobacterial

activity. Thus, substituent R^1 is considered as a fragment for further optimization of the activity/toxicity and the ADME properties.

2. The number and certain position of nitro groups on benzyl moiety had considerable influence on antimycobacterial activity of target compounds: in each series, 3,5-dinitrobenzyl derivatives had significantly higher efficiency than 2,4-dinitrobenzyl derivatives, which were more effective than 4-nitrobenzyl analogs or benzyl analogs.
3. 3,5-Dinitrobenzyl fragment was needed to be placed on heteroatom at position 5 of tetrazole, in order to reach high antimycobacterial activity.

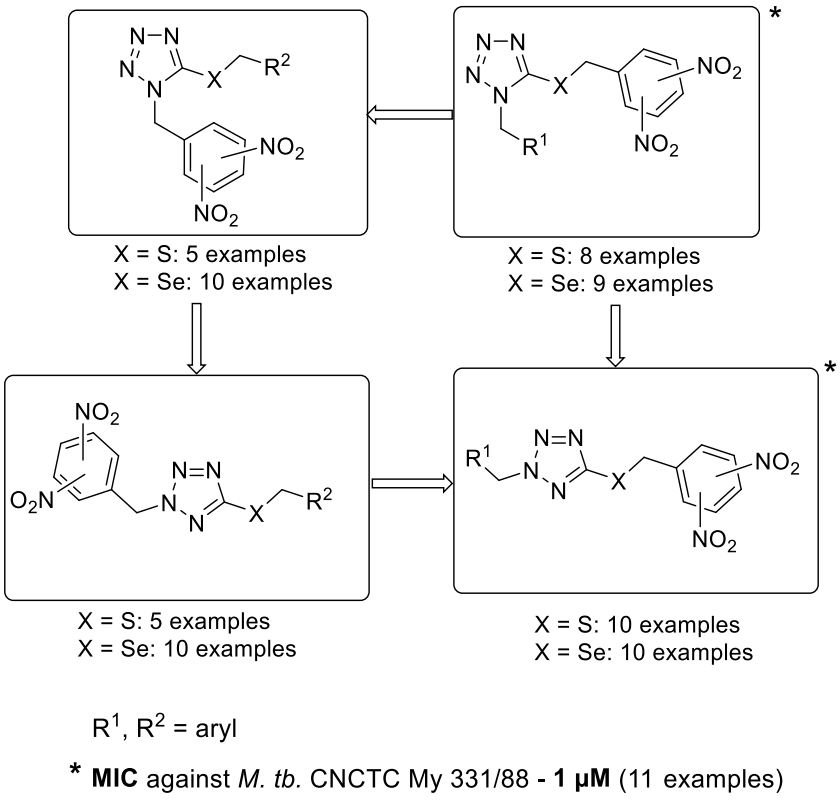


Figure 37. Sulfanyl/selanyl tetrazole regioisomers: structure – antimycobacterial activity relationships study. (**III**)

4. Less polar 2-alkyl-5-alkylsulfanyl-2*H*-tetrazoles and their selenium analogs exhibited higher activity than their 1-isomers.
5. The heteroatom in linkage between dinitrobenzyl moiety and tetrazole core had slight effect on antimycobacterial activity of prepared compounds; efficiencies of isosteric sulfur, selenium and oxygen analogs were similar.

6. Partial or full oxidation of sulfur atom in 1-alkyl/aryl-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles as well as partial or full reduction of 3,5-dinitrobenzyl fragment of corresponding tetrazoles and 1,3,4-oxadiazoles led to a significant decrease of antimycobacterial activity.
7. The heterocyclic core proved to be an important structural feature, which significantly influenced the antimycobacterial activity of prepared compounds. 2-Alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfanyl]-1,3,4-oxadiazoles and 1,3,4-thiadiazoles exhibited considerably higher antimycobacterial activity than corresponding 1*H*-and 2*H*-tetrazoles.

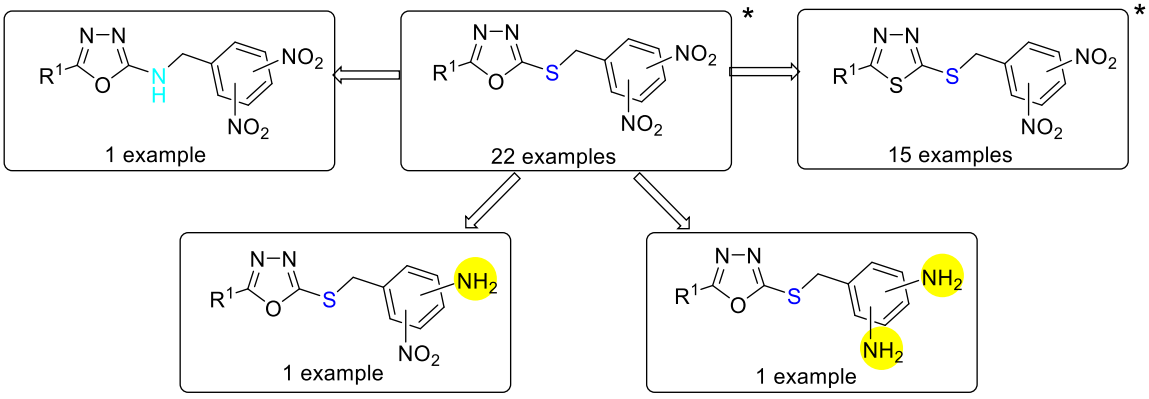


Figure 38. Structure - antimycobacterial activity relationships study of 2-alkyl-5-alkylsulfanyl-1,3,4-oxadiazoles and 2-alkyl-5-alkylsulfanyl-1,3,4-thiadiazoles. (**IV**)

8. The compounds containing 3,5-dinitrobenzylsulfanyl fragment showed the highest antimycobacterial activity in each series and proved to be highly effective against both drug-susceptible and drug-resistant strains of *M.tb*. Their action was selective for mycobacteria, while they displayed no antibacterial and antifungal activities. Moreover, 3,5-dinitrobenzyl derivatives had low cellular toxicity.

Within this work, 1-alkyl/aryl-2-[(3,5-dinitrobenzyl)sulfanyl]-1,3,4-oxadiazoles and 1,3,4-thiadiazoles were identified as new lead structures for further development of potent antiTB heterocyclic compounds. These derivatives showed the highest

antiTB activity among the studied compounds with MIC 0.03 μM against *M.tb*. The study of additional tetrazole derivatives will also be also continued as these compounds have shown good prediction of ADME properties (ADMET Predictor ™).

Furthermore, another large series of nitro group-containing heterocyclic compounds have been synthesized and studied. These compounds showed superior antiTB activities and low toxicities against human cells. As these compounds are included in patent application, which is currently under review, their structure, synthesis and antimycobacterial data cannot be revealed.

The results of these investigations were published as three research articles in peer reviewed scientific journals or were patented.

These results deal with:

1. the development and optimization of synthetic approaches to 1-alkyl/aryl-5-alkylselanyl-1*H*-tetrazoles (**I**);
2. the synthesis and structure - antimycobacterial activity relationship study of 1-alkyl/ary-5-alkylsulfanyl-1*H*-tetrazoles and their isosteric analogs (**II**).
3. the synthesis and structure - antimycobacterial activity relationship study of 1*H*- and 2*H*-regioisomers of sulfanyltetrazoles and selanyltetrazoles (**III**).
4. the synthesis and structure - antimycobacterial activity relationships study of 2-alkyl/aryl-2-alkylsulfanyl-1,3,4-oxa- and 1,3,4-thiadiazoles (**IV**).

10. References

1. Daniel, T. M. The history of tuberculosis. *Respir. Med.* **2006**, 100, 1862-1870.
2. Gagneux, S.; DeRiemer, K.; Van, T.; Kato-Maeda, M.; de Jong, B. C.; Narayanan, S.; Nicol, M.; Niemann, S.; Kremer, K.; Gutierrez, M. K.; Hilty, M.; Hopewell, P. C.; Small, P. M. Variable host–pathogen compatibility in Mycobacterium tuberculosis. *Proc. Natl. Acad. Sci USA.* **2006**, 103 (8), 2869-2873.
3. Canci, A.; Minozzi, S.; Borgognini Tarli, S.M. New evidence of Tuberculous spondylitics from Neolithic Liguria (Italy). *Inter. J. Osteoarchaeology.* **1996,** 6, 497-501.
4. Formicola, V.; Milanesi, Q.; Scarsini, C. Evidence of spinal tuberculosis at the beginning of the fourth millennium BC from Arene Candide cave (Liguria, Italy). *Am. J. Phys. Anthropol.* **1987**, 72 (1), 1-6.
5. Zink, A. R.; Sola, C.; Reischl, U.; Grabner, W.; Rastogi, N.; Wolf, H.; Nerlich, A. G. Characterization of Mycobacterium tuberculosis Complex DNAs from Egyptian Mummies by Spoligotyping. *J. Clin. Microbiol.* **2003**, 41 (1), 359-367.
6. Hippocrates. Book 1, Of the Epidemics. [trans.] Francis Adams. Written 400 B.C.E. Available at http://classics.mit.edu/Hippocrates/epidemics.1.i.html
7. Hippocrates. Epidemic, Book 1 amd 3. 400 B.C.E. Available at: http://perseus.uchicago.edu/perseus-cgi/citequery3.pl?dbname=GreekFeb2011&getid=2&query=Hipp.%20Epid.%201.1.
8. Herzog, H. History of Tuberculosis. *Respiration.* **1998**, 65, 5-15.
9. Brown, L.; Brown, M. L. H. The story of clinical pulmonary tuberculosis. Baltimore: Williams & Wilkins, 1941.
10. Blackmore, E. A practical treatise on the forms, causes, sanability and treatment of pulmonary consumption. Longman, Rees and Co. W. Curry, Jun. and Co. Dublin. Waugh and Innes, Edinburgh. Rowe, Plymouth., **1832**.
11. ARETAEUS. Causes and Symptoms of Chronic Diseases. Book I. Chapter VIII. On Phthisis. [trans.] Francis Adams. 1972. Available at: http://books.google.cz/books?id=9x8UAAAAQAAJ&pg=PR15&lpg=PR15&dq=ARETAEUS,+Causes+and+Symptoms+of+Chronic+Diseases&source=bl&ots=QL_IFFH0Zn&sig=ckT8vjHdCAUxfksQAs_iOnEOxe4&hl=cs&sa=X&ei=GfQfVK62FoWOOLvEgDA&ved=0CC4Q6AEwBQ#v=onepage&q=ARETAEUS%2C%20Causes%20and%20Symptoms%20of%20Chronic%20Diseases&f=false; http://www.perseus.tufts.edu/hopper/text?doc=Aret.+SD+1
12. Frith, J. History of Tuberculosis. Part 1 - Phthisis, consumption and the White Plague. *JMVH*, **2014**, 22 (2), 29-35

13. Pease, A.S. Some remarks on the diagnosis and treatment of tuberculosis in antiquity. *The History of Science Society.* **1940**, 31 (2), 380-393.
14. Avicenna Abu Ali Ibn Sina “The Canon of Medicine”. Completed in 1025. Available at: http://www.e-reading.me/book.php?book=24541. (In Russian); https://archive.org/details/AvicennasCanonOfMedicine (in English).
15. Trail, R. R. Richard Morton (1637-1698). *Med. Hist. Apr*. **1970**, 14 (2), 66–174.
16. Benjamin, M. A new theory of consumptions: more especially of a phthisis, or consumption of the lungs. London : **1722**. Available at: http://pds.lib.harvard.edu/pds/view/7782041?n=9&imagesize=1200&jp2Res=0.5&printThumbnails=no.
17. Wardrop, J. Works of Matthew Baillie, M. D. Morbid Anatomy of the most important parts of the Human Body. Chapter IV. Diseases appearences of the Lungs. London. **1925**, 63-68. Available at: http://books.google.cz/books?id=h1o3AQAAMAAJ&pg=RA1-PA66&lpg=RA1-PA66&dq=Matthew+Baille+phthisis&source=bl&ots=XNV_E8X6ng&sig=tvfguPXxYoE3DtmpwJVIgUnEwv4&hl=cs&sa=X&ei=V7knVOb1I8PYarH9gPgH&ved=0CBsQ6AEwAQ#v=onepage&q&f=false.
18. Garrison, F. H. An introduction to the History of Medicine with medical chronology, suggestions for study and bibliographic data. Third edition, revised and enlarged. Philadelphia and London : W. B. Saunders company, **1921**.
19. Roguin, A. René Théophile Hyacinthe Laënnec (1781-1826): the man behind the stethoscope. *Clin. Med. Res.,* **2006**, 4 (3), pp. 230-235.
20. Parke, J. L., [trans.]. Felix von Niemeyer's clinical lectures on pulmonary phthisis. New York : Moorhead, Sympson & Bond., **1868**. Available at: https://archive.org/stream/felixvonniemeyer00niem#page/n7/mode/2up.
21. Rokitansky, K. Pathological anatom. [trans.] W. E. Swaine. Volume 1. General pathological anatomy. Chapter IX. Tubercle – Tuberculosis. Philadelphia : Blanchard & Lea, **1855**, 223 -247.
22. Guthrie, D. A History of Medicine. London : Thomas Nelson, **1945.** Available at: https://archive.org/details/historyofmedicin035119mbp.
23. Robert Koch and Tuberculosis. NobelPrize.org. http://www.nobelprize.org/educational/medicine/tuberculosis/readmore.html. September 21, 2003.
24. Sakula, A. Robert Koch: centenary of the discovery of the tubercle bacillus, 1882. *Thorax*, **1982**, 37, 246-251.
25. Frith, J. History of Tuberculosis. Part 2 - the Sanatoria and the Discoveries of the Tubercle Bacillus. *JMVH*. **2014**, 22 (2), 36-41.

26. The Nobel Prize in Physiology and Medicine 1952. Selman A. Waksman - Biographical. NobelPrize.org. http://www.nobelprize.org/nobel_prizes/medicine/laureates/1952/waksman-bio.html. [Online] September 30, 2014.
27. Begg, E. J.; Barcley, M. L. Aminoglycosides-50 years on. *Br. J. Clin. Pharmac.* **1995**, 39, 597-603.
28. Mitchison, D. A. The Diagnosis and Therapy of Tuberculosis During the Past 100 Years. *Am. J. Respir. Crit. Care Med.* **2005**, 171, 699-706.
29. Iseman, M.D. Tuberculosis therapy: past, present and future. *Eur. Respir. J.* **2002**, 20 (36), 87s-94s.
30. Da Silva, P. E. A.; Palomino, J. K. Molecular basis and mechanisms of drug resistance in *Mycobacterium tuberculosis*: classical and new drugs. *J. Antimicrob. Chemother.* **2011**, 66, 1417-1430.
31. Janin, Y. L. Antituberculosis drugs: Ten years of research. *Bioorg. Med. Chem*. **2007**, 15, 2479-2513.
32. Hoelscher, M. Advances in new TB drug development. http://tb.lshtm.ac.uk/sites/tbcentre.lshtm.ac.uk/files/attachments/pages/Hoelscher%20TB%20DRUGS-London2014.pdf. October 14, 2014.
33. Tomlinson, C. TB online. Pyrazinamide. http://www.tbonline.info/posts/2011/9/1/pyrazinamide/. September 1, 2011.
34. Zhang, Y.; Mitchison, D. The curious characteristics of pyrazinamide: a review. *Int. J. Tuberc. Lung Dis.* **2003**, 7 (1), 6-21.
35. Vandal, O. H.; Nathan, C. F.; Ehrt, S. Acid Resistance in *Mycobacterium tuberculosis*. *J. Bacteriol.* **2009**, 191 (15), 4714–4721.
36. Dougherty, T. J.; Pucci, M. J. R. Antibiotic Discovery and Development. London : Springer Science+Business Media, **2012**. Available at: http://books.google.cz/books?id=E6Dv5XsXU-IC&pg=PA238&lpg=PA238&dq=kanamycin+discovery&source=bl&ots=DWpLE2qXLf&sig=5kup0lMEAJcEnz3lAzYxYdElb74&hl=cs&sa=X&ei=6WwtVPuNCMHXPMHzgJgC&ved=0CB8Q6AEwAzgK#v=onepage&q&f=false.
37. Donald, P. R.; van Helden, P. D. Progres in Respiratory Research. - Vol. 40. Antituberculosis Chemotherapy. s.l. : S. Kargel AG, R. O. Box, **2011**. Available at: http://books.google.cz/books?id=r37jwNmr4sMC&pg=PA8&lpg=PA8&dq=nicotinamide+as+antituberculotic&source=bl&ots=dPajsaXX5v&sig=wSF30fjFASECy2OHzfbCCv2eMlI&hl=cs&sa=X&ei=LpotVMbOD-XVygPFqYGICA&ved=0CB8Q6AEwAg#v=onepage&q=cycloserine&f=false.

38. Hoeprich, P. D. Alanine : Cycloserine Antagonism: VI. Demonstration of D-alanine in the serum of guinea pigs and mice. *J. Biol. Chem.* **1965**, 240, 1654-1660.
39. Zítková, L.; Toušel, J. Pharmacokinetics of Cycloserine and Terizidone: A Comparative Study. *Chemother.* **1974**, 20 (1), 18-28.
40. Poce, G.; Coczza, M.; Consalvi, S.; Biava, M. SAR analysis of new anti-TB drugs currently in pre-clinical and clinical development. *Eur. J. Med. Chem*. **2014**, 86, 335-351.
41. Jackson, M.; McNeil, M. R.; Brennan, P. J. Progress in targeting cell envelope biogenesis in *Mycobacterium tuberculosis*. *Future Microbiol.* ***2013****,* 8 (7), 855-875.
42. Mitchison, D. A. Fluoroquinolones in the Treatment of Tuberculosis. A Study in Mice. *Am. J. Respir. Crit. Care Med.* **2004**, 169, 334-335.
43. Nadler, J. P.; Berger, J.; Nord, J. A.; Cofsky, R.; Saxena, M. Amoxicillin-clavulanic acid for treating drug-resistant *Mycobacterium tuberculosis*. *Chest*. **1991**, 99 (4), 1025-1026.
44. Chambers, H. F.; Kocago, Y.; Sipit, T.; Turner, J.; Hopewell, P. C. Activity of Amoxicillin/Clavulanate in Patients with Tuberculosis. *Clinic. Infect. Dis*. **1998**, 26 (4), 874–877.
45. Hugonnet, J. M.; Tremblay, L. W.; Boshoff, H. I.; Barry, 3rd, C. E.; Blanchard, J. S. Meropenem-Clavulanate Is Effective Against Extensively Drug-Resistant *Mycobacterium tuberculosis*. Science. **2009**, 323 (5918), 1215-1218.
46. England, K.; Boshoff, H. I. M.; Arora, K.; Weiner, D.; Dayao, E.; Schimel, D.; Via, L. E.; Barry, 3rd, C. E. Meropenem-clavulanic acid shows activity against *Mycobacterium tuberculosis* in vivo. *Antimicrob. Agents Chemother*. **2012**, 56 (6), 3384-3387.
47. World Health Organization. Global Tuberculosis Repotr 2013. http://www.who.int/tb/publications/global_report/en/.
48. Stehr, M.; Elamin, A. A.; Singh, M. Filling the Pipeline - New Drugs for an Old Disease. *Curr. Top. Med. Chem*. **2014**, 14 (1), 110-119.
49. Jain, N. K.; Agnihotri, S. P.; Sharma, K. K,; Gupta, S.; Gupta, P. Extensively drug resistance (XDR) tb is not always fatal. *Indian J. Tuberc.* **2009**, 56 (1), 48-50.
50. Lu, Y.; Zheng, M.; Wang, B.; Fu, L.; Zhao, W.; Li, P.; Xu, J.; Zhu, H.; Jin, H.; Yin, D.; Huang, H.; Upton, A. M.; Ma, Z. Clofazimine Analogs with Efficacy against Experimental Tuberculosis and Reduced Potential for Accumulation. *Antimicrob. Agents Chemother.* **2011**, 55 (11), 5185–5193.
51. Arbiser, J.L.; Moschella, S. L. Clofazimine: a review of its medical uses and mechanisms of action. *J. Am. Acad. Dermatol.* **1995**, 32 (2 Pt 1), 241-247.
52. Luna-Herrera, J.; Reddy, V. M.; Daneluzzi, D.; Ggangadharam, P. R. J. Antituberculosis Activity of Clarithromycin. *Antimicrob. Agents Chemother*. **1995**, 39 (12), 2692-2695.

53. Mor, N.; Esfandiari, A. Synergistic Activities of Clarithromycin and Pyrazinamide against Mycobacterium tuberculosis in Human Macrophages. *Antimicrob. Agents Chemother.* **1997**, 41 (9), 2035-2036.
54. World Health Organization. http://www.who.int/en/.
55. Lienhardt. C.; Raviglione, M.; Spigelman, M.; Hafner, R.; Jaramillo, E.; Hoelscher, M.; Zumla, A.; Gheuens, J. New Drugs for the Treatment of Tuberculosis: Needs, Challenges, Promise, and Prospects for the Future. *J. Infect. Dis.* **2012**, 205, S241-S249.
56. Zumla, A.; Nahid, P.; Cole, S. T. Advances in the development of new tuberculosis drugs and treatment regimens. *Nat. Rev. Drug Discov.* **2013**, 12, 388–404.
57. Beena and Rawat, D. S. Antituberculosis Drug Research: A Critical Overview. *Med. Res. Rev.* **2013**, 33 (4), 693-764.
58. Yan, M.; Ma, S. Recent Advances in the Research of Heterocyclic Compounds as Antitubercular Agents. *ChemMedChem* **2012**, 7, 2063-2075.
59. The U.S. Food and Drug Administration. FAD News Release: approval of Sirturo (bedaquiline). http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm333695.htm. December 31, 2012.
60. Europen Medicines Agency. EMA recommendes approval of a new medicine for multidrug-tesistant tuberculosis (bedaquiline). http://www.ema.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2013/12/news_detail_001999.jsp&mid=WC0b01ac058004d5c1. December 20, 2013.
61. Chan, B.; Khadem, T. M.; Brown, J. A review of tuberculosis: Focus on bedaquiline. *Am. J. Health Syst. Pharm.* **2013**, 70 (15), 1984-1994.
62. Kakkar, A. K.; Dahiya, N. Bedaquiline for the treatment of resistant tuberculosis: Promises and pitfalls. *Tuberculosis*. **2014**, 94, 357-362.
63. Rivers, E. C.; Mancera, R. L. New anti-tuberculosis drugs in clinical trials with novel mechanisms of action. *Drug Discovery Today.* **2008**, 13 (23/24), 1090-1098.
64. Bioportfolio. Metronidazole for Pulmonary Tuberculosis. http://www.bioportfolio.com/resources/trial/102297/Metronidazole-For-Pulmonary-Tuberculosis-south-Korea.html. October 10, 2014.
65. Ashtekar, D. R.; Costa-Perira, R.; Nagrajan, K.; Vishvanathan, N.; Bhatt, A. D.; Rittel, W. In vitro and in vivo activities of the nitroimidazole CGI 17341 against *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **1993**, 37 (2), 183-186.
66. Mukherjee, T.; Boshoff, H. Nitroimidazoles for the treatment of TB: past, present and future. *Future Med. Chem.* **2011**, 3 (11), 1427-1454.

67. Stover, C. K.; Warrener, P.; Van Devanter, D. R.; Sherman, D. R.; Arain, T. M.; Langhorne, M. H.; Anderson, S. W.; Towell, J. A.; Yuan, Y.; McMurray, D. N.; Kreiswirth, B. N.; Barry, C. E.; Baker, W. R. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. *Nature*. **2000**, 405 (6789), 962-966.
68. Brennan, P. J.; Young, D. B.; Robertson, B. D. Handbook of Anti-Tuberculosis Agents. *Tuberculosis.* **2008**, 88 (2), 85-170. Available at: http://www.tballiance.org/newscenter/research_papers/TB_DB_Final.pdf.
69. Singh, R.; Manjunatha, U.; Boshoff, H. I.; Ha, Y.H.; Niyomrattanakit, P.; Ledwidge, R.; Dowd, C. S.; Lee, I. Y.; Kim, P.; Zhang, L.; Kang, S.; Keller, T.H.; Jiricek, J.; Barry, C. E. 3rd. PA-824 Kills Nonreplicating Mycobacterium tuberculosis by Intracellular NO Release. *Science.* **2008**, 322 (5906), 1392-1395.
70. Ginsberg, A. M.; Laurenzi, M. W.; Rouse, D. J.; Whitney, K. D.; Spigelman, M. K. Safety, Tolerability, and Pharmacokinetics of PA-824 in Healthy Subjects. *Antimicrob. Agents Chemoth.* **2009**, 53 (9), 3720-3725.
71. Manjunatha, U.; Boshoff, H.; Barry, C. E. The mechanism of action of PA-824. *Commun. Integr. Biol*. **2009**, 2 (3), 215-218.
72. Matsumoto, M.; Hashizume, H.; Tomishige, T.; Kawasaki, M.; Tsubouchi, H.; Sasaki, H.; Shimokawa, Y.; Komatsu, M. OPC-67683, a Nitro-Dihydro-Imidazooxazole Derivative with Promising Action against Tuberculosis In Vitro and in Mice. *PloS Med*. **2006**, 3 (11), 1231-1244.
73. Blaser, A.; Palmer, B. D.; Sutherland, H. S.; Kmentova, I.; Franzblau, S. G.; Wan, B.; Wang, Y.; Ma, Z.; Thompson, A. M.; Denny, W. A. Structure-Activity Relationships for Amide-, Carbamate-, And Urea-Linked Analogues of the Tuberculosis Drug (6S)-2-Nitro-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5*H*-imidazo[2,1-b][1,3]oxazine (PA-824). *J. Med. Chem.* **2012**, 55 (1), 321-326.
74. Makarov, V., Möllmann, U. and Application, Cole. S. T. Eurasian Patent. EP2029583 2007.
75. Makarov, V.; Manina, G.; Mikusova, K.; Möllmann, U.; Ryabova, O.; Saint-Joanis, B.; Dhar, N.; Pasca, M. R.; Buroni, S.; Lucarelli, A. P.; Milano, A.; De Rossi, E.; Belanova, M.; Bobovska, A.; Dianiskova, P.; Kordulakova, J.; Sala, C.; Fullam, E.; Schneider,P.;McKinney, J. D.; Brodin, P.; Christophe, T.; Waddell, S.; Butcher, F.; Albrethsen, J.; Rosenkrands, I.; Brosch, R.; Nandi, V.; Bharath, S.; Gaonkar, S.; Shandil, R. K.; Balasubramanian, V.; Balganesh, T.; Tyagi, S.; Grosset, J.; Riccardi, G.; Cole, S. T. Benzothiazinones Kill *Mycobacterium tuberculosis* by Blocking Arabinan Synthesis. *Science*. **2009**, 324, 801-804.
76. Makarov, V.; Lechartier, B.; Zhang, M.; Neres, J.; van der Sar, A. M.; Raadsen, S. A.; Hartkoorn, R. C.; Ryabova, O. B.; Vocat, A.; Decosterd, L. A.; Widmer, N.; Buclin, T.;

Bitter, W.; Andries, K.; Pojer, F.; Dyson, P. J.; Cole, S. T. Towards a new combination therapy for tuberculosis with next generation benzothiazinones. *EMBO Mol. Med.* **2014**, 6 (3), 372-383.

77. Christophe, T.; Jackson, M.; Jeon, H. K.; Fenistein, D.; Contreras-Dominguez, M.; Kim, J.; Genovesio, A.; Carralot, J. P.; Ewann, F.; Kim, E. H.; Lee, S. Y.; Kang, S.; Seo, M. J.; Park, E. J.; Škovierová, H.; Pham, H.; Riccardi, G.; Nam, J. Y.; Marsollier, L.; Kempf, M.; Joly-Guillou, M. L.; Oh, T.; Shin, W. K.; No, Z.; Nehrbass, U.; Brosch, R.; Cole, S. T.; Brodin, P. High Content Screening Identifies Decaprenyl-Phosphoribose 29 Epimerase as a Target for Intracellular Antimycobacterial Inhibitors. *PLoS Pathog.* **2009**, 5 (10), e1000645.
78. Priscille, B.; Thierry, C.; Zaesung, N.; Jaeseung, K.; Auguste, G.; Cedric, F. D. P.; Heekyoung, J.; Anne, E. F.; Sunhee, K.; Saeyeon, L.; Jung, S. M.; Eunjung, P.; Monica, C. D.; Youn, N. J.; Hye, K. E. Anti-infective Compounds. US 2011/178077.
79. Magnet, S.; Hartkoorn, R. C.; Székely, R.; Pató, J.; Triccas, J. A.; Schneider, P.; Szántai-Kis, C.; Örfi, L.; Chambon, M.; Banfi, D.; Bueno, M.; Turcatti, G.; Kéri, G.; Cole, S. T. Leads for antitubercular compounds from kinase inhibitor library screens. *Tuberculosis.* **2010**, 90, 354-360.
80. Stanley, S. A.; Grant, S. S.; Kawate, T.; Iwase, N.; Shimizu, M.; Wivagg, C.; Silvis, M.; Kazyanskaya, E.; Aquadro, J.; Golas, A.; Fitzgerald, M.; Dai, H.; Zhang, L.; Hung, D. T. Identification of Novel Inhibitors of M. tuberculosis Growth Using Whole Celll Based High-Throughput Screening. *ACS Chem. Biol.* **2012**, 7, 1377-1384.
81. Favrot, L.; Ronning, D. R. Targeting the mycobacterial envelope for tuberculosis drug development. *Expert Rev. Anti Infect Ther.* **2012**, 10 (9), 1023-1036.
82. Lamichhane, G. Novel targets in M. tuberculosis: search for new drugs. *Trends in Mol. Med.* **2011**, 17 (1), 25-33.
83. Brennan, P. J. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis*. **2003**, 83, 91-97.
84. Brennan, P. J.; Crick, D. C. The Cell Wall Core of Mycobacterium tuberculosis in the Context of Drug Discovery. *Curr. Top. Med. Chem.* **2007**, 7 (5), 475-488.
85. Dover, L. G.; Alderwick, L.J.; Brown, A. A.; Futterer, K.; Besra, G. S. Regulation of Cell Wall Synthesis and Growth. *Curr. Mol. Med*. **2007**, 7 (3), 247-276.
86. Chatterjee, D. The mycobacterial cell well: structure, biosynthesis and sites of drug action. *Curr. Opin. Chem. Biol*. **1997**, 1, 579-588.
87. Bhamidi, S.; Scherman, M. S.; Rithner, C. D.; Prenni, J. E.; Chatterjee, D.; Khoo, K. H.; McNeil, M. R. The Identification and Location of Succinyl Residues and the Characterization of the Interior Arabinan Region Allow for a Model of the Complete

Primary Structure of *Mycobacterium Tuberculosis* Mycolyl Arabinogalactan. *J. Biol. Chem.* **2008**, 283 (19), 12992-13000.

88. Herzigová, P.; Klimešová, V.; Palát, K.; Kaustová, J.; Dahse, H. M.; Mollmann, U. Preparation and in vitro Evaluation of 4-Benzylsulfanylpyridine-2-carbohaydrazides as Potential Antituberculosis Agents. *Arch. Pharm. Chem. Life Sci.* **2009**, 342, 394-404.
89. Klimešová, V.; Zahajská, L.; Waisser, K.; Kaustová, J.; Mollmann, U. Synthesis and antimycobacterial activity of 1,2,4-triazole 3-benzylsulfanyl derivatives. *IL FARMACO*. **2004**, 59, 279-288.
90. Kočí, J.; Klimešová, V.; Waisser, K.; Kaustová, J.; Dahse, H. M.; Molmann, U. Heterocyclic Benzazole Derivatives with Antimycobacterial in vitro activity. *Bioorg. Med. Chem. Lett.* **2002**, 12, 3275-3278.
91. Klimešová, V.; Kočí, J.; Waisser, K.; Kaustová, J. New benzimidazole derivatives as antimycobacterial agants. *Farmaco*. **2002**, 57, 259-265.
92. Klimešová, V.; Kočí, J.; Pour, M.; Stachel, J.; Waisser, K.; Kaustová, J. Synthesis and preliminary evaluation of benzimidazole derivatives as antimicrobial agents. *Eur. J. Med. Chem.* **2002**, 37, 409-418.
93. Klimešová, V.; Kočí, J.; Waisser, K.; Kaustová, J.; Mollmann, U. Preparation and in vitro evaluation of benzylsulfanyl benzoxazole derivatives as potential antituberculosis agents. *Eur. J. Med. Chem.* **2009**, 44, 2286-2293.
94. Klimešová, V.; Kočí, J.; Palát, K.; Stolaříková, J.; Dahs, H. M.; Mollmann, U. Structure - Activity Relationships of 2-Benzylsulfanylbenzothiazoles: Synthesis and Selective Antimycobacterial Properties. *Med. Chem*. **2012**, 8 (2), 281-292.
95. American Thoracic Society, Medicinal Section of the American Lung Associacion. Diagnosis and treatment of disease caused by nontuberculous mycobacteria. *Am. J. Respir. Crit. Care Med.* 1997, 156 (2), S1-S25.
96. Strauss, M. J. The nitroaromatic Group in Nitro Design. Pharmacology and Toxicology (for Nonpharmacologists). Technical Review. *Ind. Eng. Chem. Prod. Res. Dev.* **1979**, 18 (3), 158-166.
97. Ju, K. S. and E., Parales R. Nitroaromatic Compounds, from Synthesis to Biodegradation. *Microbiol. Mol. Biol. Rev*. **2010**, 74 (2), 250-272.
98. Patterson, S.; Wyllie, S. Nitro drugs for the treatment of trypanosomatid diseases: past, present, and future prospects. *Trends in Parasitology.* **2014**, 30 (6), 289-298.
99. Rakesh; Bruhn, D. F.; Scherman, M. S.; Woolhiser, L. K.; Madhura, D. B.; Maddox, M. M.; Singh, A. P.; Lee, R. B.; Hurdle, J. G.; McNeil, M. R.; Lenaerts, A. J.; Meibohm, B.; Lee, R. E. Pentacyclic Nitrofurans with In Vivo Efficacy and Activity against Nonreplicating Mycobacterium tuberculosis. *PloS One*. **2014**, 9 (2), e87909.

100. Myznikov, L. V.; Hrabalek, A.; Koldobskii, G. I. Drugs in the tetrazole series. Review. *Chem. Heterocycl. Comp.* **2007**, 43, 1-9.
101. Roh, J.; Vavrova, K.; Hrabalek, A. Synthesis and Functionalization of 5-Substituted Tetrazoles. *Eur. J. Org. Chem.* **2012**, 31, 6101-6118.
102. Muraglia, E.; Kinzel, O. D.; Laufer, R.; Miller, M. D.; Moyer, G.; Munshi, V.; Orvieto, F.; Palumbi, M. C.; Pescatore, G.; Rowley, M.; Williarns, P.D.; Summa, V. Tetrazole thioacetanilides: Potent non-nucleoside inhibitors of WT HIV reverse transcriptase and its K103N mutant. *Bioorg. Med. Chem. Lett.* **2006**, 16, 2748-2752.
103. Adamec, J.; Waisser, K.; Kunes, J.; Kaustova, J. A Note on the Antitubercular Activities of 1-Aryl-5-benzylsulfanyltetrazoles. *Arch. Pharm.* **2005**, 338, 385-389.
104. Adamec, J.; Beckert, R.; Weiss, D.; Klimesova, V.; Waisser, K.; Mollmann, U.; Kaustova, J.; Buchta, V. Hybrid molecules of estrone: New compounds with potential antibacterial, antifungal, and antiproliferative activities. *Bioorg. Med. Chem.* **2007**, 15, 2898-2906.
105. Shin, K. J.; Koo, K. D.; Yoo, K. H.; Kim, D. C.; Kim, D. J.; Park, S. W. Synthesis and biological properties of new 1β-methylcarbapenems having tetrazolothioether moiety. *Bioorg. Med. Chem. Lett*. **2000**, 10, 1421-1425.
106. Dhayanithi, V.; Syed, S. S.; Kumaran, K.; Sankar, K. R. J.; Ragavan, R. V.; Goud, P.S. K.; Kumari, N. S.; Pati, H. N. Synthesis of selected 5-thio-substituted tetrazole derivatives and evaluation of their antibacterial and antifungal activities. *J. Serb. Chem. Soc*. **2011**, 76, 165-175.
107. Schwarz, K.; Foltz, C. Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. *J. Am. Chem. Soc.* **1957**, 79, 3292-3293.
108. Koketsu, M.; Ishihara, H. Synthesis of 1, 3-selenazine and 1, 3-selenazole and their biological activities. *Curr. Org. Chem.* **2003**, 7, 175-185.
109. Libero, F. M.; Xavier, M. C. D.; Victoria, F. N.; Nascente, P. S.; Savegnago, L.; Perin, G.; Alves, D. Synthesis of novel selenium and tellurium-containing tetrazoles: a class of chalcogen compounds with antifungal activity. *Tetrahedron Lett.* **2012**, 53, 3091-3094.
110. Koketsu, M.; Tanaka, K.; Takenaka, Y.; Kwong, C. D.; Ishihara, H. Synthesis of 1, 3-thiazine derivatives and their evaluation as potential antimycobacterial agents. *Eur. J. Pharm. Sci*. **2002**, 15, 307-310.
111. Parnham, M. J.; Graf, E. Pharmacology of synthetic organic selenium compounds. *Prog. Drug Res*. **1991**, 36, 9-47.
112. Koketsu, M.; Ishihara, H.; Wu, W. J.; Murakami, K.; Saiki, I. 1,3-Selenazine derivatives induce cytotoxicity and DNA fragmentation in human HT-1080 fibrosarcoma cells. *Eur. J. Pharm. Sci*. **1999**, 9 (2), 157-161.

113. May, S.W.;Wang, L. Q.; Gill-Woznichak, M. M.; Browner, R. F.; Ogonowski, A. A.; Smith, J. B.; Pollock, S. H. An Orally Active Selenium-Based Antihypertensive Agent with Restricted CNS Permeability. *J. Pharm. Exp. Ther*. **1997**, 283, 470-477.
114. Fernandez-Bolanos, J. G.; Lopez, O.; Ulgar, V.; Maya, I.; Fuentes, J. Synthesis of O-unprotected glycosyl selenoureas. A new access to bicyclic sugar isoureas. *Tetrahedron Lett.* **2004**, 45, 4081-4084.
115. Goltsberg, M. A.; Koldobskii, G. I. Simple method for the preparation of 5-alkyoxy(aryloxy)-1-phenyltetrazoles. *Zhurnal Organicheskoi Khimii.* **1995**, 31, 1726-1727.
116. De Oliveira, C. S.; Lira, B. F.; Barbosa-Filho, J. M.; Lorenzo, J. G. F.; De Athayde-Filho, P. F. Synthesis Approaches and Pharmacological Actiity of 1,3,4-Oxadiazoles: A Review of the Literature from 2000-2012. *Molecules*. **2012**, 17, 10192-10231.
117. Kushwaha, N.; Kushwaha, S. K. S.; Rai, A. K. Biological Activities of Thiadiazole Derivatives: A Review. *Inter. J. ChemTech. Res.* **2012**, 4 (2), 517-531
118. Maddry, J. A.; Ananthan, S.; Goldman, R. C.; Hobrath, J. V.; Kwong, C. D.; Maddox, C.; Rasmussen, L.; Reynolds, R. C.; Secrist, J. A.; Sosa, M. I.; White, E. L.; Zhang, W. Antituberculosis activity of the molecular libraries screening center network libraries screening center network library. *Tuberculosis.* **2009**, 89, 354-363.
119. Bakal, R. L.; Gattani, S. G. Identification and development of 2,5-disubstituted oxadiazole as potential candidate for treatment of XDR and MDR tuberculosis. *Eur. J. Med. Chem.* **2012**, 47, 278-282.
120. Foroumadi, A.; Soltani, F.; Moallemzadeh-Haghighi, H.; Shafiee, A. Synthesis, in vitro-Antimycobacterial Activity and Cytotoxicity of Some Alkyl α-(5-aryl-1,3,4-thiadiazole-2-ylthio)acetates. *Arch. Pharm. Chem. Life Sci.* **2005**, 338, 112-116.
121. Mohamed Jawed Ahsan, M. J.; Samy, J. G.; Khalilullah, H.; Nomani. Md. S.; Saraswat, P.; Gaur, R.; Singh, A. Molecular properties prediction and synthesis of novel 1,3,4-oxadiazole analogues as potent antimicrobial and antitubercular agents. *Bioorg. Med. Chem. Lettю* **2011**, 21, 7246-7250.
122. Navarrete-Vazquez, G.; Molina-Salinas, G. M.; Duarte-Fajardo, Z. V.; Vargas-Villarreal, J.; Estrada-Soto, S.; Gonzalez-Salazar, F.; Hernandez-Nuneza, E.; Said-Fernandezb, S. Synthesis and antimycobacterial activity of 4-(5-substituted-1,3,4-oxadiazol-2-yl)pyridines. *Bioorg. Med. Chem.* **2007**, 15, 5502-5508.
123. Ananthan, S.; Faaleolea, E. R.; Goldman, R. C.; Hobrath, J. V.; Kwong, C. D.; Laughon, B. E.; Maddry, J. A.; Mehta, A.; Rasmussen, L.; Reynolds, R. C.; Secrist, J. A.; Shindo, N.; Showe, D. N.; Sosa, M. I.; Suling, W. J.; White, E. L. High-throughput screening for inhibitors of *Mycobacterium tuberculosis* H37Rv. *Tuberculosis*. **2009**, 89, 334-353.

124. Macaev, F.; Rusu, G.; Pogrebnoi, S.; Gudima, A.; Stingaci, E.; Vlad, L.; Shvets, N.; Kandemirli, F.; Dimogloa, A.;Reynoldsd, R. Synthesis of novel 5-aryl-2-thio-1,3,4-oxadiazoles and the study of their structure–antimycobacterial activities. *Bioorg. Med.Chem.* **2005**, 13, 4842-4850.
125. Bakal, R. L.; Gattani, S. G. Identification and development of 2,5-disubstituted oxadiazole as potential candidate for treatment of XDR and MDR tuberculosis. *Eur. J. Med. Chem.* **2012**, 47, 278-282.
126. Foroumadi A;, Sakhteman A.; Sharifzadeh Z.; Mohammadhosseini N.; Hemmateenejad B.; Moshafi MH.; Vosooghi M.; Amini M.; Shafiee A. Synthesis, antituberculosis activity and QSAR study of some novel 2-(nitroaryl)-5-nitrobenzylsulfinyl and sulfonyl)-1,3,4-thiadiazole derivatives. *DARU*. **2007**, 15 (4), 218-226.
127. Zarghi, A.; Faizi, M.; Shafaghi, B.; Ahadian, A.; Khojastehpoor, H. R.; Zanganeh, V.; Tabatabai, S.; Shafiee, A. Design and synthesis of new 2-substituted-5-(2-benzylthiophenyl)-1,3,4-oxadiazoles as benzodiazepine receptor agonists. *Bioorg. Med. Chem. Lett.* **2005**, 15, 3126-3129.
128. Efimova, Y. A.; Artamonova, T. V.; Koldobskii, G. I. Tetrazoles: LIII. Microwave-Activated Acylation of 5-Substituted Tetrazoles. *Russ. J. Org. Chem*. **2008**, 44 (9), 1345-1347.
129. Dişli, A.: Salman, M. Synthesis of Some New 5-Substituted 1H-Tetrazoles. *Russ. J. Org. Chem*. **2009**, 45 (1), 151-153.
130. Hodgkins, J. E.; Reeves, W. P. The Modified Kaluza Synthesis. III. The Synthesis of Some Aromatic Isothiocyanates. *J. Org. Chem.* **1964**, *29* (10), 3098–3099.
131. Trefzer, C.; Škovierová, H.; Buroni, S.; Bobovská, A.; Nenci, s.; Molteni, E.; Pojer, F.; Pasca, M. R.; Makarov, V.; Cole, s. T.; Riccardi, G.; Mikušová, K.; Johnsson, K. Benzothiazinones Are Suicide Inhibitors of Mycobacterial Decaprenylphosphoryl-β-D-ribofuranose 2′-Oxidase DprE1. *J. Am. Chem.Soc.* **2012**, 134, 912−915.

11. Attachments

Attachment **I**.

Karabanovich, G.; Roh, J.; Padělková, Z.; Novák, Z.; Vávrová, K.; Hrabálek, A. One-pot synthesis of 1-substituted-5-alkylselanyl-1*H*-tetrazoles from isoselenocyanates: unexpected formation of *N*-alkyl-*N*-arylcyanamides and (Z)-*Se*-alkyl-*N*-cyano-*N,N'*-diarylisoselenoureas. *Tetrahedron*. **2013**, 69, 8798-8808.

Tetrahedron 69 (2013) 8798–8808

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

One-pot synthesis of 1-substituted-5-alkylselanyl-1*H*-tetrazoles from isoselenocyanates: unexpected formation of *N*-alkyl-*N*-arylcyanamides and (*Z*)-*Se*-alkyl-*N*-cyano-*N*,*N*′-diarylisoselenoureas

Galina Karabanovich [a], Jaroslav Roh [a,*], Zdeňka Padělková [b], Zdeněk Novák [a], Kateřina Vávrová [a], Alexandr Hrabálek [a]

[a] Department of Inorganic and Organic Chemistry, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, Hradec Králové 50005, Czech Republic
[b] Department of General and Inorganic Chemistry, Faculty of Chemical Technology, University of Pardubice, Studentská 573, Pardubice 532 10, Czech Republic

ARTICLE INFO

Article history:
Received 15 April 2013
Received in revised form 11 July 2013
Accepted 22 July 2013
Available online 4 August 2013

Keywords:
Selenium
Isoselenocyanate
Tetrazole
Cyanamide
Tetrazole-5-selenol

ABSTRACT

1-Substituted-5-alkylsulfanyl-1*H*-tetrazoles are well known class of organic substances with various applications in medicinal chemistry or photographic industry. Their selenium analogues, 1-substituted-5-alkylselanyl-1*H*-tetrazoles are, however, much less explored because of the lack of suitable methods for their preparation. In this work we investigated the synthesis of 1-alkyl/aryl-5-alkylselanyl-1*H*-tetrazoles from synthetically available alkyl/arylisoselenocyanates. One-pot reactions of arylisoseleno cyanates with sodium azide and alkylating agent led to the target 5-alkylselanyl-1-aryl-1*H*-tetrazoles but also to interesting side products, namely *N*-alkyl-*N*-arylcyanamides and (*Z*)-*Se*-alkyl-*N*-cyano-*N*,*N*′-diarylisoselenoureas. Nevertheless, when alkylisoselenocyanates were utilized as the substrates, the reactions led exclusively to the formation of 1-alkyl-5-alkylselanyl-1*H*-tetrazoles in good yields. This simple one-pot procedure brings new possibilities for the preparation of variously substituted selenium compounds. It also opens the way to further investigations of selenium isosteres of the widely utilized 5-thiotetrazole moiety in biomedical applications.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Selenium is a trace element, essential for normal human development, growth, metabolism, and function of the immune system. Originally, selenium was considered as one of the most toxic elements. Its benefits were not fully appreciated until 1957, when Schwarz and Foltz described the relationship between selenium intake by food and prevention of liver necrosis in rats.[1] In 1973 it was discovered that selenium is a part of glutathione peroxidases $GSHP_x$, antioxidant proteins that reduce potentially damaging reactive oxygen species, such as hydrogen peroxide and lipid hydroperoxides, to harmless products.[2] In recent years increasing attention has been paid to selenium-containing compounds due to their use as synthetic tools,[3] and as promising biologically active substances exhibiting antifungal,[4] antimycobacterial,[5] antiviral,[6] antitumor[7] or antihypertensive properties.[8]

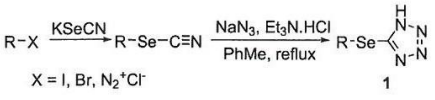
Our group has been interested in the chemistry of tetrazole-containing compounds, especially 5-substituted tetrazoles and substituted tetrazole-5-thiols. Tetrazoles are a well-described class of nitrogen-containing heterocycles with valuable properties, which have found widespread application in various fields of industry and science, mainly in medicinal chemistry as lipophilic and metabolically stable surrogates for other functional groups.[9,10] Tetrazole-5-thiols are also a frequent fragment of pharmaceutically active compounds. The best-known examples of drug containing 1-substituted-5-alkyl/arylsulfanyl-1*H*-tetrazole are the β-lactam antibiotics of the cephalosporin and cephamycin series, which exhibit low toxicity and a wide spectrum of antibacterial activity.[10] In addition, 1-aryltetrazol-5-ylthioacetanilides were identified as potent inhibitors of HIV-1RT with low nanomolar intrinsic activity on the enzyme and submicromolar antiviral activity in HIV infected cells.[11] In a series of 1-substituted 5-alkyl/arylsulfanyl-1*H*-tetrazole compounds, antitubercular,[12,13] antibacterial,[14] and antifungal activities[15] were found.

Although the chemistry of selenium compounds is quite similar to the chemistry of sulfur compounds, 1-substituted-5-alkyl/arylselanyl-1*H*-tetrazoles have been much less explored compared to

* Corresponding author. Tel.: +420 495067339; fax: +420 495067166; e-mail addresses: jaroslav.roh@faf.cuni.cz, roh_j1aa@faf.cuni.cz (J. Roh).

0040-4020/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tet.2013.07.103

their sulfur isosteres. Most publications dealing with 5-selanyl tetrazoles describe the syntheses of 5-alkyl/arylselanyl-1*H*-tetrazoles (**1**) based on the reaction of alkyl/arylselenocyanates with sodium azide under various conditions (Scheme 1).[16–18] 5-Alkyl/arylselanyl-1*H*-tetrazoles could be subsequently functionalized. However, low regioselectivity of these reactions leads to the formation of 1,5- and mainly 2,5-disubstituted derivatives.[9,19] A series of compounds **2** (Fig. 1), which have been synthesized from corresponding 5-arylselanyl-1*H*-tetrazoles and *p*-toluensulfonic acid using dicyclohexylcarbodiimide, can be shown as an example.[20] However, the resulting 1,5-regioisomer was not fully confirmed.



Scheme 1. Synthesis of 5-alkyl/arylselanyl-1*H*-tetrazoles from alkyl/arylselenocyanates.

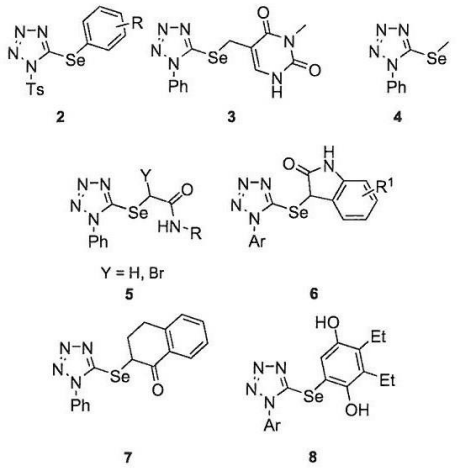


Fig. 1. Structures of known 1-substituted 5-alkyl/arylselanyl-1*H*-tetrazoles.

1-Substituted 5-alkyl/arylselanyl-1*H*-tetrazoles can be also prepared using double alkylation of sodium selenide. The major disadvantage of these reactions lies in low selectivity, which causes the formation of symmetrical selenides and low yields of the desired products. 5-{[(1-Phenyl-1*H*-tetrazol-5-yl)selanyl]methyl} uracil and its methyl derivative **3** were prepared using this protocol.[21] 5-Methylselanyl-1-phenyl-1*H*-tetrazole (**4**) was prepared from 5-chloro-1-phenyl-1*H*-tetrazole by its reaction with bis(methoxymagnesium)diselenide in methanol. However, the target product was obtained in 14% yield only.[22] Compounds with structures **5**–**8** and some of their derivatives were described in patents connected with the development of photographic light-sensitive materials.[23–25]

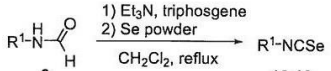
To investigate these sulfur/selenium bioisosteres in greater detail, a general method of 1-substituted 5-alkyl/arylselanyl-1*H*-tetrazoles synthesis, using easily available reactants and a simple experimental procedure, is needed.

In this study we explored the possibility of the synthesis of 1-alkyl/aryl-5-alkylselanyl-1*H*-tetrazoles starting from alkyl/arylisoselenocyanates, analogous with the simplest and most successful approaches for the synthesis of 1-substituted 5-alkyl/arylsulfanyl-1*H*-tetrazoles,[14,26–28] starting from the corresponding alkyl/arylisothiocyanates. This involved both two-step and one-pot procedures under various conditions including phase-transfer catalysis.

2. Results and discussion

The starting alkyl/arylisoselenocyanates **10**–**16** were prepared by a one-pot reaction from the corresponding *N*-alkyl/arylformamides **9** (Table 1).[29] The reaction proceeded through the formation of an isonitrile, which than reacted with selenium to form an isoselenocyanate. These compounds are an advantageous starting material for the synthesis of different organoselenium compounds because of their simple synthesis and safety handling and storage.

Table 1
Preparation of alkyl/arylisoselenocyanates **10**–**16**.



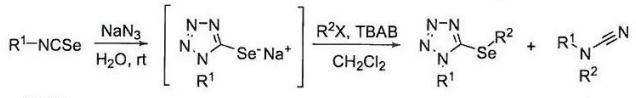
Entry	Product	R^1	Yield [%]
1	**10**	Ph–	63
2	**11**	4-MeOPh–	68
3	**12**	4-BrPh–	58
4	**13**	$CH_3(CH_2)_3$–	67
5	**14**	$CH_3(CH_2)_5$–	43
6	**15**	C_6H_{11}–	98
7	**16**	$CH_3(CH_2)_7$–	67

First, we attempted to synthesize 1-substituted-1*H*-tetrazole-5-selenols from the corresponding isoselenocyanates. To find suitable conditions for the preparation of 1-substituted-1*H*-tetrazole-5-selenols, we treated phenylisoselenocyanate (**10**) with sodium azide in various solvents (H_2O, CH_3OH, C_2H_5OH, CH_3CN, THF, DMF, CH_3NO_2, Et_3N, CH_2Cl_2/H_2O), with tetrabutylammonium azide (TBAA) or $Et_3N{\cdot}HN_3$ in toluene or CH_2Cl_2, with Me_3SnN_3 in toluene, THF, CH_3CN or CH_2Cl_2 and also with Me_3SiN_3 itself. Unfortunately all these reactions led to decomposition with precipitation of selenium powder, confirming that many nucleophilic selenium species are highly reactive and usually cannot be isolated.[30] Neverthe less, reaction of cyclohexylisoselenocyanate (**15**) with sodium azide in water was successful and sodium 1-cyclohexyl-1*H*-tetrazole-5-selenolate was isolated as a mixture with residual sodium azide. Unfortunately, acidification of sodium 1-cyclohexyl-1*H*-tetrazole-5-selenolate led to precipitation of red selenium at pH 5.5–6.

As tetrazole-5-selenolates were sensitive to acidification, we decided to alkylate them without isolation under the conditions of phase-transfer catalysis with tetrabutylammonium bromide (TBAB) as a phase-transfer catalyst (method 1, Table 2). Reaction of alkylisoselenocyanates **13**–**15** with sodium azide followed by addition of an alkylating agent under the condition of phase-transfer catalysis led to the formation of the intended 1-alkyl-5-alkyl selanyl-1*H*-tetrazoles (**23a**, **25a**, **26a**, **31a**, **33a**, and **34a**) as the only products, but only in low-to-moderate yields (11–50%). In the case of aryl derivatives, the target compounds (**17**–**19a**, **21a**) were formed in very low yields (5–14%), due to an unexpected formation of *N*-alkyl-*N*-arylcyanamides (**17**–**19b**) as the main products. Moreover, reaction of 4-bromophenylisoselenocyanate (**12**) led to complex mixture of products. In the case of alkyl derivatives, the formation of *N*,*N*-disubstituted cyanamides was not observed.

These results encouraged us to carry out one-pot reactions in an attempt to immediately alkylate tetrazole-5-selenolate before its decomposition. Reactions of phenylisoselenocyanate (**10**) and 4-methoxyphenylisoselenocyanate (**11**) with benzyl bromide and

Table 2
Synthesis of 1-alkyl/aryl-5-alkylselanyl-1*H*-tetrazoles prepared from alkyl/arylisoselenocyanates **10–15** by method 1



10-15 **a** **b**

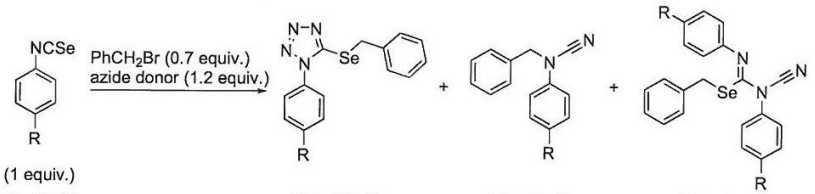
Entry	Substrate	R^1	R^2	X	Product	Yield [%]	
						a	**b**
1	**10**	Ph–	$PhCH_2$–	–Br	**17**	5	52
2	**11**	4-CH_3OPh–	$PhCH_2$–	–Br	**18**	5	49
3	**10**	Ph–	3,5-$(NO_2)_2PhCH_2$–	–Cl	**19**	14	57
4	**12**	4-BrPh–	3,5-$(NO_2)_2PhCH_2$–	–Cl	**21**	5	—
5	**13**	$CH_3(CH_2)_3$–	$PhCH_2$–	–Br	**23**	11	0
6	**14**	$CH_3(CH_2)_5$–	CH_3–	$-OSO_3CH_3$	**25**	17	0
7	**14**	$CH_3(CH_2)_5$–	$PhCH_2$–	–Br	**26**	15	0
8	**15**	C_6H_{11}–	CH_3–	$-OSO_3CH_3$	**31**	44	0
9	**15**	C_6H_{11}–	3,5-$(NO_2)_2PhCH_2$–	–Cl	**33**	47	0
10	**15**	C_6H_{11}–	2,4-$(NO_2)_2PhCH_2$–	–Cl	**34**	50	0

azide in different solvents were accomplished to find suitable conditions for the synthesis of 5-alkylselanyl-1-aryl-1*H*-tetrazoles (Table 3).

As shown in Table 3, almost all reactions of substrate **10** led again to the formation of the target product **17a** together with cyanamide **17b**, as in the case of method 1. Furthermore, another unexpected side product was found, confirmed to be (*Z*)-*Se*-benzyl-*N*-cyano-*N*,*N*′-diphenylisoselenourea (**17c**) by NMR and X-ray crystallography experiments (Fig. 2). The highest yields of 5-benzylselanyl-1-phenyl-1*H*-tetrazole (**17a**) were achieved in THF or CH_3CN, both with a small amount of water, at rt (Table 3, entries 4 and 7).

