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NEW COMPOUNDS AS PERSPECTIVE ANTITUBERCULAR AND ANTIVIRAL AGENTS

ABSTRACT OF A THESIS
Associating to a scientific degree „Doctor of Sciences“

Professional Field: 4.2. Chemical Sciences
Scientific Specialty: Organic Chemistry

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NOTES AND ACKNOWLEDGMENTS:

The presented scientific results were obtained from 2009 to 2022 and they are part of my scientific publications from this period (over 17 scientific papers). Two main research areas were included in this thesis: development of new compounds with antitubercular activity and development of new compounds with antiviral activity. Other investigated properties of the compounds were also mentioned (cytotoxicity, QSAR, genetics). The DSc thesis is written on 175 pages and contains 34 figures, 28 tables, 29 schemes and 340 references. For this concourse were selected and attached 8 scientific papers (4 in Q1 and 4 in Q2). In 5 of them Georgi Dobrikov is a corresponding/first author. The results are presented in 26 oral or poster presentations at scientific conferences in Bulgaria and abroad.

The results included in this thesis were obtained during the implementation of research projects financed under contracts funded by the Bulgarian Science Fund: B02-11 (2014-2019); DCOST-01/4 (2017-2019); KP-06-H31/7 (2019-present); KP-06-H39/7 (2019-present); KP-06-M59/8 (2021-present). The financial support of the Bulgarian Science Fund for the purchase of Bruker Avance II+ 600 NMR spectrometer in the framework of the Program 'Promotion of the Research Potential through Unique Scientific Equipment' – project UNA-17/2005 is gratefully acknowledged. The financial support of the Operational Program "Science and Education for Smart Growth" 2014-2020, co-financed by European Union through the European Structural and Investment Funds, Grant BG05M2OP001-1.002-0012, is also gratefully acknowledged. Within this project two new NMR spectrometers (used in this work) were purchased - Bruker Avance Neo 400 and Bruker Neo 600.

Some commercial reagents are used in multiple reactions in this thesis. For that reason, they have more than one number in reaction schemes, in order to facilitate reading and understanding the content.

The numbering of compounds in this abstract are the same as in dissertation.

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TABLE OF CONTENTS

1. INTRODUCTION

1.1. Tuberculosis – spreading and importance

Tuberculosis (TB) is one of the oldest and deadliest diseases that still afflicts people today TB has killed worldwide more people in the past two millennia than any other infection.

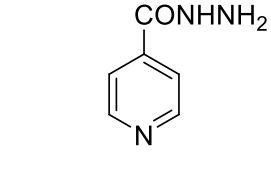
The causative agents of TB are bacteria, members of complex, represented mainly by *Mycobacterium tuberculosis* (*Mtb*). Most probably, the common ancestor of these species existed on Earth around 15 000 years ago. However, *Hayman* has speculated of Jurassic origins more than 150 million years ago.[1] The earliest clear evidence of human TB comes from skeletal remains located in a Neolithic settlement in the Eastern Mediterranean, dating from about 9000 years ago.[2]

The characteristics that would make a drug ideal for the treatment of tuberculosis are: (i) it should be more effective against MDR/XDR-TB, (ii) it should shorten treatment time for both sensitive and drug-resistant TB, (iii) it should target nonreplicating state of bacteria, and (iv) it should be compatible with current anti-TB and HIV medications (as *Mtb*/HIV co-infection is predominant). Unfortunately, even with the newest and most promising chemical structures, the development of resistance and many side effects is still observed. It is substantial to continue screening of multiple chemical libraries, including synthetic, semisynthetic and natural compounds.[3]

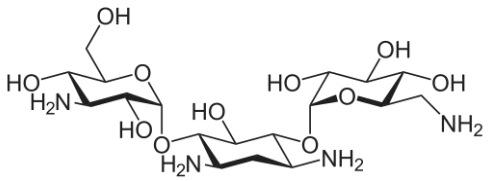
1.2. Current trends in development of antitubercular drugs

The classical treatment of tuberculosis was established since 1960`s and includes two main groups of drugs (their structures are presented in Figures 1 and 2). Isoniazid, rifampin, ethambutol (EMB), pyrazinamide and streptomycin are the essential first-line anti-tuberculosis drugs. Aminoglycosides (kanamycin, amikacin), quinolones (levofloxacin), ethionamide or prothionamide, cycloserine, para-aminosalicylic acid (PAS) and polypeptide (capreomycin) are the second-line anti-tuberculosis drugs.[4] This list was more or less unchanged until last decade, when MDR/XDR-TB extremely raised worldwide.

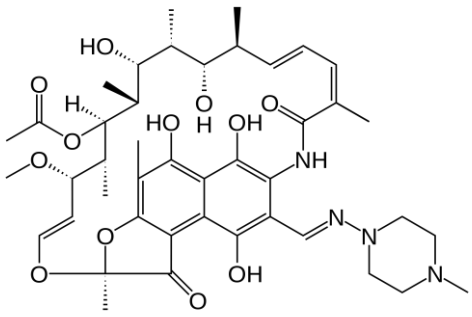
In 2019, the WHO published new guidelines that brought a major revision and reclassification to the drugs recommended for use in longer MDR-TB treatment regimens (summarized in Figure 2). This treatment includes combination of all three drugs from Group A (levofloxacin or moxifloxacin, bedaquiline and linezolid), with one or two drugs from Group B. Drugs from Group C can be used when medicines from Groups A and B are insufficient for successful cure or are not applicable for some reason.



isoniazid



kanamycin



rifampin (rifampicin)

Figure 1. Some drugs used in classical treatment of tuberculosis since 1960`s.

The latest WHO guidelines (2020)[5] have also clarified that the new 6−9 month regimen of pretomanid, bedaquiline, and linezolid can now be used for MDR-TB patients with additional resistance to fluoroquinolone antibiotics. In addition, most second-line drugs are more toxic, costly, and less effective than the first-line drugs.[2] For example, unjustified hopes have been put on the bedaquiline in particular. After its introduction into clinical practice, it proved to be not very efficient, with unwanted side effects such as compromised cardiac safety.[6,7]

All above mentioned drugs have known mechanism of action, as well as target genes and proteins (along with the associated mutations) causing resistance to these drugs.[3] Many other compounds are currently in clinical development (phases I, II or III). The most important candidates can be summarized in Table 1.[2] Cotemporary TB drug discovery is predominantly based on both target-based or phenotypic/cell-based methods, leading to completely new classes of active compounds.[8] However, other classification of drug discovery is also possible. A recent analysis reveals that 43% of published clinical candidates from the *Journal of Medicinal Chemistry* are derivatives of known compounds (DKC), and other 29% were obtained as a result of random screening (RS).[9]

It seems that RS still remains a relevant and efficient method for discovery of bioactive hit compounds, especially suitable for academic institutions with limited funding. This is why we use this method in our research of antitubercular drugs. In addition, in some molecules we incorporated fragments that are previously known from the literature to be pharmacophore. In conclusion, huge amount of *in vitro* active compounds (in any preclinical phases) are described in the scientific literature. They are divided in many different classes, based on their chemical structures or mechanism of actions.[8,10-16] However, their detailed description is not a goal of this thesis and only single examples related to our publication activity will be mentioned below, in section "Results and discussion".

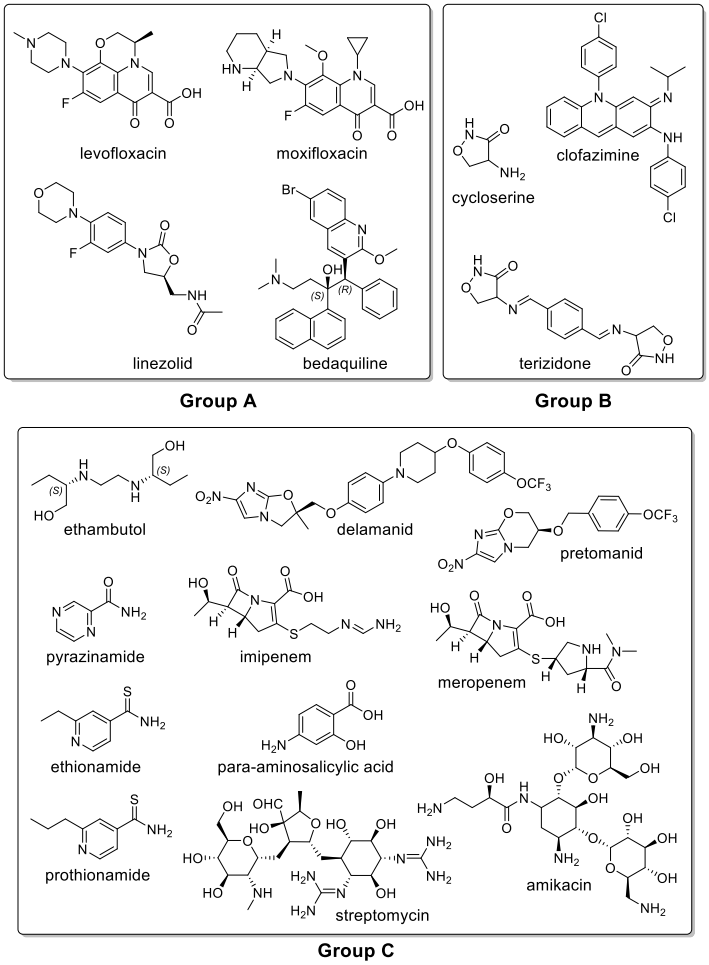
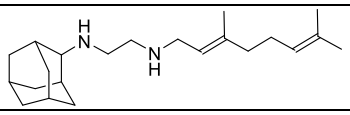
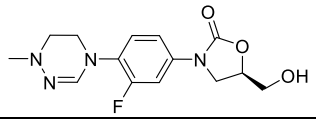
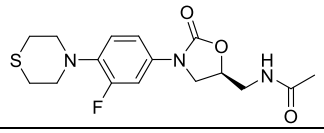
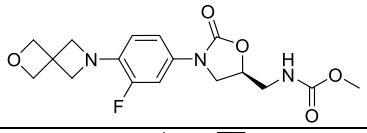
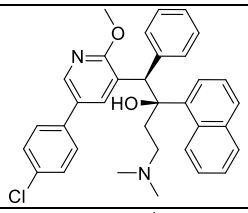
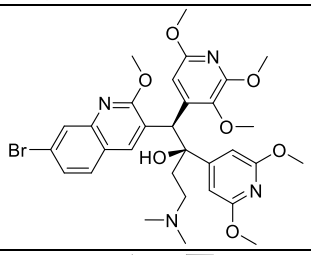
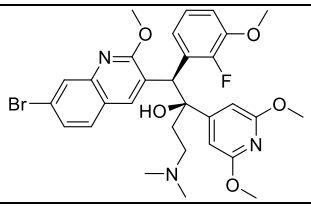
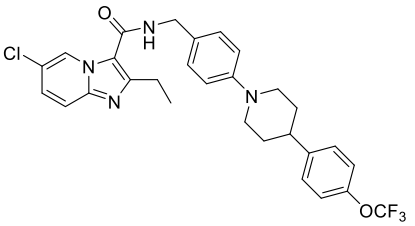
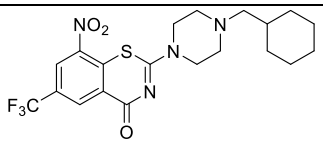
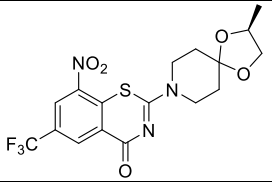
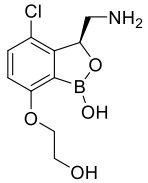
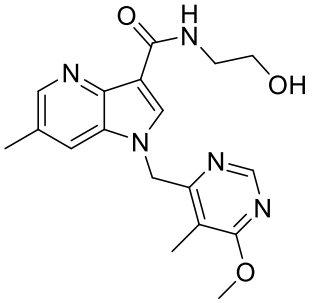
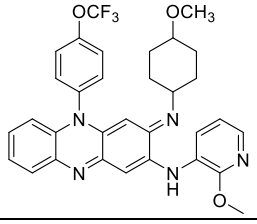
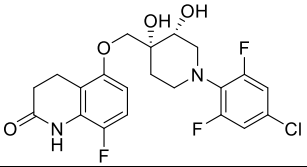
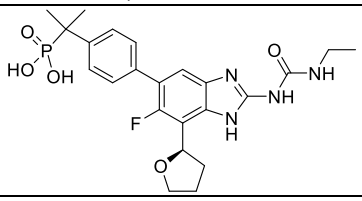
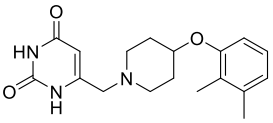
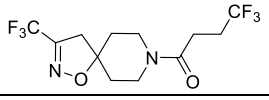


Figure 2. Drugs recommended by WHO in 2019 for longer MDR-TB treatment.

Table 1. Anti-TB drug candidates in clinical development (phases I, II or III in 2022).

Name	Structure	Chemical class	Phase
SQ109		Ethylenediamine	II/III
Delpazolid		Oxazolidinone	II
Sutezolid		Oxazolidinone	II
TBI-223		Oxazolidinone	I
Sudapyridine (WX-081)		Triarylpyridine	II
TBAJ-876		Diarylquinoline	I
TBAJ-587		Diarylquinoline	
Telacebec		Imidazopyridine amide	II
Macozinone		Benzothiazinone	II
BTZ043		Benzothiazinone	I/II
GSK3036656		Benzoxaborole	II

TBA-7371		1,4-Azaindole	II
Pyrifazimine (TBI-166)		Iminophenazine	II
OPC-167832		Carbostyril	I/II
SPR720		Benzimidazole urea	I
GSK2556286		Pyrimidine-2,4-dione	I
BVL-GSK098		Amido-piperidine	I

1.3. Current trends in development of anti-enteroviral drugs

Enteroviruses (EV) are non-enveloped, single-stranded (+) RNA viruses belonging to the Picornaviridae family. This large family includes several pathogens that are implicated in a wide range of clinical manifestations, affecting humans and animals. EV may also be linked to even more serious illnesses, which can subsequently be life-threatening. Such threads includes meningitis, encephalitis, myocarditis, polio, insulin dependent diabetes, etc.[17,18] Coxsackieviruses (class of EV), and in particular Coxsackie B group, have often been associated with the development of myocarditis, which may lead to sudden death in young adults or progress to dilated cardiomyopathy if untreated. The enterovirus life cycle is described briefly in (Figure 3).[19,20]

Modern antiviral chemotherapy has not yet achieved the same successes as antibacterial chemotherapy. Existing antivirals on chemical basis are quite limited. This is true not only for those applicable in clinical practice, but also to those showing promising activity in the preclinical phases. Described discrepancy is mainly due to two reasons – the first is the extremely frequent mutation of viruses, leading to the rapid development of resistance to antiviral drugs. The second reason is the limited number of biochemical processes in viruses that can be influenced by antivirals. Viruses do not have their own metabolism, they rely on the host's metabolism to carry out the above mentioned life cycle. Therefore, antiviral chemotherapeutics interrupt or disturb this cycle not directly, but only through a selective influence on the normal biochemical processes in the host cells.

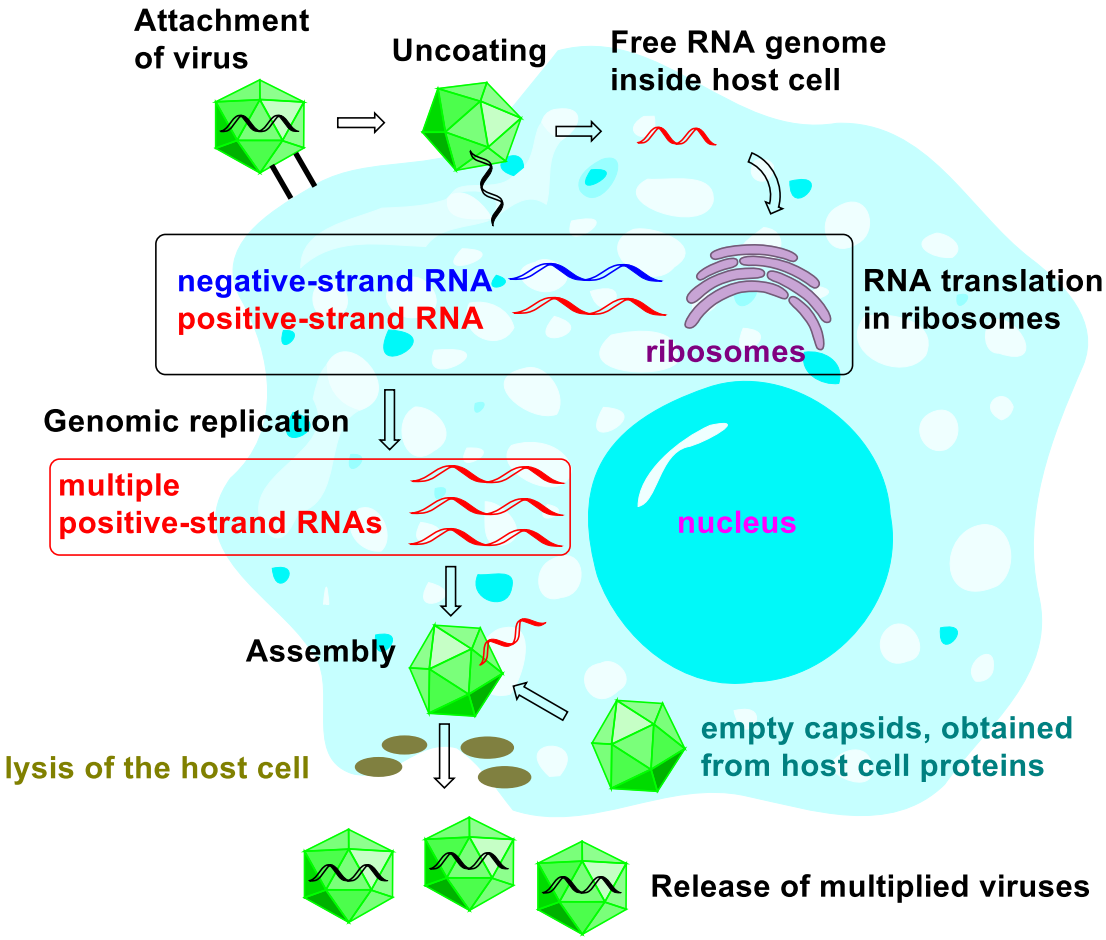


Figure 3. Key stages of enterovirus life cycle.

Over the past decades, several classes of non-peptidic compounds have been reported to be selective *in vitro* inhibitors of enterovirus replication. However, a sharp discrepancy exists also between the antiviral activity established *in vitro* and *in vivo* (experiments involving laboratory animals). In contrast with hundreds virus replication inhibitors showing *in vitro* effects less than twenty manifested some *in vivo* activity. Unfortunately, efficient anti-EV chemotherapy for clinical use is still not established. The development of drug-resistance is the main reason for the lack of antivirals in clinical use for enteroviral infections.

A limited number of synthetic organic compounds have been shown to selectively inhibit exactly one of the processes mentioned above – the replication of viral RNA. Influencing this process is a key point in the fight against viruses, which is why the search for new compounds with such a mechanism of action continues today. The main classes of synthetic compounds known to date with this action are presented below.

2. AIM AND TASKS OF THE THESIS

The aim of the present thesis is to present synthesis of novel compounds, perspective as potent *in vitro*/*in vivo* antitubercular and antiviral agents with lowered cytotoxicity and improved pharmacological properties. QSAR analyses have been performed in some cases, in order to elucidate possibilities for further development of improved bioactive compounds. A special emphasis was placed on the synthetic part of our work and characterization of obtained new compounds, without providing details of the biological and spectroscopic methods used.

The following tasks have been set for the realization of the stated aim:

- **Development of new derivatives of *R*-2-aminobutan-1-ol as antitubercular agents:**
  - *N*-acyl derivatives;
  - *N*-alkyl/aryl derivatives;
  - heterocyclic derivatives;
  - ureas, thioureas and acylthioureas.

- **Development of new derivatives of (+)-camphor as antitubercular agents:**
  - amidoalcohols with camphane skeletons;
  - other derivatives of aminoalcohols with camphene skeleton.

- **Development of new potent analogues of known antiviral agent MDL-860:**
  - new diaryl ethers bearing cyano and nitro groups;
  - new diaryl amines and diaryl thioethers bearing cyano and nitro groups;
  - other diaryl ethers and their heterocyclic analogues.

3. RESULTS AND DISCUSSION

Our publications presented here involve two specific areas of medicinal chemistry – synthesis of new antibacterial and antiviral agents. Special emphasis is placed on antitubercular agents. Besides a brief description of the synthetic methods, this section also includes results for *in vitro*/*in vivo* activity of synthesized compounds. Detailed synthetic procedures and analytical data are presented in Experimental Section.

3.1. Development of new compounds with potent *in vitro* antitubercular and antibacterial activity

3.1.1. New ethambutol analogues as perspective anti-TB drugs

The simple diamine ethambutol (Figure 2) was synthesized by reacting 1,2-dihaloethane with (*S*)-2-amino-1-butanol.[21,22] An alternative synthetic method was also described.[23] The EMB is primarily a bacteriostatic anti-tuberculosis agent with not fully known mechanism of action. It targets the arabinosyl transferases responsible for arabinogalactan biosynthesis, a key component of the unique mycobacterial cell-wall.[24-26] Despite of modest antimycobacterial activity and due to its synergy with other drugs, and lower toxicity, EMB is used in combination with more potent frontline antimycobacterial agents. Early SAR study indicates that the distance between the two nitrogens, the presence of two hydroxyl groups, and the small side chains in the molecule are key pharmacophore elements. The configuration of the molecule is decisively important for the activity, since EMB (with *S*,*S*-configuration) is approx. 200-500 fold more potent than its (*R*,*R*)-enantiomer.[27]

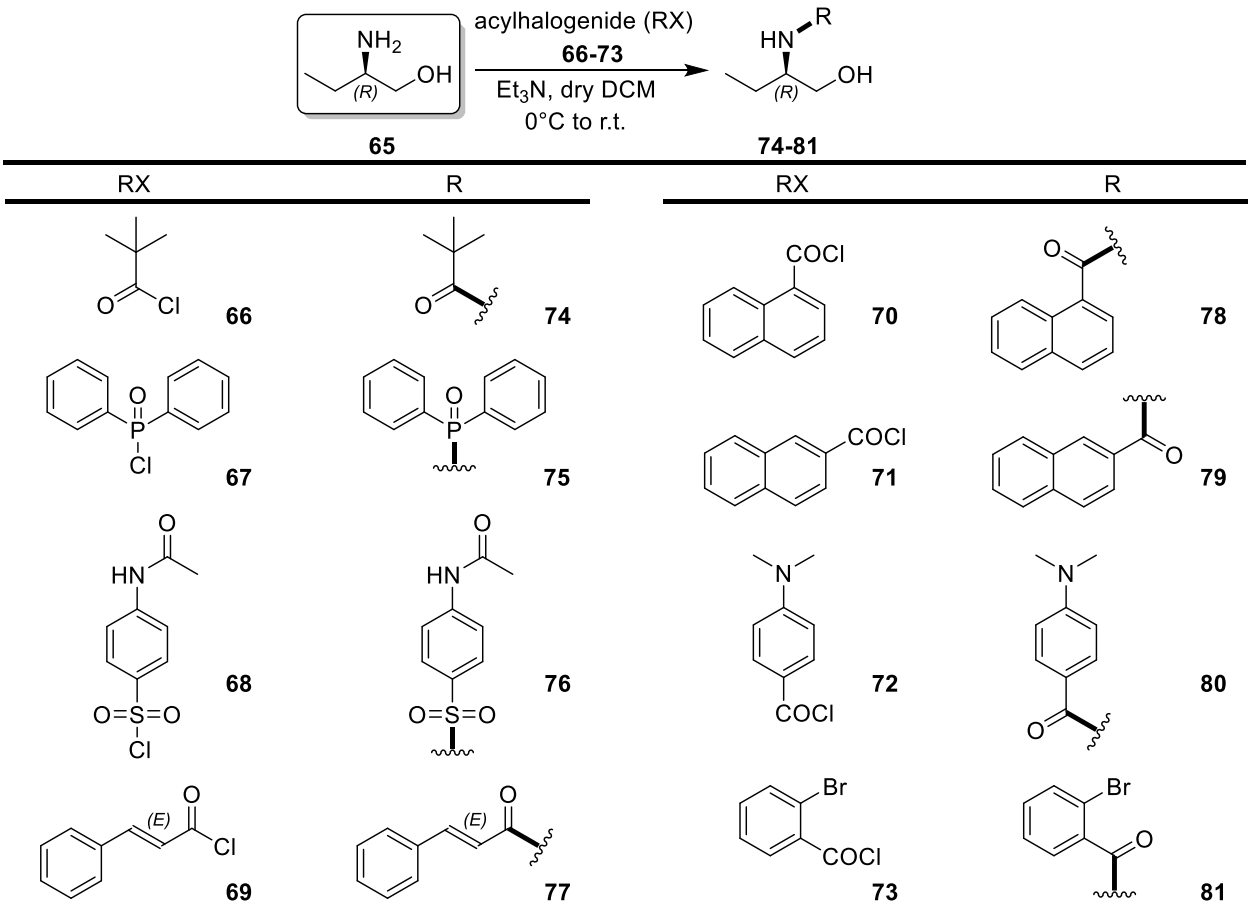
In recent years diverse derivatives of (*S*)-2-amino-1-butanol and 1,2-ethylenediamine have been synthesized and evaluated for their antimycobacterial activities and mechanisms of action.[28-32] In general, most of the compounds containing (*S*)-2-amino-1-butanol motif are showing similar but not significantly higher activity than EMB. It is important to note that the utilization of (*R*)-2-amino-1-butanol motif for the synthesis of anti TB drug candidates has been obviously neglected. There are only isolated examples of such derivatives showing results that are not encouraging enough with respect to antimycobacterial activity,[33] but possessing promising antifungal activity.[34]

Herein, we report the synthesis of small libraries of structurally diverse compounds incorporating (*R*)- and (*S*)-2-amino-1-butanol motif and the evaluation of their *in vitro* antimycobacterial, antibacterial and antifungal activities.[35,36] All compounds were obtained in excellent purity (>99%) after column chromatography and/or crystallization. They were fully characterized by NMR, MS, melting points and elemental analysis.

Synthesis of new *N*-acylated, -alkylated and -arylated derivatives (**74-81**, **85-87**, **95-101**, **108-113**, **116-118**, **125-130**, **144-156** and **158-163**) of commercial enantiopure (*R*)-2-amino-1-butanol. Evaluation of their antimycobacterial, antibacterial and antifungal activity[35]

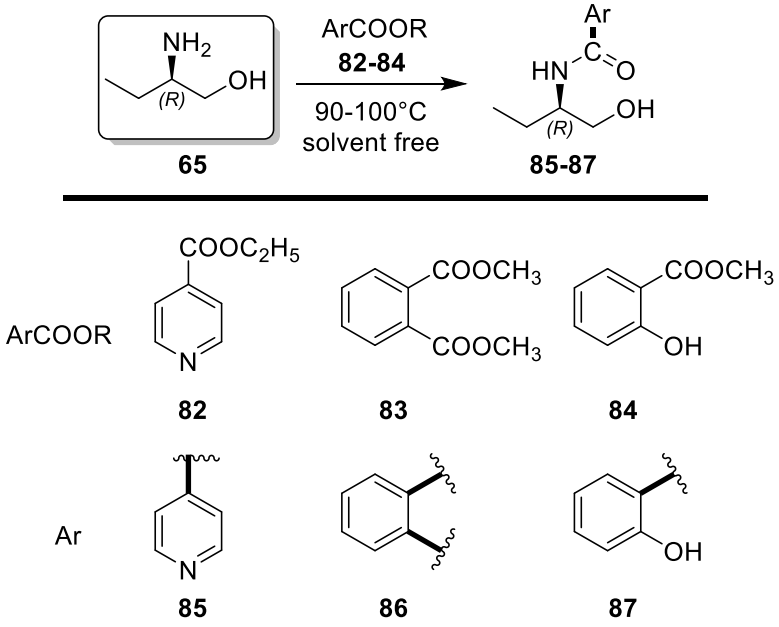
A series of *N*-monoacylated compounds **74-81** was synthesized[35] and good to excellent yields were achieved using standard conditions for acylation of (*R*)-2-amino-1-butanol (**65**) (0°C and Et_3N in dry dichloromethane (DCM)) with acid chlorides **66-73** (Scheme 1). The synthesis of **77-79** and **81** has been published earlier, with no data about the purity.[37-41] In the case of **75**, the (*S*)-enantiomer has been obtained elsewhere in enantiomerically enriched form (75-97 % ee), by using of rather complicated procedure.[37] The amide derivatives **85-87** were synthesized using simple solvent-free aminolysis of esters **82-84** with **65** by heating at 90-100°C (Scheme 2).[35]

The synthesis of compounds **95-101** (Scheme 3)[35] was performed by applying standard procedures described in literature[42-48] – heating of **65** with **88-94**, and using either solvent-free conditions or different solvents (e.g. diethylether, tetrahydrofuran, ethanol etc.). Compounds **95**, **96** and **99** have been obtained previously,[42-44,48] however, the data published were insufficient in respect of purity and of properties. It is interesting to note that the reaction of **65** with **90** under those particular conditions led to ester **97**. Compound **98** was obtained from **65** and **91** as a result of migration of the double bond. Similar rearrangement reaction has been previously observed.[49]

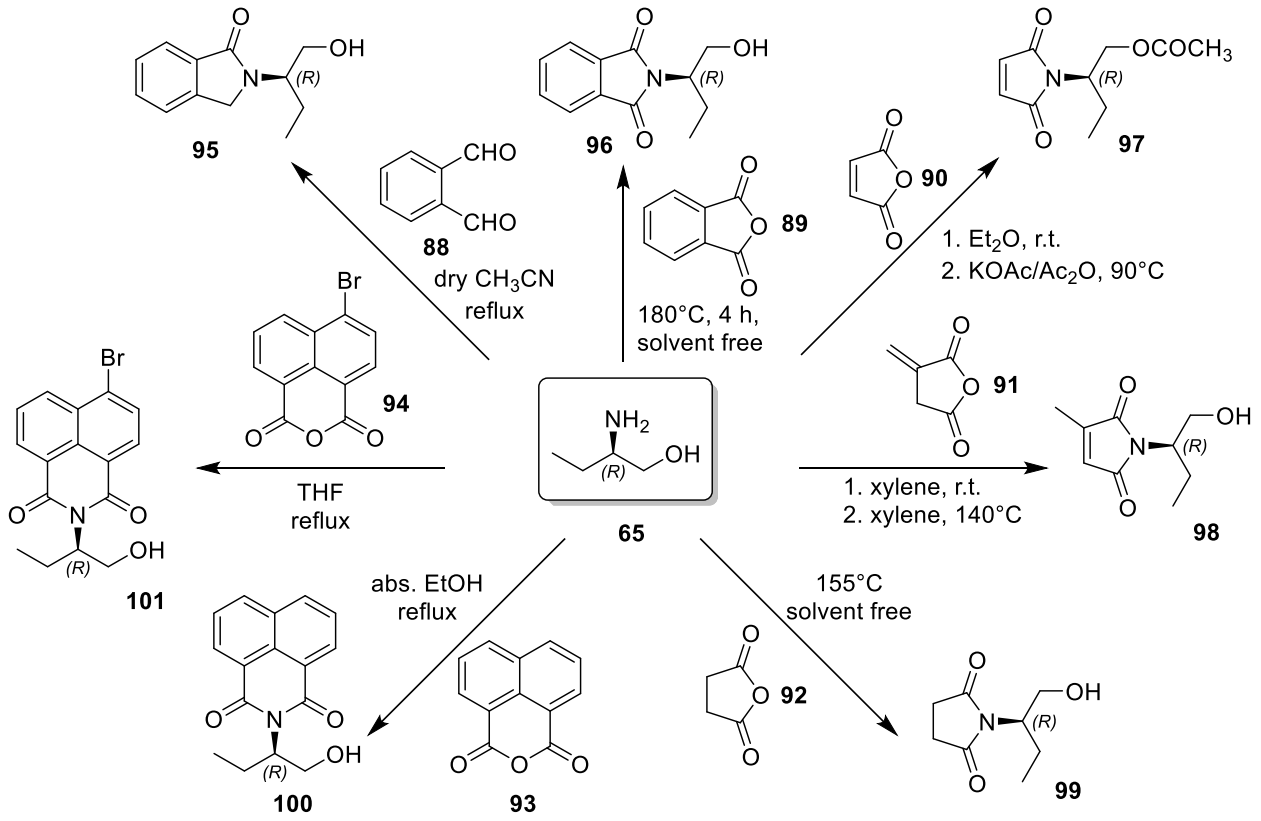


Scheme 1. Synthesis of compounds **74-81**.

