UNIVERZITA KARLOVA

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Katedra Farmaceutické chemie a Farmaceutické analýzy

DERIVÁTY PYRAZINU JAKO POTENCIÁLNÍ ANTITUBERKULOTIKA

(příprava a studium biologických vlastností)

Disertační práce

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Hradec Králové Září 2016

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Mgr. Ondřej JANĎOUREK

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OBSAH

1. SEZNAM POUŽITÝCH ZKRATEK

ACP	acyl carrier protein
AMB	amfotericin B
ATCC	American Type Culture Collection
ATP	adenosintrifosfát
ATR	Attenuated Total Reflectance (metoda měření infračerveného spektra)
BAC	bacitracin
CCM	Česká kolekce mikroorganismů
CNCTC	Česká národní sbírka typových kultur
CPX	ciprofloxacin
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DMSO	dimethylsulfoxid
DNA	deoxyribonukleová kyselina
EMA	European Medicines Agency
ETH	ethambutol
ESBL	Extended-Spectrum β-Lactamases (podtyp mikroorganismů s rezistencí k širokému spektru antibiotik)
FAS I/II	Fatty Acid Synthase I/II (enzymatický mykobakteriální komplex zapojený do syntézy mykolových kyselin)
FDA	Food and Drug Administration, U.S.A.
FLU	flukonazol
HepG2	buněčné linie odvozené z lidského hepatocelulárního karcinomu používané k testování cytotoxicity
HIV	Human Immunodeficiency Virus
IC_{50}	koncentrace způsobující 50% pokles aktivity vůči kontrole
INH	isoniazid
M. tbc	*Mycobacterium tuberculosis*
MDR-TB	Multi-drug resistant tuberculosis
MIC	minimální inhibiční koncentrace
MRSA	methicilin rezistentní *Staphylococcus aureus*
MW	mikrovlny
NAD^+	nikotinamid adenin dinukleotid (oxidovaná forma)

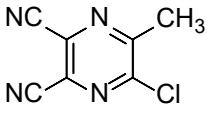
NADH	nikotinamid adenin dinukleotid (redukovaná forma)
NADPH	nikotinamid adenin dinukleotid fosfát (redukovaná forma)
NEOM	neomycin
NYS	nystatin
NIAID	National Institute of Allergy and Infectious Diseases
NMR	nukleární magnetická rezonance
OADC	Oleic acid, Albumin, Dextrose, Catalase (růstový suplement pro médium Middlebrook 7H9)
PEN	penicilin G
PHE	fenoxymethylpenicilin
POA	pyrazinkarboxylová kyselina
PZA	pyrazinamid
RFM	rifampicin
RNA	ribonukleová kyselina
RPMI	Roswell Park Memorial Institute (růstové médium pro antifungální testování)
SAR	Structure-Activity Relationships (vztah mezi strukturou a účinkem)
SI	index selektivity, parametr používaný pro určení bezpečnosti léčiva vypočítaný na základě poměru cytotoxicity a účinné koncentrace
TAACF	Tuberculosis Antimicrobial Acquisition and Coordinating Facility (program U.S.A. zaměřený na podporu výzkumu nových antituberkulotik)
TBC	tuberkulóza
TEA	triethylamin
THF	tetrahydrofuran
TLC	tenkovrstvá chromatografie
VOR	vorikonazol
WHO	Světová zdravotnická organizace
XDR-TB	Extensively drug-resistant tuberculosis

2. FORMULACE PROBLEMATIKY A ZPŮSOB ŘEŠENÍ

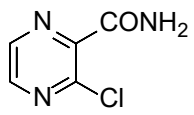
Tato práce navazuje na výzkumný záměr katedry Farmaceutické chemie a Farmaceutické analýzy a především pak na výzkum vědecké skupiny prof. Doležala, který se zabývá syntézou potenciálních antituberkuloticky účinných látek.

Cílem práce je tedy příprava nových, dosud nepopsaných sloučenin ze skupiny derivátů pyrazinamidu. Tyto sloučeniny budou popsány pomocí fyzikálně-chemických vlastností. Všechny látky budou otestovány na potenciální biologickou aktivitu, která bude zahrnovat účinky antimykobakteriální, antibakteriální, antifungální, antivirotické a u části látek i herbicidní. U podobných strukturních typů, vykazujících biologickou aktivitu, budou diskutovány vztahy mezi strukturou a aktivitou těchto sloučenin.

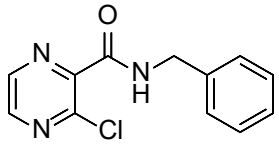
Tato práce je založena na třech výchozích látkách – 5-chlor-6-methylpyrazin-2,3-dikarbonitrilu (**I**), 3-chlorpyrazin-2-karboxamidu (**II**) a *N*-benzyl-3-chlorpyrazin-2-karboxamidu (**III**). Tyto sloučeniny byly připraveny standardními postupy popsanými v literatuře. Budou podrobeny aminodehalogenační reakci s alifatickými, alicyklickými a aromatickými aminy a dále pak s různě substituovanými benzylaminy a aromatickými fenylhydraziny. Všechny reakce budou provedeny pomocí mikrovlnného reaktoru s fokusovaným polem, jehož použití je pro tuto práci zásadní, a bude možné porovnat výsledky s metodami konvenčního ohřevu. Dle získaných výsledků biologických hodnocení budou navrženy strukturní obměny.



I



II



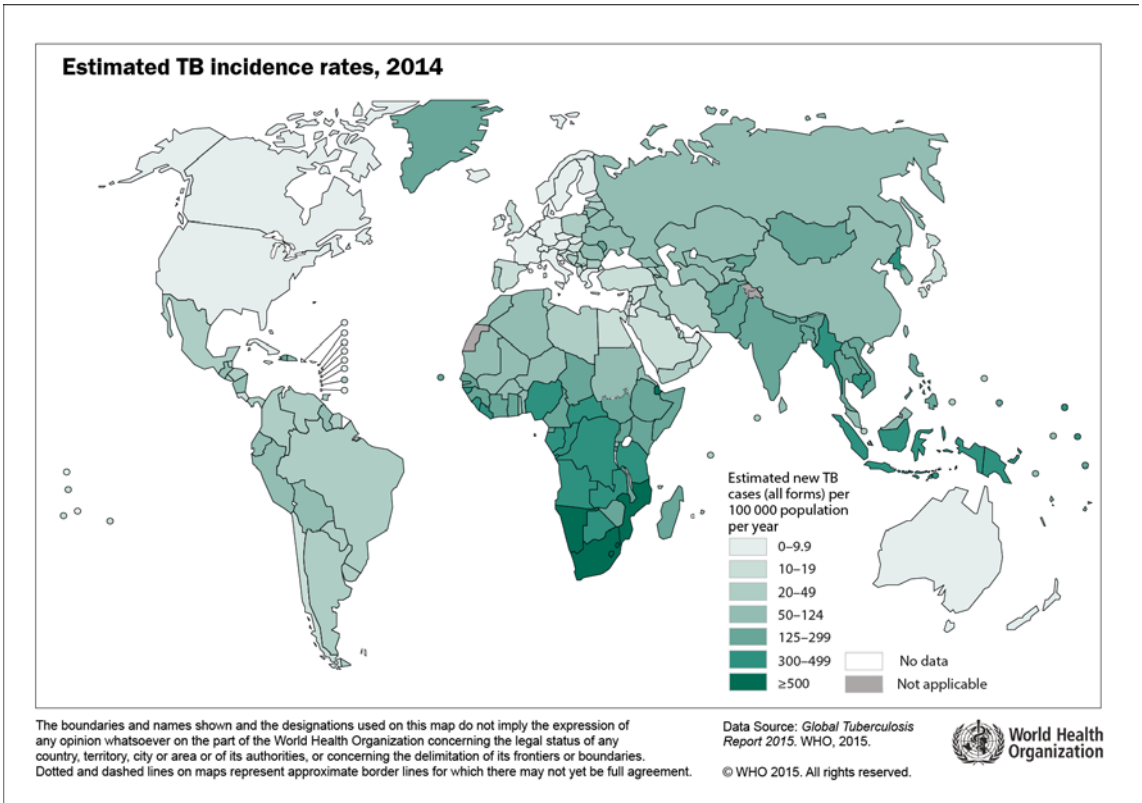
III

3. PŘEHLED SOUČASNÉHO STAVU ŘEŠENÉ PROBLEMATIKY

3.1. AKTUÁLNÍ EPIDEMIOLOGICKÁ SITUACE

Tuberkulóza (TBC) je stále velice významným epidemiologickým problémem tohoto století. Svět se neustále potýká s touto hrozbou, která zaujímá druhé místo v žebříčku úmrtnosti v kategorii infekčních chorob. Tato skutečnost v kombinaci se stále rostoucí rezistencí mikroorganismů k současné léčbě vede mnohé výzkumné skupiny k pokusům o přípravu nových, antimikrobiálně účinných sloučenin.

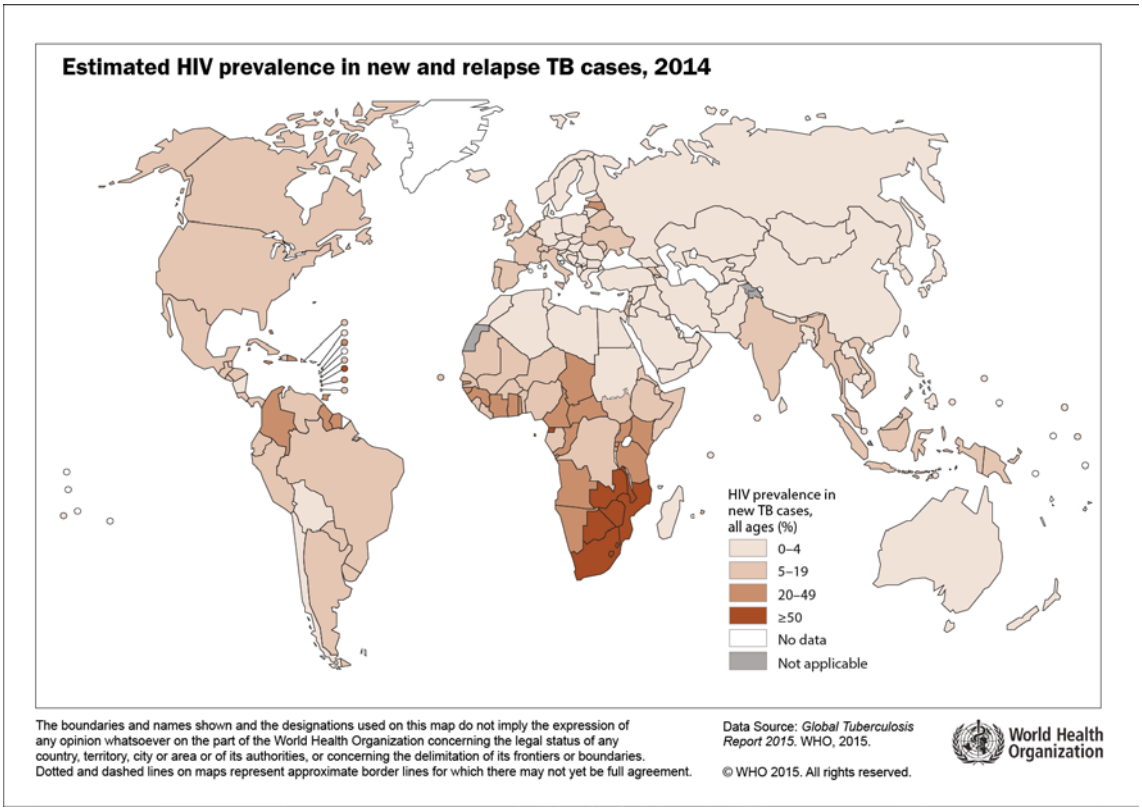
Ačkoliv byl v průběhu minulého desetiletí zaznamenán jistý pokrok v terapii a prevenci tuberkulózy, tak tento trend není a nemusí být samozřejmostí. Celková incidence nových případů tohoto onemocnění, které způsobují bakterie *Mycobacterium tuberculosis*, od roku 2006 klesá. Zároveň úmrtnost klesla o 47 % v rámci sledovaného období 1990 – 2014. Milníkem v této problematice bylo ustavení Millenium Development Goals v roce 2000, kterými Světová zdravotnická organizace (WHO) nastavila jistou hranici v hlášení výskytu, prevenci a léčbě TBC, kterou je třeba dosáhnout všemi možnými prostředky. Díky tomuto postupu bylo v období let 2000 až 2014 zachráněno 43 milionů životů. Ostatní čísla ale již tak povzbudivá nejsou. V roce 2014 onemocnělo dle zprávy WHO manifestní tuberkulózou 9,6 milionů lidí a



*Obrázek **1** – Incidence nových případů TBC ve světě za rok 2014 – převzato a publikováno se svolením WHO*[1]

1,5 milionů z nich zemřelo v důsledku této infekce (incidence ve světě znázorněna na Obr. **1**). Z tohoto počtu tvoří až 10 % dětská tuberkulóza.[1]

Dalším problémem, který přispívá ke snadnějšímu šíření této nemoci, je celosvětová pandemie HIV. Tento trend je podpořen faktem, že pacienti infikovaní virem HIV jsou imunokompromitovaní a je tedy snadnější nasednutí sekundární infekce TBC (celosvětová prevalence je znázorněna na Obr. **2**). Z celkového počtu 9,6 milionů nových případů v roce 2014 bylo 1,2 milionů ze skupiny pacientů HIV pozitivních. Smrt si vybrala svou daň u 400 tisíc z nich.