However, the main products of all these reactions were *N*-benzyl-*N*-phenylcyanamide (**17b**) or (*Z*)-Se-benzyl-*N*-cyano-*N*,*N*′-diphenylisoselenourea (**17c**). The reaction of the substrate **11** proceeded similarly, 5-benzylselanyl-1-(4-methoxyphenyl)-1*H*-tetrazole (**18a**), *N*-benzyl-*N*′-(4-methoxyphenyl)cyanamide (**18b**), and (*Z*)-*Se*-benzyl-*N*-cyano-*N*,*N*′-bis(4-methoxyphenyl)isoselenourea

Table 3
One-pot reactions of arylisoselenocyanates **10** and **11** with azide anion under various reaction conditions



10 R = H
11 R = -OCH_3

17a R = H
18a R = -OCH_3

17b R = H
18b R = -OCH_3

17c R = H
18c R = -OCH_3

Entry	Substrate	Solvent	Water [%]	Azide donor	Temp	Product	Yield [%]		
							a	**b**	**c**
1	**10**	H_2O	—	NaN_3	rt	**17**	4	0	56
2	**10**	1,4-Dioxane	5	NaN_3	rt	**17**	10	71	0
3	**10**	1,4-Dioxane	50	NaN_3	rt	**17**	3	56	0
4	**10**	THF	5	NaN_3	rt	**17**	22	47	10
5	**10**	THF	5	NaN_3	−45 °C	**17**	5	65	2
6	**10**	THF	—	TBAA	rt	**17**	5	Trace	23
7	**10**	CH_3CN	5	NaN_3	rt	**17**	17	12	19
8	**10**	CH_3CN	5	NaN_3	−45 °C	**17**	7	51	3
9	**10**	CH_3CN	—	NaN_3	rt	**17**	16	4	56
10[a]	**10**	CH_2Cl_2	50	NaN_3	rt	**17**	4	1	64
11	**10**	C_6H_5N	5	NaN_3	rt	**17**	Decomposition		
12	**10**	$(CH_3)_3SiN_3$	—	$(CH_3)_3SiN_3$	rt	**17**	Decomposition		
13	**11**	H_2O	—	NaN_3	rt	**18**	0	0	62
14[b]	**11**	H_2O	—	NaN_3	rt	**18**	1	10	23
15	**11**	THF	5	NaN_3	rt	**18**	43	47	Trace
16	**11**	THF	—	Me_3SiN_3	−60 °C	**18**	0	16	0
17	**11**	THF	—	TBAA	rt	**18**	10	28	18
18	**11**	CH_3CN	5	NaN_3	rt	**18**	45	21	20
19	**11**	DMF	5	NaN_3	rt	**18**	27	27	Trace
20[a]	**11**	CH_2Cl_2	50	NaN_3	rt	**18**	Trace	38	40

[a] Tetrabutylammonium bromide (TBAB) was used as phase-transfer catalyst.
[b] Compound **11** was reacted with sodium azide and pyridine (1:1.2:3 molar ratio) in water for 3 h at rt, followed by addition of benzyl bromide (in 0.7 molar ratio) in THF. Reaction mixture was stirred overnight.

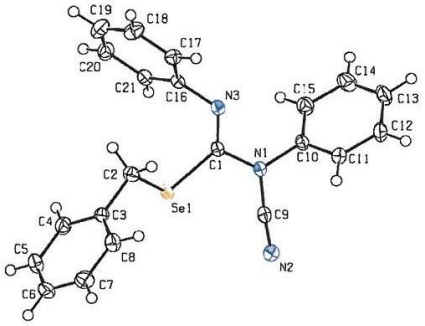
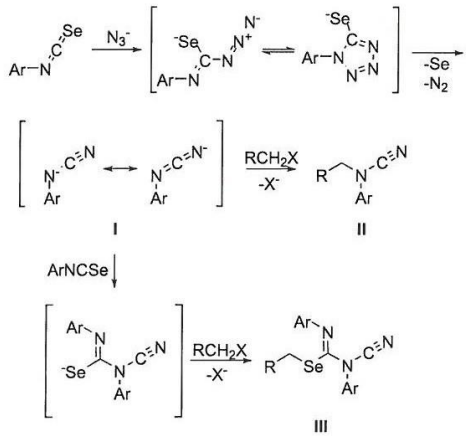


Fig. 2. The molecular structure (ORTEP 50% probability level) of isoselenourea **17c**. Selected interatomic distances [A], angles and interplanar angle [°]: Se1–C1 1.9394(16), Se1–C2 1.9830(16), C1–N1 1.419(2), C1–N3 1.257(2), N1–C9 1.346(2), C9–N2 1.147(2); C1–Se1–C2 100.29(7), Se1–C1–N1 112.29(11), Se1–C1–N3 130.25(12), C1–N1–C9 121.53(13), C1–N3–C16 123.17(14), N1–C9–N2 175.03(18); Se1–C1–N3 versus C9–N1–C10 10.90(3).

(**18c**) were formed almost under any conditions. The highest yields of the target tetrazole **18a** were again achieved in THF or CH_3CN with a small amount of water (Table 3, entries 15 and 18). Interestingly, when one-pot reactions under phase-transfer catalysis or in water were performed, the target tetrazoles **17a** and **18a** were formed either in trace amounts or were not formed at all. As Table 3 demonstrates, variations in the obtained yields are high and hardly predictable.

It should be noted that reactions in dry solvents and in a mixture of organic solvent/water, 1:1 (v/v) proceeded with slightly lower yields than in the case of using approx. 5% of water to dissolve sodium azide before its addition to reaction mixture.

The formation of the unexpected *N*-alkyl-*N*-arylcyanamides and (*Z*)-*Se*-alkyl-*N*-cyano-*N*,*N*′-diarylisoselenoureas explains the release of elemental selenium in all these reactions. We suggest that after the addition of the azide anion to arylisoselenocyanate, nitrogen together with selenium could be released and sodium *N*-cyano-*N*-arylamide (**I**) formed (Scheme 2). This anion could be subsequently alkylated to form *N*-alkyl-*N*-arylcyanamide (**II**) or it could react with another molecule of arylisoselenocyanate and, after alkylation, produce (*Z*)-*Se*-alkyl-*N*-cyano-*N*,*N*′-diarylisoselenourea (**III**).



Scheme 2. Formation of side products of the reactions of arylisoselenocyanates with azide anion.

Based on the aforementioned results we decided to use THF (method 2) or CH_3CN (method 3) as the solvents, both with 5% added water, for the synthesis of a series of 1-substituted-5-alkylselanyl-1*H*-tetrazoles. Moreover, the results achieved by these two methods were compared with yields obtained by the one-pot reactions under phase-transfer catalysis (method 4), which proceeded with the highest yields of the side products, *N*-alkyl-*N*-arylcyanamides and (*Z*)-*Se*-alkyl-*N*-cyano-*N*,*N*′-diarylisoselenoureas. Special attention was paid to the synthesis of 1-substituted-5-[(dinitrobenzyl) selanyl]-1*H*-tetrazoles, because many previous studies showed high antimycobacterial activity of related mono/dinitro substituted compounds.[31–34]

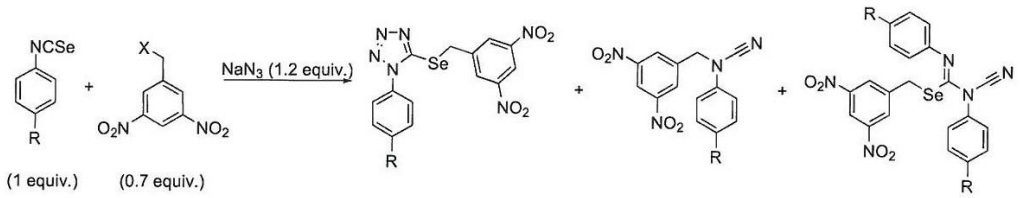
The results of the reactions of arylisoselenocyanates **10–12** are summarized in Table 4. The target 1-aryl-5-[(3,5-dinitrobenzyl) selanyl]-1*H*-tetrazoles (**19–21a**) were prepared as in previous cases in low-to-moderate yields, which were not increased even by utilization of 3,5-dinitrobenzyl iodide. Changes in the solvent system as well as in the azide donor led again to a decrease in the yields of products (e.g., product **19a** was obtained in 21% yield by the reaction of substrate **10** with 3,5-dinitrobenzyl iodide and sodium azide in the mixture of CH_3CN/H_2O, 1:1, the side products **19b** and **19c** were obtained in 39 and 11%, respectively; while the reaction of substrate **11** with 3,5-dinitrobenzyl iodide and TBAA in THF led to formation of cyanamide **20b** as the only product). In the case of the one-pot phase-transfer catalyzed reaction (Table 4, method 4), 3,5-dinitrobenzyl iodide had to be used to obtain reasonable yields. As all the reactions at temperatures below 0 °C led to the formation of cyanamides as the main products, we decided to carry out the reaction of 4-bromophenylisoselenocyanate with sodium azide and 3,5-dinitrobenzyl iodide in THF at 55 °C. In this case, 1-(4-bromo phenyl)-5-[(3,5-dinitrobenzyl)selanyl]-1*H*-tetrazole (**21a**) was isolated in 23% yield, and cyanamide **21b** in 45% yield. Unfortunately, none of the modifications of the reaction conditions or reactant ratios improved the yields of the target 5-alkylselanyl-1-aryl-1*H*-tetrazoles. Thus methods 2 and 3 are the methods of choice for the preparation of 1-substituted-5-alkylselanyl-1*H*-tetrazoles.

The results of the reactions of alkylisoselenocyanates **13–16** are summarized in Table 5. In contrast to their aryl analogues, 1-alkyl-5-alkylselanyl-1*H*-tetrazoles **22–34** were prepared in good yields using one-pot reactions (methods 2 and 3), which are complete in 30 min to 3 h. Surprisingly, neither *N*,*N*-dialkyl cyanamides nor *Se*-alkyl-*N*-cyano-*N*,*N*′-dialkylisoselenoureas were found regardless of the method used. Tetrabutylammonium azide was also used instead of sodium azide, but, as in the above-mentioned cases, this change did not increase the product yields; tetrazole **32** was prepared in 46% and its dinitro analogue **33** was not formed at all—inseparable mixture of side products was formed.

In this work we demonstrated the enhanced reactivity of alkyl/arylisoselenocyanates toward azide anion compared to alkyl/arylisothiocyanates. Whereas reactions of alkyl/arylisothiocyanates with azide anion lead exclusively to the formation of 1-alkyl/aryl-1*H*-tetrazole-5-thiols, which can be further alkylated,[28] 1-alkyl/aryl-1*H*-tetrazole-5-selenols are unstable compounds and only 1-cyclohexyl-1*H*-tetrazole-5-selenol was partially isolated as its sodium salt. The attempts to alkylate sodium 1-alkyl/aryl-1*H*-tetrazole-5-selenolates in situ under phase-transfer catalysis conditions led to the formation of 1-alkyl-5-alkylselanyl-1*H*-tetrazoles in moderate yields, but 1-aryl-5-alkylselanyl-1*H*-tetrazoles were formed only in very low yields due to unexpected formation of *N*-alkyl-*N*-arylcyanamides (Table 2).

One-pot reactions of alkyl/arylisoselenocyanates with an azide donor and alkylating agent significantly increased the yields of target 1-substituted-5-alkylselanyl-1*H*-tetrazoles. In the case of alkyl derivatives, good yields of the products **22–34** were achieved (Table 5). In the case of aryl derivatives, the target tetrazoles **17–21a** were formed in low-to-moderate yields (Tables 3 and 4),

Table 4
Reactions of arylisoselenocyanates **10**–**12** with sodium azide and 3,5-dinitrobenzyl halide by methods 2, 3, and 4



(1 equiv.) (0.7 equiv.)

10 R = H
11 R = -OCH_3
12 R = -Br

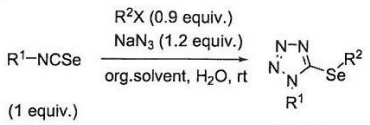
19a R = H
20a R = -OCH_3
21a R = -Br

19b R = H
20b R = -OCH_3
21b R = -Br

19c R = H
20c R = -OCH_3
21c R = -Br

Substrate	Product	Yield [%]				
		Method 2		Method 3		Method 4
		X=Cl	X=I	X=Cl	X=I	X=I
10	**19a**	15	28	28	30	16
10	**19b**	33	31	8	17	27
10	**19c**	2	15	2	32	22
11	**20a**	33	19	33	5	Trace
11	**20b**	40	62	60	44	43
11	**20c**	5	19	0	4	16
12	**21a**	12	34	5	11	17
12	**21b**	0	20	0	15	26
12	**21c**	0	0	0	36	20

Table 5
Synthesis and yields of 1-alkyl-5-alkylselanyl-1*H*-tetrazoles (**22**–**34**) prepared by methods 2, 3, and 4



(1 equiv.)
13-16 **22-34**

R^2 = CH_3, X = OSO_3CH_3; R^2 = $PhCH_2$, X = Br; R^2 = 3,5-$(NO_2)_2PhCH_2$, X = Cl

Substrate	Product	R^1	R^2	Yield [%]		
				Method 2	Method 3	Method 4
13	**22**	$CH_3(CH_2)_3$–	CH_3–	50	59	—
13	**23**	$CH_3(CH_2)_3$–	$PhCH_2$–	73	—	—
13	**24**	$CH_3(CH_2)_3$–	3,5-$(NO_2)_2PhCH_2$–	44	66	54
14	**25**	$CH_3(CH_2)_5$–	CH_3–	57	41	—
14	**26**	$CH_3(CH_2)_5$–	$PhCH_2$–	58	84	61
14	**27**	$CH_3(CH_2)_5$–	3,5-$(NO_2)_2PhCH_2$–	30	45	25
16	**28**	$CH_3(CH_2)_7$–	CH_3–	54	48	—
16	**29**	$CH_3(CH_2)_7$–	$PhCH_2$–	56	74	—
16	**30**	$CH_3(CH_2)_7$–	3,5-$(NO_2)_2PhCH_2$–	38	71	—
15	**31**	C_6H_{11}–	CH_3–	73	—	—
15	**32**	C_6H_{11}–	$PhCH_2$–	76	65	30
15	**33**	C_6H_{11}–	3,5-$(NO_2)_2PhCH_2$–	38	68	57
15	**34**	C_6H_{11}–	2,4-$(NO_2)_2PhCH_2$–	[a]	[a]	[a]

[a] Unseparable mixture. TLC showed the formation of **34** in insignificant amount only.

because these reactions again proceeded via the formation of *N*-alkyl-*N*-arylcyanamides (**17**–**21b**) and, surprisingly, (*Z*)-*Se*-alkyl-*N*-cyano-*N*,*N*′-diarylisoselenoureas (**17**–**21c**) as the main products.

In general, alkyl substituents stabilized the 1-alkyl-1*H*-tetrazole-5-selenolates formed after the addition of the azide anion to isoselenocyanates and facilitated the formation of 1-alkyl-5-alkylselanyl-1*H*-tetrazoles. On the other hand, aryl substituents facilitated the decomposition of 1-aryl-1*H*-tetrazole-5-selenolates leading to the formation of side products, *N*-alkyl-*N*-arylcyanamides and (*Z*)-*Se*-alkyl-*N*-cyano-*N*,*N*′-diarylisoselenoureas.

Furthermore, isoselenoureas **17c** and **19c** (Figs. 2 and 3) were characterized using X-ray crystallography. Both compounds crystallized in the triclinic space group *P*-1, isoselenourea **17c** with two molecules within the unit cell, while its dinitro analogue **19c** crystallized with two independent molecules and with four molecules within the triclinic unit cell. To the best of our knowledge, analogous structures are still elusive in the literature; only several cyclic isoselenoureas (e.g., A,[35,36] B,[37,38] C,[39,40] D,[41,42] E,[35,43] F,[44,45] G[46,47]), which resemble the structural motif of compounds **17c** and **19c**, were found within the crystallographic database (Fig. 4). These compounds reveal rather similar distances and angles of interest within various ring arrangements to the isoselenoureas **17c** and **19c**. The lack of conjugation is seen from the separation of C and N atoms in both molecules of **17c** and **19c**, and thus the double

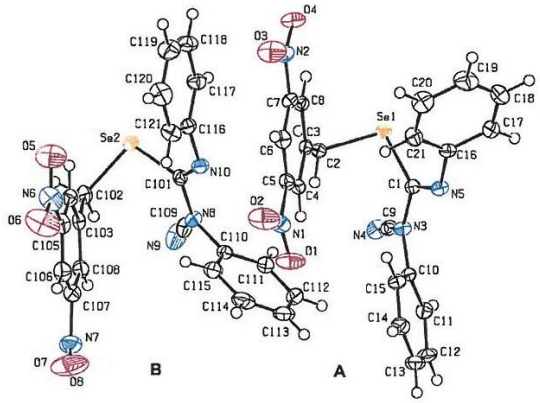


Fig. 3. The molecular structure (ORTEP 50% probability level) of isoselenourea **19c**. Selected interatomic distances [Å], angles and interplanar angle [°] for molecules **A** and **B** (appropriate parameters are given in parentheses): Se1–C1 1.934(2) (1.930(2)), Se1–C2 1.9737(19) (1.972(2)), C1–N3 1.427(2) (1.421(3)), C1–N5 1.255(3) (1.256(3)), N3–C9 1.347(3) (1.343(3)), C9–N4 1.145(3) (1.143(3)); C1–Se1–C2 97.62(8)(97.65(8)), Se1–C1–N3 114.71(14) (115.44(14)), Se1–C1 N5 127.31(15) (126.86(16)), C1–N3–C9 119.91(16) (120.78(17)), C1–N5–C16 123.19(18) (122.73(18)), N3–C9–N4 175.9(2); Se1–C1–N5 versus C9–N3–C10 22.28(2) (16.76(3)).

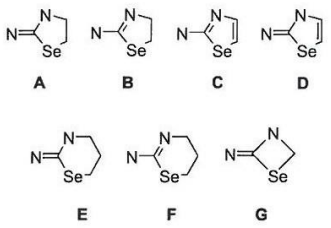


Fig. 4. Structural motifs of compounds similar to isoselenoureas **17c** and **19c** found in the crystallographic database.

and triple bonds between carbon and nitrogen can be surely assigned (Figs. 2 and 3).

In the crystal packing of isoselenoureas **17c** and **19c** (Figs. 5 and 6), no structurally interesting interatomic nor $\pi-\pi$ interactions were observed. On the other hand, along the *a*-axis, an intriguing supramolecular architecture is detected. In the case of **17c**, a dimer-like structure is formed (Fig. 5), while in the case of **19c**, its molecules are mutually oriented in layers (Fig. 6) with alternating domains of selenium and oxygen atoms.

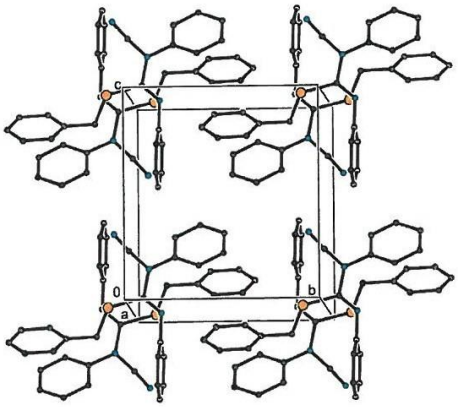


Fig. 5. Crystal packing of isoselenourea **17c** view along axis *a*.

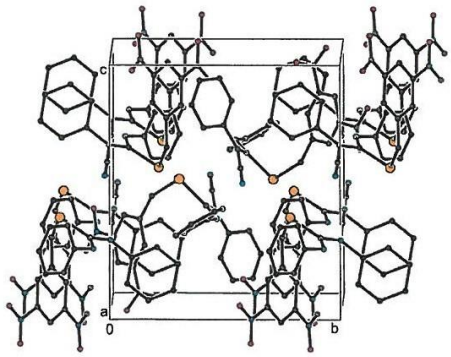


Fig. 6. Crystal packing of isoselenourea **19c** view along axis *a*.

3. Conclusions

In this work, the synthesis of 1-substituted-5-alkylselanyl-1*H*-tetrazoles from alkyl/arylisoselenocyanates has been described. One-pot reactions of arylisoselenocyanates with sodium azide and an alkylating agent led unexpectedly to the formation of *N*-alkyl-*N*-arylcyanamides and (*Z*)-*Se*-alkyl-*N*-cyano-*N*,*N*′-diaryliso selenoureas as the main products, while 1-aryl-5-alkylselanyl-1*H*-tetrazoles were formed only in low yields. Nevertheless, when alkylisoselenocyanates were utilized as the substrates, the reactions led exclusively to the formation of 1-alkyl-5-alkylselanyl-1*H*-tetrazoles in good yields.

Simple and general methods described in this work bring new possibilities for the synthesis of these selenium-containing compounds and can be used in the synthesis and further investigations of biologically active sulfur/selenium bioisosteres.

4. Experimental section

4.1. General

All chemicals for synthesis were obtained from Sigma–Aldrich (Schnelldorf, Germany) and used as received. TLC was performed on Merck aluminum sheets with silica gel 60 F_{254} and/or Merck aluminum sheets with silica gel 60 RP-18 F_{254}s. Merck Kieselgel 60 (0.040–0.063 mm) and/or Merck LiChroprep RP-18 (0.015–0.025 mm) were used for column chromatography. Melting points were recorded on a Büchi B-545 apparatus and are uncorrected. Infrared spectra were measured on a Nicolet 6700 (ATR mode). ^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury Vx BB 300 or VNMR S500 NMR spectrometers. 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 carried out on Automatic Microanalyser EA1110CE (Fisons Instruments S.p.A., Milano, Italy). Electrospray ionization mass spectroscopic (ESI MS) experiments were performed using Acquity UPLC with MS/MS Quattro Micro detection (Micromass+Waters). High-resolution mass spectra were recorded on VG-Analytical ZAB-SEQ.

4.2. Crystallography

The X-ray data for the colorless and yellowish crystals of isoselenoureas **17c** and **19c** were obtained at 150 K using an Oxford Cryostream low-temperature device on a Nonius KappaCCD diffractometer with Mo Kα radiation (λ=0.71073 Å), a graphite monochromator, and the ϕ and χ scan mode. Data reductions were performed with DENZO-SMN.[48] The absorption was corrected by integration methods.[49] Structures were solved by direct methods (Sir92)[50] and refined by full matrix least-square based on F^2 (SHELXL97).[51] Hydrogen atoms were mostly localized on a difference Fourier map, however, to ensure uniformity of treatment of crystal, all hydrogens were recalculated into idealized positions (riding model) and assigned temperature factors $H_{iso}(H)$=1.2 U_{eq}(-pivot atom) with C–H=0.97 and 0.93 Å for methylene and hydrogen atoms in aromatic rings.

$R_{int} = \sum\left|F_o^2 - F_{o,mean}^2\right| / \sum F_o^2$, GOF $= [\sum(w(F_o^2 - F_c^2)^2)/(N_{diffrs} - N_{params})]^{1/2}$ for all data, $R(F)=\sum||F_o|-|F_c||/\sum|F_o|$ for observed data, $wR(F^2) = [\sum(w(F_o^2 - F_c^2)^2)/(\sum w(F_o^2)^2)]^{1/2}$ for all data.

Crystallographic data for structural analysis have been deposited with the Cambridge Crystallographic Data Centre, CCDC no. 928734 and 928735 for **17c** and **19c**, respectively. Copies of this information may be obtained free of charge from The Director, CCDC, 12 Union Road, Cambridge CB2 1EY, UK (fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk or website: http://www.ccdc.cam.ac.uk).

Single crystals of isoselenoureas **17c** and **19c** were of sufficient quality for X-ray diffraction techniques. The crystallographic data and structure refinement parameters for compound **17c** are:

$C_{21}H_{17}N_3Se$, M=390.34, triclinic, *P*-1, *a*=9.0471(5) Å, *b*=10.6140(5) Å, *c*=10.6470(3) Å, *α*=83.622(4)°, *β*=68.472(3)°, *γ*=68.326(4)°, *Z*=2, *V*=883.47(8) Å^3, D_c=1.467 g cm^{-3}, *μ*=2.133 mm^{-1}, T_{min}/T_{max}=0.519/0.739; $-11\leq h\leq 11$, $-13\leq k\leq 13$, $-13\leq l\leq 13$; 18,954 reflections measured (θ_{max}=27.49°), 18,869 independent (R_{int}=0.0226), 3769 with $I>2\sigma(I)$, 226 parameters, *S*=1.098, *R*1(obsd data)=0.0246, *wR2*(all data)=0.0612; max., min. residual electron density=0.527, −0.775 e Å^{-3}.

Crystallographic data for compound **19c**: $C_{21}H_{15}N_5O_4Se$, *M*=480.34, triclinic, *P*-1, *a*=12.7870(9) Å, *b*=13.1701(10) Å, *c*=14.4670(9) Å, *α*=78.456(6)°, *β*=68.953(4)°, *γ*=63.744(6)°, *Z*=4, *V*=2036.7(3) Å^3, D_c=1.566 g cm^{-3}, *μ*=1.883 mm^{-1}, T_{min}/T_{max}=0.549/0.772; $-16\leq h\leq 16$, $-17\leq k\leq 17$, $-18\leq l\leq 18$; 44,792 reflections measured (θ_{max}=27.5°), 44,722 independent (R_{int}=0.0296), 7683 with $I>2\sigma(I)$, 559 parameters, *S*=1.116, *R*1(obsd data)=0.0298, *wR2*(all data)=0.0570; max., min. residual electron density=0.362, −0.382 e Å^{-3}.

4.3. Synthesis of alkyl/arylisoselenocyanates (10–16)

*4.3.1. Phenylisoselenocyanate (**10**).*[52] To a refluxing mixture of the *N*-phenylformamide (1 g, 8.25 mmol) and triethylamine (3.59 g, 4.95 ml, 35.49 mmol) in CH_2Cl_2 (35 ml) a solution of triphosgene (1.3 g, 4.37 mmol) in CH_2Cl_2 (15 ml) was added dropwise over a period of 1 h. After the addition was complete, the mixture was stirred at reflux for an additional 3 h until the starting material disappeared, as determined by TLC. Selenium powder (1.3 g, 16.5 mmol) was then added, and the mixture was refluxed for an additional 12 h. The formation of isoselenocyanate could be observed by formation of an orange spot on TLC (after oxidation on air). After completion, the mixture was cooled, filtered, and the precipitate on the filter was washed with EtOAc. The organic solution was evaporated under reduced pressure and the residue was purified by silica gel column chromatography (mobile phase: hexane) to give 0.95 g (5.2 mmol, 63%) of **10** as a yellow viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$): *δ* 7.40–7.31 (3H, m), 7.30–7.27 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$): *δ* 129.68, 129.54, 129.31 (NCSe), 128.07, 126.06. IR (KBr): 2110 (*s*, NCSe), 2049, 1588, 1487, 1070, 907, 845, 748, 683 cm^{-1}.

Isoselenocyanates **11–14** and **16** were prepared according to procedure in Section 4.3.1.

*4.3.2. 4-Methoxyphenylisoselenocyanate (**11**).*[53] The refluxing mixture was stirred for 3 h after the addition of the solution of triphosgene in CH_2Cl_2 and then for 6 h after the addition of selenium powder. Mobile phase: hexane/EtOAc, 35:1. Yield: 68% as slightly pink solid; mp 49–50 °C (lit.[53] 50–51 °C). ^{1}H NMR (500 MHz, $CDCl_3$): *δ* 7.22 (2H, d, *J*=9.0 Hz), 6.86 (2H, d, *J*=9.0 Hz), 3.81 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$): *δ* 159.13, 127.37 (2C, NCSe+Ar), 122.14, 114.79, 55.57. IR (KBr): 2993, 2955, 2930, 2834, 2116 (*s*, NCSe), 2058, 1877, 1502, 1452, 1441, 1303, 1248, 1166, 1105, 1028, 820, 759, 702 cm^{-1}.

*4.3.3. 4-Bromophenylisoselenocyanate (**12**).*[45] The refluxing mixture was stirred for 4.5 h after the addition of the solution of triphosgene in CH_2Cl_2 and then for 12 h after the addition of selenium powder. Mobile phase: hexane. Yield: 58% as white crystalline solid; mp 80–81 °C. ^{1}H NMR (500 MHz, DMSO): *δ* 7.66 (2H, d, *J*=8.6 Hz), 7.47 (2H, d, *J*=8.6 Hz); ^{13}C NMR (126 MHz, DMSO): *δ* 132.98, 129.66 (NCSe), 128.52, 128.31, 121.60. IR (KBr): 3083, 2119 (*s*, NCSe), 2050, 1479, 1398, 1069, 1011, 845, 816 cm^{-1}. Anal. Calcd for C_7H_4BrNSe: C, 32.22; H, 1.54; N, 5.37. Found: C, 32.03; H, 1.83; N, 5.45.

*4.3.4. Butylisoselenocyanate (**13**).*[54] The refluxing mixture was stirred for 4.5 h after the addition of the solution of triphosgene in CH_2Cl_2 and then for 5.5 h after the addition of selenium powder. Mobile phase: hexane/EtOAc, 30:1. Yield: 67% as slightly yellow viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) *δ* 3.59 (2H, t, *J*=6.6 Hz), 1.74–1.67 (2H, m), 1.50–1.41 (2H, m), 0.94 (3H, t, *J*=7.3 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): *δ* 121.82 (NCSe), 45.11, 31.30, 19.66, 13.13. IR (KBr): 2959, 2938, 2873, 2140 (s, NCSe), 1736, 1458, 1446, 1344, 1116, 743 cm^{-1}.

*4.3.5. Hexylisoselenocyanate (**14**).*[55] The refluxing mixture was stirred for 4.5 h after the addition of the solution of triphosgene in CH_2Cl_2 and then for 8 h after the addition of selenium powder. Mobile phase: hexane/EtOAc, 30:1. Yield: 43% as slightly yellow viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$): *δ* 3.58 (2H, t, *J*=6.6 Hz), 1.75–1.68 (2H, m), 1.45–1.37 (2H, m), 1.36–1.26 (4H, m), 0.89 (3H, t, *J*=6.9 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): *δ* 121.87 (NCSe), 45.42, 30.82, 29.35, 26.12, 22.33, 13.87. IR (KBr): 2955, 2928, 2858, 2138 (s, NCSe), 1449, 1344, 726 cm^{-1}.

*4.3.6. Cyclohexylisoselenocyanate (**15**).*[56] Cyclohexylisoselenocyanate was prepared from commercially available cyclohexyl isocyanide. A mixture of cyclohexyl isocyanide (1 g, 9.16 mmol) and selenium powder (1.45 g, 18.32 mmol) in dry CH_2Cl_2 (15 ml) was stirred at reflux for 3 h. After completion, as determined by TLC, the solvent was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography (hexane/EtOAc, 50:1). Yield: 98% (1.69 g, 8.98 mmol) as slightly yellow viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$): *δ* 3.84–3.74 (1H, m), 1.95–1.87 (2H, m), 1.79–1.63 (4H, m), 1.54–1.32 (4H, m); ^{13}C NMR (75 MHz, $CDCl_3$): *δ* 121.65 (NCSe), 55.85, 32.65, 24.88, 23.01. IR (KBr): 2933, 2855, 2114 (s, NCSe), 2065, 1448, 1361, 1318, 1126, 1048, 891, 671 cm^{-1}

*4.3.7. Octylisoselenocyanate (**16**).* The refluxing mixture was stirred for 3 h after the addition of the solution of triphosgene in CH_2Cl_2 and then for 5 h after the addition of selenium powder. Mobile phase: hexane/EtOAc, 35:1. Yield: 67% as slightly yellow viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$): *δ* 3.59 (2H, t, *J*=6.7 Hz), 1.77–1.68 (2H, m), 1.47–1.21 (10H, m), 0.89 (3H, t, *J*=6.8 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): *δ* 121.70 (NCSe), 45.42, 31.64, 29.36, 28.97, 28.65, 26.47, 22.56, 14.03. IR (KBr): 2924, 2855, 2135 (s, NCSe), 1737, 1456, 1343, 1116, 722 cm^{-1}; HRMS *m/z* (CI) calcd for $C_9H_{17}NSe+H^+$ ($M+H^+$) 220.0599, found 220.0595.

4.4. Synthesis of 1-alkyl/aryl-5-alkylselanyl-1*H*-tetrazoles

4.4.1. General procedures. Dimethylsulfate, benzyl bromide, 3,5-dinitrobenzyl chloride, and 3,5-dinitrobenzyl iodide were used as alkylating agents.

4.4.2. Method 1. Isoselenocyanate (1 mmol) and sodium azide (1.2 mmol) in water (7 ml) were stirred for 12–24 at rt until completion, as determined by TLC (for R_f values see later). Precipitated selenium was filtered off and the aqueous solution was washed with EtOAc (2×10 ml). The alkylating agent (0.7 mmol) and TBAB (0.035 mmol) in CH_2Cl_2 (7 ml) were added to the aqueous solution. The reaction mixture was gently stirred at rt overnight. The organic phase was separated, washed with water (2×10 ml), and dried over anhydrous sodium sulfate. The solvent was evaporated and the crude products were separated and purified by column chromatography on silica gel.

4.4.3. Method 2. To the solution of isoselenocyanate (1 mmol) and alkylating agent (0.7 mmol for arylisoselenocyanates/0.9 mmol for alkylisoselenocyanates) in THF (10 ml), sodium azide (1.2 mmol) in water (0.5 ml) was added. The reaction mixture was stirred until the starting isoselenocyanate disappeared, as determined by TLC. After completion, the THF was evaporated, the crude product was

dissolved in EtOAc (10 ml), and washed with water (2×10 ml). The organic phase was dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the crude products were purified by silica gel column chromatography and/or reverse phase column chromatography (see later).

4.4.4. Method 3. This method is same as method 2, only acetonitrile was used instead of THF.

4.4.5. Method 4. Isoselenocyanate (1 mmol) and alkylating agent (0.7 mmol for arylisoselenocyanates/0.9 mmol for alkylisoselenocyanates) in CH_2Cl_2 (7 ml) and sodium azide (1.2 mmol) in H_2O (7 ml) were stirred in the presence of TBAB (0.035/0.045 mmol for aryl/alkylisoselenocyanate, respectively) at rt until completion (usually overnight). The organic phase was then separated, washed with water (2×10 ml), and dried over anhydrous sodium sulfate. The solvent was evaporated and the crude product was purified by column chromatography on silica and/or reverse phase column chromatography (see later).

4.5. 5-Alkylselanyl-1-aryl-1*H*-tetrazoles (17–21b)

Yields of the products are given in Tables 2–4.

Compounds **17a**, **17b**, and **17c** were separated by reverse phase column chromatography, mobile phase: MeCN/H_2O, 2:1.

*4.5.1. 5-Benzylselanyl-1-phenyl-1H-tetrazole (**17a**).* Beige solid; mp 71 °C. R_f (hexane/EtOAc, 3:1) 0.72, R_f (RP-18; MeCN/H_2O, 2:1) 0.27. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.55–7.44 (5H, m, Ar), 7.42–7.36 (2H, m, Ar), 7.33–7.24 (3H, m, Ar), 4.68 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 147.18, 136.24, 133.94, 130.20, 129.75, 129.11, 128.80, 127.91, 123.88, 32.56. IR (KBr): 3030, 2922, 1595, 1497, 1374, 1233, 1089, 1014, 973, 765, 693 cm^{-1}. Anal. Calcd for $C_{14}H_{12}N_4Se$: C, 53.34; H, 3.84; N, 17.77. Found: C, 53.25; H, 3.98; N, 17.53.

*4.5.2. N-Benzyl-N-phenylcyanamide (**17b**).*[57] White crystalline solid; mp 62 °C (lit.[57] 59–60 °C). R_f (hexane/EtOAc, 3:1) 0.75, R_f (RP-18; MeCN/H_2O, 2:1) 0.33. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.45–7.30 (7H, m, Ar), 7.17–7.05 (3H, m, Ar), 4.81 (2H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ 139.76, 134.25, 129.62, 129.06, 128.50, 127.31, 123.67, 115.98, 113.90, 53.70. IR (KBr): 3030, 2925, 2216 (s, C≡N), 1599, 1494, 1453, 1367, 1233, 1181, 1078, 930, 878, 766, 718, 686 cm^{-1}. Anal. Calcd for $C_{14}H_{12}N_2$: C, 80.74; H, 2.81; N, 13.45. Found: C, 80.40; H, 2.52; N, 13.62. LRMS *m/z* (ESI) 208.88 (100, MH$^+$), 209.89 (15%).

*4.5.3. (Z)-Se-Benzyl-N-cyano-N,N′-diphenylisoselenourea (**17c**).* White crystalline solid; mp 97–98 °C. R_f (hexane/EtOAc, 3:1) 0.8, R_f (RP-18; MeCN/H_2O, 2:1) 0.12. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.44–7.08 (13H, m, Ar), 6.79–6.71 (2H, m, Ar), 4.05 (2H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ 147.54, 144.34, 137.35, 136.59, 129.55, 129.11, 128.91, 128.74, 128.25, 127.72, 125.03, 124.76, 120.42, 111.20, 31.96. IR (KBr): 2223 (s, C≡N), 1627, 1587, 1486, 1240, 1231, 1187, 1163, 1077, 851, 751, 690 cm^{-1}; HRMS *m/z* (ESI) calcd for $C_{21}H_{17}N_3Se$+H$^+$ (M+H$^+$) 392.06605, found 392.06597. Anal. Calcd for $C_{21}H_{17}N_3Se$: C, 64.62; H, 4.39; N, 10.77. Found: C, 64.58; H, 4.79; N, 10.86.

Compounds **18a**, **18b**, and **18c** were separated by reverse phase column chromatography, mobile phase: MeCN/H_2O, 2:1.

*4.5.4. 5-Benzylselanyl-1-(4-methoxyphenyl)-1H-tetrazole (**18a**).* Yellowish viscous oil. R_f (hexane/EtOAc, 3:1) 0.34, R_f (RP-18; MeCN/H_2O, 2:1) 0.28. ^{1}H NMR (300 MHz, acetone): δ 7.47–7.40 (4H, m, Ar), 7.32–7.20 (3H, m, Ar), 7.17–7.10 (2H, m), 4.64 (2H, s), 3.88 (3H, s); ^{13}C NMR (75 MHz, acetone): δ 161.96, 148.22, 138.71, 129.92, 129.42, 128.40, 127.62, 126.99, 115.71, 56.08, 32.53. IR (KBr): 3062, 2837, 1608, 1515, 1454, 1377, 1258, 1171, 1023, 834, 761, 698 cm^{-1}. LRMS *m/z* (ESI) 346.7 (100, MH$^+$), 344.8 (50), 342.8 (20%); HRMS *m/z* (ESI) calcd for $C_{15}H_{14}N_4OSe$+H$^+$ (M+H$^+$) 347.04056, found 347.04056.

*4.5.5. N-Benzyl-N-(4-methoxyphenyl)cyanamide (**18b**).* White crystalline solid; mp 60 °C. R_f (hexane/EtOA, 3:1) 0.43, R_f (RP-18; MeCN/H_2O, 2:1) 0.41. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.43–7.31 (5H, m), 7.05 (2H, d, *J*=9.1 Hz), 6.86 (2H, d, *J*=9.1 Hz), 4.74 (2H, s), 3.77 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.26, 134.47, 133.07, 129.00, 128.47, 127.47, 118.25, 114.83, 55.53, 54.64. IR (KBr): 2956, 2926, 2359, 2212 (s, C≡N), 1509, 1453, 1373, 1291, 1230, 1178, 1019, 821, 740, 703, 684 cm^{-1}. Anal. Calcd for $C_{15}H_{14}N_2O$: C, 75.61; H, 5.92; N, 11.76. Found: C, 75.73; H, 6.05; N, 12.03.

*4.5.6. (Z)-Se-Benzyl-N-cyano-N,N′-di(4-methoxyphenyl)isoselenourea (**18c**).* Yellow viscous oil. R_f (hexane/EtOAc, 3:1) 0.35, R_f (RP-18; MeCN/H_2O, 2:1) 0.19. ^{1}H NMR (300 MHz, acetone): δ 7.35–7.20 (7H, m), 7.00 (2H, d, *J*=9.0 Hz), 6.88 (2H, d, *J*=8.8 Hz), 6.78 (2H, d, *J*=9.0 Hz), 4.03 (2H, s), 3.83 (3H, s), 3.76 (3H, s); ^{13}C NMR (75 MHz, acetone): δ 160.69, 157.84, 145.20, 141.90, 138.39, 131.28, 129.82, 129.50, 128.66, 128.31, 122.57, 115.56, 114.86, 111.96, 55.91, 55.64, 32.01; ^{15}N NMR (50.7 MHz, $CDCl_3$): δ 296.5, 214.1, 103.4. IR (KBr): 2956, 2835, 2222 (C≡N), 1621, 1504, 1292, 1242, 1177, 1077, 1031, 831, 697 cm^{-1}. LRMS *m/z* (ESI) 451.7 (100, MH$^+$), 449.8 (52), 452.7 (28), 448.8 (21.5%); HRMS *m/z* (ESI) calcd for $C_{23}H_{21}N_3O_2Se$+H$^+$ (M+H$^+$) 452.08718, found 452.08711.

Compounds **19a**, **19b**, and **19c** were separated by silica gel column chromatography, mobile phase: hexane/EtOAc, 10:1.

*4.5.7. 5-(3,5-Dinitrobenzylselanyl)-1-phenyl-1H-tetrazole (**19a**).* Light beige crystalline solid; mp 141–143 °C (with decomposition). R_f (hexane/EtOAc, 3:1) 0.18. ^{1}H NMR (300 MHz, acetone): δ 8.85 (2H, d, *J*=2.1 Hz), 8.80 (1H, t, *J*=2.1 Hz), 7.78–7.39 (5H, m), 4.95 (2H, s); ^{13}C NMR (75 MHz, acetone): δ 149.29, 147.59, 144.56, 134.88, 131.44, 130.88, 130.46, 125.20, 118.29, 30.39. IR (KBr): 3098, 1536 (NO_2), 1499, 1342 (NO_2), 1232, 1093, 1036, 914, 809, 771, 734, 692, 672 cm^{-1}. Anal. Calcd for $C_{14}H_{10}N_6O_4Se$: C, 41.50; H, 2.49; N, 20.74. Found: C, 41.32; H, 2.71; N, 20.47.

*4.5.8. N-(3,5-Dinitrobenzyl)-N-phenylcyanamide (**19b**).* Yellowish solid; mp 174–176 °C. R_f (hexane/EtOAc, 3:1) 0.28. ^{1}H NMR (300 MHz, acetone): δ 8.92 (1H, t, *J*=2.1 Hz), 8.76 (2H, d, *J*=2.1 Hz), 7.46–7.39 (2H, m), 7.31–7.26 (2H, m), 7.18–7.11 (1H, m), 5.38 (2H, s); ^{13}C NMR (75 MHz, acetone): δ 149.76, 140.98, 140.39, 130.78, 128.85, 124.89, 119.28, 116.86, 113.66, 52.39. IR (KBr): 3115, 2921, 2360, 2221 (s, C≡N), 1597, 1538 (NO_2), 1492, 1342 (NO_2), 1234, 1188, 1083, 903, 810, 763, 723, 696, 666 cm^{-1}. Anal. Calcd for $C_{14}H_{10}N_4O_4$: C, 56.38; H, 3.38; N, 18.78. Found: C, 56.3; H, 3.56; N, 18.88.

*4.5.9. (Z)-Se-(3,5-Dinitrobenzyl)-N-cyano-N,N′-diphenylisoselenourea (**19c**).* Yellowish solid; mp 130 °C. R_f (hexane/EtOAc, 3:1) 0.39. ^{1}H NMR (300 MHz, acetone): δ 8.81 (1H, t, *J*=2.2 Hz), 8.54 (2H, d, *J*=2.2 Hz), 7.53–7.38 (5H, m), 7.32–7.24 (2H, m), 7.14–7.07 (1H, m), 6.84–6.78 (2H, m), 4.42 (2H, s); ^{13}C NMR (75 MHz, acetone): δ 149.37, 148.32, 144.24, 144.09, 138.50, 130.61, 129.97, 129.77, 129.47, 126.34, 125.59, 121.30, 118.31, 111.33, 30.26. IR (KBr): 3087, 2963, 2922, 2220 (C≡N), 1651, 1592, 1537 (NO_2), 1488, 1343 (NO_2), 1237, 1183, 1076, 912, 853, 809, 754, 695 cm^{-1}. Anal. Calcd for $C_{21}H_{15}N_5O_4Se$: C, 52.51; H, 3.15; N, 14.58. Found: C, 52.46; H, 3.32; N, 14.30; HRMS *m/z* (ESI) calcd for $C_{21}H_{15}N_5O_4Se$+H$^+$ (M+H$^+$) 482.03620, found 482.03618.

Compounds **20a**, **20b**, and **20c** were separated by silica gel column chromatography, mobile phase: hexane/EtOAc, 7:1.

*4.5.10. 5-(3,5-Dinitrobenzylselanyl)-1-(4-methoxyphenyl)-1H-tetrazole (**20a**).* Brownish solid; mp 131–132 °C (with decomposition).

R_f(hexane/EtOAc, 3:1) 0.12. ^{1}H NMR (500 MHz, acetone): δ 8.83 (2H, d, J=2.1 Hz), 8.80 (1H, t, J=2.1 Hz), 7.48 (2H, d, J=9.0 Hz), 7.14 (2H, d, J=9.0 Hz), 4.91 (2H, s), 3.89 (3H, s); ^{13}C NMR (125 MHz, acetone): δ 162.07, 149.27, 147.77, 144.63, 130.42, 127.39, 126.94, 118.26, 115.81, 56.11, 30.26. IR (KBr): 3089, 2923, 1608, 1591, 1537 (NO_2), 1511, 1446, 1342 (NO_2), 1257, 1173, 1020, 913, 839, 730, 672 cm^{-1}. Anal. Calcd for $C_{15}H_{12}N_6O_5Se$: C, 41.39; H, 2.78; N, 19.31. Found: C, 41.59; H, 2.41; N, 19.0.

*4.5.11. N-(3,5-Dinitrobenzyl)-N-(4-methoxyphenyl)cyanamide (**20b**).* Yellowish solid; mp 121–122 °C. R_f(hexane/EtOAc, 3:1) 0.24. ^{1}H NMR (500 MHz, acetone): δ 8.91 (1H, t, J=2.1 Hz), 8.73 (2H, d, J=2.1 Hz), 7.23 (2H, d, J=9.2 Hz), 6.97 (2H, d, J=9.2 Hz), 5.28 (2H, s), 3.76 (3H, s); ^{13}C NMR (125 MHz, acetone): δ 157.70, 149.73, 141.15, 133.51, 128.97, 119.37, 119.25, 115.92, 114.53, 55.83, 53.32. IR (KBr): 3091, 2839, 2218 (s, C≡N), 1596, 1545 (NO_2), 1509, 1439, 1344 (NO_2), 1238, 1180, 1025, 913, 821, 729 cm^{-1}. Anal. Calcd for $C_{15}H_{12}N_4O_5$: C, 54.88; H, 3.68; N, 17.07. Found: C, 54.65; H, 4.04; N, 16.72.

*4.5.12. (Z)-Se-(3,5-Dinitrobenzyl)-N-cyano-N,N′-di(4-methoxyphenyl)isoselenourea (**20c**).* Yellowish solid; mp 142 °C (with decomposition). R_f (hexane/EtOAc, 3:1) 0.29. ^{1}H NMR (500 MHz, acetone): δ 8.80 (1H, t, J=2.2 Hz), 8.48 (2H, d, J=2.2 Hz), 7.40 (2H, d, J=9.0 Hz), 7.01 (2H, d, J=9.0 Hz), 6.90–6.76 (4H, m), 4.32 (2H, s), 3.83 (3H, s), 3.76 (3H, s); ^{13}C NMR (125 MHz, acetone): δ 160.90, 158.05, 149.35, 144.26, 143.74, 141.20, 131.06, 129.91, 128.73, 122.76, 118.22, 115.68, 114.92, 111.56, 55.96, 55.64, 30.05. IR (KBr): 3112, 2904, 2221 (C≡N), 1625, 1542 (NO_2), 1505, 1342 (NO_2), 1245, 1175, 1076, 1038, 833, 730 cm^{-1}; HRMS m/z (ESI) calcd for $C_{23}H_{19}N_5O_6Se+H^+$ ($M+H^+$) 542.05733, found 542.05739.

Compounds **21a**, **21b**, and **21c** were separated by silica gel column chromatography, mobile phase: hexane/EtOAc, 8:1.

*4.5.13. 1-(4-Bromphenyl)-5-(3,5-dinitrobenzylselanyl)-1H-tetrazole (**21a**).* White solid; mp 154–155 °C (with decomposition). R_f (hexane/EtOAc, 3:1) 0.12. ^{1}H NMR (500 MHz, acetone) δ 8.84 (2H, d, J=2.1 Hz), 8.80 (1H, t, J=2.1 Hz), 7.84 (2H, d, J=8.8 Hz), 7.58 (2H, d, J=8.3 Hz), 4.95 (2H, s); ^{13}C NMR (125 MHz, acetone): δ 148.38, 146.76, 143.59, 133.19, 133.11, 129.57, 126.28, 123.99, 117.40, 29.71. IR (KBr): 3104, 2923, 1530 (NO_2), 1496, 1361, 1340 (NO_2), 1176, 1073, 1007, 841, 731, 674 cm^{-1}. Anal. Calcd for $C_{14}H_9BrN_6O_4Se$: C, 34.73; H, 1.87; N, 17.36. Found: C, 34.93; H, 1.68; N, 17.01.

*4.5.14. N-(4-Bromphenyl)-N-(3,5-dinitrobenzyl)cyanamide (**21b**).* Beige solid; mp 170 °C (with decomposition). R_f (hexane/EtOAc, 3:1) 0.18. ^{1}H NMR (300 MHz, acetone): δ 8.92 (1H, t, J=2.2 Hz), 8.77 (2H, d, J=2.1 Hz), 7.57 (2H, d, J=9.1 Hz), 7.26 (2H, d, J=9.1 Hz), 5.39 (2H, s); ^{13}C NMR (75 MHz, acetone) δ 149.80, 140.53, 139.92, 133.59, 128.99, 119.41, 118.95, 117.09, 113.21, 52.51. IR (KBr): 3149, 2959, 2230 (s, C≡N), 1595, 1492, 1485, 1395, 1283, 1252, 1178, 1074, 1010, 813 cm^{-1}. Anal. Calcd for $C_{14}H_9BrN_4O_4$: C, 44.58; H, 2.41; N, 14.86. Found: C, 44.2; H, 2.71; N, 14.59.

*4.5.15. (Z)-Se-(3,5-Dinitrobenzyl)-N-cyano-N,N′-di(4-bromophenyl) isoselenourea (**21c**).* Beige solid; mp 156–158 °C (with decomposition). R_f (hexane/EtOAc, 3:1) 0.33. ^{1}H NMR (300 MHz, acetone): δ 8.82 (1H, t, J=2.1 Hz), 8.58 (2H, d, J=2.1 Hz), 7.68 (2H, d, J=8.8 Hz), 7.50 (2H, d, J=8.8 Hz), 7.43 (2H, d, J=8.6 Hz), 6.76 (2H, d, J=8.6 Hz), 4.52 (s, 2H); ^{13}C NMR (75 MHz, acetone): δ 149.41, 147.57, 144.97, 144.03, 137.66, 133.68, 132.73, 129.95, 128.61, 123.53, 122.78, 118.37, 118.32, 110.95, 30.60. IR (KBr): 3101, 2923, 2234 (C≡N), 1616, 1539 (NO_2), 1487, 1344 (NO_2), 1184, 1071, 1013, 913, 825, 730, 672 cm^{-1}. Anal. Calcd for $C_{21}H_{13}Br_2N_5O_4Se$: C, 39.53; H, 2.05; N, 10.97. Found: C, 39.91; H, 2.43; N, 10.76; HRMS m/z (ESI) calcd for $C_{21}H_{13}Br_2N_5O_4Se+Na^+$ ($M+Na^+$) 659.83917, found 659.83855.

4.6. 1-Alkyl-5-alkylselanyl-1*H*-tetrazoles (22–34)

Yields of the products are given in Tables 2 and 5.

*4.6.1. 1-Butyl-5-methylselanyl-1H-tetrazole (**22**).* Yellowish viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.24 (2H, t, J=7.2 Hz), 2.72 (3H, s), 1.92–1.82 (2H, m), 1.42–1.30 (2H, m), 0.96 (3H, t, J=7.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 146.45, 47.70, 31.14, 19.57, 13.36, 8.13. IR (KBr): 2960, 2935, 2874, 2218 (w), 1458, 1428, 1383, 1275, 1186, 1078, 978, 925, 753 cm^{-1}. LRMS m/z (ESI) 220.7 (100, MH^+), 218.9 (52), 216.8 (20%); HRMS m/z (ESI) calcd for $C_6H_{12}N_4Se+H^+$ ($M+H^+$) 221.02999, found 221.02997.

*4.6.2. 5-Benzylselanyl-1-butyl-1H-tetrazole (**23**).* Yellowish viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.29–7.19 (5H, m), 4.51 (2H, s), 4.05 (2H, t, J=7.2 Hz), 1.74–1.59 (2H, m), 1.14–1.26 (2H, m), 0.85 (3H, t, J=7.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.91, 136.72, 128.93, 128.81, 127.92, 47.67, 32.91, 31.20, 19.46, 13.30. IR (KBr): 2960, 2873, 1495, 1454, 1426, 1381, 1183, 1070, 979, 760, 697 cm^{-1}. LRMS m/z (ESI) 296.8 (100, MH^+), 294.8 (49), 292.9 (18%); HRMS m/z (ESI) calcd for $C_{12}H_{16}N_4Se+H^+$ ($M+H^+$) 297.06129, found 297.06131.

*4.6.3. 1-Butyl-5-(3,5-dinitrobenzylselanyl)-1H-tetrazole (**24**).* White solid; mp 89 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.92 (1H, t, J=2.0 Hz), 8.69 (2H, d, J=2.1 Hz), 4.77 (2H, s), 4.20 (2H, t, J=7.2 Hz), 1.94–1.67 (2H, m), 1.40–1.15 (2H, m), 0.93 (3H, t, J=7.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 148.52, 145.00, 142.05, 129.43, 118.13, 47.97, 31.18, 29.66, 19.54, 13.31. IR (KBr): 3103, 2965, 2877, 1537 (NO_2), 1428, 1343 (NO_2), 1194, 1075, 987, 915, 810, 731, 677 cm^{-1}. Anal. Calcd for $C_{12}H_{14}N_6O_4Se$: C, 37.41; H, 3.66; N, 21.82. Found: C, 37.5; H, 4.0; N, 21.75.

*4.6.4. 5-Methylselanyl-1-hexyl-1H-tetrazole (**25**).* Yellowish viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.22 (2H, t, J=7.2 Hz), 2.70 (3H, s), 1.94–1.79 (2H, m), 1.41–1.15 (6H, m), 0.94–0.79 (3H, m); ^{13}C NMR (75 MHz, $CDCl_3$): δ 146.43, 47.96, 30.97, 29.12, 25.95, 22.33, 13.87, 8.12. IR (KBr): 2955, 2931, 2859, 2213 (w), 1457, 1427, 1382, 1275, 1180, 1082, 925, 728, 659 cm^{-1}. LRMS m/z (ESI) 248.8 (100, MH^+), 246.9 (52), 244.9 (21%); HRMS m/z (ESI) calcd for $C_8H_{16}N_4Se+H^+$ ($M+H^+$) 249.06129, found 249.06131.

*4.6.5. 5-Benzylselanyl-1-hexyl-1H-tetrazole (**26**).* Yellowish viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32–7.23 (5H, m), 4.55 (2H, s), 4.08 (2H, t, J=7.2 Hz), 1.78–1.68 (2H, m), 1.29–1.19 (6H, m), 0.86 (3H, t, J=6.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.93, 136.71, 128.91, 128.80, 127.92, 47.96, 32.89, 30.92, 29.20, 25, 87, 22.30, 13.87. IR (KBr): 2930, 2858, 1495, 1454, 1426, 1381, 1178, 1069, 760, 697 cm^{-1}. LRMS m/z (ESI) 324.8 (100, MH^+), 322.8 (52), 326.9 (19%); HRMS m/z (ESI) calcd for $C_{14}H_{20}N_4Se+H^+$ ($M+H^+$) 325.09260, found 325.09262.

*4.6.6. 5-(3,5-Dinitrobenzylselanyl)-1-hexyl-1H-tetrazole (**27**).* White crystalline solid; mp 57–58 °C. ^{1}H NMR (500 MHz, acetone): δ 8.82–8.79 (3H, m), 4.89 (2H, s), 4.29 (2H, t, J=7.1 Hz), 1.81–1.76 (2H, m), 1.25–1.20 (6H, m), 0.82 (3H, t, J=7.0 Hz); ^{13}C NMR (125 MHz, acetone): δ 149.28, 146.43, 144.77, 130.30, 118.25, 48.66, 31.67, 30.55, 30.32, 26.52, 22.96, 14.10. IR (KBr): 3111, 2933, 2859, 1541 (NO_2), 1460, 1426, 1385, 1343 (NO_2), 1179, 1074, 809, 729, 675 cm^{-1}. Anal. Calcd for $C_{14}H_{18}N_6O_4Se$: C, 40.69; H, 4.39; N, 20.33. Found: C, 41.01; H, 4.61; N, 20.14. LRMS m/z (ESI) 415.0 (100, MH^+), 413.1 (43), 416.9 (16%).

*4.6.7. 5-Methylselanyl-1-octyl-1H-tetrazole (**28**).* Yellowish viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.23 (2H, t, J=7.2 Hz), 2.72 (3H,

s), 1.93–1.84 (2H, m), 1.35–1.22 (10H, m), 0.91–0.84 (3H, m); ^{13}C NMR (75 MHz, $CDCl_3$): δ 146.44, 47.99, 31.65, 29.19, 28.97, 28.82, 26.30, 22.57, 14.04, 8.14. IR (KBr): 2926, 2856, 2218, 1457, 1427, 1383, 1276, 1176, 1087, 975, 924, 724 cm^{-1}. LRMS *m/z* (ESI) 276.9 (100, MH^+), 274.8 (48), 273.9 (19%); HRMS *m/z* (ESI) calcd for $C_{10}H_{20}N_4Se+H^+$ ($M+H^+$) 277.09260, found 277.09263.