It is known that some ureas and thioureas are effective drugs against a range of MDR strains of *M. tuberculosis*.[50-54] Therefore it is worthy to evaluate the activity of similar structures incorporating the (*R*)-2-aminobutanol moiety. The synthesis of compounds **108-110** (Scheme 4) was performed by mixing **65** and isothiocyanate **102** and isocyanates **103-104** respectively, in tetrahydrofuran as a solvent.[35] The formation of **108**[55-57] and **110**[58] was described elsewhere in connection with different studies, not being related to the present investigations.

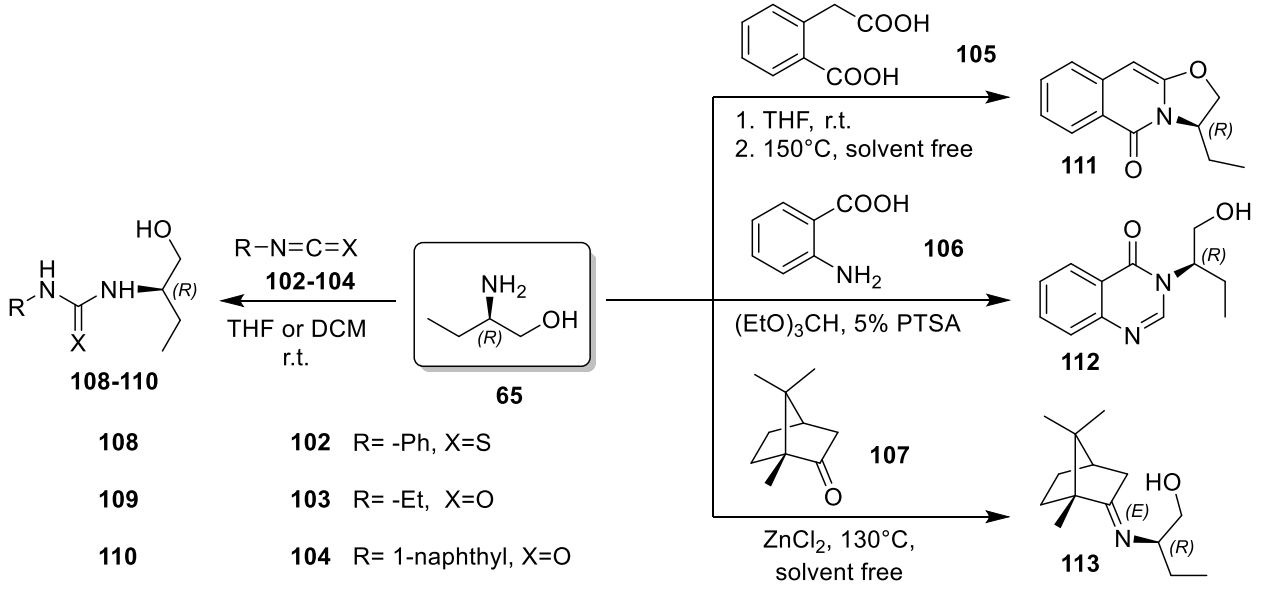


Scheme 2. Synthesis of compounds **85-87**.



Scheme 3. Synthesis of compounds **95-101**.

The heterocycle **111** was synthesized in two steps (Scheme 4). In the first step the salt of **65** was formed and isolated as precipitate after mixing of **65** with homophthalic acid (**105**) in tetrahydrofuran (THF). This intermediate was then heated without solvent for 5 h to form **111** in good yields. Structures similar to **111** have shown anti-inflammatory and analgesic activity.[59] The quinazolinone derivative **112** (Scheme 4) was prepared in acceptable yield, in boiling toluene by mixing **65**, anthranilic acid (**106**) and triethyl orthoformate, in presence of catalytic amounts of p-toluenesulfonic acid (PTSA). In the present case, the published solvent-free procedure for synthesis of similar quinazolinones[60] was not successful. Some 4(3*H*)-quinazolinones have shown antitubercular[61] and antibacterial[62] activity. Compound **113** (Scheme 4) was interesting to synthesize due to the expected lipophilicity and hydrolytic stability caused by the steric hindrance of the camphene skeleton. The reaction was carried out by condensation of (+)-camphor (**107**) and **65** in the presence of catalytic amount of anhydrous $ZnCl_2$, according to published procedure.[63]



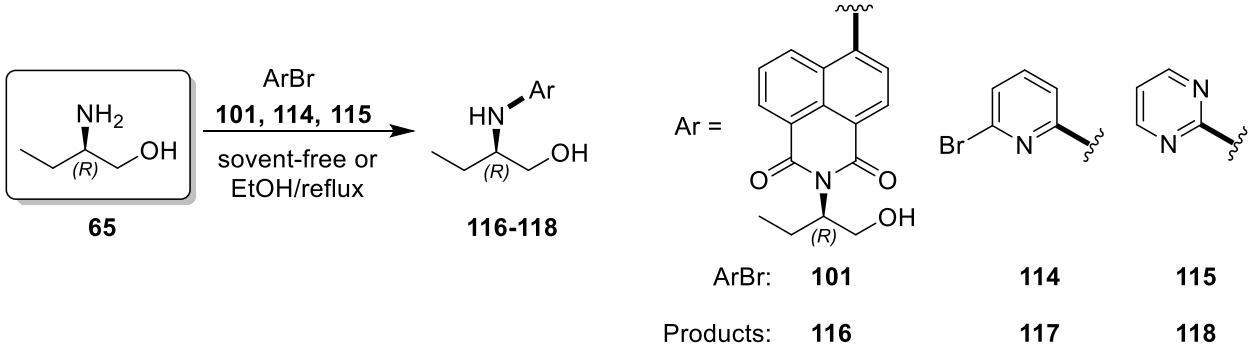
108 **102** R= -Ph, X=S

109 **103** R= -Et, X=O

110 **104** R= 1-naphthyl, X=O

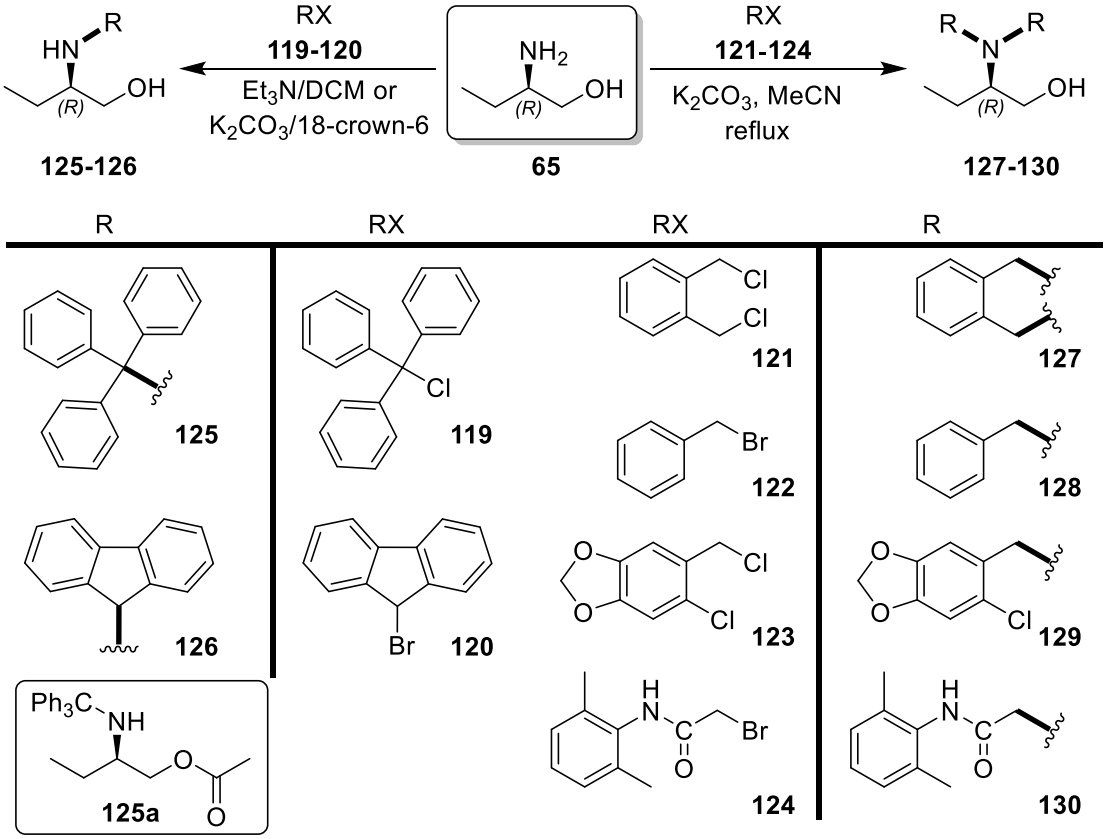
Scheme 4. Synthesis of compounds **108-113**.

Series of *N*-substituted aminoalcohols have been synthesized by using (*R*)-2-amino-1-butanol (**65**) as central chiral unit, in reaction with aryl- and alkyl- halogenides.[35] Aminoalcohol **65** reacted under solvent-free conditions with the heteroaryl bromides **101** and **114**, producing compounds **116-117** (Scheme 5). It is interesting to note that the second bromine atom in **114** was not replaced with (*R*)-2-amino-1-butanol unit. Compound **118** was easily synthesized from **65** and **115** in refluxing ethanol (EtOH) (Scheme 5).



Scheme 5. Synthesis of compounds **116-118**.

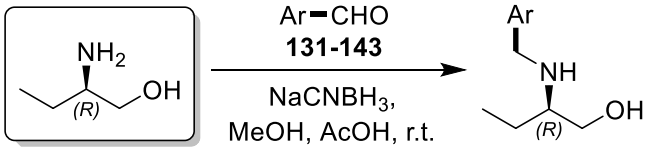
N,*N*-disubstituted aminoalcohols **127-130** (Scheme 6)[35] were prepared in good yields, under standard conditions, by using excess of K_2CO_3 in refluxing acetonitrile. Due to steric reasons, in the case of products **125** and **126** (Scheme 6) only mono-substitution of the amino group was achieved. The reactions, however, were smooth in both cases - Et_3N in dichloromethane (DCM) for **125** and K_2CO_3/18-crown-6 in acetonitrile (MeCN) for **126**. Attempts for *N*-acetylation of **125** (with acetyl chloride/Et_3N) provided only the *O*-acylated product **125a**. The synthesis of compounds **127**,[64-69] **128**[70,71] and **125** has been previously described. The latter two derivatives have been used for preparation of antitumor agents.[72,73]



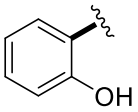
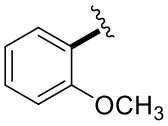
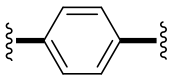
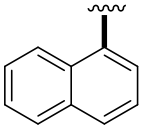
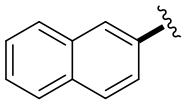
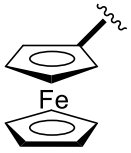
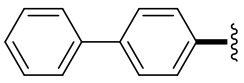
Scheme 6. Synthesis of compounds **125-130**.

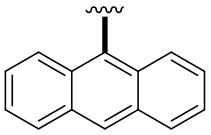
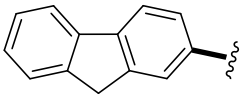
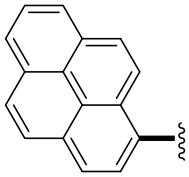
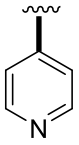
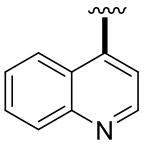
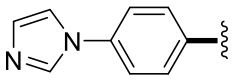
A set of benzylic type substituted aminoalcohols **144-156** was synthesized (Scheme 7)[35] by applying very efficient reductive amination of aldehydes **131-143** with aminoalcohol **65**. Instead of using the commonly applied (and more expensive) reducing agent $NaBH(OAc)_3$,[74-76] we used the cheaper $NaCNBH_3$ in methanol/acetic acid (MeOH/AcOH) and achieved good results.[77] Some

of the isolated products have been previously mentioned elsewhere. The *S*-enantiomer of **144** has been described in patents as catalyst.[78,79] Bis-substituted product **146** has been a part of study, concerning prevention of drug-induced cytotoxicity.[80] Aminoalcohol **147** and its *S*-enantiomer were used as ligands for stereoselective reduction of ketones.[81] Racemate of **149** has been mentioned as intermediate for preparation of ferrocenylmethylaminoalkoxy silanes.[82] The *S*-enantiomers of **150** and **151** have been previously prepared by reductive amination of aldehydes **137** and **138** respectively, by using resin supported cyano borohydride.[83] The formation of **153** through reductive amination has been noted in a study regarding the cytotoxicity of pyrene-containing aminoalcohols.[84]



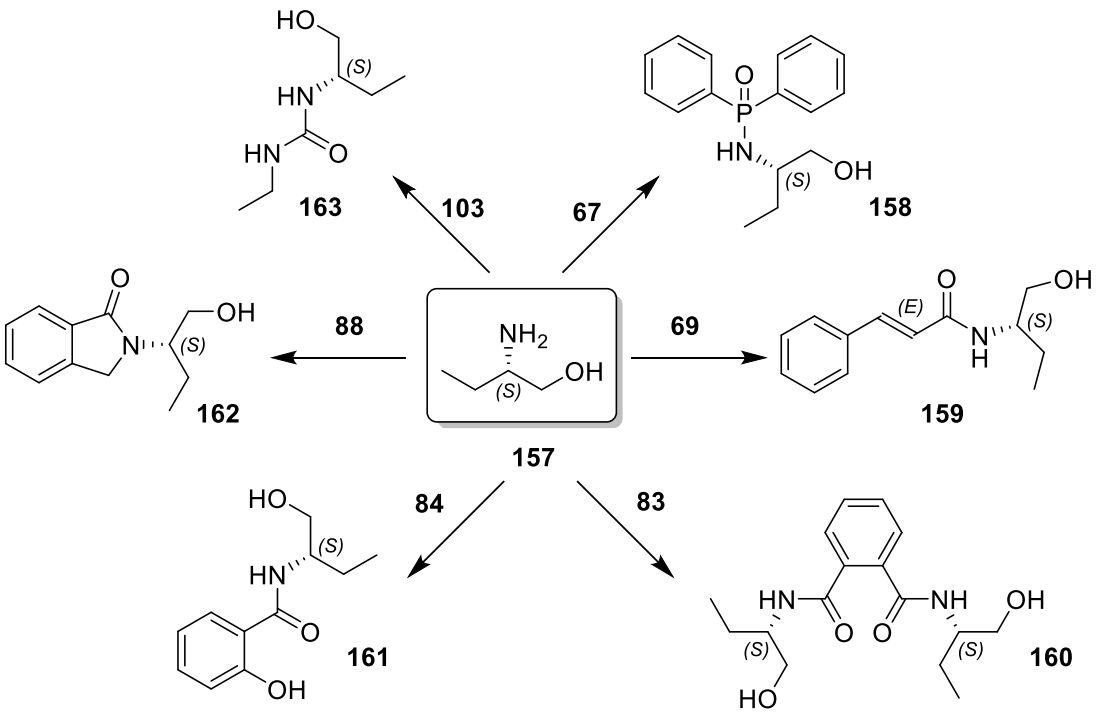
65 **144-156**

Ar	Ar−CHO	Products
	**131**	**144**
	**132**	**145**
	**133**	**146**
	**134**	**147**
	**135**	**148**
	**136**	**149**
	**137**	**150**

Ar	Ar−CHO	Products
	**138**	**151**
	**139**	**152**
	**140**	**153**
	**141**	**154**
	**142**	**155**
	**143**	**156**

Scheme 7. Synthesis of compounds **144-156**.

The evaluation of antimycobacterial activity of the compounds derived from (*R*)-2-amino-1-butanol, provided very promising results (see below, Table 2). Therefore, studying the *S*-enantiomers of the most active compounds was necessary to compare the activity data. Thus, the synthesis of compounds **158-163** (Scheme 8)[35] from (*S*)-2-amino-1-butanol (**157**) was achieved by applying the same procedures already described above for their *R*-analogues **75**, **77**, **86**, **87**, **95** and **109**, respectively.



Scheme 8. Synthesis of compounds **158-163**.

The synthesized compounds (**74-81**, **85-87**, **95-101**, **108-113**, **116-118**, **125-130**, **144-156** and **158-163**) were evaluated for their *in vitro* activity against reference strain *Mycobacterium tuberculosis* H37Rv (Table 2) using the method of *Canetti*.[85,86] Five of the compounds, namely **75**, **86**, **87**, **95** and **109** have shown remarkable high activity, which is between 5 and 11 times better than EMB used as reference. For the purpose of comparison, the most active EMB analogue, 1,2-diamine SQ 109 (Table 1), is 35 times more potent than EMB.[29] It is interesting to note that no analogue structures of **75**, **87**, **95** and **109** were found, that possess antimycobacterial activity. Known antimycobacterial activity of salicylanilide phenolic esters,[87,88] shows that the salicylamide fragment is important for the activity of **87**. Compounds **77**, **80**, **125a**, **127** and **116** have shown 50-80% activity compared to EMB. The cinnamamide group of **77** has been incorporated in diverse structures possessing antimycobacterial activity.[89,90] The remaining (*R*)-2-aminobutanol derivatives that were synthesized have no perceptible activity. All of the compounds mentioned above (except **125a**) are in agreement with the formal *Lipinski*`s rule of five (Table 2).

The evaluation of the antimycobacterial activity of the *S*-enantiomers **158-163** has shown absence of activity (100% growth) at concentrations ca. 2-3 fold higher than MIC of EMB. These results are in contrast with the high activity observed for the *R*-configured enantiomers (**75**, **77**, **86**, **87**, **95** and **109**, respectively). This is opposite to the fact that (*S*,*S*)-EMB is approximately 500 fold more active than (*R*,*R*)-EMB.[27] Therefore, the direct comparison of the structures presented in this study with EMB should be handled with care. Since **158-163** were not interesting as antimycobacterial agents, their cytotoxicity and other antibacterial activity was not investigated. There is no correlation between antimycobacterial activity and activity against other microorganisms, for the compounds in this study (see below and compare Tables 2 and 3). This indicates the specific activity of all potent derivatives of (*R*)-2-aminobutanol to the *Mycobacterium tuberculosis*, as it is established for the majority of first-line antimycobacterial agents (including EMB) in clinical use. Furthermore, since all compounds are partial structure analogues of EMB, it can be assumed that they share similar mode of antimycobacterial action,[24-26] although this needs clarification.[91] Therefore, investigating the mechanism of action and the role of chirality of these compounds would aid further discovery of more potent analogues.

Table 2. *In vitro* screening data for antimycobacterial activity and cytotoxicity of compounds **74-81**, **85-87**, **95-101**, **108-113**, **116-118**, **125-130**, **144-156** and **158-163**.

Compound	Activity toward *M. tb.* H37Rv, MIC (μM)	*In vitro* cytotoxicity toward HEK 293T,[a] IC_{50} (μM)	Selectivity index, SI[b,c]	Log*P*[d]	Solubility in deionized water at 20°C (mg/ml)[b]
74	28.86	NT	NT	0.48 +/-0.31	NT
75	0.69	155	224.6	1.82 +/-0.57	<1
76	>17.46	NT	NT	0.79 +/-0.37	NT
77	9.12	211	23.1	1.70 +/-0.35	<1
78	>20.55	NT	NT	2.48 +/-0.36	NT
79	>20.55	NT	NT	2.48 +/-0.36	NT
80	8.46	173	20.5	1.67 +/-0.39	1.5
81	>18.37	NT	NT	1.60 +/-0.46	NT
85	>25.74	NT	NT	-0.01 +/-0.37	NT
86	0.65	244	375.4	-0.27 +/-0.52	46
87	0.96	257	267.7	1.59 +/-0.45	2
95	0.97	89	91.8	0.49 +/-0.36	1
96	>22.81	NT	NT	1.81 +/-0.28	NT
97	23.67	NT	NT	0.07 +/-0.33	NT
98	27.29	NT	NT	0.66 +/-0.33	NT
99	>29.21	NT	NT	-0.70 +/-0.39	NT
100	>18.57	NT	NT	1.44 +/-0.62	NT
101	>14.36	NT	NT	2.21 +/-0.65	NT
108	>22.29	NT	NT	1.14 +/-0.33	NT
109	1.25	114	91.2	-0.23 +/-0.35	215
110	>19.36	NT	NT	2.74 +/-0.40	NT
111	23.23	NT	NT	2.28 +/-0.75	NT
112	22.91	NT	NT	0.94 +/-0.29	NT
113	>22.39	NT	NT	4.62 +/-0.47	NT
116	14.03	47	3.4	0.92 +/-0.98	<1
117	20.40	NT	NT	2.41 +/-0.62	NT
118	>29.90	NT	NT	0.47 +/-0.57	NT
125	>15.09	NT	NT	6.22 +/-0.53	NT
125a	13.39	114	8.5	6.90 +/-0.40	<1
126	19.74	NT	NT	3.36 +/-0.56	NT
127	10.46	122	11.7	2.20 +/-0.38	<1
128	>18.56	NT	NT	4.61 +/-0.42	NT
129	>11.73	NT	NT	5.79 +/-0.55	NT
130	>12.15	NT	NT	4.16 +/-0.38	NT
144	25.61	NT	NT	1.26 +/-0.35	NT
145	>23.89	NT	NT	1.91 +/-0.36	NT
146	17.83	NT	NT	1.78 +/-0.48	NT
147	21.80	NT	NT	3.23 +/-0.34	NT
148	>21.80	NT	NT	3.23 +/-0.34	NT
149	17.41	NT	NT	-[e]	NT
150	>19.58	NT	NT	3.76 +/-0.39	NT
151	17.90	NT	NT	4.46 +/-0.34	NT
152	18.72	NT	NT	3.94 +/-0.40	NT
153	16.48	NT	NT	4.95 +/-0.34	NT
154	27.74	NT	NT	0.51 +/-0.35	NT
155	21.71	NT	NT	1.86 +/-0.35	NT
156	20.38	NT	NT	1.76 +/-0.62	NT
158	>17.28	NT	NT	1.82 +/-0.57	NT
159	>22.80	NT	NT	1.70 +/-0.35	NT
160	>16.21	NT	NT	-0.27 +/-0.52	NT
161	>23.90	NT	NT	1.59 +/-0.45	NT
162	>31.21	NT	NT	-0.23 +/-0.35	NT
163	>24.36	NT	NT	0.49 +/-0.36	NT
EMB.2HCl[f]	7.22	NT	NT	0.06[g]	100[f]

[a] HEK - human embryonal kidney cell line 293T.

[b] NT – not tested; cytotoxicity, SI and water solubility were tested/calculated/measured only for selected active compounds.

[c] Selectivity index SI = IC_{50}/MIC.

[d] Log*P*, octanol-water partitioning coefficient, was calculated using ACDLabs/ChemSketch 12.01 (www.acdlabs.com).

[e] Log*P* was not calculated for this compound because of software limitations.

[f] EMB.2HCl – ethambutol dihydrochloride (reference compound).

[g] Log*P* and water solubility of EMB.2HCl are known in the literature: N.R. Budha, R.E. Lee and B. Meibohm, *Curr. Med. Chem.* **2008**, *15*, 809.

In order to examine the selectivity of the antiproliferative effects, the cytotoxic *in vitro* activity of representative compounds, exerting antimycobacterial activity, was assessed against a human embryonal kidney non-tumor cell line 293T (HEK 293T), after 72 h continuous exposure.[35] Evident from the IC_{50} values summarized in Table 2, the compounds were generally of low-to-moderate cytotoxicity against the human cells; with few exceptions the compounds induced 50% inhibition of cellular proliferation and viability at concentrations greatly exceeding 100 μM. It is noteworthy that the structural peculiarities affording the highest antimycobacterial activity within the series, were also generally associated with very low antiproliferative/cytotoxic effects against human cells with selectivity indices ranging from 91.2 to 375.4.

For the synthesized derivatives of (*R*)-2-amino-1-butanol (except **158**-**163**) a qualitative evaluation of *in vitro* antibacterial and antifungal activities against conditioned pathogenic microorganisms was performed (Table 3),[35] using agar diffusion test.[92] Antibacterial activities were examined against the Gram (+) strains: *Bacillus subtilis* ATCC 6633, *Bacillus idosus* B 241, *Bacillus megaterium* NRRL 1353895, *Bacillus mycoides* DSMZ 274, *Bacillus cereus* ATCC 11778, *Acinetobacter johnsonii* ATCC 17909, *Staphylococcus aureus* NRRL B 313, *Sarcina lutea* ATCC 9341, *Micrococcus luteus* ATCC 9631, and the Gram (-) strain *Escherichia coli* ATCC 8739. Antibiotics streptomycin and gentamicine sulphate were used as reference compounds. Antifungal activities were examined against the yeast strains *Candida tropicalis* ATCC 20336 and *Saccharomyces cerevisiae* ATCC 9763, and the fungal strain *Penicillium chrysogenum* CECT 2802. Fluconazole and itraconazole were used as antifungal reference compounds.

The most of the compounds tested were not active even at concentrations 50 mg/ml and consequently are not appropriate for antimicrobial agents (Table 3). Some of the compounds (**75**, **97**-**98**, **111**, **126**, **127**, **147**-**148**, **150**-**152**) demonstrated wide spectrum of low-to-moderate activity at lower concentrations – 25, 10, 5, 2, 1.25 or 1 mg/ml. The data in Table 3 (second values) show that almost all of the compounds are not active at concentrations 2 mg/ml or less. Only compounds **97**, **151** and **152** were active against all microorganisms (except *Escherichia coli*) in significantly lower concentrations. The most potent compound **97** exhibited emphasized activity against the fungi *Candida tropicalis*, *Saccharomyces cerevisiae* and *Penicillium chrysogenum* even at concentrations 6, 6 and 12.5 μg/ml, respectively. The intact maleimido ring of **97** is probably necessary for the observed activity. On the contrary, the substituted maleimide **98** and the dihydro analogue **99** showed significantly lower activity and absence of activity, respectively. Similar trend has been observed in recent report concerning the antifungal activity of analogous *N*-alkyl-aryl substituted maleimide derivatives.[93]

List of microorganisms (Table 3, columns 1-13): 1. *Bacillus subtilis*, 2. *Bacillus idosus*, 3. *Bacillus megaterium*, 4. *Bacillus mycoides*, 5. *Bacillus cereus*, 6. *Acinetobacter johnstonii*, 7. *Staphylococcus aureus*, 8. *Sarcina lutea*, 9. *Micrococcus luteus*, 10. *Escherichia coli*, 11. *Candida tropicalis*, 12. *Saccharomyces cerevisiae*, 13. *Penicillium chrysogenum*.

Table 3. Evaluation of *in vitro* antibacterial and antifungal activity of derivatives **74-81**, **85-87**, **95-101**, **108-113**, **116-118**, **125-130** and **144-156** against conditioned pathogenic microorganisms.

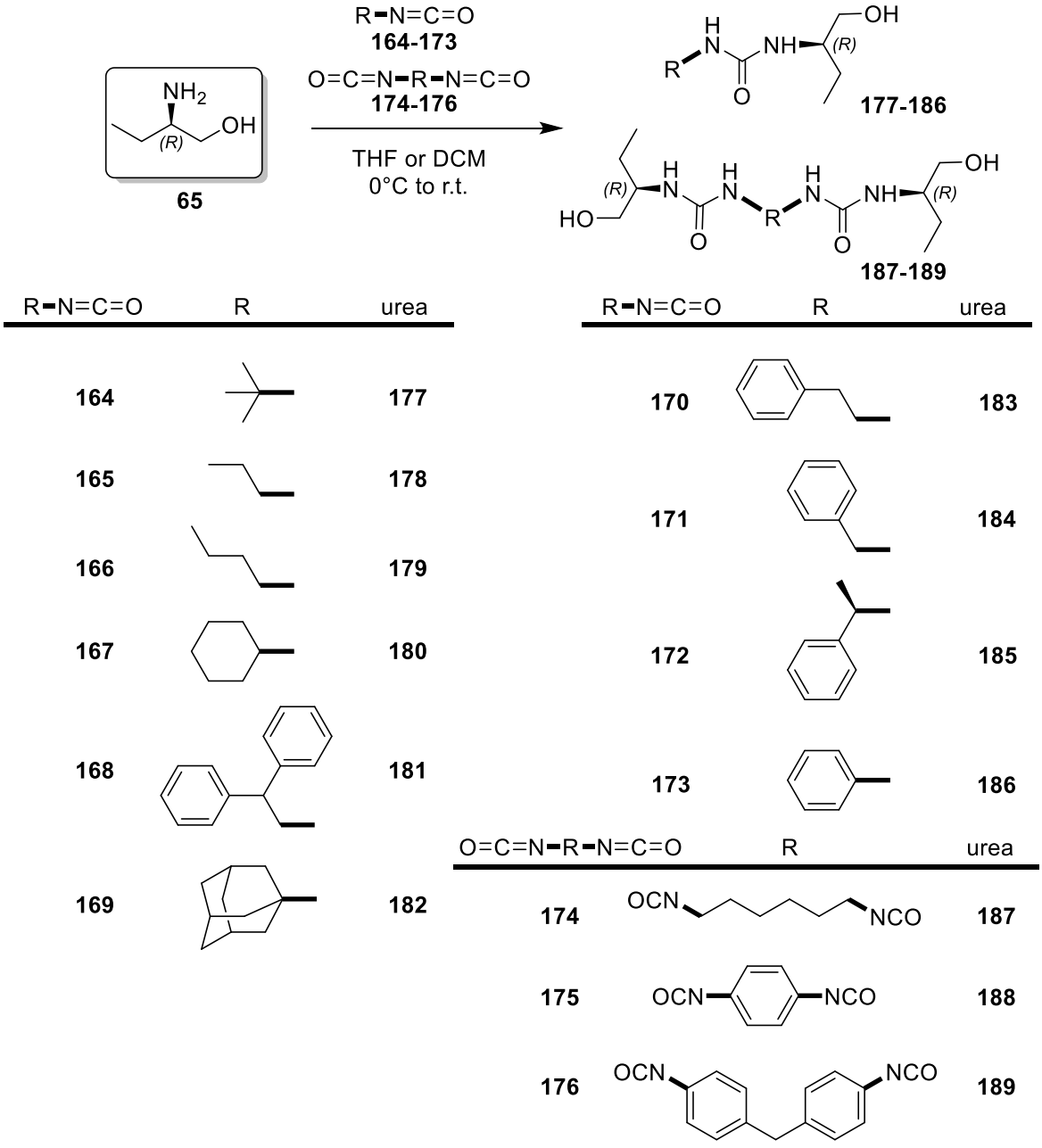
Comp.	Microorganisms												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
74	15	11	NA	11	16	NA	15	NA	NA	NA	NA	NA	NA
75	18	15	16	15	15	17	18	18	15	NA	22	16	NT
76	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NT
77	NA	NA	12	12	12	NA	12	15	NA	NA	11	13	NT
78	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
79	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
80	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
81	NA	NA	NA	NA	NA	NA	NA	NA	NA	NT	NA	NA	NA
85	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
86	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
87	NA	11	13	12	11	13	11	13	11	NA	13	15	NA
95	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
96	NA	NA	NA	NA	NA	NA	NA	NA	NA	11	NA	NA	NA
97	40 0.06	35 0.06	40 0.06	38 0.06	40 0.013	39 0.06	>40 0.06	38 0.06	38 0.3	29 1.25	>40 0.006	>40 0.006	>40 0.013
98	21	23	20	24	28	21	20	21	22	32	20	21	25
99	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
100	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	10
101	NA	NA	NA	NA	NT	NA	NA	NT	NA	NT	NA	NA	NA
108	NA	NA	13	12	14	12	NA	12	12	16	16	15	NT
109	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
110	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
111	NA	15	13	15	18	10	12	13	15	0	20	11	35
112	NA	14	NA	NA	NA	14	NA	NA	NA	NA	NA	NA	NA
113	NA	NA	10	11	NA	NA	NA	NA	NA	NA	NA	NA	NA
116	NA	NA	NA	NA	NA	NA	NA	NA	NT	NT	NT	NA	NA
117	13	17	15	13	19	14	14	17	18	17	25	10	17
118	NA	NA	NA	NA	12	NA	NA	NA	NA	NA	NA	NA	NA
125	12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
125a	13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
126	NA	15	22	17	NA	16	12	17	17	12	17	22	20
127	11	20	NT	23	25	NT	25	NT	NT	25	21	16	10
128	NA	11	NA	NA	11	11	11	12	12	NA	10	NA	NA
129	NA	NA	10	NA	NA	NA	NA	10	NT	NA	NA	NA	NA
130	NA	10	NA	12	11	NA	15	NA	12	NA	NA	NA	NA
144	17	11	12	14	14	13	12	12	11	NA	NA	NA	12
145	10	15	11	11	13	10	10	NT	11	NA	NA	16	NA
146	10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
147	17	18	10	18	15	20	16	13	17	28	30	32	22
148	19	19	17	17	14	17	19	17	19	22	10	10	35
149	15	16	16	20	15	15	18	17	17	16	12	14	12
150	18	18	19	18	15	20	20	17	17	28	18	18	40
151	18 0.3	19 1.25	20 0.6	20 0.6	19 0.3	17 0.6	19 0.03	18 0.3	19 0.3	12	31 0.013	32 0.013	16 0.06
152	18 1.25	17 0.6	16 1.25	20 1.25	16 1.25	17 1.25	17 1.25	18	16	16 1.25	21 0.06	20 1.25	30
153	14	30	13	15	14	14	13	13	14	NA	40	35	14
154	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
155	10	12	11	16	13	13	14	13	10	20	NT	NT	20
156	12	16	NA	18	13	18	16	18	16	NA	NA	NA	12
S[a]	35 30 25	30 27 21	30 25 20	35 30 25	30 28 23	30 24 20	32 26 20	27 22 19	30 25 20	35 26 23	NT	NT	NT
G[b]	30 27	35 29	36 30	32 28	29 26	39 31	35 27	34 28	38 28	31 25	NT	NT	NT
F[c]	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	35	-	-
I[d]	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	30	-

Diameter of zone of inhibition are given in mm. Second values are the lowest concentrations (in mg/ml) provoking zone of inhibition >11 mm. Second values are given only for compounds preserving their activity at concentrations 1.25 mg/ml or less.
NA – not active according first zone inhibition test (at concentration 50 mg/ml); further tests not performed;
NT – zone inhibition test not performed.
[a] Zone inhibition for reference Streptomycin (S) were measured at three concentrations – 25, 5 and 1 mg/ml.
[b] Zone inhibition for reference Gentamycin sulphate (G) were measured at concentrations 20 and 4 mg/ml.
[c] Zone inhibition for reference Fluconazole (F) was measured at concentration 25 mg/ml.
[d] Zone inhibition for reference Itraconazole (I) was measured at concentration 12.5 mg/ml, but is not completely soluble in DMSO at this concentration.