*Obrázek **2** – Prevalence HIV infekce u nově diagnostikované TBC v roce 2014 – převzato a publikováno se svolením WHO*[1]

Možná ještě závažnějším problémem spojeným s tuberkulózou je celosvětový výskyt rezistentních kmenů mykobakterií, které znamenají významný epidemiologický problém, se kterým je nutné bojovat. V roce 2014 byla u 480 tisíc pacientů diagnostikovaná takzvaná multilékově rezistentní TBC (MDR-TB; Multi-drug resistant tuberculosis) a 190 tisíc z nich na tuberkulózu zemřelo. Tento typ rezistence je charakterizován odolností minimálně vůči léčivům tzv. první linie isoniazidu a rifampicinu, příp. vůči dalším z této skupiny, do které se ještě řadí pyrazinamid, streptomycin a ethambutol. Největším zdrojem těchto kmenů jsou oblasti Jižní Afriky a Jihovýchodní Asie. Pokud se tento typ rezistence rozšíří i o léčiva 2. linie

(fluorochinolon a jeden z intravenózně podávaných léčivých přípravků např. amikacin, kanamycin), pak se jedná o extenzivně rezistentní kmeny *M. tuberculosis* (XDR-TB; Extensively drug-resistant tuberculosis). Tyto kmeny jsou většinou detekovány v rámci určení rezistence MDR-TB a v roce 2014 tvořily asi 9,7 % z celkového počtu rezistentních kmenů. V roce 2012 byl poprvé zaznamenán kmen, který vykázal totální rezistenci ke všem známým a používaným léčivům. Tento impulz částečně urychlil vývoj některých nadějných sloučenin, z nichž se ve zrychleném registračním řízení dostaly do praxe dvě molekuly – bedachilin a delamanid. Tím se termín totální rezistence dostal opět jenom do teoretické roviny.[2,3]

V České republice je prozatím vývoj poněkud příznivější (viz. Tabulka **1**). Od roku 1989 počet nově hlášených případů meziročně neustále klesá a v roce 2014 dosáhl počtu 514 případů s incidencí 4,9 nemocných na 100 tisíc obyvatel. Toto číslo je o něco málo vyšší než v roce 2013, ale rozdíl je prakticky zanedbatelný. Z celkového počtu zaznamenaných onemocnění tvoří 18,7 % cizinců s největším podílem pacientů z Ukrajiny a Vietnamu. Tyto dvě země se dají považovat z epidemiologického pohledu v ČR za rezervoáry onemocnění TBC. Z pohledu regionálního jsou nejvíce zasaženy oblasti Středních Čech, Vysočiny a Severní Moravy.[4] Tento trend by se však v budoucnu mohl změnit a to díky vyhláškám, které vstoupily v platnost v roce 2009 a 2010. První z těchto vyhlášek zrušila pravidelné přeočkování dětí v 11 letech, u kterých se projevila negativní tuberkulinová reakce. Druhá pak zrušila povinné plošné očkování všech novorozenců s výjimkou dětí, které splňují kritéria pro naočkování (aktivní TBC v rodině, pobyt v zemi s vysokým výskytem TBC, kontakt dítěte s nemocným).

*Tabulka **1** – Počet onemocnění TBC v ČR v průběhu let 1989-2014[4]*

Rok	Počet onemocnění	Na 100 000 obyv.	Cizinci
1989	1905	18,4	Nezjišťováno
1999	1631	15,9	Nezjišťováno
2009	710	6,8	136
2010	680	6,5	117
2011	609	5,8	112
2012	611	5,8	105
2013	502	4,8	79
2014	514	4,9	96

3.2. TUBERKULÓZA A JEJÍ LÉČBA

3.2.1. Tuberkulóza

Jak již bylo zmíněno, jde o infekční onemocnění, které je způsobeno mikroorganismy rodu *Mycobacterium*, takzvaným *Mycobacterium tuberculosis* (*M. tbc*) komplexem. Do tohoto komplexu se řadí kromě hlavního původce *Mycobacterium tuberculosis* také *M. bovis*, *M. africanum*, *M. microti* a *M. canetti*. V roce 2010 byl objeven nový druh bakterie, který se do tohoto komplexu zařadil také, a je jím *Mycobacterium mungi*.[5]

Primárně se jedná o plicní onemocnění, ale v jistých případech může dojít k napadení ostatních orgánů. Poté se označuje jako tzv. mimoplicní TBC.

Tuberkulóza je možná tak stará jako lidstvo samo. Díky moderním technologiím se podařilo prokázat tuberkulózní příznaky na lidských ostatcích z doby asi 7000-9000 př. n. l.[6] Další takto „poznamenané“ ostatky byly nalezeny na území starověkého Egypta při zkoumání mumií z období 3. tisíciletí př. n. l.[7] Ovšem označení tuberkulóza se datuje až do roku 1839, kdy ho poprvé použil Johann Lukas Schönlein. Do té doby se toto onemocnění označovalo různě, nejčastěji podle toho, jak nemocný vypadal – úbytě, souchotiny.

Toto onemocnění má několik fází. První kontakt (primární TBC) je ve většině případů zprostředkován formou kapének. Pro vznik nákazy stačí i pouhých pět živých mikrobů. Uvolňování a šíření infekčního agens nastává především u osob s otevřenou formou aktivní tuberkulózy. Po vniknutí do organismu dochází v plicích postiženého k fagocytóze bakterií makrofágy. Uvnitř makrofágů bohužel nedojde k usmrcení mykobakterie, ale naopak převládá růst a pomnožení. Takto napadené buňky jsou lymfatickými cestami roznášeny do mízních uzlin. Po určité době (10-14 dní) se vytvářejí specifické protilátky a aktivuje se imunitní systém. Ložiska infekce v plicích jsou ohraničována a oddělována od zdravé tkáně buňkami imunitního systému. Tyto uzlíky nebo také tuberkuly jsou naplněné nekrotickou tkání svým vzhledem připomínající sýr – dochází ke kaseifikaci (zesýrovatění). Poté dojde ke spontánnímu zhojení a vyléčení, případně přeživší mykobakterie upraví svou metabolickou aktivitu a přecházejí do dormantního stavu. Tato ložiska jsou dále kalcifikována a můžou sloužit i pro rentgenové potvrzení proběhlého onemocnění. Postprimární fáze infekce se může nejčastěji objevit u pacientů vystavených dalšímu zdroji nákazy nebo u imunokompromitovaných pacientů, u kterých se reaktivují kalcifikovaná ložiska. Imunitní odpověď hostitele je prakticky okamžitá a následně nastává fáze rychlejšího

odbourávání granulomů. Proteolytické enzymy, které se na tom podílejí, však mohou narušit i okolní tkáň a umožnit tak vylití obsahu tuberkulů do bronchoalveolárních prostor. Tímto se pacient stává aktivním přenašečem tuberkulózy a je třeba takovéto jedince okamžitě zachytit. Pokud se ložiska nacházejí v blízkosti velkých cév, může dojít i narušení cévní stěny a vylití obsahu granulomu do cévního řečiště. V tomto případě se také často projevuje vykašlávání krve (hemoptýza), což je jeden z typických příznaků TBC. Dále se touto cestou mohou mykobakterie dostat k dalším orgánům a dát tak vzniknout mimoplicní formě tuberkulózy (meningeální, kostní, urinální, apod.).[8-10]

3.2.2. *Mycobacterium tuberculosis*

M. tbc je lidský patogen, který má největší podíl na vzniku onemocnění TBC. Jedná se o nepohyblivou, obligátně aerobní tyčku, která je specifická v mnoha ohledech. Jedním z nich je generační poločas. Doba pro replikaci těchto bakterií je okolo 20 hodin. Oproti běžným patogenům je tento čas až 60x delší. Tento fakt tím pádem znesnadňuje kultivaci a včasnou detekci onemocnění. Typickým jevem je výskyt tzv. dormantních neboli spících forem mykobakterií. Tyto bakterie jsou schopné upravit svou metabolickou aktivitu na takovou úroveň, že přežívají v nehostinných podmínkách hostitelova organismu po dlouhá desetiletí. Stejně tak jsou velmi těžko zasažitelné terapeuticky. Další zvláštností je složení buněčné stěny. Ta je velice bohatá na lipidy. I složení těchto lipidů je dost specifické a u jiných mikroorganismů se v této podobě nevyskytuje. Funkcí plní tato stěna hned několik. Prvním z nich je faktor virulence tzv. Cord-faktor. Což je glykolipid dimykolát trehalosy, který je schopný maskovat mykobakterie před obrannými mechanismy hostitelského organismu.[11] Další funkcí této stěny je snížená prostupnost pro množství látek, ke kterým samozřejmě patří i antiinfektiva. V neposlední řadě slouží také jako ochrana vůči vnějšímu prostředí, ve kterém je tak mykobakteriím umožněno přežít.[12] Bližším zkoumáním buněčné stěny bylo zjištěno, že se skládá ze zesíťovaného peptidoglykanu, na který se váží arabinogalaktany, jež jsou esterifikované mykolovými kyselinami. Právě tyto kyseliny jsou typické pro mykobakterie (dále se vyskytují pouze u rodů *Nocardia* a *Corynebacteria*) a skládají se z řetězců o délce až 87 uhlíků. Kromě toho obsahují také β-hydroxy skupinu.[13] Toto složení buněčné stěny nám také snadno umožňuje identifikaci mykobakterií pomocí mikrobiologického barvení. Právě vysoký obsah lipidů neumožňuje klasické Grammovo barvení a je třeba využít metody dle Ziehl-Neelsena, kdy se bakterie barví kyselým karbolfuchsinem, který není možné vymýt ani

kyselým alkoholem (směs 1% kyseliny chlorovodíkové a 70% ethanolu). Buněčná stěna mykobakterií se tak jeví jako ideální cíl pro vývoj nových antituberkulotik.

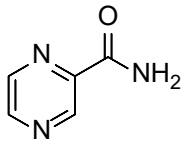
Z hlediska poznání mykobakterií se stal důležitým rok 1882, ve kterém Robert Koch poprvé popsal skutečného původce TBC. Od této doby bylo tedy možné cílit léčbu konkrétně. Dalším průlomovým rokem byl rok 1998, kdy S. T. Cole se svým kolektivem rozluštili kompletní genom *M. tbc* a určili i funkce jednotlivých genů v rámci metabolismu a fyziologie.[14,15] Na základě těchto poznatků mohla a může většina vědeckých skupin cílit svůj výzkum na určitý fyziologický nebo metabolický děj mykobakteriální buňky.

3.2.3. Terapie tuberkulózy

Farmakoterapie tuberkulózy je časově velice náročná. V ideálním případě trvá šest měsíců, v tom horším pak i dvanáct měsíců. Dalším rizikovým faktorem je adherence pacienta k léčbě. Čím déle se léčiva podávají, tím hůře pacienti léčbu snášejí. Dochází k ústupu příznaků a zároveň se objevují nežádoucí účinky podávaných preparátů. V mnoha případech se stává, že je léčba přerušena a hrozí relaps nemoci nebo vznik rezistence k aktuálně podávané kombinaci léčiv. Základní paleta antituberkulotik se dělí na dvě skupiny a dle tohoto rozdělení jsou podávány.