*4.6.8. 5-Benzylselanyl-1-octyl-1H-tetrazole (**29**).* Yellowish viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32–7.22 (5H, m), 4.55 (2H, s), 4.07 (2H, t, *J*=7.3 Hz), 1.78–1.68 (2H, m), 1.31–1.16 (10H, m), 0.86 (3H, t, *J*=6.7 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.90, 136.71, 128.91, 128.80, 127.92, 47.94, 32.89, 31.61, 29.23, 28.91, 28.74, 26.21, 22.53, 14.01. IR (KBr): 2926, 2855, 1495, 1454, 1426, 1381, 1174, 760, 723, 697 cm^{-1}. LRMS *m/z* (ESI) 352.8 (100, MH^+), 350.9 (53), 354.8 (21%); HRMS *m/z* (ESI) calcd for $C_{16}H_{24}N_4Se+H^+$ ($M+H^+$) 353.12390, found 353.12386.

*4.6.9. 5-(3,5-Dinitrobenzylselanyl)-1-octyl-1H-tetrazole (**30**).* White solid; mp 61–62 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.92 (1H, t, *J*=2.1 Hz), 8.69 (2H, d, *J*=2.1 Hz), 4.76 (2H, s), 4.19 (2H, t, *J*=7.2 Hz), 1.88–1.76 (2H, m), 1.32–1.20 (10H, m), 0.90–0.83 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 148.54, 144.97, 142.05, 129.40, 118.12, 48.23, 31.59, 29.66, 29.23, 28.91, 28.75, 26.27, 22.53, 14.01. IR (KBr): 3087, 2930, 2857, 1533 (NO_2), 1450, 1430, 1344 (NO_2), 1175, 1084, 931, 809,732, 676 cm^{-1}. Anal. Calcd for $C_{16}H_{22}N_6O_4Se$: C, 43.54; H, 5.02; N, 19.04. Found: C, 43.58; H, 5.36; N, 19.07.

*4.6.10. 1-Cyclohexyl-5-methylselanyl-1H-tetrazole (**31**).* Yellowish viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.20–4.07 (1H, m), 2.71 (3H, s), 2.10–1.87 (6H, m), 1.80–1.70 (1H, m), 1.54–1.19 (3H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.42, 58.85, 32.21, 25.14, 24.78, 7.99. IR (KBr): 2935, 2857, 1452, 1423, 1383, 1366, 1272, 1189, 1083, 1001, 894, 817, 752 cm^{-1}. LRMS *m/z* (ESI) 246.9 (100, MH^+), 244.8 (52), 243.0 (20%); HRMS *m/z* (ESI) calcd for $C_8H_{14}N_4Se+H^+$ ($M+H^+$) 247.04564, found 247.04571.

*4.6.11. 5-Benzylselanyl-1-cyclohexyl-1H-tetrazole (**32**).* White solid; mp 62 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 7.32–7.20 (5H, m), 4.53 (2H, s), 4.08–3.97 (1H, m), 1.90–1.79 (6H, m), 1.71–1.67 (1H, m), 1.37–1.20 (3H, m); ^{13}C NMR (75 MHz, $CDCl_3$): δ 144.91, 136.79, 128.91, 128.77, 127.83, 58.81, 32.77, 32.23, 25.08, 24.73. IR (KBr): 2935, 2857, 1494, 1452, 1421, 1365, 1272, 1185, 1083, 1001, 894, 817, 753, 696 cm^{-1}. Anal. Calcd for $C_{14}H_{18}N_4Se$: C, 52.34; H, 5.65; N, 17.44. Found: C, 52.27; H, 5.98; N, 17.36. LRMS *m/z* (ESI) 322.97 (100, MH^+), 321.0 (48), 319.01 (19%).

*4.6.12. 1-Cyclohexyl-5-(3,5-dinitrobenzylselanyl)-1H-tetrazole (**33**).* Yellowish crystalline solid; mp 103 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.94–8.90 (1H, m), 8.72–8.69 (2H, m), 4.77 (2H, s), 4.13–4.04 (1H, m), 2.02–1.86 (6H, m), 1.77–1.74 (1H, m), 1.46–1.22 (3H, m); ^{13}C NMR (125 MHz, $CDCl_3$): δ 148.51, 143.93, 142.17, 129.44, 118.07, 59.27, 32.29, 29.48, 25.05, 24.67. IR (KBr): 3095, 2941, 2859, 1538 (NO_2), 1452, 1424, 1383, 1361, 1342 (NO_2), 1276, 1198, 1083, 1005, 922, 817, 808, 731, 671 cm^{-1}. Anal. Calcd for $C_{14}H_{16}N_6O_4Se$: C, 40.89; H, 3.92; N, 20.43. Found: C, 40.93; H, 4.21; N, 20.74.

*4.6.13. 1-Cyclohexyl-5-(2,4-dinitrobenzylselanyl)-1H-tetrazole (**34**).* Yellowish crystalline solid; mp 98–99 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.98 (1H, d, *J*=2.3 Hz), 8.41 (1H, dd, *J*=8.6, 2.3 Hz), 8.18 (1H, d, *J*=8.6 Hz), 4.94 (2H, s), 4.07–3.98 (1H, m), 2.01–1.85 (6H, m), 1.78–1.71 (1H, m), 1.46–1.20 (3H, m); ^{13}C NMR (125 MHz, $CDCl_3$): δ 147.44, 146.94, 145.12, 141.32, 134.33, 128.21, 121.08, 59.20, 32.22, 28.08, 25.04, 24.68. IR (KBr): 3089, 2955, 2853, 1611, 1537 (NO_2), 1455, 1426, 1340 (NO_2), 1277, 1193, 1175, 1083, 997, 913, 817, 802, 744, 712, 684 cm^{-1}. Anal. Calcd for $C_{14}H_{16}N_6O_4Se$: C, 40.89; H, 3.92; N, 20.43. Found: C, 40.57; H, 4.17; N, 20.06.

Acknowledgements

This work was supported by the Grant Agency of Charles University (Project GAUK 55610/2010) and Charles University in Prague (SVV 267 001).

Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2013.07.103. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes

1. Schwarz, K.; Foltz, C. *J. Am. Chem. Soc.* **1957**, *79*, 3292–3293.
2. Dodig, S.; Cepelak, I. *Acta Pharm.* **2004**, *54*, 261–276.
3. Koketsu, M.; Ishihara, H. *Curr. Org. Chem.* **2003**, *7*, 175–185.
4. Libero, F. M.; Xavier, M. C. D.; Victoria, F. N.; Nascente, P. S.; Savegnago, L.; Perin, G.; Alves, D. *Tetrahedron Lett.* **2012**, *53*, 3091–3094.
5. Koketsu, M.; Tanaka, K.; Takenaka, Y.; Kwong, C. D.; Ishihara, H. *Eur. J. Pharm. Sci.* **2002**, *15*, 307–310.
6. Parnham, M. J.; Graf, E. *Prog. Drug Res.* **1991**, *36*, 9–47.
7. Koketsu, M.; Ishihara, H.; Wu, W. J.; Murakami, K.; Saiki, I. *Eur. J. Pharm. Sci.* **1999**, *9*, 157–161.
8. May, S. W.; Wang, L. Q.; GillWoznichak, M. M.; Browner, R. F.; Ogonowski, A. A.; Smith, J. B.; Pollock, S. H. *J. Pharm. Exp. Ther.* **1997**, *283*, 470–477.
9. Roh, J.; Vavrova, K.; Hrabalek, A. *Eur. J. Org. Chem.* **2012**, 6101–6118.
10. Myznikov, L. V.; Hrabalek, A.; Koldobskii, G. I. *Khim. Geterotsikl. Soedin.* **2007**, 3–13.
11. Muraglia, E.; Kinzel, O. D.; Laufer, R.; Miller, M. D.; Moyer, G.; Munshi, V.; Orvieto, F.; Palumbi, M. C.; Pescatore, G.; Rowley, M.; Williarns, P. D.; Summa, V. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2748–2752.
12. Adamec, J.; Waisser, K.; Kunes, J.; Kaustova, J. *Arch. Pharm.* **2005**, *338*, 385–389.
13. Adamec, J.; Beckert, R.; Weiss, D.; Klimesova, V.; Waisser, K.; Mollmann, U.; Kaustova, J.; Buchta, V. *Bioorg. Med. Chem.* **2007**, *15*, 2898–2906.
14. Shin, K. J.; Koo, K. D.; Yoo, K. H.; Kim, D. C.; Kim, D. J.; Park, S. W. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1421–1425.
15. Dhayanithi, V.; Syed, S. S.; Kumaran, K.; Sankar, K. R. J.; Ragavan, R. V.; Goud, P. S. K.; Kumari, N. S.; Pati, H. N. *J. Serb. Chem. Soc.* **2011**, *76*, 165–175.
16. Disli, A.; Salman, M. *Russ. J. Org. Chem.* **2009**, *45*, 151–153.
17. Sureshbabu, V. V.; Vasantha, B.; Hemantha, H. P. *Synthesis* **2011**, 1447–1455.
18. Kanakaraju, S.; Prasanna, B.; Chandramouli, G. V. P. *J. Chem. Pharm. Res.* **2012**, *4*, 2994–2998.
19. Koldobskii, G. I.; Kharbash, R. B. *Russ. J. Org. Chem.* **2003**, *39*, 453–470.
20. Yildirir, Y.; Us, M. F.; Colak, N.; Ozkan, H.; Yavuz, S.; Disli, A.; Ozturk, S.; Turker, L. *Med. Chem. Res.* **2009**, *18*, 91–97.
21. Bartelskeith, J. R.; Mahoney, J. B.; Puttick, A. J. *J. Org. Chem.* **1985**, *50*, 980–987.
22. Bartelskeith, J. R.; Burgess, M. T.; Stevenson, J. M. *J. Org. Chem.* **1977**, *42*, 3725–3731.
23. Kikuchi, S.; Endou, T.; Satou, K. JP52046817 (A), 1977.
24. Haniyu, T.; Ibe, Y. JP61147250 (A), Japan, 1986.
25. Barr, C. U.S. Patent 3,733,201 (A), 1973.
26. Kauer, J. C.; Sheppard, W. A. *J. Org. Chem.* **1967**, *32*, 3580–3592.
27. Lieber, E.; Ramachandran, J. *Can. J. Chem.* **1959**, *37*, 101–109.
28. Han, S. Y.; Lee, J. W.; Kim, H. J.; Kim, Y. J.; Lee, S. W.; Gyoung, Y. S. *Bull. Korean Chem. Soc.* **2012**, *33*, 55–59.
29. Fernandez-Bolanos, J. G.; Lopez, O.; Ulgar, V.; Maya, I.; Fuentes, J. *Tetrahedron Lett.* **2004**, *45*, 4081–4084.
30. Iwaoka, M.; Tomoda, S. *Top. Curr. Chem.* **2000**, *208*, 55–80.
31. Christophe, T.; Jackson, M.; Jeon, H. K.; Fenistein, D.; Contreras-Dominguez, M.; Kim, J.; Genovesio, A.; Carralot, J. P.; Ewann, F.; Kim, E. H.; Lee, S. Y.; Kang, S.; Seo, M. J.; Park, E. J.; Skovierova, H.; Pham, H.; Riccardi, G.; Nam, J. Y.; Marsollier, L.; Kempf, M.; Joly-Guillou, M. L.; Oh, T.; Shin, W. K.; No, Z.; Nehrbass, U.; Brosch, R.; Cole, S. T.; Brodin, P. *PLoS Pathog.* **2009**, *5*.
32. Makarov, V.; Manina, G.; Mikusova, K.; Mollmann, U.; Ryabova, O.; Saint-Joanis, B.; Dhar, N.; Pasca, M. R.; Buroni, S.; Lucarelli, A. P.; Milano, A.; De Rossi, E.; Belanova, M.; Bobovska, A.; Dianiskova, P.; Kordulakova, J.; Sala, C.; Fullam, E.; Schneider, P.; McKinney, J. D.; Brodin, P.; Christophe, T.; Waddell, S.; Butcher, P.; Albrethsen, J.; Rosenkrands, I.; Brosch, R.; Nandi, V.; Bharath, S.; Gaonkar, S.; Shandil, R. K.; Balasubramanian, V.; Balganesh, T.; Tyagi, S.; Grosset, J.; Riccardi, G.; Cole, S. T. *Science* **2009**, *324*, 801–804.
33. Trefzer, C.; S'Kovierova, H.; Buroni, S.; Bobovska, A.; Nenci, S.; Molteni, E.; Pojer, F.; Pasca, M. R.; Makarov, V.; Cole, S. T.; Riccardi, G.; Mikusova, K.; Johnsson, K. *J. Am. Chem. Soc.* **2012**, *134*, 912–915.

34. Koci, J.; Klimesova, V.; Waisser, K.; Kaustova, J.; Dahse, H. M.; Mollmann, U. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3275–3278.
35. Sommen, G. L.; Linden, A.; Heimgartner, H. *Eur. J. Org. Chem.* **2005**, 3128–3137.
36. Fleischhauer, J.; Beckert, R.; Gunther, W.; Kluge, S.; Zahn, S.; Weston, J.; Berg, D.; Gorls, H. *Synthesis* **2007**, 2839–2848.
37. Atanassov, P. K.; Linden, A.; Heimgartner, H. *Heterocycles* **2003**, *61*, 569–579.
38. Kaname, M.; Minoura, M.; Sashida, H. *Tetrahedron Lett.* **2011**, *52*, 505–508.
39. Zhou, Y. H.; Linden, A.; Heimgartner, H. *Helv. Chim. Acta* **2000**, *83*, 1576–1598.
40. Koketsu, M.; Kogami, M.; Ando, H.; Ishihara, H. *Synthesis* **2006**, 31–36.
41. Atanassov, P. K.; Linden, A.; Heimgartner, H. *Helv. Chim. Acta* **2010**, *93*, 395–404.
42. Fleischhauer, J.; Beckert, R.; Hornig, D.; Gunther, W.; Gorls, H.; Klimesova, V. *Z. Naturforsch., B* **2008**, *63*, 415–424.
43. Sashida, H.; Pan, C.; Kaname, M.; Minoura, M. *Synthesis* **2010**, 3091–3096.
44. Kanoh, K.; Ishihara, H.; Koketsu, M. *Synthesis* **2007**, 2617–2620.
45. Atanassov, P. K.; Linden, A.; Heimgartner, H. *Helv. Chim. Acta* **2003**, *86*, 3235–3243.
46. Koketsu, M.; Otsuka, T.; Ishihara, H. *Heterocycles* **2006**, *68*, 2107–2112.
47. Koketsu, M.; Yamamura, Y.; Ando, H.; Ishihara, H. *Heterocycles* **2006**, *68*, 1267–1273.
48. Otwinowski, Z.; Minor, W. *Methods Enzymol.* **1997**, *276*, 307–326.
49. Coppens, P. In *Crystallographic Computing*; Ahmed, F. R., Hall, S. R., Huber, C. P., Eds.; Munksgaard: Copenhagen, Denmark, 1970.
50. Altomare, A.; Cascarano, G.; Giacovazzo, C.; Guagliardi, A. *J. Appl. Crystallogr.* **1994**, *27*, 1045–1050.
51. Sheldrick, G. M. *SHELXL-97*; University of Göttingen: Göttingen, Germany.
52. Koketsu, M.; Takahashi, A.; Ishihara, H. *J. Heterocycl. Chem.* **2007**, *44*, 79–81.
53. Henriksen, L.; Ehrbar, U. *Synthesis* **1976**, 519–521.
54. Maeda, H.; Takashima, M.; Sakata, K.; Watanabe, T.; Honda, M.; Segi, M. *Tetrahedron Lett.* **2011**, *52*, 415–417.
55. Collard-Charon, C.; Huls, R.; Renson, M. *Bull. Soc. Chim. Belg.* **1962**, *71*, 541–553.
56. Lopez, O.; Maza, S.; Ulgar, V.; Maya, I.; Fernandez-Bolanos, J. G. *Tetrahedron* **2009**, *65*, 2556–2566.
57. Boyer, J. H.; Frints, P. J. A. *Tetrahedron Lett.* **1968**, *9*, 3211–3212.

Attachment **II**.

Karabanovich, G.; Roh, J.; Smutný, T.; Něměcek, J.; Vicherek, P.; Stolaříková, J.; Vejsová, M.; Dufková, I.; Vávrová, K.; Pávek, P.; Klimešová, V.; Hrabálek, A. 1-Substituted-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles and their isosteric analogs: A new class of selective antitubercular agents active against drug-susceptible and multidrug-resistant mycobacteria. *Eur. J. Med. Chem*. **2014**, 82, 324-340.

European Journal of Medicinal Chemistry 82 (2014) 324–340

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

1-Substituted-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles and their isosteric analogs: A new class of selective antitubercular agents active against drug-susceptible and multidrug-resistant mycobacteria

Galina Karabanovich [a], Jaroslav Roh [a, *], Tomáš Smutný [a], Jan Němeček [a], Petr Vicherek [a], Jiřina Stolaříková [b], Marcela Vejsová [a], Ida Dufková [a], Kateřina Vávrová [a], Petr Pávek [a], Věra Klimešová [a], Alexandr Hrabálek [a]

[a] Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Heyrovského 1203, 50005 Hradec Kralove, Czech Republic
[b] Regional Institute of Public Health, Department of Bacteriology and Mycology, Partyzánské náměstí 7, 70200 Ostrava, Czech Republic

ARTICLE INFO

Article history:
Received 22 April 2014
Received in revised form
26 May 2014
Accepted 29 May 2014
Available online 2 June 2014

Keywords:
Tuberculosis
Tetrazole
Antituberculosis drug
Selectivity
Mycobacterium
Structure–activity relationships

ABSTRACT

In this work, a new class of highly potent antituberculosis agents, 1-substituted-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles and their oxa and selanyl analogs, is described. The minimal inhibitory concentration (MIC) values reached 1 μM (0.36–0.44 μg/mL) against *Mycobacterium tuberculosis* CNCTC My 331/88 and 0.25–1 μM against six multidrug-resistant clinically isolated strains of *M. tuberculosis*. The antimycobacterial effects of these compounds were highly specific because they were ineffective against all eight bacterial strains and eight fungal strains studied. Furthermore, these compounds exhibited low in vitro toxicity in four mammalian cell lines (IC_{50} > 30 μM). We also examined the structure–activity relationships of the compounds, particularly the effects on antimycobacterial activity of the number and position of the nitro groups, the linker between tetrazole and benzyl moieties, and the tetrazole itself. Relatively high variability of substituent R^1 on the tetrazole in the absence of negative effects on antimycobacterial activity allows further structural optimization with respect to toxicity and the ADME properties of the 1-substituted-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles lead compounds.

© 2014 Elsevier Masson SAS. All rights reserved.

1. Introduction

Tuberculosis (TB) is an infectious disease that is pandemic and claims hundreds of thousands of lives every year. Although a reduction in new TB cases and deaths has been achieved over the last few years, the global dissemination of TB remains tremendous. According to the World Health Organization (WHO), 8.6 million new cases and 1.3 million deaths occurred from TB infection around the world in 2012 [1]. Although a large panel of antituberculosis drugs exists, the increasing occurrence of multidrug-resistant (MDR), extensively drug-resistant (XDR) and even totally drug-resistant (TDR) TB strains highlights the necessity of a continual search for new highly efficient antimycobacterial compounds. The importance of such investigations is further emphasized by the relative toxicity and side effects of the drugs currently used for treatment, the low tolerability of the medicaments and the potential drug–drug interactions of antituberculotics and antiretroviral drugs used in the treatment of HIV-positive TB patients. Several series of potential antituberculotics have been prepared and studied over the last few decades, and the number of these studies is still increasing (for recent reviews, see Refs. [2] and [3]). Highly effective anti-TB agents were found in groups of indole-2-carboxamides [4,5], salicylanilides [6], imidazopyridine amides targeting the respiratory cytochrome bc1 complex [7], 1,2,4-benzotriazine di-*N*-oxides [8], inhibitors of DNA gyrase B thiazolopyridine ureas [9] or inhibitors of mycobacterial FtsZ 2,5,6-trisubstituted benzimidazoles [10].

Recently, ten antituberculotic drugs have progressed to the clinical phase of development. Among them, the two

Abbreviations: ADME, absorption, distribution, metabolism, excretion; CNCTC, Czech National Collection of Type Cultures; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DprE1, decaprenyl-phosphoribose epimerase; INH, isoniazid; MDR, multidrug-resistant; MIC, minimal 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; Rf, retention factor; SDS, sodium dodecyl sulfate; TB, tuberculosis; TBAB, tetrabutylammonium bromide; TDR, totally drug-resistant; THF, tetrahydrofuran; TLC, thin layer chromatography; XDR, extensively drug-resistant.

* Corresponding author.
E-mail address: jaroslav.roh@faf.cuni.cz (J. Roh).

http://dx.doi.org/10.1016/j.ejmech.2014.05.069
0223-5234/© 2014 Elsevier Masson SAS. All rights reserved.

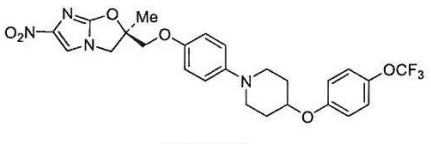
nitroimidazole based compounds PA-824 [11] and OPC-67683 (delamanid) [12] are active against drug-susceptible and drug-resistant tuberculosis strains with minimal inhibitory concentrations (MIC) for *Mycobacterium tuberculosis* $H_{37}Rv$ of 0.11 μM and 0.02 μM (Fig. 1). It is hypothesized that these compounds are activated by the bioreduction of the nitroimidazole moiety by two deazaflavin-dependent enzymes and that they act as inhibitors of mycolic acid synthesis. One of the great advantages of PA-824 and OPC-67683 is their activity against both replicating and non-replicating bacteria, thus rendering these compounds capable of treating latent forms of TB [13,14].

Another group of compounds bearing a nitro group and exhibiting high in vitro and in vivo antimycobacterial activities are the benzothiazinones. The lead compound BTZ-043 possessed an MIC value of 2.3 nM for *M. tuberculosis* $H_{37}Rv$ and its piperazinobenzothiazinone analog PBTZ 169 proceeded to preclinical development. These compounds are also activated via the bioreduction of the nitro group. The active nitroso metabolite acts as an inhibitor of decaprenyl-phosphoribose epimerase (DprE1), an essential enzyme involved in arabinan biosynthesis [15,16]. The presence of two nitro groups are vital for another promising class of compounds, the dinitrobenzamides (DNB), which also act as highly potent inhibitors of DprE1 [17] (Fig. 2).

In our previous work, a series of heterocyclic compounds, including variously substituted benzylsulfanyl benzazoles, triazoles and pyridines were synthesized, and their antitubercular activity was studied. The highest in vitro antimycobacterial activity was observed in a series of benzazole derivatives with 2,4- or 3,5-dinitrobenzylsulfanyl substituents, with MIC values ranging from 2 to 8 μM for *M. tuberculosis* CNCTC My 331/88 (Fig. 3). These substances also showed the same level of activity towards MDR strains of *M. tuberculosis* as well as non-tuberculous mycobacteria, such as *Mycobacterium kansasii* and *Mycobacterium avium* [18–23].

To further study the role of the heterocycle in the antimycobacterial activity of abovementioned 2-(dinitrobenzylsulfanyl)benzazoles, we decided to change the benzazole-2-thiol moiety for 1*H*-tetrazole-5-thiol moiety. The reason for selecting the tetrazole ring was its easy synthetic accessibility and, presumably, better ADME properties than the poorly soluble benzazoles [24,25]. Furthermore, substitution at position 1 of tetrazole would enable studying the structure–activity relationships and optimization of the properties of the target compounds.

Therefore, the aim of this work was to synthesize a series of 1-substituted-5-(dinitrobenzylsulfanyl)-1*H*-tetrazoles and their isosteres and to study their antimycobacterial activity (Fig. 4). To probe the selectivity of their action, several compounds were also evaluated in eight bacterial strains, eight fungal strains and four mammalian cell lines. To investigate the structure–activity relationships of these compounds, sulfinyl and sulfonyl analogs and oxygen and selenium isosteres of 1-substituted-5-(dinitrobenzylsulfanyl)-1*H*-tetrazoles were synthesized. Furthermore, derivatives bearing benzyl, 4-nitrobenzyl and partially or completely reduced dinitrobenzyl moieties on the heteroatom in position 5 of the tetrazole were also prepared to determine the role of double nitro-substitution in antimycobacterial activity. Finally, compounds with 3,5-dinitrobenzylsulfanyl groups attached to selected alkyl and aryl groups were synthesized to study the effect of the tetrazole cycle on antimycobacterial activity.



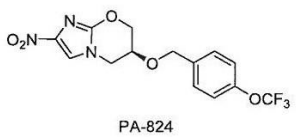


Fig. 1. Structures of the nitroimidazole based anti-TB compounds OPC-67683 and PA-824.

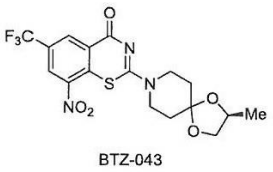


Fig. 2. Structure of the anti-TB benzothiazinone BTZ-043.

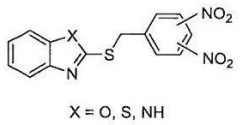


Fig. 3. Structure of the benzazole derivatives bearing a dinitrobenzylsulfanyl moiety.

2. Results and discussion

2.1. Chemistry

A series of 1-alkyl/aryl-5-alkylsulfanyl-1*H*-tetrazole compounds **2u**, **3u**, **4e**, **4g**, **4j**, **4s–u**, **5a–v** was prepared at high yields (66–97%) by the reaction of 1-substituted-1*H*-tetrazole-5-thiols (**1a–v**) with the corresponding alkyl halides under conditions of phase-transfer catalysis (**Method A**) or in acetonitrile in the presence of triethylamine (**Method B**, Scheme 1). Tetrazole-5-thiols (**1a–v**) were purchased or obtained by reacting alkyl/arylisothiocyanate with excess sodium azide in water at 80–100 °C [26].

To study the effects of substituent R^1 (Table 1) in 5-(dinitrobenzylsulfanyl)-1*H*-tetrazoles on the biological properties of the compounds, 1-alkyl (**4e**, **5b–5f**) and 1-aryl derivatives with various substitutions on the phenyl ring (**4g**, **4j**, **4s–4u**, **5g–5v**) were prepared. Methyl ester **6** was additionally prepared by Fisher

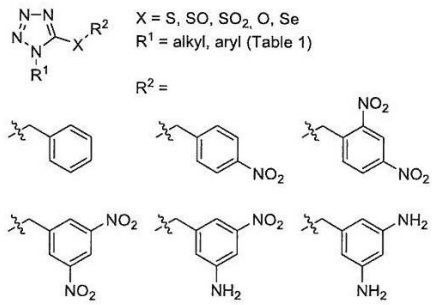
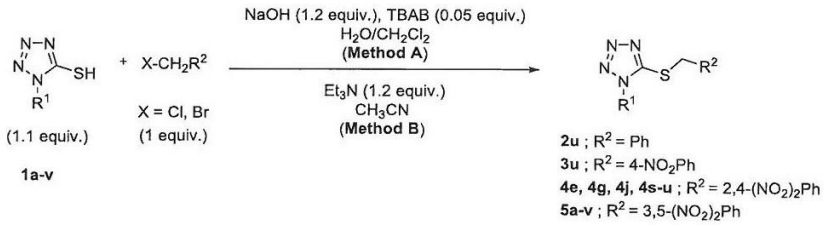


Fig. 4. General structure of the studied compounds.



Scheme 1. Synthesis of 1-alkyl/aryl-5-alkylsulfanyl-1*H*-tetrazoles (**2u**, **3u**, **4e**, **4g**, **4j**, **4s**–**u**, **5a**–**v**).

esterification of the carboxylic acid **5v**. Selected 1-alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles (**5e**, **5g**, **5j**, **5u**) were converted into the corresponding sulfinyl (**7**) and sulfonyl (**8**) derivatives by oxidation with hydrogen peroxide under mild reaction conditions (Scheme 2) [27]. Full oxidation of sulfanyltetrazoles **5e**, **5g**, **5j** and **5u** led to sulfonyl derivatives **8e**, **8g**, **8j** and **8u** in high yields (62–91%). Sulfinyl derivatives **7e**, **7g**, **7j** and **7u** were prepared only in moderate yields (40–60%) because the oxidation of sulfanyltetrazole **5e**, **5g**, **5j** and **5u** by hydrogen peroxide is not selective and sulfonyl derivatives **8e**, **8g**, **8j** and **8u** always appeared even when the substrates were still present in the reaction mixture.

Oxygen bioisosteres of the title compounds, i.e., 1-aryl-5-[(3,5-dinitrobenzyl)oxy]-1*H*-tetrazoles (**10g**, **10j**, **10k**, **10n**–**p**, **10s**–**u**), were synthesized according to Scheme 3. Starting tetrazole-5-thiols (**1g**, **1j**, **1k**, **1n**–**p**, **1s**–**u**) were methylated with dimethyl sulfate under phase-transfer catalysis conditions and then oxidized by hydrogen peroxide. Both reactions proceeded at high yields (60–98%). The resulting 1-aryl-5-(methylsulfonyl)-1*H*-tetrazoles (**9g**, **9j**, **9k**, **9n**–**p**, **9s**–**u**) underwent a nucleophilic substitution with 3,5-dinitrobenzyl alcohol in tetrahydrofuran (THF) in the presence of potassium hydroxide at room temperature. This reaction protocol was highly efficient when methanol or benzylalcohol were used as substrates [28]. However, in our case the reactions proceeded only at low yields of the target compounds **10g**, **10j**, **10k**, **10n**–**p**, **10s**–**u** (23–55%) due to the formation of a side product, (3,5-dinitrobenzyl)(methyl)sulfone (**11**) at 30–40% yield. Moreover, no 1-alkyl-5-[(3,5-dinitrobenzyl)oxy]-1*H*-tetrazoles were obtained with this method. The structure of compound **11** was verified by the comparison of its NMR spectra with a standard compound prepared by oxidation of (3,5-dinitrobenzyl)(methyl)sulfane (**19**). Formation of such byproduct (**11**) via nucleophilic attack of the released methanesulfinate on benzylic methylene of the desired products (**10**) was not observed using other alcohols [28].

Selenium derivatives, i.e., 1-alkyl/aryl-5-benzylselanyl-1*H*-tetrazoles (**13e**, **13g**, **14e**, **15c**–**e**, **15g**, **15j**, **15n**), were prepared from the alkyl/arylisoselenocyanates **12c**–**e**, **12g**, **12j**, **12n**. These precursors (**12**) were prepared in a one-pot reaction using *N*-alkyl/arylformamides and triphosgene in refluxing CH_2Cl_2 in the presence of triethylamine followed by the addition of selenium powder (Scheme 4) [29]. Briefly, alkyl/arylisoselenocyanate was stirred at room temperature with the appropriate alkylating agent and sodium azide in THF or acetonitrile, both of which contained 5% water. 1-Alkyl-5-alkylselanyl-1*H*-tetrazoles (**13e**, **15c**, **15d**, **15e**) were prepared at good yields (45–76%), while aryl derivatives (**13g**, **15g**, **15j**, **15n**) were obtained at low yields (22–34%) due to the formation of *N*-alkyl-*N*-arylcyanamides and (Z)-Se-alkyl-*N*-cyano-*N*,*N*′-diarylisoselenoureas as the main products [30]. Compound **14e** was obtained via a two-step procedure – cyclohexylisoselenocyanate **12e** was stirred in an aqueous solution of sodium azide followed by the alkylation under phase-transfer catalysis conditions [30].

Compounds with a partially or fully reduced dinitrobenzyl moiety (**16g**, **16j** and **17j**) were prepared according to Scheme 5. In the first step, 3,5-dinitrobenzyl chloride was reduced by an excess of tin(II) chloride dihydrate in ethanol. Addition of 1-substituted-1*H*-tetrazole-5-thiol (**1g**, **1j**) in THF to the aqueous solution of these partially or fully reduced ammonium salts resulted in the formation of the desired products **16g**, **16j** and **17j**.

Compounds **18**–**24** were designed to have a 3,5-dinitrobenzylsulfanyl moiety attached to selected alkyl, aryl or acyl moieties (Scheme 6) instead of the tetrazole. The *S*-(3,5-dinitrobenzyl) ethanethioate compound **18** was prepared, and a one-pot deacetylation with a Williamson reaction resulted in the formation of sulfanes **19** and **20** [31]. Reaction of 3,5-dinitrobenzyl chloride with alkyl/aryl thiols in THF in the presence of triethylamine led to the formation of sulfanes **21**, **22** and **23**. The treatment of 3,5-dinitrobenzyl chloride with an excess of potassium thiocyanate in DMF led to the formation of 3,5-dinitrobenzyl thiocyanate (**24**), which was converted to the corresponding 1*H*-tetrazole **25** via a reaction with sodium azide in the presence of triethylammonium chloride at a good yield [32], [24].

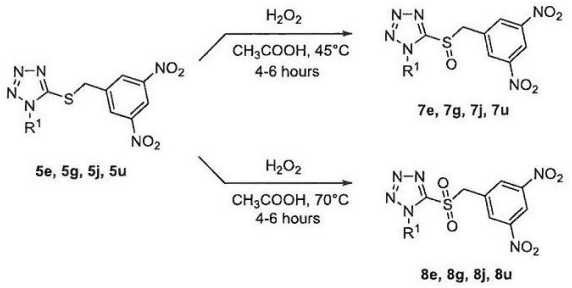
2.2. In vitro antimycobacterial activity

All synthesized compounds were evaluated for their in vitro antimycobacterial activities against *M. tuberculosis* My 331/88 and against the non-tuberculous mycobacteria *M. avium* My 330/88, *M. kansasii* My 235/80 and *M. kansasii* 6509/96. All mycobacterial strains were obtained from the Czech National Collection of Type Cultures (CNCTC), with the exception of *M. kansasii* 6509/96, which is a clinical isolate. Selected compounds were evaluated for their in vitro antimycobacterial activities against six multidrug-resistant strains of *M. tuberculosis* (234/2005, 9449/2007, 8666/2010, Praha 1, Praha 4 and Praha 131), which were clinically isolated. The antimycobacterial activities of the compounds were evaluated after incubation at 37 °C for 7/14/21 days for both strains of *M. kansasii* and after 14/21 days for *M. tuberculosis* strains and *M. avium*. Antimycobacterial activities are expressed as the MIC, which is the lowest concentration of a substance at which the inhibition of mycobacterial growth occurred (the concentration that inhibited >99% of the mycobacterial population). The first line anti-TB drug isoniazid (INH) was used as a prototype drug.

The majority of the prepared compounds exhibited significant in vitro antimycobacterial activity against mycobacterial strains. Compounds of series **2**, **3**, **4**, **7**, **8**, **13** and **14** were less effective against INH-susceptible *M. tuberculosis* and *M. kansasii* 6509/96 compared to INH and slightly more effective against INH-resistant strains *M. avium* and *M. kansasii* My 235/80. Nevertheless, 5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazole derivatives (**5**) and their bioisosteric oxygen (**10**) and selenium (**15**) analogs were found to be highly active against the collection strains of *M. tuberculosis* and *M. kansasii* and also against one INH-susceptible clinically isolated strain of *M. kansasii* 6509/96, with MIC values reaching 1 μM. They were also highly active against six multi-drug resistant clinically isolated strains of *M. tuberculosis*, with MIC values of 0.25–1 μM (Tables 2 and 4).

Table 1
Substituents R^1 in the series of compounds **1–10, 12–15**.

	a	b	c	d	e	f	g	h
R^1	NH_2	CH_3	$CH_3(CH_2)_5$	$PhCH_2$	C_6H_{11} (cyclohexyl)	$C_{10}H_{15}$ (1-adamantyl)	Ph	2-CH_3OPh
	i		**j**	**k**		**l**	**m**	**n**
R^1	3-CH_3OPh		4-CH_3OPh	3,4,5-$(CH_3O)_3Ph$		3,4-$(CH_3)_2Ph$	4-Et_2NPh	4-BrPh
	o	**p**	**q**	**r**	**s**	**t**	**u**	**v**
R^1	4-ClPh	3,4-Cl_2Ph	3-FPh	4-FPh	2-NO_2Ph	3-NO_2Ph	4-NO_2Ph	3-COOHPh



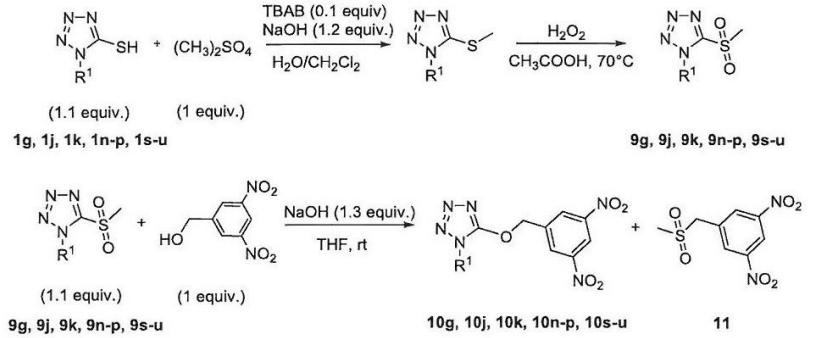
Scheme 2. Synthesis of 1-alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfinyl]-1*H*-tetrazoles (**7e, 7g, 7j, 7u**) and 1-alkyl/aryl-5-[(3,5-dinitrobenzyl)sulfonyl]-1*H*-tetrazoles (**8e, 8g, 8j, 8u**).

This study confirmed that the two nitro groups on the benzyl moiety of such compounds are necessary for high antimycobacterial effects, which is in agreement with previous observations [17–21]. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(4-nitrophenyl)-1*H*-tetrazole (**5u**) and its 2,4-dinitrobenzylsulfanyl analog **4u** exhibited several fold higher activity than benzylsulfanyl and 4-nitrobenzylsulfanyl analogs **2u** and **3u** (Table 2). The same relationships were observed in a series of selenium bioisosteres: dinitrobenzylselanyl derivatives **14e**, **15e** and **15g** proved to be highly active compared to compounds without nitro substitutions (**13e, 13g**). When the position of the nitro groups on the benzyl moiety was taken into consideration, 3,5-dinitro derivatives exhibited significantly higher antimycobacterial activity than 2,4-dinitro derivatives regardless of the substituent at position 1 of the tetrazole; all of the 1-substituted-5-[(2,4-dinitrobenzyl)sulfanyl]tetrazoles in series **4** were less effective than the corresponding 1-substituted-5-[(3,5-dinitrobenzyl)sulfanyl]tetrazoles in series **5**. The comparison between the activities of 5-(dinitrobenzylselanyl) derivatives **14e** and **15e** supported this finding. Partially and fully reduced derivatives (**16g, 16j** and **17j**) significantly lost their antimycobacterial activity, which confirmed the necessity of both nitro groups for high antimycobacterial effect.

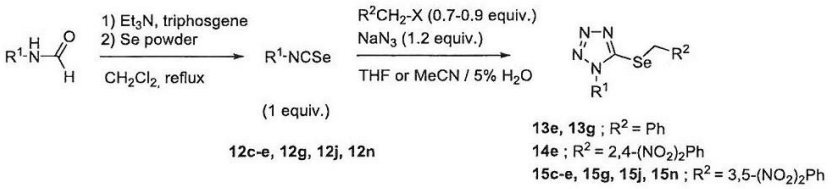
In contrast to the high importance of the number and position of the nitro groups, the type of heteroatom between the dinitrobenzyl and the tetrazole moieties had surprisingly little effect on antitubercular activity. The antimycobacterial activities of sulfur, oxygen and selenium isosteric series **5**, **10** and **15** were almost the same, regardless of the heteroatom involved.

It can be expected that 1-alkyl/aryl-5-alkylsulfanyl-1*H*-tetrazoles will be metabolized in vivo to sulfoxides or sulfones. Therefore, 1-substituted 5-[(3,5-dinitrobenzyl)sulfinyl]-1*H*-tetrazoles (**7e, 7g, 7j, 7u**) and their sulfonyl analogs **8e, 8g, 8j** and **8u** were prepared. Their MIC values showed that both partial and full oxidation of sulfanyl derivatives **5e, 5g, 5j** and **5u** led to a significant decrease in their antimycobacterial activity.

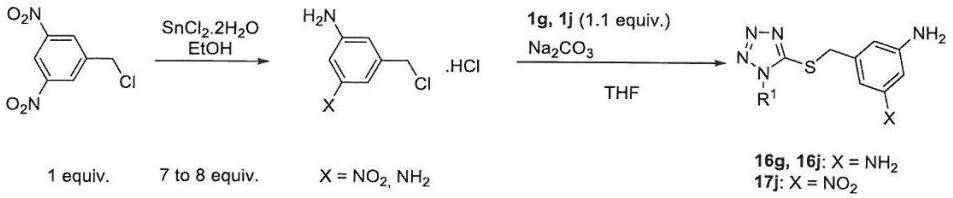
With respect to the substituent R^1, compounds bearing 4-substituted phenyl at position 1 of the tetrazole generally displayed higher activities then the 2- or 3-substituted compounds. Activities of 2-, 3- and 4-nitrophenyl (**5s, 5t, 5u**) or 2-, 3- and 4-methoxyphenyl (**5h, 5i, 5j**) derivatives are shown as examples (Table 2). The same relationships were observed in the series of oxy analogs **10**: Antitubercular activity of the 4-nitrophenyl derivative



Scheme 3. Synthesis of 1-aryl-5-[(3,5-dinitrobenzyl)oxy]-1*H*-tetrazoles (**10g, 10j, 10k, 10n–p, 10s–u**).



Scheme 4. Synthesis of 1-alkyl/aryl-5-alkylselanyl-1*H*-tetrazoles (**13e, 13g, 14e, 15c–e, 15g, 15j, 15n**).



Scheme 5. Synthesis of partially and fully reduced derivatives **16g**, **16j** and **17j**.

10u was higher than those of 3- and 2-nitrophenyl derivatives **10s** and **10t** (Table 2).

The antimycobacterial activity of the prepared compounds was highly influenced by their lipophilicity. The introduction of a carboxylic acid moiety to the molecule **5v** led to a complete loss of antimycobacterial activity. The activity against *M. tuberculosis* increased when the carboxyl group was esterified (**6**). Lower activities of compounds with amino (**5a**) and methyl (**5b**) substituents can also be associated with their lower lipophilicity. The lack of an antimycobacterial effect of the 3,4-dimethylphenyl derivative (**5l**) can be explained by its insolubility in the culture medium (which caused precipitation during the experiment) rather than by the properties of 3,4-dimethylphenyl substituent.

The R^1 substituent is the only fragment of the most active compounds obtained from the series **5** and **10** that can be changed without significant loss of their anti-TB properties. Therefore, this substituent could be used for future optimization of the activity/toxicity ratio and the ADME properties of these promising antitubercular compounds.

To probe the importance of the tetrazole moiety, we prepared a series of tetrazole-free substances **18–24** (Table 3). Their generally low antimycobacterial activity showed that tetrazole is also an important structural feature of these compounds. Nevertheless, the antimycobacterial activity of derivatives **18** and **24** were in the μM range, indicating that the electron-withdrawing group attached to the 3,5-dinitrobenzylsulfanyl moiety is beneficial for antimycobacterial activity. This finding is in agreement with the results obtained for the most active tetrazole-based compounds of series **5**, **10** and **15**. Finally, we prepared compound **25** lacking any substitution at position 1 of the tetrazole ring. Although compound **25** contained a structural motif of the highly antimycobacterial active compounds of series **5**, it was inactive. The reason for this loss of activity is most likely the ability of the unsubstituted tetrazole ring to ionize at physiological pH similar to the carboxyl compound **5v** (the pKa of the 5-substituted tetrazole is approx. 4–5) [24].

M. tuberculosis CNCTC My 331/88 strain is also known as $H_{37}Rv$ and is susceptible to the first-line anti-TB drugs rifampicin, isoniazid, ethambutol and the second-line drug streptomycin with MIC values of 0.25 μM, 0.5 μM, 1 μM and 0.25 μM, respectively. Compounds **5c–e**, **5n–p**, **5u**, **10u**, **15c–e** and **15j** described in this work reached MIC values of 1 μM, a level similar to that of the first-line anti-TB drugs (Table 2). Furthermore, no cross-resistance with either the first or second-line anti-TB drugs against MDR *M. tuberculosis* strains was observed, indicating that the series of compounds **5**, **10** and **15** possessed different modes of action than the currently prescribed anti-TB drugs (Table 4).

2.3. In vitro antibacterial and antifungal activity

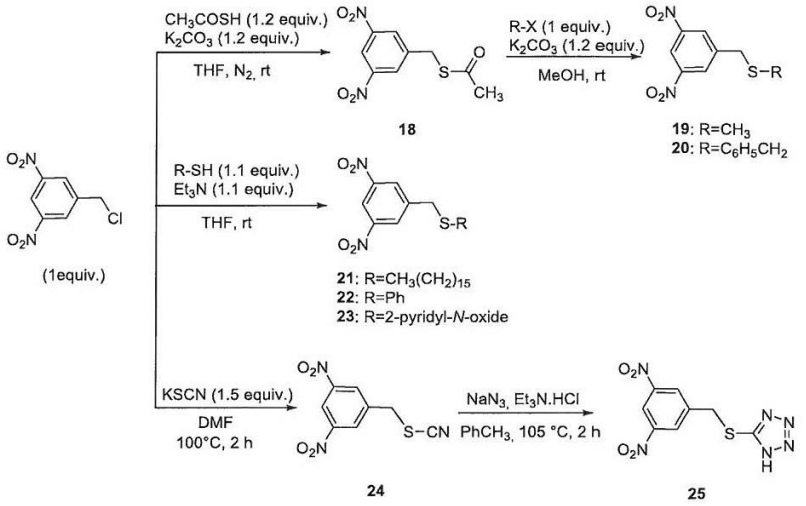
To investigate the selectivity of the studied compounds against TB, we evaluated the activity of a few selected 3,5-dinitro derivatives **5d**, **5e**, **5p**, **5u**, **10u**, **15d**, **15e** with promising anti-TB activity along with two 2,4-dinitro derivatives **4u**, **14e** and the inactive 3,5-dinitro derivatives **5v** and **23** against 8 bacterial and 8 fungal strains (Tables 5 and 6). All compounds containing the 3,5-dinitrobenzyl moiety, i.e., those with high anti-TB activity, exhibited no antibacterial or antifungal activities and no toxic effects were observed even at the highest concentrations tested for all of the studied strains. Interestingly, compounds bearing the 2,4-dinitrobenzyl moiety including 5-[(2,4-dinitrobenzyl)sulfanyl]-1-(4-nitrophenyl)-1*H*-tetrazole (**4u**) and 1-cyclohexyl-5-[(2,4-dinitrobenzyl)selanyl]-1*H*-tetrazole (**14e**) showed some activity against several bacterial and fungal strains.

2.4. In vitro cell proliferation/viability assays

Finally, to gain basic insight into the toxicity of these compounds on mammalian cell lines, the effects of selected 3,5-dinitro derivatives **5d**, **5e**, **5j**, **5p**, **5t**, **5u**, **10p**, **15d**, **15e** and **15j** with promising anti-TB activities and the 2,4-dinitro derivatives **4e**, **14e**, sulfoxide **7e** and sulfone **8e** on the viability of HeLa (human cervix epithelioid carcinoma), HepG2 (human Caucasian hepatocyte carcinoma), HuH7 (human hepatocellular carcinoma) and MDCKII (Madin–Darby canine kidney cells) cell lines were studied. Due to low solubility, some studied compounds precipitated in the cell culture medium during the experiment at concentrations above 30 μM. Therefore, when IC_{50} exceeded this point, the data are presented as percent viability when compared to control vehicle-treated samples at a concentration of 30 μM. These results suggest that the 3,5-dinitrobenzylsulfanyl derivatives **5d**, **5e**, **5j**, **5p**, **5t** and **5u** with promising anti-TB activity have limited effects on the cellular viability of these four mammalian cell lines. Compounds **4e**, **7e**, **10p**, **15d**, **15e** and **15j** showed some toxicity in at least one cell line, and compounds **8e** and **14e** were toxic in at least three cell lines (Table 7).

3. Conclusion

In this work, we observed high antimycobacterial effects of 1-substituted-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles **5** and their oxy and selanyl analogs **10** and **15**. Antimycobacterial activities reached MIC values of 1 μM against *M. tuberculosis* CNCTC My 331/88 and 0.25–1 μM against six multidrug-resistant clinically isolated strains of *M. tuberculosis*; these compounds exhibited no cross-resistance with common anti-TB drugs. Furthermore, these compounds possessed similar activity towards both INH-susceptible and INH-resistant strains of non-tuberculous *M. kansasii*. Moreover, compounds of series **5**, **10** and **15** exhibited highly selective antimycobacterial effects because they exhibit no antibacterial or antifungal effects and exhibit low toxicity on selected mammalian cell lines with IC_{50} values upwards of 30 μM. Hence, selectivity indices of the most promising compounds of series **5** are above 30. It should be noted that also selenium compounds **15d**, **15e**, **15j** displayed surprisingly high selectivity and low toxicity.



Scheme 6. Synthesis of compounds **18**–**25** bearing a 3,5-dinitrobenzyl fragment.

As a result of the structure–activity relationship studies, the role of the individual structural fragments of the studied compounds in antimycobacterial activity was elucidated. 3,5-Dinitro substitution on the benzyl moiety was necessary for the high and selective antimycobacterial effects of the studied compounds. Moving the nitro groups to positions 2 and 4 decreased the antimycobacterial effect and increased the overall in vitro toxicity against mammalian cell lines and some bacterial and fungal strains. Other changes in the structure, such as the removal of one or both nitro groups, replacement of the tetrazole moiety for alkyl/aryl/acyl substituent or the reduction of nitro groups, led to a decrease or complete loss of their antimycobacterial activity. Interestingly, the derivatives with sulfanyl, oxy and selanyl linkers showed similar antitubercular properties, whereas sulfoxide and sulfone linkers diminished the anti-TB activity of such compounds. It was shown that the presence of ionizable groups in the molecule deteriorated the antimycobacterial effect. Nevertheless, the type of a lipophilic substituent R^1 on the tetrazole group had little influence on antimycobacterial effects of the studied compounds. This finding opens the possibility of further optimization of the activity, toxicity and ADME properties of the most promising compounds, i.e., 1-substituted-5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazoles **5** and their oxy and selanyl analogs of series **10** and **15**.

4. Experimental section

4.1. General

The structural identity of the prepared compounds was confirmed by ^{1}H NMR and ^{13}C NMR spectroscopy analysis. The purity of all compounds reported was determined by elemental analysis and the results were within 0.4% of the calculated values. All chemicals used for synthesis were obtained from Sigma–Aldrich (Schnelldorf, Germany) and were used as received. 1-Aryl-1*H*-tetrazole-5-thiols (**1a–v**) were prepared via the reaction of the corresponding isothiocyanates (which are commercially available) with 1.2 M excess sodium azide in water at 80–100 °C [26]. All products were known and characterized by comparing their spectral data with those of authentic standards. TLC was performed on Merck aluminum plates with silica gel 60 F_{254}. Merck Kieselgel 60 (0.040–0.063 mm) was used for column chromatography. Melting points were recorded with a Büchi B-545 apparatus and are uncorrected. Infrared spectra were measured on Nicolet 6700 (ATR mode). ^{1}H and ^{13}C NMR spectra were recorded by Varian Mercury Vx BB 300 or VNMR S500 NMR spectrometers. 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 carried out on an Automatic Microanalyser EA1110CE (Fisons Instruments S.p.A., Milano, Italy). Electrospray ionization mass spectroscopic (ESI MS) experiments were performed using an Acquity UPLC with MS/MS Quattro Micro detection (Micromass + Waters).

4.2. General procedure for the synthesis of 1-substituted-5-alkylsulfanyl-1H-tetrazoles (2–5)

4.2.1. Method A

A solution of alkyl halide (1 mmol) and tetrabutylammonium bromide (TBAB) (0.05 mmol) in CH_2Cl_2 (7 mL) was added to the solution of 1-substituted tetrazole-5-thiol (**1**) (1.1 mmol) and sodium hydroxide (1.2 mmol) in water (4 mL). The reaction mixture was stirred at room temperature (rt) under conditions of phase-transfer catalysis until completion as determined by TLC. Next, the organic phase was separated, washed with water (2 × 10 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the crude product was purified by crystallization or silica gel column chromatography.

4.2.2. 1-(4-Nitrophenyl)-5-(4-nitrobenzylsulfanyl)-1H-tetrazole (3u)

The reaction mixture was stirred for 48 h. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 6:1). Yield: 67% (yellow solid); mp 120–122 °C. ^{1}H NMR (300 MHz, DMSO) δ 8.46 (d, J = 8.8 Hz, 2H), 8.16 (d, J = 8.2 Hz, 2H), 7.94 (d, J = 8.8 Hz, 2H), 7.72 (d, J = 8.2 Hz, 2H), 4.74 (s, 2H); ^{13}C NMR (75 MHz, DMSO) δ 154.10, 148.23, 147.08, 144.62, 137.86, 130.61, 125.76, 125.54, 123.77, 36.12. Anal. Calcd for $C_{14}H_{10}N_6O_4S$: C, 46.93; H, 2.81; N, 23.45; S, 8.95. Found: C, 47.12, H, 2.92, N, 23.57; S, 8.96.

Table 2
In vitro antimycobacterial activities of the target compounds of series **2–17** expressed as MIC (μM).

	M. tuberculosis My 331/88	*M. avium* My 330/88	*M. kansasii* My 235/80	*M. kansasii* 6509/96
	14/21 days		7/14/21 days	
2u	125/125	62/62	16/32/32	16/62/62
3u	250/250	250/250	250/250/250	250/250/250
4e	8/16	16/32	4/8/16	16/62/62
4g	8/16	32/62	16/62/62	8/32/32
4j	8/8	62/62	32/62/62	8/16/32
4s	16/32	32/62	16/62/62	16/32/62
4t	16/32	62/62	16/32/62	8/32/32
4u	4/8	62/62	8/32/32	16/32/62
5a	8/16	1000/1000	8/32/32	16/32/32
5b	4/8	500/1000	4/16/32	16/32/32
5c	1/2	32/62	2/4/8	2/4/8
5d	1/2	250/250	2/8/8	2/4/4
5e	1/1	16/32	4/4/4	2/2/4
5f	4/4	500/500	4/8/16	8/8/8
5g	4/4	62/62	2/4/16	2/2/4
5h	125/125	125/125	125/125/125	125/125/125
5i	2/4	125/125	125/125/125	125/125/125
5j	2/4	16/32	1/4/4	1/1/2
5k	16/16	250/250	16/32/32	16/16/32
5l	500/500	250/250	250/250/250	250/250/250
5m	2/2	62/62	4/8/32	8/16/62
5n	1/1	125/125	1/2/2	2/4/4
5o	1/2	125/125	2/4/4	2/4/4
5p	1/1	8/16	1/1/2	1/2/4
5q	16/32	250/250	4/4/8	4/8/8
5r	2/2	250/250	2/4/4	4/8/16
5s	4/16	62/62	4/16/16	4/4/16
5t	2/2	62/125	2/4/8	2/2/4
5u	1/1	32/32	1/1/1	1/2/2
5v	1000/1000	1000/1000	500/1000/1000	1000/1000/1000
6	8/16	250/250	500/500/500	500/500/500
7e	8/16	125/125	4/16/62	4/16/32
7g	62/62	125/125	62/250/250	125/250/250
7j	62/62	250/250	62/125/125	32/125/125
7u	32/32	125/250	62/62/62	16/62/62
8e	2/4	32/62	8/16/16	8/16/16
8g	8/8	62/125	32/62/62	32/62/62
8j	8/8	125/125	125/250/250	125/125/125
8u	16/32	250/500	32/32/62	16/32/32
10g	2/4	125/125	8/16/32	8/16/32
10j	2/2	16/32	4/8/16	4/16/16
10k	16/16	32/32	8/16/32	16/32/62
10n	2/4	125/125	8/16/16	4/16/16
10o	2/4	32/62	8/16/32	8/16/32
10p	4/4	32/62	4/16/32	4/16/32
10s	16/16	125/125	32/62/125	62/125/250
10t	4/8	250/250	125/125/125	125/125/125
10u	1/1	125/125	1/1/1	1/1/2
13e	125/125	125/125	8/32/32	62/250/500
13g	125/125	62/125	2/8/16	32/125/250
14e	4/8	32/32	4/16/16	16/62/62
15c	1/1	16/32	1/2/2	2/4/4
15d	1/2	32/32	2/4/4	4/8/8
15e	1/2	32/32	1/1/2	2/4/4
15g	8/8	32/32	8/8/16	16/16/32
15j	1/1	32/32	1/2/2	2/4/8
15n	2/2	125/125	2/4/4	2/4/8
16g	250/500	1000/1000	250/1000/1000	250/1000/1000
16j	125/125	1000/1000	250/1000/1000	250/1000/1000
17j	250/250	250/250	250/250/250	250/250/250
INH	0.5/1	>250/>250	>250/>250	4/4/4

*4.2.3. 5-[(2,4-Dinitrobenzyl)sulfanyl]-1-(2-nitrophenyl)-1H-tetrazole (**4s**)*

The reaction mixture was stirred overnight. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 5:1). Yield: 74% (yellow solid); mp 147–148 °C (with decomposition). ^{1}H NMR (500 MHz, Acetone) δ 8.87 (d, J = 2.4 Hz, 1H), 8.59 (dd, J = 8.5, 2.4 Hz, 1H), 8.37 (dd, J = 8.2, 1.5 Hz, 1H), 8.20 (d, J = 8.5 Hz, 1H), 8.12–8.01 (m, 2H), 7.87 (dd, J = 7.6, 1.5 Hz, 1H), 5.07 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 156.07, 148.90, 148.59, 139.82, 136.24, 135.38, 134.08, 130.44, 128.75, 127.34, 126.63, 121.51, 35.00. Anal. Calcd for $C_{14}H_9N_7O_6S$: C, 41.69; H, 2.25; N, 24.31; S, 7.95. Found: C, 41.57; H, 2.37; N, 24.48; S, 8.25.

*4.2.4. 5-[(2,4-Dinitrobenzyl)sulfanyl]-1-(4-nitrophenyl)-1H-tetrazole (**4u**)*

The reaction mixture was stirred overnight. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 10:1). Yield: 83% (light beige solid); mp 145–148 °C (with decomposition). ^{1}H NMR (500 MHz, DMSO): δ 8.76–8.70 (m, 1H), 8.56–8.43 (m, 3H), 8.13–8.06 (m, 1H), 7.98–7.89 (m, 2H), 4.97 (s, 2H); ^{13}C NMR (126 MHz, DMSO): δ 153.99, 148.28, 148.12, 147.23, 138.84, 137.77, 134.51, 128.20, 125.87, 125.57, 120.65, 34.11. Anal. Calcd for $C_{14}H_9N_7O_6S$: C, 41.69; H, 2.25; N, 24.31; S, 7.95. Found: C, 41.86; H, 2.07; N, 24.63; S, 8.29.