Synthesis and antitubercular activity of new (*R*)-2-amino-1-butanol derived ureas, thioureas and acylthioureas (**177-189**, **194-197** and **203-207**)[36]

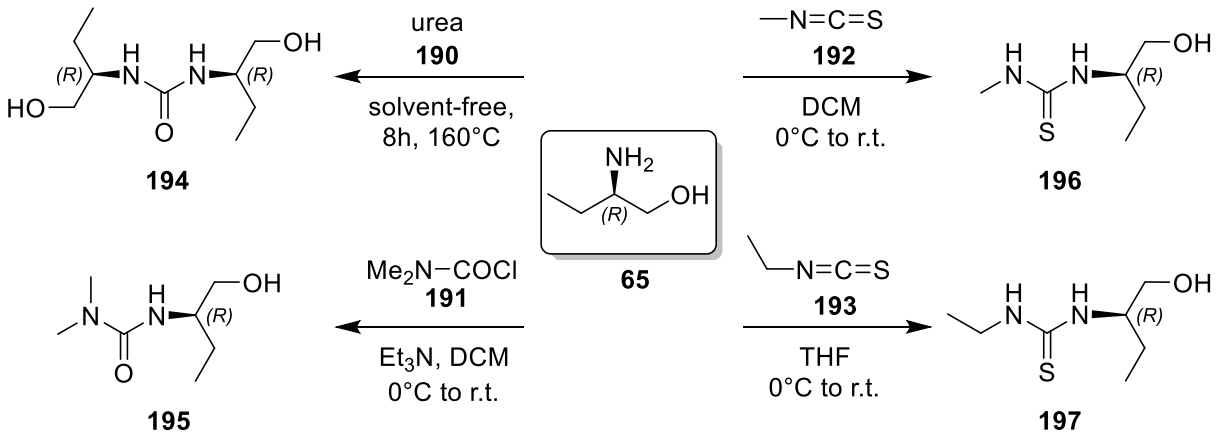
A variety of structurally related thioureas (*N*-aryl-*N*`-alkyl and *N*,*N*`-diaryl substituted) have been extensively evaluated against different strains of *Mtb* showing valuable activities,[54,94-98] sometimes greatly exceeding EMB. One representative example is isoxyl (thiocarlide; 4,4-diisoamyloxydiphenylthiourea), efficiently clinically used drug since 1960`s.[51,52] Recently, the subclass of acylthioureas have been also an object of interest showing promising anti-TB activity, as summarized by *S. Ananthan et al*.[50,99]

Taking into account the above presented results, we were encouraged to perform the synthesis of new series of ureas, thioureas and acylthioureas incorporating the (*R*)-2-amino-1-butanol motif (Schemes 9-11) and to evaluate their *in vitro* antimycobacterial activity.



Scheme 9. Synthesis of compounds **177-189**.

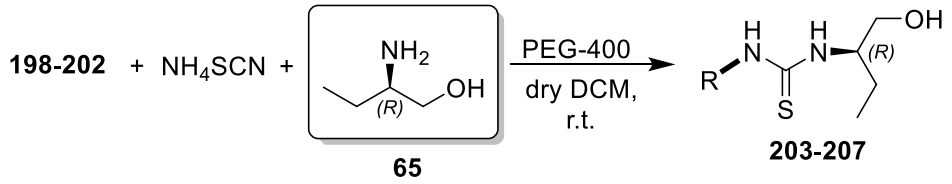
The synthesis of compounds **177-189** was performed by mixing **65** with the corresponding mono- (**164-173**) and bis- (**174-176**) isocyanates in dry tetrahydrofuran (THF) or DCM as a solvent (Scheme 9). Desired ureas were obtained in very high yields and excellent purity. The preparation of **183-184**,[58] and **186**[100,101] has been described previously, however as racemic mixtures and without evaluation of their bioactivity.

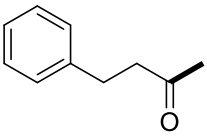
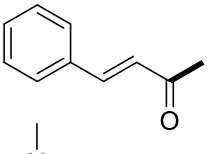
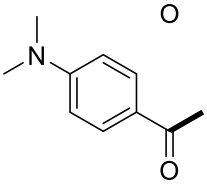


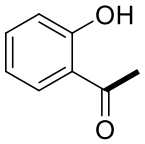
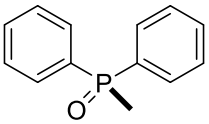
Scheme 10. Synthesis of compounds **194-197**.

The urea derivative **194** was synthesized from **65** and urea (Scheme 10) using a published solvent-free procedure.[102] Compound **195** was obtained by reacting **65** with **191** under standard acylation conditions (0°C and Et_3N in dry DCM). The thioureas **196** and **197** were obtained in high yields in the same manner as ureas **177-189**, by mixing **65** with the isothiocyanates **192** and **193**, respectively (Scheme 10). The preparation of **196** has been mentioned earlier.[103]

Compounds **203-207** (Scheme 11) were obtained by using of recently published procedure[104] - one-pot reaction of acylchlorides **198-202** with NH_4SCN in the presence of catalytic amounts of polyethylene glycol 400 (PEG-400), followed by addition of **65** to the reaction mixture. The yields were moderate, however the application of this method was easy to perform and convenient in respect of the purification of the desired products (simple filtration through a pad of silica provided excellent pure products).



acyl halogenides **198-200**	R	acyl thioureas
198		**203**
199		**204**
200		**205**

acyl halogenides **201-202**	R	acyl thioureas
201		**206**
202		**207**

Scheme 11. Synthesis of compounds **203-207**.

The synthesized compounds were evaluated for their in vitro activity against *M. tuberculosis* H37Rv and MDR strain 43 (Table 4) using the method of *Canetti*.[85,86] All the compounds synthesized are in agreement with the formal *Lipinski's* rule of five. The first 17 derivatives of (*R*)-2-amino-1-butanol - ureas **177-189**, **194-195** and thioureas **196-197** (Schemes 9 and 10) were inactive even at concentrations of 5 μg/ml (100% growth of the bacteria was observed). The only observed exception was compound **181**, showing activity close to EMB. It is interesting to point

out, that even a small structural change in the molecule of **109** (Scheme 4)[35] induce lack of activity. For example, its inactive near homologues possess propyl (**178**), *n*-butyl (**179**) and *t*-butyl (**177**) groups. Replacement of the carbonyl group with thiocarbonyl (thioureas **196**-**197**) leads to the same consequences. Similar negative trend was observed for many derivatives of EMB.[27,85]

Table 4. *In vitro* screening data for antimycobacterial activity and cytotoxicity of compounds **177**-**189**, **194**-**197** and **203**-**207**.

Compound	Activity toward *M. tb.* H37Rv, MIC (μM)	Activity toward *M. tb.* strain 43 MIC (μM)[a]	*In vitro* cytotoxicity toward HEK 293T, IC_{50} (μM)[a]	Selectivity index, SI[b]	Log*P*[c]
177	>26.56	NT	NT	NT	0.46 +/-0.36
178	>28.71	NT	NT	NT	0.30 +/-0.35
179	>26.56	NT	NT	NT	0.83 +/-0.35
180	>23.33	NT	NT	NT	1.30 +/-0.35
181	4.80	NT	NT	NT	3.04 +/-0.38
182	>18.77	NT	NT	NT	2.12 +/-0.39
183	>21.16	NT	NT	NT	1.43 +/-0.36
184	>22.49	NT	NT	NT	1.01 +/-0.37
185	>21.16	NT	NT	NT	1.36 +/-0.38
186	>24.01	NT	NT	NT	1.51 +/-0.40
187	>14.43	NT	NT	NT	-0.16 +/-0.49
188	>14.76	NT	NT	NT	0.41 +/-0.56
189	>11.67	NT	NT	NT	2.80 +/-0.56
194	>24.48	NT	NT	NT	-0.51 +/-0.39
195	>31.21	NT	NT	NT	0.05 +/-0.39
196	>30.82	NT	NT	NT	-0.09 +/-0.27
197	>28.36	NT	NT	NT	0.44 +/-0.27
203	7.13	>17.83	66.2	9.3	1.75 +/-0.60
204	0.36	>17.96	42.9	119.2	2.29 +/-0.61
205	3.39	>16.92	12.8	3.8	1.73 +/-0.61
206	7.46	7.46	104.4	14.0	1.58 +/-0.62
207	5.74	11.49	179.6	31.3	1.86 +/-0.62
EMB.2HCl[d]	7.22	NT	NT	NT	0.06[e]

[a] NT – not tested - for low active compounds against H37Rv strain; cytotoxicity and SI were tested/calculated only for selected active compounds. [b] selectivity index: SI = IC_{50}/MIC (H37Rv). [c] Log*P*, octanol-water partitioning coefficient, was calculated using ACDLabs/ChemSketch 12 (www.acdlabs.com). [d] EMB.2HCl – ethambutol dihydrochloride (reference compound). [e] Log*P* and water solubility of EMB.2HCl are known in the literature: N.R. Budha, R.E. Lee and B. Meibohm, *Curr. Med. Chem.* **2008**, *15*, 809.

Other series of five new acylthioureas **203**-**207** (Scheme 11) was designed to contain important pharmacophore groups (discovered in our previous study),[35] attached to acylthioureas containing (*R*)-2-amino-1-butanol moiety. Compounds **203** and **205**-**207** showed activity against *M. tuberculosis* H37Rv comparable to EMB. Cinnamic derivative **204** is the most active compound in this study with MIC 0.36 μM. Besides, relatively low cytotoxicity and excellent selectivity index (119.2) of **204** suggest that this compound is a good lead structure for further investigations. Encouraging from above results, we tested **203**-**207** against MDR strain 43 (resistant to rifampin and isoniazid). In this case compounds **203**-**205** lose their activity (100% growth of bacteria at concentrations 5 μM). On the other hand, **206** preserved its activity and **207** was only 2 fold less active.

The findings that the acylthioureas **203**-**207** exerted good antimycobacterial activity gave us a good reason for further more detailed evaluation of the cytotoxic effect of those compounds. The cytotoxic activity of the tested compounds was investigated against HEK 293T cell line using the MTT dye reduction assay.[105] The corresponding IC_{50} values of the tested compounds were calculated using nonlinear regression analysis and summarized in Table 4. As it could be seen the compounds demonstrated a wide range of cytotoxicity with IC_{50} between 12.8 and 179.6 μM. Perceptible cytotoxicity to the cells was exhibited by the substances **203**-**204** while compound **205** showed strong cytotoxic effect. It is noteworthy that the most potent antimycobacterial sample within this series (**204**) showed excellent SI (119.2). Therefore it turned out that compound **204** is of particular interest due to its highly favorable features – low cytotoxicity and significant antimycobacterial activity which makes it a good candidate for an efficient therapeutic candidate.

3.1.2. New (-)-fenchone derivatives as perspective anti-TB drugs

(-)-Fenchone derived enantiopure antituberculosis candidates (compounds **218**-**226**, **230**-**232**, **236**-**238** and **241**-**242**)[106]

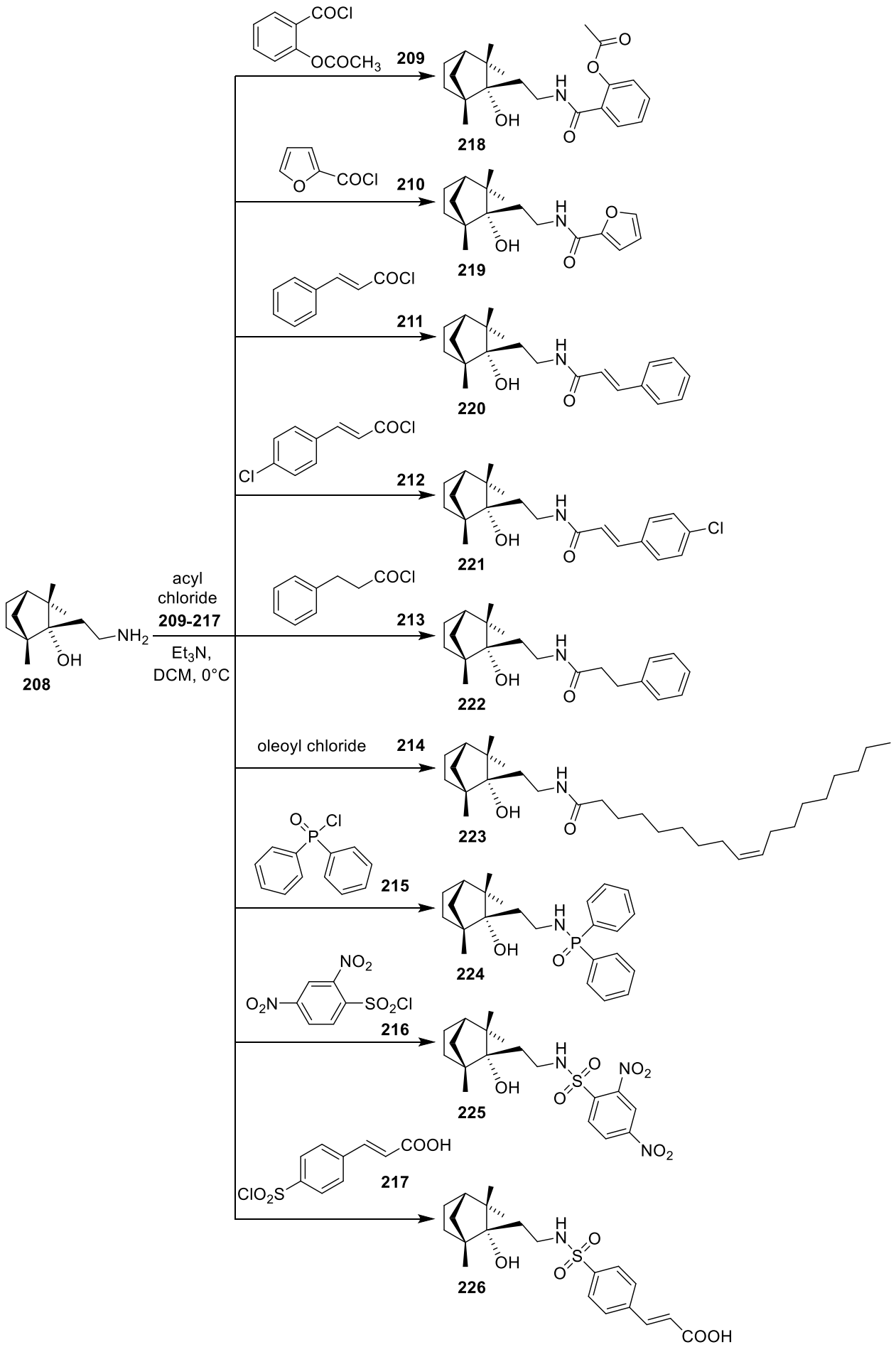
An urgent need for highly potent and effective antitubercular drugs with fewer or no side effects and shorter treatment periods is still apparent. Potential anti-TB drugs such as diarylquinolones (bedaquiline), nitroimidazoles (delamanid, pretomanid), pyrroles (sudoterb) and diamines[107] are in different stages of clinical trials or were already approved.[99,108] The incorporation of lipophilic polycyclic aliphatic compounds into drug structures with promising anti-TB applications has enjoyed much attention from researchers for several years starting with the discovery of polycyclic diamine SQ 109.[29] The studies of *Onajole et al*. confirm[32,107] the importance of further investigations of polycyclic compounds as potent anti-TB agents.

In the course of our research for novel anti-tubercular compounds, we demonstrated the role of chirality and wide spectrum of substituted acyl groups, attached to nitrogen atom of (*R*)-2-aminobutanol.[35,36] The present study[106] aims at investigating of new subclass diastereomerically pure bicyclic *N*-substituted compounds with fenchane skeletons and the possibility of further enhancing/improving their anti-TB activity. Based on this, novel amidoalcohols and other *N*-carbonyl derivatives bearing the lipophilic fenchane skeleton were synthesized and screened.

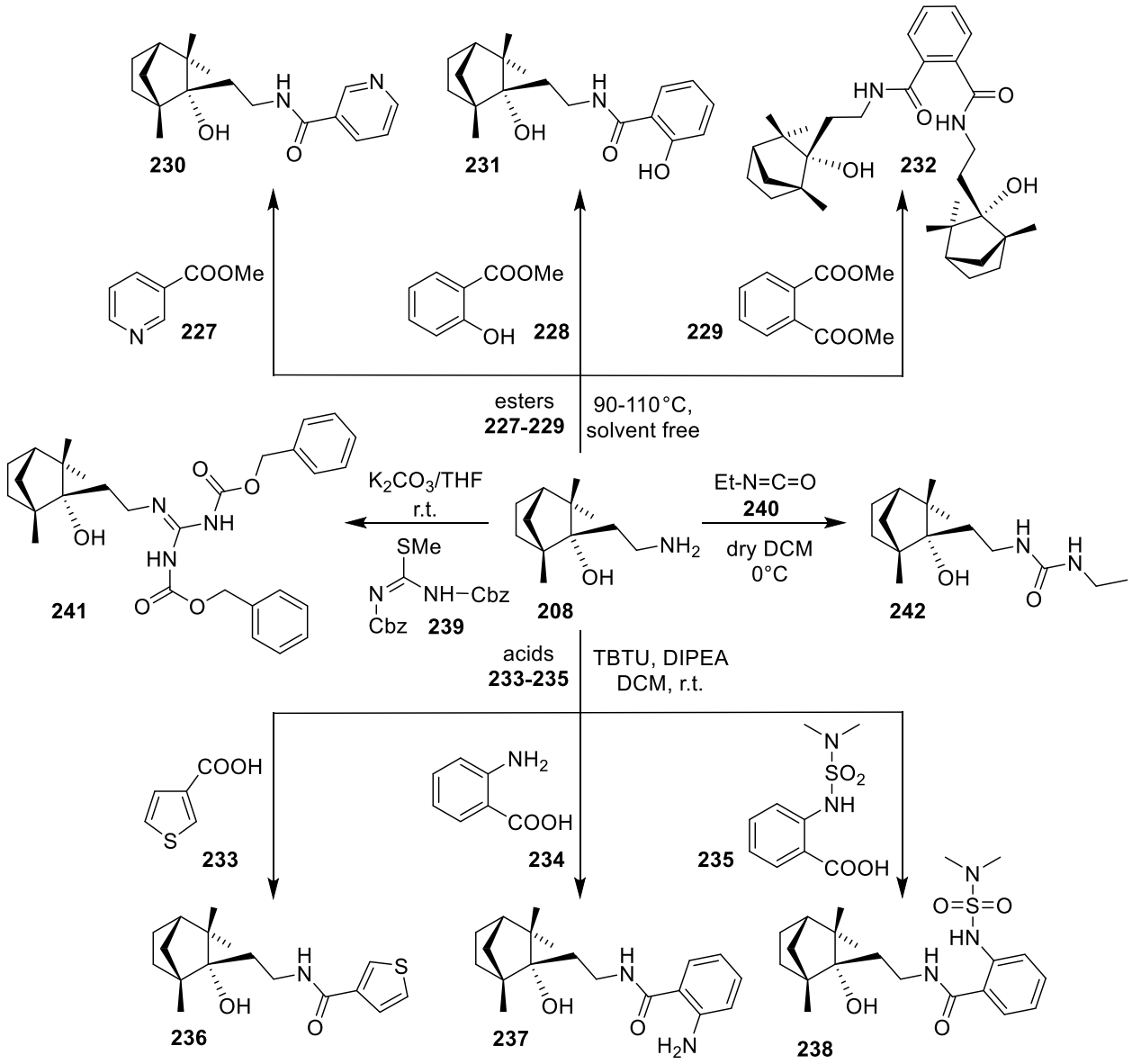
The starting enantiopure aminoalcohol **208** (Scheme 12) was prepared in two steps from natural (-)-fenchone, as described in our previous study.[109] Series of *N*-acyl derivatives (**218**-**226**) was obtained in good to excellent yields using standard conditions for acylation of **208** (0°C and Et_3N in dry DCM) with commercial acid chlorides **209**-**216**. Intermediate **217** was prepared from cinnamic acid as described.[110]

The amidoalcohols **230**-**232** were synthesized using simple solvent-free aminolysis of esters **227**-**229** with **208** at 90-100°C (Scheme 13). Compounds **236**-**238** were prepared through the coupling reaction between **208** and acids **233**-**235**, respectively, in presence of *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate (TBTU) as activating agent[111]. In case of anthranilic acid (**234**) preliminary protection of the aromatic amino group was not necessary, due to its low reactivity in comparison to the high nucleophilic amino group of **208**. To the best of our knowledge, there are no data published concerning intermediate **235**. This compound we prepared in one step from commercial chemicals (**234** and $Me_2N\text{-}SO_2Cl$). Urea **242** was synthesized through reaction between **208** and ethyl isocyanate (**240**) at 0°C (Scheme 13). The *N*-carbobenzyloxy (Cbz) protected guanidine derivative **241** was prepared in quantitative yield from **208** and commercially available 1,3-bis(benzyloxycarbonyl)-2-methyl-isothiourea (**239**) according analogous procedure described in literature.[112]

The above mentioned compounds were evaluated for their *in vitro* activity against *M. tuberculosis* H37Rv (Table 5) using the method of *Canetti*.[85,86] Most compounds are in agreement with the formal *Lipinski's* rule of five and were designed in order to investigate their antimycobacterial activity by using of EMB and aminoalcohol **208** as references. Most of the amides (**220**, **222**, **224**, **230**-**232** and **242**) contain pharmacophore groups known in our previous studies.[35,36] It is interesting to point out that the introduction of various substituents at N-atom of **208** doesn't affect dramatically the activity of the derivatives. As a whole, the compounds in this series (including **208**) showed moderate but stable level of antimycobacterial activity (in most cases 30-70% of the activity of reference EMB). Compounds **225**-**226** and **232** demonstrated MIC near to EMB. The activity of sulfonamide **225** was expected since studies of *Malwal et al.* revealed[113,114] the importance of 2,4-dinitrosulfonamide group as *in vivo* source of sulfur dioxide that is a key agent for selective radical damaging of bacterial biomacromolecules. In this context, it is interesting to note that the activity of cinnamic sulfonamide **226** (MIC 6.14 μM) is comparable with that of **225** although the structures are rather different in respect of the aromatic moieties. Compound **241** is a rare example of synthetic antitubercular agent possessing in its structure the guanidine motif. This moiety is common in antibiotics like streptomycin, capreomycin, viomycin[108,115] and some synthetic polyamines.[116]



Scheme 12. Synthesis of compounds **218-226**.



Scheme 13. Synthesis of compounds **230-232**, **236-238** and **241-242**.

The stable level of antimycobacterial activity gave us a good reason for further more detailed evaluation of the cytotoxic effect of those compounds and SI calculation. The cytotoxic activity of the tested compounds was investigated against human embryonic kidney cell line (HEK 293T) using the MTT dye reduction assay.[105] The corresponding IC_{50} values (in μM) of the tested compounds were calculated using nonlinear regression analysis and summarized in Table 5. As it could be seen the compounds demonstrated a wide range of cytotoxicity with IC_{50} between 18.8 and 335.6. Almost all of the substances have shown acceptable low to moderate cytotoxicity to the cells with only four exceptions (**218**, **220**, **223** and **230**) which showed high cytotoxicity. Compounds **225-226** and **241** demonstrated MIC near to EMB and low cytotoxic effect. It is important to note that the most potent antimycobacterial sample - the cinnamic sulfonamide **226** showed good SI (54.7) and therefore it is of particular interest due to its highly favorable features low cytotoxicity and significant antimycobacterial activity which makes it a good candidate for an efficient therapeutic agent.

Table 5. *In vitro* screening data for antimycobacterial activity and cytotoxicity of compounds **218**-**226**, **230**-**232**, **236**-**238** and **241**-**242**.

Compound	Activity toward *M. tb.* $H_{37}Rv$, MIC (μM)	Cytotoxicity toward HEK 293T, IC_{50} (μM)	Selectivity index, SI[a]	Log*P*[b]
208	15.20	42.3	2.8	2.13 +/- 0.28
218	13.92	18.9	1.4	2.80 +/- 0.40
219	10.30	62.3	6.0	2.91 +/- 0.42
220	15.27	23.8	1.6	4.24 +/- 0.38
221	22.15	72.5	3.3	4.76 +/- 0.40
222	18.22	178.6	9.8	3.86 +/- 0.42
223	13.00	21.6	1.7	9.72 +/- 0.39
224	17.62	64.8	3.7	4.36 +/- 0.60
225	7.02	97.9	13.9	3.91 +/- 0.44
226	6.14	335.6	54.7	3.96 +/- 0.45
230	19.87	18.8	0.9	2.79 +/- 0.40
231	18.92	83.9	4.4	3.99 +/- 0.46
232	11.44	106.7	9.3	4.53 +/- 0.55
236	19.52	70.3	3.6	3.42 +/- 0.49
237	18.96	81.5	4.3	3.41 +/- 0.43
238	14.17	62.1	4.4	3.14 +/- 0.68
241	14.91	121.2	8.1	2.31 +/- 0.40
242	9.85	82.4	8.4	6.16 +/- 0.65
EMB.2HCl[c]	7.22	not tested	-	0.06[d]

[a] selectivity index: SI = IC_{50}/MIC.
[b] Log*P*, octanol-water partitioning coefficient, was calculated using ACDLabs/ChemSketch 2012 (www.acdlabs.com).
[c] EMB.2HCl – ethambutol dihydrochloride (reference compound).
[d] Log*P* of EMB.2HCl is known in the literature: N.R. Budha, R.E. Lee and B. Meibohm, *Curr. Med. Chem.* **2008**, *15*, 809.

(-)-Fenchone derived cinnamamides as antitubercular agents (compounds **245**-**246** and **276**-**304**)[117]

Cinnamic acid and its derivatives were used in the earliest efforts for treating tuberculosis, starting from the late 19th century.[118-121] The potential of these compounds has been extensively examined for the past 30 years. Their synergic activity in combination with classic anti-TB drugs is well established.[122] Furthermore, the attachment of a cinnamyl moiety to the antibiotic rifamicin results in a product Rifacinna® (Figure 4), which is more active than rifamicin against susceptible *M. tuberculosis* strains, and also shows ability to overcome resistance.[123] Many other natural[124-127] and synthetic[89,90,126] products, bearing the cinnamic moiety, have also been proven effective against *M. tuberculosis*.

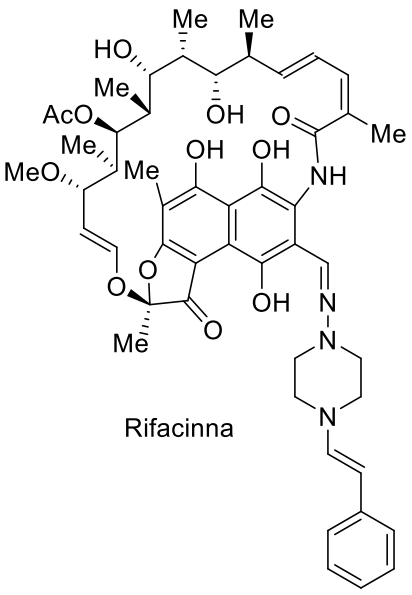
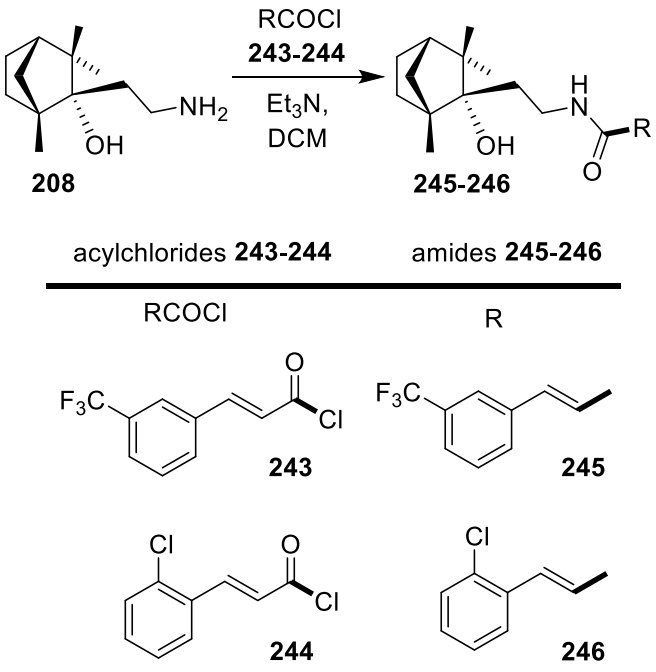


Figure 4.



Scheme 14. Synthesis of compounds **245**-**246**.

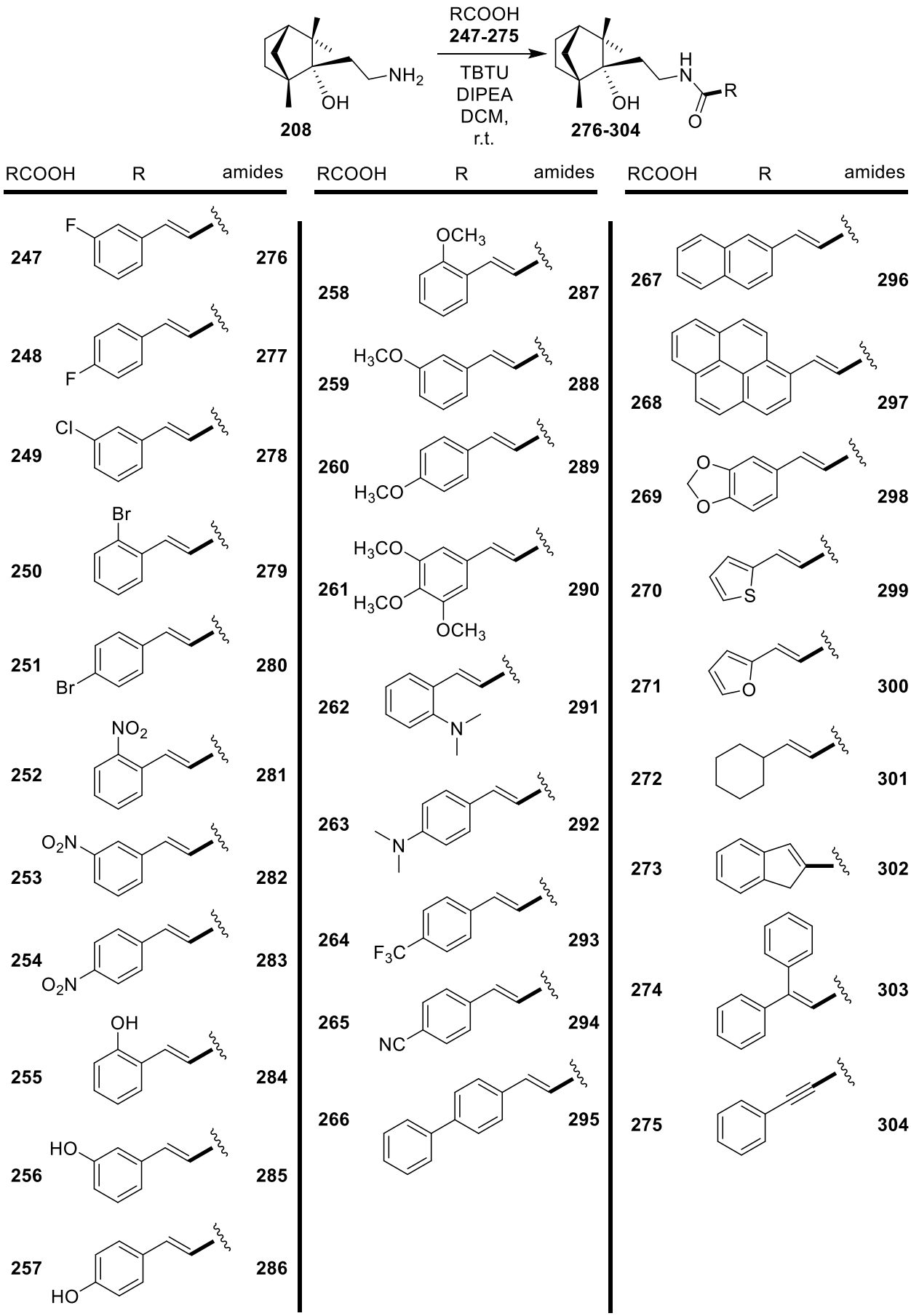
As a general rule, the presence of large lipophilic fragments within an active structure improves the anti-TB action. Especially promising results demonstrate compounds containing geranyl moiety.[29,90,128] In some works[129,130] it was proven that compounds incorporating bicyclic monoterpene moiety also possess a significant antimycobacterial activity. In our previous research,[106] a fenchone-based series (Scheme 12 and Table 5), including unsubstituted cinnamamide, showed moderate ability to inhibit the growth of *M. tuberculosis* and some examples even matched the activity of the classic anti-TB agent ethambutol. The investigation of new compounds bearing bulk lipophilic polycyclic fragments is getting increasingly popular since the discovery of SQ109, which now shows promising results.