3.2.3.1. Antituberkulotika 1. linie

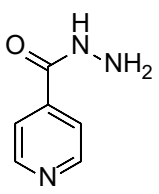
Pyrazinamid



pyrazinamid

Chemicky pyrazin-2-karboxamid, který má nezastupitelné místo při léčbě TBC. Blíže je o něm pojednáno v samostatné kapitole.

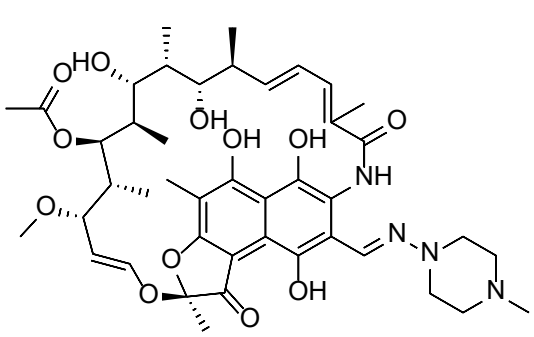
Isoniazid



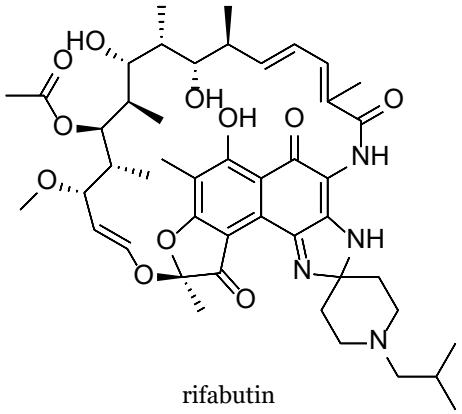
isoniazid

Systematické pojmenování je hydrazid kyseliny isonikotinové, který je analogem nikotinamidu. Mechanismus účinku je dán aktivací samotného isoniazidu pomocí katalasy-peroxidasy KatG.[16] Aktivovaný meziprodukt se váže na NADH a vytváří s ním adukt. Tento intermediát inhibuje enzymové systémy mykobakterií, které jsou závislé na využívání NADH. Nejznámějším z těchto enzymových systémů je Fatty Acid Synthasa II (FAS II). Blokovaným enzymem je enoyl-ACP reduktasa (InhA) a tato blokáda zapříčiňuje zástavu syntézy mykolových kyselin.[17] Mezi další inhibované enzymy patří β-keto-ACP reduktasa a dihydrofolátreduktasa. Rezistence na toto léčivo je dána právě mutací genů kódujících tyto enzymové systémy.

Rifampicin/Rifabutin



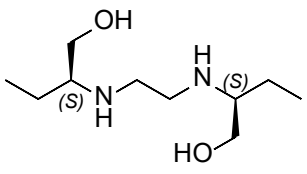
rifampicin



rifabutin

Jedná se o látky ze skupiny ansamycinových antibiotik, jejichž součástí je makrocyklický laktam. Vyznačují se vysokou baktericidní aktivitou vůči všem formám *M. tbc*. Umožňují tak urychlit léčbu tuberkulózy a současně jsou součástí všech základních léčebných režimů. Díky fyzikálním vlastnostem (především lipofilitě) tyto molekuly dobře pronikají přes vysoce lipofilní membrány, jako je buněčná stěna mykobakterií či hematoencefalická bariéra. V mykobakteriu inhibují prokaryotickou DNA dependentní RNA polymerasu, čímž prakticky zastavují syntézu RNA (viz Obr. **4**).[18] Rezistence je opět spojená s mutací genu, který je zodpovědný za syntézu RNA polymerasy.[19]

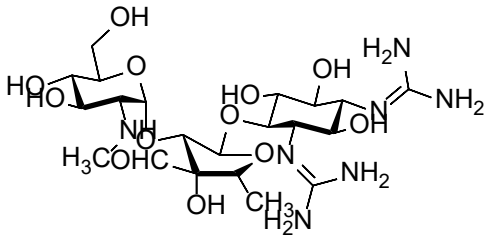
Ethambutol



ethambutol

Aminoalkohol (2*S*)-2-{2-[((2*S*)-1-hydroxybut-2-yl)amino]ethylamino}butan-1-ol s bakteriostatickým efektem je účinný pouze jako *S*,*S*-(+)-izomer. Mechanismus účinku je založen na inhibici arabinosyl transferasy, tedy enzymu zodpovědného za syntézu arabinanu a dalších součástí mykobakteriální stěny (viz Obr. **3**).[20,21] Rezistence vzniká opět mutací genů zodpovědných za proteosyntézu transferas EmbB.[22]

Streptomycin



streptomycin

Řadí se mezi přírodní antibiotika a je produkován bakteriemi *Streptomyces griseus*. Je řazen do skupiny aminoglykosidů a je známý přítomností atypických aminosacharidů. Menší nevýhodou je značná polarita a obsah bazických skupin. To neumožňuje klasické perorální podání a je třeba aplikovat intramuskulárně či intravenózně. Mechanismus účinku je u těchto látek založen na inhibici proteosyntézy blokací ribozomální podjednotky 30S (viz Obr. **3**).[23]

3.2.3.2. Antituberkulotika 2. linie

Do této kategorie spadá široké spektrum látek s různou strukturou a různou cestou podání. Využívají se v případě rezistence na některé z antituberkulotik 1. linie. Patří sem fluorochinolony (ciprofloxacin, ofloxacin, levofloxacin, moxifloxacin), další aminoglykosidy (amikacin, kanamycin), polypeptidová antibiotika (kapreomycin, viomycin, enviomycin), thioamidy (ethionamid, prothionamid) a nakonec kyselina *p*-aminosalicylová, cykloserin, terizidon a linezolid (viz. Tabulka **2**).[24] Mechanismus účinku některých z nich je znázorněn na Obr. **3** a **4**.

*Tabulka **2** – Základní rozdělení antituberkulotik 2. linie*

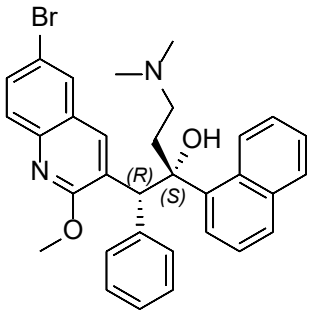
Skupina	Účinné látky	Podání	Mechanismus účinku
Fluorochinolony	ciprofloxacin, ofloxacin, levofloxacin, moxifloxacin	p.o., i.v.	Inhibice ATP dependentní gyrasy (topoisomerasy II)[25]
Aminoglykosidy	amikacin, kanamycin	i.v., i.m.	Inhibice proteosyntézy blokací ribozomální podjednotky 30S[23]

Polypeptidy	kapreomycin, viomycin, enviomycin	i.v., i.m.	Inhibice proteosyntézy vazbou na RNA[26]
Thioamidy	ethionamid, prothionamid	p.o.	Inhibice NADH závislých mechanismů[27,28]
	kys. *p*-aminosalicylová	p.o.	Inhibice syntézy kys. listové[29] či interakce v metabolismu železa[30]
	cykloserin, terizidon	p.o.	Inhibice syntézy peptidoglykanu[31,32]
	linezolid	p.o., i.v.	Inhibice proteosyntézy blokací ribozomální podjednotky 50S[33]

3.2.3.3. Nejnovější antituberkulotika

V posledních letech došlo k překotnému vývoji nových léčiv a z důvodu výskytu totálně rezistentních kmenů *M. tbc* se přistoupilo ke zrychlení klinických testů některých nadějných sloučenin. Avšak i pro tato léčiva platí náročné podmínky uplatňované ve vývoji nových, potenciálně aktivních sloučenin (vyšší účinnost, ideálně nový cíl zásahu, dobrá snášenlivost v rámci jiných terapií, apod.).[34]

Bedachilin

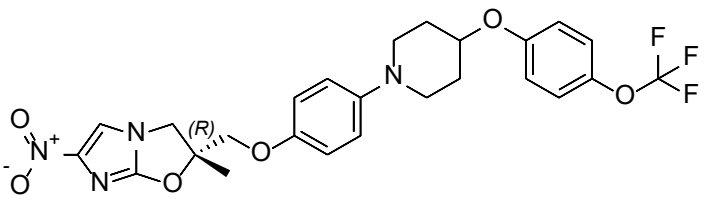


bedachilin

Vývojově označovaná látka TMC207 se chemicky řadí mezi diarylchinoliny a je považována za prvního zástupce nových, vysoce účinných sloučenin, jejichž minimální inhibiční koncentrace je vyjádřena v desítkách až stovkách nanogramů na mililitr. Jde o tuberkulocidní látku, kterou lze použít v monoterapii i kombinované léčbě. Projevila zároveň i dostatečnou bezpečnost.[35,36] Mechanismus účinku je založen na inhibici ATP synthasy (viz Obr. **3**). Následkem toho dochází k ovlivnění energetického metabolismu a smrti mykobakterie. Výhodou je vysoká selektivita k mykobakteriální ATP synthase,

která je oproti lidské formě až 20000krát vyšší. Pravděpodobně největším přínosem je aktivita vůči rezistentním kmenům, která vedla ke zrychlené registraci u FDA a později i u EMA pro použití v terapii MDR-TB.

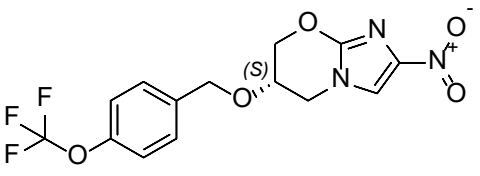
Delamanid



delamanid

OPC-67683, takto je také označován nitroimidazooxazolový derivát, který je v organismu metabolizován na aktivní sloučeniny, které blokují syntézu mykolových kyselin. Dále nastává uvolňování oxidu dusnatého, který způsobuje respirační otravu a smrt bakterie.[37] Minimální inhibiční koncentrace jsou opět velice nízké, dosahující desítek až jednotek nanogramů na mililitr. S výhodou se využívá v kombinaci s antiretrovirovou terapií u pacientů s ko-infekcí HIV, jelikož neovlivňuje jaterní enzymy. Tím je možné využít širokou paletu antivirotik, která často vykazují hepatotoxický účinek stejně jako některá ostatní antituberkulotika, a jejich kombinace tak není z hlediska bezpečnosti možné v praxi využít. Použití delamanidu je prozatím limitováno na pacienty, pro které není možné využít standardní léčebné postupy.