*4.2.5. 1-Amino-5-[(3,5-dinitrobenzyl)sulfanyl]-1H-tetrazole (**5a**)*

The reaction mixture was stirred overnight. The product was purified by crystallization (CH_3CN/H_2O). Yield: 90% (white solid); mp 129–131 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.80 (s, 2H), 8.72 (s, 1H), 6.95 (s, 2H), 4.75 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 153.59, 148.02, 142.48, 129.75, 117.91, 33.55. Anal. Calcd for $C_8H_7N_7O_4S$: C, 32.32; H, 2.37; N, 32.98; S, 10.79. Found: C, 32.49; H, 2.45; N, 33.01; S, 11.03.

*4.2.6. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(3,4,5-trimethoxyphenyl)-1H-tetrazole (**5k**)*

The reaction mixture was stirred 3 h. The product was purified by crystallization ($EtOH/H_2O$). Yield: 85% (yellowish solid); mp 139–140 °C. ^{1}H NMR (300 MHz, Acetone) δ 8.87 (d, J = 2.1 Hz, 2H), 8.84 (t, J = 2.1 Hz, 1H), 6.90 (s, 2H), 4.93 (s, 2H), 3.88 (s, 6H), 3.79 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 154.92, 154.33, 149.29, 143.0, 140.44, 130.52, 129.57, 118.61, 103.28, 60.71, 56.83, 35.91. Anal. Calcd for $C_{17}H_{16}N_6O_7S$: C, 45.53; H, 3.60; N, 18.74; S, 7.15. Found: C, 45.45; H, 3.95; N, 18.7; S, 7.14.

*4.2.7. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-[4-(N,N-diethylamino) phenyl]-1H-tetrazole (**5m**)*

The reaction mixture was stirred overnight. The product was purified by column chromatography (Mobile phase: Hexane/EtOH, 7:1). Yield: 66% (yellow solid); mp 169–170 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.77 (s, 2H), 8.71 (t, J = 1.9 Hz, 1H), 7.25 (d, J = 9.2 Hz, 2H), 6.74 (d, J = 9.2 Hz, 2H), 4.78 (s, 2H), 3.37 (q, J = 7.1 Hz, 4H), 1.10 (t, J = 7.1 Hz, 6H); ^{13}C NMR (126 MHz, DMSO) δ 153.57, 148.72, 148.01, 142.09, 129.86, 125.97, 119.85, 117.93, 111.26, 44.00, 34.87, 12.38. Anal. Calcd for $C_{17}H_{16}N_6O_7S$: C, 50.34; H, 4.46; N, 22.83; S, 7.47. Found: C, 50.44; H, 4.54; N, 22.89; S, 7.07.

*4.2.8. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(4-chlorophenyl)-1H-tetrazole (**5o**)*

The reaction mixture was stirred 5 h. The product was purified by crystallization (CH_3CN/H_2O). Yield: 83% (white solid); mp 161–162 °C. ^{1}H NMR (300 MHz, DMSO) δ 8.78 (d, J = 2.2 Hz, 2H), 8.72 (t, J = 2.2 Hz, 1H), 7.71 (d, J = 9.0 Hz, 2H), 7.65 (d, J = 9.0 Hz, 2H), 4.81 (s, 2H); ^{13}C NMR (75 MHz, DMSO) δ 153.80, 148.03, 141.91, 135.52, 131.89, 130.21, 129.95, 126.71, 118.0, 35.15. Anal. Calcd for $C_{14}H_9ClN_6O_4S$: C, 42.81; H, 2.32; N, 21.40; S, 8.16. Found: C, 43.2; H, 2.23; N, 21.62; S, 8.27.

Table 3
In vitro antimycobacterial activities of compounds **18–25**; expressed as MIC (μM).

	M. tuberculosis My 331/88	*M. avium* My 330/88	*M. kansasii* My 235/80	*M. kansasii* 6509/96
	14/21 days		7/14/21 days	
18	8/16	62/125	16/32/125	62/125/500
19	62/125	250/250	62/125/250	125/250/500
20	250/250	125/125	125/125/125	125/125/125
21	1000/1000	1000/1000	500/500/500	500/500/500
22	250/250	250/250	250/250/250	500/500/500
23	62/62	500/1000	16/32/32	125/125/125
24	4/4	32/62	2/8/16	4/8/16
25	1000/1000	1000/1000	250/1000/1000	250/500/500
INH	0.5/1	>250/>250	>250/>250	4/4/4

Table 4
In vitro antimycobacterial activities of selected compounds **5e**, **5u**, **10u**, **15c**, **15e** and **15j** and common anti-TB drugs against MDR strains of *M. tuberculosis*. The results are expressed as MIC (μM) after 14/21 days of incubation and 14 days of incubation for anti-TB drugs.

	MDR *M. tuberculosis* strains					
	234/2005	**9449/2007**	**8666/2010**	**Praha 1**	**Praha 4**	**Praha 131**
5e	0.5/1	1/1	0.5/0.5	1/1	0.5/1	1/1
5u	0.25/0.5	0.5/0.5	0.25/0.5	0.5/0.5	0.25/0.5	0.5/0.5
10u	0.5/1	1/2	0.5/0.5	1/2	0.5/1	1/2
15c	0.5/1	0.5/1	0.25/0.5	0.5/1	0.5/1	0.5/1
15e	0.5/1	0.5/1	0.5/0.5	0.5/1	0.5/1	0.5/1
15j	1/1	1/1	0.5/0.5	0.5/1	0.5/1	1/1
Streptomycin	32 (R)	>32 (R)	>32 (R)	16 (R)	>32 (R)	>32 (R)
Isoniazid	16 (R)	32 (R)	64 (R)	16 (R)	16 (R)	16 (R)
Ethambutol	16 (R)	16 (R)	8 (S)	32 (R)	16 (R)	32 (R)
Rifampin	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)
Ofloxacin	0.5 (S)	8 (R)	2 (S)	1 (S)	>16 (R)	16 (R)
Gentamicin	0.25 (S)	2 (S)	1 (S)	1 (S)	0.5 (S)	>8 (R)
Clofazimine	0.125 (S)	2 (R)	0.125 (S)	0.5 (R)	0.5 (R)	0.25 (S)
Amikacin	0.5 (S)	2 (S)	0.5 (S)	0.5 (S)	1 (S)	>32 (R)

S – Strain susceptible to the given antibiotic drug.
R – Strain resistant to the given antibiotic drug.

4.2.9. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(3,4-dichlorophenyl)-1H-tetrazole (**5p**)

The reaction mixture was stirred overnight. The product was purified by column chromatography (Mobile phase: Hexane/EtOH, 7:1). Yield: 90% (light beige solid); mp 147–148 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.77 (s, 2H), 8.72 (s, 1H), 8.01 (d, J = 2.8 Hz, 1H), 7.91 (d, J = 8.7 Hz, 1H), 7.68–7.63 (m, 1H), 4.79 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 153.93, 147.99, 141.87, 133.83, 132.61, 132.47, 132.01, 129.90, 127.04, 125.33, 117.93, 35.35. Anal. Calcd for $C_{14}H_8Cl_2N_6O_4S$: C, 39.36; H, 1.89; N, 19.67; S, 7.51. Found: C, 39.01; H, 1.80; N, 19.74; S, 7.83.

4.2.10. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(3-fluorophenyl)-1H-tetrazole (**5q**)

The reaction mixture was stirred overnight. The product was purified by crystallization (CH_3CN/H_2O). Yield: 74% (white solid); mp 141–143 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.79 (d, J = 2.0 Hz, 2H), 8.72 (t, J = 2.0 Hz, 1H), 7.72–7.66 (m, 1H), 7.63–7.58 (m, 1H), 7.53–7.46 (m, 2H), 4.82 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 162.08 (d, J = 247.1 Hz), 153.87, 148.01, 141.83, 134.15 (d, J = 10.5 Hz), 132.07 (d, J = 9.0 Hz), 129.92, 121.15 (d, J = 3.2 Hz), 117.97, 117.95 (d, J = 20.9 Hz), 112.64 (d, J = 25.7 Hz), 35.13. Anal. Calcd for $C_{14}H_9FN_6O_4S$: C, 44.68; H, 2.41; N, 22.33; S, 8.52. Found: C, 44.43; H, 2.57; N, 22.18; S, 8.63.

4.2.11. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(3-nitrophenyl)-1H-tetrazole (**5t**)

The reaction mixture was stirred overnight. The product was purified by column chromatography (Mobile phase: Hexane/EtOH, 7:1). Yield: 84% (white solid); mp 159–160 °C. ^{1}H NMR (300 MHz, Acetone) δ 8.88 (d, J = 2.1 Hz, 2H), 8.84 (t, J = 2.1 Hz, 1H), 8.55–8.52 (m, 1H), 8.52–8.47 (m, 1H), 8.18–8.12 (m, 1H), 8.01 (t, J = 8.2 Hz, 1H), 5.00 (s, 2H); ^{13}C NMR (75 MHz, Acetone) δ 154.58, 149.66, 149.32, 142.74, 135.22, 132.44, 131.19, 130.60, 125.89, 120.24, 118.70, 36.25. Anal. Calcd for $C_{14}H_9N_7O_6S$: C, 41.69; H, 2.25; N, 24.31; S, 7.95. Found: C, 41.61; H, 2.45; N, 24.56; S, 8.26.

4.2.12. Method B

The corresponding alkyl halide (1 mmol) was added to the solution of 1-substituted tetrazole-5-thiol (**1**) (1.1 mmol) and triethylamine (1.2 mmol) in CH_3CN (7 mL). The reaction mixture was stirred until completion as determined by TLC. The solvent was evaporated under reduced pressure. The crude product was dissolved in EtOAc (10 mL) and washed with 1% NaOH (10 mL) and water (2 × 10 mL). The organic phase was separated and dried over anhydrous sodium sulfate. The solvent was evaporated under

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

Strains		Tested compounds – MIC (IC_{95}; μM)												
		4u	**5d**	**5e**	**5p**	**5u**	**5v**	**10u**	**14e**	**15d**	**15e**	**23**	**VAN**	**GEN**
SA	24 h	62.5	>500	>500	>125	>125	>500	>125	31.25	>500	>500	>500	0.35	–
	48 h	>125	>500	>500	>125	>125	>500	>125	31.25	>500	>500	>500	–	–
MRSA	24 h	62.5	>500	>500	>125	>125	>500	>125	31.25	>500	>500	>500	0.35	–
	48 h	>125	>500	>500	>125	>125	>500	>125	31.25	>500	>500	>500	–	–
SE	24 h	1.95	>500	>500	>125	>125	500	>125	15.62	>500	>500	>500	0.35	–
	48 h	1.95	>500	>500	>125	>125	>500	>125	15.62	>500	>500	>500	–	–
EF	24 h	>125	>500	>500	>125	>125	>500	>125	15.62	>500	>500	>500	0.7	–
	48 h	>125	>500	>500	>125	>125	>500	>125	15.62	>500	>500	>500	–	–
EC	24 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	–	0.26
	48 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	–	–
KP	24 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	–	0.26
	48 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	–	–
KP-E	24 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	–	0.26
	48 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	–	–
PA	24 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	–	1
	48 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	–	–

SA – Staphylococcus aureus ATCC 6538; **MRSA** – methicillin resistant Staphylococcus aureus H 5996/08; **SE** – Staphylococcus epidermidis H 6966/08; **EF** – Enterococcus faecalis J 14365/08; **EC** – Escherichia coli ATCC 8739; **KP** – Klebsiella pneumoniae D 11750/08; **KP-E** – ESBL positive Klebsiella pneumoniae J 14368/08; **PA** – Pseudomonas aeruginosa ATCC 9027; **VAN** – Vancomycin; **GEN** – Gentamicin.

Table 6
In vitro antifungal activities of selected compounds expressed as MIC (μM).

Strains		Tested compounds – MIC (IC_{80}/IC_{50}; μM)[a]												
		4u	**5d**	**5e**	**5p**	**5u**	**5v**	**10u**	**14e**	**15d**	**15e**	**23**	**FLU**	**AMB**
CA	24 h	>125	>500	>500	>125	>125	>500	>125	15.62	>500	>500	>500	0.82	0.54
	48 h	>125	>500	>500	>125	>125	>500	>125	15.62	>500	>500	>500	–	–
CT	24 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	1.6	0.54
	48 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	–	–
CK	24 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	105	1
	48 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	–	–
CG	24 h	>125	>500	>500	>125	>125	>500	>125	62.5	>500	>500	>500	26	0.54
	48 h	>125	>500	>500	>125	>125	>500	>125	62.5	>500	>500	>500	–	–
TA	24 h	>125	>500	>500	>125	>125	>500	>125	62.5	>500	>500	>500	210	0.27
	48 h	>125	>500	>500	>125	>125	>500	>125	250	>500	>500	>500	–	–
AF	24 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	>500	0.54
	48 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	–	–
AC	24 h	>125	>500	>500	>125	>125	>500	>125	500	>500	>500	>500	>500	1
	48 h	>125	>500	>500	>125	>125	>500	>125	>500	>500	>500	>500	–	–
TM	72 h	>125	>500	>500	>125	>125	>500	>125	31.25	>500	>500	>500	105	0.54
	120 h	>125	>500	>500	>125	>125	>500	>125	31.25	>500	>500	>500	–	–

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** – Fluconazole; **AMB** – Amphotericin B.
[a] IC_{50} for **AF**, **AC**, **TM**; IC_{80} for **CA**, **CT**, **CK**, **CG**, **TA**.

reduced pressure, and the crude product was purified by crystallization or by silica gel column chromatography.

*4.2.13. 5-(Benzylsulfanyl)-1-(4-nitrophenyl)-1H-tetrazole (**2u**)*

The reaction mixture was stirred at rt for 1 h. Product was purified by crystallization (CH_3CN/H_2O). Yield: 81% (yellowish solid); mp 106–107 °C. ^{1}H NMR (500 MHz, Acetone) δ 8.51 (d, J = 9.0 Hz, 2H), 7.97 (d, J = 9.0 Hz, 2H), 7.50–7.46 (m, 2H), 7.36–7.27 (m, 3H), 4.68 (s, 2H); ^{13}C NMR (125 MHz, Acetone) δ 155.08, 149.19, 139.32, 136.84, 130.09, 129.53, 128.85, 126.10, 125.94, 38.23. Anal. Calcd for $C_{14}H_{11}N_5O_2S$: C, 53.66; H, 3.54; N, 22.35; S, 10.23. Found: C, 53.88; H, 3.91; N, 22.31; S, 10.37.

*4.2.14. 1-Cyclohexyl-5-[(2,4-dinitrobenzyl)sulfanyl]-1H-tetrazole (**4e**)*

Reaction mixture was stirred at 60 °C for 5 h. Product was purified by crystallization (CH_3CN/H_2O). Yield: 77% (white solid); mp 98–100 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.96 (d, J = 2.3 Hz, 1H), 8.42 (dd, J = 8.5, 2.3 Hz, 1H), 8.22 (d, J = 8.5 Hz, 1H), 4.98 (s, 2H), 4.12–3.85 (m, 1H), 2.09–1.16 (m, 10H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 151.71, 147.65, 147.50, 139.29, 134.90, 127.91, 121.02, 58.52, 33.97, 31.94, 25.02, 24.68. Anal. Calcd for $C_{14}H_{16}N_6O_4S$: C, 46.15; H, 4.43; N, 23.06; S, 8.80. Found: C, 46.22; H, 4.77; N, 22.82; S, 8.94.

*4.2.15. 5-[(2,4-Dinitrobenzyl)sulfanyl]-1-phenyl-1H-tetrazole (**4g**)*

The reaction mixture was stirred at rt overnight. Product was purified by crystallization (CH_3CN/H_2O). Yield: 85% (white solid); mp 115–116 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.96 (d, J = 2.4 Hz, 1H), 8.46 (dd, J = 8.5, 2.4 Hz, 1H), 8.30 (d, J = 8.5 Hz, 1H), 7.58–7.53 (m, 3H), 7.50–7.46 (m, 2H), 5.03 (s, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 153.28, 147.67, 147.46, 139.15, 134.96, 133.10, 130.45, 129.97, 127.93, 123.58, 121.06, 34.27. Anal. Calcd for $C_{14}H_{10}N_6O_4S$: C, 46.93; H, 2.81; N, 23.45; S, 8.95. Found: C, 46.82; H, 3.10; N, 23.23; S, 9.21.

*4.2.16. 5-[(2,4-Dinitrobenzyl)sulfanyl]-1-(4-methoxyphenyl)-1H-tetrazole (**4j**)*

The reaction mixture was stirred at rt for 1 h. The product was purified by crystallization ($EtOH/H_2O$). Yield: 69% (yellowish solid); mp 123–125 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.96 (d, J = 2.4 Hz, 1H), 8.45 (dd, J = 8.6, 2.4 Hz, 1H), 8.29 (d, J = 8.6 Hz, 1H), 7.35 (d, J = 9.0 Hz, 2H), 7.02 (d, J = 9.0 Hz, 2H), 5.00 (s, 2H), 3.87 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 160.94, 153.42, 147.64, 147.45, 139.25, 134.94, 127.91, 125.66, 125.33, 121.04, 115.01, 55.67, 34.18. Anal. Calcd for $C_{15}H_{12}N_6O_5S$: C, 46.39; H, 3.11; N, 21.64; S, 8.26. Found: C, 46.53; H, 3.38; N, 21.56; S, 8.38.

*4.2.17. 5-[(2,4-Dinitrobenzyl)sulfanyl]-1-(3-nitrophenyl)-1H-tetrazole (**4t**)*

The reaction mixture was stirred at rt overnight. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 4:1). Yield: 70% (yellowish solid); mp 126–128 °C (with decomposition). ^{1}H NMR (500 MHz, Acetone) δ 8.85 (d, J = 2.4 Hz, 1H), 8.57 (dd, J = 8.5, 2.4 Hz, 1H), 8.54–8.48 (m, 2H), 8.25 (d, J = 8.5 Hz, 1H), 8.14–8.11 (m, 1H), 8.00 (t, J = 8.1 Hz, 1H), 5.11 (s, 2H); ^{13}C NMR (126 MHz, Acetone) δ 154.81, 149.65, 149.07, 148.54, 139.88, 135.46, 132.43, 131.24, 128.67, 125.94, 121.43, 120.38, 35.08. Anal. Calcd for $C_{14}H_9N_7O_6S$: C, 41.69; H, 2.25; N, 24.31; S, 7.95. Found: C, 41.43; H, 2.46; N, 24.70; S, 8.13.

*4.2.18. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-methyl-1H-tetrazole (**5b**)*

The reaction mixture was stirred at rt for 3 h. The product was purified by crystallization (CH_3CN/H_2O). Yield: 89% (white solid); mp 107–108 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.77 (s, 2H), 8.72 (s, 1H), 4.77 (s, 2H), 3.89 (s, 3H); ^{13}C NMR (126 MHz, DMSO) δ 152.97, 148.04, 142.20, 129.81, 117.98, 34.86, 33.86. Anal. Calcd for $C_9H_8N_6O_4S$: C, 36.49; H, 2.72; N, 28.37; S, 10.82. Found: C, 36.70; H, 2.88; N, 28.48; S, 10.49.

*4.2.19. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-hexyl-1H-tetrazole (**5c**)*

The reaction mixture was stirred at rt overnight. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 4:1). Yield: 91% (light beige solid); mp 59–60 °C. ^{1}H NMR (300 MHz, DMSO) δ 8.77 (d, J = 2.1 Hz, 2H), 8.72 (t, J = 2.1 Hz, 1H), 4.81 (s, 2H), 4.22 (t, J = 7.0 Hz, 2H), 1.76–1.63 (m, 2H), 1.27–1.04 (m, 6H), 0.88–0.73 (m, 3H); ^{13}C NMR (75 MHz, DMSO) δ 152.61, 148.03, 142.19, 129.77, 117.97, 47.23, 34.89, 30.58, 28.35, 25.40, 21.98, 13.92. Anal. Calcd for $C_{14}H_{18}N_6O_4S$: C, 45.89; H, 4.95; N, 22.94; S, 8.75. Found: C, 46.03; H, 4.99; N, 23.08; S, 8.4.

*4.2.20. 1-Benzyl-5-[(3,5-dinitrobenzyl)sulfanyl]-1H-tetrazole (**5d**)*

The reaction mixture was stirred at rt overnight. The product was purified by crystallization (CH_3CN/H_2O). Yield: 76% (beige solid); mp 154–156 °C (with decomposition). ^{1}H NMR (500 MHz, DMSO) δ 8.72–8.65 (m, 3H), 7.33–7.27 (m, 3H), 7.17–7.12 (m, 2H),

Table 7
Viability determined by proliferation/viability cell assays after a 48-h treatment with test compounds for four cell lines. Vehicle-treated control viability was set to be 100%.

	HuH7[a]		HeLa		MDCKII		HepG2	
	IC50 (μM)	Viability at 30 μM	IC50 (μM)	Viability at 30 μM	IC50 (μM)	Viability at 30 μM	IC50 (μM)	Viability at 30 μM
4e	>30	97	>30	61	17	28	16	47
5d	>30	112	>30	91	>30	87	>30	94
5e	>30	128	>30	84	>30	98	>30	88
5j	>30	86	n.d.	39	>30	123	>30	90
5p	>30	90	>30	67	>30	53	26	42
5t	>30	95	>30	62	>30	95	>30	84
5u	>30	90	>30	65	>30	116	>30	100
7e	>30	84	>30	62	>30	52	23	42
8e	28	43	21	22	<7.5	18	9	10
10p	>30	111	>30	69	22	22	25	27
14e	>30	49	29	32	<7.5	11	11	4
15d	>30	70	>30	54	22	41	16.5	33
15e	>30	125	>30	66	19	7	>30	87
15j	>30	72	>30	78	<7.5	9	19	17

n.d. — not determined.
Standard deviations were <10% of the means.
[a] MTS assay.

5.54 (s, 2H), 4.77 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 153.06, 147.97, 141.97, 133.92, 129.70, 128.99, 128.62, 127.95, 117.99, 50.51, 35.00. Anal. Calcd for $C_{15}H_{12}N_6O_4S$: C, 48.38; H, 3.25; N, 22.57; S, 8.61. Found: C, 48.50; H, 3.38; N, 22.79; S, 8.76.

*4.2.21. 1-Cyclohexyl-5-[(3,5-dinitrobenzyl)sulfanyl]-1H-tetrazole (**5e**)*

The reaction mixture was stirred at rt overnight. The product was purified by crystallization (CH_3CN/H_2O). Yield: 84% (white solid); mp 111–112 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.95 (t, J = 2.1 Hz, 1H), 8.72 (d, J = 2.1 Hz, 2H), 4.74 (s, 2H), 4.15–4.05 (m, 1H), 2.05–1.71 (m, 7H), 1.48–1.22 (m, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 150.76, 148.51, 140.95, 129.39, 118.34, 58.65, 35.25, 31.99, 25.05, 24.69. Anal. Calcd for $C_{14}H_{16}N_6O_4S$: C, 46.15; H, 4.43; N, 23.06; S, 8.80. Found: C, 46.35; H, 4.75; N, 22.80; S, 8.92.

*4.2.22. 1-(1-Adamantyl)-5-[(3,5-dinitrobenzyl)sulfanyl]-1H-tetrazole (**5f**)*

The reaction mixture was stirred at rt overnight. The product was purified by crystallization (CH_3CN/H_2O). Yield: 95% (white solid); mp 166–167 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.82 (s, 2H), 8.72 (s, 1H), 4.87 (s, 2H), 2.25–2.21 (m, 6H), 2.18 (m, 3H), 1.72–1.66 (m, 6H); ^{13}C NMR (126 MHz, DMSO) δ 151.24, 148.04, 141.98, 129.92, 117.97, 61.81, 40.45, 35.51, 35.12, 29.07. Anal. Calcd for $C_{18}H_{20}N_6O_4S$: C, 51.91; H, 4.84; N, 20.18; S, 7.70. Found: C, 51.67; H, 5.01; N, 20.47; S, 7.51.

*4.2.23. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-phenyl-1H-tetrazole (**5g**)*

The reaction mixture was stirred at rt overnight. The product was purified by crystallization (CH_3CN/H_2O). Yield: 86% (white solid); mp 155–156 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.96 (t, J = 2.0 Hz, 1H), 8.73 (d, J = 2.0 Hz, 2H), 7.65–7.43 (m, 5H), 4.76 (s, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 152.28, 148.50, 140.72, 133.10, 130.53, 130.00, 129.46, 123.60, 118.38, 35.51. Anal. Calcd for $C_{14}H_{10}N_6O_4S$: C, 46.93; H, 2.81; N, 23.45; S, 8.95. Found: C, 46.68; H, 3.13; N, 23.16; S, 9.31.

*4.2.24. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(2-methoxyphenyl)-1H-tetrazole (**5h**)*

The reaction mixture was stirred at 80 °C for 2 h. The product was purified by crystallization ($CHCl_3/Et_2O$). Yield: 59% (white solid); mp 182–183 °C (with decomposition). ^{1}H NMR (500 MHz, DMSO) δ 8.79–8.70 (m, 3H), 7.68–7.59 (m, 1H), 7.46 (d, J = 7.9 Hz, 1H), 7.33 (d, J = 8.5 Hz, 1H), 7.19–7.09 (m, 1H), 4.77 (s, 2H), 3.76 (s, 3H); ^{13}C NMR (126 MHz, DMSO) δ 155.20, 153.67, 148.05, 142.03, 133.32, 129.73, 128.20, 121.23, 120.96, 118.02, 113.33, 56.28, 34.79. Anal. Calcd for $C_{15}H_{12}N_6O_5S$: C, 46.39; H, 3.11; N, 21.64; S, 8.26. Found: C, 46.41; H, 3.20; N, 21.87; S, 8.43.

*4.2.25. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(3-methoxyphenyl)-1H-tetrazole (**5i**)*

The reaction mixture was stirred at 80 °C for 2 h. The product was purified by crystallization ($CHCl_3$/Hexane). Yield: 70% (white solid); mp 146–147 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.84–8.67 (m, 3H), 7.52 (s, 1H), 7.22–7.11 (m, 3H), 4.81 (s, 2H), 3.80 (s, 3H); ^{13}C NMR (126 MHz, DMSO) δ 160.15, 153.69, 148.02, 141.92, 133.98, 131.05, 129.91, 117.96, 116.66, 116.54, 110.52, 55.89, 35.05. Anal. Calcd for $C_{15}H_{12}N_6O_5S$: C, 46.39; H, 3.11; N, 21.64; S, 8.26. Found: C, 46.24; H, 3.06; N, 21.91; S, 8.57.

*4.2.26. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(4-methoxyphenyl)-1H-tetrazole (**5j**)*

The reaction mixture was stirred at rt for 1 h. The product was purified by crystallization (CH_3CN/H_2O). Yield: 85% (white solid); mp 134–135 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.95 (t, J = 2.0 Hz, 1H), 8.72 (d, J = 2.0 Hz, 2H), 7.40 (d, J = 9.0 Hz, 2H), 7.04 (d, J = 9.0 Hz, 2H), 4.74 (s, 2H), 3.88 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.02, 152.43, 148.50, 140.82, 129.44, 125.67, 125.36, 118.36, 115.04, 55.69, 35.45. Anal. Calcd for $C_{15}H_{12}N_6O_5S$: C, 46.39; H, 3.11; N, 21.64; S, 8.26. Found: C, 46.63; H, 3.26; N, 21.61; S, 8.41.

*4.2.27. 1-(3,4-Dimethylphenyl)-5-[(3,5-dinitrobenzyl)sulfanyl]-1H-tetrazole (**5l**)*

The reaction mixture was stirred at 70 °C for 6 h. The product was purified by crystallization (CH_3CN/H_2O). Yield: 45% (white solid); mp 182–183 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.76 (s, 2H), 8.71 (t, J = 2.2 Hz, 1H), 7.38–7.27 (m, 3H), 4.79 (s, 2H), 2.28 (s, 3H), 2.27 (s, 3H); ^{13}C NMR (126 MHz, DMSO) δ 153.50, 147.99, 141.96, 139.65, 138.66, 130.75, 130.69, 129.87, 125.29, 121.97, 117.94, 35.07, 19.38, 19.29. Anal. Calcd for $C_{16}H_{14}N_6O_4S$: C, 49.74; H, 3.65; N, 21.75; S, 8.30. Found: C, 49.94; H, 3.70; N, 22.02; S, 8.24.

*4.2.28. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(4-bromophenyl)-1H-tetrazole (**5n**)*

The reaction mixture was stirred at 80 °C for 2 h. The product was purified by crystallization (CH_3CN/H_2O). Yield: 75% (yellowish solid); mp 168–171 °C (with decomposition). ^{1}H NMR (300 MHz, DMSO) δ 8.78 (d, J = 2.0 Hz, 2H), 8.72 (s, 1H), 7.84 (d, J = 8.5 Hz, 2H),

7.58 (d, J = 8.5 Hz, 2H), 4.82 (s, 2H); ^{13}C NMR (75 MHz, DMSO) δ 153.73, 148.01, 141.87, 133.13, 132.29, 129.92, 126.84, 124.07, 117.97, 35.17. Anal. Calcd for $C_{14}H_9BrN_6O_4S$: C, 38.46; H, 2.07; N, 19.22; S, 7.33. Found: C, 38.17; H, 2.32; N, 19.18; S, 7.67.

*4.2.29. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(4-fluorophenyl)-1H-tetrazole (**5r**)*

The reaction mixture was stirred at rt overnight. The product was purified by crystallization (CH_3CN/H_2O). Yield: 97% (beige solid); mp 123–125 °C (with decomposition). ^{1}H NMR (500 MHz, DMSO) δ 8.79 (d, J = 2.2 Hz, 2H), 8.72 (t, J = 2.2 Hz, 1H), 7.72–7.65 (m, 2H), 7.52–7.45 (m, 2H), 4.81 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 162.95 (d, J = 248.8 Hz), 153.92, 148.03, 141.92, 129.93, 129.37 (d, J = 3.1 Hz), 127.64 (d, J = 9.3 Hz), 117.99, 117.18 (d, J = 23.5 Hz), 35.06. Anal. Calcd for $C_{14}H_9FN_6O_4S$: C, 44.68; H, 2.41; N, 22.33; S, 8.52. Found: C, 45.05; H, 2.55; N, 22.66; S, 8.62.

*4.2.30. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(2-nitrophenyl)-1H-tetrazole (**5s**)*

The reaction mixture was stirred at 60 °C for 12 h. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 4:1). Yield: 71% (light beige solid); mp 141–142 °C (with decomposition). ^{1}H NMR (500 MHz, Acetone) δ 8.86 (t, J = 2.1 Hz, 1H), 8.83 (d, J = 2.1 Hz, 2H), 8.37 (dt, J = 8.1, 1.1 Hz, 1H), 8.12–8.07 (m, 1H), 8.06–8.01 (m, 1H), 7.89 (dt, J = 7.7, 1.1 Hz, 1H), 4.95 (s, 2H); ^{13}C NMR (126 MHz, Acetone) δ 155.66, 149.30, 145.23, 142.59, 136.23, 134.06, 130.49, 130.47, 127.33, 126.69, 118.73, 36.00. Anal. Calcd for $C_{14}H_9N_7O_6S$: C, 41.69; H, 2.25; N, 24.31; S, 7.95. Found: C, 41.73; H, 2.47; N, 24.34; S, 8.15.

*4.2.31. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(3-nitrophenyl)-1H-tetrazole (**5t**)*

The reaction mixture was stirred at 60 °C for 10 h. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 7:1). Yield: 83% (white solid); mp 159–160 °C. ^{1}H NMR (300 MHz, Acetone) δ 8.88 (d, J = 2.1 Hz, 2H), 8.84 (t, J = 2.1 Hz, 1H), 8.55–8.52 (m, 1H), 8.52–8.47 (m, 1H), 8.18–8.12 (m, 1H), 8.01 (t, J = 8.2 Hz, 1H), 5.00 (s, 2H); ^{13}C NMR (75 MHz, Acetone) δ 154.58, 149.66, 149.32, 142.74, 135.22, 132.44, 131.19, 130.60, 125.89, 120.24, 118.70, 36.25. Anal. Calcd for $C_{14}H_9N_7O_6S$: C, 41.69; H, 2.25; N, 24.31; S, 7.95. Found: C, 41.61; H, 2.45; N, 24.46; S, 8.26.

*4.2.32. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(4-nitrophenyl)-1H-tetrazole (**5u**)*

The reaction mixture was stirred at 60 °C for 6 h. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 5:1). Yield: 83% (beige solid); mp 125–127 °C (with decomposition). ^{1}H NMR (500 MHz, acetone) δ 8.89 (d, J = 2.2 Hz, 2H), 8.84 (t, J = 2.2 Hz, 1H), 8.53 (d, J = 9.0 Hz, 2H), 8.01 (d, J = 9.0 Hz, 2H), 5.02 (s, 2H); ^{13}C NMR (126 MHz, Acetone) δ 154.54, 149.32, 149.30, 142.68, 139.13, 130.62, 126.19, 126.00, 118.71, 36.21. Anal. Calcd for $C_{14}H_9N_7O_6S$: C, 41.69; H, 2.25; N, 24.31; S, 7.95. Found: C, 41.46; H, 2.14; N, 24.65; S, 8.04.

*4.2.33. 3-{5-[(3,5-Dinitrobenzyl)sulfanyl]-1H-tetrazol-5-yl}benzoic acid (**5v**)*

The reaction mixture was stirred at 60 °C for 12 h. The product was purified by crystallization (CH_3CN/H_2O). Yield: 95% (white solid); mp 183–184 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.78 (s, 2H), 8.70 (s, 1H), 8.14–8.09 (m, 1H), 8.08–8.05 (m, 1H), 7.87–7.82 (m, 1H), 7.76–7.69 (m, 1H), 4.82 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 166.18, 153.66, 148.02, 141.93, 133.29, 131.20, 130.55, 129.94, 128.45, 124.97, 117.97, 35.25. Anal. Calcd for $C_{14}H_9N_7O_6S$: C, 44.78; H, 2.51; N, 20.89; S, 7.97; Found: C, 44.60; H, 2.69; N, 21.12; S, 7.75.

*4.2.34. Methyl 3-{5-[(3,5-dinitrobenzyl)sulfanyl]-1H-tetrazol-5-yl} benzoate (**6**)*

3-{5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazol-5-yl}benzoic acid (0.25 mmol) and catalytic amount of sulfuric acid was refluxed in methanol (5 mL) for 20 h. The precipitate was filtered and crystallized from $EtOH/H_2O$. Yield: 72% (white solid); mp 178–179 °C (with decomposition). ^{1}H NMR (300 MHz, DMSO) δ 8.76 (d, J = 2.1 Hz, 2H), 8.70 (t, J = 2.1 Hz, 1H), 8.15 (dt, J = 7.8, 1.4 Hz, 1H), 8.11–8.09 (m, 1H), 7.94–7.89 (m, 1H), 7.78 (t, J = 7.9 Hz, 1H), 4.81 (s, 2H), 3.89 (s, 3H). ^{13}C NMR (75 MHz, DMSO) δ 165.00, 153.69, 148.01, 141.93, 133.50, 131.37, 131.14, 130.88, 129.91, 129.42, 125.09, 117.95, 52.86, 35.33. Anal. Calcd for $C_{16}H_{12}N_6O_6S$: C, 46.15; H, 2.90; N, 20.18; S, 7.70. Found: C, 46.34; H, 2.70; N, 19.92; S, 8.08.

*4.3. General procedure for the synthesis of 1-substituted 5-[(3,5-dinitrobenzyl)sulfinyl]-1H-tetrazoles (**7**)*

30% Hydrogen peroxide (0.5 mL, 5 mmol) was added to the suspension of 1-substituted 5-[3,5-(dinitrobenzyl)sulfanyl]-1*H*-tetrazole (**5**) (1 mmol) in acetic acid (98%, 7 mL). The reaction mixture was stirred at 45 °C for 4–6 h. When sulfonyl derivatives began to form (as determined by TLC), the reaction was stopped by pouring the reaction mixture into cold water (10 mL). The precipitate was filtered, dissolved in EtOAc, and dried over anhydrous sodium sulfate. The product (**7**) was separated by silica gel column chromatography (Mobile phase: Hexane/EtOAc, 5:1).

*4.3.1. 1-Cyclohexyl-5-[(3,5-dinitrobenzyl)sulfinyl]-1H-tetrazole (**7e**)*

The reaction mixture was stirred for 6 h. Yield: 53% (light beige solid); mp 130–133 °C. ^{1}H NMR (300 MHz, Acetone) δ 8.96 (t, J = 2.1 Hz, 1H), 8.66 (d, J = 2.1 Hz, 2H), 5.26 (d, J = 13.4 Hz, 1H), 5.15 (d, J = 13.4 Hz, 1H), 4.94–4.64 (m, 1H), 2.15–2.01 (m, 2H), 1.98–1.62 (m, 5H), 1.50–1.21 (m, 3H); ^{13}C NMR (75 MHz, Acetone) δ 155.02, 149.22, 134.65, 132.06, 119.53, 60.20, 58.17, 33.87, 33.34, 25.65, 25.59, 25.40. Anal. Calcd for $C_{14}H_{16}N_6O_5S$: C, 44.21; H, 4.24; N, 22.09; S, 8.4. Found: C, 44.60; H, 4.17; N, 21.87; S, 8.75.

*4.3.2. 5-[(3,5-Dinitrobenzyl)sulfinyl]-1-phenyl-1H-tetrazole (**7g**)*

The reaction mixture was stirred for 4 h. Yield: 60% (light beige solid); mp 149–150 °C (with decomposition). ^{1}H NMR (300 MHz, Acetone) δ 8.93 (t, J = 2.1 Hz, 1H), 8.69 (d, J = 2.1 Hz, 2H), 7.77–7.52 (m, 5H), 5.30–5.20 (m, 2H); ^{13}C NMR (75 MHz, Acetone) δ 157.36, 149.27, 134.64, 134.02, 132.15, 131.99, 130.68, 126.05, 119.59, 57.49. Anal. Calcd for $C_{14}H_{10}N_6O_5S$: C, 44.92; H, 2.69; N, 22.45; S, 8.57. Found: C, 45.28; H, 3.02; N, 22.12; S, 8.36.

*4.3.3. 5-[(3,5-Dinitrobenzyl)sulfinyl]-1-(4-methoxyphenyl)-1H-tetrazole (**7j**)*

The reaction mixture was stirred for 6 h. Yield: 40% (light beige solid); mp 132–135 °C (with decomposition). ^{1}H NMR (300 MHz, Acetone) δ 8.93 (t, J = 2.1 Hz, 1H), 8.69 (d, J = 2.1 Hz, 2H), 7.57 (d, J = 9.0 Hz, 2H), 7.14 (d, J = 9.0 Hz, 2H), 5.27–5.17 (m, 2H), 3.90 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 162.46, 157.34, 149.27, 134.69, 132.11, 127.63, 126.53, 119.57, 115.64, 57.41, 56.17. Anal. Calcd for $C_{15}H_{12}N_6O_6S$: C, 44.55; H, 2.99; N, 20.78; S, 7.93. Found: C, 44.84; H, 3.25; N, 20.71; S, 7.90.

*4.3.4. 5-[(3,5-Dinitrobenzyl)sulfinyl]-1-(4-nitrophenyl)-1H-tetrazole (**7u**)*

The reaction mixture was stirred for 6 h. Yield: 57% (light beige solid); mp 134–136 °C. ^{1}H NMR (300 MHz, Acetone) δ 8.93 (t, J = 2.1 Hz, 1H), 8.68 (d, J = 2.1 Hz, 2H), 8.48 (d, J = 9.1 Hz, 2H), 7.99 (d, J = 9.1 Hz, 2H), 5.32 (d, J = 13.4 Hz, 1H), 5.24 (d, J = 13.4 Hz, 1H); ^{13}C NMR (75 MHz, Acetone) δ 157.51, 149.87, 149.23, 138.68, 134.36,

132.17, 127.60, 125.77, 119.66, 57.84. Anal. Calcd for $C_{14}H_9N_7O_7S$: C, 40.10; H, 2.16; N, 23.38; S, 7.65. Found: C, 40.44; H, 2.37; N, 23.01; S, 7.70.

4.4. General procedure for the synthesis of 1-substituted 5-[(3,5-dinitrobenzyl)sulfonyl]-1H-tetrazoles (**8**)

30% Hydrogen peroxide (0.5 mL, 5 mmol) was added to the suspension of 1-substituted 5-[3,5-(dinitrobenzyl)sulfanyl]-1*H*-tetrazole (**5**) (1 mmol) in acetic acid (98%, 5 mL). The reaction mixture was stirred at 70 °C for 4–6 h until the starting material was consumed, as determined by TLC. The reaction mixture was poured into cold water (10 mL). The precipitate was filtered, dissolved in EtOAc, dried over anhydrous sodium sulfate and purified by silica gel column chromatography (Mobile phase: Hexane/EtOAc, 2:1).

4.4.1. 1-Cyclohexyl-5-[(3,5-dinitrobenzyl)sulfonyl]-1H-tetrazole (**8e**)

The reaction mixture was stirred for 5 h. Yield: 91% (white solid); mp 150–152 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.10 (t, J = 2.1 Hz, 1H), 8.77 (d, J = 2.1 Hz, 2H), 5.22 (s, 2H), 4.78 (tt, J = 11.2, 3.9 Hz, 1H), 2.17–2.07 (m, 2H), 2.04–1.91 (m, 4H), 1.81–1.72 (m, 1H), 1.50–1.40 (m, 2H), 1.36–1.25 (m, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 151.90, 148.72, 131.77, 129.59, 119.99, 61.40, 60.23, 32.80, 24.95, 24.55. Anal. Calcd for $C_{14}H_{16}N_6O_6S$: C, 42.42; H, 4.07; N, 21.20; S, 8.09. Found: C, 42.68; H, 4.31; N, 21.39; S, 8.03.

4.4.2. 5-[(3,5-Dinitrobenzyl)sulfonyl]-1-phenyl-1H-tetrazole (**8g**)

The reaction mixture was stirred for 6 h. Yield: 85% (white solid); mp 179–183 °C. ^{1}H NMR (300 MHz, Acetone) δ 8.99 (t, J = 2.1 Hz, 1H), 8.83 (d, J = 2.1 Hz, 2H), 7.77–7.51 (m, 5H), 5.63 (s, 2H); ^{13}C NMR (75 MHz, Acetone) δ 154.07, 149.40, 134.07, 132.83, 132.36, 131.56, 130.30, 126.75, 120.32, 60.80. Anal. Calcd for $C_{14}H_{10}N_6O_6S$: C, 43.08; H, 2.58; N, 21.53; S, 8.21. Found: C, 43.29; H, 2.78; N, 21.59; S, 8.41.

4.4.3. 5-[(3,5-Dinitrobenzyl)sulfonyl]-1-(4-methoxyphenyl)-1H-tetrazole (**8j**)

The reaction mixture was stirred for 4 h. Yield: 78% (white solid); mp 192–193 °C. ^{1}H NMR (300 MHz, Acetone) δ 8.99 (t, J = 2.1 Hz, 1H), 8.81 (d, J = 2.1 Hz, 2H), 7.55 (d, J = 9.0 Hz, 2H), 7.12 (d, J = 9.0 Hz, 2H), 5.60 (s, 2H), 3.90 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 162.71, 154.08, 149.39, 132.80, 131.63, 128.24, 126.51, 120.29, 115.25, 60.75, 56.15. Anal. Calcd for $C_{15}H_{12}N_6O_7S$: C, 42.86; H, 2.88; N, 19.99; S, 7.63. Found: C, 42.97; H, 3.12; N, 20.19; S, 7.73.

4.4.4. 5-[(3,5-Dinitrobenzyl)sulfonyl]-1-(4-nitrophenyl)-1H-tetrazole (**8u**)

The reaction mixture was stirred for 6 h. Yield: 62% (white solid); mp 159–161 °C. ^{1}H NMR (300 MHz, Acetone) δ 9.00 (t, J = 2.1 Hz, 1H), 8.84 (d, J = 2.1 Hz, 2H), 8.52 (d, J = 9.0 Hz, 2H), 8.04 (d, J = 9.0 Hz, 2H), 5.67 (s, 2H); ^{13}C NMR (75 MHz, Acetone) δ 154.21, 150.36, 149.43, 138.67, 132.88, 131.39, 128.52, 125.59, 120.40, 60.95. Anal. Calcd for $C_{14}H_9N_7O_8S$: C, 38.63; H, 2.08; N, 22.52; S, 7.37. Found: C, 39.01; H, 2.26; N, 22.75; S, 6.98.

4.5. General procedure for the synthesis of 1-aryl-5-methylsulfonyl-1H-tetrazoles (**9**)

1-Aryl-1*H*-tetrazole-5-thiol (**1**) (1 mmol) was stirred with dimethyl sulfate (1.1 mmol) in the water/dichloromethane (7 mL/7 mL) in the presence of NaOH (1.2 mmol) and TBAB (0.1 mmol) under conditions of phase-transfer catalysis overnight. Upon completion, the organic phase was separated, washed with water (2 × 20 mL) and dried over anhydrous sodium sulfate. 1-Aryl-5-methylsulfanyl-1*H*-tetrazoles were formed at yields of 75–98% and were used in the following step without further purification. Oxidation of methylsulfanyl derivatives by 30% hydrogen peroxide (1:5 M ratio) in acetic acid was carried out at 70–75 °C for 3–6 h. Upon completion, the reaction mixture was poured into cold water and the precipitate was filtered and crystallized from EtOH/H_2O.

4.5.1. 1-Phenyl-5-methylsulfonyl-1H-tetrazole (**9g**)

Yield: 90%. ^{1}H NMR (300 MHz, DMSO) δ 7.97–7.46 (m, 5H), 3.67 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 154.36, 133.13, 131.71, 129.66, 126.39, 44.07.

4.5.2. 1-(4-Methoxyphenyl)-5-methylsulfonyl-1H-tetrazole (**9j**)

Yield: 93%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.59 (d, J = 9.0 Hz, 2H), 7.06 (d, J = 9.0 Hz, 2H), 3.88 (s, 3H), 3.61 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.67, 153.90, 126.36, 125.48, 114.79, 55.67, 43.73.

4.5.3. 5-Methylsulfonyl-1-(3,4,5-trimethoxyphenyl)-1H-tetrazole (**9k**)

Yield: 51%. ^{1}H NMR (300 MHz, acetone) δ 7.12 (s, 2H), 3.88 (s, 6H), 3.82 (s, 3H), 3.63 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 155.38, 154.53, 141.1, 129.39, 104.76, 60.75, 56.8, 44.19.

4.5.4. 1-(4-Bromophenyl)-5-methylsulfonyl-1H-tetrazole (**9n**)

Yield: 64%. ^{1}H NMR (300 MHz, Acetone) δ 7.89 (d, J = 8.8 Hz, 2H), 7.75 (d, J = 8.8 Hz, 2H), 3.66 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 155.34, 133.58, 133.54, 128.82, 125.96, 44.3.

4.5.5. 1-(4-Chlorophenyl)-5-methylsulfonyl-1H-tetrazole (**9o**)

Yield: 70%. ^{1}H NMR (300 MHz, Acetone) δ 7.83 (d, J = 9.0 Hz, 2H), 7.74 (d, J = 9.0 Hz, 2H), 3.67 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 155.38, 137.79, 133.1, 130.52, 128.65, 44.29.

4.5.6. 1-(3,4-Dichlorophenyl)-5-methylsulfonyl-1H-tetrazole (**9p**)

Yield: 60%. ^{1}H NMR (300 MHz, Acetone) δ 8.10 (d, J = 2.4 Hz, 1H), 7.93 (d, J = 8.7, 1H), 7.82 (dd, J = 8.7, 2.4 Hz, 1H), 3.67 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 155.37, 136.17, 133.74, 133.52, 132.3, 129.18, 127.18, 44.37.

4.5.7. 5-Methylsulfonyl-1-(2-nitrophenyl)-1H-tetrazole (**9s**)

Yield: 62%. ^{1}H NMR (300 MHz, acetone) δ 8.55–8.49 (m, 1H), 8.19–8.06 (m, 3H), 3.62 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 155.69, 144.68, 136.26, 134.46, 131.22, 127.42, 127.21, 44.25.

4.5.8. 5-Methylsulfonyl-1-(3-nitrophenyl)-1H-tetrazole (**9t**)

Yield: 70%. ^{1}H NMR (300 MHz, DMSO) δ 8.75 (t, J = 2.2 Hz, 1H), 8.56–8.51 (m, 1H), 8.28–8.22 (m, 1H), 7.96 (t, J = 8.2 Hz, 1H), 3.68 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 154.45, 147.93, 133.94, 133.35, 131.18, 126.51, 122.32, 44.38.

4.5.9. 5-Methylsulfonyl-1-(4-nitrophenyl)-1H-tetrazole (**9u**)

Yield: 80%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.48 (d, J = 8.8 Hz, 2H), 7.98 (d, J = 8.8 Hz, 2H), 3.69 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 154.13, 149.15, 137.34, 125.90, 125.26, 43.85.

4.6. General procedure for the synthesis of 1-aryl-5-[(3,5-dinitrobenzyl)oxy]-1H-tetrazoles (**10**)

Sodium hydroxide (1.3 mmol) was added to the solution of 1-aryl-5-methylsulfonyl-1*H*-tetrazole (1.1 mmol) (**9**) and 3,5-dinitrobenzyl alcohol (1 mmol) in THF (10 mL). The reaction mixture was stirred at room temperature for 15–48 h. After completion, THF was removed, and the crude product was dissolved in EtOAc (20 mL) and washed with water (2 × 20 mL). The

solvent was evaporated under reduced pressure, and products **10g**, **10j**, **10k**, **10n–p**, **10s–u** and a byproduct **11** were separated by silica gel column chromatography.

*4.6.1. 5-[(3,5-Dinitrobenzyl)oxy]-1-phenyl-1H-tetrazole (**10g**)*

The reaction mixture was stirred for 48 h. The product was purified by silica gel column chromatography (Mobile phase: $CHCl_3$). Yield: 46% (yellowish solid); mp 145–147 °C (with decomposition). ^{1}H NMR (300 MHz, Acetone) δ 8.97–8.92 (m, 3H), 7.85–7.78 (m, 2H), 7.65–7.50 (m, 3H), 6.00 (s, 2H); ^{13}C NMR (75 MHz, Acetone) δ 160.96, 149.58, 140.15, 134.24, 130.57, 130.15, 129.71, 123.15, 119.65, 73.30. Anal. Calcd for $C_{14}H_{10}N_6O_5$: C, 49.13; H, 2.94; N, 24.55. Found: C, 48.94; H, 3.12; N, 24.27.

*4.6.2. 5-[(3,5-Dinitrobenzyl)oxy]-1-(4-methoxypheny)l-1H-tetrazole (**10j**)*

The reaction mixture was stirred for 24 h. The product was purified by silica gel column chromatography (Mobile phase: $CHCl_3$). Yield: 45% (yellowish solid); mp 149–150 °C. ^{1}H NMR (300 MHz, Acetone) δ 8.95 (t, J = 2.2 Hz, 1H), 8.90 (d, J = 2.2 Hz, 2H), 7.68 (d, J = 9.0 Hz, 2H), 7.13 (d, J = 9.0 Hz, 2H), 5.96 (s, 2H), 3.87 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 161.22, 160.93, 149.57, 140.23, 129.66, 126.91, 125.20, 119.62, 115.58, 73.13, 56.02. Anal. Calcd for $C_{15}H_{10}N_6O_6$: C, 48.39; H, 3.25; N, 22.57. Found: C, 48.38; H, 3.51; N, 22.34.

*4.6.3. 5-[(3,5-Dinitrobenzyl)oxy]-1-(3,4,5-trimethoxyphenyl)-1H-tetrazole (**10k**)*

The reaction mixture was stirred for 48 h. The product was purified by silica gel column chromatography (Mobile phase: Hexane/EtOAc, 2:1). Yield: 23% (white solid); mp 143–144 °C. ^{1}H NMR (300 MHz, Acetone) δ 8.95 (s, 3H), 7.09 (s, 2H), 5.97 (s, 2H), 3.87 (s, 6H), 3.77 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 160.85, 154.85, 149.58, 140.24, 139.63, 129.48, 119.65, 101.49, 73.18, 60.69, 56.72. Anal. Calcd for $C_{17}H_{16}N_6O_8$: C, 47.23; H, 3.73; N, 19.44. Found: C, 47.20; H, 3.45; N, 19.27.

*4.6.4. 5-[(3,5-Dinitrobenzyl)oxy]-1-(4-bromophenyl)-1H-tetrazole (**10n**)*

The reaction mixture was stirred for 48 h. The product was purified by silica gel column chromatography (Mobile phase: Hexane/EtOAc, 8:1). Yield: 32% (light beige solid); mp 179–181 °C (with decomposition). ^{1}H NMR (300 MHz, Acetone) δ 8.96 (t, J = 2.1 Hz, 1H), 8.93 (d, J = 2.1 Hz, 2H), 7.80 (s, 4H), 6.01 (s, 2H); ^{13}C NMR (75 MHz, Acetone) δ 160.94, 149.56, 140.01, 133.67, 133.48, 129.81, 124.90, 123.27, 119.69, 73.48. Anal. Calcd for $C_{14}H_9BrN_6O_5$: C, 39.93; H, 2.15; N, 19.95. Found: C, 40.03; H, 2.34 N, 20.12.

*4.6.5. 5-[(3,5-Dinitrobenzyl)oxy]-1-(4-chlorophenyl)-1H-tetrazole (**10o**)*

The reaction mixture was stirred for 15 h. The product was purified by silica gel column chromatography (Mobile phase: Hexane/EtOAc, 6:1). Yield: 53% (yellowish solid); mp 162–164 °C (with decomposition). ^{1}H NMR (300 MHz, Acetone) δ 8.96 (t, J = 2.1 Hz, 1H), 8.93 (d, J = 2.1 Hz, 2H), 7.86 (d, J = 8.9 Hz, 2H), 7.64 (d, J = 8.9 Hz, 2H), 6.01 (s, 2H); ^{13}C NMR (75 MHz, Acetone) δ 160.96, 149.57, 140.02, 135.29, 133.01, 130.65, 129.80, 124.71, 119.69, 73.47. Anal. Calcd for $C_{14}H_9ClN_6O_5$: C, 44.64; H, 2.41; N, 22.31. Found: C, 44.71; H, 2.26; N, 22.47.

*4.6.6. 5-[(3,5-Dinitrobenzyl)oxy]-1-(3,4-dichlorophenyl)-1H-tetrazole (**10p**)*

The reaction mixture was stirred for 15 h. The product was purified by silica gel column chromatography (Mobile phase: Hexane/EtOAc, 5:1). Yield: 55% (yellowish solid); mp 160–162 °C (with decomposition). ^{1}H NMR (500 MHz, Acetone) δ 8.96 (t, J = 2.1 Hz, 1H), 8.93 (d, J = 2.1 Hz, 2H), 8.07 (d, J = 2.4 Hz, 1H), 7.85 (dd, J = 8.8, 2.4 Hz, 1H), 7.82 (d, J = 8.8 Hz, 1H), 6.03 (s, 2H); ^{13}C NMR (126 MHz, Acetone) δ 160.99, 149.56, 139.95, 133.79, 133.76, 133.46, 132.53, 129.75, 124.78, 122.88, 119.71, 73.67. Anal. Calcd for $C_{14}H_8Cl_2N_6O_5$: C, 40.90; H, 1.96; N, 20.44. Found: C, 40.98; H, 2.06; N, 20.31.

*4.6.7. 5-[(3,5-Dinitrobenzyl)oxy]-1-(2-nitrophenyl)-1H-tetrazole (**10s**)*

The reaction mixture was stirred for 48 h. The product was purified by silica gel column chromatography (Mobile phase: Hexane/Et_2O, 1:1). Yield: 26% (yellowish solid); mp 147–149 °C (with decomposition). ^{1}H NMR (300 MHz, acetone) δ 8.96 (t, J = 2.1 Hz, 1H), 8.82 (d, J = 2.1 Hz, 2H), 8.32 (dd, J = 8.4, 1.5 Hz, 1H), 8.11–7.92 (m, 3H), 5.95 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 161.59, 149.49, 139.54, 135.94, 133.14, 129.84, 129.74, 126.94, 125.95, 119.81, 73.56. Anal. Calcd for $C_{14}H_9N_7O_7$: C, 43.42; H, 2.34; N, 25.32. Found: C, 43.53; H, 2.49; N, 25.27.

*4.6.8. 5-[(3,5-Dinitrobenzyl)oxy]-1-(3-nitrophenyl)-1H-tetrazole (**10t**)*

The reaction mixture was stirred for 48 h. The product was purified by silica gel column chromatography (Mobile phase: Hexane/$CHCl_3$, 1:2). Yield: 47% (white solid); mp 176–183 °C (with decomposition). ^{1}H NMR (500 MHz, acetone) δ 8.98–8.95 (m, 3H), 8.70 (t, J = 2.2 Hz, 1H), 8.40 (ddd, J = 8.2, 2.2, 1.0 Hz, 1H), 8.32 (ddd, J = 8.2, 2.2, 1.0 Hz, 1H), 7.95 (t, J = 8.2 Hz, 1H), 6.07 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 161.17, 149.59, 139.96, 135.10, 132.18, 129.78, 128.70, 124.50, 119.75, 117.74, 73.77. Anal. Calcd for $C_{14}H_9N_7O_7$: C, 43.42; H, 2.34; N, 25.32. Found: C, 43.34; H, 2.59; N, 25.59.

*4.6.9. 5-[(3,5-Dinitrobenzyl)oxy]-1-(4-nitrophenyl)-1H-tetrazole (**10u**)*

The reaction mixture was stirred for 36 h. The product was purified by silica gel column chromatography (Mobile phase: $CHCl_3$). Yield: 48% (yellow solid); mp 168–170 °C (with decomposition). ^{1}H NMR (500 MHz, DMSO) δ 8.89 (d, J = 2.2 Hz, 2H), 8.84 (t, J = 2.2 Hz, 1H), 8.44 (d, J = 9.1 Hz, 2H), 8.07 (d, J = 9.1 Hz, 2H), 5.91 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 160.27, 148.33, 147.22, 138.77, 137.72, 129.39, 125.5, 123.04, 119.09, 72.91. Anal. Calcd for $C_{14}H_9N_7O_7$: C, 43.42; H, 2.34; N, 25.32. Found: C, 43.58; H, 2.54; N, 25.02.

*4.6.10. (3,5-Dinitrobenzyl)(methyl) sulfone (**11**)*

Yield: 30–40% (beige solid); mp 139–140 °C. ^{1}H NMR (300 MHz, Acetone) δ 8.97 (t, J = 2.1 Hz, 1H), 8.79 (d, J = 2.1 Hz, 2H), 4.87 (s, 2H), 3.02 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 149.46, 134.63, 132.02, 119.41, 59.12, 40.29. Anal. Calcd for $C_8H_8N_2O_6S$: C, 36.93; H, 3.10; N, 10.77; S, 12.32. Found: C, 36.91; H, 3.2; N, 10.99; S, 12.49.