As part of our continuous program of developing new anti-TB agents we designed a series of new hybrid structures. They consist of cinnamoyl-like motifs (containing condensed, substituted aryl and heteroaryl moieties), linked through an amide bond to functionalized fenchone-derived bicyclic system. Based on the results and argumentation presented above,[106] it is expected for some of these molecular hybrids increased anti-TB activity. Herein, we are reporting the synthesis of 31 novel amidoalcohols and their screening for activity against drug sensitive (H37Rv) strain of *M. tuberculosis*. The chemistry was directed to the synthesis of structurally diverse compounds consisting of aminoethyl-fenchol scaffold and substituted cinnamoyl residue attached through amide bond. To the best of our knowledge, there is only limited number of studies concerning the investigation of antitubercular activity of differently substituted cinnamoyl derivatives.[89,90,124-127] The structures planned were designed in order to find more active cinnamamides through varying the type and positions of the substituents in the aromatic ring, as well as to ascertain possible structure-activity relationships (SAR). In addition, the pharmacophoric contribution of the cinnamoyl moiety itself was evaluated by replacement with different groups possessing structural similarity. In all cases the fenchane part of the molecules was introduced through aminoalcohol **208** by using relatively simple but efficient synthetic strategy.[106,109]

Initially the reaction of aminoalcohol **208** with the acid chlorides **243** and **244** was performed to obtain amidoalcohols **245** and **246** in very good yields. Standard conditions for acylation of **208** have been applied (0°C and Et_3N in dry DCM, Scheme 14). The synthesized compounds were isolated in pure form by column chromatography.

The reaction of aminoalcohol **208** and readily available cinnamic acids offers more opportunities for obtaining structurally diverse series of compounds (Scheme 15). Amidoalcohols **276**-**304** were prepared through the coupling reaction between **208** and cinnamic acids (or their analogues) **247**-**275** in the presence of TBTU as activating agent and base *N*,*N*-diisopropylethylamine (DIPEA). Commercially available TBTU was chosen as one of the most effective and selective coupling reagent in peptide synthesis.[111] Some of the acids have been prepared by known procedures (**262**,[131] **268**[132] and **272**[133]), especially significantly improving the yield of **262** (70%). In the case of the hydroxyl substituted cinnamic acids **255**-**257** protection of the hydroxyl-functionality was not performed, which was the reason for moderate yields of the products **284**-**286** (34, 57 and 23 %, respectively). Other desired products were isolated in high yields (68-96 %) and all of them were obtained in pure form after column chromatography.

Most of the compounds synthesized (except **295**-**297** and **303**) are in agreement with the formal *Lipinski's* rule of five (Table 6), but are practically insoluble in deionized water at 20 °C (significantly less than 1 mg/ml). They were evaluated for *in vitro* activity against *Mycobacterium tuberculosis* H37Rv (Table 6) by using the method of *Canetti*.[85,86] As a whole, the compounds in this study[117] show moderate level of antimycobacterial activity, which for the most of them varies between 30 and 70 % of that of EMB (and 80-140% of that of **208**, respectively).[106] In general, the presence of different substituents and the variation of their positions in the aromatic ring, as well as the introduction of heterocyclic or condensed aromatic rings of the cinnamoyl moiety, doesn't impact the activity significantly. More serious modification of the cinnamoyl moiety (triple instead of double bond; saturated instead of aromatic ring; another phenyl ring at β-position, etc.) causes the same weak effect.



Scheme 15. Synthesis of compounds **276-304**.

Compounds **281**, **290**, **292**, and **297**-**298** can be highlighted as most active (up to 10 times the activity of EMB). Interestingly, the activity of the most potent compound **292** (MIC 0.2 μg/ml) is much higher than the activity of its ortho-isomer **291** (MIC 5.0 μg/ml). The activity of the tri- and di-alkoxy derivatives **290** and **298**, respectively is higher than compared with the corresponding mono-methoxy analogues **287**-**289**. The somewhat better results for **297** could be due to its higher lipophilicity. According to the obtained results, some conclusions regarding SAR can be drawn. The modified fenchane moiety inherited from **208** is mainly responsible for the antimycobacterial activity, while the cinnamoyl moiety itself contributes to a lesser extent. Some *N*- and *O*-containing substituents at the aromatic ring can increase the activities (**281**, **290**, **292** and **298**). The presence of halogen or CF_3 substituents (compounds **276**-**280** and **293**) doesn't have any noticeable effect on the activity. Cinnamamide analogues bearing condensed aromatic rings (**296**-**297**) need further investigation.

Table 6. *In vitro* screening data for antimycobacterial activity and cytotoxicity of synthesized compounds **245**-**246** and **276**-**304**.

Compound	Yield (%)	Activity toward *M. tb.* $H_{37}Rv$, MIC (μg/ml)	Cytotoxicity toward HEK 293T, IC_{50} (μg/ml)	Selectivity index, SI[a]	Log*P*[b]
245	82	7.0	NT	NT	5.10 +/- 0.59
246	71	5.0	NT	NT	4.62 +/- 0.40
276	81	5.0	NT	NT	4.36 +/- 0.49
277	84	4.5	NT	NT	4.25 +/- 0.50
278	78	5.0	NT	NT	4.85 +/- 0.40
279	80	6.0	NT	NT	5.01 +/- 0.44
280	87	>6.0	NT	NT	5.13 +/- 0.53
281	80	2.5	94.2	37.7	3.79 +/- 0.47
282	83	4.0	NT	NT	4.14 +/- 0.40
283	73	6.0	NT	NT	4.02 +/- 0.42
284	34	4.0	NT	NT	4.26 +/- 0.55
285	57	6.0	NT	NT	3.65 +/- 0.40
286	23	4.0	NT	NT	3.70 +/- 0.40
287	87	6.5	NT	NT	4.25 +/- 0.39
288	92	4.5	NT	NT	4.22 +/- 0.39
289	86	3.5	NT	NT	4.19 +/- 0.39
290	68	1.0	10.9	10.9	3.83 +/- 0.41
291	89	5.0	NT	NT	4.35 +/- 0.40
292	80	0.2	13.4	67.0	4.75 +/- 0.48
293	76	6.0	NT	NT	5.20 +/- 0.59
294	85	4.5	NT	NT	3.81 +/- 0.50
295	80	4.5	NT	NT	6.00 +/- 0.41
296	88	5.0	NT	NT	5.47 +/- 0.39
297	73	4.0	NT	NT	7.19 +/- 0.39
298	80	2.5	11.5	4.6	4.36 +/- 0.48
299	78	5.0	NT	NT	4.06 +/- 0.57
300	96	4.0	NT	NT	3.86 +/- 0.40
301	90	6.0	NT	NT	4.69 +/- 0.34
302	74	5.0	NT	NT	4.67 +/- 0.39
303	82	5.0	NT	NT	6.40 +/- 0.75
304	51	5.0	NT	NT	5.35 +/- 0.60
EMB.2HCl	-	2.0	NT	NT	0.06[d]

[a] selectivity index: SI = IC_{50}/MIC; NT – not tested/calculated

[b] Log*P*, octanol-water partitioning coefficient, was calculated using ACDLabs/ChemSketch 2012 (www.acdlabs.com).

[d] Log*P* of ethambutol dihydrochloride (EMB.2HCl) is known in the literature: N.R. Budha, R.E. Lee and B. Meibohm, *Curr. Med. Chem.* **2008**, *15*, 809.

Four of compounds synthesized, which have shown activities higher that of EMB, were selected for further evaluation. The cytotoxic activity of the tested compounds was investigated onto two different human embryonic cell lines from kidney (HEK 293T) and from umbilical vein/vascular endothelium (HUVEC) (data not shown) by using the MTT dye reduction assay.[105] The corresponding IC_{50} values of the tested compounds were calculated using nonlinear regression analysis. As the calculated values for both tested cell lines were quite similar, only these for HEK 293T are summarized in Table 6. It could be seen that three (**290**, **292** and **298**) of the four tested

compounds have demonstrated relatively high cytotoxicity with IC_{50} in the range 10.9-13.4 μg/ml. However one of them (**292**) has shown high selectivity index 67 due to its MIC value (10 times higher activity than that of EMB) that makes it a promising hit structure for further optimization. The last of the tested compounds **281** demonstrated MIC similar to EMB and very low cytotoxic effect of 94.2 μg/ml, which also results in a good selectivity index (37.7). Consequently this structure could be used for further optimization trough synthetic variations.

3.1.3. Antibacterial and antitubercular activity of new arylmethylidene ketones and pyrimidines with camphane skeletons[134]

Camphor analogues, like other monoterpenes in general, demonstrate a variety of biological activities including antiviral, antimicrobial, and anticonvulsant properties. The introduction of such type of moieties to different chemical entities via structural modifications is known to significantly impact their biological activity. Several reports applied this strategy in order to enhance the performance of their bioactive compounds.[135]

Recently, several studies revealed antibacterial and antitubercular activity of camphor derivatives.[129,130,136,137] Few series of acylated amines with camphane skeleton were synthesized (Figure 5, compounds **306** and **307**). Depending on the acyl substituents, the former compounds demonstrated moderate to high *in vitro* antitubercular activity. Activity of diastereomerically pure camphor aminoalcohols (compounds **308**) strongly dependent on the presence of six-membered heterocyclic substituents in their molecules.[136] Some quaternary salts of camphor sulfonamides (compounds **309**) revealed significant antibacterial and antifungal activity (up to 100-fold higher than the conventional disinfectant benzalkonium bromide).[138]

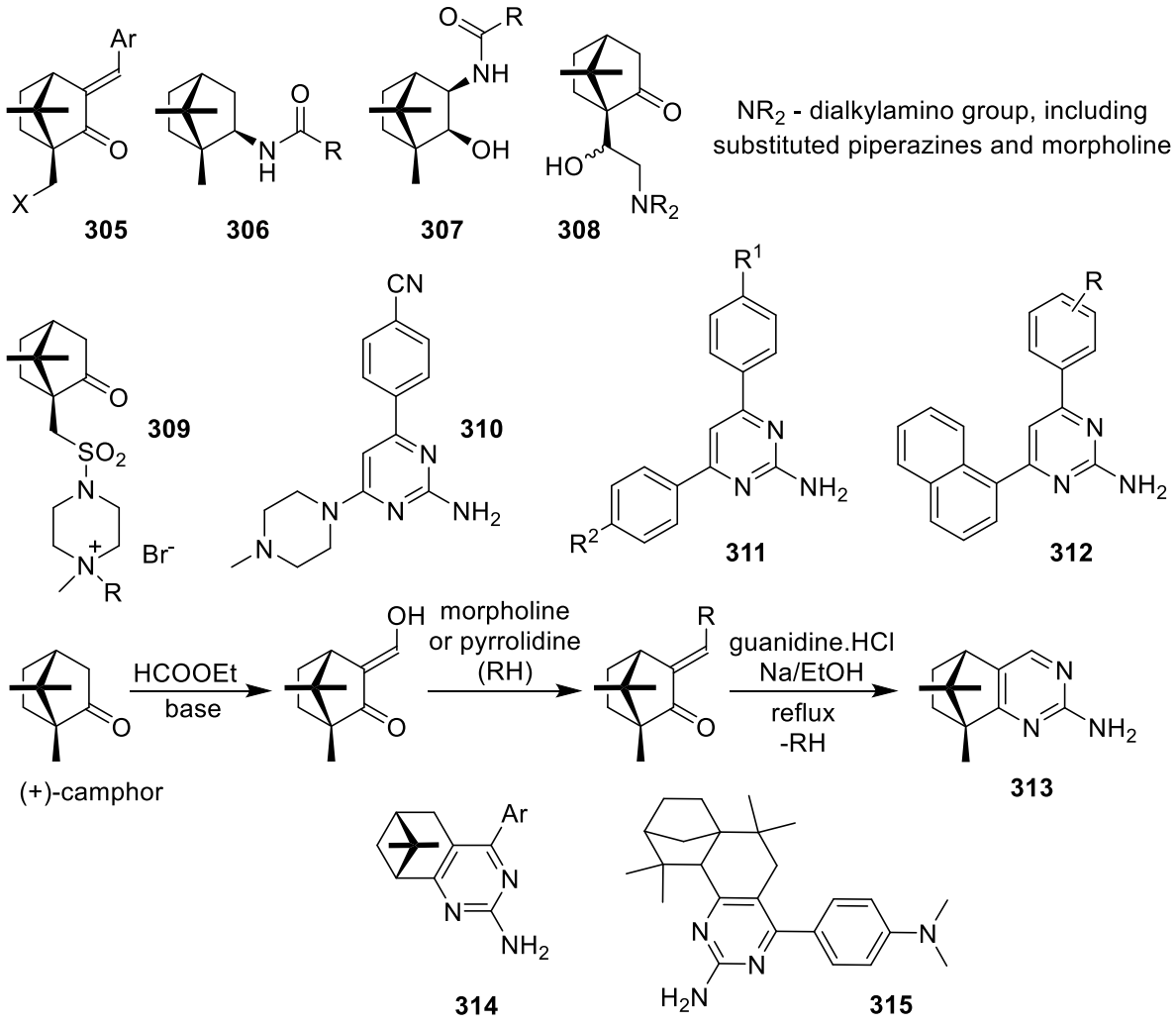


Figure 5. Camphor and 2-aminopyrimidine derivatives demonstrated bioactivity.

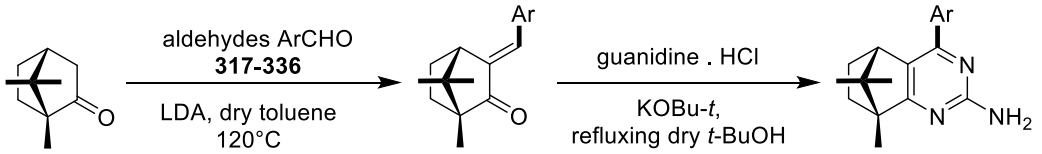
Among all heterocyclic compounds, pyrimidines are of prime interest. They exhibit a broad spectrum of biological activities, mainly due to its occurrence in deoxyribonucleic acid bases. The pyrimidine moiety has a broad therapeutic profile as it is a vital component of many natural compounds and chemotherapeutic drugs.[139,140] In particular, different substituted 2-aminopyrimidines were synthesized and appear to be bioactive in several different ways.[141] For example, some aryl substituted 2-aminopyrimidines (Figure 17, compound **310**) have been identified as promising histamine H4 antagonists.[142] The H4 receptor (H4R), was shown to be present within cells of the hematopoietic lineage. There is a growing body of evidence based on animal models in which the H4R plays a role in immune and inflammatory responses[143] and modulates itch responses as well. Thus, agents that interact with the H4R have been proposed to be useful in the treatment of conditions such as asthma, rheumatoid arthritis, and pruritus.[144]

Some 2-aminopyrimidine derivatives were examined for adenosine A3 antagonist activity.[145] Several studies indicate that adenosine A3 receptors execute an important role in pathologies, such as asthmatic and inflammatory syndromes. Adenosine A3 receptor agonists have been found to cause bronchoconstriction and promote the release of inflammatory mediators from mast cells whereas selective antagonists for the human A3 receptor could be anti-asthmatic and anti-inflammatory agents.[146]

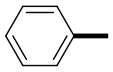
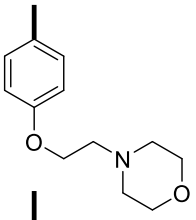
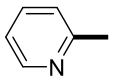
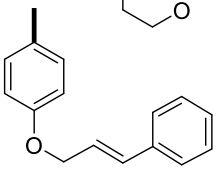
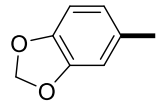
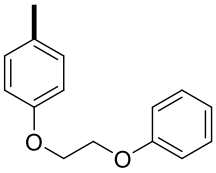
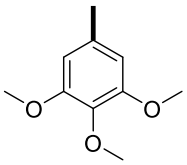
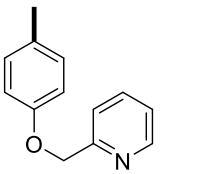
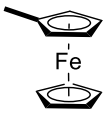
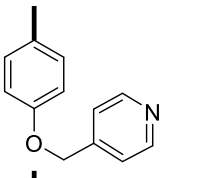
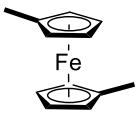
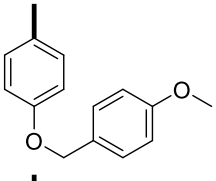
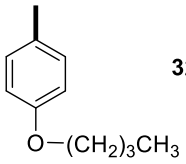
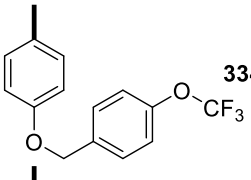
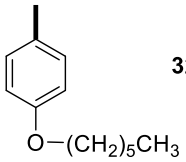
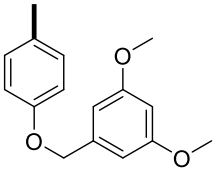
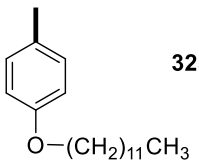
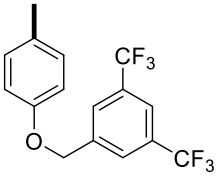
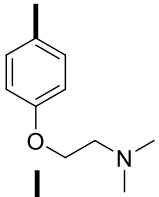
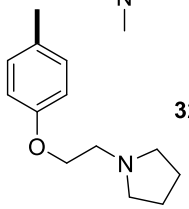
Aryl-substituted 2-aminopyridines exhibit significant antioxidant activity. These compounds were found to be active in scavenging of nitric oxide radical, scavenging of hydrogen peroxide, and lipid peroxidation inhibition (compounds of type **311**).[147] Both antimicrobial and antifungal activities are proven for 2-aminopyrimidine derivatives, such as compounds of type **312**.[148] Many other studies reveal the antimicrobial properties of the 2-aminopyrimidines.[149-152] To the best of our knowledge few examples of bioactive compounds that combine both monoterpene and 2-aminopyrimidine moieties exist. A series of similar camphor derived heterocyclic compounds (e.g. compound **313**) demonstrated strong central nervous system stimulation activity.[153] A series of pinanyl-2-amino pyrimidines (compounds **314**) were tested for antibacterial and antifungal activity,[154] however, the obtained results were found to be less promising. Compound **315** was synthesized starting from isolongifolanone in two steps, and its fluorescence properties have been studied.[155]

In the light of the above mentioned, in our study[134] we focused on the synthesis of new camphor derivatives, bearing pyrimidine and arylidene moieties. We designed the molecules bearing such combination of fragments in two series, expecting their potential for antibacterial (particularly, antitubercular) activity.

The first stage of our synthetic strategy was based on the transformation of readily available enantiomerically pure (+)-camphor (**316**) to series of conjugated ketones **337**-**356** (Scheme 16). Reactions were carried out by gently refluxing in dry toluene (under inert atmosphere), using lithium diisopropylamide (LDA) as a base (Scheme 1), and using aldehydes **317**-**336** as reagents. Some of intermediate aldehydes were not commercially available and were synthesized. Thus, dialdehyde **322** was synthesized starting from ferrocene.[156] Intermediates **323**,[157] **324**,[158] **325**,[159] **326**,[160] **327**,[161] **328**,[162] **330**,[163] and **324**[164] were easily prepared from 4-hydroxybenzaldehyde, according to above cited synthetic protocols. Since information about the preparation of aldehydes **329** and **335** is absent from the literature, we developed our own methods. Other aldehydes (**317**-**321** and **331**-**333**) were used as commercial products. The formation and characterization of some desired ketones (**337**-**338**, **341**-**342**) was described elsewhere, in different studies not related to the present investigation: *Grošelj et al.*[165] (for **337**), *Nevalainen et al.*[166] (for **338**), *Salisova et al.*[167] (for **341**), *Kamenova-Nacheva et al.*[168] (for **342**). Some evidence exists indicating that ketones **339** and **343** were mentioned in some early patents or publications before the 1950`s. However reliable data for their preparation and characterization are not accessible. Within the frames of this study,[134] we consider these compounds as new and described them in details, together with other new ketones. Ketones **337**-**356** were isolated only as pure *E*-isomers after purification. *Z*-isomers were not observed even by thin-layer chromatography (TLC) of the crude products, and this is not surprising.[168]



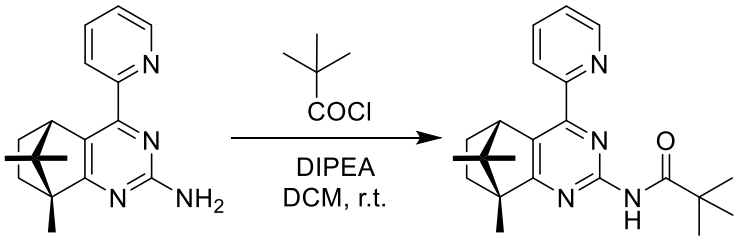
316 337-356 357-370

Ar	aldehydes 317-336	ketones 337-356	pyrimidines 357-370	Ar	aldehydes 317-336	ketones 337-356	pyrimidines 357-370
	317	337	357		328	348	-
	318	338	358		329	349	-
	319	339	359		330	350	365
	320	340	360		331	351	366
	321	341	361		332	352	-
	322	342	-		333	353	367
	323	343	362		334	354	368
	324	344	363		335	355	369
	325	345	364		336	356	370
	326	346	-				
	327	347	-				

Scheme 16. Synthesis of compounds **337-370**.

In a second stage, most of the ketones **337**-**356** were converted in one step to the corresponding aryl substituted 2-aminopyrimidines **357**-**370**, via condensation with an excess of guanidine hydrochloride and potassium *tert*-butoxyde (KOBu-*t*) in refluxing dry *tert*-butanol (*t*-BuOH) at open-air conditions. In fact, we enhanced application of methodology, developed by *Nagai et al.*[153] (Figure 17, compound **313**), by using of stronger base. In our case, process of cyclisation and aromatization was accomplished without leaving of the aromatic moieties from the arylidene ketones (Scheme 16). This is not surprising, since these aromatics are not in role of leaving groups (in contrast with pyrrolidine and morfoline in procedure for synthesis of compound **313**). Moreover, the original procedure requires three steps (starting from (+)-camphor), but only one non-substituted camphor 2-aminopyrimidine is the possible product (**313**). Other examples of substituted 2-aminopyridines were also synthesized by similar methodology by *Wu et al.* and *Wang et al.*,[154,155] however they could be considered as less hindered (Figure 17, compounds **314** and **315**). Thus, we opened a simple two-step way for preparation of highly sterically hindered 2-aminopyridines with camphor skeletons, bearing different substituents to the pyrimidine ring.

All compounds **337**-**356** and **357**-**370** were obtained in good to excellent yields after purification by column chromatography and characterized using 1D/2D NMR, MS, melting point temperatures and elemental analysis. In addition, x-ray analysis of single crystals was performed for compounds **349**, **355** and **368** (Figure 18). Our attempts to convert ketones **342**, **346**, **347**-**349** and **352** to their corresponding 2-aminopyrimidines were unsuccessful. In all cases, complicated and inseparable mixture of products was observed. In order to prove the synthetic conception for further conversion of compounds **357**-**370** to their *N*-acyl derivatives, acylated aminopyrimidine **371** was successfully prepared from **358** (Scheme 17). It is important to point out, that in all reactions the configuration of the camphene skeleton was untouched. Thus, the enantiomeric purity of the target compounds is ensured (confirmed by x-ray).



358 **371**

Scheme 17. Synthesis of compound **371**.

Compounds **349**, **355** and **368** were chosen as representative for classes of compounds, synthesized in this study. Suitable single crystals of **349**, **355** and **368** were mounted on nylon loops. The coordinates and intensities of the diffraction peaks were collected at room temperature on Agilent SupernovaDual diffractometer equipped with Atlas CCD detector using micro-focus Mo*K*α radiation (λ = 0.71073 Å). Data collection and reduction for all compounds were performed using the CrysAlisPro,[169] multi-scan absorption correction method was applied. The structures were solved by intrinsic phasing and refined by the full-matrix least-squares method on F^2 with ShelxT and ShelxL[170] programs using Olex2 GUI.[171] The non-hydrogen atoms were located successfully from Fourier map and were refined anisotropically. The nitrogen hydrogen atoms were located from difference Fourier map while all remaining hydrogen atoms were placed on calculated positions and a riding model $U_{iso(H) = 1.2}U_{eq\ (C\ or\ N)}$. ORTEP[172] views of the molecules present in the asymmetric unit (ASU) of compounds **349**, **355** and **368** are shown in Figure 18.

Compounds **349**, **355** and **368** crystallize in noncentrosymmetric manner in the monoclinic space groups $P2_1$, $P2$ and triclinic $P1$ respectively (Figure 6) The refinement of the structures showed the anticipated configuration (*5S*,*8R*) as the camphane moiety must remain unchanged. Indeed, from structural point of view the overlay of the molecules based on the camphane fragment reveals a highly conserved geometry. Although the bond lengths and bond angles of the molecules are comparable some variations, even between chemically identical molecules forming the

asymmetric unit (compounds **355** and **368**) are detected. The variations are mainly related to rotation of the aromatic rings e.g. the presence of rotamers is identified.

The x-ray data for **349** and **355** unambiguously confirmed, that configuration of ketones **337**-**356** in respect of double bonds is always *E*. This is in accordance with our previous investigation of such type camphor derived ketones.[168]

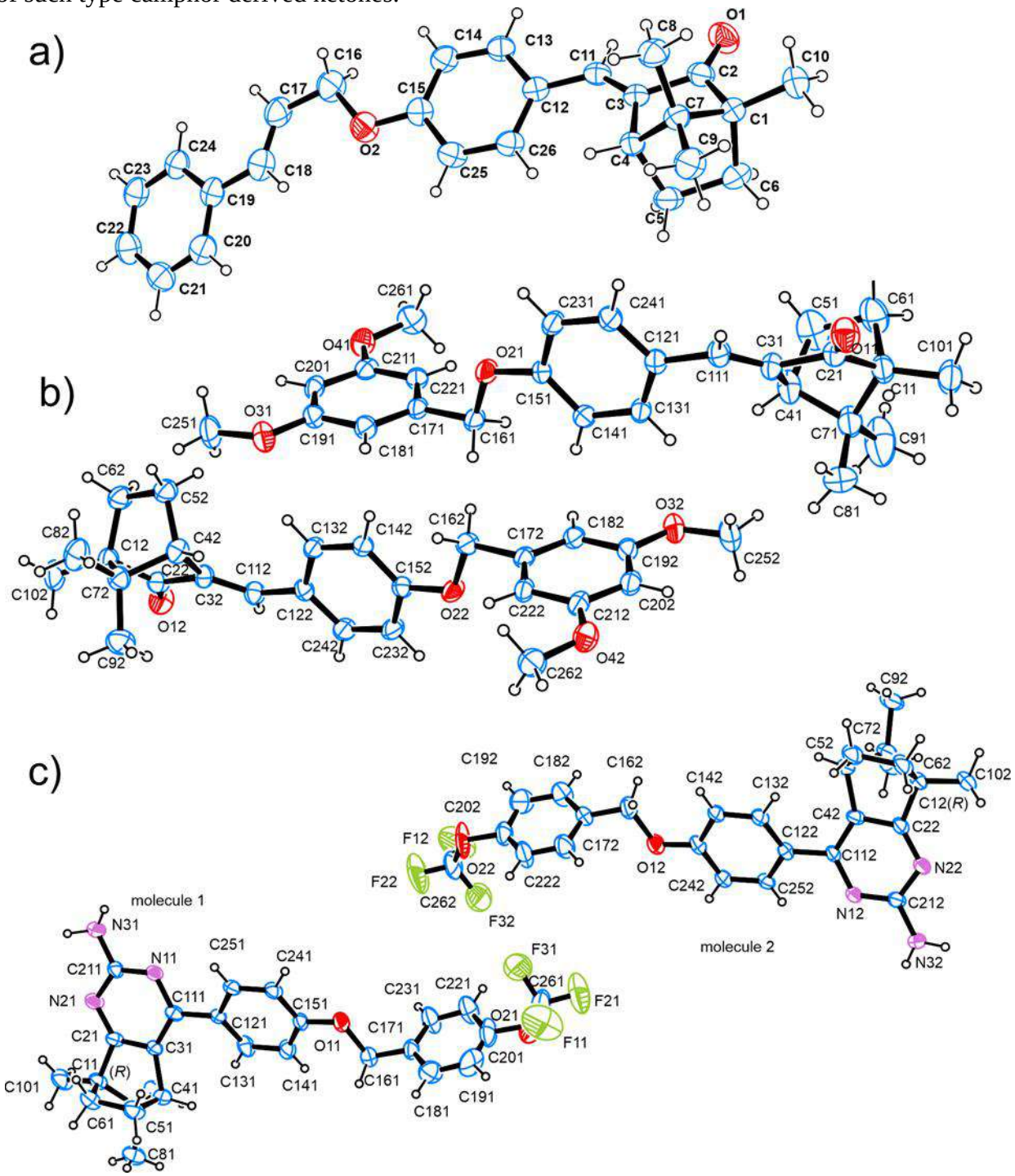


Figure 6. View of the molecular structure obtained by single-crystal X-ray analysis and atom-numbering scheme of compounds **a) 349**, **b) 355** and **c) 368**; displacement ellipsoids are drawn at the 50% probability level (the hydrogen atoms are represented by circles of arbitrary radii).

Evaluation of antibacterial activity and cytotoxicity of compounds **337-371**

The *in vitro* antibacterial activity and cytotoxicity of the synthesized compounds (**337-371**) was evaluated. Antimycobacterial activity is presented in Table 7. MIC values for each compound was determined against reference strain *M. tuberculosis* H37Rv (ATCC 27294) by broth microdilution in Middlebrook 7H9 (mycobacterial culture media) as per the current guidelines of EUCAST.[173] Among all tested compounds, four of them could be considered as *in vitro* active, namely compounds **344**, **349**, **350** and **354**. Compared to isoniazid, they exhibit activity of the same order, whereas **354** was found to feature superior activity. To the best of our knowledge, there are no reported analogues of these compounds in the literature with antitubercular activity. Therefore, any suggestions about possible mechanism of action could be quite speculative. Intriguingly, likewise the well-known antitubercular drug pretomanid[174] (currently in clinical use), compound **354** possesses 4-trifluoromethoxybenzyl moiety. All 2-aminopyrimidine derivatives (**357-371**) are completely inactive (including **368**, which contains the same moiety). It can be concluded that such type of 2-aminopyrimidines are not suitable for antitubercular agents.

Table 7. MIC values of compounds **337-371** toward *M. tb.* H37Rv.

Entry	Compound	*In vitro* activity toward *M. tb.* H37Rv, MIC (μM)	Calculated Log*P*
1	**337**	>200	4.19
2	**338**	>200	3.28
3	**339**	>88	3.97
4	**340**	76	3.81
5	**341**	144	-[c]
6	**342**	>100	-[c]
7	**343**	>160	5.31
8	**344**	9.18	6.14
9	**345**	>120	8.65
10	**346**	76	4.06
11	**347**	71	4.38
12	**348**	68	3.67
13	**349**	4.19	6.31
14	**350**	8.23	5.73
15	**351**	>145	4.88
16	**352**	72	4.46
17	**353**	16.60	5.67
18	**354**	0.91	7.32
19	**355**	>120	5.54
20	**356**	>100	7.64
21	**357**	89	4.65
22	**358**	>90	3.74
23	**359**	>75	4.43
24	**360**	135	4.28
25	**361**	65	-[c]
26	**362**	71	5.77
27	**363**	66	6.60
28	**364**	>100	9.11
29	**365**	>120	6.19
30	**366**	NT[b]	5.35
31	**367**	30	6.13
32	**368**	>50	7.79
33	**369**	14	6.01
34	**370**	96	8.10
35	**371**	>70	5.38
36	**INH**[a]	1.47	0.69

[a] INH – isoniazid.