Pretomanid



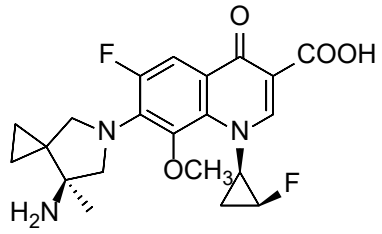
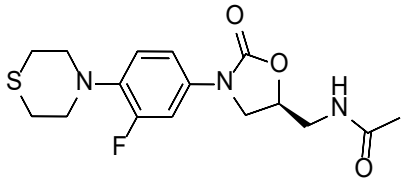
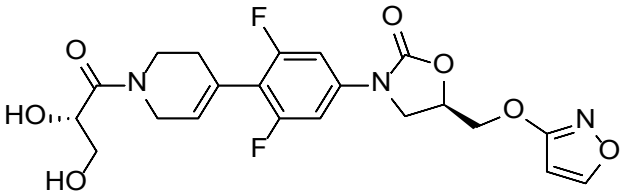
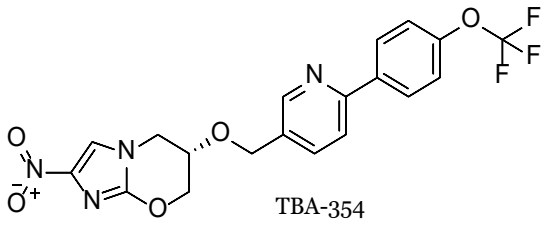
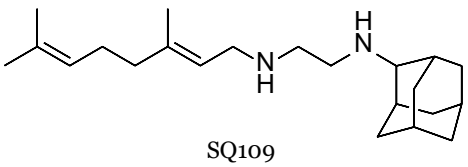
pretomanid

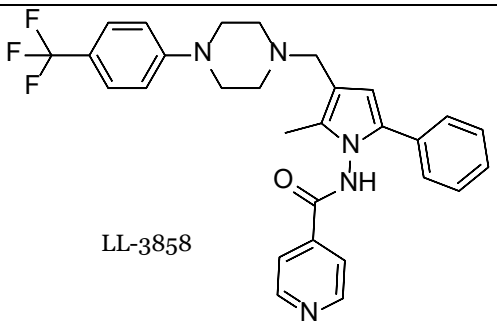
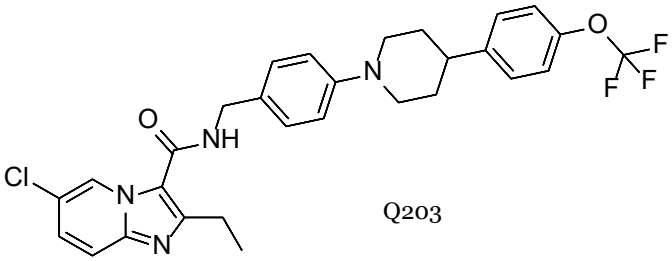
Podobně jako delamanid je tato sloučenina řazena mezi nitroimidazoly, konkrétně nitroimidazooxaziny. Její původní označení, uváděné v literatuře, je PA-824. Má podobný mechanismus účinku jako předchozí sloučenina, a tím je ovlivnění syntézy mykolových kyselin, především keto varianty těchto látek (viz Obr. **4**). Uvolňování oxidu dusnatého zasahuje anaerobní fázi mykobakterií. Tím je zajištěno působení jak na aktivní mykobakterie, tak na dormantní formy.[38] MIC odpovídá přibližně hodnotám isoniazidu, tedy desítky až stovky ng/ml.

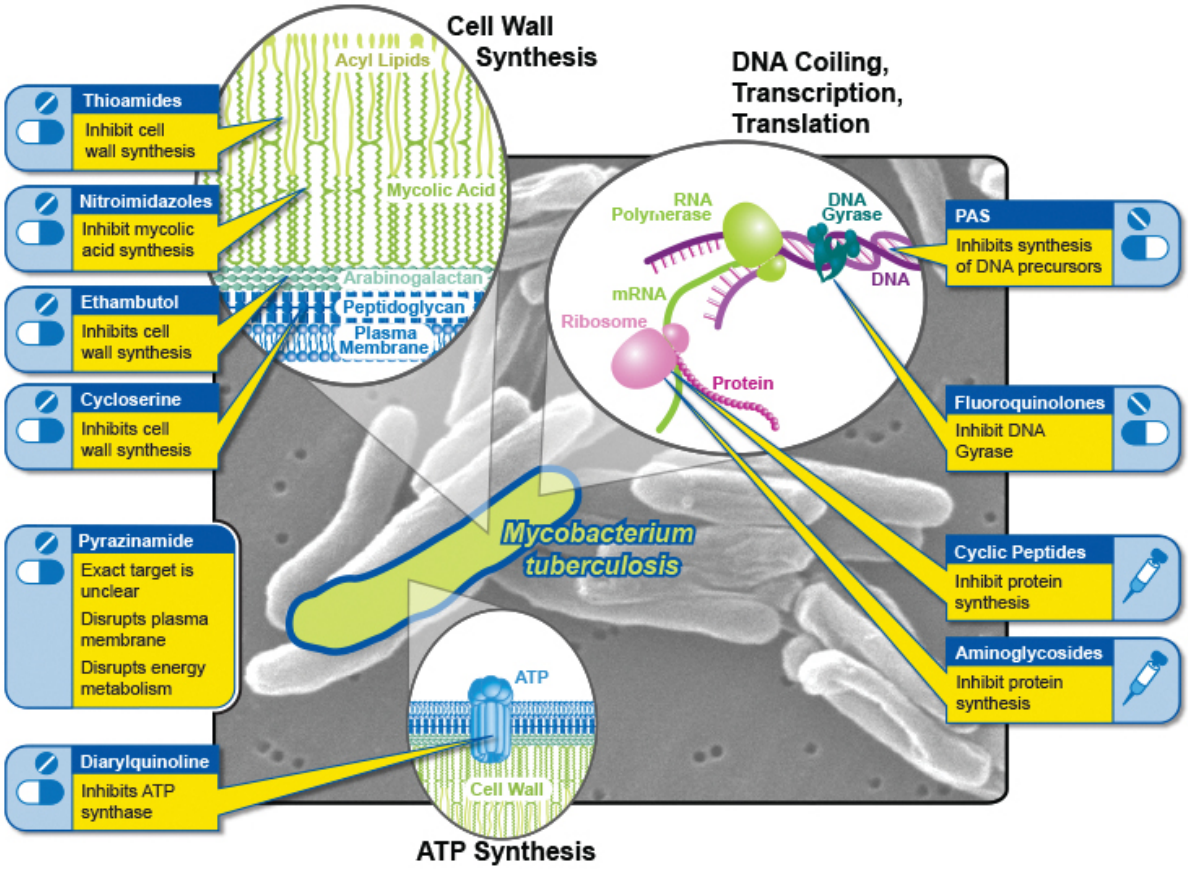
3.2.3.4. Nadějné sloučeniny ve vývoji

Existují řady studií se sloučeninami, které jsou v různých stádiích klinického hodnocení. Ty asi nejnadějnější jsou shrnuty v Tabulce **3**. Mechanismus účinku některých těchto sloučenin je uvedený na Obrázku **3** či **4**.

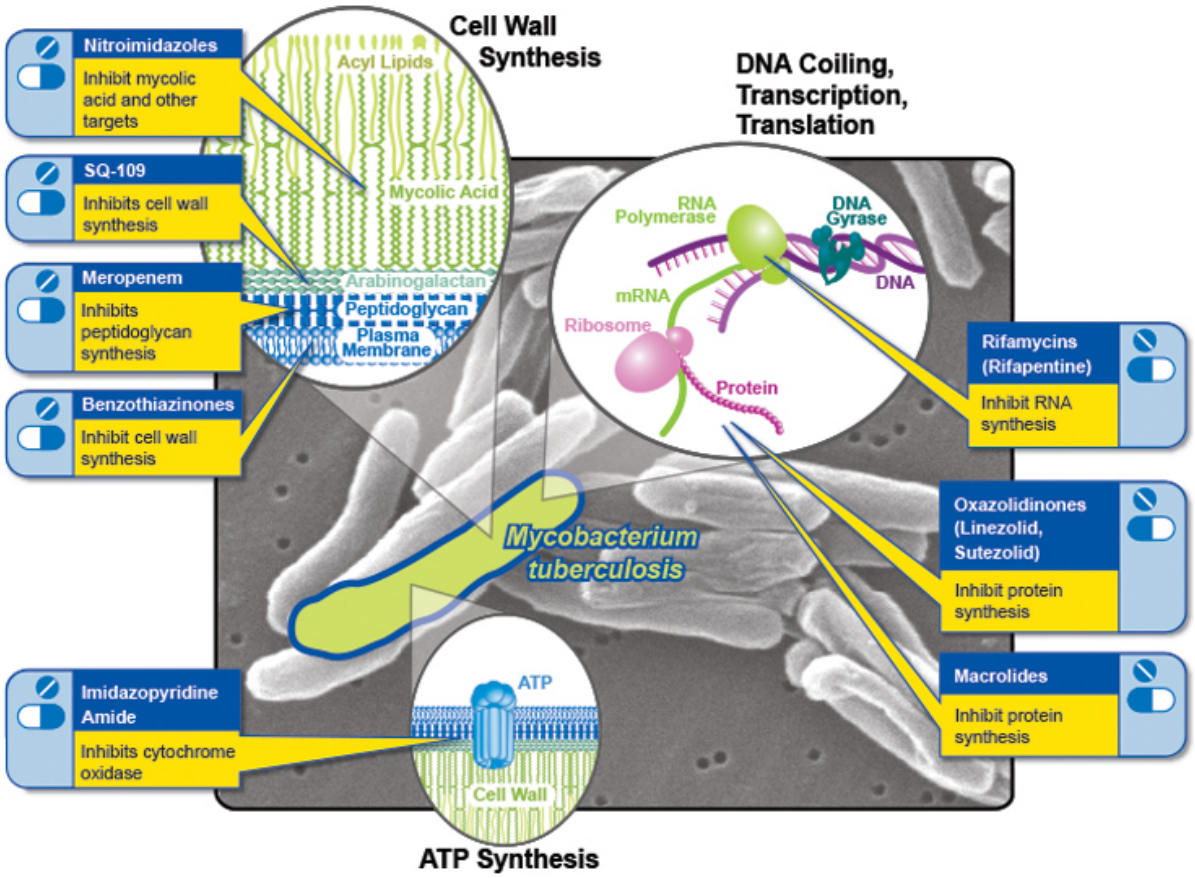
*Tabulka **3** – Souhrn aktuálně vyvíjených molekul v dalších fázích klinických studií*

Fluorochinolony	DC-159a[39]	 DC-159a
1,3-Oxazolidinony	PNU-100480 (Sutezolid)[40]	 PNU-100480
1,3-Oxazolidinony	AZD5847[41]	 AZD5847
Nitroimidazoly	TBA-354[42]	 TBA-354
Ethylendiaminy	SQ109[43]	 SQ109

Pyrroly	LL-3858 (Sudoterb)[44]	 LL-3858
Imidazopyridiny	Q203[45]	 Q203



*Obrázek **3** – Místa terapeutického zásahu vybraných antituberkulotik používaných v praxi – převzato z National Institute of Allergy and Infectious Diseases (NIAID).*



Obrázek 4 – Místa terapeutického zásahu používaných i vyvíjených antituberkulotik – převzato z National Institute of Allergy and Infectious Diseases (NIAID).

3.2.4. Léčebné postupy

Postupy pro léčbu tuberkulózy jsou celosvětově standardizovány a WHO tyto postupy shrnuje ve svých guidelines.[46] Jednotlivé státy pak v určité podobě tato doporučení přejímají a využívají pro boj s TBC na národní úrovni.

V rámci standardních režimů se používají kombinace několika léčiv 1. linie. Ideálně se během zahajovací/intenzivní fáze podává kombinace všech čtyř základních účinných látek (INH/RFM/PZA/ETH) po dobu dvou měsíců, která je následována fází pokračovací s podáváním kombinace dvou až tří léčiv z této skupiny (INH/RFM či INH/RFM/ETH) v závislosti na výskytu rezistence v dané oblasti po dobu dalších čtyř měsíců. Délka terapie však závisí na citlivosti mikroorganismu a terapeutické odpovědi organismu.

Pro kmeny, které vykáží multilékovou rezistenci, se stanovuje většinou individuální režim, který je založen na vyšetření citlivosti daného klinického izolátu vůči používaným antituberkulotikům první volby a následné využití kombinace s léčivy 2. linie podle doporučených postupů. Celkový počet použitých léčiv je v těchto případech v rozmezí 5-7 účinných látek a minimální doba léčby je 18 měsíců, 24 měsíců

pak v případech chronických pacientů s opakovanou infekcí a poškozením plicní tkáně nekrózou. Léčbu je třeba neustále monitorovat a ideálně sledovat pacienty i po ukončení farmakoterapie.