*4.7. General method for the synthesis of 1-alkyl/aryl-5-alkylselanyl-1H-tetrazoles (**13–15**) [30]*

Alky/arylisoselenocyanates (**12**) were prepared by reacting *N*-alkyl/arylformamides with triphosgene in refluxing CH_2Cl_2 in the presence of triethylamine followed by the addition of selenium powder.

4.7.1. Method C

Sodium azide (1.2 mmol) as a saturated solution in water (0.5 mL) was added to the solution of alkylisoselenocyanate **12c** or **12d** (1 mmol) and alkyl halide (0.9 mmol) in 10 mL of an organic solvent (THF or CH_3CN). The reaction was completed in 0.5–3 h depending on the substituent. Upon completion, the organic

solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography (Hexane/EtOAc).

*4.7.2. 5-Benzylselanyl-1-cyclohexyl-1H-tetrazole (**13e**)*

The reaction was carried out in THF. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 6:1). Yield: 76% (white solid); mp 62 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32–7.20 (m, 5H), 4.53 (s, 2H), 4.08–3.97 (m, 1H), 1.90–1.79 (m, 6H), 1.71–1.67 (m, 1H), 1.37–1.20 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 144.91, 136.79, 128.91, 128.77, 127.83, 58.81, 32.77, 32.23, 25.08, 24.73. IR (KBr): 2935, 2857, 1494, 1452, 1421, 1365, 1272, 1185, 1083, 1001, 894, 817, 753, 696 cm^{-1}. Anal. Calcd for $C_{14}H_{18}N_4Se$: C, 52.34; H, 5.65; N, 17.44. Found: C, 52.27; H, 5.98; N, 17.36. MS (ESI): *m/z* 322.97 $[M + H]^+$.

*4.7.3. 5-[(3,5-Dinitrobenzyl)selanyl]-1-hexyl-1H-tetrazole (**15c**)*

The reaction was carried out in CH_3CN. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 10:1). Yield: 45% (white solid); mp 57–58 °C. ^{1}H NMR (500 MHz, Acetone) δ 8.82–8.79 (m, 3H), 4.89 (s, 2H), 4.29 (t, J = 7.1 Hz, 2H), 1.81–1.76 (m, 2H), 1.25–1.20 (m, 6H), 0.82 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, Acetone) δ 149.28, 146.43, 144.77, 130.30, 118.25, 48.66, 31.67, 30.55, 30.32, 26.52, 22.96, 14.10. IR (KBr): 3111, 2933, 2859, 1541, 1460, 1426, 1385, 1343 (NO_2), 1179, 1074, 809, 729, 675 cm^{-1}. Anal. Calcd for $C_{14}H_{18}N_6O_4Se$: C, 40.69; H, 4.39; N, 20.33. Found: C, 41.01; H, 4.61; N, 20.14. MS (ESI): *m/z* 415.0 $[M + H]^+$.

*4.7.4. 1-Benzyl-5-[(3,5-dinitrobenzyl)selanyl]-1H-tetrazole (**15d**)*

The reaction was carried out in CH_3CN. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 5:1). Yield: 33% (light brown solid); mp 152–154 °C. ^{1}H NMR (500 MHz, Acetone) δ 8.75 (t, J = 2.2 Hz, 1H), 8.68 (d, J = 2.2 Hz, 2H), 7.33–7.29 (m, 3H), 7.22–7.19 (m, 2H), 5.57 (s, 2H), 4.81 (s, 2H); ^{13}C NMR (126 MHz, Acetone) δ 149.21, 146.86, 144.55, 134.90, 130.16, 129.72, 129.45, 128.81, 118.26, 52.09, 30.51. Anal. Calcd for $C_{15}H_{12}N_6O_4Se$: C, 42.97; H, 2.88; N, 20.05. Found: C, 43.19; H, 2.88; N, 19.75.

*4.7.5. 1-Cyclohexyl-[(3,5-dinitrobenzyl)selanyl]-1H-tetrazole (**15e**)*

The reaction was carried out in CH_3CN. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 5:1). Yield: 68% (yellowish solid); mp 103 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.94–8.90 (m, 1H), 8.72–8.69 (m, 2H), 4.77 (s, 2H), 4.13–4.04 (m, 1H), 2.02–1.86 (m, 6H), 1.77–1.74 (m, 1H), 1.46–1.22 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 148.51, 143.93, 142.17, 129.44, 118.07, 59.27, 32.29, 29.48, 25.05, 24.67. IR (KBr): 3095, 2941, 2859, 1538, 1452, 1424, 1383, 1361, 1342, 1276, 1198, 1083, 1005, 922, 817, 808, 731, 671 cm^{-1}. Anal. Calcd for $C_{14}H_{16}N_6O_4Se$: C, 40.89; H, 3.92; N, 20.43. Found: C, 40.93; H, 4.21; N, 20.74.

4.7.6. Method D

A saturated solution of sodium azide (1.2 mmol) in water (0.5 mL) was added to the solution of arylisoselenocyanate **12g**, **12j** or **12n** (1 mmol) and alkyl halide (0.7 mmol) in 10 mL of organic solvent (THF or CH_3CN). The reaction proceeded with the release of nitrogen and the precipitation of elemental selenium and was completed in 15–30 min. Upon completion, selenium powder was filtered off. The solvent was evaporated and the residue was adsorbed onto silica gel and purified by column chromatography or reverse phase column chromatography.

*4.7.7. 5-Benzylselanyl-1-phenyl-1H-tetrazole (**13g**)*

The reaction was carried out in THF. The product was purified by reverse phase column chromatography (Mobile phase: CH_3CN/H_2O, 2:1) Yield: 17% (beige solid); mp 71 °C. R_f (Hexane/EtOAc, 3:1) 0.72, R_f (RP-18; CH_3CN/H_2O, 2:1) 0.27. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.55–7.44 (m, 5H), 7.42–7.36 (m, 2H), 7.33–7.24 (m, 3H), 4.68 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 147.18, 136.24, 133.94, 130.20, 129.75, 129.11, 128.80, 127.91, 123.88, 32.56. IR (KBr): 3030, 2922, 1595, 1497, 1374, 1233, 1089, 1014, 973, 765, 693 cm^{-1}. Anal. Calcd for $C_{14}H_{12}N_4Se$: C, 53.34; H, 3.84; N, 17.77. Found: C, 53.25; H, 3.98; N, 17.53.

*4.7.8. 1-Cyclohexyl-5-[(2,4-dinitrobenzyl)selanyl]-1H-tetrazole (**14e**)*

Cyclohexylisoselenocyanate (**12e**) (1 mmol) and sodium azide (1.2 mmol) in water (5 mL) were stirred at rt overnight. The aqueous solution was filtered and washed with EtOAc (2 × 7 mL). 2,4-Dinitrobenzyl chloride (1 mmol) and TBAB (0.1 mmol) in CH_2Cl_2 (7 mL) were added to the aqueous solution. The reaction mixture was stirred at rt overnight. The organic layer was separated and dried over anhydrous sodium sulfate; the solvent was evaporated under reduced pressure. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 10:1). Yield: 50% (yellowish solid); mp 98–99 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.98 (d, J = 2.3 Hz, 1H), 8.41 (dd, J = 8.6, 2.3 Hz, 1H), 8.18 (d, J = 8.6 Hz, 1H), 4.94 (s, 2H), 4.07–3.98 (m, 1H), 2.01–1.85 (m, 6H), 1.78–1.71 (m, 1H), 1.46–1.20 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 147.44, 146.94, 145.12, 141.32, 134.33, 128.21, 121.08, 59.20, 32.22, 28.08, 25.04, 24.68. IR (KBr): 3089, 2955, 2853, 1611, 1537, 1455, 1426, 1340, 1277, 1193, 1175, 1083, 997, 913, 817, 802, 744, 712, 684 cm^{-1}. Calcd for $C_{14}H_{16}N_6O_4Se$: C, 40.89; H, 3.92; N, 20.43. Found: C, 40.57; H, 4.17; N, 20.06.

*4.7.9. 5-[(3,5-Dinitrobenzyl)selanyl]-1-phenyl-1H-tetrazole (**15g**)*

The reaction was carried out in CH_3CN. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 10:1). Yield: 28% (light beige solid); mp 141–143 °C (with decomposition). R_f (Hexane/EtOAc, 3:1) 0.18. ^{1}H NMR (300 MHz, Acetone) δ 8.85 (d, J = 2.1 Hz, 2H), 8.80 (t, J = 2.1 Hz, 1H), 7.78–7.39 (m, 5H), 4.95 (s, 2H); ^{13}C NMR (75 MHz, Acetone) δ 149.29, 147.59, 144.56, 134.88, 131.44, 130.88, 130.46, 125.20, 118.29, 30.39. IR (KBr): 3098, 1536, 1499, 1342, 1232, 1093, 1036, 914, 809, 771, 734, 692, 672 cm^{-1}. Anal. Calcd for $C_{14}H_{10}N_6O_4Se$: C, 41.50; H, 2.49; N, 20.74. Found: C, 41.32; H, 2.71; N, 20.47.

*4.7.10. 5-[(3,5-Dinitrobenzyl)selanyl]-1-(4-methoxyphenyl)-1H-tetrazole (**15j**)*

The reaction was carried out in THF. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 7:1). Yield: 33% (brownish solid); mp 131–132 °C (with decomposition). R_f (Hexane/EtOAc, 3:1) 0.12. ^{1}H NMR (500 MHz, Acetone) δ 8.83 (d, J = 2.1 Hz, 2H), 8.80 (t, J = 2.1 Hz, 1H), 7.48 (d, J = 9.0 Hz, 2H), 7.14 (d, J = 9.0 Hz, 2H), 4.91 (s, 2H), 3.89 (s, 3H); ^{13}C NMR (125 MHz, Acetone) δ 162.07, 149.27, 147.77, 144.63, 130.42, 127.39, 126.94, 118.26, 115.81, 56.11, 30.26. IR (KBr): 3089, 2923, 1608, 1591, 1537, 1511, 1446, 1342, 1257, 1173, 1020, 913, 839, 730, 672 cm^{-1}. Anal. Calcd for $C_{15}H_{12}N_6O_5Se$: C, 41.39; H, 2.78; N, 19.31. Found: C, 41.59; H, 2.41; N, 19.0.

*4.7.11. 1-(4-Bromophenyl)-5-[(3,5-dinitrobenzyl)selanyl]-1H-tetrazole (**15n**)*

The reaction was carried out in THF, 3,5-dinitrobenzyl iodide was used as an alkylating agent. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 8:1). Yield: 34% (white solid); mp 154–155 °C (with decomposition). R_f (Hexane/EtOAc, 3:1) 0.12. ^{1}H NMR (500 MHz, Acetone) δ 8.84 (d, J = 2.1 Hz, 2H), 8.80 (t, J = 2.1 Hz, 1H), 7.84 (d, J = 8.8 Hz, 2H), 7.58 (d, J = 8.8 Hz, 2H), 4.95 (s, 2H); ^{13}C NMR (125 MHz, Acetone) δ 148.38, 146.76, 143.59, 133.19, 133.11, 129.57, 126.28, 123.99, 117.40, 29.71. IR (KBr): 3104, 2923, 1530, 1496, 1361, 1340, 1176, 1073, 1007, 841, 731,

674 cm^{-1}. Anal. Calcd for $C_{14}H_9BrN_6O_4Se$: C, 34.73; H, 1.87; N, 17.36. Found: C, 34.93; H, 1.68; N, 17.01.

*4.7.12. 5-[(3,5-diaminobenzyl)sulfanyl]-1-phenyl-1H-tetrazole (**16g**)*

A yellow solution of 3,5-dinitrobenzyl chloride (0.217 g, 1 mmol) and tin (II) chloride dihydrate (1.8 g, 8 mmol) in 10 mL of ethanol was stirred at reflux for 10 min. Upon completion, the solvent was evaporated under reduced pressure, 5 mL of water was added to the residue and it was washed with EtOAc (2 × 10 mL). Then, 1-phenyl-1*H*-tetrazole-5-thiol **1g** (1.1 mmol) in 7 mL of THF was added to the aqueous layer followed by the addition of sodium carbonate to reach pH 8. The reaction mixture was stirred overnight at rt. Upon completion, the reaction mixture was washed with EtOAc (2 × 15 mL). The organic layer was separated, dried over Na_2SO_4 and evaporated. The product was purified by column chromatography (Mobile phase: EtOAc/Hexane/Et_3N, 50:25:1). Yield: 70% (light brown solid); R_f 0.47. ^1H NMR (500 MHz, $CDCl_3$) δ 7.56–7.48 (m, 5H), 6.13 (d, J = 2.1 Hz, 2H), 5.93 (t, J = 2.1 Hz, 1H), 4.43 (s, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 154.22, 147.94, 137.27, 133.74, 129.98, 129.67, 123.83, 106.48, 101.34, 37.88. Anal. Calcd for $C_{14}H_{14}N_6S$: C, 56.36; H, 4.37; N, 28.17; S, 10.75. Found: C, 56.16; H, 4.14; N, 27.94; S, 10.50.

*4.7.13. 5-[(3,5-Diaminobenzyl)sulfanyl]-1-(4-methoxyphenyl)-1H-tetrazole (**16j**) and 3-nitro-5-{[(1-(4-methoxyphenyl)-1H-tetrazol-5-yl)thio]methyl}aniline (**17j**)*

The yellow solution of 3,5-dinitrobenzyl chloride (0.217 g, 1 mmol) and tin (II) chloride dihydrate (1.58 g, 7 mmol) in 10 mL of ethanol was stirred at reflux for 10 min. Upon completion, the solvent was evaporated under reduced pressure, 5 mL of water was added to the residue and it was washed with EtOAc (2 × 10 mL). Then, 1-(4-methoxyphenyl)-1*H*-tetrazole-5-thiol **1j** (1.1 mmol) in 7 mL of THF was added to the aqueous layer followed by sodium carbonate to reach pH 8. The reaction mixture was stirred at rt overnight. Upon completion, the reaction mixture was washed with EtOAc (2 × 15 mL). The organic layer was separated, dried over Na_2SO_4 and evaporated. Products were separated and purified by column chromatography (Mobile phase: EtOAc/Hexane/Et_3N, 50:25:1).

*4.7.14. 5-[(3,5-Diaminobenzyl)sulfanyl]-1-(4-methoxyphenyl)-1H-tetrazole (**16j**)*

Yield: 40% (light brown solid); R_f (EtOAc/Hexane/Et_3N, 50:25:1) 0.44. ^1H NMR (300 MHz, $CDCl_3$) δ 7.41 (d, J = 8.9 Hz, 2H), 7.00 (d, J = 8.9 Hz, 2H), 6.12 (d, J = 2.0 Hz, 2H), 5.93 (t, J = 2.0 Hz, 1H), 4.40 (s,2H), 3.86 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 160.64, 154.33, 147.87, 137.29, 126.28, 125.49, 114.74, 106.42, 101.24, 55.62, 37.67. Anal Calcd for $C_{15}H_{16}N_6OS$: C, 54.86; H, 4.91; N, 25.59; S, 9.76. Found: C, 54.91; H, 5.3; N, 25.58; S, 9.49.

*4.7.15. 3-Nitro-5-{[(1-(4-methoxyphenyl)-1H-tetrazol-5-yl)thio]methyl}aniline (**17j**)*

Yield: 10% (yellow solid); R_f (EtOAc/Hexane/Et_3N, 50:25:1) 0.82. ^1H NMR (300 MHz, Acetone) δ 7.54–7.46 (m, 3H), 7.40 (t, J = 2.2 Hz, 1H), 7.19–7.11 (m, 3H), 5.43 (s, 2H), 4.58 (s, 2H), 3.88 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 161.91, 154.62, 150.80, 150.28, 140.03, 127.03, 126.89, 121.12, 115.71, 112.11, 108.03, 56.07, 37.02. Anal Calcd for $C_{15}H_{14}N_6O_3S$: C, 50.27; H, 3.94; N, 23.45; S, 8.95. Found: C, 50.06; H, 4.09; N, 23.14; S, 8.62.

*4.7.16. S-(3,5-dinitrobenzyl) ethanethioate (**18**)*

Thioacetic acid (0.21 g, 0.20 mL, 2.78 mmol) was added dropwise to a suspension of 3,5-dinitrobenzyl chloride (0.5 g, 2.31 mmol) and potassium carbonate (0.38 g, 2.78 mmol) in 10 mL of THF. The reaction mixture was stirred for 2 h at room temperature. The solvent was evaporated, and the crude product was dissolved in chloroform (20 mL) and washed with water (3 × 20 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 7:1). Yield: 84% (beige solid); mp 70–72 °C. ^1H NMR (300 MHz, $CDCl_3$) δ 8.92 (t, J = 2.1 Hz, 1H), 8.51 (d, J = 2.1 Hz, 2H), 4.25 (s, 2H), 2.40 (s, 3H); ^{13}C NMR (125 MHz, Acetone) δ 193.85, 148.49, 142.78, 129.06, 117.69, 32.33, 30.31. Anal. Calcd for $C_9H_8N_2O_5S$: C, 42.19; H, 3.15; N, 10.93; S, 12.51. Found: C, 42.34; H, 3.17; N, 10.81; S, 12.7.

*4.7.17. (3,5-dinitrobenzyl)(methyl)sulfane (**19**)*

Potassium carbonate (0.1 g, 0.72 mmol) and dimethyl sulfate (0.08 g, 0.63 mmol) were added to the solution of ethanethioate **18** (0.15 g, 0.58 mmol) in 7 mL CH_3OH, and the reaction was stirred for 30 min. The solvent was evaporated, and the crude product was crystallized from CH_3CN/H_2O. Yield: 75% (brown solid); mp 59–60 °C. ^1H NMR (300 MHz, Acetone) δ 8.83 (t, J = 2.2 Hz, 1H), 8.66 (d, J = 2.2 Hz, 2H), 4.06 (s, 2H), 2.05 (s, 3H); ^{13}C NMR (75 MHz, Acetone) δ 149.45, 145.11, 129.87, 117.84, 37.03, 14.56. Anal. Calcd for $C_8H_8N_2O_4S$: C, 42.1; H, 3.53; N, 12.27; S, 14.05. Found: C, 42.32; H, 3.90; N, 12.03; S, 14.0.

*4.7.18. Benzyl(3,5-dinitrobenzyl)sulfane (**20**)*

Potassium carbonate (0.1 g, 0.72 mmol) and benzyl bromide (0.11 g, 0.63 mmol) were added to the solution of ethanethioate **18** (0.15 g, 0.58 mmol) in 7 mL of CH_3OH, and the reaction was stirred for 30 min. The solvent was evaporated, and the crude product was crystallized from CH_3CN/H_2O. Yield: 48% (yellow solid); mp 105–107 °C. ^1H NMR (300 MHz, $CDCl_3$) δ 8.86 (t, J = 2.1 Hz, 1H), 8.37 (d, J = 2.1 Hz, 2H), 7.42–7.13 (m, 5H), 3.75 (s, 2H), 3.69 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 148.34, 143.36, 136.76, 128.94, 128.88, 128.73, 127.50, 117.34, 36.38, 34.70. Anal. Calcd for $C_{14}H_{12}N_2O_4S$: C, 55.25; H, 3.97; N, 9.21; S, 10.54. Found: C, 55.44; H, 4.15; N, 9.23; S, 10.47.

*4.8. General procedure for the synthesis of sulfanes **21–23***

3,5-Dinitrobenzyl chloride (0.125 g, 0.58 mmol) was added to a solution of thiol (0.64 mmol) and triethylamine (0.064 g, 0.64 mmol) in 7 mL of THF, and the reaction was stirred for 15 min. The reaction mixture was diluted with EtOAc (15 mL) and was washed with 2 N NaOH (2 × 15 mL) and water (1 × 15 mL). The organic layer was dried over Na_2SO_4. The solvent was evaporated, and the crude product was crystallized from CH_3CN/H_2O.

*4.8.1. (3,5-Dinitrobenzyl)(hexadecyl)sulfane (**21**)*

Yield: 84% (light gray solid); mp 46–48 °C. ^1H NMR (500 MHz, DMSO) δ 8.70 (s, 1H), 8.62 (s, 2H), 4.02 (s, 2H), 2.46–2.37 (m, 2H), 1.54–1.42 (m, 2H), 1.33–1.08 (m, 26H), 0.83 (t, J = 6.7 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 148.13, 144.62, 129.28, 117.06, 33.70, 33.58, 31.48, 30.68, 29.23, 29.20, 29.16, 29.14, 29.08, 28.90, 28.76, 28.69, 28.28, 27.94, 23.93, 22.28, 14.11. Anal. Calcd for $C_{23}H_{38}N_2O_4S$: C, 62.98; H, 8.73; N, 6.39; S, 7.31. Found: C, 62.64; H, 8.91; N, 6.56; S, 7.5.

*4.8.2. (3,5-Dinitrobenzyl)(phenyl)sulfane (**22**)*

Yield: 85% (yellow solid); mp 123–124 °C. ^1H NMR (500 MHz, Acetone) δ 8.78 (t, J = 2.1 Hz, 1H), 8.56 (d, J = 2.1 Hz, 2H), 7.40–7.36 (m, 2H), 7.31–7.20 (m, 3H), 4.52 (s, 2H); ^{13}C NMR (126 MHz, Acetone) δ 149.25, 144.30, 134.78, 131.75, 130.01, 129.77, 128.15, 117.96, 37.77. Anal. Calcd for $C_{13}H_{10}N_2O_4S$: C, 53.79; H, 3.47; N, 9.65; S, 11.05. Found: C, 54.07; H, 3.48; N, 9.73; S, 10.74.

*4.8.3. 2-[(3,5-Dinitrobenzyl)sulfanyl]pyridine-1-oxide (**23**)*

Yield: 96% (yellow solid); mp 180–182 °C (with decomposition). ^{1}H NMR (500 MHz, DMSO) δ 8.79 (d, J = 2.1 Hz, 2H), 8.71 (t, J = 2.1 Hz, 1H), 8.30 (d, J = 6.4, 1H), 7.54–7.49 (m, 1H), 7.36–7.30 (m, 1H), 7.23–7.18 (m, 1H), 4.58 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 149.50, 148.26, 141.72, 138.51, 129.43, 125.77, 122.45, 121.96, 117.83, 32.10. Anal. Calcd for $C_{12}H_9N_3O_5S$: C, 46.9; H, 2.95; N, 13.67; S, 10.44. Found: C, 46.96; H, 3.16; N, 13.92; S, 10.4.

*4.8.4. 3,5-Dinitrobenzyl thiocyanate (**24**)*

A mixture of 3,5-dinitrobenzyl chloride (0.5 g, 2.31 mmol) and potassium thiocyanate (3.45 mmol) in 8 mL of DMF was heated at 100 °C for 2 h. Upon completion, the reaction mixture was diluted with EtOAc (30 mL) and the organic layer was washed with water (2 × 30 mL). The organic solvent was dried over Na_2SO_4 and evaporated. The crude product was crystallized from CH_3CN/H_2O. Yield: 82% (white solid); mp 120–121 °C. ^{1}H NMR (500 MHz, Acetone) δ 8.95 (t, J = 2.1 Hz, 1H), 8.82 (d, J = 2.1 Hz, 2H), 4.74 (s, 2H); ^{13}C NMR (126 MHz, Acetone) δ 149.66, 141.82, 130.29, 119.37, 111.87, 36.31. Anal. Calcd for $C_8H_5N_3O_4S$: C, 40.17; H, 2.11; N, 17.57; S, 13.40. Found: C, 40.15; H, 2.06; N, 17.42; S, 13.54.

*4.8.5. 5-[(3,5-Dinitrobenzyl)sulfanyl]-1H-tetrazole (**25**)*

3,5-Dinitrobenzyl thiocyanate **24** (0.47 g, 1.95 mmol) was added to a suspension of sodium azide (0.14 g, 2.15 mmol) and triethylammonium chloride (0.3 g, 2.15 mmol) in 10 mL of toluene. The reaction mixture was heated at 105 °C for 2 h. Upon completion, the reaction mixture was diluted with 1% NaOH (30 mL) and washed with EtOAc (2 × 20 mL). The aqueous layer was acidified by HCl to pH = 2, and the precipitate was filtered and washed with water. Yield: 68% (white solid); mp 151–152 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.75 (t, J = 2.1 Hz, 1H), 8.71 (d, J = 2.1 Hz, 2H), 4.75 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 148.09, 142.46, 129.7, 126.96, 117.92, 34.39. Anal. Calcd for $C_8H_6N_6O_4S$: C, 34.04; H, 2.14; N, 29.78; S, 11.36. Found: C, 34.06; H, 2.10; N, 29.46; S, 11.34.

4.9. In vitro antimycobacterial assay

The in vitro antimycobacterial activity of the prepared compounds was evaluated against mycobacterial strains *M. 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), and clinically isolated *M. kansasii* 6509/96, *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 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 and 0.125 μmol/L. 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 *M. tuberculosis* and *M. avium*. Isoniazid (INH) was used as a prototype drug.

4.10. General procedure for cell proliferation/viability assays

4.10.1. Cell lines and cell culture

The HeLa cell line (human cervix epithelioid carcinoma) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% (v/v) non-essential amino acids and 10% (v/v) fetal bovine serum (FBS). HepG2 (human Caucasian hepatocyte carcinoma) and HuH7 (human hepatocellular carcinoma) cells were cultivated in DMEM medium supplemented with 10% FBS and 1% non-essential amino acids. The MDCKII Madin–Darby canine kidney cell line was maintained in DMEM medium with 5% FBS.

The cell lines were seeded into 96-well cultivation plates (30×10^3 cell per well) for 24 h and then treated with test compounds for 48 h at concentrations ranging from 1 μM to 30 μM. Stock solutions of all test compounds were prepared in DMSO at a concentration of 30 mM (the final DMSO concentration in the culture media was 0.1%).

4.10.2. Cell proliferation/viability assays

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Cell Proliferation Kit I, Roche) or MTS ([3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega, Hercules, CA, USA) assays were used to determine the effects of the test compounds on the viability of four mammalian cell lines. The assays are based on the conversion of tetrazolium salts into colored formazan products by mitochondrial reductases of the living cells and were quantified spectrophotometrically. All of the tests were performed as described in the manufacturer's protocols.

Briefly, the cells were incubated with the test compounds or vehicle alone (DMSO; 0.1%) under standard cultivation conditions for 48 h. After the treatment period, either 10 μL of MTT reagent (final concentration 0.5 mg/mL) or 20 μL of MTS reagent were added to each well containing phenol red-free medium. After 4 h, the MTT reagent was replaced by 100 μL of solubilization solution (10% sodium dodecyl sulfate (SDS) in 0.01 M HCl) and incubated overnight. In the case of the MTS reagent, the assay was directly evaluated after 4 h. The absorbance of the solubilized formazan crystals was measured at 570 or 490 nm by a plate reader (BioTec Synergy 2, Bio Tek, Winooski, VZ, USA). Cells treated with 10% SDS solution for 48 h were used as a toxic control and their absorbance was subtracted from each sample's absorbance as a background. The half maximal inhibitory concentration (IC_{50}) was determined using at least four concentration points. When IC_{50} was not reached, the data were presented as the percent of cell viability at a concentration of 30 μM (cell viability of control vehicle-treated samples was set as 100%). All of the experiments were repeated three times.

4.11. In vitro antibacterial and antifungal assays

For the assessment of in vitro antibacterial and antifungal activity of the synthesized substances, the broth microdilution method was used. 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), *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 medium buffered to pH 7.0 with 0.165 mol of 3-morpholinopropane-1-sulfonic acid. Compounds were dissolved in DMSO, and the final concentrations of the substances ranged from 500 to 0.488 μM. Drug-free controls were included. The MIC was defined as an 80% or

greater (for yeasts and yeasts-like organisms – IC_{80}) or 50% or greater (for molds – IC_{50}) reduction of the fungal growth compared with the control. 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 *S. aureus* H 5996/08 (MRSA), *Staphylococcus epidermidis* H 6966/08 (SE) and *Enterococcus faecalis* J 14365/08 (EF)) and 4 strains of Gram negative rods (*Escherichia coli* ATCC 8739 (EC), *Klebsiella pneumoniae* D 11750/08 (KP), *K. 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. Mueller Hinton broth was used as the culture medium for testing the bacteria. The MIC was defined as a 95% or greater reduction of growth compared with the control. 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 as a prototype drug for Gram negative rods.

Acknowledgments

This work was supported by the Czech Science Foundation project 14-08423S. G.K., J.N. and T.S. thank Charles University in Prague (SVV 260 062 and 260 063).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2014.05.069. These data include MOL files and InChiKeys of the most important compounds described in this article.

References

[1] World Health Organization, Global Tuberculosis Report 2012. http://www.who.int/tb/.
[2] A. Zumla, P. Nahid, S.T. Cole, Advances in the development of new tuberculosis drugs and treatment regimens, Nat. Rev. Drug. Discov. 12 (2013) 388–404.
[3] Beena, D.S. Rawat, Antituberculosis drug research: a critical overview, Med. Res. Rev. 33 (2013) 693–764.
[4] R.R. Kondreddi, J. Jiricek, S.P.S. Rao, S.B. Lakshminarayana, L.R. Camacho, R. Rao, M. Herve, P. Bifani, N.L. Ma, K. Kuhen, A. Goh, A.K. Chatterjee, T. Dick, T.T. Diagana, U.H. Manjunatha, P.W. Smith, Design, synthesis, and biological evaluation of indole-2-carboxamides: a promising class of antituberculosis agents, J. Med. Chem. 56 (2013) 8849–8859.
[5] O.K. Onajole, M. Pieroni, S.K. Tipparaju, S. Lun, J. Stec, G. Chen, H. Gunosewoyo, H.D. Guo, N.C. Ammerman, W.R. Bishai, A.P. Kozikowski, Preliminary structure-activity relationships and biological evaluation of novel antitubercular indolecarboxamide derivatives against drug-susceptible and drug-resistant Mycobacterium tuberculosis strains, J. Med. Chem. 56 (2013) 4093–4103.
[6] M. Kratky, J. Vinsova, V. Buchta, K. Horvati, S. Bosze, J. Stolarikova, New amino acid esters of salicylanilides active against MDR-TB and other microbes, Eur. J. Med. Chem. 45 (2010) 6106–6113.
[7] K. Pethe, P. Bifani, J.C. Jang, S. Kang, S. Park, S. Ahn, J. Jiricek, J.Y. Jung, H.K. Jeon, J. Cechetto, T. Christophe, H. Lee, M. Kempf, M. Jackson, A.J. Lenaerts, H. Pham, V. Jones, M.J. Seo, Y.M. Kim, M. Seo, J.J. Seo, D. Park, Y. Ko, I. Choi, R. Kim, S.Y. Kim, S. Lim, S.A. Yim, J. Nam, H. Kang, H. Kwon, C.T. Oh, Y. Cho, Y. Jang, J. Kim, A. Chua, B.H. Tan, M.B. Nanjundappa, S.P.S. Rao, W.S. Barnes, R. Wintjens, J.R. Walker, S. Alonso, S. Lee, S. Oh, T. Oh, U. Nehrbass, S.J. Han, Z. No, J. Lee, P. Brodin, S.N. Cho, K. Nam, Discovery of Q203, a potent clinical candidate for the treatment of tuberculosis, Nat. Med. 19 (2013) 1157–1160.
[8] S. Chopra, G.A. Koolpe, A.A. Tambo-ong, K.N. Matsuyama, K.J. Ryan, T.B. Tran, R.S. Doppalapudi, E.S. Riccio, L.V. Iyer, C.E. Green, B.J. Wan, S.G. Franzblau, P.B. Madrid, Discovery and optimization of benzotriazine di-N-oxides targeting replicating and nonreplicating mycobacterium tuberculosis, J. Med. Chem. 55 (2012) 6047–6060.
[9] M.G. Kale, A. Raichurkar, P.S. Hameed, D. Waterson, D. McKinney, M.R. Manjunatha, U. Kranthi, K. Koushik, L.K. Jena, V. Shinde, S. Rudrapatna, S. Barde, V. Humnabadkar, P. Madhavapeddi, H. Basavarajappa, A. Ghosh, V.K. Ramya, S. Guptha, S. Sharma, P. Vachaspati, K.N.M. Kumar, J. Giridhar, J. Reddy, V. Panduga, S. Ganguly, V. Ahuja, S. Gaonkar, C.N.N. Kumar, D. Ogg, J.A. Tucker, P.A. Boriack-Sjodin, S.M. de Sousa, V.K. Sambandamurthy, S.R. Ghorpade, Thiazolopyridine ureas as novel antitubercular agents acting through inhibition of DNA gyrase B, J. Med. Chem. 56 (2013) 8834–8848.
[10] D. Awasthi, K. Kumar, S.E. Knudson, R.A. Slayden, I. Ojima, SAR studies on trisubstituted benzimidazoles as inhibitors of Mtb FtsZ for the development of novel antitubercular agents, J. Med. Chem. 56 (2013) 9756–9770.
[11] A.J. Lenaerts, V. Gruppo, K.S. Marietta, C.M. Johnson, D.K. Driscoll, N.M. Tompkins, J.D. Rose, R.C. Reynolds, I.M. Orme, Preclinical testing of the nitroimidazopyran PA-824 for activity against mycobacterium tuberculosis in a series of in vitro and in vivo models, Antimicrob. Agents Chemother. 49 (2005) 2294–2301.
[12] M. Matsumoto, H. Hashizume, T. Tomishige, M. Kawasaki, H. Tsubouchi, H. Sasaki, Y. Shimokawa, M. Komatsu, OPC-67683, a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis in vitro and in mice, Plos Med. 3 (2006) 2131–2144.
[13] Z.K. Ma, C. Lienhardt, H. McIlleron, A.J. Nunn, X.X. Wang, Global tuberculosis drug development pipeline: the need and the reality, Lancet 375 (2010) 2100–2109.
[14] A. Matteelli, R. Centis, L. D'Ambrosio, G.B. Migliori, Multidrug-resistant tuberculosis today, Bull. World Health Organ. 90 (2012) 78.
[15] V. Makarov, G. Manina, K. Mikusova, U. Mollmann, O. Ryabova, B. Saint-Joanis, N. Dhar, M.R. Pasca, S. Buroni, A.P. Lucarelli, A. Milano, E. De Rossi, M. Belanova, A. Bobovska, P. Dianiskova, J. Kordulakova, C. Sala, E. Fullam, P. Schneider, J.D. McKinney, P. Brodin, T. Christophe, S. Waddell, P. Butcher, J. Albrethsen, I. Rosenkrands, R. Brosch, V. Nandi, S. Bharath, S. Gaonkar, R.K. Shandil, V. Balasubramanian, T. Balganesh, S. Tyagi, J. Grosset, G. Riccardi, S.T. Cole, Benzothiazinones kill mycobacterium tuberculosis by blocking arabinan synthesis, Science 324 (2009) 801–804.
[16] C. Trefzer, H. Skovierova, S. Buroni, A. Bobovska, S. Nenci, E. Molteni, F. Pojer, M.R. Pasca, V. Makarov, S.T. Cole, G. Riccardi, K. Mikusova, K. Johnsson, Benzothiazinones are suicide inhibitors of mycobacterial decaprenylphosphoryl-beta-d-ribofuranose 2'-oxidase DprE1, J. Am. Chem. Soc. 134 (2012) 912–915.
[17] T. Christophe, M. Jackson, H.K. Jeon, D. Fenistein, M. Contreras-Dominguez, J. Kim, A. Genovesio, J.P. Carralot, F. Ewann, E.H. Kim, S.Y. Lee, S. Kang, M.J. Seo, E.J. Park, H. Skovierova, H. Pham, G. Riccardi, J.Y. Nam, L. Marsollier, M. Kempf, M.L. Joly-Guillou, T. Oh, W.K. Shin, Z. No, U. Nehrbass, R. Brosch, S.T. Cole, P. Brodin, High content screening identifies decaprenyl-phosphoribose 2' epimerase as a target for intracellular antimycobacterial inhibitors, PLoS Pathog. 5 (2009) e1000645.
[18] J. Koci, V. Klimesova, K. Waisser, J. Kaustova, H.M. Dahse, U. Mollmann, Heterocyclic benzazole derivatives with antimycobacterial in vitro activity, Bioorganic Med. Chem. Lett. 12 (2002) 3275–3278.
[19] V. Klimesova, J. Koci, K. Waisser, J. Kaustova, U. Mollmann, Preparation and in vitro evaluation of benzylsulfanyl benzoxazole derivatives as potential antituberculosis agents, Eur. J. Med. Chem. 44 (2009) 2286–2293.
[20] V. Klimesova, J. Koci, K. Palat, J. Stolarikova, H.M. Dahse, U. Mollmann, Structure-activity relationships of 2-benzylsulfanylbenzothiazoles: synthesis and selective antimycobacterial properties, Med. Chem. 8 (2012) 281–292.
[21] V. Klimesova, J. Koci, M. Pour, J. Stachel, K. Waisser, J. Kaustova, Synthesis and preliminary evaluation of benzimidazole derivatives as antimicrobial agents, Eur. J. Med. Chem. 37 (2002) 409–418.
[22] V. Klimesova, M. Svoboda, K. Waisser, M. Pour, J. Kaustova, New pyridine derivatives as potential antimicrobial agents, Farmaco 54 (1999) 666–672.
[23] P. Herzigova, V. Klimesova, K. Palat, J. Kaustova, H.M. Dahse, U. Mollmann, Preparation and in-vitro evaluation of 4-Benzylsulfanylpyridine-2-carbohydrazides as potential antituberculosis agents, Arch. Pharm. 342 (2009) 394–404.
[24] J. Roh, K. Vavrova, A. Hrabalek, Synthesis and functionalization of 5-substituted tetrazoles, Eur. J. Org. Chem. (2012) 6101–6118.
[25] L.V. Myznikov, A. Hrabalek, G.I. Koldobskii, Medicinal preparations in the series of tetrazoles, Khimia Geterotsiklicheskikh Soedin. (2007) 3–13.
[26] H.W. Altland, Smiles rearrangement of 2-tetrazolylthio-3-aminopyridines, J. Org. Chem. 41 (1976) 3395–3399.
[27] U.N. Dmitrieva, Y.E. Zevatskii, S.M. Ramsh, T.V. Artamonova, L.V. Myznikov, Synthesis of 5-(4-methylphenyl)sulfinyl- and 5-(4-methylphenyl)sulfonyl-1-phenyl-1H-tetrazoles, Chem. Heterocycl. Compd. 48 (2012) 382–383.
[28] M.A. Goltsberg, G.I. Koldobskii, Simple method for the preparation of 5-alkyoxy(aryloxy)-1-phenyltetrazoles, Zhurnal Organicheskoi Khimii 31 (1995) 1726–1727.
[29] J.G. Fernandez-Bolanos, O. Lopez, V. Ulgar, I. Maya, J. Fuentes, Synthesis of O-unprotected glycosyl selenoureas. A new access to bicyclic sugar isoureas, Tetrahedron Lett. 45 (2004) 4081–4084.
[30] G. Karabanovich, J. Roh, Z. Padelkova, Z. Novak, K. Vavrova, A. Hrabalek, One-pot synthesis of 1-substituted-5-alkylselanyl-1H-tetrazoles from iso-selenocyanates: unexpected formation of N-alkyl-N-arylcyanamides and (Z)-Se-alkyl-N-cyano-N,N'-diarylisoselenoureas, Tetrahedron 69 (2013) 8798–8808.
[31] C.C. Han, R. Balakumar, Mild and efficient methods for the conversion of benzylic bromides to benzylic thiols, Tetrahedron Lett. 47 (2006) 8255–8258.
[32] R. Welz, S. Muller, 5-(Benzylmercapto)-1H-tetrazole as activator for 2'-O-TBDMS phosphoramidite building blocks in RNA synthesis, Tetrahedron Lett. 43 (2002) 795–797.

Attachment **III**.

Karabanovich, G.; Roh, J.; Soukup, O.; Pávková, I.; Pasdiorová, M.; Tambor, V.; Stolaříková, J.; Vejsová, M.; Vávrová, K.; Klimešová, V.; Hrabálek, A. Tetrazole Regioisomers in the Development of Nitro Group-Containing Antitubercular Agents. *Med. Chem. Commun*. **2014**. DOI: 10.1039/c4md00301b

MedChemComm

CONCISE ARTICLE

View Article Online
View Journal

Published on 25 September 2014. Downloaded by Charles University in Prague on 24/10/2014 18:32:27.

Cite this: DOI: 10.1039/c4md00301b

Tetrazole regioisomers in the development of nitro group-containing antitubercular agents†

Galina Karabanovich,[a] Jaroslav Roh,*[a] Ondřej Soukup,[b] Ivona Pávková,[c] Markéta Pasdiorová,[b] Vojtěch Tambor,[b] Jiřina Stolaříková,[d] Marcela Vejsová,[a] Kateřina Vávrová,[a] Věra Klimešová[a] and Alexandr Hrabálek[a]

Received 10th July 2014
Accepted 23rd September 2014

DOI: 10.1039/c4md00301b

www.rsc.org/medchemcomm

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^{-1}) 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, the 3,5-dinitrobenzyl moiety *per se* is not the carrier of mutagenicity. These findings encourage further optimization of the 2-alkyl chain to improve the 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 of 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*) have become the biggest challenges 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.

This establishes the need for the discovery of new highly efficient antimycobacterial agents (for recent reviews, see ref. 2–5). 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] 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 concentrations (MICs) from 2 to 8 μM against *M. tb* (Fig. 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 (Fig. 1). The antimycobacterial activities of these compounds confirmed that

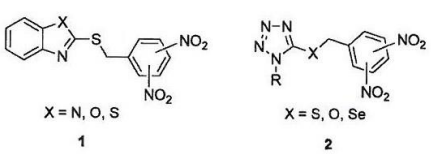


Fig. 1 Dinitrobenzyl-bearing benzazole and tetrazole derivatives with high and selective antimycobacterial activity.

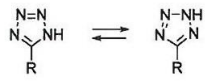
[a]Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Heyrovského 1203, 50005 Hradec Králové, Czech Republic. E-mail: jaroslav.roh@faf.cuni.cz
[b]Biomedical Research Center, University Hospital Hradec Králové, Sokolská 581, 50005 Hradec Králové, Czech Republic
[c]Department of Molecular Pathology and Biology, Faculty of Military Health Sciences, University of Defence, Třebešská 1575, 50005 Hradec Králové, Czech Republic
[d]Regional Institute of Public Health, Department of Bacteriology and Mycology, Partyzánské náměstí 7, 70200 Ostrava, Czech Republic

† 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/c4md00301b

Published on 25 September 2014. Downloaded by Charles University in Prague on 24/10/2014 18:32:27.

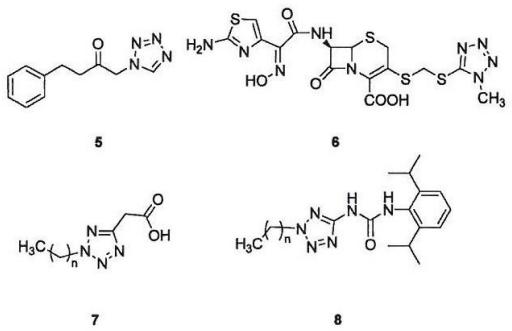
the presence of two nitro groups on the benzylsulfanyl moiety is vital for increased antimycobacterial activity. 3,5-Dinitro substituted tetrazole derivatives of series **2** exhibited higher activities compared to the 2,4-dinitro analogs, with MIC values of 1 μM (0.36–0.44 μg mL^{-1}) against *M. tb*, *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, the 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 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**, Fig. 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**, Fig. 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



3 **4**

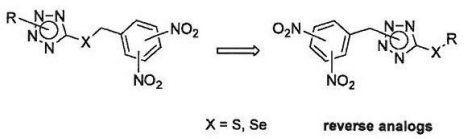
Fig. 2 1*H*- and 2*H*-tautomers of 5-substituted tetrazoles.



5 **6**

7 **8**

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



X = S, Se reverse analogs

Fig. 4 General structure of the studied compounds.

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 (Fig. 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, 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 (Fig. 4).

Results and discussion

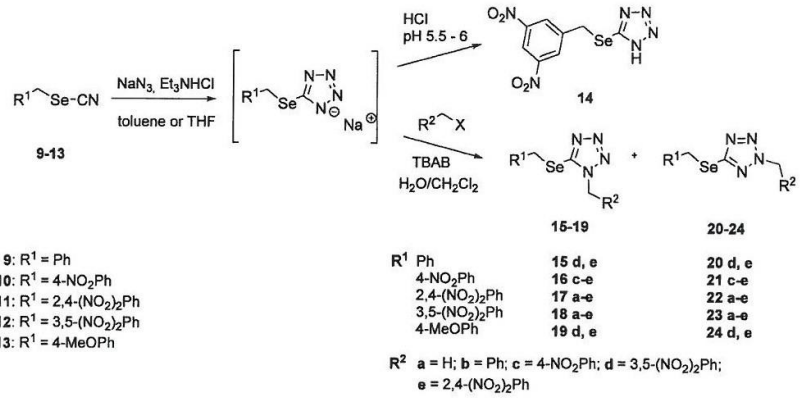
Chemistry

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. 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.

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

This journal is © The Royal Society of Chemistry 2014

Published on 25 September 2014. Downloaded by Charles University in Prague on 24/10/2014 18:32:27.



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

to the 1-isomers (Table 1). Correlations between methylene hydrogens in 1,5-isomer **19d** in 1D NOESY experiments and the absence of such correlations in 2,5-isomer **24d** confirmed their regioisomeric identities (see ESI†). 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 on 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]

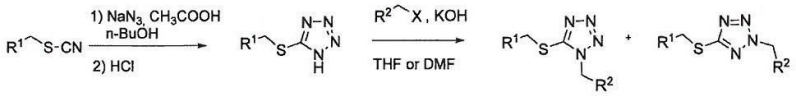
The synthetic procedure for 1-alkyl-5-(alkylsulfanyl)-1*H*-tetrazoles (**33–36**) and 2-alkyl-5-(alkylsulfanyl)-2*H*-tetrazoles (**37–40**) is shown in Scheme 2. Unlike selenium analogs, 5-(alkylsulfanyl)-1*H*-tetrazoles (**29–32**) were isolated in moderate yields (50–68%). Alkylation of the tetrazoles (**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 their 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

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[a]

			1-Isomer		2-Isomer	
1-/2-Isomers	R^1-CH_2Se	R^2-CH_2N	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)

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

Published on 25 September 2014. Downloaded by Charles University in Prague on 24/10/2014 18:32:27.



25-28 **29-32** **33-36** **37-40**

25: R^1 = Ph
26: R^1 = 4-NO_2Ph
27: R^1 = 3,5-$(NO_2)_2$Ph
28: R^1 = 2,4-$(NO_2)_2$Ph

29: R^1 = Ph
30: R^1 = 4-NO_2Ph
31: R^1 = 3,5-$(NO_2)_2$Ph
32: R^1 = 2,4-$(NO_2)_2$Ph

R^1	**33**	**37**
Ph	**33 d**	**37 d**
4-NO_2Ph	**34 c, d**	**38 c, d**
2,4-$(NO_2)_2$Ph	**35 a-d**	**39 a-d**
3,5-$(NO_2)_2$Ph	**36 a-d, f, g**	**40 a-d, f, g**

R^2 **a** = H; **b** = Ph; **c** = 4-NO_2Ph; **d** = 3,5-$(NO_2)_2$Ph; **f** = 4-ClPh; **g** = 3,4-Cl_2Ph

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

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-Isomer		2-Isomer	
1-/2-Isomers	R^1-CH_2S	R^2-CH_2N	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)

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 ESI†).

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 the Sula semisynthetic medium. Minimum inhibitory concentrations (MICs), *i.e.*, the lowest concentrations that inhibit 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 MICs 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]-1*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]-2*H*-tetrazoles (**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 of 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

Published on 25 September 2014. Downloaded by Charles University in Prague on 24/10/2014 18:32:27.

Table 3 *In vitro* antimycobacterial activities of selanyltetrazoles **15–24** expressed as MICs (μM)[a]

				M. tb My 331/88	*M. avium* My 330/88	*M. kansasii* My 235/80	*M. kansasii* 6509/96
		R^1	R^2	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

[a] n.d. not determined.

type of substituent on the tetrazole than 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**, and **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) substituent (**18c**, **36c**) on tetrazole. The MIC values of benzylselanyl/3,5-dinitrobenzyl and 3,5-dinitrobenzylselanyl/benzyl reverse analog pairs **15d**/**18b** and **20d**/**23b** and their 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 3,5-dinitrophenyl fragments, such 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 (Tables S1 and S2, see ESI†).

Published on 25 September 2014. Downloaded by Charles University in Prague on 24/10/2014 18:32:27.

Table 4 *In vitro* antimycobacterial activities of sulfanyltetrazoles **33–40** expressed as MICs (μM)[a]

				M. tb My 331/88	*M. avium* My 330/88	*M. kansasii* My 235/80	*M. kansasii* 6509/96
		R^1	R^2	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

[a] n.d. not determined.

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 5).

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} value 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 chlorine atoms, as observed in compounds **36g** and **40g**.

Table 5 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[a]

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

[a] n.d. not determined.

This journal is © The Royal Society of Chemistry 2014

Published on 25 September 2014. Downloaded by Charles University in Prague on 24/10/2014 18:32:27.

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 mutation in both strains. This indicates that the 3,5-dinitrobenzyl moiety is not generally connected with frame shift or base-exchange mutagenicity (see ESI†).

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 to 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 the 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.

Acknowledgements

This work was supported by the Czech Science Foundation project 14-08423S and MH CZ – DRO (UHHK, 00179906). G.K. thanks Charles University in Prague (SVV 260 062).

Notes and references

1 World Health Organization, *Global tuberculosis report*, 2012, http://www.who.int/tb.
2 A. Zumla, P. Nahid and S. T. Cole, *Nat. Rev. Drug Discovery*, 2013, **12**, 388–404.
3 Beena and D. S. Rawat, *Med. Res. Rev.*, 2013, **33**, 693–764.
4 M. Yan and S. T. Ma, *ChemMedChem*, 2012, **7**, 2063–2075.
5 C. Lienhardt, M. Raviglione, M. Spigelman, R. Hafner, E. Jaramillo, M. Hoelscher, A. Zumla and J. Gheuens, *J. Infect. Dis.*, 2012, **205**, S241–S249.
6 A. J. Lenaerts, V. Gruppo, K. S. Marietta, C. M. Johnson, D. K. Driscoll, N. M. Tompkins, J. D. Rose, R. C. Reynolds and I. M. Orme, *Antimicrob. Agents Chemother.*, 2005, **49**, 2294–2301.
7 M. Matsumoto, H. Hashizume, T. Tomishige, M. Kawasaki, H. Tsubouchi, H. Sasaki, Y. Shimokawa and M. Komatsu, *PLoS Med.*, 2006, **3**, 2131–2144.
8 M. T. Gler, V. Skripconoka, E. Sanchez-Garavito, H. P. Xiao, J. L. Cabrera-Rivero, D. E. Vargas-Vasquez, M. Q. Gao, M. Awad, S. K. Park, T. S. Shim, G. Y. Suh, M. Danilovits, H. Ogata, A. Kurve, J. Chang, K. Suzuki, T. Tupasi, W. J. Koh, B. Seaworth, L. J. Geiter and C. D. Wells, *N. Engl. J. Med.*, 2012, **366**, 2151–2160.
9 T. Christophe, M. Jackson, H. K. Jeon, D. Fenistein, M. Contreras-Dominguez, J. Kim, A. Genovesio, J. P. Carralot, F. Ewann, E. H. Kim, S. Y. Lee, S. Kang, M. J. Seo, E. J. Park, H. Skovierova, H. Pham, G. Riccardi, J. Y. Nam, L. Marsollier, M. Kempf, M. L. Joly-Guillou, T. Oh, W. K. Shin, Z. No, U. Nehrbass, R. Brosch, S. T. Cole and P. Brodin, *PLoS Pathog.*, 2009, **5**, e1000645.
10 V. Makarov, G. Manina, K. Mikusova, U. Mollmann, O. Ryabova, B. Saint-Joanis, N. Dhar, M. R. Pasca, S. Buroni, A. P. Lucarelli, A. Milano, E. De Rossi, M. Belanova, A. Bobovska, P. Dianiskova, J. Kordulakova, C. Sala, E. Fullam, P. Schneider, J. D. McKinney, P. Brodin, T. Christophe, S. Waddell, P. Butcher, J. Albrethsen, I. Rosenkrands, R. Brosch, V. Nandi, S. Bharath, S. Gaonkar, R. K. Shandil, V. Balasubramanian, T. Balganesh, S. Tyagi, J. Grosset, G. Riccardi and S. T. Cole, *Science*, 2009, **324**, 801–804.
11 C. Trefzer, H. Skovierova, S. Buroni, A. Bobovska, S. Nenci, E. Molteni, F. Pojer, M. R. Pasca, V. Makarov, S. T. Cole, G. Riccardi, K. Mikusova and K. Johnsson, *J. Am. Chem. Soc.*, 2012, **134**, 912–915.
12 V. Makarov, B. Lechartier, M. Zhang, J. Neres, A. M. van der Sar, S. A. Raadsen, R. C. Hartkoorn, O. B. Ryabova, A. Vocat, L. A. Decosterd, N. Widmer, T. Buclin, W. Bitter, K. Andries,

Published on 25 September 2014. Downloaded by Charles University in Prague on 24/10/2014 18:32:27.

F. Pojer, P. J. Dyson and S. T. Cole, *EMBO Mol. Med.*, 2014, **6**, 372–383.
13 Z. Kazimierczuk, M. Andrzejewska, J. Kaustova and V. Klimesova, *Eur. J. Med. Chem.*, 2005, **40**, 203–208.
14 V. Klimesova, J. Koci, M. Pour, J. Stachel, K. Waisser and J. Kaustova, *Eur. J. Med. Chem.*, 2002, **37**, 409–418.
15 J. Koci, V. Klimesova, K. Waisser, J. Kaustova, H. M. Dahse and U. Mollmann, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 3275–3278.
16 V. Klimesova, J. Koci, K. Palat, J. Stolarikova, H. M. Dahse and U. Mollmann, *Med. Chem.*, 2012, **8**, 281–292.
17 G. Karabanovich, J. Roh, T. Smutný, J. Němeček, P. Vicherek, J. Stolaříková, M. Vejsová, I. Dufková, K. Vávrová, P. Pávek, V. Klimešová and A. Hrabálek, *Eur. J. Med. Chem.*, 2014, **82**, 324–340.
18 G. I. Koldobskii and R. B. Kharbash, *Russ. J. Org. Chem.*, 2003, **39**, 453–470.
19 G. I. Koldobskii, *Russ. J. Org. Chem.*, 2006, **42**, 469–486.
20 J. Roh, K. Vavrova and A. Hrabalek, *Eur. J. Org. Chem.*, 2012, 6101–6118.
21 G. Ortar, M. G. Cascio, A. S. Moriello, M. Camalli, E. Morera, M. Nalli and V. Di Marzo, *Eur. J. Med. Chem.*, 2008, **43**, 62–72.
22 M. Sabbah, F. Fontaine, L. Grand, M. Boukraa, M. L. Efrit, A. Doutheau, L. Soulere and Y. Queneau, *Bioorg. Med. Chem.*, 2012, **20**, 4727–4736.
23 J. Garfunkle, C. Ezzili, T. J. Rayl, D. G. Hochstatter, I. Hwang and D. L. Boger, *J. Med. Chem.*, 2008, **51**, 4392–4403.
24 G. Roman, M. N. Rahman, D. Vukomanovic, Z. C. Jia, K. Nakatsu and W. A. Szarek, *Chem. Biol. Drug Des.*, 2010, **75**, 68–90.
25 M. Kume, T. Kubota, Y. Kimura, H. Nakashimizu and K. Motokawa, *J. Pharm. Soc. Jpn.*, 1992, **112**, 622–637.
26 U. Muh, M. Schuster, R. Heim, A. Singh, E. R. Olson and E. P. Greenberg, *Antimicrob. Agents Chemother.*, 2006, **50**, 3674–3679.
27 A. D. White, M. W. Creswell, A. W. Chucholowski, C. J. Blankley, M. W. Wilson, R. F. Bousley, A. D. Essenburg, K. L. Hamelehle, B. R. Krause, R. L. Stanfield, M. A. Dominick and M. Neub, *J. Med. Chem.*, 1996, **39**, 4382–4395.
28 H. Ozkan, S. Yavuz, A. Disli, Y. Yildirir and L. Turker, *Heteroat. Chem.*, 2007, **18**, 255–258.
29 A. Disli and M. Salman, *Russ. J. Org. Chem.*, 2009, **45**, 151–153.
30 G. Karabanovich, J. Roh, Z. Padelkova, Z. Novak, K. Vavrova and A. Hrabalek, *Tetrahedron*, 2013, **69**, 8798–8808.
31 R. E. Trifonov and V. A. Ostrovskii, *Russ. J. Org. Chem.*, 2006, **42**, 1585–1605.
32 N. N. Sveshnikov and J. H. Nelson, *Magn. Reson. Chem.*, 1997, **35**, 209–212.
33 U. Manjunatha, H. I. Boshoff and C. E. Barry, *Commun. Integr. Biol.*, 2009, **2**, 215–218.

This journal is © The Royal Society of Chemistry 2014

Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications.
This journal is © The Royal Society of Chemistry 2014

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

Galina Karabanovich,[a] Jaroslav Roh,[a]* Ondřej Soukup,[b] Ivona Pávková,[c] Markéta Pasdiorová,[b] Vojtěch Tambor,[b] Jiřina Stolaříková,[d] Marcela Vejsová,[a] Kateřina Vávrová,[a] Věra Klimešová,[a] and Alexandr Hrabálek[a]

[a] *Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Heyrovského 1203, 50005 Hradec Králové, Czech Republic, e-mail: jaroslav.roh@faf.cuni.cz*

[b] *Biomedical Research Center, University Hospital Hradec Králové, Sokolská 581, 50005 Hradec Králové, Czech Republic*

[c] *Department of Molecular Pathology and Biology, Faculty of Military Health Sciences, University of Defence, Třebešská 1575, 50005 Hradec Králové, Czech Republic*

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

CONTENT

Table S1 2

Table S2 3

Experimental section 4

Chemistry 4

NOESY experiments 27

In vitro antimycobacterial assay 35

In vitro antibacterial and antifungal assays 36

In vitro cell proliferation/viability assessment 37

Ames Fluctuation test 38

References 40

Table S1. In vitro antibacterial activities of compounds **18b**, **23b**, **36b**, **40b** and **40d** expressed as MIC (μM).