[b] NT – not tested.

[c] log*P* was not calculated for compounds **341**, **342** and **361** due to software limitations.

Table 8. *In vitro* MIC values[a] of compound **350** for drug-resistant *M. tb.* isolates from Russia.

Strain ID	Subtype of *M. tb.* Beijing genotype	Strain origin (region in Russia)	Type[b]	Drug resistance profile[c]	MIC (μM)
20	Central Asian Russian clade	Murmansk	PR	HE	>260
98	Ancient sublineage, 1071-32-cluster	Komi	XDR	SHEKROfl	8.23
228	B0/W148-cluster	Komi	MDR	HR	8.23
336	CAO-cluster	Komi	PR	SH	>260
575	Central Asian Russian clade	Vologda	MDR	SHREPR	>260
1702	CAO-cluster	Vologda	PR	SH	>260
2321M	Central Asian Russian clade	Murmansk	MDR	HRE	>260
3135	B0/W148-cluster	Vologda	PR	SHE	>260
4179	B0/W148-cluster	Vologda	MDR	HRE	>260
6665	B0/W148-cluster	Murmansk	MDR	HRE	8.23
7069	B0/W148-cluster	Murmansk	MDR	HRE	8.23

[a] MIC for reference INH is >15 μM for all strains and is not given in this table.

[b] Type of resistance: PR – polyresistant; XDR - extensively drug resistant; MDR - multidrug-resistant.

[c] Single letter code for resistance to streptomycin (S), isoniazid (H), rifampin (R), ofloxacin (Ofl), kanamycin (K), ethambutol (E) and pyrazinamide (P).

MIC for above mentioned active compounds was also tested for 11 *M. tuberculosis* clinical isolates by using REMA assay[175] in the reference laboratory at St. Petersburg Research Institute of Phthisiopulmonology (Table 8). Rather moderate, but sustainable activity showed only **350** – this compound preserved its activity (8.23 μM) toward four of strains. The clinical isolates were of different genotypes from tuberculosis patients in Northwestern Russia. Clinical isolates represented different clusters within the clinically/epidemiologically significant Beijing genotype (B0/W148, Central Asian Russian, Central Asia Outbreak, ancient sublineage).[176-178] The isolates differed in phenotypic drug resistance (one extensively drug-resistant, six multidrug-resistant, four polyresistant). Thus, compound **350** showed a wide MIC range, which varied from 8.23 μM to >260 μM. This highlights the key importance of testing new anti-TB compounds not only with reference laboratory strain H37Rv that belongs to the phylogenetically marginal branch of *M. tuberculosis*, but on the panel of diverse clinical drug-resistant isolates circulating in the countries with high burden of MDR-TB, such as Russia.

Table 9. *In vitro* MIC values of compounds **337-371** toward selected bacteria and fungi strains.

Compound	MIC (μM)				
	S. aureus ATCC 29213	*MRSA*	*E. coli* ATCC 35218	*P. aeruginosa* ATCC 27853	*C. albicans 562*
337	133	266	133	266	266
338	133	265	66	133	133
339	28	56	28	16	225
340	12	24	6	12	48
341	46	92	92	92	184
342	7.8	31	31	63	63
343	102	102	51	102	205
344	94	94	188	94	188
345	2.35	9.42	4.71	18.80	38
346	6.11	12.21	12.21	6.11	49
347	1.41	5.66	2.83	22.63	91
348	1.35	5.41	5.41	21.65	43
349	21	42	21	86	42
350	10.60	21.30	21.30	43	85
351	11.51	23	23	46	5.76
352	2.88	5.76	2.88	11.52	46
353	42	85	42	85	85
354	19	37	19	19	74
355	4.92	9.84	9.84	20	20
356	8.29	33	16.58	16.58	1.04
357	7.16	14.32	14.32	7.16	1.79
358	3.57	7.13	14.27	14.27	28
359	0.77	1.55	1.55	12.37	25
360	2.71	2.71	1.35	5.42	2.71
361	21	84	84	42	2.58
362	46	92	46	92	92
363	0.66	2.63	5.27	10.54	42
364	8.63	17.25	17.25	35	4.31
365	0.60	2.40	2.40	9.60	38
366	21	42	42	84	42
367	4.81	9.62	9.62	38	4.81
368	34	68	68	34	68
369	18	36	18	72	4.49
370	7.67	15.34	15.34	7.67	31
371	5.49	11	11	22	44
G[a]	**0.52**	**1.04**	**1.04**	**2.08**	NT[c]
AB[b]	NT[c]	NT[c]	NT[c]	NT[c]	**0.54**

[a] G – Gentamicin (reference). [b] AB - Amphotericin B (reference). [c] NT – not tested.

In vitro antimicrobial activity of all synthesized compounds was tested against *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans* (as a fungal representative), by determining their MIC (Table 9). Obtained results are quite promising. Many of the compounds (especially among 2-aminopyridines **357**-**371**) exhibited good activity against different strains of bacteria and fungi, compared with reference antibiotics (gentamicin and amphotericin B). Compound **360** can be outlined as broad-spectrum antibiotic, active against all tested bacteria and fungi, with MIC values comparable to those of gentamicin. Other synthesized compounds demonstrated various selectivity against tested bacteria. Compounds **345**, **347**, **348**, **352**, **355**, **363** and **365** are markedly active against *Staphylococcus aureus*, but 2-3 fold less active against MRSA. However, **359** is the most active against MRSA in this series (similar to gentamicin). Remarkable antifungal activity demonstrate **356**, **357** and **361**. Only compounds **347** and **365** possess strong activity against *Escherichia coli*. Moderate activity against *Pseudomonas aeruginosa* showed **346**, **357**, **360** and **370** (2-4 fold less active than gentamicin).

In order to explore the therapeutic window for future applications of these compounds as antimicrobial/anti-tubercular therapeutics, initially we analyzed the possible cytotoxic effect for most of the synthesized compounds on the viability of human primary fibroblasts cells over different periods of treatment (24, 48 and 72 h). Compounds **337**, **338**, **340**, **342**, **346**, **347**, **348**, **361**, **364** and **365** show significant cytotoxic activity at 24 hours treatment that increases over longer period of treatment (Figure 7). This is presumably due to the higher concentration tested (100 μM). The same conclusion can be drawn for **352**, **361** and **365** because they demonstrated high cytotoxicity at 72 h treatment. In general, 2-aminopyrimidines with camphene skeletons (**357**-**371**) can be outlined as less toxic, in comparison with ketones **337**-**356**. Bearing in mind our previous studies, it is noteworthy that arylidene camphanes exhibit enhanced cytotoxicity, depending on the nature of the arylidene moiety.[168] Thus, only compounds **355** and **370** from this study can be considered as safe for further application as antimicrobial agents (Table 9), since they preserve its low cytotoxicity even at long time treatment (72 h). Other active compounds in Table 9 need further elucidation of their cytotoxicity and therapeutic windows.

Furthermore, we determined cell viability in other cancer and non transformed cell types for selected 3 most active compounds *in vitro* against *M. tuberculosis* H37Rv, namely compounds **344**, **349** and **354** (Figure 8). We measured the cell viability using a colorimetric MTT-assay.[179] Compound 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (known as MTT) indicates the activity of cellular enzymes that reduce MTT to formazan, which present a purple color. MTT assay measures mitochondrial metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes and proportionally reflects the number of viable cells. We used seven different human cell lines including cancer cells and non-cancerous cells. Hela cells are derived from cervical cancer, HepG2 (ATCC® HB-8065™) are liver cancer derived cells, MIA PaCa-2 (ATCC® CRL-1420™) is a human pancreatic cancer cell line, KMST is a nontumorigenic immortalized human fibroblast cell line, human embryonic Wisconsin-Madison stem cells are termed WA13, human embryonic kidney 293 cells are termed HEK293 and WI-38 are diploid human fibroblasts-derived from lung tissue of a female fetus. Cells were seeded in 96-multiwell plates and exposed to four different concentrations of three different compounds for 72 hours. As shown in Figure 7, the investigated compounds display none or only very mild cytotoxicity, regardless of the cancerous properties of the analyzed human cell lines, indicating that cytotoxicity of these compounds is low and their use as antitubercular drugs might provide a significant therapeutic window.

To gain insight into the metabolic stability, the most potent against *M. tuberculosis* H37Rv compounds (**349** and **354**) were incubated in mouse liver microsomes at 37ºC. Samples were analyzed by HPLC to determine the percentage of compounds remaining after 60 min of incubation. Inspection of the results presented in Table 10 reveals that these compounds were metabolically stable, with 7-12% of metabolization after 1 h of incubation.

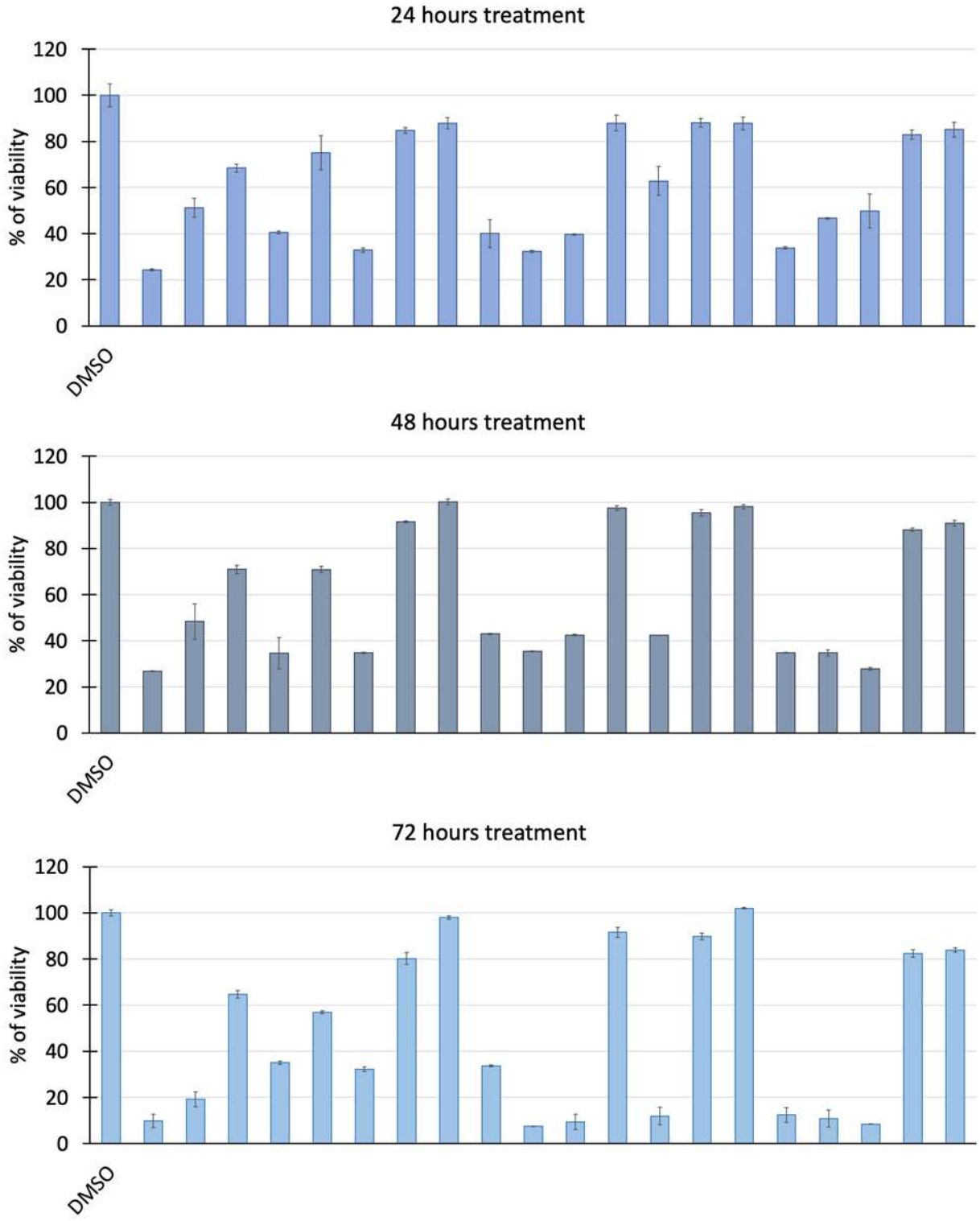


Figure 7. Cytotoxicity evaluation of compounds **357-371** in human primary fibroblast cell line CC-2509 upon treatment for 72 hours with 100 μM of the tested compounds. Values are expressed as percentage of activity and standard deviation compared to vehicle treatment with DMSO (bars after DMSO are for compounds **357-371** and were not labeled).

Table 10. Analytical conditions and metabolic stability for compounds **349** and **354** when incubated in mouse liver microsomes.

Compound	Eluent ($MeOH:H_2O$), λ (nm)	Retention time, RT (min)	% metaboliz. after 60 min incubation
349	90:10, 310 nm	5.9	12
354	90:10, 310 nm	5.0	7

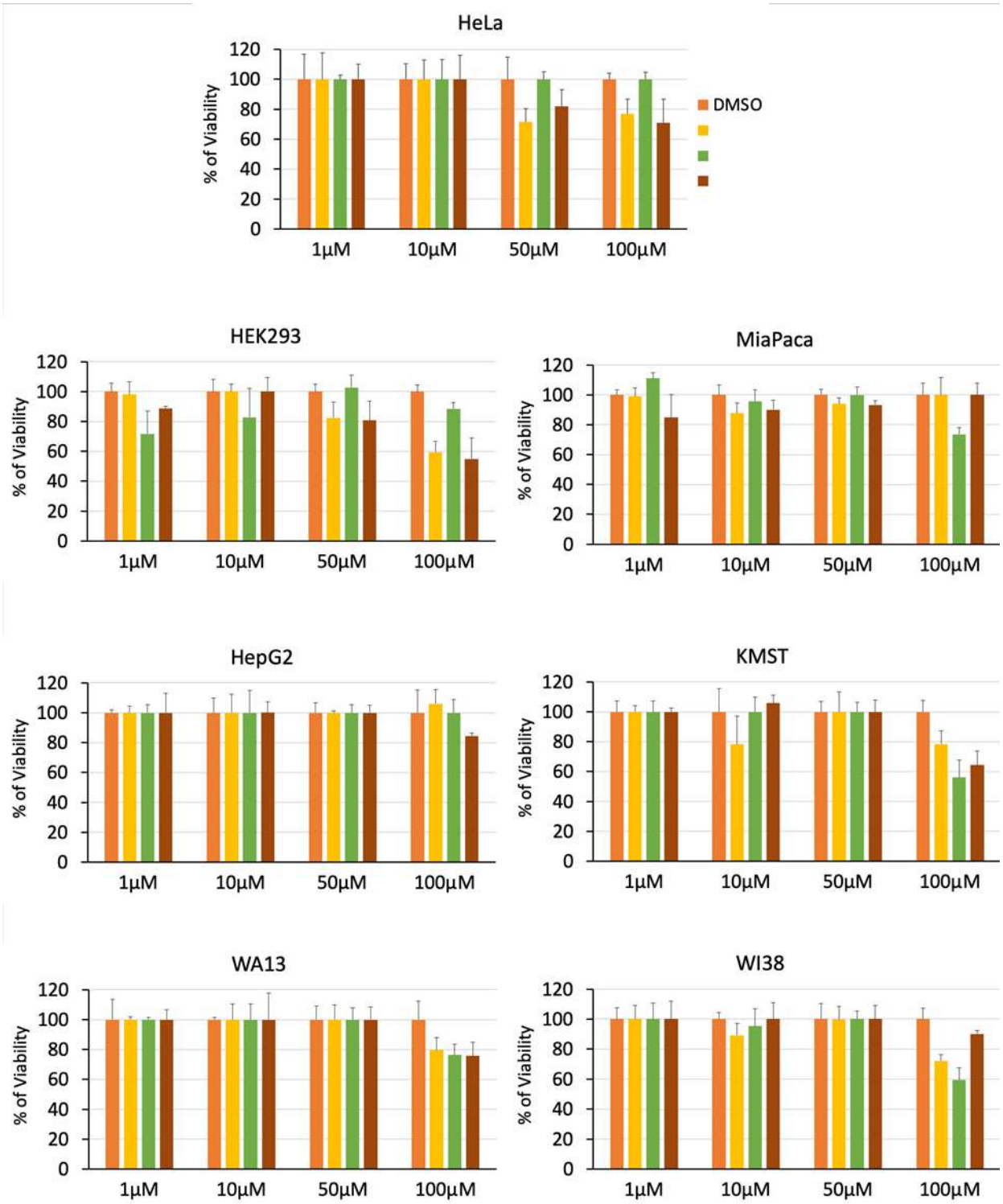


Figure 8. Cell viability upon treatment with selected compounds of human cell lines: Hela, MIA PaCa, HepG2, HEK293, WA13, KMST and WI-38. Cells were exposed to 1, 10, 50 or 100 µM of the tested compound, namely **344** (green bars), **349** (yellow bars) and **354** (brown bars) for 72 hours. Values are expressed as percentage of activity compared to vehicle treatment with DMSO for 72 hours.

Prehistory of bioactivity studies of (+)- camphor and (-)-fenchone derivatives (short description)

Our earliest research regarding synthesis of new arylidene camphor ketones and (-)-fenchone derivatives started in 2001. At that time they were used only as catalysts for asymmetric synthesis or intermediates for their preparation. Some of these products were included in my PhD thesis (2001-2006), but they were published years later.[180-182] After 2011 we started screening of these compounds for biological activity and expanded number of newly obtained compounds.

During the years, we performed in parallel enhanced studies over cytotoxicity of small series arylmethylidene ketones with camphor skeletons. Since this thematic is indirectly related to the topic of this thesis, here we present only short description. The synthetic approach towards all compounds in these series (Figure 9) was based on a procedure, similar to above presented in Scheme 16. In 2017 we described[168] the synthesis of (+)-camphor derivatives containing sulfonamide groups, ferrocenylmethylidene or arylidene moieties. The obtained derivatives were tested *in vitro* against seven human cancer cells lines, namely BV-173 (leukemia), K-256a (leukemia), NB-4 (leukemia), A549 (lung adenocarcinoma), H1299 (lung adenocarcinoma), MCF-7 (breast adenocarcinoma), and MDA-MB231 (breast adenocarcinoma), and two normal human cell lines (HEK293 and HUVEC). Compound **F21-1** can be outlined in this series as most potent anticancer agent with good selectivity index (SI up to 11.5) toward normal human cells. Later, this compound was studied in depth (in respect of its cytotoxicity).[183] Our last study[184] was dedicated to discover active water soluble analogues of **F21-1**. Thus, we synthesized **F21-2**, which possesses excellent solubility and high anticancer activity.

It can be concluded that presence of ferrocenylmethylidene group in presented ketones with camphor skeletons, is essential for their cytotoxicity. However, sulfonamide moiety can significantly modify their activity and pharmacological properties. Thus, ferrocene containing ketones with camphene skeletons demonstrate higher cytotoxicity and are most appropriate for anticancer agents, but not for antibacterials. Ketones with other aryl groups demonstrate enhanced antibacterial activity, respectively.

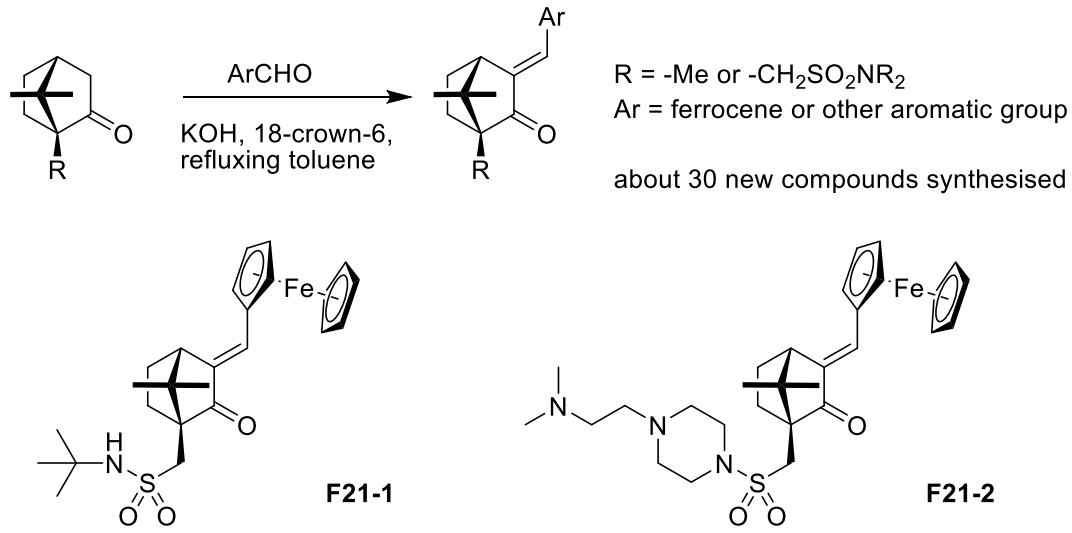


Figure 9. Synthesis and cytotoxicity of arylmethylidene ketones with camphor skeletons.

3.1.4. Molecular insight into *Mycobacterium tuberculosis* resistance to new nitrofuranyl amides[185]

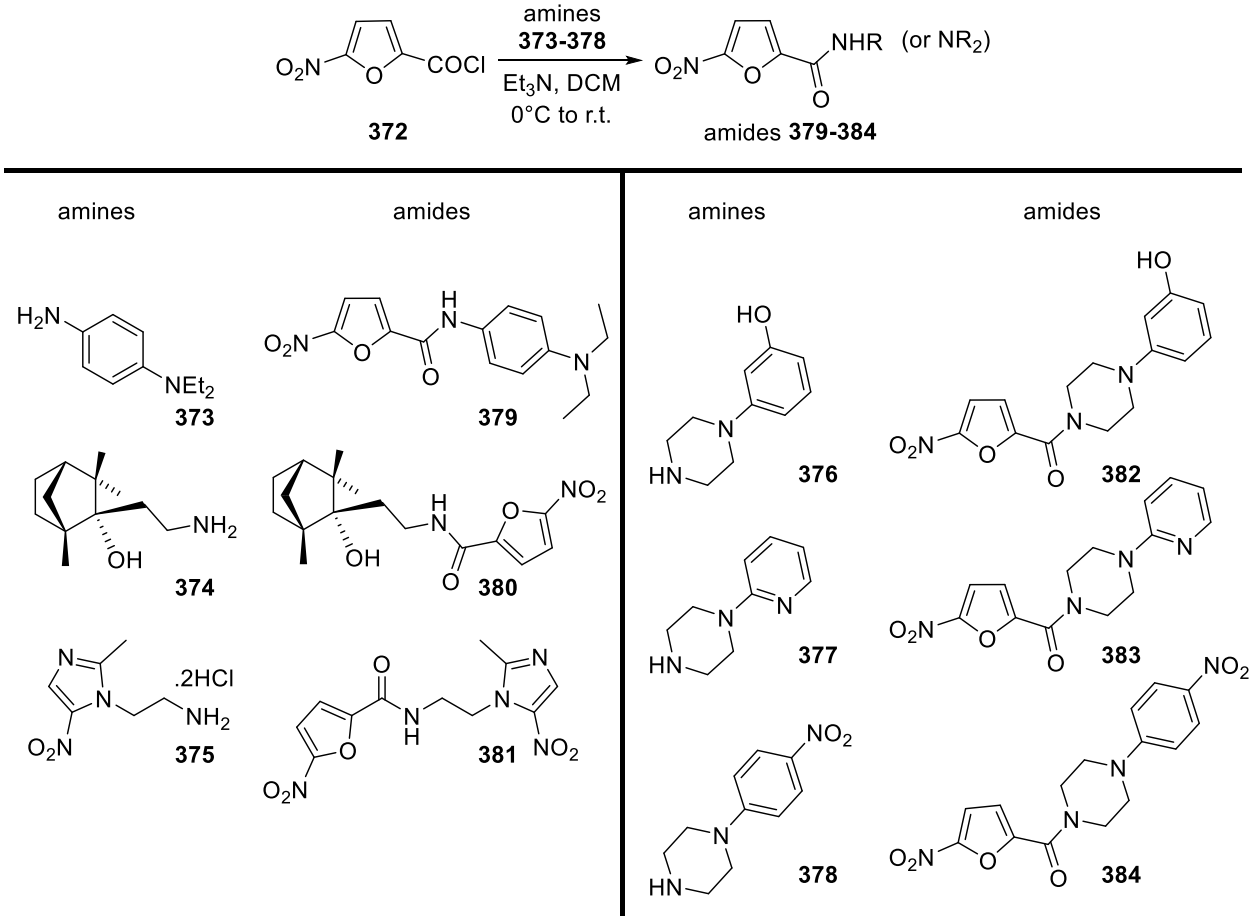
M. tuberculosis has a complex and chemically resistant cell wall. Therefore, anti-tubercular drugs are specific and do not act on other pathogenic bacteria, and vice versa - many available antibiotics do not (with a few exceptions) act on mycobacteria. Many anti-TB antibiotics are functionally unique to *M. tuberculosis* as they target the cell wall that is structurally distinct from that of other bacteria. Recently, the most active synthesized antitubercular agents have been classified by their chemical structures - amines, amino alcohols, hydrazides, ureas, thioureas, heterocycles, etc.[174,186] Natural products or their semi-synthetic derivatives also represent an interesting option in the search for new anti-tuberculosis drugs.[187,188] However, in recent decades only a few new drugs (bedaquiline, delamanid, linezolid, and clofazimine) have been approved for use in treatment regimens for patients with MDR TB. Although this may seem great, unjustified hopes have been put on the bedaquiline in particular. After its introduction into clinical practice, it proved to be not very effective, with unwanted side effects such as the QT interval prolongation and its cardiac safety.[6,7]

Nitroimidazoles (e.g., delamanid) and nitrofurans are bioreducible drugs whose action is based on the reduction in the nitro group by reductase enzymes. As an alternative to the nitroimidazoles, nitrofuranes (nitrofuranylamides, nitrofuranylpiperazines, nitrofuranylisoxazolines) also demonstrate *in vitro* and *in vivo* anti-TB activity.[189] Some new nitrofurans were efficient against actively growing and latent mycobacteria with unique modes of action.[190] Some structure-activity relationship studies showed that the nitro group is essential for the anti-TB activity. Replacement of the furan moiety with another ring leads to a decrease in or lack of activity, while nitroaromatic systems significantly increase the activity against latent or anoxic bacteria.[190-192]

The main known mechanism of action of nitrofurans as shown for *E. coli*, relies on the activation of the nitrofuran prodrug by nitroreductases leading to oxidative stress due to bactericidal reactive oxygen and nitrogen species.[193] In *E. coli* type I nitroreductases NsfA and NsfB are oxygen insensitive and catalyze the reduction in the nitro moiety into reactive nitroso and hydroxylamino derivatives. More potential unknown enzymes may exist with less pronounced effect due to low protein expression or low affinity for the nitrofurans.[193] De novo selected nitrofurantoin resistant *E. coli* strain with wild type nfsA and nfsB contained an in-frame deletion in ribE that encodes an enzyme in the biosynthesis of flavin mononucleotide, an essential NfsA/NfsB cofactor.[194] The information on the nitrofuran mode of action on mycobacteria and the molecular mechanism of mycobacterial resistance to nitrofurans is limited. *M. tuberculosis* lacks plasmids and horizontal gene transfer and most of its diversity is driven by the chromosomal point mutations or short indels, including the development of drug resistance. Different spontaneous mutations emerge in the *M. tuberculosis* population and may be selected and fixed if they are sufficiently beneficial for bacterial survival, adaptation, and fitness. In this sense, culturing the bacteria on a medium containing an active compound under elevated concentrations may permit the identification of such resistance-associated mutations and gain insight into the mode of action of the compound.

In this study,[185] we describe the synthesis of the new nitrofuranyl amides and investigate their anti-TB activity and possible mechanism of action/resistance through whole-genome sequencing of *M. tuberculosis* spontaneous mutants. We focused on nitrofuranyl amides since they possess strong antitubercular and antibacterial activity. However, especially in the case of antitubercular activity, their mechanism of action is still largely unknown. A classical design of this kind of mutagenesis study is based on individual resistant clones that are additionally recultured on a drug-containing medium and each individual clone is separately submitted to DNA extraction and whole-genome sequencing. In contrast, in this study we have purposefully pursued another approach that may be seen as a kind of "metagenomics-like" one. We performed a Whole Genome Sequencing analysis (WGS analysis) of the originally grown pooled colonies, rather than single colonies. In this way, we expected to dissect the primary genetic response of mycobacteria to the inhibiting action of the compound.

A series of six new nitrofuranyl amides (**379-384**) was synthesized (Scheme 18)[185] by the implementation of a classic methodology for the preparation of amides - namely the reaction of 5-nitrofuran-2-carbonyl chloride (**372**) with different amines (**373-378**) in dry dichloromethane (DCM) at basic conditions (ensured by an excess of triethylamine). Amines **373-378** (except aminoalcohol **374**)[106] are commercial products. All target compounds **379-384** were isolated in high purity after column chromatography in moderate to high yields. The choice of the amide moieties as pharmacophore groups in this study is not accidental. Different nitrofuranyl amides are known to be active *in vitro* against *M. tuberculosis*, but their activity can be significantly influenced by other part of their molecules. Synthesis of **379** was inspired by other active nitrofuranyl anilides.[195] The design of **380** was suggested by our previous studies[106,117] revealing that some fenchone derivatives possess antitubercular activity. Compound **381** combines both nitrofuran and nitroimidazole moieties in one molecule. It is known that bicyclic nitroimidazole pretomanid (see Figure 2) is a pro-drug with a very complex mechanism of action active against both replicating and hypoxic, non-replicating *Mycobacterium tuberculosis*.[196] The other three compounds (**382-384**) in this study contain aryl piperazine moieties, which can contribute significantly to their antitubercular activity.[197]



Scheme 18. Synthesis of compounds **379-384**.

The MIC values of the synthesized compounds were determined for reference strain H37Rv. The compounds were initially tested using both whole-cell microdilution (WCMD)[198] and Resazurin Microtiter Assay (REMA)[175] methods (Table 11). The three most efficient substances with low MIC were further retested using the REMA method in replicated experiments under different ranges of concentrations (Table 12). Replication of experiments was used to eliminate any possible mistakes in the results. To compare compounds **379-384** (which possess different molecular weights), all MIC results were presented and commented only in μM. Compounds **383** and **384** showed high efficiency in only one experiment. Repeated testing revealed a very large heterogeneity in the MIC results. This might be due to low solubility or instability of compounds **383** and **383** in the testing media. However, our experiments were oriented only to find an

appropriate compound in this series with a low and reproducible MIC value, regardless of the reasons for such heterogeneity. Compound **382** was the only one that showed both low MIC with high reproducibility and concordance of results in different REMA experiments. For this reason, **382** was selected as a model compound for further genetic experiments. The REMA MIC determination was also performed for a known antibiotic, isoniazid (MIC 0.062 μg/mL) and confirmed that the condition used to determine MIC was appropriate.

Table 11. MIC values of the tested compounds by two different methods, WCMD and REMA.

Compound	MIC (WCMD), μM	MIC (REMA), μM
379	0.66	16.48
380	4.64	29.73
381	>80	162
382	0.19	0.50
383	0.026	0.20
384	<0.012	0.36

Table 12. Results of replicated REMA experiments for MIC testing of three more efficient compounds.

Compound	MIC (REMA), μM
382	<1.26; 0.50; 0.50
383	<1.32; <0.13; 0.33
384	<1.16; <0.12; >0.58; 1.44

The *in vitro* mutagenesis was performed on *M. tuberculosis* reference strain H37Rv subcultures grown under increasing concentrations of compound **382** (MIC 0.50 μM, Table 12). Whole-genome sequencing (WGS) of the resistant mutants identified mutations in six genes (Table 13). Strain H37Rv is known to have undergone laboratory evolution and its subcultures are not genomically identical in different laboratories worldwide. Since the first whole-genome sequence of this strain was published in 1987,[199] more H37Rv strains from different laboratories have been sequenced and deposited in GenBank. For this reason, and to avoid false single nucleotide polymorphism calling (SNP calling), we performed WGS and SNP mapping, not only of the treated with 11 bacterial subcultures, but also of their parental substrain H37Rv.

None of the six mutations were present in the parental strain herein used. These mutations emerged in response to the nitrofuran action (see example in Figure 22). Sufficiently high sequencing depth permitted us to quantitatively and statistically assess the coexistence of the wild type and mutant alleles in the same genome position. In all instances, the mutant reads constituted a minority of all reads, but in all cases, there was a clear increase in the proportion of mutant reads with increasing concentration of the compound, and in some cases, it was a two-fold increase. In four cases, the higher percentage of mutant reads was significant (Table 13).

Table 13. Characteristics of the mutant positions in subcultures of *M. tuberculosis* H37Rv grown under different concentrations of **382**.