3.3. PYRAZINAMID

3.3.1. Chemie pyrazinu

Pyrazin představuje jako dusíkatý heterocyklus molekulu velice vhodnou pro různé chemické obměny. Samotná molekula představuje strukturu, kterou je možné znázornit několika rezonančními vzorci. Tento heteroaromát dále vykazuje aromaticitu nižší, než je aromaticita benzenu. Hodnotové vyjádření odpovídá 75 %. Ostatní 6-ti členné aromatické dusíkaté heterocykly mají hodnotu podobnou. Co se týče acidobazických vlastností, tak je pyrazin velice slabou dvojsytnou bází s hodnotou disociačních konstant p*Ka*=0,65 a p*Ka*=-5,78.[47]

Rozložení náboje resp. hustoty π-elektronů je u pyrazinu podobné jako například u pyridinu. Toto rozložení tak ovlivňuje reaktivitu samotného jádra (viz. Schéma **1**). Elektrofilní substituce neprobíhají příliš snadno, což je dáno právě částečným kladným nábojem na atomu uhlíku sousedícího s atomem dusíku (indukční efekt, který je ovlivněn existencí výše zmiňovaných rezonančních struktur). Naopak nukleofilní substituce jsou pro chemické obměny velice vhodné. Reaktivitu v těchto případech zvyšuje také převedení pyrazinu na *N*-oxid.[48,49]

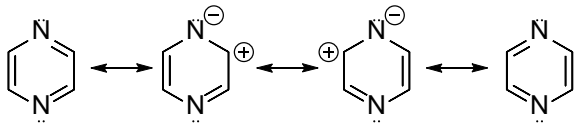


*Schéma **1** – Rozložení elektronové hustoty na pyrazinovém jádře*

3.3.2. Vlastnosti pyrazinamidu

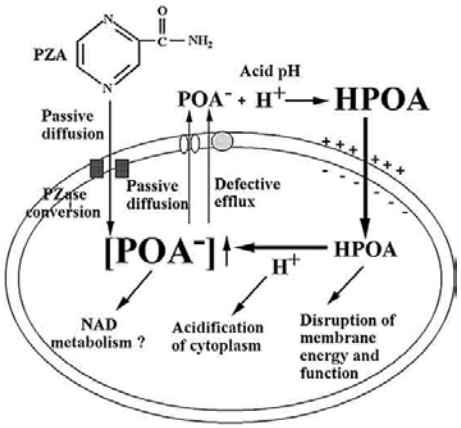
Pyrazinamid je bílá krystalická látka s teplotou tání 190-191 °C.[50] Poprvé byl připraven firmou Merck v roce 1939, avšak jeho antimykobakteriální aktivita byla objevena až o mnoho let později, tedy v roce 1952.[51-53] Toto poznání bylo založeno především na podobnosti s nikotinamidem, jehož antituberkulotické vlastnosti byly využity poprvé v roce 1948.[54]

3.3.3. Mechanismus účinku pyrazinamidu

O mechanismu účinku tohoto léčiva se již léta vedou spory. První teorie se zabývají nespecifickým místem působení, založeným na fyzikálně-chemických vlastnostech PZA a pyrazinkarboxylové kyseliny (POA). Novější výzkum osvětluje již konkrétní cíle zásahu této heterocyklické sloučeniny.

3.3.3.1. Enzymatická konverze PZA na POA a zásah do energetického metabolismu mykobakteriální buňky

Při tomto mechanismu se předpokládá pasivní difuze pyrazinamidu do mykobakteriální buňky. Zde se následně enzymatickou cestou pomocí nikotinamidasy/pyrazinamidasy (EC 3.5.1.19) metabolizuje na vlastní účinnou látku kyselinu pyrazinkarboxylovou. Vnímavost kmene vůči PZA je závislá na aktivitě tohoto enzymu.[55] POA je z buňky zpět uvolňována pasivní difuzí či aktivním, energeticky náročným transportem. Část pyrazinkarboxylátových iontů je v kyselém extracelulárním prostředí protonizována na nábojově neutrální molekulu kyseliny pyrazinkarboxylové a pasivní difuzí se opět dostává do buňky. Tím se posouvá acidobazická rovnováha v cytoplazmě a její acidifikací dochází k inaktivaci a poškození enzymatického aparátu buňky. Spolu s tím jsou ovlivněny a narušeny membránové transportní mechanismy, u nichž je pH gradient základem funkce.[56] Každý druh a každý kmen rodu *Mycobacterium* má tento efluxní transport různě vyvinutý. Tento rozdíl je asi nejvíce patrný právě u *M. tbc*, kdy snížená aktivita efluxního systému zvyšuje intracelulární koncentraci POA rychleji. Zároveň se tohoto rozdílu úspěšně využívá při terapii dormantních forem *M. tbc*, které jsou velice těžce zasažitelné. Tyto ‚spící' mikroorganismy jsou metabolicky méně aktivní včetně aktivity efluxní, což



Obrázek 5 – Aktivace PZA a mechanismus účinku spojený s narušením energetického metabolismu mykobaketrie – převzato z Wade, M. M.; Zhang, Y., 2004 se souhlasem editora Frontiers in bioscience: a journal and virtual library [61]

přispívá ke kumulaci POA v intracelulárním prostoru. Celý proces je znázorněný na Obr. **5**.[57,58]

Vůči tomuto mechanismu byla zaznamenána rezistence, která je dána mutací genu zodpovědného za proteosyntézu pyrazinamidasy. Tímto genem je *pncA* a právě tento gen snadno podléhá mutacím v celé délce a zapříčiňuje největší procento (v průměru 85 %) všech rezistencí vůči PZA.[59-62]

Nejnovější výzkum však prokázal, že vliv kyselého prostředí není pro účinek PZA/POA důležitý. Zkoumáním pH cytoplazmy mykobakterií bylo zjištěno, že k intracelulárnímu okyselení nedochází v takové míře, která by ovlivnila životaschopnost těchto mikroorganismů. Tento nespecifický mechanismus lze tedy považovat za vedlejší s menším vlivem na celkový účinek.[63]

3.3.3.2. Inhibice syntézy NAD⁺ a ovlivnění energetického metabolismu buňky

Tato teorie využívá strukturní podobnosti pyrazinamidu s nikotinamidem. Ten je spolu s kyselinou nikotinovou součástí syntézy nikotinamid adenin dinukleotidu (NAD^+), který slouží jako kofaktor redoxních reakcí. Nikotinamidasa v tomto cyklu aktivuje nikotinamid, potažmo pyrazinamid, na korespondující kyseliny. Ty jsou poté začleněny do NAD^+. V případě PZA by tak vznikal nefunkční derivát. Bohužel, tato teorie nebyla prokázána a zároveň proti ní svědčí i fakt, že k syntéze NAD^+ bakterie včetně mykobakterií využívají i jiné syntetické cesty včetně syntézy *de novo*.[64]

3.3.3.3. Inhibice Fatty Acid Synthasy I

Dalším potenciálním místem zásahu je syntéza mykolových kyselin. Mechanismus účinku byl prokázán na enzymu Fatty Acid Synthasa I (FAS I, EC 2.3.1.85). Tento enzymatický komplex je zodpovědný za syntézu prekurzorů mykolových kyselin s kratším řetězcem. Ty jsou následně prodlužovány pomocí FAS II. Narušení tohoto řetězce vede k poruchám tvorby buněčné stěny a smrti patogenu. Účinek byl však nejprve prokázán pro derivát PZA – 5-chlorpyrazinamid.[65,66] Později se tento mechanismus účinku rozšířil i na samotný PZA a POA, bohužel tyto látky inhibovaly FAS I pouze ve vysokých koncentracích. Dalším zkoumáním se podařilo demonstrovat vazbu PZA a POA do aktivních míst enzymu. Pomocí NMR spektroskopie byly určeny jednotlivé vazebné interakce. PZA například obsadil vazebné místo NADPH.[67]

3.3.3.4. Inhibice trans-translace

Trans-translace je vitální mechanismus sloužící pro přežití buňky. Je to jakýsi systém kontroly proteosyntézy. Pokud by došlo k nějaké chybě v translaci (zlomy, chybné triplety, apod.), dojde současně i k zastavení tvorby peptidu a zablokování ribozomu. Takto zablokovaný ribozom je neschopný další proteosyntézy a byl by pro buňku nepoužitelný. Během *trans*-translace dochází k uvolnění defektního aminokyselinového řetězce, jeho degradaci a především k obnovení funkce ribozomu. K tomu slouží tmRNA (transfer-messenger RNA), která se navazuje na poškozenou mRNA (mediátorovou RNA), umožní odpojení defektního proteinu a zároveň ho označí kodonem pro degradaci.[68] PZA prokázal inhibiční účinek na tento záchranný proces u *M. tbc*. Prvním krokem je navázání POA (musí tedy dojít k aktivaci PZA pomocí pyrazinamidasy) na ribozomální protein SA (RpsA). Tento protein, který je zodpovědný za navázání tmRNA na zablokovaný ribozom, je vitální pro přežití mikroorganismu. Exprimaci tohoto záchranného mechanismu podporuje většinou vystavení extrémním podmínkám, kdy je větší šance k translačním chybám.[69]

I zde se může vyskytnout rezistence, která je spojená s mutací genu *rpsA*, který kóduje právě ribozomální protein SA na podjednotce 30S.[70,71]

3.3.3.5. Inhibice aspartát dekarboxylasy

Nejnovější výzkum ukazuje na další mechanismus účinku pyrazinamidu. Ten je vázán na enzym aspartát dekarboxylasu (EC 4.1.1.11), který je zodpovědný za konverzi *L*-aspartátu na β-alanin. Ten je základem pro syntézu panthotenátu, jenž je nezbytnou součástí koenzymu A. Koenzym A poté slouží jako významný mediátor při získávání energie. Narušení této metabolické dráhy dává možnost k depleci energetických zásob a odumření buňky. Pro prokázání této hypotézy bylo použito metody, kdy bylo do živné půdy přidáno určité množství β-alaninu či panthotenátu. Mykobakterie, které byly kultivovány na takto obohacené půdě, se vyznačovaly mnohem nižší citlivost vůči POA. Tím byla dokázána inhibice aspartát dekarboxylasy, jelikož u půdy s přídavkem *L*-aspartátu ke snížení citlivosti nedošlo.[72]

I u tohoto mechanismu může dojít ke vzniku rezistence. Ten je opět spojen s mutací genu, který tentokrát kóduje aspartát dekarboxylasu – *panD*.[73]

3.3.4. Farmakologie pyrazinamidu

Z hlediska farmakokinetiky je u pyrazinamidu důležitý fakt, že *in vitro* je PZA méně účinný než jeho metabolit POA. *In vivo* je tento jev opačný. POA nevykazuje aktivitu žádnou, zatímco PZA je vysoce účinný. Vše je dáno schopností se vstřebávat. Po perorálním podání dochází tedy k snadné absorpci PZA. Následný metabolismus je poměrně rychlý. U části podaného léčiva nastává hydrolýza PZA na kyselinu, a ta je z organismu vyloučena. Dále může dojít oxidaci cestou xanthinoxidasy a vznikající 5-hydroxy metabolity jsou opět vyloučeny. Exkrece se v těchto případech děje ledvinami a močí.[74]

Obvyklé dávkování pro dospělé osoby trpící tuberkulózou je 20-30 mg/kg tělesné hmotnosti při režimu denního podávání a 30-40 mg/kg tělesné hmotnosti při režimu podávání 3x týdně, který se ovšem z hlediska kompliance a kontroly nedoporučuje využívat.[46] S takto vysokým dávkováním souvisí i výskyt nežádoucích účinků. PZA jako hepatotoxická sloučenina vyžaduje sledování funkce jaterních enzymů. Zvlášť při podávání kombinací s rifampicinem a isoniazidem, jež jsou sami o sobě také hepatotoxickými sloučeninami. Dalším, velmi častým nežádoucím účinkem je zvýšená hladina kyseliny močové. Tu PZA ovlivňuje kompeticí s kyselinou močovou o transportní systém v tubulech nefronu. Zvýšená urikémie může vyústit až v dnavý záchvat, který lze zvládnout běžnou protizánětlivou léčbou a nepředstavuje tak důvod pro vysazení léčby.[75]

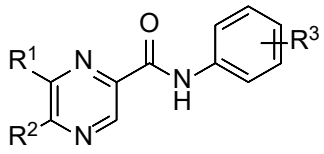
3.3.5. Publikované deriváty PZA s antituberkulotickou aktivitou

Derivátů s účinností na *M. tbc* bylo publikováno mnoho. Bylo vytvořeno již několik přehledů, které shrnují vývoj těchto sloučenin. Jedním z nich je Burger´s Medicinal Chemistry, Drug Discovery, and Development.[76] V této kapitole budou zmíněny deriváty připravené v rámci pracovní skupiny prof. Doležala, která se syntézou derivátů PZA zabývá již několik desítek let.