Strains		Studied compounds – MIC (IC_{95}; μM)						
		18b	**23b**	**36b**	**40b**	**40d**	**VAN**	**GEN**
SA	24 h	>500	>500	>500	>500	>500	0.35	-
	48 h	>500	>500	>500	>500	>500	-	-
MRSA	24 h	>500	>500	>500	>500	>500	0.35	-
	48 h	>500	>500	>500	>500	>500	-	-
SE	24 h	>500	>500	>500	>500	>500	0.35	-
	48 h	>500	>500	>500	>500	>500	-	-
EF	24 h	>500	>500	>500	>500	>500	0.7	-
	48 h	>500	>500	>500	>500	>500	-	-
EC	24 h	>500	>500	>500	>500	>500	-	0.26
	48 h	>500	>500	>500	>500	>500	-	-
KP	24 h	>500	>500	>500	>500	>500	-	0.26
	48 h	>500	>500	>500	>500	>500	-	-
KP-E	24 h	>500	>500	>500	>500	>500	-	0.26
	48 h	>500	>500	>500	>500	>500	-	-
PA	24 h	>500	>500	>500	>500	>500	-	1
	48 h	>500	>500	>500	>500	>500	-	-

SA - *Staphylococcus aureus* ATCC 6538; **MRSA** - methicillin resistant *Staphylococcus aureus* H 5996/08; **SE** - *Staphylococcus epidermidis* H 6966/08; **EF** - *Enterococcus faecalis* J 14365/08; **EC** - *Escherichia coli* ATCC 8739; **KP** - *Klebsiella pneumoniae* D 11750/08; **KP-E** - ESBL positive *Klebsiella pneumoniae* J 14368/08; **PA** - *Pseudomonas aeruginosa* ATCC 9027; **VAN** – Vancomycin; **GEN** – Gentamicin

Table S2. In vitro antifungal activities of compounds **18b**, **23b**, **36b**, **40b** and **40d** expressed as MIC (μM).

Strains		Studied compounds - MIC* (IC_{80}/IC_{50}; μM)						
		18b	**23b**	**36b**	**40b**	**40d**	**FLU**	**AMB**
CA	24 h	>500	>500	>500	>500	>125	0.82	0.54
	48 h	>500	>500	>500	>500	>125	-	-
CT	24 h	>500	>500	>500	>500	>125	1.6	0.54
	48 h	>500	>500	>500	>500	>125	-	-
CK	24 h	>500	>500	>500	>500	>125	105	1
	48 h	>500	>500	>500	>500	>125	-	-
CG	24 h	>500	>500	>500	>500	>125	26	0.54
	48 h	>500	>500	>500	>500	>125	-	-
TA	24 h	>500	>500	>500	>500	>125	210	0.27
	48 h	>500	>500	>500	>500	>125	-	-
AF	24 h	>500	>500	>500	>500	>125	>500	0.54
	48 h	>500	>500	>500	>500	>125	-	-
AC	24 h	>500	>500	>500	>500	>125	>500	1
	48 h	>500	>500	>500	>500	>125	-	-
TM	72 h	>500	>500	>500	>500	>125	105	0.54
	120 h	>500	>500	>500	>500	>125	-	-

*IC_{50} for **AF**, **AC**, **TM**; IC_{80} for **CA**, **CT**, **CK**, **CG**, **TA**

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** – Fluconazole; **AMB** – Amphotericin B

Experimental section

Chemistry

The structures of the prepared compounds were identified using ^{1}H-NMR and ^{13}C-NMR spectroscopy. The purity of all compounds reported was determined by elemental analysis or HPLC-HRMS. All chemicals were obtained from Sigma-Aldrich (Schnelldorf, Germany) and were used without further purification. TLC was performed on Merck aluminum plates with silica gel 60 F_{254}. Merck Kieselgel 60 (0.040-0.063 mm) was used for column chromatography. Melting points were recorded with a Büchi B-545 apparatus (BUCHI Labortechnik AG, Flawil, Switzerland) and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded by 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 carried out on an Automatic Microanalyser EA1110CE (Fisons Instruments S.p.A., Milano, Italy). HPLC-HRMS (ESI+) experiments were performed using the UltiMate3000 Rapid Separation Liquid Chromatography System with a Q-Exactive Plus Mass Spectrometer (Thermo Scientific, Bremen, Germany).

Although we did not observe any explosive or shock-sensitive properties of studied compounds, proper safety precautions should be applied while synthesizing and handling them!

Synthesis of alkyl selenocyanates (9-13):

A suspension of alkyl halide (10 mmol) and potassium selenocyanate (11 mmol) in 7 mL DMF or THF was stirred until completion under the conditions given below. The reaction mixture was then diluted with EtOAc (10 mL) and the organic layer was washed with 1%

aqueous NaOH (2 × 10 mL) and H_2O (1 × 10 mL). The organic solvent was dried over Na_2SO_4. If necessary, the crude product was purified by column chromatography (Mobile phase: Hexane/EtOAc).

Benzyl selenocyanate (9): Benzyl bromide was used as a substrate. The reaction mixture was stirred in THF at room temperature (rt) for 1 h. Yield: 84% as a malodorous white solid; mp 68-69 °C (lit.[1] mp 68-70 °C). ^{1}H NMR (300 MHz, acetone) δ 7.53 – 7.24 (m, 5H), 4.43 (s, 2H); ^{13}C NMR (75 MHz, Acetone) δ 138.62, 129.80, 129.57, 128.91, 103.04, 33.04.

4-Nitrobenzyl selenocyanate (10):[2] 4-Nitrobenzyl chloride was used as a substrate. The reaction mixture was heated in DMF at 80 °C for 8 h. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 4:1). Yield: 66% as a yellow solid; mp 121-122 °C (lit.[3] mp 122 °C). ^{1}H NMR (300 MHz, acetone) δ 8.26 (d, J = 8.8 Hz, 2H), 7.73 (d, J = 8.8 Hz, 2H), 4.55 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 148.48, 146.63, 131.02, 124.64, 102.75, 31.48.

2,4-Dinitrobenzyl selenocyanate (11): 2,4-Dinitrobenzyl chloride was used as a substrate. The reaction mixture was heated in THF at 60 °C for 10 h. Yield: 90% as a yellowish solid; mp 69-70 °C (lit.[4] mp 70-72 °C) . ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.06 (d, J = 2.4 Hz, 1H), 8.55 (dd, J = 8.5, 2.4 Hz, 1H), 7.76 (d, J = 8.5 Hz, 1H), 4.51 (s, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 148.01, 146.60, 139.55, 133.32, 128.70, 121.70, 101.46, 29.29.

3,5-Dinitrobenzyl selenocyanate (12): 3,5-Dinitrobenzyl chloride was used as a substrate. The reaction mixture was heated in DMF at 80 °C for 5 h. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 10:1). Yield: 82% as a yellow solid; mp 128-129 °C. ^{1}H NMR (300 MHz, acetone) δ 8.90 (t, J = 2.1 Hz, 1H), 8.77 (d, J = 2.1 Hz, 2H), 4.75 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 149.54, 143.87, 130.02, 118.85, 102.67, 30.54. Anal. Calcd for $C_8H_5N_3O_4Se$: C, 33.58; H, 1.76; N, 14.64. Found: C, 33.62; H, 1.97; N, 14.55.

4-Methoxybenzyl selenocyanate (13): 4-Methoxybenzyl chloride was used as a substrate. The reaction mixture was stirred in THF at rt for 10 h. Yield: 97% as a brownish solid; mp 53-55 °C (lit.[5] mp 54-55 °C). ^{1}H NMR (300 MHz, acetone) δ 7.37 (d, J = 8.8 Hz, 2H), 6.92 (d, J = 8.8 Hz, 2H), 4.41 (s, 2H), 3.79 (s, 3H); ^{13}C NMR (75 MHz, acetone) δ 160.59, 131.14, 130.22, 114.96, 103.20, 55.55, 33.19.

Synthesis of 5-[3,5-(dinitrobenzyl)selanyl]-1*H*-tetrazole (14):

3,5-Dinitrobenzyl selenocyanate **12** (0.286 g, 1 mmol) was added to a suspension of sodium azide (0.065 g, 1 mmol) and triethylammonium chloride (0.138 g, 1 mmol) in toluene (7 mL). The reaction mixture was stirred at 50 °C for 10 h. The reaction mixture was cooled to rt and extracted with 1% aqueous NaOH (15 mL). The aqueous layer then was washed with EtOAc (2 × 15 mL) and carefully acidified by HCl to pH 5.5-6, and the product was filtered and washed with water. Rapid acidification of the aqueous phase or acidification to a lower pH led to precipitation of selenium. Yield: 46% as white solid. ^{1}H NMR (500 MHz, acetone) δ 8.80 – 8.77 (m, 3H), 4.85 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 149.28, 144.96, 130.23, 118.14, 29.33. Anal. Calcd for $C_8H_6N_6O_4Se$: C, 29.19; H, 1.84; N, 25.53. Found: C, 29.55; H, 2.14; N, 25.49.

General procedure for the preparation of 1-alkyl-5-(alkylselanyl)-1*H*-tetrazoles (15-19) and 2-alkyl-5-(alkylselanyl)-2*H*-tetrazoles (20-24).

Alkyl selenocyanate **9**, **10**, **12** or **13** (1 mmol) was added to a suspension of sodium azide (1 mmol) and triethylammonium chloride (1 mmol) in toluene (7 mL). The reaction mixture was stirred at 80-90 °C for 2-6 h until completion, as determined by TLC. The reaction mixture was cooled to rt and extracted with 1% aqueous NaOH (15 mL). The aqueous layer then was

washed with EtOAc (2 × 15 mL). Alkyl halide (0.75 mmol) and TBAB (0.038 mmol) in CH_2Cl_2 (15 mL) were added to the aqueous phase. The reaction mixture was stirred at rt for 48 h. Subsequently, the organic layer was separated, washed with water (2 × 15 mL) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the products, 1- and 2-isomers, were separated by silica gel column chromatography using the mobile phase hexane/EtOAc.

When 2,4-dinitrobenzyl selenocyanate **11** was used as a substrate, the reaction with sodium azide and triethylammonium chloride was stirred in THF at rt overnight and then THF was evaporated. Subsequently, the residue was dissolved in 1% aqueous NaOH (15 mL), and the procedure continued as described previously.

Dimethyl sulfate, benzyl bromide, 4-nitrobenzyl iodide, 3,5-dinitrobenzyl iodide and 2,4-dinitrobenzyl iodide were used as alkylating agents.

1-Alkyl-5-(alkylselanyl)-1*H*-tetrazoles (15-19):

*5-(Benzylselanyl)-1-(3,5-dinitrobenzyl)-1H-tetrazole (**15d**)*: Yield: 32% as a yellowish solid; mp 111-112 °C. ^{1}H NMR (500 MHz, acetone) δ 8.88 (t, J = 2.1 Hz, 1H), 8.54 (d, J = 2.1 Hz, 2H), 7.27 – 7.24 (m, 2H), 7.19 – 7.12 (m, 3H), 5.87 (s, 2H), 4.54 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 149.54, 147.66, 139.12, 138.80, 129.59, 129.57, 129.32, 128.35, 119.57, 50.40, 33.39. Anal. Calcd for $C_{15}H_{12}N_6O_4Se$: C, 42.97; H, 2.88; N, 20.05. Found: C, 43.28; H, 2.92; N, 19.69.

*5-(Benzylselanyl)-1-(2,4-dinitrobenzyl)-1H-tetrazole (**15e**)*: Yield: 12% as a beige solid; mp 78-82 °C. ^{1}H NMR (500 MHz, acetone) δ 8.91 (d, J = 2.4 Hz, 1H), 8.44 (dd, J = 8.6, 2.4 Hz, 1H), 7.34 – 7.32 (m, 2H), 7.30 – 7.23 (m, 3H), 7.11 (d, J = 8.6 Hz, 1H), 6.03 (s, 2H), 4.58 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 148.77, 148.75, 148.32, 138.87, 136.61, 132.10, 129.77, 129.48, 129.02, 128.49, 121.50, 48.98, 33.34. HRMS (ESI+) m/z calcd for $C_{15}H_{13}N_6O_4Se^+$:

421.01580 (100.0%), 419.01659 (47.9%), 417.01849 (18.9%), 423.01598 (17.6%); found 421.0141 (100%), 419.0155 (50%), 417.0173 (19%), 423.0139 (18%).

5-[(4-Nitrobenzyl)selanyl]-1-(4-nitrobenzyl)-1H-tetrazole ***(16c)***: Yield: 16% as a yellowish solid; mp 132-133 °C (with decomposition). ^{1}H NMR (300 MHz, acetone) δ 8.18 (d, J = 8.8 Hz, 2H), 8.09 (d, J = 8.8 Hz, 2H), 7.61 (d, J = 8.8 Hz, 2H), 7.46 (d, J = 8.8 Hz, 2H), 5.74 (s, 2H), 4.68 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 148.84, 148.06, 147.30, 147.16, 142.02, 130.96, 129.91, 124.70, 124.34, 51.12, 31.69. Anal. Calcd for $C_{15}H_{12}N_6O_4Se$: C, 42.97; H, 2.88; N, 20.05. Found: C, 42.68; H, 3.05; N, 19.73.

1-(3,5-Dinitrobenzyl)-5-[(4-nitrobenzyl)selanyl]-1H-tetrazole ***(16d)***: Yield: 30% as a light beige solid; mp 135-136 °C (with decomposition). ^{1}H NMR (500 MHz, acetone) δ 8.87 (t, J = 2.1 Hz, 1H), 8.56 (d, J = 2.1 Hz, 2H), 8.06 (d, J = 8.7 Hz, 2H), 7.59 (d, J = 8.7 Hz, 2H), 5.94 (s, 2H), 4.69 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 149.53, 148.00, 147.46, 147.19, 139.06, 130.88, 129.62, 124.31, 119.53, 50.47, 31.85. Anal. Calcd for $C_{15}H_{11}N_7O_6Se$: C, 38.81; H, 2.39; N, 21.12. Found: C, 38.84; H, 2.94; N, 20.59.

1-(2,4-Dinitrobenzyl)-5-[(4-nitrobenzyl)selanyl]-1H-tetrazole ***(16e)***: Yield: 20% as an amorphous yellow solid. ^{1}H NMR (500 MHz, acetone) δ 8.91 (d, J = 2.4 Hz, 1H), 8.47 (dd, J = 8.6, 2.4 Hz, 1H), 8.13 (d, J = 8.7 Hz, 2H), 7.65 (d, J = 8.7 Hz, 2H), 7.25 (d, J = 8.6 Hz, 1H), 6.09 (s, 2H), 4.71 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 148.83, 148.10, 148.05, 147.18, 136.42, 132.44, 131.02, 128.98, 124.41, 121.52, 49.13, 31.79. Anal. Calcd for $C_{15}H_{11}N_7O_6Se$: C, 38.81; H, 2.39; N, 21.12. Found: C, 38.87; H, 2.54; N, 21.43. HRMS (ESI+) m/z calcd for $C_{15}H_{12}N_7O_6Se^{+}$: 466.00088 (100.0%), 464.00167 (47.9%), 462.00357 (18.9%), 468.00106 (17.6%); found: 466.0009 (100%), 464.0021 (49%), 462.0037 (19%), 468.0010 (18%).

5-[(2,4-Dinitrobenzyl)selanyl]-1-methyl-1H-tetrazole ***(17a)***: Yield: 16% as a yellow solid; mp 88-89 °C. ^{1}H NMR (500 MHz, acetone) δ 8.87 (d, J = 2.4 Hz, 1H), 8.53 (dd, J = 8.6, 2.4 Hz, 1H), 8.03 (d, J = 8.6 Hz, 1H), 4.91 (s, 2H), 3.98 (s, 3H); ^{13}C NMR (126 MHz, acetone) δ

148.37, 148.28, 147.14, 142.27, 134.82, 128.86, 121.57, 34.67, 28.89. Anal. Calcd for $C_9H_8N_6O_4Se$: C, 31.50; H, 2.35; N, 24.49. Found: C, 31.63; H, 2.69; N, 24.78.

*1-Benzyl-5-[(2,4-dinitrobenzyl)selanyl]-1H-tetrazole (**17b**)*: Yield: 13% as a light beige solid; mp 159-160 °C. ^{1}H NMR (500 MHz, acetone) δ 8.82 (d, J = 2.4 Hz, 1H), 8.46 (dd, J = 8.5, 2.4 Hz, 1H), 7.94 (d, J = 8.5 Hz, 1H), 7.35 – 7.30 (m, 3H), 7.25 – 7.23 (m, 2H), 5.57 (s, 2H), 4.89 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 148.23, 147.14, 142.15, 134.84, 134.69, 129.77, 129.46, 128.89, 128.83, 121.61, 52.13, 29.26. Anal. Calcd for $C_{15}H_{12}N_6O_4Se$: C, 42.97; H, 2.88; N, 20.05. Found: C, 43.28; H, 3.18; N, 19.81.

*1-[(2,4-Dinitrobenzyl)selanyl]-1-(4-nitrobenzyl)-1H-tetrazole (**17c**):* Yield: 7% as an oil. ^{1}H NMR (300 MHz, acetone) δ 8.81 (d, J = 2.4 Hz, 1H), 8.48 (dd, J = 8.5, 2.4 Hz, 1H), 8.20 (d, J = 8.7 Hz, 2H), 7.96 (d, J = 8.5 Hz, 1H), 7.51 (d, J = 8.7 Hz, 2H), 5.79 (s, 2H), 4.91 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 148.87, 148.23 (2C), 147.54, 142.01, 141.90, 134.76, 130.02, 128.85, 124.75, 121.55, 51.21, 29.44. HRMS (ESI+) m/z calcd for $C_{15}H_{12}N_7O_6Se^+$: 466.00088 (100.0%), 464.00167 (47.9%), 462.00357 (18.9%), 468.00106 (17.6%); found 466.0011 (100%), 464.0018 (47%), 462.0038 (19%), 468.0013 (18%).

*5-[(2,4-Dinitrobenzyl)selanyl]-1-(3,5-dinitrobenzyl)-1H-tetrazole (**17d**)*: Yield: 10% as an amorphous yellow solid. ^{1}H NMR (300 MHz, acetone) δ 8.89 (t, J = 2.1 Hz, 1H), 8.82 (d, J = 2.4 Hz, 1H), 8.60 (d, J = 2.1 Hz, 2H), 8.48 (dd, J = 8.5, 2.4 Hz, 1H), 7.98 (d, J = 8.5 Hz, 1H), 6.00 (s, 2H), 4.92 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 149.63, 148.30 (2C), 147.79, 142.05, 139.03, 134.82, 129.69, 128.91, 121.55, 119.63, 50.59, 29.58. Anal. Calcd for $C_{15}H_{10}N_8O_8Se$: C, 35.38; H, 1.98; N; 22.0. Found: C, 35.66; H, 1.87; N, 22.04. HRMS (ESI+) m/z calcd for $C_{15}H_{11}N_8O_8Se^+$: 510.98596 (100.0%), 508.98674 (47.9%), 506.98865 (18.9%), 512.98613 (17.6%); found 510.9857 (100%), 508.9868 (49%), 506.9886 (19%), 512.9860 (19%).

*1-(2,4-Dinitrobenzyl)-5-[(2,4-dinitrobenyl)selanyl]-1H-tetrazole (**17e**):* Yield: 6% as an amorphous yellow solid. ^{1}H NMR (500 MHz, acetone) δ 8.92 (d, J = 2.4 Hz, 1H), 8.85 (d, J = 2.5 Hz, 1H), 8.55 – 8.51 (m, 2H), 8.04 (d, J = 8.4 Hz, 1H), 7.35 (d, J = 8.6 Hz, 1H), 6.14 (s, 2H), 4.95 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 148.92, 148.84, 148.36, 148.31, 142.03, 136.37, 134.88, 132.60, 129.07, 128.94, 121.60 (2c), 49.39, 29.54. Anal. Calcd for $C_{15}H_{10}N_8O_8Se$: C, 35.38; H, 1.98; N; 22.0. Found: C, 35.55; H, 1.98; N, 21.72. HRMS (ESI+) m/z calcd for $C_{15}H_{11}N_8O_8Se^+$: 510.98596 (100.0%), 508.98674 (47.9%), 506.98865 (18.9%), 512.98613 (17.6%); found 510.9854 (100%), 508.9867 (48%), 506.9883 (18%), 512.9857 (18%).

*5-[(3,5-Dinitrobenzyl)selanyl]-1-methyl-1H-tetrazole (**18a**)*: Yield: 15% as a light grey solid; mp 118-119 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.93 (t, J = 2.1 Hz, 1H), 8.70 (d, J = 2.1 Hz, 2H), 4.76 (s, 2H), 3.94 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 148.54, 145.62, 141.90, 129.39, 118.19, 34.12, 29.67. Anal. Calcd for $C_9H_8N_6O_4Se$: C, 31.50; H, 2.35; N, 24.49. Found: C, 31.42; H, 2.54; N, 24.74.

*1-Benzyl-5-[(3,5-dinitrobenzyl)selanyl]-1H-tetrazole (**18b**)*:[17] Yield: 12% as a light beige solid; mp 154-156 °C (with decomposition). ^{1}H NMR (500 MHz, acetone) δ 8.75 (t, J = 2.1 Hz, 1H), 8.68 (d, J = 2.1 Hz, 2H), 7.35 – 7.29 (m, 3H), 7.22 – 7.19 (m, 2H), 5.57 (s, 2H), 4.81 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 149.21, 146.86, 144.55, 134.90, 130.16, 129.72, 129.45, 128.81, 118.26, 52.09, 30.51. Anal. Calcd for $C_{15}H_{12}N_6O_4Se$: C, 42.97; H, 2.88; N, 20.05. Found: C, 43.05; H, 2.80; N, 20.15.

*5-[(3,5-Dinitrobenzyl)selanyl]-1-(4-nitrobenzyl)-1H-tetrazole (**18c**):* Yield: 15% as a beige solid; mp 156-158 °C. ^{1}H NMR (500 MHz, acetone) δ 8.75 (t, J = 2.1 Hz, 1H), 8.69 (d, J = 2.1 Hz, 2H), 8.19 (d, J = 8.7 Hz, 2H), 7.49 (d, J = 8.7 Hz, 2H), 5.79 (s, 2H), 4.85 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 148.34, 148.01, 146.45, 143.66, 141.13, 129.34, 129.10, 123.87, 117.39, 50.34, 29.86. Anal. Calcd for $C_{15}H_{11}N_7O_6Se$: C, 38.81; H, 2.39; N, 21.12. Found: C,

39.1; H, 2.71; N, 20.8. HRMS (ESI+) m/z calcd for $C_{15}H_{12}N_7O_6Se^+$: 466.00088 (100.0%), 464.00167 (47.9%), 462.00357 (18.9%), 468.00106 (17.6%); found 466.0011 (100%), 464.0019 (46%), 462.0036 (18%), 468.0012 (18%).

1-(3,5-Dinitrobenzyl)-5-[(3,5-dinitrobenzyl)selanyl]-1H-tetrazole ***(18d)****:* Yield: 12% as a beige solid; mp 140-143 °C. ^{1}H NMR (300 MHz, acetone) δ 8.89 (t, J = 2.1 Hz, 1H), 8.77 – 8.72 (m, 4H), 8.59 (d, J = 2.1 Hz, 1H), 5.99 (s, 2H), 4.88 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 149.59, 149.24, 147.54, 144.53, 139.00, 130.25, 129.64, 119.59, 118.26, 50.52, 30.77. Anal. Calcd for $C_{15}H_{10}N_8O_8Se$: C, 35.38; H, 1.98; N; 22.0. Found: C, 35.74; H, 2.66; N, 21.51.

1-(2,4-Dinitrobenzyl)-5-[(3,5-Dinitrobenzyl)selanyl]-1H-tetrazole ***(18e)****:* Yield: 12% as a beige solid; mp 137-138 °C (with decomposition). ^{1}H NMR (500 MHz, acetone) δ 8.91 (d, J = 2.4 Hz, 1H), 8.80 (t, J = 2.1 Hz, 1H), 8.75 (d, J = 2.1 Hz, 2H), 8.51 (dd, J = 8.6, 2.4 Hz, 1H), 7.36 (d, J = 8.6 Hz, 1H), 6.12 (s, 2H), 4.89 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 149.27, 148.93, 148.87, 148.00, 144.51, 136.30, 132.77, 130.31, 129.02, 121.55, 118.31, 49.29, 30.81. Anal. Calcd for $C_{15}H_{10}N_8O_8Se$: C, 35.38; H, 1.98; N; 22.0. Found: C, 35.67; H, 2.26; N, 21.63.

1-(3,5-dinitrobenzyl)-5-[(4-methoxybenzyl)selanyl]-1H-tetrazole ***(19d)***: Yield: 12% yield as a yellowish solid; mp 121-123 °C. ^{1}H NMR (500 MHz, acetone) δ 8.88 (t, J = 2.1 Hz, 1H), 8.54 (d, J = 2.1 Hz, 2H), 7.14 (d, J = 8.7 Hz, 2H), 6.69 (d, J = 8.7 Hz, 2H), 5.87 (s, 2H), 4.50 (s, 2H), 3.71 (s, 3H); ^{13}C NMR (126 MHz, acetone) δ 160.04, 149.49, 147.74, 139.13, 130.79, 130.45, 129.57, 119.46, 114.60, 55.43, 50.38, 33.36. Anal. Calcd for $C_{16}H_{14}N_6O_5Se$: C, 42.77; H, 3.14; N, 18.71. Found: C, 42.72; H, 3.34; N, 18.48.

1-(2,4-dinitrobenzyl)-5-[(4-methoxybenzyl)selanyl]-1H-tetrazole ***(19e)***: Yield: 11% as a yellow solid; 122-124 °C (with decomposition). ^{1}H NMR (500 MHz, acetone) δ 8.91 (d, J = 2.4 Hz, 1H), 8.42 (dd, J = 8.5, 2.4 Hz, 1H), 7.22 (d, J = 8.6 Hz, 2H), 7.03 (d, J = 8.5 Hz, 1H),

6.79 (d, J = 8.6 Hz, 2H), 6.02 (s, 2H), 4.53 (s, 2H), 3.77 (s, 3H); ^{13}C NMR (126 MHz, acetone) δ 160.22, 148.67, 148.38, 136.64, 131.97, 130.97, 130.53, 128.92, 121.46, 114.80, 55.51, 48.90, 33.32. Anal. Calcd for $C_{16}H_{14}N_6O_5Se$: C, 42.77; H, 3.14; N, 18.71. Found: C, 42.72; H, 3.43; N, 18.57.

2-Alkyl-5-(alkylselanyl)-2*H*-tetrazoles (20-24):

*5-(Benzylselanyl)-2-(3,5-dinitrobenzyl)-2H-tetrazole (**20d**)*: Yield: 34% as a yellow solid; mp 102-103 °C. ^{1}H NMR (500 MHz, acetone) δ 8.97 (t, J = 2.1 Hz, 1H), 8.78 (d, J = 2.1 Hz, 2H), 7.44 – 7.31 (m, 2H), 7.27 – 7.06 (m, 3H), 6.31 (s, 2H), 4.47 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 157.68, 149.68, 139.27, 138.74, 130.17, 129.76, 129.23, 128.04, 119.80, 55.55, 30.53. Anal. Calcd for $C_{15}H_{12}N_6O_4Se$: C, 42.97; H, 2.88; N, 20.05. Found: C, 42.92; H, 3.17; N, 19.79.

*5-(Benzylselanyl)-2-(2,4-dinitrobenzyl)-2H-tetrazole (**20e**)*: Yield: 15% as a beige solid; mp 75-77 °C. ^{1}H NMR (500 MHz, acetone) δ 8.97 (d, J = 2.4 Hz, 1H), 8.60 (dd, J = 8.6, 2.4 Hz, 1H), 7.55 (d, J = 8.6 Hz, 1H), 7.39 – 7.32 (m, 2H), 7.28 – 7.19 (m, 3H), 6.48 (s, 2H), 4.48 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 157.60, 149.11, 149.03, 139.26, 136.04, 133.59, 129.79, 129.29, 129.07, 128.08, 121.50, 54.19, 30.58. Anal. Calcd for $C_{15}H_{12}N_6O_4Se$: C, 42.97; H, 2.88; N, 20.05. Found: C, 43.17; H, 3.39; N, 19.74.

*2-(4-Nitrobenzyl)-5-[(4-nitrobenzyl)selanyl]-2H-tetrazole (**21c**)*: Yield: 28% as a white solid; 132-133 °C. ^{1}H NMR (500 MHz, acetone) δ 8.28 (d, J = 8.7 Hz, 2H), 8.06 (d, J = 8.7 Hz, 2H), 7.68 (d, J = 8.7 Hz, 2H), 7.62 (d, J = 8.7 Hz, 2H), 6.10 (s, 2H), 4.58 (s, 2H). ^{13}C NMR (126 MHz, acetone) δ 156.75, 149.11, 147.85, 141.79, 130.98, 130.56, 124.77, 124.20, 56.41, 29.33. Anal. Calcd for $C_{15}H_{12}N_6O_4Se$: C, 42.97; H, 2.88; N, 20.05. Found: C, 42.96; H, 3.07; N, 19.99. HRMS (ESI+) m/z calcd for $C_{15}H_{13}N_6O_4Se^+$: 421.01580 (100.0%), 419.01659 (47.9%), 417.01849 (18.9%), 423.01598 (17.6%); found 421.0156 (100%), 419.0164 (48%), 417.0184 (19%), 423.0155 (18%).

*2-(3,5-Dinitrobenzyl)-5-[(4-nitrobenzyl)selanyl]-2H-tetrazole (**21d**)*: Yield: 31% as a beige solid; mp 110-112 °C. ^{1}H NMR (500 MHz, acetone) δ 8.96 (t, J = 2.1 Hz, 1H), 8.77 (d, J = 2.1 Hz, 2H), 8.07 (d, J = 8.8 Hz, 2H), 7.65 (d, J = 8.8 Hz, 2H), 6.32 (s, 2H), 4.59 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 157.08, 149.66, 147.85, 138.64, 130.98, 130.16, 124.20, 119.81, 55.62, 29.30. Anal. Calcd for $C_{15}H_{11}N_7O_6Se$: C, 38.81; H, 2.39; N, 21.12. Found: C, 38.81; H, 2.61; N, 21.25.

*2-(2,4-Dinitrobenzyl)-5-[(4-nitrobenzyl)selanyl]-2H-tetrazole (**21e**)*: Yield: 37% as an oil. ^{1}H NMR (500 MHz, acetone) δ 8.96 (d, J = 2.4 Hz, 1H), 8.61 (dd, J = 8.6, 2.4 Hz, 1H), 8.09 (d, J = 8.8 Hz, 2H), 7.64 – 7.61 (m, 3H), 6.47 (s, 2H), 4.58 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 156.91, 149.17, 149.13, 147.87, 147.83, 135.76, 133.86, 130.98, 129.07, 124.24, 121.51, 54.26, 29.37. HRMS (ESI+) m/z calcd for $C_{15}H_{12}N_7O_6Se^+$: 466.00088 (100.0%), 464.00167 (47.9%), 462.00357 (18.9%), 468.00106 (17.6%); found 466.0004 (100%), 464.0015 (50%), 462.0032 (19%), 468.0005 (18%).

*5-[(2,4-Dinitrobenzyl)selanyl]-2-methyl-2H-tetrazole (**22a**)*: Yield: 30% as a yellow solid; mp 91-93 °C. ^{1}H NMR (500 MHz, acetone) δ 8.84 (d, J = 2.5 Hz, 1H), 8.47 (dd, J = 8.5, 2.5 Hz, 1H), 8.00 (d, J = 8.5 Hz, 1H), 4.83 (s, 2H), 4.39 (s, 3H); ^{13}C NMR (126 MHz, acetone) δ 155.86, 148.58, 148.02, 142.97, 134.79, 128.55, 121.48, 40.09, 26.66. Anal. Calcd for $C_9H_8N_6O_4Se$: C, 31.50; H, 2.35; N, 24.49. Found: C, 31.58; H, 2.57; N, 24.3.

*2-Benzyl-5-[(2,4-dinitrobenzyl)selanyl]-2H-tetrazole (**22b**)*: Yield: 24% as an oil. ^{1}H NMR (500 MHz, acetone) δ 8.81 (d, J = 2.4 Hz, 1H), 8.33 (dd, J = 8.5, 2.4 Hz, 1H), 7.86 (d, J = 8.5 Hz, 1H), 7.44 – 7.39 (m, 5H), 5.90 (s, 2H), 4.80 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 156.24, 148.50, 147.95, 142.80, 134.78, 134.68, 129.74, 129.66, 129.45, 128.38, 121.51, 57.52, 26.81. HRMS (ESI+) m/z calcd for $C_{15}H_{13}N_6O_4Se^+$: 421.01580 (100.0%), 419.01659 (47.9%), 417.01849 (18.9%), 423.01598 (17.6%); found 421.0149 (100%), 419.0162 (49%), 417.0181 (19%), 423.0147 (18%).

*5-[(2,4-Dinitrobenzyl)selanyl]-2-(4-nitrobenzyl)-2H-tetrazole (**22c**):* Yield: 11% as an oil. ^{1}H NMR (300 MHz, acetone) δ 8.81 (d, *J* = 2.4 Hz, 1H), 8.39 (dd, *J* = 8.5, 2.4 Hz, 1H), 8.28 (d, *J* = 8.7 Hz, 2H), 7.94 (d, *J* = 8.5 Hz, 1H), 7.70 (d, *J* = 8.7 Hz, 2H), 6.12 (s, 2H), 4.83 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 156.73, 149.09, 148.58, 148.00, 142.76, 141.70, 134.77, 130.59, 128.45, 124.78, 121.50, 56.47, 26.84. HRMS (ESI+) m/z calcd for $C_{15}H_{12}N_7O_6Se^+$: 466.00088 (100.0%), 464.00167 (47.9%), 462.00357 (18.9%), 468.00106 (17.6%); found 466.0006 (100%), 464.0019 (52%), 462.0035 (19%), 468.0007 (19%).

*5-[(2,4-Dinitrobenzyl)selanyl]-2-(3,5-dinitrobenzyl)-2H-tetrazole (**22d**)*: Yield: 22% as a yellow solid; mp 142-144 °C. ^{1}H NMR (300 MHz, acetone) δ 8.97 (t, *J* = 2.1 Hz, 1H), 8.81 (d, *J* = 2.4 Hz, 1H), 8.79 (d, *J* = 2.1 Hz, 2H), 8.42 (dd, *J* = 8.6, 2.4 Hz, 1H), 7.99 (d, *J* = 8.6 Hz, 1H), 6.33 (s, 2H), 4.84 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 157.07, 149.69, 148.61, 148.00, 142.78, 138.56, 134.81, 130.21, 128.49, 121.47, 119.84, 55.70, 26.83. Anal. Calcd for $C_{15}H_{10}N_8O_8Se$: C, 35.38; H, 1.98; N; 22.0. Found: C, 35.49; H, 2.14; N, 21.81.

*2-(2,4-Dinitrobenzyl)-5-[(2,4-dinitrobenyl)selanyl]-2H-tetrazole (**22e**):* Yield: 23% as a yellow solid; mp 94-97 °C. ^{1}H NMR (500 MHz, acetone) δ 8.97 (d, *J* = 2.4 Hz, 1H), 8.81 (d, *J* = 2.4 Hz, 1H), 8.62 (dd, *J* = 8.5, 2.4 Hz, 1H), 8.42 (dd, *J* = 8.5, 2.4 Hz, 1H), 7.95 (d, *J* = 8.5 Hz, 1H), 7.64 (d, *J* = 8.5 Hz, 1H), 6.49 (s, 2H), 4.83 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 156.92, 149.19, 149.11, 148.55, 148.03, 142.70, 135.74, 134.77, 133.85, 129.11, 128.52, 121.53, 121.52, 54.36, 26.96. Anal. Calcd for $C_{15}H_{10}N_8O_8Se$: C, 35.38; H, 1.98; N; 22.0. Found: C, 35.56; H, 2.16; N, 21.65.

*5-[(3,5-Dinitrobenzyl)selanyl]-2-methyl-2H-tetrazole (**23a**):* Yield: 23% as a beige solid; mp 102-103 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.89 (t, *J* = 2.1 Hz, 1H), 8.60 (d, *J* = 2.1 Hz, 2H), 4.54 (s, 2H), 4.35 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 154.74, 148.40, 143.24, 129.21, 117.67, 39.78, 27.70. Anal. Calcd for $C_9H_8N_6O_4Se$: C, 31.50; H, 2.35; N, 24.49. Found: C, 31.61; H, 2.51; N, 24.74.

2-Benzyl-5-[(3,5-dinitrobenzyl)selanyl]-2H-tetrazole ***(23b)***: Yield: 20% as a beige solid; mp 96-97 °C. ^{1}H NMR (300 MHz, acetone) δ 8.77 – 8.69 (m, 3H), 7.36 (s, 5H), 5.87 (s, 2H), 4.74 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 156.18, 149.19, 145.24, 134.74, 130.19, 129.70, 129.28, 128.92, 117.96, 57.50, 28.39. Anal. Calcd for $C_{15}H_{12}N_6O_4Se$: C, 42.97; H, 2.88; N, 20.05. Found: C, 43.2; H, 3.0; N, 19.82.

5-[(3,5-Dinitrobenzyl)selanyl]-2-(4-nitrobenzyl)-2H-tetrazole ***(23c)****:* Yield: 44% as a light grey solid; mp 101-103 °C. ^{1}H NMR (300 MHz, acetone) δ 8.74 (t, J = 2.1 Hz, 1H), 8.70 (d, J = 2.1 Hz, 2H), 8.24 (d, J = 8.6 Hz, 2H), 7.64 (d, J = 8.6 Hz, 2H), 6.10 (s, 2H), 4.75 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 156.72, 149.22, 149.09, 145.24, 141.72, 130.46, 130.22, 124.79, 118.01, 56.50, 28.52. Anal. Calcd for $C_{15}H_{11}N_7O_6Se$: C, 38.81; H, 2.39; N, 21.12. Found: C, 39.16; H, 2.35; N, 21.25.

2-(3,5-Dinitrobenzyl)-5-[(3,5-dinitrobenzyl)selanyl]-2H-tetrazole ***(23d)****:* Yield: 31% as a beige solid; mp 162-164 °C. ^{1}H NMR (300 MHz, acetone) δ 8.96 (t, J = 2.1 Hz, 1H), 8.77 – 8.69 (m, 5H), 6.32 (s, 2H), 4.76 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 155.68, 149.69, 149.21, 145.22, 138.55, 130.17, 130.10, 119.82, 117.96, 55.71, 28.48. Anal. Calcd for $C_{15}H_{10}N_8O_8Se$: C, 35.38; H, 1.98; N; 22.0. Found: C, 35.68; H, 2.34; N, 22.18.

2-(2,4-Dinitrobenzyl)-5-[(3,5-dinitrobenzyl)selanyl]-2H-tetrazole ***(23e)***: Yield: 35% as a white solid; mp 124-125 °C. ^{1}H NMR (500 MHz, acetone) δ 8.95 (d, J = 2.4 Hz, 1H), 8.75 (t, J = 2.1 Hz, 1H), 8.70 (d, J = 2.1 Hz, 2H), 8.57 (dd, J = 8.6, 2.4 Hz, 1H), 7.58 (d, J = 8.6 Hz, 1H), 6.47 (s, 2H), 4.75 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 156.82, 149.19, 149.16, 145.14, 135.69, 133.73, 130.19, 129.5, 121.5, 117.99, 54.34, 28.52. Anal. Calcd for $C_{15}H_{10}N_8O_8Se$: C, 35.38; H, 1.98; N; 22.0. Found: C, 35.53; H, 2.25; N, 21.84.

2-(3,5-Dinitrobenzyl)-5-[(4-methoxybenzyl)selanyl]-2H-tetrazole ***(24d)****:* Yield: 18% as a yellow solid; mp 93-97 °C. ^{1}H NMR (300 MHz, acetone) δ 8.97 (t, J = 2.1 Hz, 1H), 8.78 (d, J = 2.1 Hz, 2H), 7.27 (d, J = 8.7 Hz, 2H), 6.75 (d, J = 8.7 Hz, 2H), 6.31 (s, 2H), 4.43 (s, 2H),

3.72 (s, 3H); ^{13}C NMR (75 MHz, acetone) δ 159.88, 157.84, 149.67, 138.77, 130.96, 130.86, 130.16, 119.79, 114.58, 55.52, 55.42, 30.28. Anal. Calcd for $C_{16}H_{14}N_6O_5Se$: C, 42.77; H, 3.14; N, 18.71. Found: C, 43.1; H, 3.46; N, 18.78.

*2-(2,4-dinitrobenzyl)-5-[(4-methoxybenzyl)selanyl]-2H-tetrazole (**24e**):* Yield: 5% as an oil. ^{1}H NMR (500 MHz, acetone) δ 8.97 (d, J = 2.4 Hz, 1H), 8.60 (dd, J = 8.6, 2.4 Hz, 1H), 7.50 (d, J = 8.6 Hz, 1H), 7.28 (d, J = 8.7 Hz, 2H), 6.79 (d, J = 8.7 Hz, 2H), 6.47 (s, 2H), 4.44 (s, 2H), 3.75 (s, 3H); ^{13}C NMR (126 MHz, acetone) 159.90, 157.70, 149.09, 149.05, 136.11, 133.49, 131.01, 130.87, 129.06, 121.49, 114.64, 55.46, 54.15, 30.36. HRMS (ESI+) m/z calcd for $C_{16}H_{15}N_6O_5Se^+$: 451.02636 (100.0%), 449.02715 (47.9%), 447.02906 (18.9%), 453.02654 (17.6%); found 451.0265 (100%), 449.0273 (48%), 447.0293 (19%), 453.0266 (18%).

Synthesis of alkyl thiocyanates (25-28):

A suspension of alkyl halide (10 mmol) and potassium thiocyanate (11 mmol) in 7 mL DMF or THF was stirred until completion, as determined by TLC, under the conditions given below. The reaction mixture was then diluted with EtOAc (10 mL) and the organic layer was washed with 1% aqueous NaOH (2 × 10 mL) and H_2O (1 × 10 mL). The organic solvent was dried over Na_2SO_4. If necessary, the crude product was purified by column chromatography (Mobile phase: Hexane/EtOAc).

*Benzyl thiocyanate (**25**)*:[6] Benzyl bromide was used as the starting material. The reaction mixture was stirred in DMF at 100 °C for 1 h. Yield: 95% as a white solid; mp 40-41 °C (lit.[7] mp 39-40 °C). ^{1}H NMR (300 MHz, DMSO) δ 7.48 – 7.28 (m, 5H), 4.35 (s, 2H); ^{13}C NMR (75 MHz, DMSO) δ 136.33, 129.21, 128.93, 128.46, 113.15, 37.18.

*4-Nitrobenzyl thiocyanate (**26**)*:[8] 4-Nitrobenzyl chloride was used as a substrate. The reaction mixture was heated in DMF at 100 °C for 6 h. Yield: 96% as a yellowish solid; mp 79-81 °C (lit.[9] mp 85-86 °C). ^{1}H NMR (300 MHz, DMSO) δ 8.26 (d, J = 8.7 Hz, 2H), 7.69 (d, J = 8.7

Hz, 2H), 4.48 (s, 2H); ^{13}C NMR (75 MHz, DMSO) δ 147.44, 144.24, 130.56, 124.11, 112.82, 35.94.

3,5-Dinitrobenzyl thiocyanate (27): 3,5-Dinitrobenzyl chloride was used as a substrate. The reaction mixture was heated in DMF at 100 °C for 3 h. The product was purified by column chromatography (Mobile phase: Hexane/EtOAc, 7:1). Yield: 82% as a white solid; mp 120-122 °C (lit.[10] mp 120-121 °C). ^{1}H NMR (500 MHz, acetone) δ 8.95 (t, J = 2.1 Hz, 1H), 8.82 (d, J = 2.1 Hz, 2H), 4.74 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 149.66, 141.82, 130.29, 119.37, 111.87, 36.31. Anal. Calcd for $C_8H_5N_3O_4S$: C, 40.17; H, 2.11; N, 17.57; S, 13.40. Found: C, 40.15; H, 2.06; N, 17.42; S, 13.53.

2,4-Dinitrobenzyl thiocyanate (28): 2,4-Dinitrobenzyl chloride was used as the starting material. The reaction mixture was refluxed in THF for 10 h. Yield: 75% as a yellowish solid; mp 84-86 °C (lit.[8] mp 86-87 °C). ^{1}H NMR (300 MHz, DMSO) δ 8.82 (d, J = 2.4 Hz, 1H), 8.66 (dd, J = 8.5, 2.4 Hz, 1H), 7.99 (d, J = 8.5 Hz, 1H), 4.74 (s, 2H); ^{13}C NMR (75 MHz, DMSO) δ 147.71, 147.62, 137.98, 134.32, 128.64, 121.11, 112.39, 33.83.

General procedure for the synthesis of 5-(alkylsulfanyl)-1*H*-tetrazoles (29-31):

Acetic acid (0.39 g, 6.5 mmol) was added to a suspension of alkyl thiocyanate **25-27** (5 mmol) and sodium azide (0.42 g, 6.5 mmol) in *n*-butanol (15 mL). The reaction mixture was heated at 100 °C for 4-8 h. Subsequently, the solvent was evaporated under reduced pressure. The obtained solid was dissolved in 3% NaOH (10 mL) and washed with EtOAc (2 × 15 mL). The aqueous layer was acidified to pH 2-3, and the product was filtered and washed with water.

5-(Benzylsulfanyl)-1H-tetrazole (29):[11] Yield: 50% as a white solid; mp 138-139 °C (lit.[12] mp 134-136 °C). ^{1}H NMR (300 MHz, DMSO) δ 7.44 – 7.20 (m, 5H), 4.50 (s, 2H); ^{13}C NMR (75 MHz, DMSO) δ 153.88, 136.85, 129.09, 128.73, 127.82, 36.17.

5-[(4-Nitrobenzyl)sulfanyl]-1H-tetrazole (30): Yield: 51% as a yellow solid; mp 147-148 °C. ^{1}H NMR (300 MHz, DMSO) δ 8.16 (d, J = 8.7 Hz, 2H), 7.67 (d, J = 8.7 Hz, 2H), 4.62 (s, 2H); ^{13}C NMR (75 MHz, DMSO) δ 153.74, 146.97, 145.35, 130.37, 123.80, 35.21. Anal. Calcd for $C_8H_7N_5O_2S$: C, 40.5; H, 2.97; N, 29.52; S, 13.52. Found: C, 40.66; H, 3.23; N, 29.81; S, 13.85.

5-[(3,5-Dinitrobenzyl)sulfanyl]-1H-tetrazole (31): Yield: 68% as a white solid; mp 151-152 °C. ^{1}H NMR (500 MHz, DMSO) δ 8.75 (t, J = 2.1 Hz, 1H), 8.71 (d, J = 2.1 Hz, 2H), 4.75 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 148.09, 142.46, 129.7, 126.96, 117.92, 34.39. Anal. Calcd for $C_8H_6N_6O_4S$: C, 34.04; H, 2.14; N, 29.78; S, 11.36. Found: C, 34.06; H, 2.1; N, 29.46; S, 11.34.

Synthesis of 5-[(2,4-dinitrobenzyl)sulfanyl]-1H-tetrazole (32)

2,4-Dinitrobenzyl thiocyanate **28** (0.72 g, 3 mmol) was added to a suspension of sodium azide (0.23 g, 3.6 mmol) and triethylammonium chloride (0.5 g, 3.6 mmol) in THF. The reaction mixture was stirred at rt overnight. Subsequently, the solvent was evaporated under reduced pressure. The crude product was dissolved in 1% NaOH (10 mL), washed with EtOAc (2 × 15 mL) and the aqueous layer was acidified to pH 1-2. The product was filtered and washed with water. Yield: 80% as a yellowish solid; mp 167-168 °C. ^{1}H NMR (300 MHz, DMSO) δ 8.75 (d, J = 2.4 Hz, 1H), 8.52 (dd, J = 8.5, 2.4 Hz, 1H), 7.99 (d, J = 8.5 Hz, 1H), 4.87 (s, 2H); ^{13}C NMR (75 MHz, DMSO) δ 153.80, 148.10, 147.15, 139.47, 134.11, 128.15, 120.72, 33.19. Anal. Calcd for $C_8H_6N_6O_4S$: C, 34.04; H, 2.14; N, 29.78; S, 11.36. Found: C, 34.28; H, 2.31; N, 29.66; S, 11.68.

General procedure for the preparation of 1-alkyl-5-(alkylsulfanyl)-1*H*-tetrazoles (33-36) and 2-alkyl-5-(alkylsulfanyl)-2*H*-tetrazoles (37-40).

An alkylating agent (1 mmol) was added to a solution of 5-(alkylsulfanyl)-1*H*-tetrazole **29-32** (1.1 mmol) and KOH (1.2 mmol) in 7 mL of THF (for 2,4-dinitrobenzyl derivatives) or DMF (for other derivatives). The reaction mixture was heated at 75 °C for 2-10 h until completion, as determined by TLC. The solvent was evaporated under reduced pressure and the resulting mass was dissolved in EtOAc (20 mL), washed with 1% NaOH (2 × 20 mL) and water (1 × 20 mL) and dried over sodium sulfate Na_2SO_4. The solvent was evaporated and the products, 1- and 2-isomers, were separated using silica gel column chromatography (Mobile phase: Hexane/EtOAc, 10:1 - 5:1).

Dimethyl sulfate, benzyl bromide, 4-nitrobenzyl iodide, 3,5-dinitrobenzyl iodide, 4-chlorobenzyl chloride and 3,4-dichlorobenzyl chloride were used as alkylating agents.

1-Alkyl-5-(alkylsulfanyl)-1*H*-tetrazoles (33-36):

*5-(Benzylsulfanyl)-1-(3,5-dinitrobenzyl)-1H-tetrazole (**33d**):* The reaction mixture was heated for 2 h. Yield: 25% as a white solid; mp 112-113 °C.^{1}H NMR (300 MHz, acetone) δ 8.90 (t, *J* = 2.1 Hz, 1H), 8.56 (d, *J* = 2.1 Hz, 2H), 7.37 – 7.32 (m, 2H), 7.27 – 7.19 (m, 3H), 5.89 (s, 2H), 4.56 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 154.65, 149.60, 138.99, 137.32, 129.78, 129.54, 129.40, 128.66, 119.60, 49.80, 38.31. Anal. Calcd for $C_{15}H_{12}N_6O_4S$: C, 48.38; H, 3.25; N, 22.57; S, 8.61. Found: C, 48.63; H, 3.38; N, 22.93; S, 8.75.

*1-(4-Nitrobenzyl)-5-[(4-nitrobenzyl)sulfanyl]-1H-tetrazole (**34c**):* The reaction mixture was heated for 4 h. Yield: 28% as a white solid; mp 126-127 °C. ^{1}H NMR (300 MHz, acetone) δ 8.21 (d, *J* = 8.7 Hz, 2H), 8.13 (d, *J* = 8.7 Hz, 2H), 7.69 (d, *J* = 8.7 Hz, 2H), 7.49 (d, *J* = 8.7 Hz, 2H), 5.74 (s, 2H), 4.71 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 154.10, 148.87, 148.31, 145.41, 141.84, 131.10, 129.92, 124.74, 124.36, 50.54, 36.94. Anal. Calcd for $C_{15}H_{12}N_6O_4S$: C, 48.38; H, 3.25; N, 22.57; S, 8.61. Found: C, 48.59; H, 3.59; N, 22.76; S, 8.69.

*5-[(4-Nitrobenzyl)sulfanyl]-1-(3,5-dinitrobenzyl)-1H-tetrazole (**34d**):* The reaction mixture was heated for 5 h. Yield: 27% as a white solid; mp 160-161 °C. ^{1}H NMR (300 MHz, acetone) δ 8.91 (t, *J* = 2.1 Hz, 1H), 8.59 (d, *J* = 2.1 Hz, 2H), 8.13 (d, *J* = 8.8 Hz, 2H), 7.70 (d, *J* = 8.8 Hz, 2H), 5.95 (s, 2H), 4.74 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 154.31, 149.63, 148.33, 145.45, 138.92, 131.10, 129.59, 124.36, 119.61, 49.92, 37.04. Anal. Calcd for $C_{15}H_{11}N_7O_6S$: C, 43.17; H, 2.66; N, 23.49; S, 7.68. Found: C, 42.9; H, 2.74; N, 23.14; S, 7.83.

*5-[(2,4-Dinitrobenzyl)sulfanyl]-1-methyl-1H-tetrazole (**35a**)*: The reaction mixture was heated for 4 h. Yield: 15% as a white solid; mp 111-112 °C .^{1}H NMR (300 MHz, acetone) δ 8.87 (d, *J* = 2.4 Hz, 1H), 8.55 (dd, *J* = 8.5, 2.4 Hz, 1H), 8.15 (d, *J* = 8.5 Hz, 1H), 5.00 (s, 2H), 3.94 (s, 3H); ^{13}C NMR (75 MHz, acetone) δ 153.77, 149.00, 148.51, 140.28, 135.27, 128.63, 121.47, 34.77, 33.96. Anal. Calcd for $C_9H_8N_6O_4S$: C, 36.49; H, 2.72; N, 28.37; S, 10.82. Found: C, 36.72; H, 3.01; N, 27.99; S, 10.51.

*1-Benzyl-5-[(2,4-dinitrobenzyl)sulfanyl]-1H-tetrazole (**35b**):* The reaction mixture was heated for 7 h. Yield: 19% as a yellowish solid; mp 145-146 °C .^{1}H NMR (300 MHz, acetone) δ 8.82 (d, *J* = 2.4 Hz, 1H), 8.48 (dd, *J* = 8.6, 2.4 Hz, 1H), 8.03 (d, *J* = 8.6 Hz, 1H), 7.37 – 7.29 (m, 3H), 7.26 – 7.21 (m, 2H), 5.53 (s, 2H), 4.98 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 153.72, 148.85, 148.48, 140.14, 135.12, 134.71, 129.75, 129.42, 128.82, 128.60, 121.51, 51.51, 35.08. Anal. Calcd for $C_{15}H_{12}N_6O_4S$: C, 48.38; H, 3.25; N, 22.57; S, 8.61. Found: C, 48.16; H, 3.34; N, 22.22; S, 8.48.

*5-[(2,4-Dinitrobenzyl)sulfanyl]-1-(4-nitrobenzyl)-1H-tetrazole (**35c**):* The reaction mixture was heated for 8 h. Yield: 10% as a brownish solid. ^{1}H NMR (300 MHz, acetone) δ 8.81 (d, *J* = 2.4 Hz, 1H), 8.50 (dd, *J* = 8.5, 2.4 Hz, 1H), 8.20 (d, *J* = 8.9 Hz, 2H), 8.07 (d, *J* = 8.5 Hz, 1H), 7.50 (d, *J* = 8.9 Hz, 2H), 5.75 (s, 2H), 5.00 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 154.15, 148.89, 148.81, 148.48, 141.75, 140.01, 135.22, 129.95, 128.65, 124.76, 121.46, 50.61, 35.12. Anal. Calcd for $C_{15}H_{11}N_7O_6S$: C, 43.17; H, 2.66; N, 23.49; S, 7.68. Found: C,

43.6; H, 2.82; N, 23.73; S, 7.48. HRMS (ESI+) m/z calcd for $C_{15}H_{12}N_7O_6S^+$: 418.05643 (100.0%), 419.05978 (16.2%); found 418.0557 (100%), 419.0580 (17%).

5-[(2,4-Dinitrobenzyl)sulfanyl]-1-(3,5-dinitrobenzyl)-1H-tetrazole ***(35d)****:* The reaction mixture was heated for 10 h. The product was purified by preparative TLC. Yield: 16% as a brownish solid. ^{1}H NMR (300 MHz, acetone) δ 8.91 (t, J = 2.0 Hz, 1H), 8.81 (d, J = 2.4 Hz, 1H), 8.58 (d, J = 2.0 Hz, 2H), 8.51 (dd, J = 8.5, 2.4 Hz, 1H), 8.10 (d, J = 8.5 Hz, 1H), 5.96 (s, 2H), 5.02 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 154.38, 149.64, 148.91, 148.53, 139.96, 138.82, 135.27, 129.58, 128.64, 121.42, 119.61, 49.94, 35.14. Anal. Calcd for $C_{15}H_{10}N_8O_8S$: C, 38.97; H, 2.18; N, 24.24; S, 6.94. Found: C, 39.17; H, 2.55; N, 24.29; S, 6.7. HRMS (ESI+) m/z calcd for $C_{15}H_{11}N_8O_8S^+$: 463.04151 (100.0%), 464.04486 (16.2%); found 463.0408 (100%), 464.0440 (18%).

5-[(3,5-Dinitrobenzyl)sulfanyl]-1-methyl-1H-tetrazole ***(36a)***: The solution of dimethyl sulfate (0.126g, 1 mmol) and TBAB (0.02 g, 0.05 mmol) in CH_2Cl_2 (5 mL) was added to the solution of 5-[(3,5-dinitrobenzyl)sulfanyl]-1*H*-tetrazole **31** (0.31 g, 1.1. mmol) and NaOH (0.048 g, 1.2 mmol) in water (5 mL). The reaction mixture was stirred 48 h at rt. The organic layer was separated, washed with 1M NaOH (1 × 5 mL) and water (1 × 10 mL) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the product was isolated by column chromatography (hexane/EtOAc, 3:1). Yield: 30% as a white solid; mp 106-108 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.96 (t, J = 2.1 Hz, 1H), 8.72 (d, J = 2.1 Hz, 2H), 4.73 (s, 2H), 3.92 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.33, 148.56, 140.71, 129.35, 118.45, 35.37, 33.51. Anal. Calcd for $C_9H_8N_6O_4S$: C, 36.49; H, 2.72; N, 28.37; S, 10.82. Found: C, 36.70; H, 2.88; N, 28.48; S, 11.09.

1-Benzyl-5-[(3,5-dinitrobenzyl)sulfanyl]-1H-tetrazole ***(36b)***:[17] Compound was prepared analogously to **36a**. Yield: 21% as a beige solid; mp 154-156 °C (with decomposition). ^{1}H NMR (500 MHz, DMSO) δ 8.72 – 8.64 (m, 3H), 7.33 – 7.27 (m, 3H), 7.17 – 7.11 (m, 2H),

5.54 (s, 2H), 4.77 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 153.06, 147.97, 141.97, 133.92, 129.70, 128.99, 128.62, 127.95, 117.99, 50.51, 35.00. Anal. Calcd for $C_{15}H_{12}N_6O_4S$: C, 48.38; H, 3.25; N, 22.57; S, 8.61. Found: C, 48.26; H, 3.21; N, 22.49; S, 8.58.