Position in Genome	Ref	Mut	Gene	Amino acid change	PAM1[a]	SIFT *p*[b]	% of Mutant reads in H37Rv cultured with 382 (1.00 μM)	% of Mutant reads in H37Rv cultured with 382 (2.00 μM)	% of Mutant reads in H37Rv cultured with 382 (4.00 μM)	*p* Value for the most contrasting pairs
268,560	A	T	*Rv0224c*	Phe23Tyr	21	0.01	9.6 (13/136)	15.8 (29/183)	20.0 (25/125)	0.02
411,895	A	T	*Rv0342 (iniA)*	Gln353Leu	6	0.02	12.7 (21/165)	8.9 (16/180)	23.8 (35/147)	0.0004
1,305,250	C	G	*Rv1173 (fbiC)*	Arg774Gly	1	0.00	14.0 (18/129)	12.8 (21/164)	17.2 (22/128)	0.3
1,783,849	G	C	*Rv1580c*	Ala15Gly	21	0.00[c]	22.2 (43/193)	16.7 (32/192)	26.0 (44/169)	0.03
1,793,445	T	G	*Rv1592c*	Glu99Ala	17	0.32	6.2 (7/112)	6.0 (7/117)	20.0 (23/115)	0.002
1,847,247	G	C	*Rv1639c*	silent Thr404	9871	-	13.3 (15/113)	17.2 (25/145)	19.8 (23/116)	0.2

[a] PAM1 (Point Accepted Mutation 1) gives the probability (multiplied with 10,000) for the particular aa exchange to occur, given that 1% of the amino acids are changed. [b] Amino acid changes with SIFT p probabilities < 0.05 are predicted to affect protein function, based on search in Uni-Prot-SwissProt + TrEMBL 2010_09 databases. [c] low confidence prediction.

3.2. Development of new diaryl ethers and related compounds with anti-enteroviral activity

Over the past decades, several classes of non-peptidic compounds have been reported to be selective inhibitors of enterovirus replication after *in vitro* testing (cell culture experiments). However, a sharp discrepancy exists between the antiviral activity established *in vitro* and *in vivo* (experiments involving laboratory animals). In contrast with hundreds virus replication inhibitors showing *in vitro* effects less than twenty manifested some *in vivo* activity. Unfortunately, the efficient anti-enteroviral chemotherapy for clinical use is still not established. The development of drug-resistance is the main reason for the lack of antivirals in clinical use for enteroviral infections. Nevertheless, some anti-enteroviral compounds have entered clinical trials – isoxazoles (''WIN compounds" – disoxaril, pleconaryl), pirodavir and its analogues, imidazolidinones, chalcones, flavanes, diaryl ethers etc. (See Introduction).[200]

Diaryl ether derived compound MDL-860 (2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile, also known as DNB) was first reported in 1980`s. Indeed MDL-860 possesses a broad-spectrum of *in vitro* activity against picornaviruses, by inhibiting an early event in virus replication, after initial uncoating.[201,202] MDL-860 mechanism of action was elucidated - the identified target being the host phosphatidylinositol-4 kinase III beta (PI4KB).[203] MDL-860 also elicited in vivo efficacy in a model of coxsackievirus B3 (CVB3) induced myocarditis.[204] The promising results inspired the development of many analogues of MDL-860 over the past decades. For example, *Markley et al.* synthesized and tested[205] over than 70 diaryl ethers (and their isosteric analogues) against several piconaviruses. The latest work of *Pürstinger et al.* reports on the synthesis of 60 new diaryl ethers and their activity against CVB3 replication.[206] All obtained results clearly showed that the 2-cyano-4-nitrophenoxy group is an essential building block for the existence of antiviral activity of this class of compounds. However, varying the substituents in the other aromatic ring can have a significant impact on both the antiviral activity and cytotoxicity. Identified hit compounds, usually contain two to three halogen atoms in the second aromatic ring.

Synthesis and anti-enteroviral activity of compounds **398**-**409**

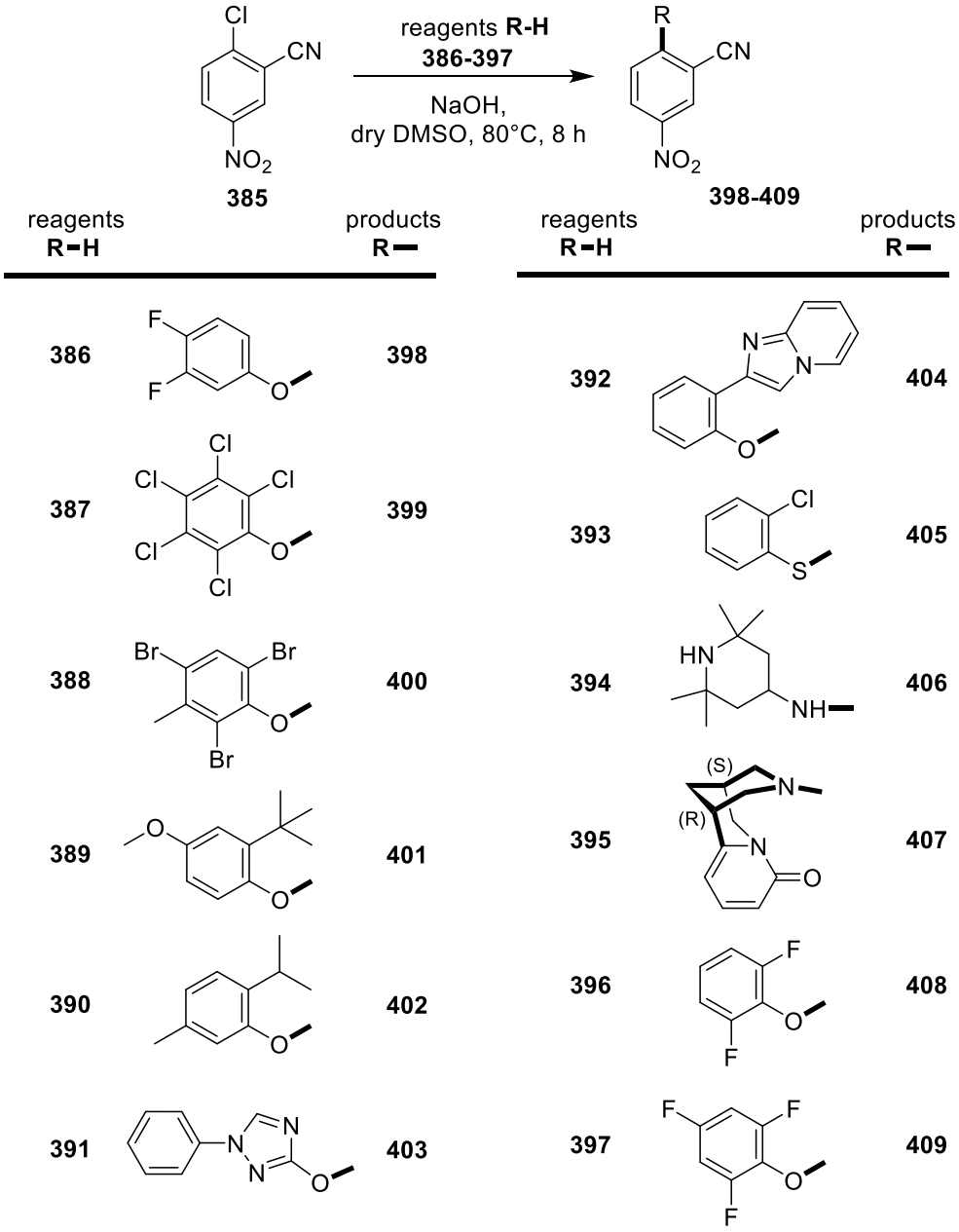
Despite the reported promising results, recently this class of compounds has been neglected in terms of further investigation with respect to their antiviral activity. Thus, our first study on antivirals was focused on the synthesis and evaluation of the antiviral activity and cytotoxicity of a series of new compounds bearing 2-cyano-4-nitrophenyl moiety bridged to various aromatic or aliphatic groups via O, N and S atoms.[18]

The target compounds (**398**-**409**) were obtained in high yields via single step synthesis as shown in Scheme 19, by using of procedures similar to those of *Markley et al.*[205] and *Pürstinger et al.*[206] Diaryl ethers **398**-**404**, **408**-**409** and the thioether **405** were synthesized in dry dimethylsulfoxyde (DMSO) by heating the corresponding phenols **386**-**392**, **386**-**397** and 2-chlorothiophenol (**405**) with 2-chloro-5-nitrobenzonitrile (**385**) in the presence of NaOH. Arylamines **406** and **407** were obtained through reaction of **385** with 2.2 equivalents of the amines **394** and **395**, respectively, in excess of *N*-methylmorpholine utilized as a basic agent. All starting compounds (except phenols **391** and **392**) are commercially available products. The synthesis of intermediates **391**[207] and **392**[208] was previously described. All target compounds were isolated and purified by column chromatography. Chemical structure elucidation was achieved by NMR and MS spectroscopy. Additionally, the structure of compound **398** was confirmed by X-ray crystallography. The obtained information could be helpful for further molecular docking studies of this class of compounds.

Compounds **398**-**409** were evaluated for their *in vitro* cytotoxicity and antiviral activities in cell culture experiments against three enteroviruses, namely polio virus 1 (PV1), coxsackievirus B1 (CVB1) and CVB3. Compound MDL-860 was used as a reference (Table 14). Diaryl ether **398** manifested a strong activity against PV1 and CVB1 (SI values 118 and 405, respectively), being inactive against CVB3. Compounds **399** and **400** showed a moderate effect (SI 20.5 and 19.6, respectively) against CVB3. A borderline activity toward CVB1was established for **399** (SI 10.9).

Compounds **400**–**407** could be considered as inactive against the three viruses included in the screening (except for a low activity of **400** against CVB3). A remarkable activity of **408** and especially **409** was demonstrated against PV1 and CVB1 viruses. None of the tested compounds replicated MDL-860 antiviral spectrum and effects embracing all the three enteroviruses (MDL-860 SI values of 72.5, 586.9 and 182 vs PV1, CVB1 and CVB3, respectively). The remarkable insusceptibility of CVB3 to the active compounds has to be emphasized. Moreover, compound **398** (with closest structural resemblance to MDL-860) demonstrated one of the highest activities to two other viruses.

Regarding the cytotoxicity (against HEp-2 cells) of the compounds studied, all were non-toxic in general (except **401**). Some of them (**400**, **403**, **404**, **405** and **407**) demonstrated even lower toxicity than MDL-860.



Scheme 19. Synthesis of compounds **398**-**409**.

Unfortunately, the number of compounds in this study is not enough to provide adequate QSAR analysis, but some structure-activity relations can be commented. An initial structure – activity analysis showed that a marked efficiency is usually demonstrated by two- or three halogen-substituted diaryl ether analogues of MDL-860.[205,206] The results of compounds **398**, **408** and **409** confirmed this deduction. Other substituents (aromatic heterocycles in **403** and **404**) or replacements of bridged oxygen atom with other heteroatom (**405**-**407**) led to lack of activity.

Similar effect was observed when halogen substituents were replaced with other (**401**-**402**). It is interesting to note, that compound **398** (structurally nearest difluoro analogue of MDL-860) lack the activity only against CVB3. On the other hand, analogues of MDL-860, containing more than three halogen atoms in the second ring were not known. In this study we provided only one pentachloro substituted compound (**399**), which demonstrated moderate antiviral effect. Of course, this single example is not representative, so this could be one of the directions for further investigations.

It can be concluded that the future synthesis of new active diaryl ethers could be promising and has to be directed to other halogen-substituted analogues of MDL-860. In addition, QSAR and some other issues (for example insolubility of the MDL-860 and its analogues in water) could be targeted in future investigations.

Table 14. *In vitro* data for anti-enterovirus activity and cytotoxicity of compounds **398**-**409**.

Compound	Cytotoxicity, CC_{50} (μM)	PV1		CVB1		CVB3	
		IC_{50} (μM)*	SI	IC_{50} (μM)*	SI	IC_{50} (μM)*	SI
398	320.0	2.7	118.0	0.8	405.0	NA	-
399	119.1	NA	-	10.9	10.9	5.8	20.5
400	570.3	NA	-	256.1	2.2	29.6	19.6
401	22.0	NA	-	NA	-	NA	-
402	94.3	NA	-	16.2	5.8	NA	-
403	570.8	NA	-	190.1	3.0	NA	-
404	718.0	NA	-	NA	-	NA	-
405	53.8	NA	-	NA	-	9.9	5.4
406	675.0	NA	-	NA	-	426.0	1.5
407	492.2	234.0	2.1	152.2	3.2	NA	-
408	355.0	6.8	52.2	0.7	507.1	NA	-
409	517.5	2.7	191.6	0.75	690	NA	-
MDL-860	493.0	6.8	72.5	0.8	586.9	2.7	182.0

*NA-not active

The selected in the *in vitro* screening compounds **398**, **408** and **409** were tested in experimental CVB1 neuroinfection in newborn mice of ICR random bred line, infected with a massive virus inoculum (20 LD_{50}). They were administered in 12-days treatment course starting on the day of virus inoculation. The subcutaneous daily dose of the compounds was as follow: compounds **398** and **408** - 50 mg/kg, compound **409** – 25 mg/kg, and MDL-860 – 75 mg/kg. The results obtained are presented in Table 15 and Figure 10.

Table 15. Study of the activity of compounds **398**, **408** and **409** at CVB1 experimental neuro infection in newborn mice.

Compound	Survivors/ Total	MST±SD days[a]	Δ days	Mortality, %	PI, %
398	22/24	8.1±1.0***	+5.0	50.0	50.0
408	9/27	8.5±1.0***	+5.4	66.7	33.3
409	3/26	5.7±1.2*	+2.6	88.4	11.6
MDL-860	0/27	6.1±1.6**	+3.0	100	0
Placebo	0/17	3.1±0.3	-	100	0

Data are from three independent experiments (average). [a] One-way ANOVA (Bonferroni's multiple comparison post-test); MST - mean survival time; PI - protection index; SD - standard deviation; $^{***}p < 0{,}0001$ vs. placebo group; $^{**}p < 0{,}01$vs. placebo group; $^{*}p < 0{,}05$ vs. placebo group.

As seen, compound **398** demonstrated the highest activity, attaining a protection effect of 50% and a very pronounced lengthening of the MST by 5 days. A marked activity was established at the course with compound **408** (PI=33.3 % and MST lengthened by 5.4 days). Compound **409** showed a weak protective effect (PI=11.6 % and Δ days of 2.6). In contrast, MDL-860 activity was marked by a lengthening of MST only, by 3 days. *Markley et al.* tested *in vivo* MDL-860 and five other analogues (against Coxsackievirus A21 in mice),[205] however it is not correct to compare

results obtained in this study (by using of subcutaneous administration) with these of *Markley et al.* (by using of oral administration against different virus). Evidently, compounds **398** and **408** could be characterized as a perspective anti-CVB agents which need further study. In previous work we have established the very high activity in experimental Coxsackievirus B1 neuroinfection in mice of the consecutive alternating administration (CAA) treatment course of a triple combination of enterovirus inhibitors including MDL-860 as a component.[209] It would be of great interest the testing the effect of CAA course by a triple combination in which MDL-860 is replaced by compound **398**.

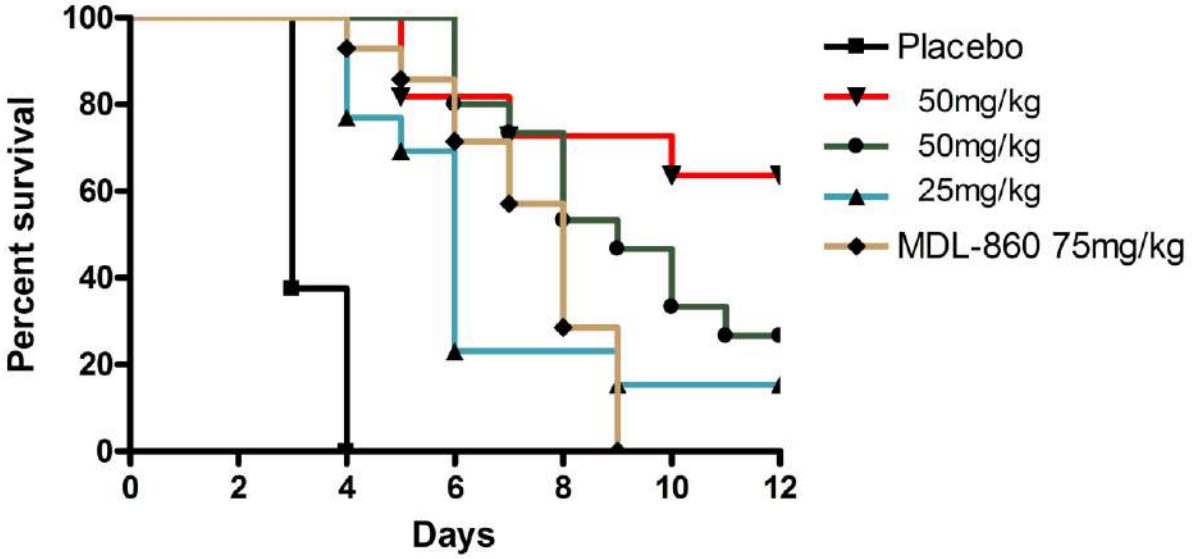


Figure 10. Individual effects of compounds **398** (red), **408** (dark green), **409** (blue) and MDL-860 in experimental neurotropic infection with Coxsackievirus B1 in newborn mice.

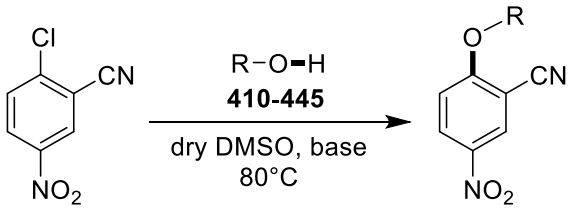
In the light of current research, it is clear that most promising is the development of new MDL-860 analogues bearing unaltered 2-cyano-4-nitro ring. In our previous work,[18] initial screening of twelve MDL-860 analogues (Scheme 19) resulted in three compounds (**398**, **408** and **409**) with high activity against PV1 and CVB1 and only two compounds (**399** and **400**) with moderate activity towards CVB3. In addition, compounds **398**, **408** and **409** exhibited activity towards CVB1 experimental neuroinfection in newborn mice.

Synthesis and anti-enteroviral activity of compounds **446-481**, **491-500**, **512-522**, **526-528** and **531**

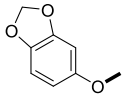
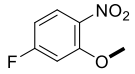
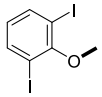
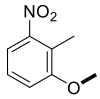
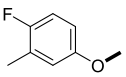
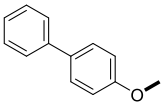
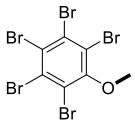
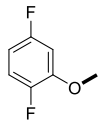
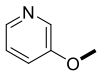
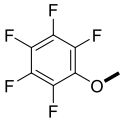
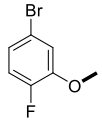
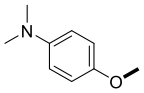
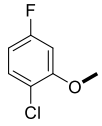
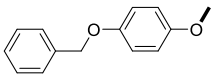
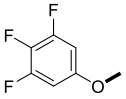
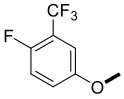
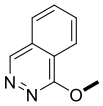
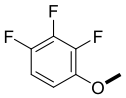
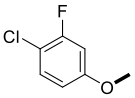
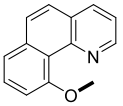
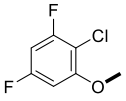
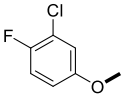
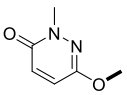
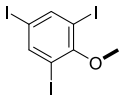
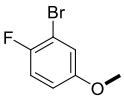
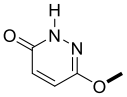
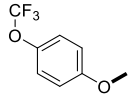
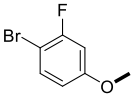
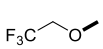
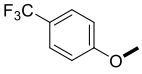
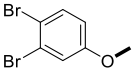
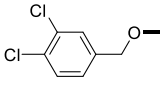
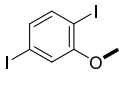
The aim of our further study[210] is the synthesis of new MDL-860 analogues possessing unaltered 2-cyano-5-nitro substituted benzene ring as a common fragment, in order to prove limits of possible variations in the other ring of MDL-860, leading to improved antiviral activity. Thus, four series of MDL-860 analogues were synthesized and evaluated for anti-viral activity. All target compounds were obtained through simple one-step nucleophilic aromatic substitution reactions of series of phenols (**410-445**), thiols (**482-490**), amines (**501-511**) and *N*-heterocycles (**523-525**) with 2-chloro-5-nitrobenzonitrile (**385**) in presence of a base. Due to the electron-deficient nature of **385**, chlorine substitution was performed in relatively mild conditions with no catalyst needed.[211] All compounds were purified by column chromatography and/or recrystallization.

The synthesis of aryl ethers **446-481** (Scheme 20) and aryl thioethers **491-500** (Scheme 21) from **385** and the corresponding phenols (**410-445**) and thiophenols (**482-490**) was performed at 80°C in dry DMSO generally using powdered KOH as a base. In some cases NaOH or K_2CO_3 were used instead (**460**, **465**, **466**, **481**, **495** and **498**). Reaction progress was monitored by TLC. Compound **456** was obtained from **385** and *in situ* generated CF_3CH_2ONa (from dry CF_3CH_2OH and NaH) in refluxing CF_3CH_2OH. Similar procedure was applied for the preparation of benzyl

ether **457**. Compound **500** was obtained as a single product through spontaneous cyclisation during the reaction between **385** and mercaptobenzimidazole (**490**).[212]



385 **446-481**

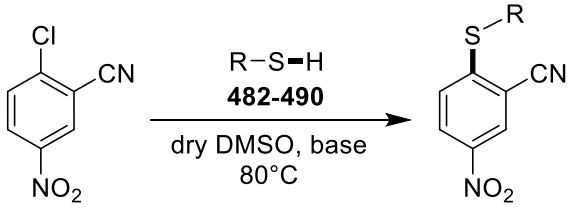
ROH	RO—	Ether	ROH	RO—	Ether	ROH	RO—	Ether
410		**446**	**422**		**458**	**434**		**470**
411		**447**	**423**		**459**	**435**		**471**
412		**448**	**424**		**460**	**436**		**472**
413		**449**	**425**		**461**	**437**		**473**
414		**450**	**426**		**462**	**438**		**474**
415		**451**	**427**		**463**	**439**		**475**
416		**452**	**428**		**464**	**440**		**476**
417		**453**	**429**		**465**	**441**		**477**
418		**454**	**430**		**466**	**442**		**478**
419		**455**	**431**		**467**	**443**		**479**
420		**456**	**432**		**468**	**444**		**480**
421		**457**	**433**		**469**	**445**		**481***

*Compound **481** contains ca. 5% of the 3,4-diiodo isomer.

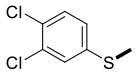
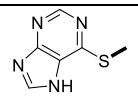
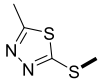
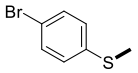
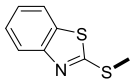
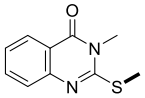
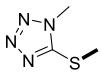
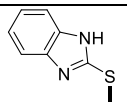
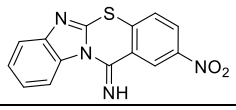
Scheme 20. Synthesis of diaryl ethers **446**-**481**.

The aryl thioether **499** was therefore not isolated. It should be mentioned that the preparation of compound **481** (2-(2,5-diiodophenoxy)-5-nitrobenzonitrile) was initially not aimed. Instead, we had planned to obtain a 3,4-diiodo-analogue of MDL-860. Unfortunately, the synthesis of this derivative from 3-iodophenol turned out to be quite challenging. All attempts led to isolation of 2,5-diiodophenol (**445**) with ca. 95% purity. It was not possible to further purify compound **445**, thus it was used for the synthesis of **481** as it was. The purification of compound **481** was a serious

challenge as well. Column chromatography purification followed by several successive recrystallizations led to isolation of **481** with ca 95% purity. According to X-ray data obtained on crystal grown form that mixture, compound **481** was found to contain 5% of its 3,4-diiodo substituted analogue, thus indicating the presence of 3,4-diiodophenol in **445**.



385 **491-500**

RSH	RS–	Thio-ether	RSH	RS–	Thio-ether	RSH	RS–	Thio-ether
482		**491**	**485**		**494**	**488**		**497**
483		**492**	**486**		**495**	**489**		**498**
484		**493**	**487**		**496**	**490**		**499***
								**500**

*Not isolated

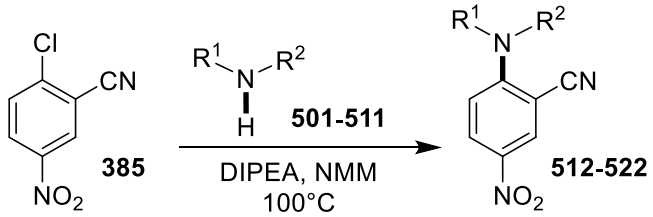
Scheme 21. Synthesis of thioethers **491**-**500**.

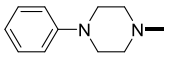
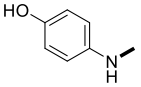
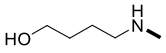
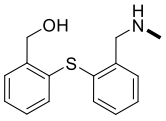
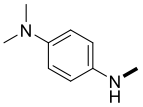
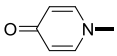
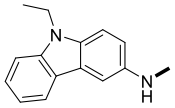
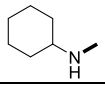
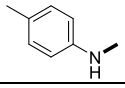
A series of arylamines **512-522** (Scheme 22) were synthesized through reaction of **385** with amines **501-511** at 100°C in a mixture of dry DIPEA and *N*-methylmorpholine (NMM). Amine **512** was synthesized from **385** and dry dymethylformamide (DMF) (as convenient *in situ* source of dimethylamine) at 130°C according to a described procedure.[213] In some cases (**517**-**519**) decomposition products (black tar) and unreacted **385** were observed.

N-arylation of heterocycles **523-525** applying common conditions (NaH/dry DMSO) afforded the corresponding compounds **526-528** in good to excellent yields (Scheme 23).

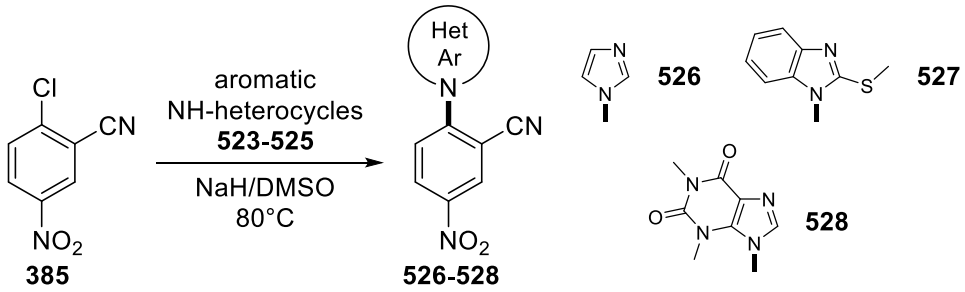
A trifluoromethyl substituted analogue (**531**) of MDL-860 was prepared from 1-fluoro-4-nitro-2-(trifluoromethyl)benzene (**529**) and 3,4-dichlorophenol (**530**) in DMSO (Scheme 24).

Application of single crystal X-ray diffraction was used in this study. This method was necessary in order to confirm the structure of **481**, on the other hand the results obtained were useful to elucidate the structure of the impurity in **481**. Thus, the crystal structure of compound **481** was elucidated by single crystal X-ray diffraction (Figure 11). Single crystals were obtained by slow evaporation of a concentrated solution of **481** in isopropanol. The most important crystallographic data and refinement parameters for **481** are shown in Table 16. The crystal structure revealed the presence of impurity (ca. 5%) of 2-(3,4-diiodophenoxy)-5-nitrobenzonitrile. It is interesting to note that I4 shifts from its "original" I2 position and the distance between iodines I3 and I4 from the minor component is 3.918 Å The hypothetical I2…I3 distance being 3.232 Å. Both ring systems (diiodo substituted and nitrobenzonitrile) are essentially planar (*rmsd* of 0.01 Å for both) though the angle between their mean planes is 81.5° e.g. the bridging O1 allows rotation of the ring systems along C–O1 bond.

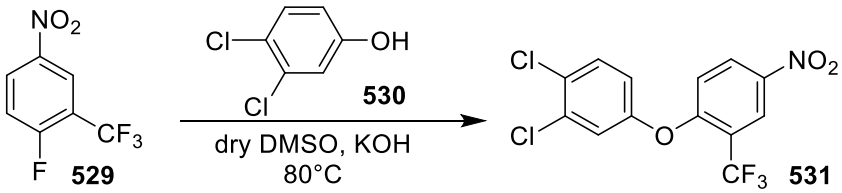


Amine	R^1R^2N–	Aryl-amine	Amine	R^1R^2N–	Aryl-amine	Amine	R^1R^2N–	Aryl-amine
501		**512**	**505**		**516**	**509**		**520**
502		**513**	**506**		**517**	**510**		**521**
503		**514**	**507**		**518**	**511**		**522**
504		**515**	**508**		**519**			

Scheme 22. Synthesis of arylamines **512-522**.



Scheme 23. Synthesis of heterocycles **512-522**.



Scheme 24. Synthesis of compound **531**.

Table 16. Most important crystallographic and data refinement parameters for compound **481**.

Empirical formula	$C_{13}H_6I_2N_2O_3$		
Formula weight	492.00	*F*(000)	912.0
Temperature/K	290	Crystal size/mm^3	0.3 × 0.25 × 0.12
Crystal system	Monoclinic	Radiation	Cu*K*α (λ = 1.54184)
Space group	$P2_1/c$	2Θ range for data collection/°	8.684 to 148.524
a/Å	21.1900(7)	Index ranges	-22 ≤ h ≤ 26, -6 ≤ k ≤ 6, -15 ≤ l ≤ 16
b/Å	5.60500(10)	Reflections collected	9013
c/Å	13.2171(4)	Independent reflections	3015 [R_{int} = 0.0544, R_{sigma} = 0.0431]
α/°	90	Data/restraints/parameters	3015/0/202
β/°	106.026(4)	Goodness-of-fit on F^2	1.026
γ/°	90	Final *R* indexes [*I*>=2σ (*I*)]	R_1 = 0.0511, wR_2 = 0.1304
Volume/Å^3	1508.79(8)	Final *R* indexes [all data]	R_1 = 0.0706, wR_2 = 0.1456
Z	4	Largest diff. peak/hole / e Å^{-3}	1.11/-1.32
ρ_{calc} g/cm^3	2.166	CCDC number	1876618
μ/mm^{-1}	32.829		

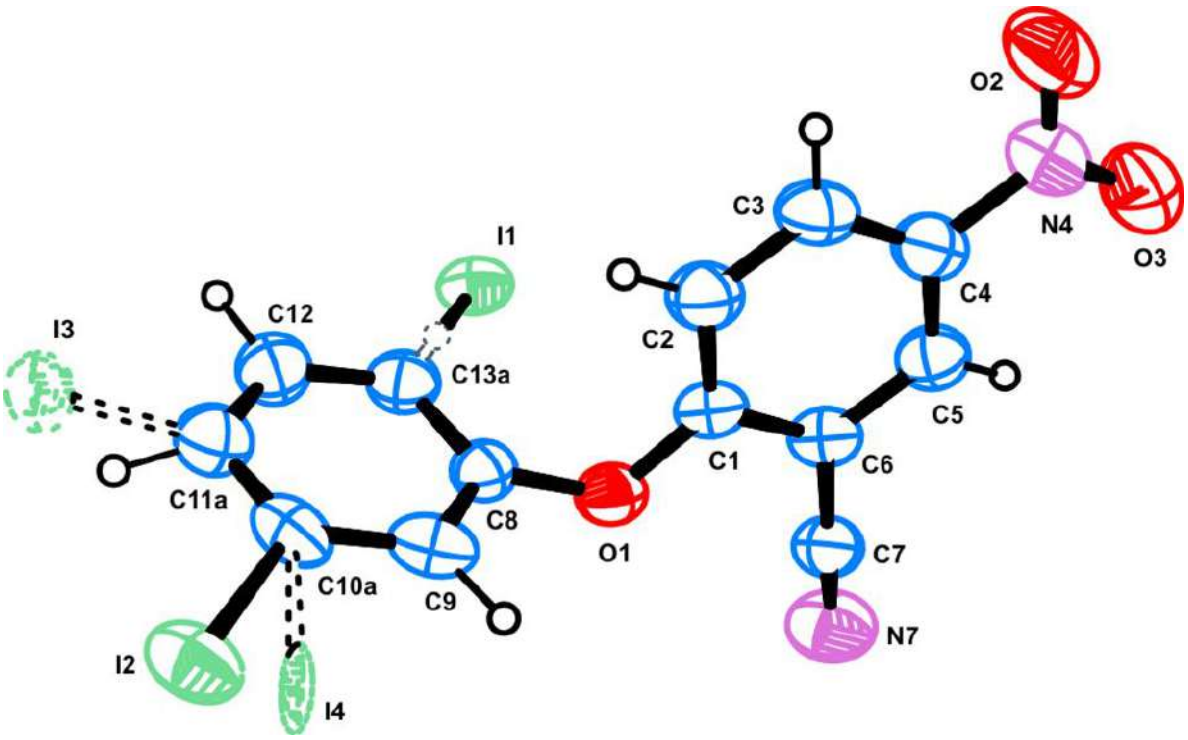


Figure 11. ORTEP drawing of compound **481** showing the atomic numbering system and the observed disorder of the iodines (minor component of 5.58% is shown as dashed lines).