První zajímavou skupinu tvoří anilidy kyseliny pyrazinkarboxylové, jejichž karboxamidová funkce je substituována fenylovým jádrem s různými obměnami. Byly připraveny celé série těchto sloučenin, z nichž u některých byla zaznamenána vynikající *in vitro* aktivita. Ta byla prokázána v rámci testování TAACF (Tuberculosis Antimicrobial Acquisition and Coordinating Facility; program probíhající v letech 1994-2010 v U.S.A., jehož účelem byla podpora výzkumu nových antituberkulotik na

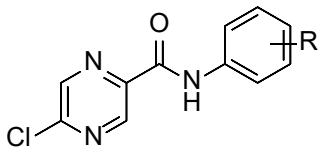
akademické úrovni), kdy pět nejaktivnějších sloučenin inhibovalo růst *M. tbc* v rozmezí 88-99% při koncentraci 6,25 μg/ml (shrnuto v Tabulce **4**).[77-81]

*Tabulka **4** – Přehled in vitro nejaktivnějších derivátů ze skupiny anilidů publikovaných prof. Doležalem a kol.*

	**R¹**	**R²**	**R³**	**% inhibice růstu *M. tbc***
	H	H	3-CF_3	99[80]
	Cl	*tert*-butyl	4-OH	97[81]
	Cl	*tert*-butyl	2-OH	96[81]
	Cl	*tert*-butyl	3-CF_3	95[80]
	Cl	*tert*-butyl	3-F	88[79]

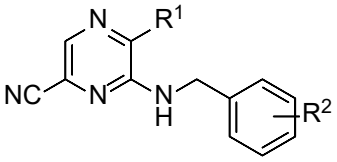
Další skupinou navazující na tuto práci jsou podobné deriváty, které obsahují v poloze 5- pyrazinového jádra chlor, čímž se tyto deriváty podobají již zmíněnému enzymovému inhibitoru FAS I – 5-chlorpyrazinkarboxylové kyselině, potažmo 5-chlorpyrazinamidu. Karboxamidová skupina je opět derivatizována fenylovým jádrem či benzylamino skupinou, která je různě substituovaná (viz. Tabulka **5**). Ty nejlepší deriváty vykázaly aktivitu vůči *M. tbc* lepší než samotný PZA. Hodnota pro nejslibnější sloučeniny byla 1,56 μg/ml. Substituce fenylového jádra byla v tomto případě také velice různorodá.[82,83]

*Tabulka **5** – Série doplňující předchozí skupinu látek s výraznou in vitro aktivitou vůči M. tbc*

	**R**	**MIC pro *M. tbc***
	2,5-CH_3	1,56 μg/ml[83]
	4-C_2H_5	
	4-*i*Pr	
	2-Cl, 5-CH_3	
	5-Cl, 2-OH	
	2-OH, 5-NO_2	
	4-CF_3	

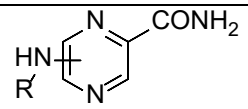
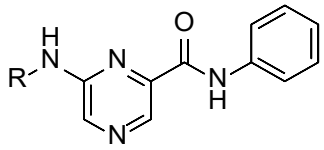
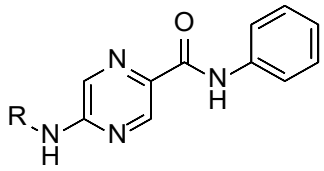
Deriváty, kdy je zachována karboxamidová funkce, případně je nahrazena analogickou skupinou karbonitrilovou, již takovou aktivitu nezopakovaly. Zde byla benzylaminová či fenylová skupina vázána přes sekundární aminoskupinu na jádro v poloze 3-. Ty nejlepší aktivity vyšly v rozmezí 6,25-12,5 μg/ml a u obou derivátů PZA se částečně shodovala i substituce na aromatickém jádře (viz Tabulka **6**).[84]

*Tabulka **6** – Deriváty s obměnou funkčních skupin na pyrazinovém jádře vedoucí ke snížení in vitro aktivity*

	R¹	**R²**	**MIC pro *M. tbc***
	CN	4-CH_3	6,25 μg/ml
	CN	H	12,5 μg/ml
	CN	3-Cl	12,5 μg/ml
	$CONH_2$	4-CH_3	12,5 μg/ml
	$CONH_2$	4-NH_2	12,5 μg/ml

Nejslibnější skupinu aktivních látek utvořila skupina s postranním alkylamino řetězcem, který byl vázaný v poloze 5- či 6- pyrazinového jádra. Deriváty, které obsahovaly volnou karboxamidovou skupinu, byly aktivní o něco méně. Zde byla nejlepší aktivita dosažena 8-uhlíkatým řetězcem. Sloučeniny, u nichž byla karboxamidová funkce převedena na anilid s fenylovým jádrem, svou aktivitu ještě zlepšily. Poloha 5- projevila oproti poloze 6- lepší aktivitu s kulminací u 6-ti a 7-mi členného řetězce s aktivitami až 0,78 μg/ml (viz. Tabulka 7).[85-87]

Tabulka 7 – Přehled alifaticky substituovaných derivátu PZA vykazujících významnou in vitro aktivitu

	R	**MIC pro *M. tbc***
	5-oktyl	6,25 μg/ml[85]
	6-oktyl	1,56 μg/ml[85]
	pentyl	3,13 μg/ml[86]
	hexyl	1,56 μg/ml[86]
	heptyl	1,56 μg/ml[86]
	oktyl	3,13 μg/ml[86]
	pentyl	3,13 μg/ml[87]
	hexyl	0,78 μg/ml[87]
	heptyl	0,78 μg/ml[87]
	oktyl	3,13 μg/ml[87]

3.4. MIKROVLNAMI ASISTOVANÉ REAKCE

V dnešní době je využití mikrovlnné technologie velikou výhodou. Praktické využití v posledních letech prudce stoupá. Je to dáno také tím, že stoupá obliba takzvané „zelené" chemie (green chemistry). Použití mikrovlnných reaktorů tento trend umožňuje. Je zde možnost snižování spotřeby rozpouštědel, spotřeby energie.

Další nespornou výhodou je urychlení celé reakce, zvyšování výtěžků a jednotný ohřev celého objemu reakční směsi. Samozřejmě i tato technologie má své nevýhody. Ne vždy je pro daný typ reakce vhodná a je zde častější výskyt vedlejších produktů, které by při konvenční syntéze nevznikly. To je však možné omezit použitím moderních purifikačních metod.[88] Využití uzavřených přetlakových systémů poskytuje další výhodu. Je totiž možné dosáhnout daleko vyšší teploty rozpouštědla či reakční směsi, než je běžná teplota varu pro tuto sloučeninu za atmosférického tlaku.

Mikrovlnné záření je typem elektromagnetického vlnění. Rozmezí vlnových délek pro mikrovlny je poměrně široké – 1 milimetr až 1 metr, které odpovídá frekvenci 0,3-300 GHz. Pro použití v praxi je třeba tuto vlnovou délku potažmo frekvenci více specifikovat, neboť v těchto vlnových délkách operují i jiná zařízení (vysílačky, rádia, a jiné). Průmyslové reaktory využívají frekvenci 2,455 GHz, což by mělo omezit interference s ostatními přístroji.

Mikrovlnné zahřívání urychluje, dle některých autorů, chemické reakce dvěma skupinami efektů. Jsou to termické a netermické efekty. Schopnost některých sloučenin či rozpouštědel přeměnit elektromagnetickou energii na teplo je dáno právě efektem termickým. Je nezbytné, aby molekula byla schopná vytvořit dipólový moment, či se byla schopná ionizovat. Dipól je schopný interagovat s externím elektrickým polem, které dodává energii pro rotaci samotných molekul. Fázový rozdíl mezi oscilujícím elektrickým polem přístroje a molekul způsobuje energetické ztráty, což zvyšuje teplotu. Totéž platí i pro iontovou vodivost. Dalším důležitým parametrem pro interakci s elektromagnetickým polem je ztrátový úhel, který vyjadřuje rozsah schopnosti přeměnit energii na teplo. To ovlivňuje rychlost růstu teploty. U netermálních efektů je to poněkud složitější. Zde se značně různí názory odborné společnosti, zda vůbec tento typ efektů existuje. Teorií a modelů existuje několik a v posledních letech se množí poznatky, že jsou tyto efekty méně důležité pro celkový průběh mikrovlnami asistované reakce.[89-92]

4. ZVOLENÉ METODY ŘEŠENÍ

4.1. OBECNÉ POSTUPY

Všechny použité chemikálie byly zakoupeny od společnosti Sigma-Aldrich (Sigma-Aldrich, Co., Steinheim, Německo) či Fluorochem (Fluorochem Ltd., Hadfield, Derbyshire, Velká Británie) a byly minimálně v syntetické kvalitě. Tenkovrstvá chromatografie byla prováděna na TLC deskách Merck Sillica gel F_{254} s UV detekcí při 254 nm (Merck KGaA, Darmstadt, Německo). K syntéze finálních produktů byl využit mikrovlnný reaktor CEM Discover vybavený podavačem Explorer 24 (CEM Corporation, Matthews, Severní Karolína, U.S.A.). Získané produkty byly přečištěny pomocí preparativního flash chromatografu CombiFlash® Rf (Teledyne Isco, Inc., Lincoln, Nebraska, U.S.A.). Byla použita gradientová eluce v systému hexan-ethylacetát s detekční vlnovou délkou 260 nm. Jako stacionární fáze byl použit silikagel s velikostí zrn 40-63 μm.

^{1}H a ^{13}C NMR spektra byla měřena na přístrojích Varian Mercury-VxBB 300 a Varian VNMR S500 (Varian corp., Palo Alto, Kalifornie, U.S.A.). Měření probíhalo při laboratorní teplotě a chemické posuny byly uváděny jako δ v jednotkách ppm, které byly vztaženy k tetramethylsilanu jako standardu. Infračervená spektra byla zaznamenána metodou ATR na přístroji FT-IR Nicolet 6700 (Thermo Scientific, Waltham, Massachusetts, U.S.A.). Elementární analýza byla provedena na přístroji EA 1110 CHNS Analyser (Fisons Instruments S. p. A., Carlo Erba, Milano, Itálie) a výsledky byly vyjádřeny v procentech. Teplota tání byla měřena na přístroji SMP3 Stuart Scientific (Bibby Sterling LTD, Staffordshire, Velká Británie) v otevřené kapiláře a získané hodnoty jsou nekorigované.

4.2. SYNTETICKÉ POSTUPY

Výchozí látky byly připraveny podle předchozích postupů, dříve publikovaných v odborných časopisech.

4.2.1. Příprava 5-chlor-6-methylpyrazin-2,3-dikarbonitrilu

V prvním kroku dochází ke kondenzaci kyseliny pyruvové (0,025 mol) a diaminomaleonitrilu (0,025 mol). Obě sloučeniny se rozpustily v methanolu (60 ml) a postupně se přidalo 10 ml 15% kyseliny chlorovodíkové. Celá směs se nechala reagovat po dobu tří hodin při laboratorní teplotě a za stálého míchání. Poté se oddestilovaly

2/3 rozpouštědla, přidalo se 80 ml horké vody a nechal se odpařit zbytek methanolu. Vodný roztok se umístil do lednice. Vzniklé krystaly se odsály a vysušily.