5-[(3,5-Dinitrobenzyl)sulfanyl]-1-(4-nitrobenzyl)-1H-tetrazole ***(36c)****:* The reaction mixture was heated for 10 h. Yield: 35% as a yellowish solid; mp 140-143 °C. ^{1}H NMR (500 MHz, acetone) δ 8.80 (t, J = 2.1 Hz, 1H), 8.76 (d, J = 2.1 Hz, 2H), 8.21 (d, J = 8.7 Hz, 2H), 7.51 (d, J = 8.7 Hz, 2H), 5.77 (s, 2H), 4.89 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 153.91, 149.25, 148.89, 142.87, 141.77, 130.32, 129.90, 124.75, 118.63, 50.61, 36.13. Anal. Calcd for $C_{15}H_{11}N_7O_6S$: C, 43.17; H, 2.66; N, 23.49; S, 7.68. Found: C, 43.46; H, 2.86; N, 23.22; S, 7.95.

1-(3,5-Dinitrobenzyl)-5-[(3,5-dinitrobenzyl)sulfanyl]-1H-tetrazole ***(36d)****:* The reaction mixture was heated for 7 h. Yield: 22% as a yellowish solid; mp 165-168 °C (with decomposition). ^{1}H NMR (500 MHz, acetone) δ 8.91 (t, J = 2.1 Hz, 1H), 8.81 (t, J = 2.1 Hz, 1H), 8.79 (d, J = 2.1 Hz, 2H), 8.60 (d, J = 2.1 Hz, 2H), 5.97 (s, 2H), 4.92 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 153.30, 148.77, 148.42, 142.02, 137.93, 129.53, 128.79, 118.78, 117.80, 49.11, 35.28. Anal. Calcd for $C_{15}H_{10}N_8O_8S$: C, 38.97; H, 2.18; N, 24.24; S, 6.94. Found: C, 38.87; H, 2.52; N, 23.94; S, 7.01.

1-(4-chlorobenzyl)-5-[(3,5-dinitrobenzyl)sulfanyl]-1H-tetrazole ***(36f)****:* The reaction mixture was heated for 5 h. Yield: 14% as a white solid; mp 130-131 °C. ^{1}H NMR (500 MHz, acetone) δ 8.81 (t, J = 2.1 Hz, 1H), 8.76 (d, J = 2.1 Hz, 2H), 7.37 (d, J = 8.5 Hz, 2H), 7.27 (d, J = 8.5 Hz, 2H), 5.57 (s, 2H), 4.87 (s, 2H). ^{13}C NMR (126 MHz, acetone) δ 153.58, 149.28, 142.95, 134.97, 133.60, 130.63, 130.32, 129.81, 118.61, 50.78, 36.12. Anal.Calcd for $C_{15}H_{11}ClN_6O_4S$: C, 44.29; H, 2.73; N, 20.66; S, 7.88. Found: C, 44.6; H, 2.88; N, 20.67; S, 7.63.

1-(3,4-dichlorobenzyl)-5-[(3,5-dinitrobenzyl)sulfanyl]-1H-tetrazole ***(36g)****:* The reaction mixture was heated for 5 h. Yield: 15% as a white solid; mp 121-124 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.95 (t, *J* = 2.0 Hz, 1H), 8.65 (d, *J* = 2.0 Hz, 2H), 7.45 (d, *J* = 8.2 Hz, 1H), 7.33 (d, *J* = 2.0 Hz, 1H), 7.08 (dd, *J* = 8.2, 2.0 Hz, 1H), 5.35 (s, 2H), 4.69 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.15, 148.56, 140.41, 133.89, 133.55, 132.16, 131.29, 130.03, 129.30, 127.30, 118.52, 50.00, 35.66. Anal.Calcd for $C_{15}H_{10}Cl_2N_6O_4S$: C, 40.83; H, 2.28; N, 19.05; S, 7.27. Found: C, 41.19; H, 2.68; N, 18.78; S, 7.26.

2-Alkyl-5-(alkylsulfanyl)-2*H*-tetrazoles (37-40).

5-(Benzylsulfanyl)-2-(3,5-dinitrobenzyl)-2H-tetrazole ***(37d)****:* Yield: 55% as a white solid; mp 116-117 °C. ^{1}H NMR (300 MHz, acetone) δ 8.97 (t, *J* = 2.1 Hz, 1H), 8.76 (d, *J* = 2.1 Hz, 2H), 7.56 – 7.33 (m, 2H), 7.29 – 7.10 (m, 3H), 6.27 (s, 2H), 4.44 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 165.02, 149.66, 138.66, 138.00, 130.16, 129.78, 129.24, 128.28, 119.79, 55.61, 36.50. Anal. Calcd for $C_{15}H_{12}N_6O_4S$: C, 48.38; H, 3.25; N, 22.57; S, 8.61. Found: C, 48.53; H, 3.39; N, 22.71; S, 8.57.

2-(4-Nitrobenzyl)-5-(4-nitrobenzyl)sulfanyl-2H-tetrazole ***(38c)****:* Yield: 52% as a white solid; mp 131-132 °C. ^{1}H NMR (300 MHz, acetone) δ 8.26 (d, *J* = 8.7 Hz, 2H), 8.11 (d, *J* = 8.7 Hz, 2H), 7.72 – 7.62 (m, 4H), 6.06 (s, 2H), 4.57 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 164.06, 149.07, 148.09, 146.30, 141.70, 131.03, 130.53, 124.74, 124.21, 56.45, 35.64. Anal. Calcd for $C_{15}H_{12}N_6O_4S$: C, 48.38; H, 3.25; N, 22.57; S, 8.61. Found: C, 48.45; H, 3.32; N, 22.77; S, 8.84.

5-[(4-Nitrobenzyl)sulfanyl]-2-(3,5-dinitrobenzyl)-2H-tetrazole ***(38d)****:* Yield: 53% as a light beige solid; mp 111-112 °C. ^{1}H NMR (300 MHz, acetone) δ 8.95 (t, *J* = 2.0 Hz, 1H), 8.75 (d, *J* = 2.0 Hz, 2H), 8.11 (d, *J* = 8.7 Hz, 2H), 7.70 (d, *J* = 8.7 Hz, 2H), 6.27 (s, 2H), 4.58 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 164.37, 149.66, 148.11, 146.29, 138.56, 131.01, 130.16,

124.21, 119.81, 55.69, 35.57. Anal. Calcd for $C_{15}H_{11}N_7O_6S$: C, 43.17; H, 2.66; N, 23.49; S, 7.68. Found: C, 43.42; H, 2.88; N, 23.55; S, 7.82.

*5-[(2,4-Dinitrobenzyl)sulfanyl]-2-methyl-2H-tetrazole (**39a**):* Yield: 32% as a yellowish solid; 87-88 °C. ^{1}H NMR (300 MHz, acetone) δ 8.82 (d, J = 2.4 Hz, 1H), 8.50 (dd, J = 8.5, 2.4 Hz, 1H), 8.09 (d, J = 8.5 Hz, 1H), 4.89 (s, 2H), 4.33 (s, 3H); ^{13}C NMR (75 MHz, acetone) δ 162.96, 149.39, 148.23, 141.00, 135.01, 128.31, 121.31, 40.14, 33.31. Anal. Calcd for $C_9H_8N_6O_4S$: C, 36.49; H, 2.72; N, 28.37; S, 10.82. Found: C, 36.71; H, 2.89; N, 28.02; S, 10.91.

*2-Benzyl-5-[(2,4-dinitrobenzyl)sulfanyl]-2H-tetrazole (**39b**):* Yield: 44% as a yellowish solid; 62-64 °C. ^{1}H NMR (300 MHz, acetone) δ 8.78 (d, J = 2.4 Hz, 1H), 8.37 (dd, J = 8.6, 2.4 Hz, 1H), 7.97 (d, J = 8.6 Hz, 1H), 7.38 (s, 5H), 5.83 (s, 2H), 4.86 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 163.35, 149.30, 148.15, 140.86, 134.92, 134.67, 129.70, 129.42, 128.16, 121.33, 57.57, 33.43. Anal. Calcd for $C_{15}H_{12}N_6O_4S$: C, 48.38; H, 3.25; N, 22.57; S, 8.61. Found: C, 48.69; H, 3.37; N, 22.42; S, 8.61.

*5-[(2,4-Dinitrobenzyl)sulfanyl]-2-(4-nitrobenzyl)-2H-tetrazole (**39c**):* Yield: 44% as an oil. ^{1}H NMR (500 MHz, acetone) δ 8.78 (d, J = 2.4 Hz, 1H), 8.42 (dd, J = 8.6, 2.4 Hz, 1H), 8.26 (d, J = 8.7 Hz, 2H), 8.03 (d, J = 8.6 Hz, 1H), 7.66 (d, J = 8.7 Hz, 2H), 6.05 (s, 2H), 4.88 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 163.83, 149.36, 149.10, 148.19, 141.58, 140.79, 134.98, 130.57, 128.21, 124.74, 121.30, 56.53, 33.41. HRMS (ESI+) m/z calcd for $C_{15}H_{12}N_7O_6S^+$: 418.05643 (100.0%), 419.05978 (16.2%); found 418.0562 (100%), 419.0594 (17%).

*5-[(2,4-Dinitrobenzyl)sulfanyl]-2-(3,5-dinitrobenzyl)-2H-tetrazole (**39d**):* Yield: 42% as a white solid; mp 127-130 °C. ^{1}H NMR (500 MHz, acetone) δ 8.96 (t, J = 2.1 Hz, 1H), 8.78 (d, J = 2.3 Hz, 1H), 8.75 (d, J = 2.1 Hz, 2H), 8.44 (dd, J = 8.6, 2.3 Hz, 1H), 8.08 (d, J = 8.6 Hz, 1H), 6.26 (s, 2H), 4.89 (s, 2H).; ^{13}C NMR (126 MHz, acetone) δ 163.28, 148.81, 148.55,

147.35, 139.93, 137.61, 134.15, 129.32, 127.38, 120.39, 118.97, 54.90, 32.47. Anal. Calcd for $C_{15}H_{10}N_8O_8S$: C, 38.97; H, 2.18; N, 24.24; S, 6.94. Found: C, 38.7; H, 2.44; N, 23.91; S, 7.01.

5-[(3,5-Dinitrobenzyl)sulfanyl]-2-methyl-2H-tetrazole ***(40a)****:* Yield: 58% as a light beige solid; mp 94-96 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.92 (t, J = 2.1 Hz, 1H), 8.66 (d, J = 2.1 Hz, 2H), 4.55 (s, 2H), 4.30 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.02, 148.45, 142.07, 129.21, 117.99, 39.82, 34.62. Anal. Calcd for $C_9H_8N_6O_4S$: C, 36.49; H, 2.72; N, 28.37; S, 10.82. Found: C, 36.54; H, 2.82; N, 28.46; S, 10.52.

2-Benzyl-5-[(3,5-dinitrobenzyl)sulfanyl]-2H-tetrazole ***(40b)****:* Yield: 47% as a beige solid; mp 109-111 °C. ^{1}H NMR (300 MHz, acetone) δ 8.80 – 8.75 (m, 3H), 7.34 (s, 5H), 5.83 (s, 2H), 4.74 (s, 2H); ^{13}C NMR (75 MHz, acetone) δ 163.34, 149.20, 143.78, 134.64, 130.24, 129.66, 129.60, 129.24, 118.31, 57.54, 34.94. Anal. Calcd for $C_{15}H_{12}N_6O_4S$: C, 48.38; H, 3.25; N, 22.57; S, 8.61. Found: C, 48.6; H, 3.39; N, 22.43; S, 8.7.

5-[(3,5-dinitrobenzyl)sulfanyl]-2-(4-nitrobenzyl)-2H-tetrazole ***(40c)****:* Yield: 58% as a light beige solid; mp 130-132 °C. ^{1}H NMR (500 MHz, acetone) δ 8.77 (t, J = 2.1 Hz, 1H), 8.74 (d, J = 2.1 Hz, 2H), 8.22 (d, J = 8.7 Hz, 2H), 7.61 (d, J = 8.7 Hz, 2H), 6.05 (s, 2H), 4.75 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 163.83, 149.20, 149.03, 143.72, 141.60, 130.39, 130.24, 124.71, 118.32, 56.51, 34.98. Anal. Calcd for $C_{15}H_{11}N_7O_6S$: C, 43.17; H, 2.66; N, 23.49; S, 7.68. Found: C, 43.58; H, 2.89; N, 23.14; S, 7.68.

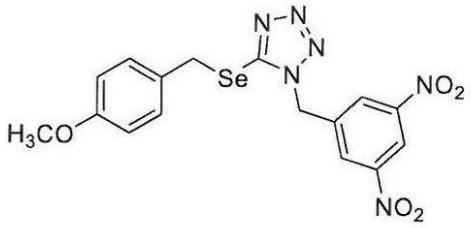
2-(3,5-dinitrobenzyl)-5-[(3,5-dinitrobenzyl)sulfanyl]-2H-tetrazole ***(40d)****:* Yield: 51% as a white solid; mp 156-159 °C (with decomposition). ^{1}H NMR (500 MHz, acetone) δ 8.95 (t, J = 2.1 Hz, 1H), 8.77 (t, J = 2.1 Hz, 1H), 8.74 (d, J = 2.1 Hz, 2H), 8.72 (d, J = 2.1 Hz, 2H), 6.27 (s, 2H), 4.77 (s, 2H); ^{13}C NMR (126 MHz, acetone) δ 163.25, 148.78, 148.37, 142.86, 137.61, 129.37, 129.22, 118.94, 117.46, 54.92, 34.09. Anal. Calcd for $C_{15}H_{10}N_8O_8S$: C, 38.97; H, 2.18; N, 24.24; S, 6.94. Found: C, 39.04; H, 2.43; N, 24.05; S, 7.02.

2-(4-chlorobenzyl)-5-[(3,5-dinitrobenzyl)sulfanyl]-2H-tetrazole ***(40f)****:* Yield: 52% as a white solid; mp 113-114 °C. ^{1}H NMR (500 MHz, acetone) δ 8.79 (t, J = 2.1 Hz, 1H), 8.75 (d, J = 2.1 Hz, 2H), 7.38 (s, 4H), 5.85 (s, 2H), 4.75 (s, 2H). ^{13}C NMR (126 MHz, acetone) δ 163.52, 149.24, 143.78, 135.16, 133.51, 131.13, 130.24, 129.77, 118.32, 56.74, 34.99. Anal.Calcd for $C_{15}H_{11}ClN_6O_4S$: C, 44.29; H, 2.73; N, 20.66; S, 7.88. Found: C, 44.40; H, 2.85; N, 20.57; s, 8.27.

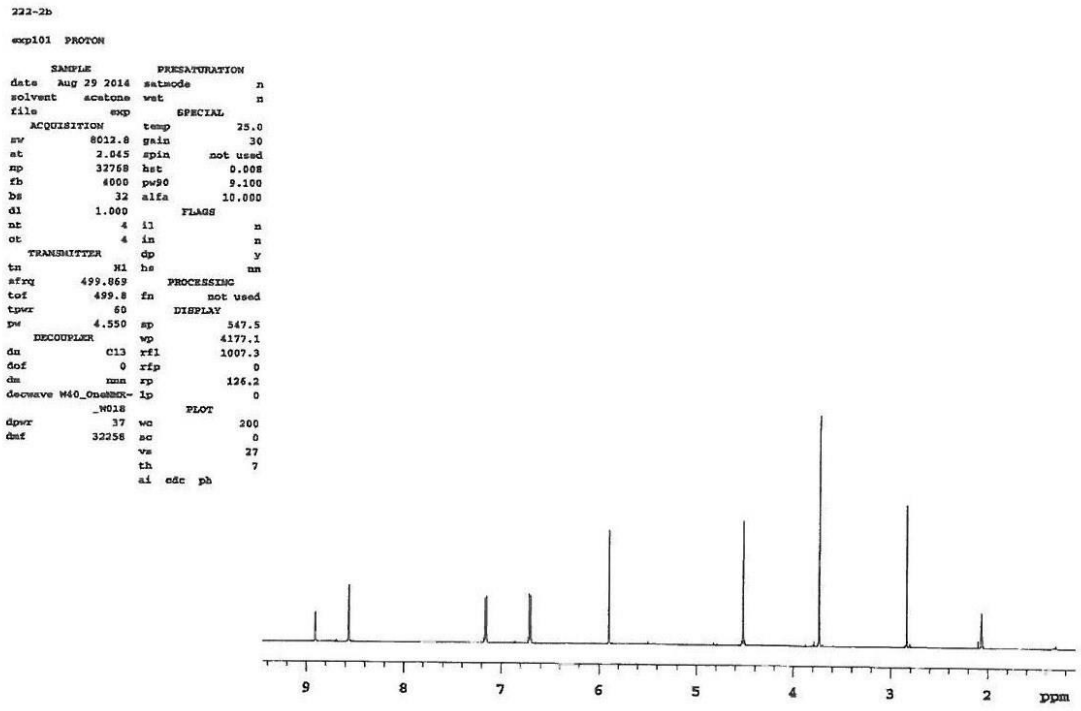
2-(3,4-dichlorobenzyl)-5-[(3,5-dinitrobenzyl)sulfanyl]-2H-tetrazole ***(40g)****:* Yield: 40% as a white solid; mp 112-113 °C. ^{1}H NMR (500 MHz, acetone) δ 8.79 (t, J = 2.1 Hz, 1H), 8.76 (d, J = 2.1 Hz, 2H), 7.60 (d, J = 2.1 Hz, 1H), 7.56 (d, J = 8.3 Hz, 1H), 7.34 (dd, J = 8.3, 2.1 Hz, 1H), 5.89 (s, 2H), 4.76 (s, 2H). ^{13}C NMR (126 MHz, acetone) δ 163.70, 149.24, 143.75, 135.34, 133.28, 133.04, 131.87, 131.49, 130.24, 129.43, 118.32, 56.13, 34.97. Anal.Calcd for $C_{15}H_{10}Cl_2N_6O_4S$: C, 40.83; H, 2.28; N, 19.05; S, 7.27. Found: C, 40.76; H, 2.45; N, 18.87; S, 7.49.

NOESY experiments

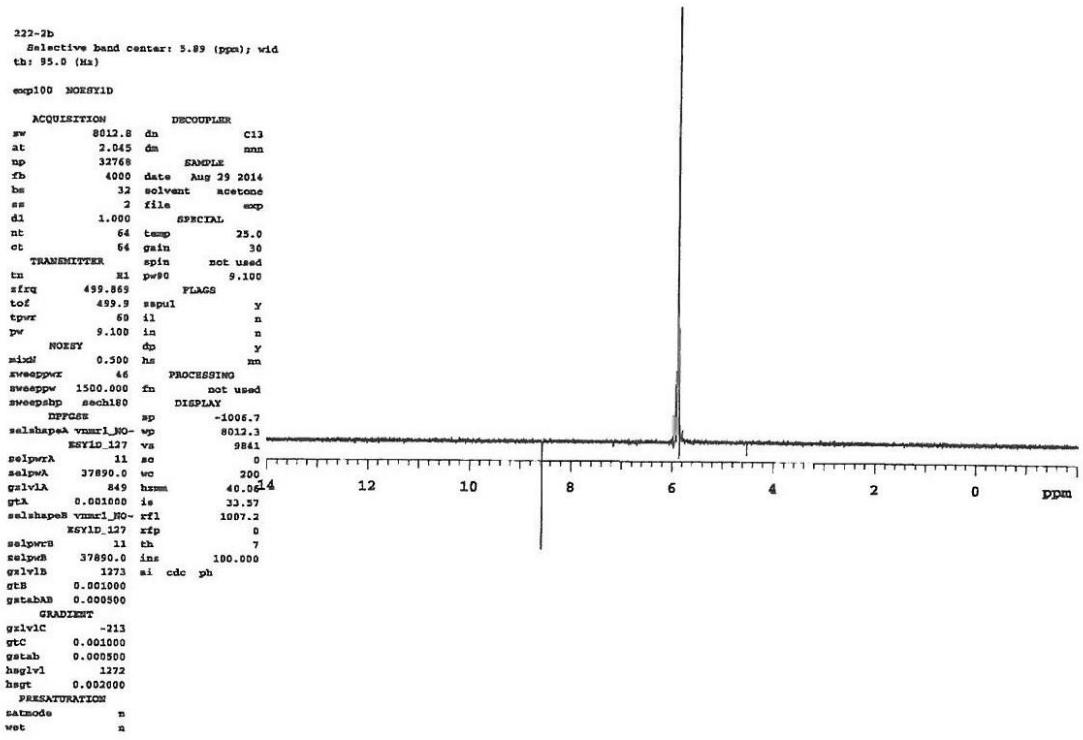
*1-(3,5-dinitrobenzyl)-5-[(4-methoxybenzyl)selanyl]-1H-tetrazole (**19d**)*



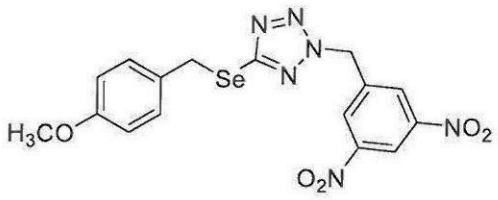
^{1}H NMR (**19d**)



1D NOESY (**19d**)


222-2b
Selective band center: 5.89 (ppm); wid
th: 95.0 (Hz)
exp100 NOESY1D
ACQUISITION
sw 8012.8
at 2.045
np 32768
fb 4000
bs 32
ss 2
d1 1.000
nt 64
ct 64
TRANSMITTER
tn H1
sfrq 499.869
tof 499.9
tpwr 59
pw 9.100
NOESY
mix 0.500
sweeppwr 46
sweeppw 1500.000
sweepshp sech180
DPFGSE
selshapeA vnmrl_NO-
ESY1D_127
selpwrA 11
selpwA 37890.0
gzlvlA 849
gtA 0.001000
selshapeB vnmrl_NO-
ESY1D_127
selpwrB 11
selpwB 37890.0
gzlvlB 1273
gtB 0.001000
gstabAB 0.000500
GRADIENT
gzlvlC -213
gtC 0.001000
gstab 0.000500
hsglvl 1272
hsgt 0.002000
PRESATURATION
satmode n
wet n
DECOUPLER
dn C13
dm nnn
SAMPLE
date Aug 29 2014
solvent acetone
file exp
SPECIAL
temp 25.0
gain 30
spin not used
pw90 9.100
FLAGS
sspul y
il n
in n
dp y
hs nn
PROCESSING
fn not used
DISPLAY
sp -1006.7
wp 8012.3
vs 9841
sc 0
wc 200
hzmm 40.06
is 33.57
rfl 1087.2
rfp 0
th 7
ins 100.000
ai cdc ph
14
12
10
8
6
4
2
0
ppm

*2-(3,5-Dinitrobenzyl)-5-[(4-methoxybenzyl)selanyl]-2H-tetrazole (**24d**)*

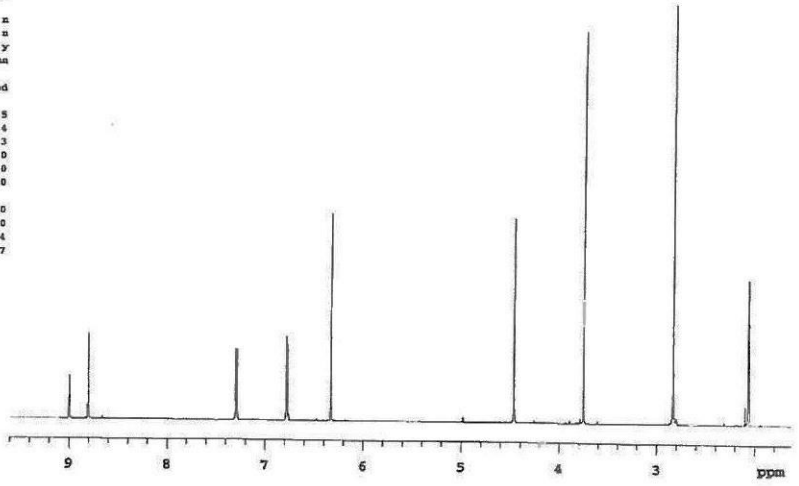


^{1}H NMR (**24d**)

222-2a

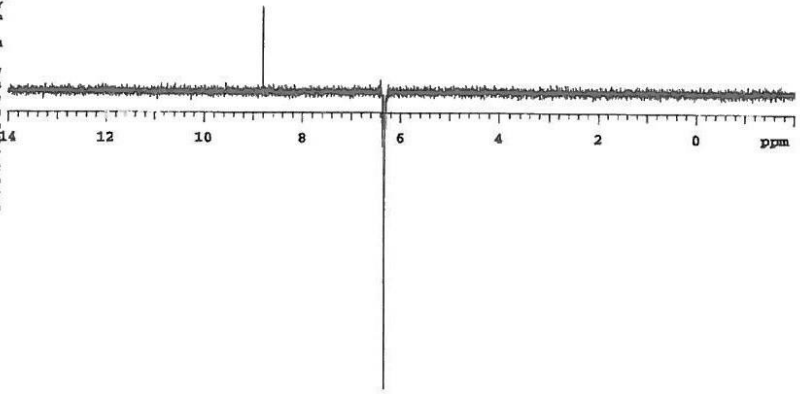
exp100 PROTON

SAMPLE		PRESATURATION	
date	Aug 29 2014	satmode	n
solvent	acetone	wet	n
file	exp	SPECIAL	
ACQUISITION		temp	25.0
sw	8012.8	gain	44
at	2.045	spin	not used
np	32768	hst	0.000
fb	4000	pw90	9.100
bs	32	alfa	10.000
dl	1.000	FLAGS	
nt	8	il	n
ct	8	in	n
TRANSMITTER		dp	y
tn	H1	hs	nn
sfrq	499.869	PROCESSING	
tof	499.8	fn	not used
tpwr	60	DISPLAY	
pw	4.550	sp	813.5
DECOUPLER		wp	3985.4
dn	C13	rf1	1007.3
dof	0	rfp	0
dm	nnn	rp	127.0
decwave	W40_OneNMR-_W018	lp	0
		PLOT	
dpwr	37	wc	200
dmf	32258	sc	0
		vs	24
		th	7
		si	cdc ph

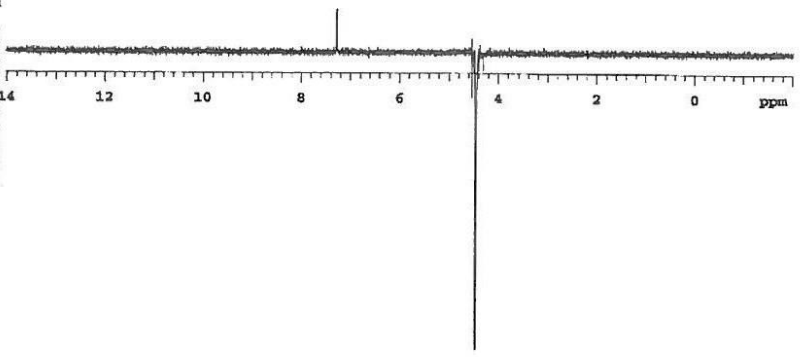


1D NOESY (**24d**)

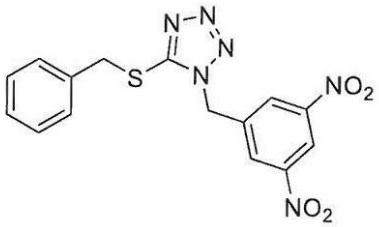
```
222-2a
  Selective band center: 6.33 (ppm); wid
th: 89.0 (Hz)

exp101  NOESY1D

   ACQUISITION          DECOUPLER
sw            8012.8  dn              C13
at             2.045  dm              nnn
np            32768          SAMPLE
fb             4000  date   Sep  2 2014
bs               32  solvent    acetone
ss                2  file            exp
d1            1.000         SPECIAL
nt               64  temp           25.0
ct               64  gain             30
   TRANSMITTER       spin       not used
tn               H1  pw90          9.100
sfrq        499.869          FLAGS
tof           499.9  ssqul            y
tpwr             60  il               n
pw            9.100  in               n
      NOESY         dp               y
mixN          0.500  hs              nn
sweeppwr         46       PROCESSING
sweeppw    1500.000  fn         not used
sweepshp    sech180        DISPLAY
      DPFGSE         sp         -1006.7
selshapeA vnmr1_NO~  wp          8012.3
          NSY1D_129  vs           23667
selpwrA          10  sc               0
selpwA       40450.0  wc             200
gzlvlA           849  hzmm         40.06
gtA        0.001000  is           33.57
selshapeB vnmr1_NO~  rfl          1007.2
          NSY1D_129  rfp               0
selpwrB          10  th               7
selpwB       40450.0  ins        100.000
gzlvlB          1273  ai  cdc  ph
gtB        0.001000
gstabAB    0.000500
    GRADIENT
gzlvlC          -213
gtC        0.001000
gstab      0.000500
hsglvl          1272
hsgt       0.002000
  PRESATURATION
satmode             n
wet                 n
```




```
222-2a
  Selective band center: 4.45 (ppm); wid
th: 119.0 (Hz)

exp101  NOESY1D

   ACQUISITION          DECOUPLER
sw            8013.8  dn              C13
at             2.045  dm              nnn
np            32768          SAMPLE
fb             4000  date   Sep  2 2014
bs               32  solvent    acetone
ss                2  file            exp
d1            1.000         SPECIAL
nt               64  temp           25.0
ct               64  gain             30
   TRANSMITTER       spin       not used
tn               H1  pw90          9.100
sfrq        499.869          FLAGS
tof           499.9  ssqul            y
tpwr             60  il               n
pw            9.100  in               n
      NOESY         dp               y
mixN          0.500  hs              nn
sweeppwr         46       PROCESSING
sweeppw    1500.000  fn         not used
sweepshp    sech180        DISPLAY
      DPFGSE         sp         -1006.7
selshapeA vnmr1_NO~  wp          8012.3
          NSY1D_128  vs           15534
selpwrA          12  sc               0
selpwA       30251.0  wc             200
gzlvlA           849  hzmm         40.06
gtA        0.001000  is           33.57
selshapeB vnmr1_NO~  rfl          1007.2
          NSY1D_128  rfp               0
selpwrB          12  th               7
selpwB       30251.0  ins        100.000
gzlvlB          1273  ai  cdc  ph
gtB        0.001000
gstabAB    0.000500
    GRADIENT
gzlvlC          -213
gtC        0.001000
gstab      0.000500
hsglvl          1272
hsgt       0.002000
  PRESATURATION
satmode             n
wet                 n
```

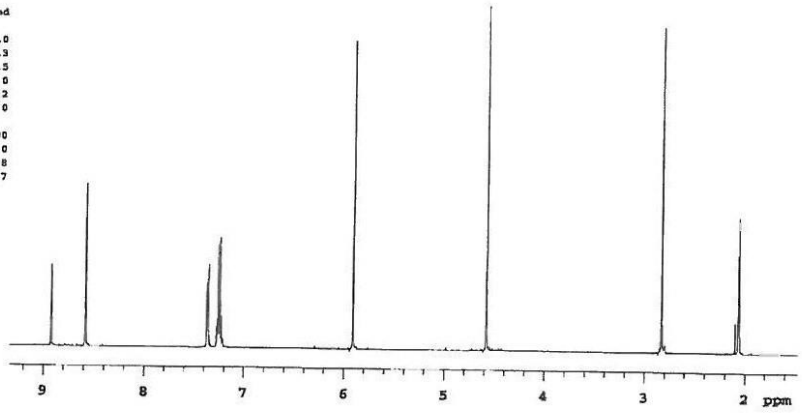


*5-(Benzylsulfanyl)-1-(3,5-dinitrobenzyl)-1H-tetrazole (**33d**)*



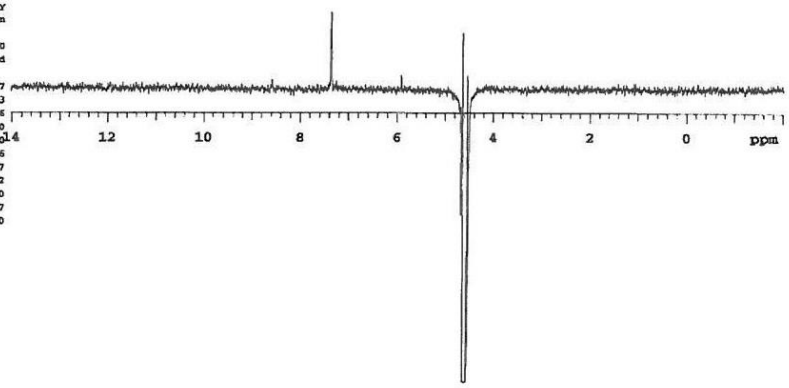
^{1}H NMR (**33d**)

```
GK237-1b

exp70  PROTON

      SAMPLE              PRESATURATION
date    Aug 26 2014  satmode          n
solvent    acetone   wet              n
file          exp        SPECIAL
   ACQUISITION       temp          25.0
sw          3930.8   gain            30
at             2.084 spin            20
np           16384   hst           0.008
fb            4000   pw90          9.100
bs              32   alfa         10.000
d1           1.000        FLAGS
nt              4   il               n
ct              4   in               n
   TRANSMITTER      dp               y
tn             H1   hs              nn
sfrq      499.869      PROCESSING
tof         200.6   fn       not used
tpwr           60      DISPLAY
pw          4.550   sp           735.0
    DECOUPLER       wp          3930.3
dn             C13  rfl         -734.5
dof              0  rfp             0
dm             nnn  rp            98.2
decwave W40_OneNMR- lp              0
               _W01H
dpwr            37       PLOT
dmf          32258   wc            200
                     sc              0
                     vs            188
                     th              7
                     ai  cdc  ph
```

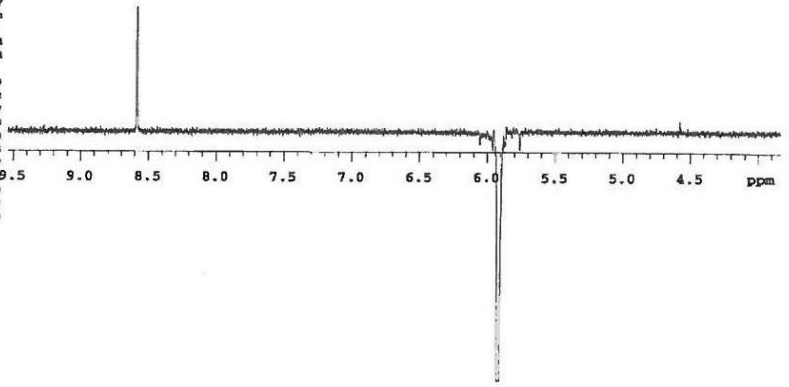


1D NOESY (**33d**)

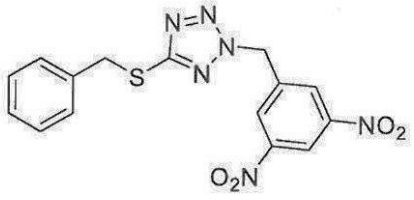
```
237-1b
  Selective band center: 4.59 (ppm); wid
th: 77.0 (Hz)

exp101  NOESY1D

   ACQUISITION
sw          8012.8
at            2.045
np           32768
fb            4000
bs              32
ss               2
d1           1.000
nt              64
ct              64
   TRANSMITTER
tn              H1
sfrq       499.869
tof          499.9
tpwr            60
pw           9.100
      NOESY
mixN         0.500
sweppwr         46
sweppw    1500.000
sweepshp   sech180
      DPFGSE
selshapeA vnmr1_NO-
        ESY1D_135
selpwrA          9
selpwA       46759.4
gzlvl1A          849
gtA         0.001000
selshapeB vnmr1_NO-
        ESY1D_135
selpwrB          9
selpwB       46759.4
gzlvl1B         1273
gtB         0.001000
gstabAB     0.000500
    GRADIENT
gzlvlC          -213
gtC         0.001000
gstab       0.000500
hsglvl          1272
hsgt        0.002000
  PRESATURATION
satmode          n
wet              n

   DECOUPLER
dn              C13
dm              nnn
      SAMPLE
date    Sep  2 2014
solvent    acetone
file             exp
     SPECIAL
temp           25.0
gain             30
spin       not used
pw90          9.100
     FLAGS
sspul            y
il               n
in               n
dp               y
hs              nn
  PROCESSING
lb            2.00
fn        not used
    DISPLAY
sp          -1006.7
wp           8012.3
vs           83516
sc               0
wc             200
hzmm         40.06
is           33.57
rfl          1007.2
rfp              0
th               7
ins          100.000
ai  cdc  ph
```



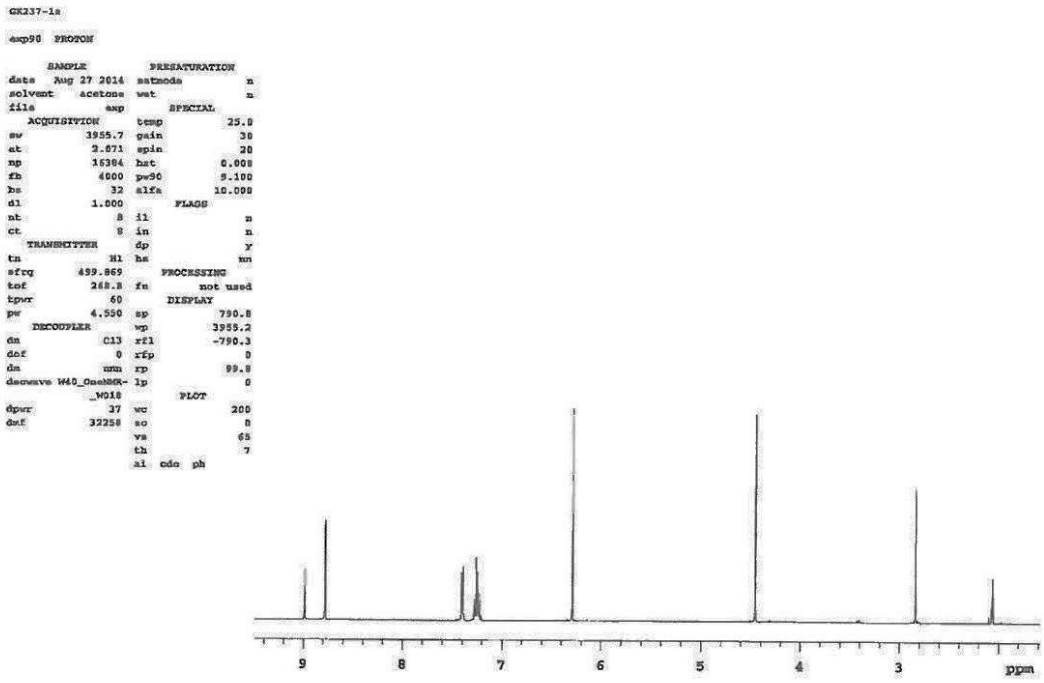

```
GX237-1b
  Selective band center: 5.90 (ppm); wid
th: 130.0 (Hz)

exp75  NOESY1D

   ACQUISITION
sw          8012.8
at            2.045
np           32768
fb            4000
bs              32
ss               2
d1           1.000
nt              64
ct              64
   TRANSMITTER
tn              H1
sfrq       499.869
tof          499.9
tpwr            60
pw           9.100
      NOESY
mixN         0.700
sweppwr         46
sweppw    1500.000
sweepshp   sech180
      DPFGSE
selshapeA vnmr1_NO-
        ESY1D_123
selpwrA         13
selpwA      27690.0
gzlvl1A          849
gtA         0.001000
selshapeB vnmr1_NO-
        ESY1D_123
selpwrB         13
selpwB      27690.0
gzlvl1B         1273
gtB         0.001000
gstabAB     0.000500
    GRADIENT
gzlvlC          -213
gtC         0.001000
gstab       0.000500
hsglvl          1272
hsgt        0.002000
  PRESATURATION
satmode          n
wet              n

   DECOUPLER
dn              C13
dm              nnn
      SAMPLE
date    Aug 27 2014
solvent    acetone
file             exp
     SPECIAL
temp           25.0
gain             30
spin              0
pw90          9.100
     FLAGS
sspul            y
il               n
in               n
dp               y
hs              nn
  PROCESSING
lb         not used
fn         not used
    DISPLAY
sp          1916.9
wp          2852.2
vs          16517
sc               0
wc             200
hzmm         14.26
is           33.57
rfl          1007.2
rfp              0
th               7
ins          100.000
ai  cdc  ph
```



5-(Benzylsulfanyl)-2-(3,5-dinitrobenzyl)-2H-tetrazole (37d)

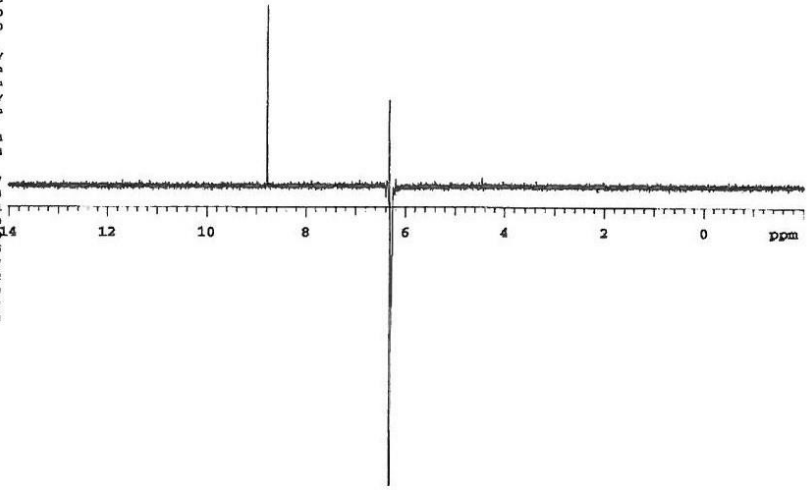


^{1}H NMR (**37d**)



1D NOESY (**37d**)

```
GK237-1a
  Selective band center: 6.28 (ppm); wid
th: 91.0 (Hz)

exp94  NOESY1D

   ACQUISITION          DECOUPLER
sw          8012.8  dn              C13
at           2.045  dm              nnn
np           32768          SAMPLE
fb            4000  date   Aug 28 2014
bs              32  solvent     acetone
ss               2  file             exp
d1           1.000          SPECIAL
nt              64  temp          25.0
ct              64  gain            30
   TRANSMITTER      spin             0
tn              H1  pw90         9.100
sfrq       499.869          FLAGS
tof          499.9  wspul            y
tpwr            60  il               n
pw           9.100  in               n
      NOESY         dp               y
mixN         0.500  hs              nn
sweeppwr        46       PROCESSING
sweeppw    1500.000  lb       not used
sweepshp   sechl80  fn       not used
      DPFGSE              DISPLAY
selshapeA vnmrl_HO-  sp         -1006.7
        NSY1D_124    wp          8013.3
selpwrA         10   vs           16151
selpwA      39560.0   sc               0
gzlvlA          849  wc             200
gtA         0.001000  hzmm         40.06
selshapeB vnmrl_HO-  is           33.57
        NSY1D_124    rf1         1007.2
selpwrB         10   rfp              0
selpwB      39560.0   th               7
gzlvlB          1273 ins         100.000
gtB         0.001000  ai  cdc  ph
gstabAB     0.000500
     GRADIENT
gzlvlC          -213
gtC         0.001000
gstab       0.000500
hsglvl          1272
hsgt        0.002000
  PRESATURATION
satmode             n
wet                 n
```



In vitro antimycobacterial assay.

Mycobacterial strains *M.tb* CNCTC My 331/88, *M. kansasii* CNCTC My 235/80 and *M. avium* CNCTC My 330/88 from the Czech National Collection of Type Cultures (CNCTC) and the clinical isolate *M. kansasii* 6509/96 were used to evaluate the antimycobacterial activity of the prepared compounds. Basic suspensions of the mycobacterial strains were prepared according to the 1.0 McFarland standard. From the basic suspension, subsequent dilutions of each strain were made: *M.tb* 10^{-3}, *M. avium* 10^{-5}, and *M. kansasii* 10^{-4}. The appropriate dilutions of the strains (0.1 mL) were added to each well of the microtiter plates containing the studied compounds.

The compounds were dissolved in dimethyl sulfoxide (DMSO) and added to the medium at concentrations of 1000, 500, 250, 125, 62, 32, 16, 8, 4, 2 and 1 μmol/L. The activities were determined via the micromethod for the determination of the minimum inhibitory concentration in Sula's semisynthetic medium (SEVAC, Prague). 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, and 21 days for the *M. kansasii* strains and after 14 and 21 days for *M.tb* and *M. avium*. Isoniazid (INH) was used as a prototype drug.

In vitro antibacterial and antifungal assays.

The broth microdilution method was used for the evaluation of in vitro antibacterial and antifungal activity. The bacteria strains included 4 Gram positive cocci (*Staphylococcus aureus* ATCC 6538 (SA), Methicillin resistant *Staphylococcus aureus* H 5996/08 (MRSA), *Staphylococcus epidermidis* H 6966/08 (SE) and *Enterococcus faecalis* J 14365/08 (EF)) and 4 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 compounds were dissolved in DMSO, and the final concentrations of the substances ranged from 0.488 to 500 μM. Mueller Hinton broth was used as the culture medium. The MIC was defined as a 95% or greater reduction of growth compared with the control. 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 for Gram negative rods.

The fungi strains 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), *Trichosporon asahii* 1188 (TA)) and 3 molds (*Aspergillus fumigatus* 231 (AF), *Absidia corymbifera* 272 (AC), and *Trichophyton mentagrophytes* 445 (TM)). The procedure was performed in RPMI 1640 medium buffered to pH 7.0 with 0.165 mol of 3-morpholinopropane-1-sulfonic acid. The concentration range was the same as that used for the aforementioned bacteria. The MIC for yeasts and yeasts-like organisms was defined as an 80% or greater (IC_{80}), and for molds 50% or greater (IC_{50}), reduction of the fungal growth compared with the control. The MIC values were determined after 24 and 48 h of incubation at 35 °C. For *T. mentagrophytes*, the final MICs were determined after 72 and 120 h of incubation. Amphotericin B and fluconazole were used as prototype drugs.

In vitro cell proliferation/viability assessment.

The standard MTT assay (Sigma Aldrich) was used according to the manufacturer´s protocol to evaluate the effects of the studied compounds on the viability of CHO-K1 cells (Chinese hamster ovary, ECACC, Salisbury, UK). The cells were cultured according to the conditions recommended by ECACC (Ham's F12 with 2 mM glutamine and 10% (v/v) fetal bovine serum) and seeded at a density of 8,000 cells per well, under standard conditions, i.e., at 37 °C under 5% CO_2. The tested compounds were dissolved in DMSO and added to the growth medium with a final concentration of DMSO below 0.5% (v/v). The cells were incubated with the compounds or vehicle alone for 24 h. Then, the medium was replaced by 10 μM MTT and the cells were allowed to convert MTT into the colored formazan product for approximately 3 h. Thereafter, the MTT-containing medium was removed and the crystals of formazan were dissolved in DMSO (100 μL). The cell viability was measured as the amount of formazan produced by mitochondria and was assessed spectrophotometrically at 570 nm with 650 nm reference wavelength on Synergy HT (BioTek, USA). Cells treated with 0.1% Triton X-100 solution for at least 15 minutes were used as a toxic control. The half maximal inhibitory concentration (IC_{50}) was then calculated using non-linear regression of at least four concentration points (in triplicate, values of the toxic controls were subtracted from each sample´s absorbance) using GraphPad Prism 5 software. The final IC_{50} value is reported as the mean and SEM of 2-4 independent measurements.

Ames Fluctuation test

The mutagenic activity of the selected substances was detected using the commercial available Muta-ChromoPlate™ Bacterial strain Kit (ebpi, Mississauga, Ontario, Canada), which is the 96-well micro-plate version of the *Salmonella typhimurium* Ames Test. The test was performed and evaluated according to the manufacturer´s instructions. The final concentration of tested substances was 50 µM (the highest concentration that enables full solubility and that showed limited cytotoxicity in mammalian cell lines). *Salmonella typhimurium* tester strains TA 98 (detection of frame shift mutagens) and TA 100 (detection of base-exchange mutations) were used. The substances were dissolved in DMSO and the final amounts of DMSO in the whole reaction mixture was 0,1%. The following standard direct-acting mutagens were used as positive controls: sodium azide for use with the strain TA100 (final amount 0.5 µg, 25ng/mL) and 2-nitrofluorene for use with the strain TA98 (final amount 30 µg; 1.5 ng/mL). A blank plate as a sterility control and plates without any tested compounds for each strain were used as a spontaneous mutation control (background control).

Results

Table S3: Evaluation of mutagenicity in Ames fluctuation assay performed with selected substances on *Salmonella typhimurium* TA100 and TA98 strains

Substance tested	Ames fluctuation assay	
	TA100	**TA98**
Sodium azide	+	n.d.
2-nitrofluorene	n.d.	+
18b	-	+
23b	-	+
36b	-	-
40b	-	+
36c	-	+
40c	-	+
36f	-	+
40f	-	+

- negative mutagenicity
+ positive mutagenicity
n.d. not determined

Statistical significance of mutagenicity for all the positive substances was at the level of 0.001.

Using the Ames fluctuation assay, except the **36b**, all the tested chemicals displayed statistically significant mutagenic effects on the strain TA98 as indicated by the number of reverse-mutated colonies. Thus, the selected chemical agents seem to act as frame-shift mutagens although we observed significant difference in potency (a number of reverse-mutated colonies) to induce reverse mutation by tested substances. On the other hand, none of the selected substances revealed genotoxic effect in strain S. typhimurium TA100.

References

1. M. Iwaoka, T. Katsuda, H. Komatsu and S. Tomoda, *J. Org. Chem.*, 2005, **70**, 321-327.
2. D. Plano, Y. Baquedano, D. Moreno-Mateos, M. Font, A. Jimenez-Ruiz, J. A. Palop and C. Sanmartin, *Eur. J. Med. Chem.*, 2011, **46**, 3315-3323.
3. C. L. Jackson, *Justus Liebigs Ann.*, 1875, **179**, 1-20.
4. P. T. Meinke and G. A. Krafft, *J. Am. Chem. Soc.*, 1988, **110**, 8671-8679.
5. T. Otsubo, F. Ogura, H. Yamaguchi, H. Higuchi and S. Misumi, *Synth. Commun.* 1980, **10**, 595-601.
6. H. M. Meshram, P. B. Thakur, B. M. Babu and V. M. Bangade, *Tetrahedron Lett.*, 2012, **53**, 1780-1785.
7. D. N. Harpp, B. T. Friedlander and R. A. Smith, *Synthesis-Stuttgart*, 1979, 181-182.
8. C. L. Jackson and F. C. Whitmore, *J. Am. Chem. Soc.*, 1915, **37**, 1915-1934.
9. C. C. Palsuledesai, S. Murru, S. K. Sahoo and B. K. Patel, *Org. Lett.*, 2009, **11**, 3382-3385.
10. G. Karabanovich, J. Roh, T. Smutný, J. Němeček, P. Vicherek, J. Stolaříková, M. Vejsová, I. Dufková, K. Vávrová, P. Pávek, V. Klimešová and A. Hrabálek, *Eur. J. Med. Chem.*, 2014, **82**, 324-340.
11. S. Vorona, T. Artamonova, Y. Zevatskii and L. Myznikov, *Synthesis-Stuttgart*, 2014, **46**, 781-786.
12. V. Aureggi and G. Sedelmeier, *Angew. Chem. Int. Ed.*, 2007, **46**, 8440-8444.

Attachment **IV**.

Hrabálek, A.; Roh, J.; Karabanovich, G.; Klimešová, V.; Němeček, J.; Pávek, P. Oxa- and thia-diazoles useful in the treatment of Tuberculosis. Patent WO2014/161516 A1.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 October 2014 (09.10.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/161516 A1

(51) International Patent Classification:
C07D 413/04 (2006.01) *C07D 285/135* (2006.01)
C07D 417/04 (2006.01) *A61K 31/4245* (2006.01)
C07D 271/113 (2006.01) *A61K 31/433* (2006.01)
C07D 285/125 (2006.01) *A61P 31/06* (2006.01)

(21) International Application Number:
PCT/CZ2013/000131

(22) International Filing Date:
16 October 2013 (16.10.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PV 2013-263 4 April 2013 (04.04.2013) CZ

(71) Applicant: **UNIVERZITA KARLOVA V PRAZE FARMACEUTICKA FAKULTA V HRADCI KRALOVE** [CZ/CZ]; Heyrovského 1203/08, 500 05 Hradec Králové (CZ).

(72) Inventors: **HRABALEK, Alexandr**; Durychova 1389, 500 12 Hradec Kralové (CZ). **ROH, Jaroslav**; S.K. Neumana 7, 586 01 Jihlava (CZ). **KARABANOVICH, Galina**; c/o Farmaceuticka fakulta University Karlovy, Heyrovského 1203/8, 500 05 Hradec Králové (CZ). **KLIMESOVA, Vera**; K Biricce 1659, 500 08 Hradec Kralove (CA). **NEMECEK, Jan**; Okruzni 237, 503 04 Cernozice (CZ). **PAVEK, Petr**; Divisova 881, 500 02 Hradec Kralove (CZ).

(74) Agent: **GUTTMANN, Michal**; Rott, Ruzicka & Guttmann, Patent, Trademark & Law Office, Vinohradska 37, 120 00 Praha 2 (CZ).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

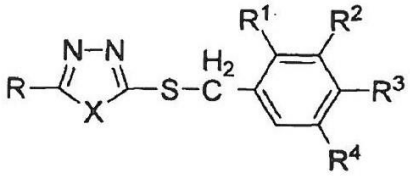
Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:

— *with international search report (Art. 21(3))*

(54) Title: OXA- AND THIA-DIAZOLES USEFUL IN THE TREATMENT OF TUBERCULOSIS



(I)

(57) Abstract: A substituted diazole of genera l formula (1) wherein X is 0 or S; R is selected from the group consisting of: H, NH2-, C_1-C_{11}n alkyl, cyclohexyl-, benzyl-, phenyl-, pyridyl- or phenyl- substituted, in positions 2, 3, 4 or 5, by one or more electron-acceptor groups comprising -N02, -N(a lkyl)3, -CF_3, CC_{13}, -CN, -COOH, -COOAlk, -COOAr, -CHO, -COAlk, -COAr, -F, -CI, -Br, -I, and/or electron-donor groups comprising -NH_2, -N Halkyl, -N(alkyl)2, -OH, -Oalkyl, -Oaryl, -$NHCOCH_3$, -NHCOalkyl; -NH-COaryl; -alkyl, -aryl, wherein when R_1 and R_3 is -$N0_2$, then R_2 a R_4 is -H, or when R_1 and R_3 is -H, then R_2 and R_4 is -$N0_2$. These compounds can bc prepared by easy synthesis and havc low toxicity and significant activity against mycobacteria including their multiresistant strains. The invention provides also a pharmaceutical preparation having substituted diazole of formula (I) as the active ingredient, as well as the use of this substituted diazole as antituberculotic.

WO 2014/161516 A1

OXA- AND THIA-DIAZOLES USEFUL IN THE TREATMENT OF TUBERCULOSIS

TECHNICAL FILED

The present invention relates to the new antituberculotics on the basis of nitro compounds, which are active against multidrug-resistant strains of mycobacteria.

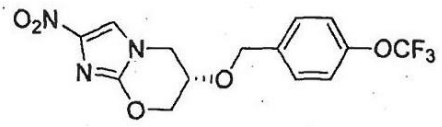
BACKGROUND ART

Tuberculosis (TB), caused by *Mycobacterium tuberculosis complex* (M.tb), belongs to the most spread infection disease worldwide for many years. Despite the present antituberculotic treatment, incidence and mortality of TB is still high (World Health Organization, Stop TB Partnership. Tuberculosis Global Facts 2011/12). Combinations of pharmaceuticals with antimycobacterial effect are used for the treatment and they are administered usually for 6 - 9 months. For many years there are at disposal only 5 effective preparations referred as antituberculotics first-line. There are namely bactericidal active isoniazid, rifampicin, pyrazinamide, streptomycin, and bacteriostatic active ethambutol. The increasing number of multidrug-resistant strain is profound public threat nowadays. Drug-resistant strains (multidrug-resistant – MDR and extensively drug resistant – XDR) require prolonging the treatment period for further up to two years with second-line drugs that are highly toxic, more expensive and less active that standard first-line drugs. The treatment includes fluoroquinolones (ofloxacin, moxifloxacin), other members of rifamycin group (rifabutin, rifapentine), kanamycin, capreomycin, amikacin and older agents such as ethionamide, capreomycin, p-aminosalicylic acid. In 2009, totally drug-resistance (TDR) strains were described. TDR strains have further resistance to second-line drugs and are virtually incurable. The great problem becomes the co-infection TB with HIV/AIDS. According to the World Health Organization one-third of world´s population has latent form of TB. Infection with HIV suppresses the immune system and allows a latent infection to reactivate into an active form of TB. Furthermore, common HIV antiretroviral therapies are not compatible with the current TB regimen. Co-infection TB and HIV is practically incurable.

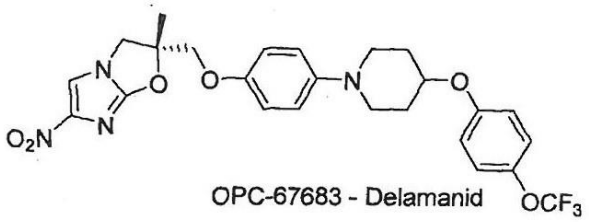
All the above mentioned reasons make a compelling case for the urgent need for new anti-TB drugs. New drugs have to be active against MDR strains, have to be compatible with

antiretroviral therapy and to cope with the latent, non-replicating form of mycobacteria. These demands can carry out the new antituberculotic active drugs with novel mechanism of action.

New potential antituberculotics in preclinical and clinical phase of development are often compounds that contain a nitro group in their molecule. For example nitroimidazol-oxazine - PA-824 (Singh, R.; Manjunatha, U.; Boshoff, H. I. M.; Ha, Y. H.; Niyomrattanakit, P.; Ledwidge, R.; Dowd, C. S.; Lee, I. Y.; Kim, P.; Zhang, L.; Kang, S.; Keller, T. H.; Jiricek, J. Barry, C. E. 3rd.