The newly synthesized MDL-860 derivatives (**446-481**, **491-500**, **512-522**, **526-528** and **531**) were subjected to *in vitro* screening study for activity towards PV1, CVB1 and CVB3 (Table 17). The CPE inhibition test was used, following the procedure of *Borenfreund* and *Puerner*.[214] Previously published data for compounds **398**-**409** (see Table 16) are commented for comparison.[18] It was demonstrated that compound **472** (by analogy with MDL-860) possesses the broadest spectrum of activity in this study (against PV1, CVB1 and CVB3). Compound **480** was active against CVB1 and CVB3. Significant activity towards PV1 and CVB1 demonstrated by **473**. Compounds **465** and **474** were effective only against CVB1 and **467**, **475** and **478** – only against PV1. Moderate activity against PV1 and CVB1 was demonstrated by compound **449**; against PV1 – only by **462**, **463**, **469** and **477**; compound **458** was active against CVB1 and **459** – against CVB3. It should be mentioned that the anti-viral activity of **462**, **463**, **468** and **491** was investigated in the early 1980`s against rhinoviruses and Coxsackie A21 virus.[205] Their activities were generally higher compared to those observed against PV1, CVB1 and CVB3 in this study (Table 17). Only MDL-860 could be considered to possess wide spectrum of activity – it demonstrated high *in vitro* activity against all tested rhinoviruses and coxsackie viruses.

Among the thioethers, **493** and **498** demonstrated weak activity against CVB1. The main disadvantage of **498** appears to be its high cytotoxicity (CC_{50} = 18.7 μM). Since thioethers are able to oxidize easily in biological media, it is not clear whether **493** and **498** are the active compounds or just prodrugs. Thus, further investigation of sulfone analogues of **493** and **498** is necessary.

The data presented in Table 17 unambiguously show that even small changes in the MDL-860 molecule dramatically influence the *in vitro* activity. Interestingly, new active compounds could be found exclusively among the diaryl ethers. Exploring in more detail the group of 2-cyano-5-nitro substituted ethers (compounds **398-402**, **404**, **405**, **407**, **408** and **446-481**), it is clear that the presence of two and three halogen substituents at different positions in the secondary benzene ring is optimal for high activity. On the other hand, poly-halogenated ethers (**399**, **448-450**) are inactive. Most of the diaryl ethers (with a few exceptions: **457**, **460**, **463**, **469**, **477**) are showing very low toxicity. In the light of the recently discovered mechanism of action of MDL-860,[203] one could argue that all active diaryl ethers have a similar mechanism of action, namely, an irreversible covalent modification of phosphatidylinositol-4 kinase III beta (PI4KB).

Table 17. *In vitro* screening data for anti-enteroviral activity of compounds **446-481**, **491-500**, **512-522**, **526-528** and **531**.

Compound[a]	Cytotoxicity CC_{50} (μM)	PV1		CVB1		CVB3	
		IC_{50} (μM)	SI	IC_{50} (μM)	SI	IC_{50} (μM)	SI
MDL-860	493.0	6.8	72.5	0.8	586.9	2.7	182.0
446	123.0	NA	–	NA	–	NA	–
447	175.0	NA	–	NA	–	NA	–
448	123.0	NA	–	NA	–	NA	–
449	332.0	30.6	11.5	22.8	14.5	NA	–
450	346.6	NA	–	NA	–	NA	–
451	280.0	NA	–	NA	–	NA	–
452	450.0	NA	–	NA	–	NA	–
453	576.0	NA	–	NA	–	NA	–
454	423.0	NA	–	NA	–	NA	–
455	367.0	NA	–	142.0	2.5	NA	–
456	165.0	NA	–	NA	–	NA	–
457	10.1	NA	–	NA	–	NA	–
458	291.0	NA	–	12.7	22.9	NA	–
459	680.0	NA	–	NA	–	54.0	12.5
460	47.1	NA	–	NA	–	NA	–
461	195.0	79.0	2.4	NA	–	NA	–
462	287.0	22.8	12.6	NA	–	NA	–
463	18.7	1.0	18.7	NA	–	NA	–
464	95.0	11.0	8.6	NA	–	NA	–
465	199.0	32	6.2	2.1	95	NA	–
466	572.0	NA	–	NA	–	NA	–
467	219.0	6.8	32.2	NA	–	NA	–
468	272.0	NA	–	NA	–	NA	–
469	30.7	1.8	17.0	NA	–	NA	–
470	92.0	NA	–	NA	–	NA	–
471	547.0	NA	–	NA	–	NA	–
472	785.0	11.0	71.3	6.4	122.6	6.8	115.4
473	234.0	2.7	86.6	6.1	38.3	NA	–
474	342.0	NA	–	2.9	117.9	NA	–
475	200.0	4.3	46.0	NA	–	NA	–
476	155.0	10.0	15.5	NA	–	NA	–
477	30.5	5.2	5.8	NA	–	NA	–
478	107.0	1.0	107	NA	–	NA	–
479	215.0	NA	–	NA	–	NA	–
480	493.0	NA	–	3.7	133.2	1.0	493.0
481	273.0	NA	–	NA	–	NA	–
491	132.0	NA	–	NA	–	NA	–
492	211.0	NA	–	NA	–	NA	–
493	187.5	NA	–	24.9	7.5	NA	–
494	161.3	NA	–	NA	–	NA	–
495	13.6	NA	–	NA	–	NA	–
496	14.6	NA	–	NA	–	NA	–
497	16.5	NA	–	NA	–	NA	–
498	18.7	NA	–	3.1	6.0	NA	–
500	349.2	NA	–	NA	–	NA	–
512	651.0	NA	–	NA	–	NA	–
513	332.6	NA	–	NA	–	NA	–
514	336.7	NA	–	NA	–	NA	–
515	495.4	NA	–	NA	–	NA	–
516	617.0	255.0	2.4	NA	–	NA	–
517	12.6	NA	–	NA	–	NA	–
518	346.6	NA	–	NA	–	NA	–
519	199.0	NA	–	NA	–	NA	–
520	55.4	NA	–	NA	–	NA	–
521	336.4	NA	–	NA	–	NA	–
522	454.0	NA	–	NA	–	NA	–
526	332.8	NA	–	NA	–	NA	–
527	94.2	NA	–	NA	–	NA	–
528	339.9	NA	–	NA	–	NA	–
531	22.8	NA	–	NA	–	NA	–

[a] MDL860 was used as a reference compound.
NA – not active

PI4KB is one of the most important enzymes in mammals, responsible for replication of enteroviruses in the host cells.[215] It could be assumed that alteration of the halogen substituents in diaryl ethers is important. This may cause small changes in the shape and geometry of the molecules but may impact significantly the PI4KB modification and *in vitro* activity, respectively.

Other type of substituents (e.g. **401**, **402**, **446-448**, **450**, **451**, **458**, **459**), or the presence of heterocyclic moieties (**407**, **408**, **449**, **452-455**) instead of benzene ring, generally led to lack of activity. Other series of compounds (**491-500**, **512-522**, **526-528** and **531**, containing different bridge heteroatoms, i.e. *S*, *N*) were completely inactive (except for **498**). It is noteworthy that even very close isosteric analogues of MDL-860 (like thioether **491** or ether **531**) are also inactive. Probably these compounds are not able to modify PI4KB or they undergo biochemical transformations before reaching the enzyme.

Compound **531** is the only MDL-860 analogue in this study, possessing a different substituent in the primary aromatic ring ($-CF_3$ instead of $-CN$). The role of the substituents in this ring is still unclear and further studies of such series of compounds is necessary. For example, some published results[205] show that replacement of the cyano group with carboxyl group in MDL-860 leads to carboxylic acid with good antiviral activity. Moreover, this replacement automatically allows improvement of water solubility through possible formation of salts.

The most active derivatives (**472**, **474** and **480**) against CVB1 were tested for *in vivo* activity in newborn mice experimentally infected with 20 MLD_{50} CVB1. Compounds **472**, **474** and **480** were administered subcutaneously as daily doses of 25 and 50 mg/kg following 12-days course since the day of viral inoculation. The results obtained showed moderate protective effects of **472** and **474**. A marked lengthening of the mean survival time was observed for **474** (25 mg/kg) and **480** (25 mg/kg) (Table 18 and Figure 12). Taking into account this lack of activity, along with the previously reported promising results for compounds (Table 17) **398** (PI 50%), **408** (PI 33%) and **409** (PI 11%), it could be suggested that there is no correlation between *in vitro* and *in vivo* activity. Since the pharmacological properties and especially the mechanisms of transport across the cell membranes for these diaryl ethers are unknown, it is difficult to explain these results. Moreover, the diaryl ether structures imply extremely poor solubility in water. The nature of the substituents does not allow chemical modification of the active compounds in order to improve solubility and/or membrane transport (e.g. conversion to prodrugs – salts, esters, etc.). Further formulation of the *in vitro* active compounds through preparation of nanoparticles or complexes with water soluble polymers could increase significantly the *in vivo* effects.

Table 18. Study of the *in vivo* activity of compounds **472**, **474** and **480** against CVB1 experimental neuroinfection in newborn mice. Data are from three independent experiments (average).

Compound/ Dose	Survivors/ Total	MST±SD, days[a]	Δ, days	Mortality, %	PI, %
472/25 mg/kg	0/16	3.2±0.5^{ns}	0.2	100	0
472/50 mg/kg	3/21	5.4±0.5^{ns}	+2.4	86	14.2
474/25 mg/kg	6/23	7.0±1.0**	+4.0	74	26
474/50 mg/kg	0/26	3.1±0.4^{ns}	+0.1	100	0
480/25 mg/kg	0/17	6.1±0.8*	+3.1	100	0
480/50 mg/kg	1/23	4.6±0.6^{ns}	+1.6	96	4.3
MDL-860	0/27	6.1±0.9*	+3.1	100	0
Placebo	0/16	3.0 ±0.3	−	100	0

[a] One-way ANOVA (Bonferroni's multiple comparison post-test); MST - mean survival time; PI – protection index; SD – standard deviation; $^{**}p<0.01$vs. placebo group; $^{*}p<0.05$ vs. placebo group; ns – not significant

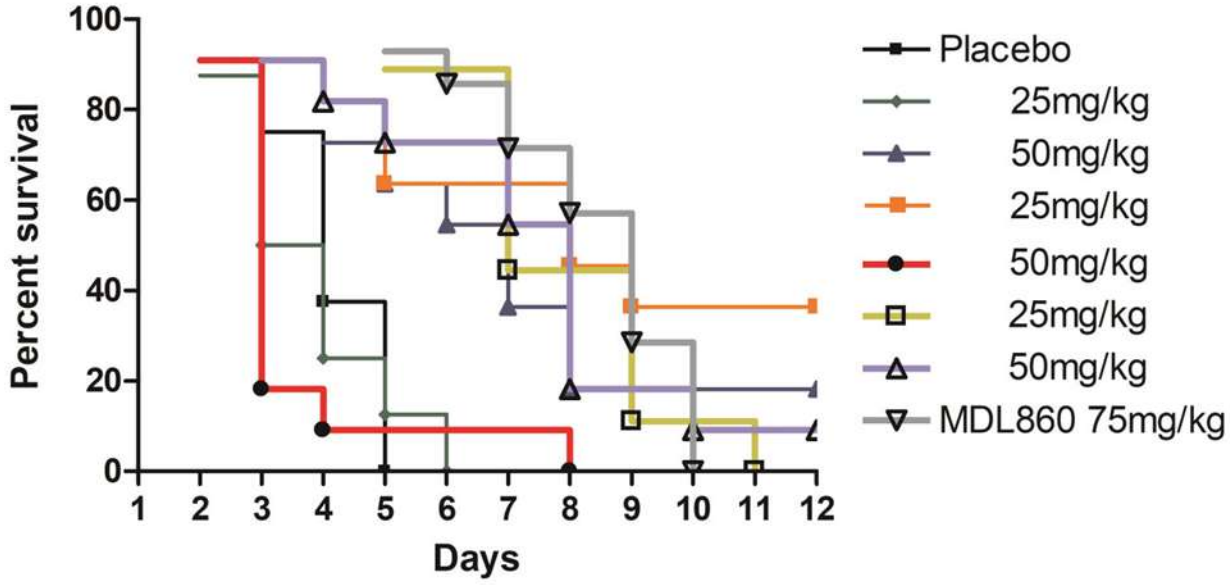


Figure 12. Individual effects of compounds **472** (green and gray), **474** (orange and red) and **480** (yellow and gray) in experimental neurotropic infection with Coxsackievirus B1 in newborn mice. MDL-860 was used as reference.

Synthesis and QSAR analysis of diaryl ethers and their analogues as broad spectrum antivirals

Coronaviruses are a type of single stranded positive sense RNA viruses that cause disease in birds and mammals. In humans, they cause respiratory tract infections that can range from mild, such as the common cold, especially Human Coranaviruses 229E and OC43 (HCoV-229E and HCoV-OC43), to life threatening conditions such as severe acute respiratory syndrome (SARS-CoV). The ability of this type of virus to develop into global epidemics, affecting a huge percentage of the world's population and causing enormous material and economic damage, is well known. At the end of 2019, a new epidemic of coronavirus SARS began in the city of Wuhan, China, the causative agent of which is described as SARS-CoV-2, and the specific form of the disease is called COVID-19. In a few months, the contagion took over the planet and a pandemic began, unprecedented in the number of victims and the economic damage it caused, and this was the first pandemic of this magnitude since 1918, when the so-called "Spanish flu" raged. The relatively higher mortality rate compared to other viral infections, the enormous economic damage, the disrupted functioning of all societies around the world, make the coronavirus pandemic the greatest challenge to humanity today.

The struggle against these viral pathogens includes different approaches, but to date they are insufficient to provide effective eradication of the illnesses caused. Herpes simplex virus (HSV) infections are incurable, but there are some antiviral drugs showing activity against the virus in the form of reducing the severity and the duration of the outbreak-associated lesions. Antiviral agents such as acyclovir[216] and valacyclovir can reduce reactivation rates.[217] Many natural products have also been found to exhibit promising anti-HSV activity.[218] Currently Human adenovirus has no accepted chemotherapeutic treatment. Instead, the different symptoms, which may develop from a human adenovirus (HAdV) infection are treated independently, while antibiotics can be prescribed to battle secondary bacterial infections. In severe cases, the antiviral drug cidofovir[219] can be applied, but its effectiveness is questionable. A vaccine against some types of HAdV is available for the United States military, however not for the other population.

A tremendous progress in the fight with PV1 has been made with the introduction of the polio vaccine. However, the polio virus has not been fully eradicated yet in countries like Afghanistan, Pakistan and Tajikistan, where because of different factors, the vaccine is not available for around 5-25% of the children.[220] No efficient and approved chemotherapy against PV1 infections exist. The same is true for the Coxsackie B infections.

The possibilities for chemotherapeutic treatment of coronavirus infections are extremely limited. Only the antiviral agent remdesivir has an official authorization from the US Food and Drug Administration (FDA) for use against COVID-19.[221] However, this drug is also quite controversial due to its limited effectiveness and many side effects. The lack of effective and universally recognized anti-covid drugs remains a serious problem and a major source of uncertainty regarding the future development of the pandemic. Great efforts are being made in the field of immunology, which is where some remarkable results come from - mass vaccination of the population is underway, which so far contributes to limiting the pandemic, but still does not give prospects for its imminent end.

Diaryl ethers have long been known to exhibit antiviral properties. Great number of their analogues have been synthetized within frames of our previous studies.[18,210] Their *in vitro* and *in vivo* antiviral activity has been determined. Many hit compounds with excellent activities against different enteroviruses have been discovered. Despite the promising results, this class of compounds has never been tested against a broader spectrum of viruses, outside the picornavirus/enterovirus group. The aim of the present study is the synthesis of new diaryl ethers (MDL-860 analogues), and screening of their *in vitro* activity against a panel of various viruses. Furthermore QSAR models are derived for a panel of the newly synthesized compounds as well as compounds reported in our previous studies. The models provide valuable insight into structural/physicochemical features of the compounds essential for the array of antiviral activities investigated. The molecular design of compounds in this study was based on our previous experience in preparation of new diaryl ethers as antivirals.[18,210] We accomplished several small series of compounds (Figure 13), containing two aromatic fragments, bridged by heteroatom X.

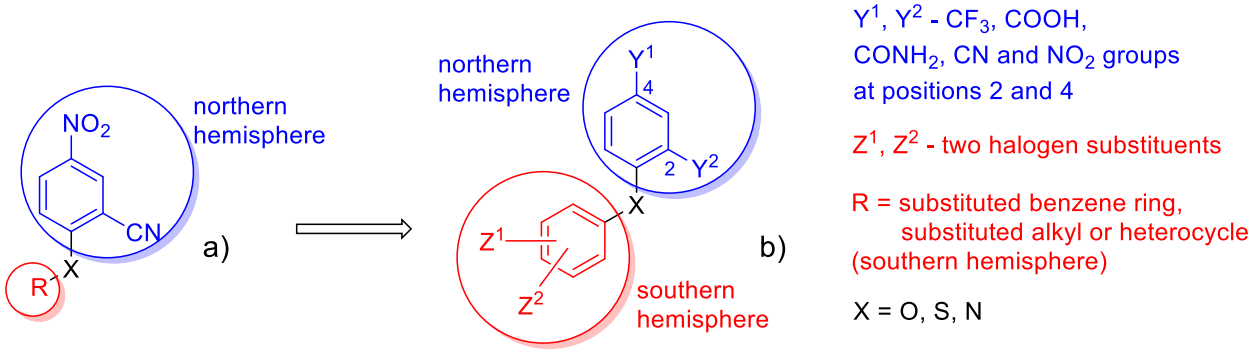
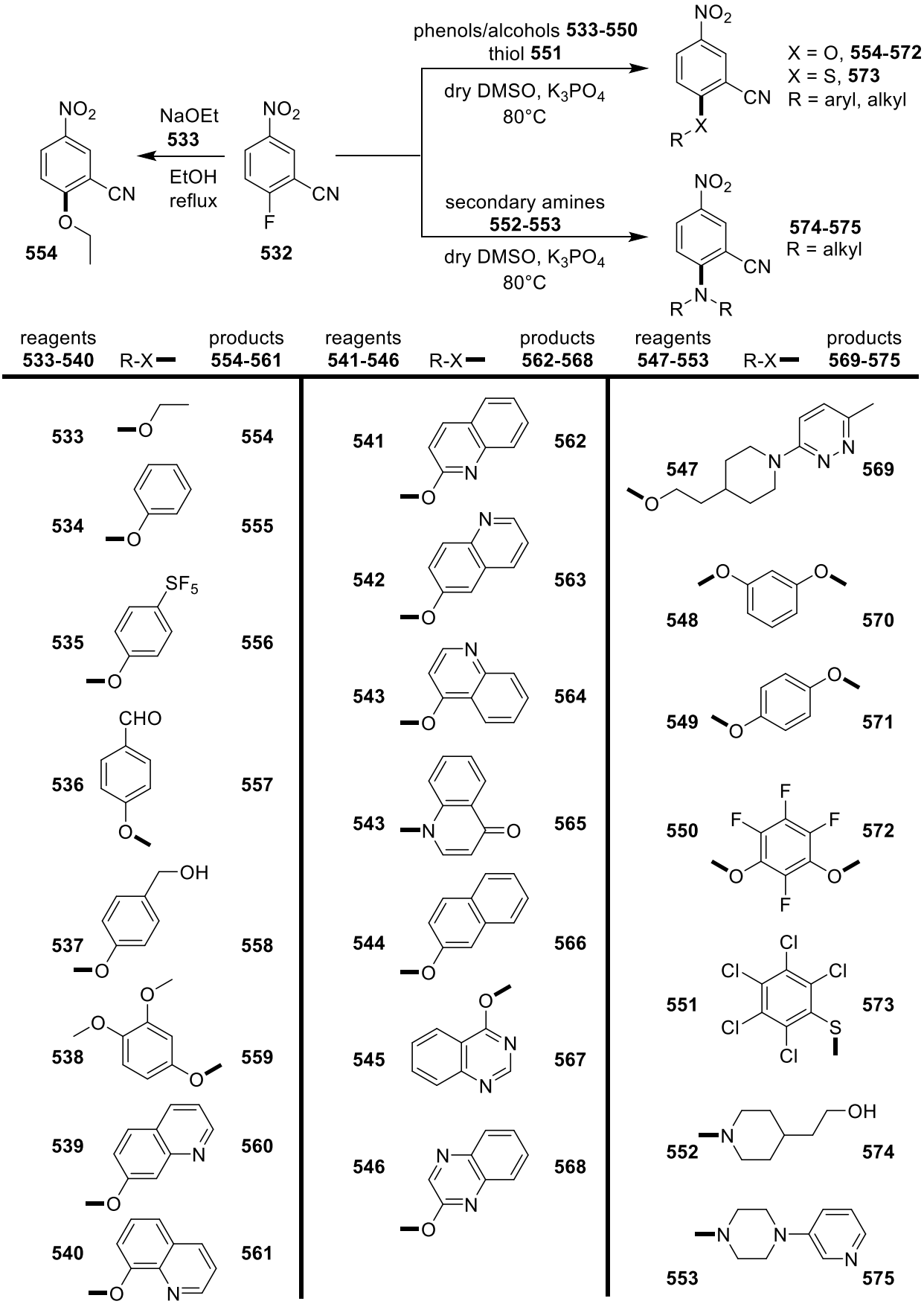


Figure 13. Molecular design of MDL-860 analogues.

The first series was based on the continuation of the synthesis of new diaryl ethers, possessing the classical position of nitro- and cyano- groups in one aromatic nucleus, as in MDL-860 (X=O, Figure 13a). The other series was based on the dihalo substituted southern hemisphere, and northern hemisphere with two electron withdrawing groups at positions 2 and 4 (Figure 13b).

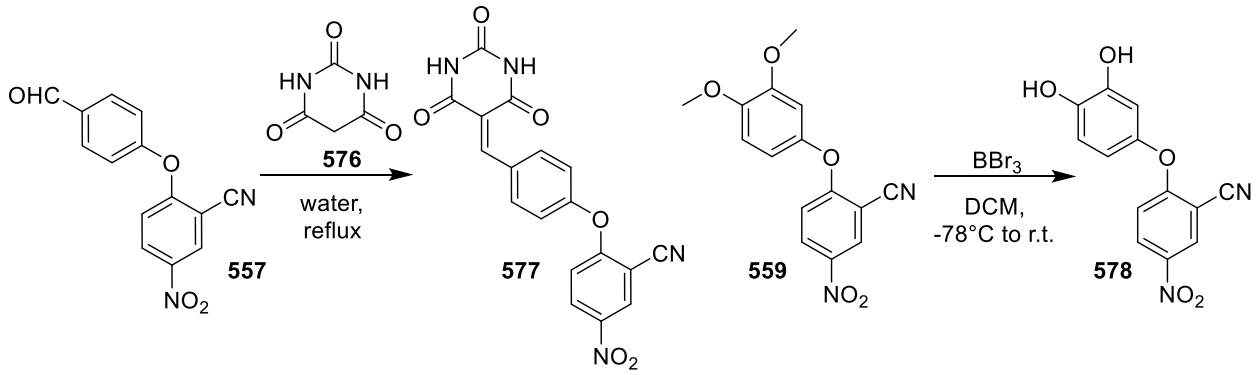


Scheme 25. Synthesis of compounds **554**-**575**.

Reaction of commercially available 2-fluoro-5-nitrobenzonitrile (**532**) with different phenols and alcohols (**533-550**) was performed in dry DMSO, by using of anhydrous K_3PO_4 as a base. Reaction times were different, depending on the phenols. As a single exception in this series, only compound **554** was synthesized by using of different procedure – reaction of **532** with NaOEt in

EtOH. Aryl ethers **554-572** were obtained in good to excellent yields. Compound **565** was obtained as a by-product of *N*-arylation of **543**.

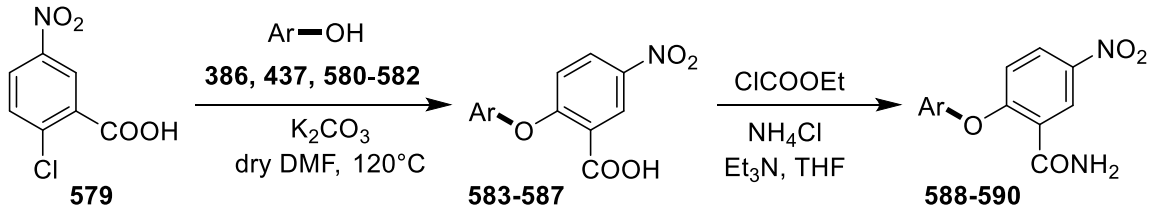
The same procedure was used for preparation of diaryl thioether **573** from thiophenol **551** and amines **574-575** from secondary amines **552-553**, respectively. Diaryl ethers **577** and **578** were obtained subsequently from products **557** and **559** by using of different synthetic procedures (Scheme 26). Condensation of **557** with barbituric acid (**576**) in hot water leads to diaryl ether **577** in excellent yield. Diol **578** was synthesized by reaction of **559** with BBr_3 solution at -78°C.

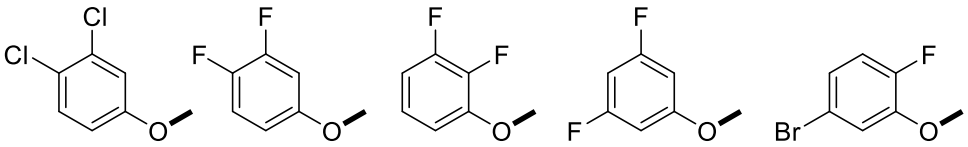


Scheme 26. Synthesis of compounds **577-578**.

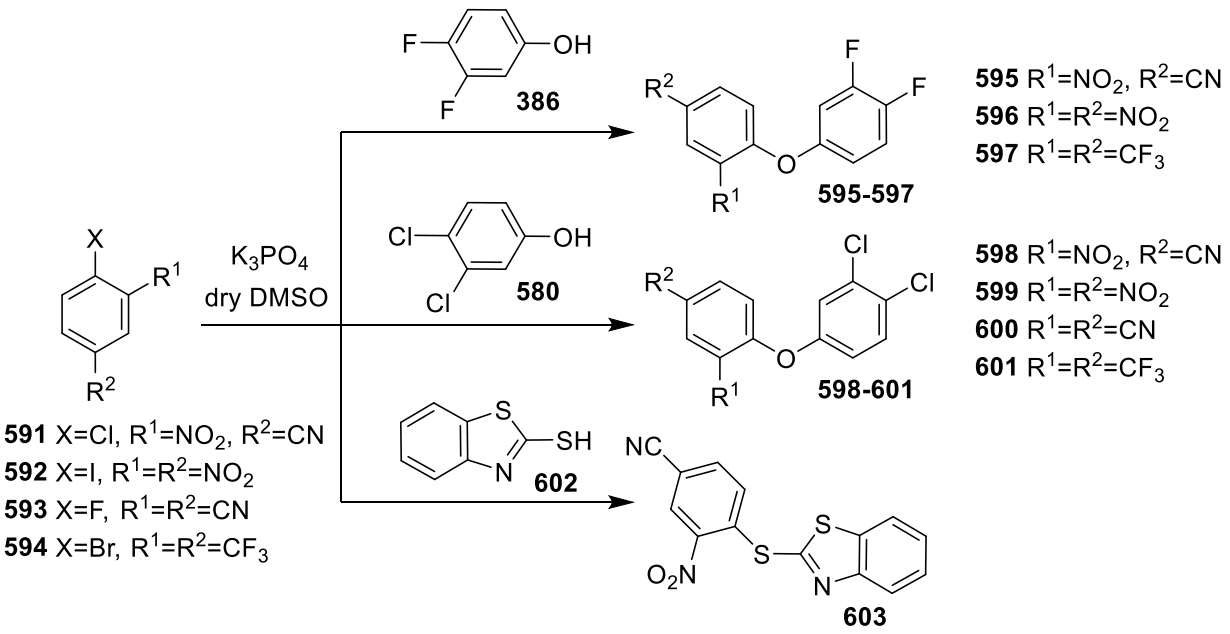
A small series of diaryl ether-acids **583-587** was accomplished through reaction of 2-chloro-5-nitrobenzoic acid (**579**) with halophenols **386, 437** and **580-582**, respectively (Scheme 27). Reactions were carried out in dry DMF at 120°C and presence of K_2CO_3 as a base. Acids **583-585** were subsequently converted to their primary amides **588-590** with ethyl chloroformate and NH_4Cl in Et_3N/THF media. As in case of compounds from Scheme 25, synthesis of diaryl ethers **595-601** and diary thioether **603** was realized by using of K_3PO_4 in dry DMSO (Scheme 28).

Compound **554** was previously synthesized and used as intermediate.[222] Synthesis of **559** and **583**, and their activity against some picorna viruses was also mentioned.[205,223] *Pürstinger et al.* obtained **558** and tested its activity against CVB3 virus.[206]



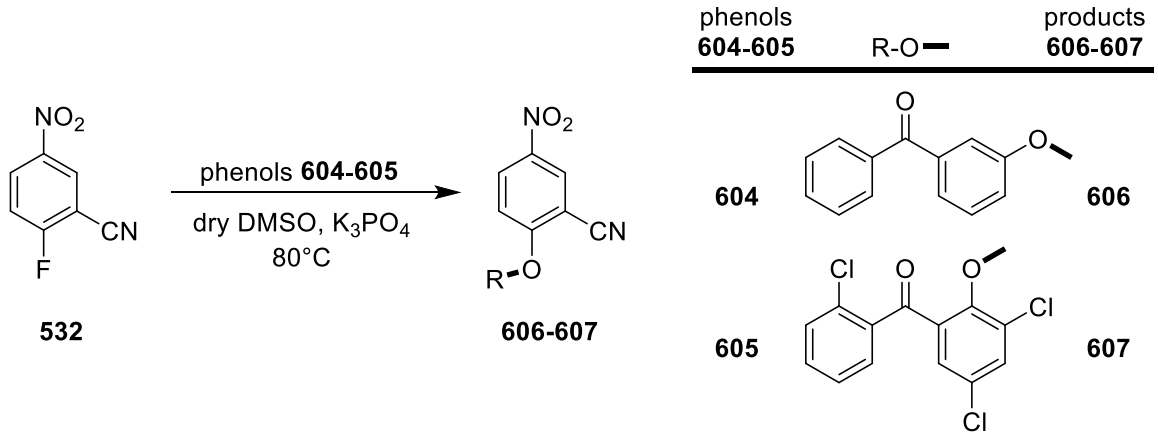
Ar—					
phenols **386, 437, 580-582**	**580**	**386**	**581**	**582**	**437**
acids **583-587**	**583**	**584**	**585**	**586**	**587**
amides **588-590**	**588**	**589**	**590**	-	-

Scheme 27. Synthesis of compounds **583-590**.



Scheme 28. Synthesis of compounds **595**-**601**, **603**.

As a result of our QSAR analysis (see below), compounds **606**-**607** were synthesized subsequently (Scheme 29). Their design was inspired not only by conclusions of QSAR, but also they were chosen to be analogues of fenofibrate – a drug used to treat abnormal blood lipid levels. Interestingly, the recent studies revealed that this compound can significantly reduce SARS-CoV-2 infection *in vitro*.[224]



Scheme 29. Synthesis of compounds **606**-**607**.

<u>*In vitro* antiviral activity of compounds **554**-**575**, **577**-**578**, **583**-**590**, **595**-**601**, **603** and **606**-**607**</u>

All 42 synthesized compounds[225] were tested *in vitro* against six viruses (Table 19), namely PV1, CVB1, CVB3, Human adenovirus C serotype 5 (HAdV-5), herpes Simplex Virus Type 1 (HSV-1) and human coronavirus OC43 (HCoV-OC43). Compound MDL-860 was synthesized according described procedure[206] and used as reference in this study. Two compounds in this series can be outlined as remarkably active - **555** toward HCoV-OC43 and **556** toward HAdV-5 (SI 97.4 and 99.7, respectively). Interestingly, **556** demonstrates quite different cytotoxicity toward different cell lines – from 20 to 698 μM. Amide **588** and benzophenone **606** are moderately active toward HCoV-OC43 (SI 57 and 48.7, respectively). As in our previous studies,[18,210] compounds demonstrated strong selectivity toward different viruses and no broad-spectrum activity was observed. It is interesting to point out, that MDL-860 is inactive toward HAdV-5, HSV-1 and HCoV-OC43, and vice versa - all synthesized compounds in this study[225] are not active against PV1, CVB1 and CVB3.