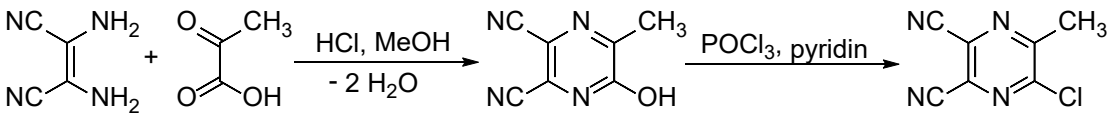


*Schéma **2** – Příprava 5-chlor-6-methylpyrazin-2,3-dikarbonitrilu kondenzací a následnou chlorací aromatické hydroxy skupiny pomocí chloridu fosforylu*

5-Hydroxy-6-methylpyrazin-2,3-dikarbonitril (0,015 mol) se postupně rozmíchal v chloridu fosforylu (0,060 mol) a celá směs se ochladila na 0 °C. Po kapkách se přidal pyridin (0,020 mol) a po ukončení exotermní reakce se celá směs zahřála na 90 °C. Po dvou hodinách se reakce ukončila a odpařil se přebytečný chlorid fosforylu. Zbylá směs se extrahovala třikrát až čtyřikrát toluenem, spojené toluenové podíly se odpařily do sucha a vzniklá krystalická látka se překrystalizovala z chloroformu. Postup je shrnut ve Schématu **2**.[93]

4.2.2. Příprava 3-chlorpyrazin-2-karboxamidu

První metodou pro přípravu této výchozí látky je homolytická amidace pyrazinového jádra (Schéma **3**). 2-Chlorpyrazin (170 mmol) byl rozpuštěn ve formamidu (146 ml) a celá směs se zahřála na 90 °C. Po částech se přidal peroxodisulfát amonný (180 mmol) a reakce pokračovala při 85 °C další tři hodiny. Směs se nechala stát při pokojové teplotě 24 hodin, přidala se destilovaná voda a odfiltroval se pevný podíl. Filtrát se nechal kontinuálně extrahovat chloroformem po dobu 16 hodin. Směs tří vznikajících polohových isomerů byla rozdělena pomocí preparativní chromatografie a 3-chlorpyrazin-2-karboxamid byl překrystalizován z ethanolu. Výtěžnost této reakce se pohybuje mezi 15 a 20 %.[94,95]

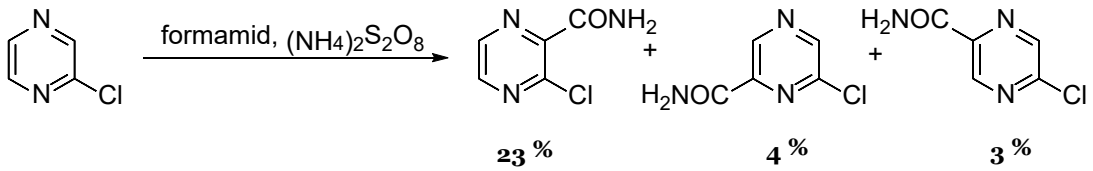


*Schéma **3** – Méně výhodná syntéza 3-chlorpyrazin-2-karboxamidu homolytickou amidací pyrazinového jádra a zastoupení jednotlivých polohových izomerů*

Druhý postup je založen na parciální hydrolýze karbonitrilové skupiny (Schéma **4**). 3-Chlorpyrazin-2-karbonitril (104 mmol) se postupně přidal do směsi koncentrovaného peroxidu vodíku (29 ml) a destilované vody (195 ml) zahřáté na

50 °C, u které bylo upraveno pH na hodnotu 9 pomocí 8% roztoku hydroxidu sodného. Po přidání celého množství karbonitrilu se směs zahřála na 55 °C a nechala reagovat dvě hodiny. Během reakce je nutné neustále monitorovat hodnotu pH a případně upravovat pomocí 8% roztoku NaOH. Při nedodržení těchto podmínek může docházet k úplné hydrolýze karbonitrilové skupiny na karboxylovou skupinu, resp. na její sodnou sůl. Po skončení reakce byla celá směs ochlazena v lednici k iniciaci krystalizace. Surový produkt byl překrystalizován z ethanolu. Výtěžnost tohoto postupu je asi 80 %.[94]

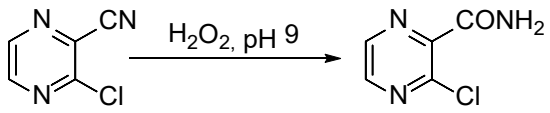


*Schéma **4** – Výhodnější příprava 3-chlorpyrazin-2-karboxamidu částečnou hydrolýzou karbonitrilové skupiny za pečlivě kontrolovaných podmínek*

4.2.3. Příprava *N*-benzyl-3-chlorpyrazin-2-karboxamidu

V prvním kroku bylo třeba nahradit aminoskupinu skupinou hydroxylovou pomocí diazotační reakce. 3-Aminopyrazin-2-karboxylová kyselina (50 mmol) se po částech za stálého míchání přidala do vychlazené koncentrované kyseliny sírové (50 ml). Nitrosylsírová kyselina se připravila vychlazením koncentrované kyseliny sírové (37,5 ml) na 0 °C a postupným přidáváním dusitanu sodného (50 mmol) po dobu 15 minut za stálého chlazení. Tato nitrosylační směs se poté po částech přidávala k vychlazenému roztoku 3-aminopyrazin-2-karboxylové kyseliny za stálé kontroly teploty (0-2 °C). Po 15 minutách se tato směs po částech nalila na ledovou tříšť a za stálého míchání se vyčkalo do ukončení vývoje dusíku. Tato silně kyselá masa se zfiltrovala, pevný podíl se promyl vodou do neutrální reakce, látka se vysušila a překrystalizovala z vody.[96] Poté byla 3-hydroxypyrazin-2-karboxylová kyselina (7 mmol) rozpuštěna v chloridu fosforylu (13 ml), bylo přidáno pár kapek pyridinu a celá směs se za stálého míchání zahřívala za refluxu dvě hodiny. Přebytečný $POCl_3$ se vakuově odpařil.[97] Připravený chlorid kyseliny 3-chlorpyrazin-2-karboxylové (7 mmol) se rozpustil v bezvodém tetrahydrofuranu (THF) a do tohoto roztoku se po částech vnesla směs benzylaminu (35 mmol) a triethylaminu (14 mmol) rozpuštěného v malém množství bezvodého THF. Celá masa se nechala reagovat za stálého míchání tři hodiny při laboratorní teplotě. Následovalo přečištění pomocí preparativní chromatografie. Jednotlivé kroky jsou znázorněny ve Schématu **5**. Jako vedlejší produkt posledního kroku této reakce vznikal také *N*-benzyl-3-(benzylamino)pyrazin-2-karboxamid

(disubstituovaný derivát). Vznikal však pouze v poměru 1:8 a zároveň byl velmi dobře oddělitelný pomocí flash chromatografie.

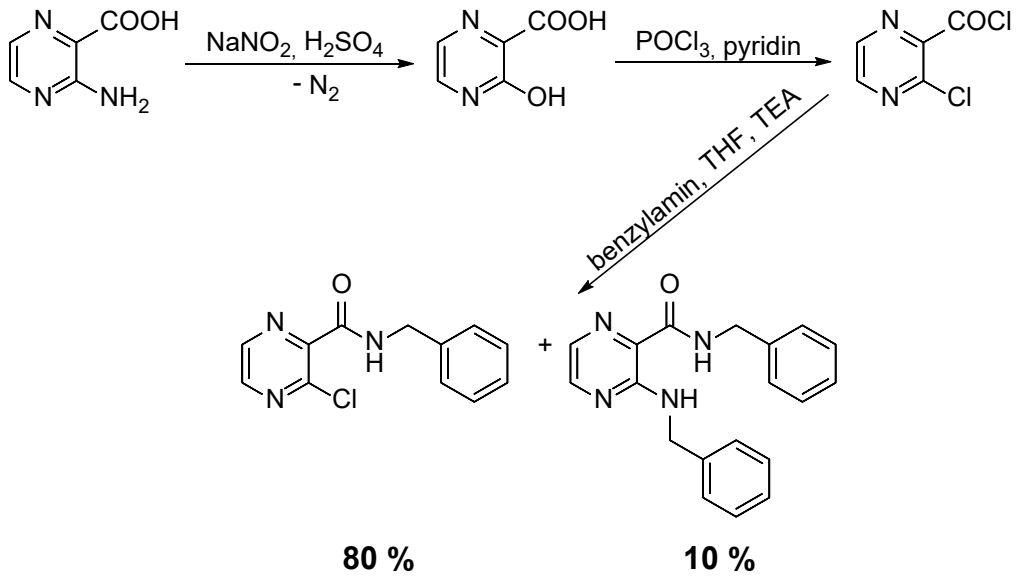


Schéma 5 – Příprava N-benzyl-3-chlorpyrazin-2-karboxamidu diazotační reakcí, chlorací a aminodehalogenační reakcí s nesubstituovaným benzylaminem a vznik vedlejšího produktu

4.2.4. Obecný postup aminodehalogenace

Výchozí látka (200 mg) se navážila do speciální silnostěnné zkumavky, přidaly se dva ekvivalenty příslušného aminu, ekvimolární množství pyridinu a 3 ml methanolu. Přidalo se míchadlo a zkumavka se uzavřela septem. Směs se umístila do mikrovlnného reaktoru. Podmínky reakce byly experimentálně určeny a jsou následující: reakční teplota 140 °C, doba reakce 30 min, výkon 120 až 200 W. Dále bylo použita metoda stálého chlazení, čím bylo dosaženo většího výkonu během celé doby reakce.

4.3. BIOLOGICKÁ HODNOCENÍ

4.3.1. Antimykobakteriální hodnocení

Toto testování bylo prováděno ve spolupráci s Ústavem klinické mikrobiologie Fakultní nemocnice v Hradci Králové.

Základem těchto testů byla mikrodiluční bujonová metoda v mikrotitračních deskách. Odečet byl vizuální s barevnou indikací aktivity pomocí metody Alamar Blue. Paleta testovaných kmenů byla v průběhu času mírně upravena. K testování byly zakoupeny kmeny z České národní sbírky typových kultur (označení CNCTC) při Státním zdravotním ústavu.[98] Jednalo se o *Mycobacterium tuberculosis* H37Rv CNCTC My 331/88, *M. kansasii* Hauduroy CNCTC My 235/80, *M. avium* CNCTC

My 152/73. Zpočátku byl ještě používán kmen *M. avium* CNCTC My 80/72, ale z důvodu časové a finanční náročnosti se od testování na tomto kmeni ustoupilo, jelikož výsledky získané v průběhu testování byly u obou kmenů *M. avium* totožné. Kmeny jsou kultivovány při 37 °C v temnu a vlhku.

Standardem pro antimykobakteriální testování byl zpočátku pyrazinamid a později se začal využívat isoniazid, který vykazoval aktivitu i vůči kmenům *M. kansasii* a *M. avium*.

Jako kultivační médium byla ze začátku používána Šulova tekutá půda (Trios, Praha, ČR) o pH v rozmezí 5,5-5,7. Takto nízké pH bylo zvoleno z důvodu aktivity standardu PZA, jelikož s rostoucí hodnotou pH klesá aktivita PZA, a to až stokrát. Zároveň je zde patrná i podobnost připravených sloučenin s PZA. Díky nižšímu pH docházelo bohužel občas k problémům s růstem mykobakterií a dále bylo nutné toto pH neustále monitorovat. Z tohoto důvodu se začalo využívat médium Middlebrook 7H9 (Sigma-Aldrich). Tento bujón je chemicky přesně definovaný a jeho hodnota pH je 6,6±0,2. Dále je obohacen o růstový suplement OADC (kyselina olejová, albumin, dextrosa, katalasa) (Himedia, Mumbai, Indie) a glycerol (Sigma-Aldrich), čímž je zajištěn dostatečný přísun živin pro růst mykobakterií.

Testované sloučeniny jsou rozpouštěny v DMSO a ředí se ve dvojkové řadě v koncentracích 20000, 10000, 5000, 2500, 1250, 625 a 313 μg/ml. U isoniazidu bylo přistoupeno ještě k nižším koncentracím a to 156, 78, 39 a 20 μg/ml. Tyto základní roztoky byly poté naředěny médiem v poměru 1:99, čímž se dosáhlo koncentrace 100x nižší. Takto naředěné látky byly napipetovány do mikrotitrační destičky. Koncentrace DMSO dosáhla 0,5 % (V/V) a neovlivnila růst mykobakterií.

Mykobakteriální suspenze byla připravena ve vodném prostředí a před použitím byla denzitometricky upravena na hodnotu 0,5-1,0 dle McFarlanda. Z této suspenze byly připraveny další s ředěním 10^{-1} a 10^{-3}. Těmi se inokulovaly mikrotitrační destičky, čímž se dosáhlo finálních koncentrací testovaných látek 100, 50, 25, 12,5, 6,25, 3,13 a 1,56 μg/ml resp. až do koncentrace 0,1 μg/ml pro isoniazid. Při použití Šulovy půdy byla inkubační doba 14-21 dní. Přesná doba kultivace byla určena nárůstem mykobakterií v kontrolní jamce. Po zavedení média Middlebrook 7H9 a detekce pomocí Alamar Blue se doba nárůstu zkrátila na 5-7 dní, čímž se výrazně zkrátila doba potřebná pro testování.