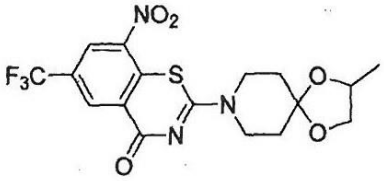


PA-824



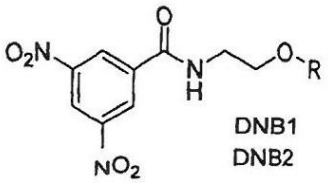
OPC-67683 - Delamanid

Bicyclic nitroimidazoles act as intracellular NO donors and kill non-replicating Mycobacterium tuberculosis. Science 2008, 322, 1392-1395), nitro-dihydroimidazo-oxazole OPC-67683 - Delamanid (Matsumoto, M.; Hashizume, H.; Tomishige, T.; Kawasaki, M.; Tsubouchi, H.; Sasaki, H.; Shimokawa, Y.; Komatsu, M. OPC-67683, and nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis in vitro and in mice. PLOSMedicine 2006, 3, 2131-2143),



BTZ038; BTZ043 (S); BTZ044 (R)

BTZ043 benzothiazinone (Makarov, V.; Manina, G.; Mikusova, K.; Möllmann, U.; Ryabova, O.; Saint-Joanis, B.; Dhar, N.; Pasca, M.R.; Buroni, S.; Lucarelli, A.P.; Milano, A.; De Rossi, E.; Belanova, M.; Bobovska, A.; Dianiskova, P.; Kordulakova, J.; Sala, C.; Fullnm, E.; Schneder, P.; McKinney, J.D.; Brodin, P.; Christophe, T.; Waddell, S.; Butcher, P.; Albrethesen, J.; Rosenkrands, I.; Brosch, R.; Nandi, V.; Bharath, S.; Gaonkar, S.; Shandil, R.K.; Balasubramanian, V.; Balganesh, T.; Tyagi, S.; Grosset, J.; Riccardi, G.; Cole, S.T. Benzothiazinones kill Mycobacterium tuberculosis by blocking arabinan synthesis. Science 2009, 324, 801-804). Further published dinitrobenzamides are following compounds



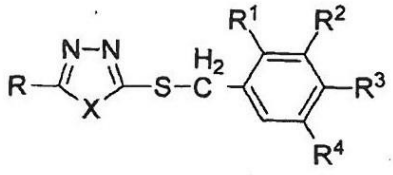
DNB1
DNB2

wherein R = 4-methoxyphenyl (DNB1) or benzyl (DNB2). (Christophe, T.; Jackson, M.; Jeon, H.K.; Fenistein, D.; Contreras-Dominguez, M.; Kim, J.; Genovesio, A.; Carralot, J.P.; Ewann, F.; Kim, E.H.; Lee, S.Y.; Kang, S.; Seo, M.S.; Park, E.J.; Škovierová, H.; Pham, H.; Riccardi, G.; Nam, J.Y.; Marsollier, L.; Kempf, M.; Joly-Guillou, M.L.; Oh, T.; Shin, W.K.; No, Z.; Nehrbass, U.; Brosch, R.; Cole, S.T.; Brodin, P. High content screening identifies decaprenyl-phosphoribose 2′epimerase as a target for intracellular antimycobacterial inhibitors. *PLOS Pathog* 2009, *5*, 1-10).

In all above mentioned compounds, nitro group has been shown to be essential for antimycobacterial activity but the mechanism of action of these molecules is different.
The Article Klimešová V. et al: Synthesis and antimycobacterial activity of 1,2,4-triazole-3-benzylsulfanyl derivatives; Il Farmaco 2004, Vol. 59, Issue 4, 279-288 discloses a compound having 4-nitrobenzylsulfanyl group, which has absolutely no antituberculotic effect. Thus, this publication contradicts the former opinion that 4-nitrobenzylsulfanyl group is a pharmacophore of the antituberculotic activity, as described in the article Foroumadi A. et al: Antituberculosis agents. V. Synthesis, evaluation of in vitro antituberculosis activity and cytotoxicity of some 2-(5-nitro-2-furyl)-1,3,4-thiadiazole derivatives; Il Farmaco 2002, Vol. 57, Issue 9, 765-769.

SUMMARY OF THE INVENTION

New compounds of general formula (I) show significant activity against *Mycobacterium tuberculosis*, against non-tuberculous mycobacteria and also against clinical isolates.
Compounds of general formula (I)



(I)

wherein

X is O or S;

R is selected from the group consisting of: H, NH_2-, C_1–C_{11} alkyl, cyclohexyl-, benzyl-, phenyl-, pyridyl- or phenyl- substituted, in positions 2, 3, 4 or 5, with one or more electron-acceptor groups comprising -NO_2, -N(alkyl)$_3$, -CF_3, CCl_3, -CN, -COOH, -COOAlk, -COOAr, -CHO, -COAlk, -COAr, -F, -Cl, -Br, -I, and/or electron-donor groups comprising -NH_2, -NHalkyl, -N(alkyl)$_2$, -OH, -Oalkyl, -Oaryl, -$NHCOCH_3$, -NHCOalkyl; -NHCOaryl; -alkyl, -aryl,
wherein when R_1 and R_3 is –NO_2, then R_2 a R_4 is -H, or when R_1 and R_3 is –H, then R_2 and R_4 is –NO_2.

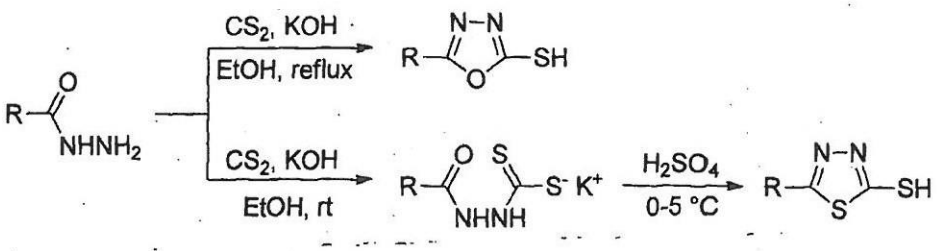
The term „electron-donor groups" as used herein, refers to substituents that increase electron density on the phenyl substituent R. Examples of electron-donor groups include especially -NH_2, -NHAlk, -NAlk_2, -OH, -OAlk, -OAr, -$NHCOCH_3$, -NHCOAlk, -NHCOAr, -Alk, -Ar, wherein Alk = Alkyl, Ar = Aryl. The term „electron-acceptor groups" as used herein, refers to substituents that decrease electron density on the phenyl substituent R. Examples of electron-donor groups include - NO_2, -NAlk_3, -CF_3, CCl_3, -CN, -COOH, -COOAlk, -COOAr, -CHO, -COAlk, -COAr, -F, -Cl, -Br, -I, wherein Alk = alkyl, Ar = aryl. (Source: (a) John McMurry: Organic Chemistry, Sixth edition, 2004, Brooks/Cole, a Thomson Learning Company; b) L. G. Wade, Jr.: Organic Chemistry, Sixth edition, 2006, Pearson Prentice Hall Inc.; c) J. Clayden, N. Greeves, S. Warren, P. Wothers: Organic Chemistry, 2001, Oxford University Press)

Another aspect of the invention is the use of the above mentioned substituted diazole of general formula (I) according to the current invention as antituberculotic agent.

Further aspect of the invention also relates to a pharmaceutical preparation containing the substituted diazole of general formula (I) as the active ingredient.

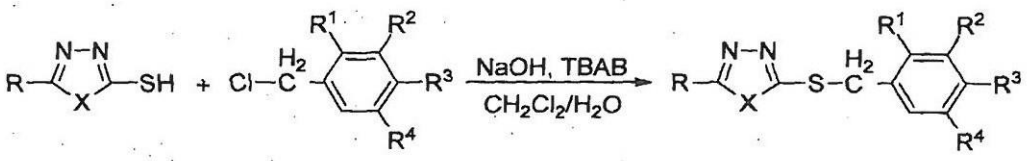
Compounds of general formula (I) can be obtained by routine methods of organic synthesis. The starting compounds for synthesis of the compounds of general formula (I) were prepared by synthetic procedures as described in a) Baron, M.; Wilson, C. V.: J. Org. Chem. 1958, 23 (7), 1021-1023; b) Zarghi, A.; Faizi, M.; Shafaghi, B.; Ahadian, A.; Khojastehpoor, H. R.; Zanganeh, V.; Tabatabai, S. A.; Shafiee, A.: Bioorg. Med. Chem. Lett. 2005, 15 (12), 3126-3129; c) Wei, M. X.; Feng, L.; Li, X. Q.; Zhou, X. Z.; Shao, Z. H.: Eur. J. Med. Chem. 2009,

44 (8), 3340-3344; d)Hasan, A.; Thomas, N. F.; Gapil, S.: Molecules 2011, 16 (2), 1297-1309. (Scheme 1)



Scheme 1.

The final products of general formula (I) were prepared by Williamson synthesis from the relevant precursor with the appropriate substituted benzyl halides (Scheme 2). The synthetic route is very simple and raw materials for them are easily accessible and also cheap.



I

Scheme 2.

TBAB = tetra-n-butylamonium bromide

The prepared compounds corresponding to general formula (I) were evaluated in Regional Institute of Public Health, Ostrava (Department for Diagnostic of Mycobacteria, Partyzánské náměstí 7, 702 00 Ostrava) in *in vitro* conditions in Šula´s semisynthetic liquid medium (SEVAC, Prague) and the minimum inhibitory concentrations (MIC) were determined. The antimycobacterial activity was tested against Czech National Collection strains Mycobacterium tuberculosis CNCTC My 331/88, M. avium CNCTC My 330/88, and M. kansasii CNCTC My 235/80, and a clinical isolate M. kansasii 6509/96. Isoniazid (INH) was used as a standard in each assay. The results are shown in Table 4.

The most active compounds of general formula (I) were also evaluated for their activity against multidrug resistant strains of *Mycobacterium tuberculosis* (MDR strains). The strains are labelled as *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. These strains were clinically isolated from patients and are deposited in Regional Institute of Public Health, Ostrava (Department for Diagnostic of Mycobacteria, Partyzánské náměstí 7, 702 00 Ostrava). The sensitivity/resistance of the mentioned clinically isolated strains to common antituberculotics and antibiotics used are summarized in Table 5a. The activity of compounds of general formula (I) was expressed as minimum inhibitory concentration (MIC). The results were obtained under the same conditions as described above. Isoniazid (INH) was used as a standard in each assay. The results are shown in Table 5b.

The most active compounds of general formula (I) that exhibited significant activity against sensitive and multiresistant strain of *Mycobacterium tuberculosis* were evaluated in basic toxicity tests on human hepatotocytes. The results show that even at the highest concentration tested (20 μmol/l) of the evaluated potential antituberculotics of general formula (I) viability of hepatocytes has not been influenced.

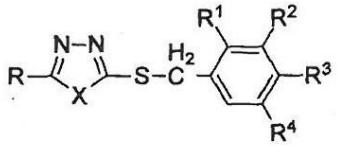
The most active compounds by general formula (I) were also evaluated for cytotoxicity according to the method CellTiter96® on cell line JEG3. Cell viability has not been influenced even at concentrations of the evaluated compounds of 10 μmol/l. In both toxicity tests, 100 times up to 200 times higher concentrations than the antituberculotic activity concentration were used.

All compounds of general formula (I) contain in their molecule at least one nitro group but their toxicity in the tests made is very low. These compounds are exceptional for their low toxicity because the nitro group in drug molecules is usually the carrier of higher toxicity or excitability. According to the knowledge of medicinal chemistry, the substitution with nitro group in the synthesis of novel potential drugs has been accounted as undesirable. In addition, the selected five-membered heterocycles from the group of 1,3,4-oxadiazole and 1,3,4-thiadiazole play a substantial role because the similarly substituted 1,3,4- triazoles, that were previously prepared by our group, exhibited virtually no antituberculotic activity.

Accordingly, the subject matter of the present invention comprises a combination of a certain five-membered nitrogen-containing heterocycle and the dinitrophenyl group which is bound to said heterocycle by a short methylsulfanyl linker group.

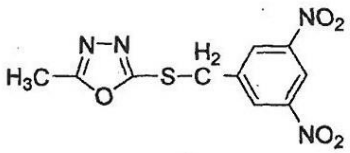
Examples

The following examples describe how to prepare substituted diazoles of general formula (I)



wherein X, R, R^1, R^2, R^3, R^4 have the above mentioned meaning.

Example 1: 2-(3,5-dinitrobenzylsulfanyl)-5-methyl-1,3,4-oxadiazole (**1**)

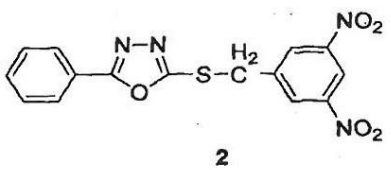


1

2-(3,5-Dinitrobenzylsulfanyl)-5-methyl-1,3,4-oxadiazole (**1**) was prepared by reaction of 5-methyl-1,3,4-oxadiazole-5-thiol (0.1 g; 0,76 mmol) with 3,5-dinitrobenzyl chloride (0.15 g; 0.69 mmol) in the system of 5 ml CH_2Cl_2/ 5 ml H_2O, in the presence of tetrabutylammonium bromide (22 mg; 0.07 mmol) and NaOH (33 mg; 0.83 mmol) at room temperature for 12 hours. After completion of the reaction, the organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated in a vacuum evaporator. The crude product 1 was isolated and purified by column chromatography on silica gel (mobile phase: hexane/ethyl acetate 4:1).

The starting 3,5-dinitrobenzyl chloride is a commercially available compound. The starting 5-methyl-1,3,4-oxadiazole-5-thiol was prepared according to published procedures: a) Hoggarth, E. J. Chem. Soc. 1952, 4811. b) Lacasse, G.; Muchowski, J. M. Can. J. Chem. 1972, 50, 3082.)

Example 2: 2-(3,5-dinitrobenzylsulfanyl)-5-phenyl-1,3,4-oxadiazole (**2**)



2

2-(3,5-Dinitrobenzylsulfanyl)-5-phenyl-1,3,4-oxadiazole (**2**) was prepared by reaction of 5-phenyl-1,3,4-oxadiazole-5-thiol (0.1g; 0.56 mmol) with 3,5-dinitrobenzyl chloride (0.11 g;

0.51 mmol) in the system of 5 ml CH_2Cl_2/ 5 ml H_2O, in the presence of tetrabutylammonium bromide (16 mg; 0.05 mmol) and NaOH (25 mg; 0.61 mmol) at room temperature for 24 hours. After completion of the reaction, the organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated in a vacuum evaporator. The crude product **2** was isolated and purified by column chromatography on silica gel (mobile phase: hexane/ethyl acetate 4:1).

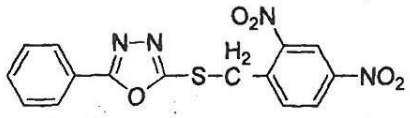
The starting 3,5-dinitrobenzyl chloride is a commercially available compound. The starting 5-phenyl-1,3,4-oxadiazole-5-thiol was prepared according to published procedures: a) Baron, M.; Wilson, C. V.: J. Org. Chem. 1958, 23 (7), 1021-1023; b) Zarghi, A.; Faizi, M.; Shafaghi, B.; Ahadian, A.; Khojastehpoor, H. R.; Zanganeh, V.; Tabatabai, S. A.; Shafiee, A.: Bioorg. Med. Chem. Lett. 2005, 15 (12), 3126-3129; c) Wei, M. X.; Feng, L.; Li, X. Q.; Zhou, X. Z.; Shao, Z. H.: Eur. J. Med. Chem. 2009, 44 (8), 3340-3344; d) Hasan, A.; Thomas, N. F.; Gapil, S.: Molecules 2011, 16 (2), 1297-1309.).

Numerous other compounds of general formula (I) (compounds 3 - 15) can be prepared using the above mentioned synthetic procedure.

Table 1. Examples of compounds of general formula (I) (compounds **2 - 15**)

	X	R^1	R^2	R^3	R^4	R
2	O	H	NO_2	H	NO_2	C_6H_5
3	O	H	NO_2	H	NO_2	2-ClC_6H_4
4	O	H	NO_2	H	NO_2	3-ClC_6H_4
5	O	H	NO_2	H	NO_2	4-ClC_6H_4
6	O	H	NO_2	H	NO_2	2,4-$(Cl)_2C_6H_3$
7	O	H	NO_2	H	NO_2	4-$CH_3C_6H_4$
8	O	H	NO_2	H	NO_2	3-$CH_3OC_6H_4$
9	O	H	NO_2	H	NO_2	4-$CH_3OC_6H_4$
10	O	H	NO_2	H	NO_2	3,5$(CH_3)_2OC_6H_3$
11	O	H	NO_2	H	NO_2	3-$NO_2C_6H_4$
12	O	H	NO_2	H	NO_2	4-$NO_2C_6H_4$
13	O	H	NO_2	H	NO_2	3,5$(NO_2)_2C_6H_3$
14	O	H	NO_2	H	NO_2	Pyridin-4-yl
15	O	H	NO_2	H	NO_2	*n*-$C_{11}H_{23}$

Example 3: 2-(2,4-dinitrobenzylsulfanyl)-5-phenyl-1,3,4-oxadiazole (**16**)



16

2-(2,4-Dinitrobenzylsulfanyl)-5-phenyl-1,3,4-oxadiazole (**16**) was prepared by reaction of 5-phenyl-1,3,4-oxadiazole-5-thiol (0.1 g; 0.56 mmol) with 2,4-dinitrobenzyl chloride (0.11 g; 0.51 mmol) in the system of 5 ml CH_2Cl_2/ 5 ml H_2O, in the presence of tetrabutylammonium bromide (16 mg; 0.05 mmol) and NaOH (25 mg; 0.61 mmol) at room temperature for 24 hours. After completion of the reaction, the organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated in a vacuum evaporator. The crude product **16** was isolated and purified by column chromatography on silica gel (mobile phase: hexane/ethyl acetate 4:1).

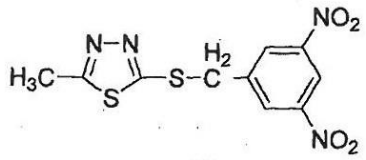
The starting 2,4-dinitrobenzyl chloride is a commercially available compound. The starting 5-phenyl-1,3,4-oxadiazole-5-thiol was prepared according to published procedures: a) Baron, M.; Wilson, C. V.: J. Org. Chem. 1958, 23 (7), 1021-1023; b) Zarghi, A.; Faizi, M.; Shafaghi, B.; Ahadian, A.; Khojastehpoor, H. R.; Zanganeh, V.; Tabatabai, S. A.; Shafiee, A.: Bioorg. Med. Chem. Lett. 2005, 15 (12), 3126-3129; c) Hasan, A.; Thomas, N. F.; Gapil, S.: Molecules 2011, 16 (2), 1297-1309.).

Numerous other compounds of general formula (I) (compounds **17** - **21**) can be prepared according to the above mentioned synthetic procedures.

Table 2. Examples of compounds of general formula (I) (compounds **16** - **21**)

	X	R^1	R^2	R^3	R^4	R
16	O	NO_2	H	NO_2	H	C_6H_5
17	O	NO_2	H	NO_2	H	4-$CH_3OC_6H_4$
18	O	NO_2	H	NO_2	H	4-ClC_6H_4
19	O	NO_2	H	NO_2	H	3-$NO_2C_6H_4$
20	O	NO_2	H	NO_2	H	4-$NO_2C_6H_4$
21	O	NO_2	H	NO_2	H	Pyridin-4-yl

Example 4: 2-(3,5-dinitrobenzylsulfanyl)-5-methyl-1,3,4-thiadiazole (**22**)

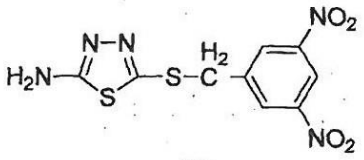


22

2-(3,5-Dinitrobenzylsulfanyl)-5-methyl-1,3,4-thiadiazole (**22**) was prepared by reaction of 5-methyl-1,3,4-thiadiazole-5-thiol (0.165 g; 1.24 mmol) with 3,5-dinitrobenzyl chloride (0.246 g; 1.14 mmol) in the system of 5 ml CH_2Cl_2/ 5 ml H_2O, in the presence of tetrabutylammonium bromide (32 mg; 0.1 mmol) and NaOH (54 mg; 1.35 mmol) at room temperature for 12 hours. Then, the organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated in a vacuum evaporator. The crude product **22** was isolated and purified by column chromatography on silica gel (mobile phase: hexane/ethyl acetate 4:1).

The starting 3,5-dinitrobenzyl chloride and 5-methyl-1,3,4-thiadiazole-5-thiol are commonly commercially available compounds.

Example 5: 2-amino-5-(3,5-dinitrobenzylsulfanyl)-1,3,4-thiadiazole (**23**)

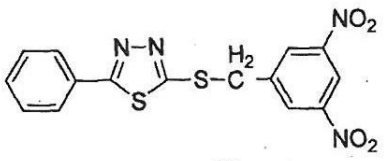


23

2-Amino-5-(3,5-dinitrobenzylsulfanyl)-1,3,4-thiadiazole (**23**) was prepared by reaction of 5-amino-1,3,4-thiadiazole-5-thiol (0.165 g; 1.24 mmol) with 3,5-dinitrobenzyl chloride (0.246 g; 1.14 mmol) in the system of 5 ml CH_2Cl_2/ 5 ml H_2O, in the presence of tetrabutyl-ammonium bromide (32 mg; 0.1 mmol) and NaOH (54 mg; 1.35 mmol) at room temperature for 12 hours. Then, the organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated in a vacuum evaporator. The crude product **23** was isolated and purified by column chromatography on silica gel (mobile phase: hexane/ethyl acetate 4:1).

The starting 3,5-dinitrobenzyl chloride and 5-amino-1,3,4-thiadiazole-5-thiol are commercially available compounds.

Example 6: 2-(3,5-dinitrobenzylsulfanyl)-5-phenyl-1,3,4-thiadiazole (**25**)



25

2-(3,5-Dinitrobenzylsulfanyl)-5-phenyl-1,3,4-thiadiazole (**25**) was prepared by reaction of 5-phenyl-1,3,4-thiadiazole-5-thiol (0.1 g; 0.51 mmol) with 3,5-dinitrobenzyl chloride (0.1 g; 0.46 mmol) in the system of 5 ml CH_2Cl_2/ 5 ml H_2O, in the presence of tetrabutyl-ammonium bromide (16 mg; 0.05 mmol) and NaOH (23 mg; 0.57 mmol) at room temperature for 24 hours. After completion of the reaction, the organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated in a vacuum evaporator. The crude product **25** was isolated and purified by column chromatography on silica gel (mobile phase: hexane/ethyl acetate 4:1).

The starting 3,5-dinitrobenzyl chloride is a commercially available compound. The starting 5-phenyl-1,3,4-thiadiazole-5-thiol was prepared according to published procedures: (a) Baron, M.; Wilson, C. V.: J. Org. Chem. **1958,** 23 (7), 1021-1023; b) Wei, M. X.; Feng, L.; Li, X. Q.; Zhou, X. Z.; Shao, Z. H.: Eur. J. Med. Chem. **2009,** 44 (8), 3340-3344).

Numerous other compounds of general formula (I) (compounds **24, 26 - 34**) can be prepared using the above mentioned synthetic procedures.

Table 3: Examples of compounds of general formula (I) (compounds **24 – 34**)

	X	R^1	R^2	R^3	R^4	R
24	S	H	NO_2	H	NO_2	Pyridin-4-yl
25	S	H	NO_2	H	NO_2	C_6H_5
26	S	H	NO_2	H	NO_2	2-ClC_6H_4
27	S	H	NO_2	H	NO_2	3-ClC_6H_4
28	S	H	NO_2	H	NO_2	4-ClC_6H_4
29	S	H	NO_2	H	NO_2	4-$CH_3C_6H_4$
30	S	H	NO_2	H	NO_2	3,5-$(CH_3)_2C_6H_3$
31	S	H	NO_2	H	NO_2	3-$CH_3OC_6H_4$
32	S	H	NO_2	H	NO_2	4-$CH_3OC_6H_4$
33	S	H	NO_2	H	NO_2	3-$NO_2C_6H_4$
34	S	H	NO_2	H	NO_2	4-$NO_2C_6H_4$

Table 4: The minimum inhibitory concentration *in vitro* (expressed in μmol.l^{-1}) of compounds of general formula (I) (micromethod for determination of minimum inhibitory concentration in Šula´s semisynthetic medium on plastic P-microplates; MICs determined after incubation at 37 °C for 14 and 21 days for *M. tuberculosis* and *M. avium*, for 7, 14 and 21 days for *M. kansasii).*

	M. tuberculosis	**M. avium**	**M. kansasii**	**M. kansasii**
	My 331/88	**My 330/88**	**My 235/80**	**6509/96**
1	4 / 8	500 / 500	4 / 8 / 16	16 / 16 / 32
2	0.06 / 0.06	16 / 32	0.5 / 1 / 1	0.5 / 1 / 2
3	0.125 / 0.125	16 / 32	0.125 / 0.25 / 0.5	0.125 / 0.5 / 1
4	0.03 / 0.06	16 / 16	0.125 / 0.25 / 0.5	0.125 / 0.5 / 1
5	0.125 / 0.125	>125 / >125	0.125 / 0.25 / 0.25	0.125 / 0.25 / 0.5
6	0.03 / 0.06	16 / 32	0.125 / 0.5 / 0.5	0.125 / 0.25 / 0.5
7	0.03 / 0.06	8 / 16	0.125 / 0.125 / 0.125	0.06 / 0.125 / 0.125
8	0.125 / 0.125	16 / 16	0.125 / 0.25 / 0.5	0.125 / 0.5 / 1
9	0.125 / 0.125	16 / 32	0.125 / 0.25 / 0.25	0.125 / 0.25 / 0.5
10	0.125 / 0.125	16 / 16	0.125 / 0.25 / 0.5	0.125 / 0.5 / 1
11	0.125 / 0.125	16 / 16	0.25 / 0.5 / 0.5	0.5 / 1 / 2
12	0.125 / 0.125	>125 / >125	0.25 / 0.25 / 0.5	0.25 / 0.5 / 1
13	0.25 / 0.5	4 / 4	0.5 / 1 / 1	0.5 / 1 / 1
14	0.5 / 0.5	62.5 / 62.5	1 / 2 / 2	2 / 2 / 4
15	0.125 / 0.25	8 / 16	0.5 / 1 / 1	0.5 / 1 / 1
16	2 / 4	>16 / >16	4 / 8 / 16	2 / 8 / 8
17	0.5 / 1	4 / 4	2 / 8 / 16	2 / 8 / 16
18	2 / 2	8 / 16	2 / 4 / 4	4 / 8 / 8
19	16 / 16	32 / 32	8 / 16 / 32	16 / 16 / 32
20	2 / 2	>125/>125	2 / 4 / 8	2 / 4 / 8
21	8 / 8	62.5 / 62.5	16 / 32 / 32	32 / 32 / 32
22	4 / 4	125 / 125	4 /4 / 8	8 / 8 / 16
23	16 / 32	1000 / 1000	16 / 32 / 32	32 / 32 / 62.5
24	0.125 / 0.125	>125/>125	0.5 / 1 / 1	0.5 / 1 / 1

25	0.06 / 0.125	8 / 16	0.25 / 0.5 / 1	0.25 / 0.5 / 1
26	0.125 / 0.125	16 / >16	0.5 / 1 / 1	0.5 / 1 / 1
27	0.125 / 0.125	8 / 16	0.25 / 1 / 1	0.25 / 0.5 / 0.5
28	0.125 / 0.125	8 / 16	0.25 / 1 / 1	0.25 / 0.5 / 1
29	0.125 / 0.125	>125/>125	0.5 / 0.5 / 1	0.5 / 1 / 2
30	0.125 / 0.125	>16 / >16	0.25 / 1 / 1	0.25 / 0.5 / 0.5
31	0.03 / 0.06	8 / 16	0.25 / 0.5 / 0.5	0.25 / 0.5 / 0.5
32	0.125 / 0.125	8 / 16	0.25 / 1 / 1	0.25 / 1 / 1
33	0.125 / 0.125	>16 / >16	0.25 / 1 / 1	0.5 / 1 / 1
34	0.125 / 0.125	>16 / >16	0.25 / 0.5 / 1	0.25 / 0.5 / 1
INH	**0.5 / 1**	**>250 / >250**	**>250 / >250 />250**	**2 /4 / 4**

Table 5a: The minimum inhibitory concentration *in vitro* (expressed in $\mu mol.l^{-1}$) of selected antibiotics and antituberculotics (micromethod for determination of minimum inhibitory concentration in Šula´s semisynthetic medium on plastic P-microplates after incubation for 14 and 21 days) for multidrug resistant strains of *M. tuberculosis*

M.tuberculosis	**7357/1998**	**234/2005**	**8666/2010**	**9449/2007**	**Praha 1**	**Praha 4**	**Praha 131**
Streptomycin	>27.5 R	27.5 R	>27.5 R	>27.5 R	13.7 R	>27.5 R	>27.5 R
Isoniazid	14.6 R	14.6 R	29.2 R	58.3 R	14.6 R	14.6 R	14.6 R
Etambutol	19.6 R	19.6 R	19.6 R	9.8 C	39.2 R	19.6 R	39.2 R
Rifampicin	>9.7 R	>9.7 R	>9.7 R	>9.7 R	>9.7 R	>9.7 R	>9.7 R
Ofloxacin	11.1 R	0.69 C	11.1 R	2.75 C	1.38 C	>22.2 R	22.2 R
Gentamicin	1.05 C	0.26 C	2.09 C	1.05 C	1.05 C	0.52 C	>8.37 R
Clofazimine	0.13 C	0.06 C	2.11 R	0.13 C	0.53 R	0.53 R	0.26 C
Amikacin	0.85 C	0.43 C	1.7 C	0.43 C	0.43 C	0.85 C	>27.2 R

R - strain resistant to mentioned antituberculotic

C - strain sensitive to mentioned antituberculotic

Table 5b: The minimum inhibitory concentration *in vitro* (expressed in $\mu mol.l^{-1}$) of compounds by general formula (I) (micromethod for determination of minimum inhibitory concentration in Šula´s semisynthetic medium on plastic P-microplates) after incubation at 37 °C for 14 and 21 days for multidrug resistant strains of *M. tuberculosis*

	M. tuberculosis (MDR strains)					
	234/2005	**9449/2007**	**8666/2010**	**Praha 1**	**Praha 4**	**Praha 131**
2	0.03/0.06	0.06/0.06	0.03/0.06	0.06/0.06	0.03/0.06	0.03/0.06
9	0.06/0.125	0.06/0.125	0.06/0.06	0.06/0.125	0.06/0.125	0.06/0.125
12	0.06/0.125	0.06/0.125	0.06/0.06	0.06/0.125	0.06/0.125	0.06/0.125
32	0.125/0.25	0.125/0.25	0.06/0.125	0.125/0.25	0.125/0.25	0.125/0.25
34	0.06/0.06	0.06/0.06	0.03/0.06	0.06/0.06	0.06/0.06	0.06/0.06

Table 6. NMR spectra of compounds of general formula (I).

	^{1}H NMR	^{13}C NMR
1	^{1}H NMR (500 MHz, Acetone) δ 8.85 (s, 3H), 4.81 (s, 2H, SCH_2), 2.45 (s, 3H, CH_3).	^{13}C NMR (126 MHz, Acetone) δ 166.23, 163.04, 149.31, 143.18, 130.41, 118.62, 35.08, 10.71.
2	^{1}H NMR (300 MHz, Acetone) δ 8.92 (d, J = 2.1 Hz, 2H), 8.86 (t, J = 2.1 Hz, 1H), 8.02 – 7.92 (m, 2H), 7.62 – 7.52 (m, 3H), 4.92 (s, 2H, SCH_2).	^{13}C NMR (75 MHz, Acetone) δ 166.77, 163.63, 149.37, 143.19, 132.71, 130.49, 130.09, 127.28, 124.41, 118.70, 35.27.
3	^{1}H NMR (300 MHz, DMSO) δ 8.84 (d, J = 2.1 Hz, 2H), 8.74 (t, J = 2.1 Hz, 1H), 7.91 (dd, J = 7.7, 1.7 Hz, 1H), 7.73 – 7.45 (m, 3H), 4.82 (s, 2H, SCH_2).	^{13}C NMR (75 MHz, DMSO) δ 163.69, 163.64, 148.11, 142.16, 133.51, 131.82, 131.36, 131.30, 129.90, 128.01, 122.19, 118.06, 34.36.
4	^{1}H NMR (300 MHz, DMSO) δ 8.96 – 8.64 (m, 3H), 7.97 – 7.84 (m, 2H), 7.72 – 7.53 (m, 2H), 4.83 (s, 2H, SCH_2).	^{13}C NMR (75 MHz, DMSO) δ 164.49, 163.49, 148.10, 142.27, 134.23, 132.05, 131.56, 129.89, 126.11, 125.27, 124.98, 118.03, 34.22.
5	^{1}H NMR (500 MHz, $CDCl_3$) δ 8.97 (t, J = 2.1 Hz, 1H), 8.74 (d, J = 2.1 Hz, 2H), 7.91 (d, J = 8.7 Hz, 2H), 7.48 (d, J = 8.7 Hz, 2H), 4.67 (s, 3H, SCH_2).	^{13}C NMR (126 MHz, $CDCl_3$) δ 165.70, 162.33, 148.57, 140.82, 138.35, 129.54, 129.33, 127.95, 121.58, 118.43, 34.90.

6	^{1}H NMR (300 MHz, DMSO) δ 8.84 (d, J = 2.0 Hz, 2H), 8.74 (t, J = 2.1 Hz, 1H), 7.95 (d, J = 8.5 Hz, 1H), 7.88 (d, J = 2.0 Hz, 1H), 7.62 (dd, J = 8.5, 2.0 Hz, 1H), 4.82 (s, 2H, SCH_2).	^{13}C NMR (75 MHz, DMSO) δ 163.81, 163.03, 148.10, 142.10, 137.39, 132.89, 132.47, 130.92, 129.89, 128.31, 121.19, 118.06, 34.33.
7	^{1}H NMR (300 MHz, DMSO) δ 8.84 (d, J = 2.1 Hz, 2H), 8.73 (t, J = 2.1 Hz, 1H), 7.81 (d, J = 8.1 Hz, 2H), 7.36 (d, J = 8.1 Hz, 2H), 4.81 (s, 2H, SCH_2), 2.37 (s, 3H, CH_3).	^{13}C NMR (75 MHz, DMSO) δ 165.70, 162.55, 148.09, 142.47, 142.34, 130.09, 129.87, 126.54, 120.31, 118.02, 34.24, 21.29.
8	^{1}H NMR (300 MHz, DMSO) δ 8.84 (d, J = 2.1 Hz, 2H), 8.73 (t, J = 2.1 Hz, 1H), 7.52 – 7.38 (m, 3H), 7.20 – 7.13 (m, 1H), 4.82 (s, 2H, SCH_2), 3.82 (s, 3H, OCH_3).	^{13}C NMR (75 MHz, DMSO) δ 165.50, 163.01, 159.80, 148.08, 142.27, 130.86, 129.87, 124.20, 118.89, 118.30, 118.03, 111.32, 55.62, 34.25.
9	^{1}H NMR (300 MHz, DMSO) δ 8.83 (d, J = 2.1 Hz, 2H), 8.72 (t, J = 2.1 Hz, 1H), 7.86 (d, J = 8.8 Hz, 2H), 7.09 (d, J = 8.8 Hz, 2H), 4.80 (s, 2H, SCH_2), 3.83 (s, 3H, OCH_3).	^{13}C NMR (75 MHz, DMSO) δ 165.58, 162.26, 162.10, 148.08, 142.35, 129.86, 128.47, 118.02, 115.39, 114.98, 55.71, 34.29.
10	^{1}H NMR (300 MHz, DMSO) δ 8.83 (d, J = 2.1 Hz, 2H), 8.72 (t, J = 2.1 Hz, 1H), 7.01 (d, J = 2.3 Hz, 2H), 6.71 (t, J = 2.3 Hz, 1H), 4.82 (s, 2H, SCH_2), 3.80 (s, 6H, OCH_3).	^{13}C NMR (75 MHz, DMSO) δ 165.47, 163.06, 161.12, 148.08, 142.23, 129.85, 124.62, 118.01, 104.33, 104.18, 55.78, 34.23.
11	^{1}H NMR (300 MHz, DMSO) δ 8.86 (d, J = 2.1 Hz, 2H), 8.74 (t, J = 2.1 Hz, 1H), 8.58 (t, J = 2.0 Hz, 1H), 8.46 – 8.33 (m, 2H), 7.87 (t, J = 8.1 Hz, 1H), 4.85 (s, 2H, SCH_2).	^{13}C NMR (75 MHz, DMSO) δ 164.14, 163.96, 148.36, 148.12, 142.21, 132.66, 131.48, 129.92, 126.59, 124.58, 121.10, 118.05, 34.29.
12	^{1}H NMR (500 MHz, DMSO) δ 8.87 (d, J = 2.1 Hz, 2H), 8.74 (t, J = 2.1	^{13}C NMR (126 MHz, DMSO) δ 164.40, 164.30, 149.33, 148.11, 142.12, 129.92,

	Hz, 1H), 8.38 (d, J = 8.8 Hz, 2H), 8.20 (d, J = 8.8 Hz, 2H), 4.85 (s, 2H, SCH_2).	128.61, 127.98, 124.70, 118.08, 34.27.
13	^{1}H NMR (300 MHz, DMSO) δ 8.97 (t, J = 2.1 Hz, 1H), 8.92 (d, J = 2.1 Hz, 2H), 8.88 (d, J = 2.2 Hz, 2H), 8.75 (t, J = 2.2 Hz, 1H), 4.89 (s, 2H, SCH_2).	^{13}C NMR (75 MHz, DMSO) δ 164.86, 163.00, 148.87, 148.12, 142.08, 129.93, 126.40, 125.87, 121.23, 118.05, 34.25.
14	^{1}H NMR (500 MHz, DMSO) δ 8.86 (d, J = 2.1 Hz, 2H), 8.78 (d, J = 6.1 Hz, 2H), 8.73 (t, J = 2.1 Hz, 1H), 7.86 (d, J = 6.1 Hz, 2H), 4.84 (s, 2H, SCH_2).	^{13}C NMR (126 MHz, DMSO) δ 164.41, 164.10, 151.04, 148.09, 142.10, 130.12, 129.91, 120.15, 118.05, 34.24.
15	^{1}H NMR (500 MHz, dmso) δ 8.78 (d, J = 2.2 Hz, 2H), 8.73 (t, J = 2.2 Hz, 1H), 4.72 (s, 2H), 2.76 (t, J = 7.5 Hz, 2H), 1.64 – 1.55 (m, 2H), 1.31 – 1.13 (m, 16H), 0.83 (t, J = 6.8 Hz, 3H).	^{13}C NMR (126 MHz, dmso) δ 168.43, 162.15, 148.04, 142.31, 129.76, 117.92, 34.19, 31.44, 29.11, 28.95, 28.85, 28.59, 28.38, 25.75, 24.64, 22.24, 14.08.
16	^{1}H NMR (300 MHz, DMSO) δ 8.78 (d, J = 2.4 Hz, 1H), 8.57 (dd, J = 8.6, 2.4 Hz, 1H), 8.10 (d, J = 8.6 Hz, 1H), 7.97 – 7.88 (m, 2H), 7.66 – 7.51 (m, 3H), 4.93 (s, 2H, SCH_2).	^{13}C NMR (75 MHz, DMSO) δ 165.83, 162.76, 148.06, 147.27, 139.17, 134.33, 132.32, 129.61, 128.25, 126.64, 123.06, 120.75, 33.17.
17	^{1}H NMR (300 MHz, DMSO) δ 8.77 (d, J = 2.4 Hz, 1H), 8.62 – 8.51 (m, 1H), 8.08 (d, J = 8.6 Hz, 1H), 7.86 (d, J = 8.4 Hz, 2H), 7.11 (d, J = 8.4 Hz, 2H), 4.91 (s, 2H, SCH_2), 3.83 (s, 3H, OCH_3).	^{13}C NMR (75 MHz, DMSO) δ 165.79, 162.30, 161.90, 148.04, 147.25, 139.24, 134.30, 128.52, 128.22, 120.74, 115.38, 115.04, 55.71, 33.18.
18	^{1}H NMR (300 MHz, Acetone) δ 8.88 (d, J = 2.4 Hz, 1H), 8.58 (dd, J = 8.5, 2.4 Hz, 1H), 8.24 (d, J = 8.5 Hz, 1H), 7.99 (d, J = 8.7 Hz, 2H), 7.61 (d, J =	^{13}C NMR (75 MHz, Acetone) δ 166.08, 164.14, 148.55, 140.14, 138.24, 135.30, 130.35, 129.03, 128.97, 128.69, 123.19, 121.53, 34.09.

	8.6 Hz, 2H), 5.03 (s, 2H, SCH_2).	
19	^{1}H NMR (300 MHz, DMSO) δ 8.79 (d, J = 2.4 Hz, 1H), 8.60 – 8.55 (m, 2H), 8.48 – 8.42 (m, 1H), 8.39 – 8.33 (m, 1H), 8.13 (d, J = 8.6 Hz, 1H), 7.89 (t, J = 8.1 Hz, 1H), 4.97 (s, 2H, SCH_2).	^{13}C NMR (75 MHz, DMSO) δ 164.33, 163.77, 148.39, 148.11, 147.31, 139.11, 134.41, 132.70, 131.55, 128.25, 126.64, 124.57, 121.19, 120.78, 33.18.
20	^{1}H NMR (500 MHz, DMSO) δ 8.78 (d, J = 2.6 Hz, 1H), 8.59 (dd, J = 8.5, 2.6 Hz, 1H), 8.40 (d, J = 8.8 Hz, 2H), 8.19 (d, J = 8.8 Hz, 2H), 8.13 (d, J = 8.5 Hz, 1H), 4.97 (s, 2H, SCH_2).	^{13}C NMR (126 MHz, DMSO) δ 164.46, 164.20, 149.35, 148.06, 147.31, 139.02, 134.38, 128.59, 128.27, 128.02, 124.77, 120.75, 33.15.
21	^{1}H NMR (500 MHz, DMSO) δ 8.81 (d, J = 6.0 Hz, 2H), 8.78 (d, J = 2.4 Hz, 1H), 8.58 (dd, J = 8.6, 2.4 Hz, 1H), 8.12 (d, J = 8.6 Hz, 1H), 7.87 (d, J = 6.0 Hz, 1H), 4.96 (s, 2H, SCH_2).	^{13}C NMR (126 MHz, DMSO) δ 164.29, 164.24, 151.10, 148.06, 147.30, 138.99, 134.37, 130.14, 128.27, 120.74, 120.20, 33.12.
22	^{1}H NMR (500 MHz, DMSO) δ 8.77 (s, 2H), 8.71 (s, 1H), 4.80 (s, 2H, SCH_2), 2.64 (s, 3H, CH_3).	^{13}C NMR (126 MHz, DMSO) δ 166.36, 163.54, 148.06, 142.39, 129.75, 117.88, 35.41, 15.40.
23	^{1}H NMR (500 MHz, DMSO) δ 8.71 (s, 1H), 8.65 (s, 2H), 7.33 (s, 2H, NH_2), 4.59 (s, 3H, SCH_2).	^{13}C NMR (126 MHz, DMSO) δ 170.36, 148.28, 148.04, 142.91, 129.63, 117.71, 36.52.
24	^{1}H NMR (500 MHz, DMSO) δ 8.83 (d, J = 2.1 Hz, 2H), 8.77 – 8.70 (m, 3H), 7.84 (d, J = 6.1 Hz, 2H), 4.91 (s, 2H, SCH_2).	^{13}C NMR (126 MHz, DMSO) δ 166.67, 166.17, 151.06, 148.11, 142.06, 136.13, 129.82, 121.45, 117.96, 35.69.
25	^{1}H NMR (300 MHz, DMSO) δ 8.81 (d, J = 2.1 Hz, 2H), 8.72 (t, J = 2.1 Hz, 1H), 7.91 – 7.82 (m, 2H), 7.60 – 7.47 (m, 3H), 4.88 (s, 2H, SCH_2).	^{13}C NMR (75 MHz, DMSO) δ 168.76, 163.94, 148.10, 142.23, 131.63, 129.79, 129.66, 129.26, 127.70, 117.92, 35.69.
26	^{1}H NMR (300 MHz, Acetone) δ 8.93	^{13}C NMR (75 MHz, Acetone) δ 165.92,

	– 8.82 (m, 3H), 8.23 – 8.15 (m, 1H), 7.67 – 7.47 (m, 3H), 5.00 (s, 2H, SCH_2).	164.88, 149.36, 143.29, 133.11, 132.76, 131.66, 131.49, 130.48, 129.32, 128.63, 118.57, 36.56.
27	^{1}H NMR (300 MHz, Acetone) δ 8.88 (d, J = 2.2 Hz, 2H), 8.84 (t, J = 2.2 Hz, 1H), 7.94 – 7.91 (m, 1H), 7.86 – 7.80 (m, 1H), 7.60 – 7.50 (m, 2H), 4.98 (s, 2H, SCH_2).	^{13}C NMR (75 MHz, Acetone) δ 168.14, 165.06, 149.37, 143.23, 135.56, 132.42, 131.95, 131.90, 130.45, 127.78, 127.07, 118.57, 36.58.
28	^{1}H NMR (300 MHz, Acetone) δ 8.88 (d, J = 2.1 Hz, 2H), 8.85 (t, J = 2.1 Hz, 1H), 7.93 (d, J = 8.6 Hz, 2H), 7.57 (d, J = 8.6 Hz, 2H), 4.98 (s, 2H, SCH_2).	^{13}C NMR (75 MHz, Acetone) δ 167.58, 164.46, 148.11, 142.20, 136.24, 129.80, 129.73, 129.40, 128.14, 117.94, 35.69
29	^{1}H NMR (500 MHz, $CDCl_3$) δ 8.87 (t, J = 2.1 Hz, 1H), 8.65 (d, J = 2.1 Hz, 2H), 7.65 (d, J = 8.1 Hz, 2H), 7.21 – 7.15 (m, 2H), 4.68 (s, 2H, SCH_2), 2.33 (s, 3H, CH_3).	^{13}C NMR (126 MHz, $cdcl_3$) δ 169.53, 161.51, 148.50, 141.95, 141.67, 129.91, 129.30, 127.65, 126.69, 118.09, 35.87, 21.49.
30	^{1}H NMR (500 MHz, DMSO) δ 8.81 (d, J = 2.2 Hz, 2H), 8.71 (t, J = 2.2 Hz, 1H), 7.46 (s, 2H), 7.16 (s, 1H), 4.86 (s, 2H, SCH_2), 2.31 (s, 6H, CH_3).	^{13}C NMR (126 MHz, DMSO) δ 168.95, 163.56, 148.05, 142.23, 138.95, 133.03, 129.78, 129.12, 125.29, 117.88, 35.65, 20.83.
31	^{1}H NMR (300 MHz, DMSO) δ 8.81 (d, J = 2.1 Hz, 2H), 8.72 (t, J = 2.1 Hz, 1H), 7.53 – 7.31 (m, 3H), 7.19 – 7.03 (m, 1H), 4.87 (s, 2H, SCH_2), 3.81 (s, 3H, OCH_3).	^{13}C NMR (75 MHz, DMSO) δ 168.55, 164.07, 159.87, 148.09, 142.24, 130.90, 130.45, 129.79, 120.29, 117.93, 117.48, 112.29, 55.58, 35.69.
32	^{1}H NMR (300 MHz, DMSO) δ 8.80 (d, J = 2.1 Hz, 2H), 8.71 (t, J = 2.1 Hz, 1H), 7.79 (d, J = 8.9 Hz, 2H), 7.05 (d, J = 8.9 Hz, 2H), 4.85 (s, 2H, SCH_2), 3.81 (s, 3H, OCH_3).	^{13}C NMR (75 MHz, DMSO) δ 168.55, 162.65, 161.84, 148.07, 142.31, 129.76, 129.35, 121.78, 117.89, 115.02, 55.66, 35.71.

33	^{1}H NMR (300 MHz, DMSO) δ 8.83 (d, J = 2.1 Hz, 2H), 8.72 (t, J = 2.1 Hz, 1H), 8.59 (t, J = 2.0 Hz, 1H), 8.36 (dd, J = 8.3, 2.3 Hz, 1H), 8.32 – 8.24 (m, 1H), 7.82 (t, J = 8.0 Hz, 1H), 4.90 (s, 2H, SCH_2).	^{13}C NMR (75 MHz, DMSO) δ 166.59, 165.57, 148.42, 148.11, 142.11, 134.03, 131.40, 130.65, 129.83, 125.84, 121.77, 117.96, 35.71.
34	^{1}H NMR (500 MHz, DMSO) δ 8.83 (s, 2H), 8.73 (s, 1H), 8.34 (d, J = 9.0 Hz, 2H), 8.15 (d, J = 9.0 Hz, 2H), 4.91 (s, 2H, SCH_2).	^{13}C NMR (126 MHz, DMSO) δ 166.63, 166.19, 148.89, 148.11, 142.07, 134.87, 129.83, 128.93, 124.77, 117.97, 35.69.

Table 7. Elemental analysis of compounds of general formula (I).

	calculated	found
1	C, 40.54; H, 2.72; N, 18.91; S, 10.82	C, 40.21; H, 2.49; N, 18.65; S, 11.21
2	C, 50.28; H, 2.81; N, 15.64; S, 8.95	C, 50.71; H, 3.14; N, 15.45; S, 9.21
3	C, 45.87; H, 2.31; N, 14.26; S, 8.16	C, 45.58; H, 2.62; N, 14.07; S, 8.35
4	C, 45.87; H, 2.31; N, 14.26; S, 8.16	C, 46.12; H, 2.68; N, 14.10; S, 8.39
5	C, 45.87; H, 2.31; N, 14.26; S, 8.16	C, 46.02; H, 2.28; N, 14.41; S, 8.39
6	C, 42.17; H, 1.89; N, 13.11; S, 7.51	C, 42.63; H, 2.28; N, 13.14; S, 7.88
7	C, 51.61; H, 3.25; N, 15.05; S, 8.61	C, 51.60; H, 3.47; N, 14.97; S, 8.95
8	C, 49.48; H, 3.11; N, 14.43; S, 8.26	C, 49.26; H, 3.30; N, 14.30; S, 8.49
9	C, 49.48; H, 3.11; N, 14.43; S, 8.26	C, 49.58; H, 3.30; N, 14.41; S, 8.61
10	C, 48.80; H, 3.37; N, 13.39; S, 7.66	C, 48.89; H, 3.55; N, 13.36; S, 8.02
11	C, 44.67; H, 2.25; N, 17.36; S, 7.95	C, 44.69; H, 2.52; N, 17.39; S, 8.21
12	C, 44.67; H, 2.25; N, 17.36; S, 7.95	C, 44.28; H, 2.39; N, 17.68; S, 8.19
13	C, 40.19; H, 1.80; N, 18.75; S, 7.15	C, 40.43; H, 2.12; N, 18.92; S, 7.48
14	C, 46.80; H, 2.52; N, 19.49; S, 8.92	C, 46.28; H, 2.74; N, 19.30; S, 9.19
15	C, 55.03; H, 6.47; N, 12.83; S, 7.35	C, 55.12; H, 6.18; N, 12.75; S, 7.29
16	C, 50.28; H, 2.81; N, 15.64; S, 8.95	C, 50.38; H, 2.86; N, 15.81; S, 9.06
17	C, 49.48; H, 3.11; N, 14.43; S, 8.26	C, 49.45; H, 3.35; N, 14.43; S, 8.69
18	C, 45.87; H, 2.31; N, 14.26; S, 8.16	C, 45.58; H, 2.72; N, 14.13; S, 8.59
19	C, 44.67; H, 2.25; N, 17.36; S, 7.95	C, 44.88; H, 2.53; N, 17.16; S, 8.03

20	C, 44.67; H, 2.25; N, 17.36; S, 7.95	C, 45.00; H, 2.42; N, 17.20; S, 8.15
21	C, 46.80; H, 2.52; N, 19.49; S, 8.92	C, 47.06; H, 2.77; N, 19.43; S, 9.26
22	C, 38.46; H, 2.58; N, 17.94; S, 20.53	C, 38.26; H, 2.78; N, 18.03; S, 20.91
23	C, 34.50; H, 2.25; N, 22.35; S, 20.47	C, 34.60; H, 2.33; N, 22.01; S, 20.69
24	C, 44.79; H, 2.42; N, 18.66; S, 17.08	C, 45.04; H, 2.61; N, 18.70; S, 17.10
25	C, 48.12; H, 2.69; N, 14.96; S, 17.13	C, 48.06; H, 2.89; N, 15.15; S, 17.40
26	C, 44.07; H, 2.22; N, 13.70; S, 15.69	C, 44.49; H, 2.21; N, 13.53; S, 15.38
27	C, 44.07; H, 2.22; N, 13.70; S, 15.69	C, 44.39; H, 2.52; N, 13.78; S, 15.49
28	C, 44.07; H, 2.22; N, 13.70; S, 15.69	C, 44.21; H, 2.21; N, 13.40; S, 15.33
29	C, 49.47; H, 3.11; N, 14.42; S, 16.51	C, 49.46; H, 3.31; N, 14.51; S, 16.62
30	C, 50.74; H, 3.51; N, 13.92; S, 15.93	C, 50.71; H, 3.46; N, 13.77; S, 16.06
31	C, 47.52; H, 2.99; N, 13.85; S, 15.86	C, 47.27; H, 2.88; N, 13.75; S, 15.95
32	C, 47.52; H, 2.99; N, 13.85; S, 15.86	C, 47.38; H, 2.88; N, 13.83; S, 15.90
33	C, 42.96; H, 2.16; N, 16.70; S, 15.29	C, 43.00; H, 2.35; N, 17.22; S, 15.99
34	C, 42.96; H, 2.16; N, 16.70; S, 15.29	C, 42.91; H, 2.19; N, 16.20; S, 15.02

Examples of the pharmaceutical compositions - tablets

In manufacture of solid dosage forms, the technology common in the given art is used, i.e., dry or wet granulation, which is well-known to a person skilled in the art. There are used routine and well proven excipients and suitable additives that give to the dosage form the desired physical characteristics.

Examples for dry granulation:

Example 1 (content of active substance 100 mg)

Medicinal substance of general formula (I) **1**	100.0 mg
Cellulose, microcrystaline	75.0 mg
Sodium carboxymethylstarch	3.5 mg
Magnesium Stearate	0.5 mg
Silicon Dioxide, Colloidal	0.5 mg

Example 2 (content of active substance 200 mg)

Medicinal substance of general formula (I) **13**	200.0 mg
Cellulose, microcrystaline	95.0 mg

Sodium carboxymethylstarch	7.0 mg
Magnesium Stearate	1.0 mg
Silicon Dioxide, Colloidal	1.0 mg

Example 3 (content of active substance 300 mg)

Medicinal substance of general formula (I) **9**	300.0 mg
Cellulose, microcrystaline	115.0 mg
Sodium carboxymethylstarch	10.5 mg
Magnesium Stearate	1.5 mg
Silicon Dioxide, Colloidal	1.5 mg

Example 4 (content of active substance 400 mg)

Medicinal substance of general formula (I) **31**	400.0 mg
Cellulose, microcrystaline	130.0 mg
Sodium carboxymethylstarch	14.5 mg
Magnesium Stearate	2.0 mg
Silicon Dioxide, Colloidal	2.0 mg

Example 5 (content of active substance 500 mg)

Medicinal substance of general formula (I) **24**	500.0 mg
Cellulose, microcrystaline	140.0 mg
Sodium carboxymethylstarch	17.5 mg
Magnesium Stearate	2.5 mg
Silicon Dioxide, Colloidal	2.5 mg

The medicinal substance is mixed together with other individual excipients and the obtained mixture is compressed by regular manner using a conventional tablet machine.

Examples for wet granulation

Example 6 (content of active substance 100 mg)

Medicinal substance of general formula (I) **19**	100.0 mg
Potato starch	48.0 mg
Lactose	27.0 g
Povidone	3.0 g

Sodium carboxymethylstarch	4.0 g
Magnesium Stearate	0.2 g
Talc	1.8 g

Example 7 (content of active substance 200 mg)

Medicinal substance of general formula (I) **6**	200.0 mg
Potato starch	60.8 mg
Lactose	34.2 g
Povidone	6.0 g
Sodium carboxymethylstarch	8.0 g
Magnesium Stearate	0.4 g
Talc	3.6 g

Example 8 (content of active substance 300 mg)

Medicinal substance of general formula (I) **33**	300.0 mg
Potato starch	73.6 mg
Lactose	41.4 g
Povidone	9.0 g
Sodium carboxymethylstarch	12.0 g
Magnesium Stearate	0.6 g
Talc	5.4 g

Example 9 (content of active substance 400 mg)

Medicinal substance of general formula (I) **15**	400.0 mg
Potato starch	82.3 mg
Lactose	46.8 g
Povidone	12.0 g
Sodium carboxymethylstarch	16.0 g
Magnesium Stearate	0.8 g
Talc	7.2 g

Example 10 (content of active substance 500 mg)

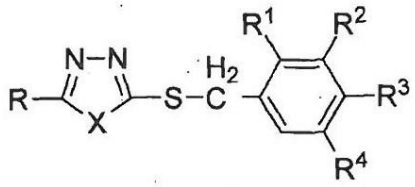
Medicinal substance of general formula (I) **3**	500.0 mg
Potato starch	96.0 mg
Lactose	54.0 g

Povidone	15.0 g
Sodium carboxymethylstarch	20.0 g
Magnesium Stearate	1.0 g
Talc	9.0 g

The therapeutically effective compound is mixed with lactose, potato starch and this mixture is granulated with povidone. The dried granulate is mixed then with sodium carboxymethyl-starch, magnesium stearate, and talc. The obtained mixture is compressed by regular manner using a conventional tablet machine.

CLAIMS

1. A substituted diazole of general formula (I)



(I)

wherein

X is O or S;

R is selected from the group consisting of: H, NH_2-, C_1–C_{11} alkyl, cyclohexyl-, benzyl-, phenyl-, pyridyl- or phenyl- substituted, in positions 2, 3, 4 or 5 by one or more electron-acceptor groups comprising -NO_2, -N(alkyl)$_3$, -CF_3, CCl_3, -CN, -COOH, -COOAlk, -COOAr, -CHO, -COAlk, -COAr, -F, -Cl, -Br, -I, and/or electron-donor groups comprising -NH_2, -NHalkyl, -N(alkyl)$_2$, -OH, -Oalkyl, -Oaryl, -$NHCOCH_3$, -NHCOalkyl; -NHCOaryl; -alkyl, -aryl,

wherein when R_1 and R_3 is –NO_2, then R_2 a R_4 is -H, or when R_1 and R_3 is –H, then R_2 and R_4 is –NO_2.

2. The substituted diazole of general formula (I) according to claim 1, wherein X is O.

3. The substituted diazole of general formula (I) according to claim 1, wherein X is S.

4. The substituted diazole of general formula (I) according to anyone of claims 1 to 3 for use as antituberculotic.

5. A use of the substituted diazole of general formula (I) according to any one of claims 1 to 3 for the manufacture of a medicament for the treatment of tuberculosis.

6. A pharmaceutical preparation characterized in that it contains the substituted diazole of formula (I) according to claim 1 as the active ingredient.

7. The pharmaceutical preparation according to claim 6, characterized in that it contains one or more pharmaceutically acceptable excipients.