Table 19. *In vitro* screening data for antiviral activity of compounds **554-575**, **577-578**, **583-590**, **595-601**, **603** and **606-607**.[a]

Comp.	HEp-2 CC_{50}[b]	PV1		CVB1		CVB3		HAdV-5		MDBK CC_{50}[b]	HSV-1		HCT-8 CC_{50}[b]	HCoV-OC43	
		IC_{50}[b]	SI	IC_{50}[b]	SI	IC_{50}[b]	SI	IC_{50}[b]	SI		IC_{50}[b]	SI		IC_{50}[b]	SI
MDL-860	493±27	6.8±2.0	72.5	0.8±0.02	616.3	2.7±0.1	182.6	NA	-	90±2.5	NA	-	174±11	69±3.3	2.5
554	658±30	NA	-	NA	-	NA	-	123±4.3	5.3	490±22	170±11	2.9	416±34	NA	-
555	402±31	NA	-	NA	-	60±2.0	6.7	17.9±1.0	22.5	400±33	NA	-	341±25	3.5±0.01	97.4
556	698±25	NA	-	NA	-	129±4.0	5.4	7.0±0.2	99.7	20±1.5	NA	-	253±23	NA	-
557	187±5.9	NA	-	NA	-	NA	-	70±2.1	2.7	260±25	NA	-	579±50	300±22	1.9
558	196±10	NA	-	NA	-	NA	-	22±1.3	8.9	235±21	NA	-	570±55	NA	-
559	750±32	NA	-	NA	-	NA	-	NA	-	520±34	NA	-	320±26	NA	-
560	443±29	NA	-	78±3.0	5.7	NA	-	33.9±2.1	13.1	550±26	32±1.8	17.2	262±22	90±4.7	2.9
561	667±34	NA	-	NA	-	431±21	1.5	77±3.1	8.7	615±33	NA	-	333±24	39±2.7	8.5
562	420±12	NA	-	107±8.0	3.9	NA	-	87±2.3	4.8	530±24	230±12	2.3	381±30	NA	-
563	460±26	NA	-	NA	-	NA	-	20.7±1.2	22.2	600±56	24±1.8	25.0	363±30	NA	-
564	160±8.2	NA	-	NA	-	NA	-	NA	-	580±46	30±1.8	19.3	3.1±0.1	NA	-
565	170±10	NA	-	NA	-	NA	-	NA	-	170±10	55±4.7	3.1	80±4.6	NA	-
566	231±11	NA	-	33.2±2.0	7.0	NA	-	NA	-	540±35	NA	-	438±34	NA	-
567	200±15	NA	-	NA	-	NA	-	73.5±3.4	2.7	360±24	18±0.9	20.0	135±11	42±33	3.2
568	214±15	NA	-	NA	-	25±1.3	8.6	100±8.2	2.1	215±19	NA	-	82±5.4	NA	-
569	405±21	NA	-	NA	-	NA	-	NA	-	610±34	NA	-	160±10	11±0.9	14.5
570	581±32	NA	-	NA	-	NA	-	NA	-	610±45	NA	-	570±45	100±8.9	5.7
571	624±34	NA	-	NA	-	NA	-	NA	-	680±45	67±3.7	10.1	654±56	NA	-
572	724±41	NA	-	NA	-	267±18	2.7	67±3.1	10.8	610±44	70±5.8	8.7	495±40	NA	-
573	210±11	NA	-	NA	-	100±6	2.1	NA	-	255±21	NA	-	196±10	7.5±0.6	26.1
573	17.0±0.5	NA	-	NA	-	NA	-	NA	-	680±46	NA	-	196±12	NA	-
575	520±29	NA	-	NA	-	NA	-	NA	-	550±34	NA	-	561±45	60±4.2	9.4
577	302±16	NA	-	NA	-	NA	-	NA	-	640±49	250±21	2.6	447±40	122±10	3.7
578	183±9.4	NA	-	NA	-	NA	-	NA	-	100±4.9	66±5.5	1.5	356±30	NA	-
583	287±13	NA	-	NA	-	NA	-	NA	-	380±23	NA	-	192±12	NA	-
584	581±23	NA	-	NA	-	NA	-	NA	-	520±45	NA	-	698±57	NA	-
585	597±31	NA	-	NA	-	NA	-	NA	-	490±34	NA	-	597±46	NA	-
586	589±27	NA	-	NA	-	NA	-	102±7.2	5.8	545±41	NA	-	256±20	NA	-
587	404±30	NA	-	NA	-	NA	-	NA	-	520±49	NA	-	597±50	86±7.9	6.9
588	581±34	NA	-	NA	-	NA	-	NA	-	530±45	NA	-	627±58	11±1.0	57.0
589	505±35	NA	-	NA	-	NA	-	NA	-	580±50	NA	-	623±58	NA	-
590	637±38	NA	-	NA	-	NA	-	NA	-	620±56	NA	-	723±66	NA	-
595	476±28	NA	-	NA	-	63±2.0	7.6	180±5.9	2.6	120±9.4	NA	-	161±14	NA	-
596	9.1±0.8	NA	-	NA	-	NA	-	NA	-	9.3±0.4	NA	-	205±18	51±2.9	4.0
597	205±11	NA	-	NA	-	205±12	-	NA	-	100±7.8	NA	-	170±11	170±9	-
598	200±14	NA	-	NA	-	25±1.0	8.0	18.0±0.9	11.1	620±45	NA	-	152 12	NA	-
599	58±2.0	NA	-	NA	-	34±1.2	1.7	32±2.1	1.8	8.5±0.5	4.7±0.2	1.8	654±60	170±10	3.8
600	245±10	NA	-	NA	-	NA	-	81±4.5	3.0	580±35	NA	-	192±11	77±6.0	2.5
601	74±3.0	NA	-	NA	-	NA	-	61±3.2	1.2	73±4.8	NA	-	209±18	73±4.6	2.9
603	27±1.0	NA	-	NA	-	NA	-	NA	-	48±3.7	NA	-	47±2.7	NA	-
606	530±14	NA	-	NA	-	100±4	5.3	NA	-	38±2.5	NA	-	151±9	3.1±0.2	48.7
607	105±3.1	NA	-	NA	-	NA	-	NA	-	5±0.3	0.3±0.02	16.6	64±2	NA	-

[a] NA – not active; CC_{50} – *in vitro* cytotoxicity (in μM); IC_{50} – *in vitro* antiviral activity in cell culture experiments (in μM); SI – selectivity index, calculated as a ratio between CC_{50} and IC_{50}; [b] CC_{50} and IC_{50} values represent the mean ± SD of three independent experiments.

Mechanism of action of MDL-860 (short description)

In order to understand mechanism of action, we first analyzed the specificity of antiviral effect of MDL-860. This compound showed moderate anti-PV activity (EC_{50} of 6.8 μM) with apparent no cytotoxicity in the concentrations examined (CC_{50} >100 μM, and SI > 14). This compound did not inhibit encephalomyocarditis virus (EMCV) replication, suggesting that MDL-860 is an atypical enviroxime-like compound possibly targeting enzyme phosphatidylinositol-4 kinase III beta (PI4KB). Moreover, we found that MDL-860 affects PI4KB activity only *in vivo*. We also found an irreversible anti-PV effect to the cells and activates some antioxidant pathways.

Despite the effectiveness, the target and the mechanism of action of MDL-860 remain unknown. In this study,[203] we have characterized antipoliovirus activity of MDL-860 and identified host (PI4KB) as the target. This work reveals the mechanism of action of this class of PI4KB inhibitors and offers insights into novel allosteric regulation of PI4KB activity. Briefly, MDL-860 treatment caused covalent modification and irreversible inactivation of PI4KB (Figure 14). A cysteine residue at amino acid 646 of PI4KB, which locates at the bottom of a surface pocket apart from the active site, was identified as the target site of MDL-860.

PI4KB is one of the four mammalian PI4 kinases (PI4K2A, PI4K2B, PI4KA, and PI4KB). This enzyme is essential in human cells and produces phosphatidylinositol 4-phosphate (PI4P) and involves in ceramid transport, membrane trafficking from the Golgi, and normal function of lysosome. The importance of PI4KB in the replication of enterovirus was first recognized by *Hsu et al.* with a potent PI4KB inhibitor PIK93.[215,226] Subsequently, PI4KB was identified as the target of a group of anti-picornavirus drug candidates known as enviroxime-like compounds - i.e. enviroxime, Ro 09-0179, oxoglaucine, GW5074, T-00127-HEV1, and BF-738735 (see Figure 15).[227-232]

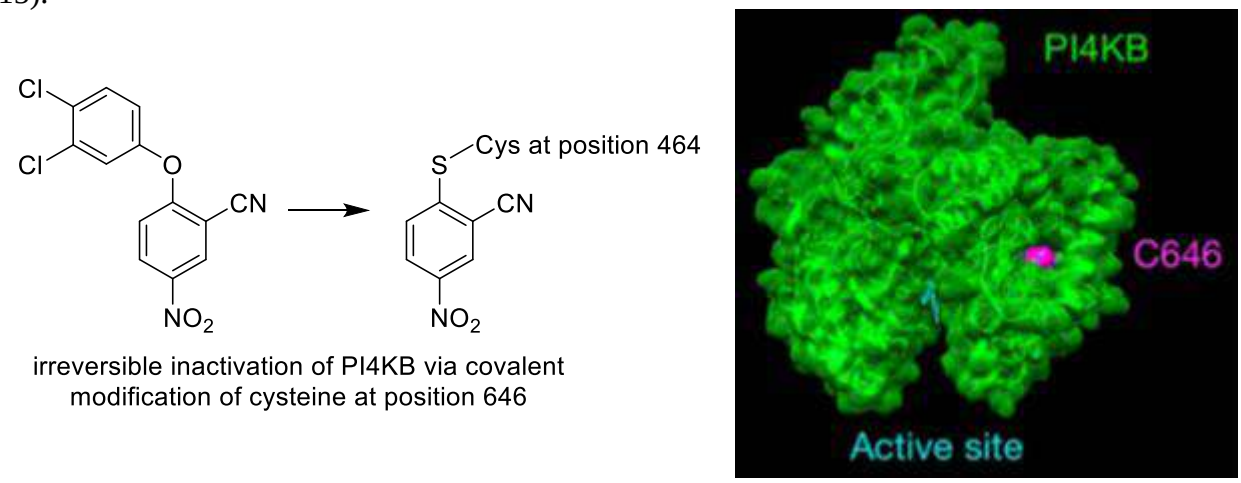


Figure 14. Mechanism of action of MDL-860.

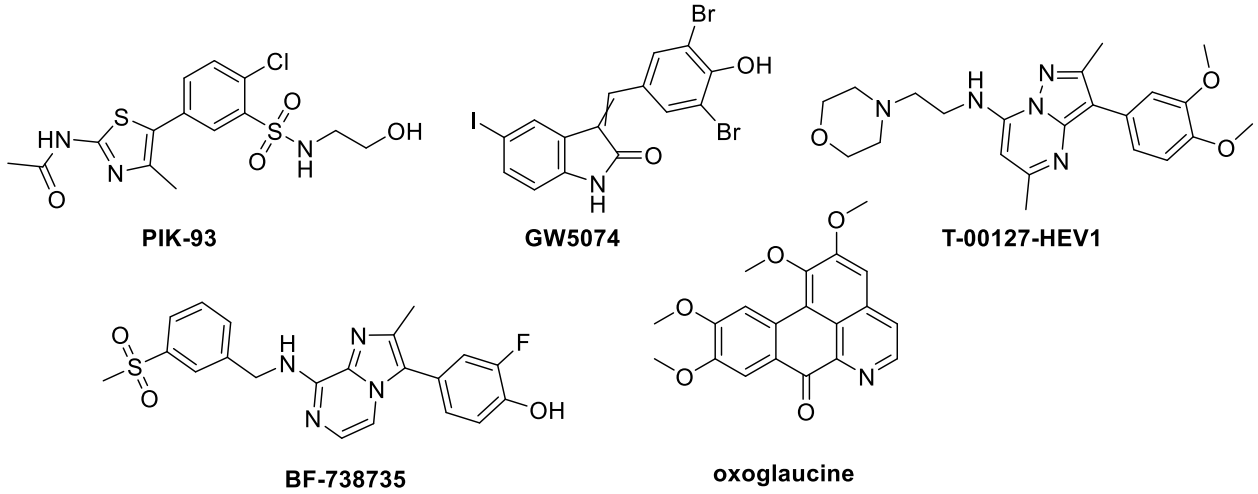


Figure 15. Potent PI4KB inhibitors.

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5. CONCLUSIONS AND CONTRIBUTIONS OF THE THESIS

5.1. Conclusions

Development of new compounds with antitubercular activity:

- Series of 50 new *N*-acylated, *N*-alkylated and *N*-arylated derivatives of commercial enantiopure (*R*)-2-amino-1-butanol was synthesized. Their *in vitro* antitubercular activity was evaluated and 4 compounds demonstrated excellent activity and low cytotoxicity (selectivity indexes between 91.8 and 375.5. In addition, their *in vitro* antibacterial activity toward 13 fungi and pathogenic bacteria was tested. Wide spectrum of activity demonstrated 3 compounds in this series.
- Series of 22 new (*R*)-2-amino-1-butanol derived ureas, thioureas and acylthioureas was synthesized. Their *in vitro* antitubercular activity was evaluated and one compound demonstrated excellent activity and low cytotoxicity (selectivity index 104.4).
- Series of 17 new (-)-fenchone derived enantiopure amides was synthesized. Their *in vitro* antitubercular activity was evaluated and one compound demonstrated moderate activity and low cytotoxicity (selectivity index 54.7).
- Series of 33 new cinnamamide analogues with fenchane skeletons were synthesized. Their *in vitro* antitubercular activity was evaluated and one compound demonstrated moderate activity and low cytotoxicity (selectivity index 67).
- Series of 20 arylmethylidene ketones (16 of them new) and 15 new pyrimidines with camphane skeletons were synthesized. Their *in vitro* antitubercular activity and cytotoxicity was evaluated. Enhanced antitubercular tests were performed toward 11 multi-resistant and cross-resistant strains of *M. tuberculosis*. Two compounds demonstrated excellent antitubercular activity and metabolic stability. In addition, their *in vitro* antibacterial activity toward 4 fungi and pathogenic bacteria was tested. One compound can be outlined as broad-spectrum antibiotic, active against all tested bacteria and fungi. More than 10 compounds showed selective activity toward individual bacteria.
- Small series of 6 new nitrofuranoyl amides was synthesized and 3 of compounds demonstrated excellent antitubercular activity. *In vitro* mutagenesis was performed on *M. tuberculosis* reference strain H37Rv subcultures, grown under increasing concentrations of one selected compound. Six mutations were identified in 6 genes.

Development of new compounds with antiviral activity:

- Few series of new analogues of diarylether MDL-860 were synthesized (137 compounds). All compounds were tested toward enteroviruses: Coxsackie 1 and 3, Poliovirus. Some compounds were evaluated toward human corona virus OC43, herpes Simplex Virus Type 1 and Human adenovirus C serotype 5. More than 10 compound can be outlined as very active and non-toxic *in vitro* (selectivity indexes over 97). Most active compounds were selected for *in vivo* experiments, showing high percentage of survived animals (mice). In depth QSAR analysis of synthesized compounds was performed, in order to understand further possible variations in their molecules toward more active analogues.

5.2. Contributions

- New subclass analogues of classical antitubercular drug ethambutol was synthesized. Some of these analogues demonstrated higher activity and lower cytotoxicity, than ethambutol.

- New class of antitubercular drug-like molecules, bearing fenchane skeleton was synthesized.

- New class of antitubercular drug-like molecules, bearing camphane skeleton was synthesized, showing strong antitubercular and antibacterial activity.

- New drug-like nitrofuranoyl compounds were synthesized and their possible mechanism of strong antitubercular activity was investigated by using of *in vitr*o provoked mutagenesis.

- Different new analogues of known diarylether MDL-860 were synthesized. Many of them demonstrated stronger activity toward 6 enteroviruses. Mechanism of action for MDL-860 was revealed.

- Large number of promising bioactive compounds (so called “hit compounds) among above mentioned groups were found. They are suitable for further drug development in next preclinical phases.

6. APPENDIX

6.1. List of publications selected for this thesis (first/corresponding author is underlined)

NOTE: Selected publications in this list were used only for this concourse!

Development of new compounds with antitubercular activity:

1. Mokrousov, I., Slavchev, I., Solovieva, N., Dogonadze, M., Vyazovaya, A., Valcheva, V., Masharsky, A., Belopolskaya, O., Dimitrov, S., Zhuravlev, V., Portugal, I., Perdigão, J., Dobrikov, G. M. Molecular insight into *Mycobacterium tuberculosis* resistance to nitrofuranyl amides gained through metagenomics-like analysis of spontaneous mutants. *Pharmaceuticals*, **2022**, *15*, 1136.
   Q1, IF: 5.215, first/corresponding author, no citations

2. Schröder, M., Petrova, M., Vlahova, Z., Dobrikov, G. M., Slavchev, I., Pasheva, E., Ugrinova, I. *In vitro* anticancer activity of two ferrocene-containing camphor sulfonamides as promising agents against lung cancer cells. *Biomedicines*, **2022**, *10*, 1353.
   Q1, IF: 4.757, 1 citation

3. Slavchev, I., Mitrev, Y., Shivachev, B., Valcheva, V., Dogonadze, M., Solovieva, N., Vyazovaya, A., Mokrousov, I., Link, W., Jiménez, L., Cautain, B., Mackenzie, T. A., Portugal, I., Lopes, F., Capela, R., Perdigão, J., Dobrikov, G. M. Synthesis, characterization and complex evaluation of antibacterial activity and cytotoxicity of new arylmethylidene ketones and pyrimidines with camphane skeletons. *ChemistrySelect*, **2022**, *7*, e202201339.
   Q2, IF: 2.307, first/corresponding author, no citations

Development of new compounds with antiviral activity:

4. Stoyanova, A.; Nikolova, I.; Pürstinger, G.; Dobrikov, G.; Dimitrov, V.; Philipov, S.; Galabov, A. S. Anti-enteroviral triple combination of viral replication inhibitors: activity against coxsackievirus B1 neuroinfection in mice. *Antiviral Chemistry and Chemotherapy*, **2015**, *24*, 136.
   Q2, IF: 1.89, 4 citations

5. Dobrikov, G. M., Slavchev, I., Nikolova, I., Stoyanova, A., Nikolova, N., Mukova, L., Nikolova, R., Shivachev, B., Galabov, A. S. Synthesis and anti-enterovirus activity of new analogues of MDL-860. *Bioorganic & Medicinal Chemistry Letters*, **2017**, *27*, 4540.
   Q2, IF: 2.454, first/corresponding author, 4 citations

6. Arita, M., Dobrikov, G., Pürstinger, G., Galabov, A.S. Allosteric regulation of Phosphatidylinositol 4 Kinase III Beta by an antipicornavirus compound MDL-860. *ACS Infectious Diseases*, **2017**, *3*, 585.
   Q1, IF: 4.325, 9 citations

7. Nikolova, I., Slavchev, I., Ravutsov, M., Dangalov, M., Nikolova, Y., Zagranyarska, I., Stoyanova, A., Nikolova, N., Mukova, L., Grozdanov, P., Nikolova, R., Shivachev, B., Kuz'min, V. E., Ognichenko, L. N., Galabov, A. S., Dobrikov, G. M. Anti-enteroviral activity of new MDL-860 analogues: Synthesis, *in vitro/in vivo* studies and QSAR analysis. *Bioorganic Chemistry*, **2019**, *85*, 487.
   Q1, IF: 4.831, first/corresponding author, 6 citations

8. Nikolova, I., Slavchev, I., Zagranyarska, I., Nikolova, N., Vilhelmova, N., Stoyanova, A., Grozdanov, P., Mukova, L., Galabov, A.S., Lessigiarska, I., Tsakovska, I., Dobrikov, G.M. Synthesis and QSAR analysis of diaryl ethers and their analogues as potential antiviral agents. *ChemistrySelect*, **2022**, *7*, e202203088.
   Q2, IF: 2.307, first/corresponding author, no citations

Total number of selected papers for this thesis: 8 (4 Q1 and 4 Q2)
Total IF of selected papers: 28.086
Average IF of selected papers: 3.51
In 5 out of 8 papers Georgi Dobrikov is first/corresponding author (2 Q1 and 3 Q2)
Total number of citations (according SONIX, citations for PhD excluded): 303

6.2. Participation in conferences

1. Violeta Valcheva, Georgi M. Dobrikov. In vitro antimycobacterial activity of series new potent (R)-2-aminobutanol derivatives. 4th Congress of European Microbiologists, 26.06.2011 - 30.06.2011, Geneva, Switzerland (poster).
2. Violeta Valcheva, Georgi M. Dobrikov. In vitro antimycobacterial activity of new potent (R)-2-aminobutanol derived acyl thioureas. 43rd Union World Conference on Lung Health, 13.11.2012 - 17.11.2012, Kuala Lumpur, Malaysia (poster).
3. Adelina Stoyanova, Ivanka Nikolova, Gerhard Puerstinger, Georgi M. Dobrikov, Vladimir Dimitrov, Stefan Philipov, Angel S. Galabov. Effect of the anti-enteroviral combination of Pleconaril, MDL-860 and Oxoglaucine applied in consecutive alternating administration (CAA) course in Coxsackievirus B1 neuroinfection in mice. 28th International Conference on Antiviral Research, 11.05.2015 - 15.05.2015, Rome, Italy (poster).
4. Georgi M. Dobrikov, Vladimir Dimitrov, Yana Nikolova, Ivailo Slavchev, Zhanina Petkova. Antimycobacterial activity of small molecules generated by synthetic transformations of natural products. COST Action CM1407 - Challenging organic syntheses inspired by nature - from natural products chemistry to drug discovery, 05.10.2015 - 06.10.2015, Rome, Italy (poster).
5. Georgi M. Dobrikov, Ivailo Slavchev, Milka Mileva, Ivanka Nikolova, Almira Georgieva, Elina Tzvetanova. Antiviral and antioxydant activity of new substituted 2-cyano-4-nitro benzenes. 2nd International conference on natural products utilization: from plants to pharmacy shelf, Plovdiv, Bulgaria, 14.10.2015 - 17.10.2015 (poster).
6. Ivailo Slavchev, Georgi M. Dobrikov, Vladimir Dimitrov, Violeta Valcheva, Iva Ugrinova, Evdokia Pasheva. Antimycobacterial activity generated by the amide coupling of (-)-fenchone derived aminoalcohol with cinnamic acids and analogues. 2nd International conference on natural products utilization: from plants to pharmacy shelf, Plovdiv, Bulgaria, 14.10.2015 - 17.10.2015 (poster).
7. Yana Nikolova, Georgi M. Dobrikov, Vladimir Dimitrov, Violeta Valcheva, Iva Ugrinova, Evdokia Pasheva. Efficient synthesis of enantiopure antituberculosis candidates derived from (-)-fenchone. 2nd International conference on natural products utilization: from plants to pharmacy shelf, Plovdiv, Bulgaria, 14.10.2015 - 17.10.2015 (poster).
8. V. Jordanova, D. Borisova, Ts. Paunova-Krasteva, Georgi M. Dobrikov, Yana Nikolova, Stoyanka Stoitsova. Anti-biofilm activity of (-)-fenchone and synthetic derivatives. 2nd International conference on natural products utilization: from plants to pharmacy shelf, Plovdiv, Bulgaria, 14.10.2015 - 17.10.2015 (poster).
9. Adelina Stoyanova, Georgi M. Dobrikov, Vladimir Dimitrov, Stefan Philipov, Ivanka Nikolova, Gerhard Puerstinger, Angel S. Galabov. Effect of a triple combination of Pleconaril, MDL-860 and Oxoglaucine administered by consecutive alternating treatment scheme against Coxsackievirus B1 neuroinfection in mice. 9th Balkan Congress of Microbiology (Microbiologia Balkanica 2015), 22.10.2015 - 24.10.2015, Thessaloniki, Greece (report).
10. Violeta Ruseva, Georgi Dobrikov. The urgency of effective antitubercular drug development – new promising structures derived from natural terpenoids. 17th International Congress on Infectious Diseases, 02.03.2016 - 05.03.2016, Hyderabad, India (poster).
11. Adelina Stoyanova, Ivanka Nikolova, Angel Galabov, Gerhard Pürstinger, Georgi Dobrikov, Vladimir Dimitrov, Stefan Philipov. Triple combination of viral replication inhibitors against Coxsackievirus B1 neuroinfection in mice. 29th International Conference on Antiviral Research, 17.04.2016 - 21.04.2016, La Jolla, USA (poster).
12. Yana Nikolova, Georgi M. Dobrikov, Violeta Valcheva, Iva Ugrinova, Evdokia Pasheva, Vladimir Dimitrov. Antimycobacterial activity of small molecules generated by synthetic transformations of (R)-2-amino-1-butanol and (1S)-(-)-fenchone. 6th European Workshop in Drug Synthesis, 15.05.2016 - 19.05.2016, Siena, Italy (poster).
13. Георги Добриков, Мартин Равуцов, Ивайло Славчев, Яна Николова, Иванка Николова, Аделина Стоянова, Ангел Гълъбов. Синтез на нови диарилетери с анти-ентеровирусна активност. 4th National Congress of Virology with International Participation, Days of Virology in Bulgaria, 18.05.2016 - 20.05.2016, Sofia, Bulgaria (report).
14. Иванка Николова, Аделина Стоянова, Надя Николова, Петър Грозданов, Георги Добриков, Ангел Гълъбов. Скрининг за анти-ентеровирусна активност на новосинтезирани диарил етери. 4th National Congress of Virology with International Participation, Days of Virology in Bulgaria, 18.05.2016 - 20.05.2016, Sofia, Bulgaria (report).

15. Ivanka Nikolova, Adelina Stoyanova, Nadya Nikolova, Lucia Mukova, Petar Grozdanov, Georgi Dobrikov, Angel S. Galabov. Antiviral activity of series of derivatives of MDL-860 against enteroviruses. Power of Viruses, 16.05.2018 - 18.05.2018, Poreč, Croatia (poster).
16. Adelina Stoyanova, Lucia Mukova, Ivanka Nikolova, Nadya Nikolova-Velislavova, Georgi Dobrikov, Stefan Filipov, Angel Galabov. Combined effects of newly synthesized diaryl ethers and some enteroviral inhibitors against Coxsackievirus B1. 6th Congress of the Microbiologists of Macedonia with international participation, 01.06.2018 - 02.06.2018, Ohrid, Macedonia (poster).
17. Ivanka Nikolova, Adelina Stoyanova, Nadia Nikolova, Lucia Mukova, Peter Grozdanov, Georgi Dobrikov, Angel Galabov. Antienteroviral activity of newly synthesized diaryl ethers. 14th Congress of Microbiologists in Bulgaria with International Participation, 10.10.2018 - 13.10.2018, Hisarya, Bulgaria (report).
18. Yana Nikolova, Georgi M. Dobrikov, Violeta Valcheva, Iva Ugrinova, Evdokia Pasheva, Vladimir Dimitrov. Efficient synthesis of small molecules derived from (−)-fenchone and evaluation of their antimycobacterial activity. 3rd Training School of COST Action CM1407 - Computational modeling tools in drug discovery with natural products, 10.12.2018 - 12.12.2018, Tenerife, Spain (poster).
19. Yana Nikolova, Martin Ravutsov, Ivaylo Slavchev, Irena Zagranyarska, Miroslav Dangalov, Ivanka Nikolova, Adelina Stoyanova, Nadya Nikolova, Lucia Mukova, Petar Grozdanov, Rosica Nikolova, Boris Shivachev, Viktor E. Kuz'min, Liudmila N. Ognichenko, Angel S. Galabov, Georgi M. Dobrikov. Novel derivatives of the anti-enteroviral agent MDL-860: Synthesis, in vitro/in vivo studies and QSAR analysis. 10th Jubilee National Conference on Chemistry, 26.09.2019 - 28.09.2019, Sofia, Bulgaria (poster).
20. Ana Vyazovaya, Ivaylo Slavchev, Violeta Valcheva, Marine Dogonadze, Maya Zaharieva, Natalia Solovieva, Olga Narvskaya, Viacheslav Zhuravlev, Georgi Dobrikov. Synthesis and evaluation of new compounds efficient against mycobacterium tuberculosis isolates circulating in high-burden country, Russian federation. 51st World Conference on Lung Health of the International Union Against Tuberculosis and Lung Disease (The Union), 20.10.2020 - 24.10.2020, Virtual Event, (poster).
21. Anna Vyazovaya, Ivaylo Slavchev, Yana Nikolova, Marine Dogonadze, Violeta Valcheva, Maya Zaharieva, Natalia Solovieva, Daria Starkova, Neliya Vasilieva, Igor Mokrousov, Viacheslav Zhuravlev, Georgi Dobrikov. Screening of compounds containing aminobutanoland camphane moieties against Mycobacterium tuberculosis clinical isolates of different genotypes. 3rd Asian African Congress of Mycobacteriology, 27.01.2021 - 29.01.2021, Virtual Event, (report).
22. Violeta Valcheva, Anna Vyazovaya, Georgi Dobrikov, Ivaylo Slavchev, Joao Perdigão. In vitro antimycobacterial activity of new synthetic (+)-camphor derivatives. 52nd Union World Conference on Lung Health, 19.10.2021 - 22.10.2021, Virtual Event, (report).
23. Irena Zagranyarska, Ivaylo Slavchev, Ivanka Nikolova, Petar Grozdanov, Iglika Lessigiarska, Ivanka Tsakovska, Georgi M. Dobrikov. Synthesis and QSAR analysis of diarylethers and their analogues as potential antiviral agents. National conference with international participation “Innovations in drug molecules”, 19.07.2022 - 22.07.2022, Hisarya Spa Resort, Bulgaria (poster).
24. Zhanina Petkova, Ivaylo Slavchev, Yavor Mitrev, Violeta Valcheva, Georgi M. Dobrikov. Synthesis, characterization and complex evaluation of antibacterial activity and cytotoxicity of new arylmethylidene ketones and pyrimidines with camphane skeletons. National conference with international participation “Innovations in drug molecules”, 19.07.2022 - 22.07.2022, Hisarya Spa Resort, Bulgaria (poster).
25. Irena Zagranyarska, Ivaylo Slavchev, Ivanka Nikolova, Petar Grozdanov, Iglika Lessigiarska, Ivanka Tsakovska, Georgi M. Dobrikov. Diarylethers and their analogues as potential antiviral agents – synthesis and computational studies. 8th International Black Sea coastline countries scientific research conference, 29.08.2022 - 30.08.2022, Sofia, Bulgaria (poster).
26. Zhanina Petkova, Ivaylo Slavchev, Yavor Mitrev, Violeta Valcheva, Georgi M. Dobrikov. Synthesis of new arylidenecamphors and pyrimidines with camphane skeleton – structural characterization and complex evaluation of their antibacterial activity and cytotoxicity. 8th International Black Sea coastline countries scientific research conference, 29.08.2022 - 30.08.2022, Sofia, Bulgaria (poster).

6.3. Projects related to the thesis' topic

1. Фонд Научни изследвания № ДФНИ Б02/11. Синтез и анти-ентеровирусна активност на нови диарил етери и техни комплекси с циклодекстрини. 2014-2018 г. 81900 лв. за базовата организация. Ръководител Георги Добриков.
2. COST Action CM1407 - Challenging organic syntheses inspired by nature - from natural products chemistry to drug discovery. 2015-2019 г. 12800 лв. за базовата организация.
3. Фонд Научни изследвания № ДКОСТ 01/4. Използване на природните продукти (+)-камфор и (-)-фенхон като източници за получаване на биологично активни съединения чрез синтетични трансформации. 2017-2019 г. 40 000 лв. за базовата организация. Ръководител Георги Добриков.
4. Russian Science Foundation № RNF-19-15-00028. Development of new efficient compounds against drug resistant Mycobacterium tuberculosis taking into account the population structure of the pathogen. 2019-2021 г. 72100 лв. за базовата организация.
5. Фонд Научни изследвания № КП-06-Н31/7. Нови производни на 2-циано-4-нитробензена и негови аналози с обещаваща антивирусна активност. 2019-2024 г. 60000 лв. за базовата организация. Ръководител Георги Добриков.
6. Фонд Научни изследвания № КП-06-Н39/7. Откриване на нови лекарствени кандидати чрез синтетични модификации на природна шикимова киселина. 2019-2024 г. 60000 лв. за базовата организация.
7. Фонд Научни изследвания КП-06-Китай/5. Откриване на нови ковалентни инхибитори на ензима Р/4КВ с потенциален антивирусен ефект. 2020-2023 г. 40000 лв. за базовата организация. Ръководител Георги Добриков.
8. Operational Program "Science and Education for Smart Growth" 2014-2020, co-financed by European Union through the European Structural and Investment Funds, Grant BG05M2OP001-1.002-0012.