Hodnoty minimální inhibiční koncentrace (MIC) byly odečteny vizuálně jako nejnižší koncentrace potřebná k potlačení růstu mykobakterií. Tato hodnota byla platná, pokud se nelišila pro obě ředění suspenzí o více jak jeden řád. Dále byl nutný nárůst v kontrolní jamce obsahující pouze DMSO (pozitivní kontrola) a inhibice růstu v jamkách se standardy (negativní kontrola). Zpočátku byl odečet založen na změně zákalu a vytvoření viditelných kolonií. Později se přistoupilo k využití barevného indikátoru růstu. Tím je takzvané barvení Alamar Blue. Chemicky se jedná o resazurin (modrá barva, nevykazuje fluorescenci), který je redoxními systémy rostoucích mykobakterií redukován na resorufin dle Schématu **6**. Tato forma je růžová a vykazuje silnou fluorescenci (především v zeleném světle) (viz. Obrázek **6** a **7**). Je tedy možné odečítat vizuálně, spektrofotometricky a s výhodou i spektrofluorimetricky (vysoká citlivost). Další nespornou výhodou tohoto barvení je i fakt, že toto barvivo je pro bakterie netoxické a nedochází tak k usmrcení, čímž by mohlo dojít ke zkreslení výsledků.

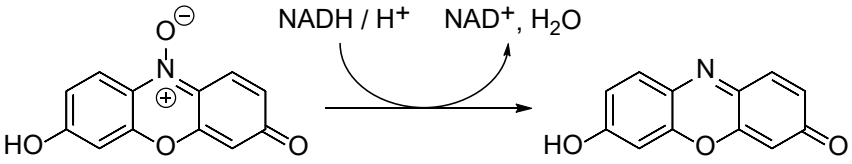
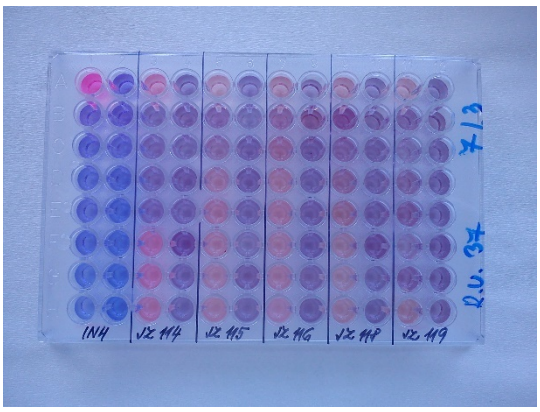
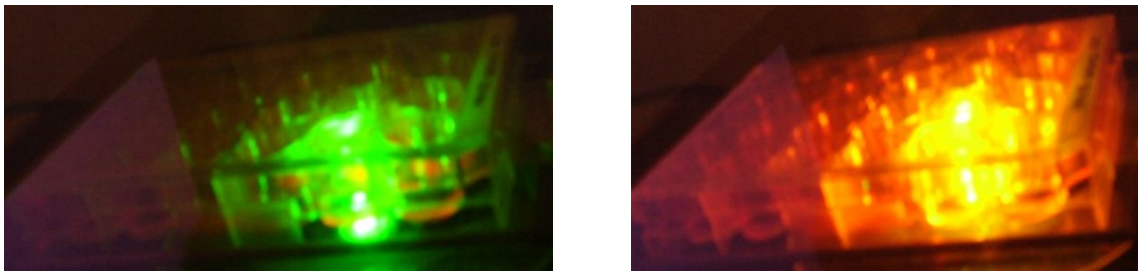


*Schéma **6** – Redukce modrého resazurinu na růžový, fluoreskující resorufin*



*Obrázek **6** – Rozložení mikrotitrační destičky a barvení pomocí Alamar Blue*



*Obrázek **7** – Ukázka fluorescence resazurinu (vlevo) a resorufinu (vpravo) v zeleném světle*

4.3.2. Hodnocení aktivity vůči *Mycobacterium smegmatis*

Toto hodnocení probíhá na katedře Farmaceutické chemie a Farmaceutické analýzy. Bylo zavedeno v rámci této disertační práce po absolvování měsíční výzkumné stáže v irském Cork Institute of Technology. Prakticky se testuje aktivita sloučenin na rychle rostoucím nepatogenním druhu rodu *Mycobacterium*. Pro testování se používá zakoupený kmen *Mycobacterium smegmatis* CCM 4622 (ATCC 607) z České kolekce mikroorganismů.[99] Metoda pro tento skríning je opět mikrodiluční bujonová v 96-ti jamkových mikrotitračních deskách. Jako médium byl použit Middlebrook 7H9 s přídavkem růstového suplementu OADC a glycerolu. Standardy jsou isoniazid, rifampicin a ciprofloxacin (CPX).

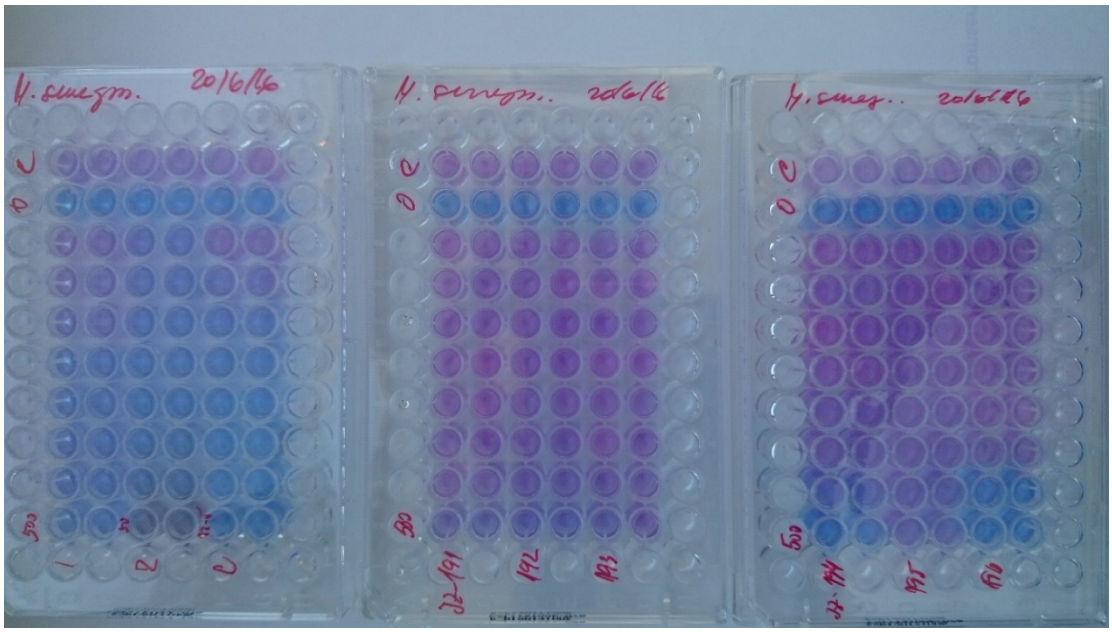
Destičky se připraví napipetováním sterilní vody do okrajových jamek (zabránění vysychání) a do ostatních jamek je přidáno 100 µl média, vyjma negativní kontroly, kde se pipetuje 200 µl. Testované sloučeniny se rozpustí v DMSO v koncentraci 20000 µg/ml. Následně se zředí bujonem v poměru 1:9 a vnáší se do první jamky v sérii v objemu 100 µl. Dojde tak k dalšímu zředění (1000 µg/ml). Dvojkovým ředěním se přenáší objem 100 µl do dalších jamek. Následuje přidání 100 µl mykobakteriální suspenze, připravené upravením denzity inokula na hodnotu 1,0 dle McFarlanda a zředění bujonem v poměru 1:20, čímž je dosaženo finálních koncentrací 500, 250, 125, 62,5, 31,25, 15,63, 7,81 a 3,91 µg/ml. Výjimkou jsou standardy rifampicin a ciprofloxacin, u kterých jsou finální koncentrace díky jejich aktivitě v hodnotách 12,5, 6,25, 3,13, 1,56, 0,78, 0,39, 0,20 a 0,10 µg/ml. Všechny látky jsou testovány dvojitě. Rozložení mikrotitrační destičky je znázorněno v Tabulce **8**. Finální koncentrace DMSO nepřekročila 2,5 % (V/V) a neovlivnila růst mykobakterií. K tomuto účelu je zavedena pozitivní kontrola, která obsahuje pouze mykobakteriální suspenzi v bujónu s přídavkem DMSO, tak aby jeho finální koncentrace činila 2,5 % (V/V). Suspenze mykobakterií a mikrotitrační destičky se inkubují při teplotě 37 °C v temnu.

Po 48 hodinách inkubace je přidáno barvivo Alamar Blue (sodná sůl resazurinu) v množství 10 % objemu jamky a MIC se odečítá po dalších čtyřech hodinách inkubace. Jako hodnota minimální inhibiční koncentrace je považována první jamka se změnou zbarvení z modré na růžovou. Příklad vykultivovaných mikrotitračních destiček je znázorněn na Obrázku **8**.

*Tabulka **8** – Rozložení mikrotitrační destičky při testování potenciálně aktivních sloučenin*

	1	2	3	4	5	6	7	8	9	10	11	12
A	V	V	V	V	V	V	V	V	V	V	V	V
B	V	500	250	125	62,5	31,25	15,63	7,81	3,91	0	K	V
C	V	500	250	125	62,5	31,25	15,63	7,81	3,91	0	K	V
D	V	500	250	125	62,5	31,25	15,63	7,81	3,91	0	K	V
E	V	500	250	125	62,5	31,25	15,63	7,81	3,91	0	K	V
F	V	500	250	125	62,5	31,25	15,63	7,81	3,91	0	K	V
G	V	500	250	125	62,5	31,25	15,63	7,81	3,91	0	K	V
H	V	V	V	V	V	V	V	V	V	V	V	V

V – voda; 0 – negativní kontrola (čistý bujón); K – pozitivní kontrola (mykobakteriální suspenze v bujónu s přídavkem DMSO v koncentraci 2,5 %); 500-3,91 µg/ml konc. testovaných látek



*Obrázek **8** – Ukázka antimykobakteriálního testování (vlevo standardy, uprostřed a vpravo test. sloučeniny)*

Využití tohoto modelu je založeno na některých vlastnostech, které jsou pro rod *Mycobacterium* společné. Nelze říct, že je tento model stoprocentně odpovídající *M. tbc*, ale jsou zde jisté výhody. Mezi ně v první řadě patří generační doba bakterií, dále nepatogenita a podobné enzymové systémy, především existence pyrazinamidasy.

Existuje několik pokusů o srovnání těchto dvou modelů. Výzkumné skupiny zabývající se genomem mykobakterií určily, že zde existuje vysoká podobnost u genů, které jsou exprimovány při hypoxických podmínkách (např. v makrofázích). Pokud je *M. smegmatis* vystaveno hypoxii, dochází také k exprimaci podobných funkcí. Zároveň i exprese genů, kódujících enzymy vyskytující se u dormantních forem *M. tbc* (nitrát

reduktasa, isocitrát lyasa, alanin dehydrogenasa), je podobná. Z výše uvedeného vyplývá, že tento model je vhodný pro použití při studiu aktivity metabolicky neaktivních mykobakterií.[100,101] Existuje i studie zabývající se hodnocením *M. smegmatis* jako modelu vhodného pro testování sloučenin s potencionální aktivitou vůči *M. tbc*. Bylo otestováno přes 5000 sloučenin a u 50 % z nich bylo zjištěno, že látky, které inhibují *M. smegmatis*, inhibují zároveň i *M. tbc*. Tato hodnota není úplně nejvyšší a někteří ji nepovažují za dostatečnou, ale díky skríningu na M. smegmatis se podařilo objevit např. sloučeninu TMC207, dnes známou pod označením Bedachilin, která je nyní nenahraditelnou složkou v terapii rezistentní TBC.[102]

4.3.3. Hodnocení antibakteriální aktivity

Antibakteriální aktivita byla hodnocena na katedře Biologických a lékařských věd Farmaceutické fakulty v Hradci Králové. Pro testování bylo použito mikrodiluční bujonové metody v 96-ti jamkových mikrotitračních deskách. Použité bakteriální kmeny pocházely z České kolekce mikroorganismů (CCM) nebo byly získány jako klinické izoláty z Fakultní nemocnice v Hradci Králové.[99] Jednalo se o kmeny *Staphylococcus aureus* CCM 4516/08, *Escherichia coli* CCM 4517, *Pseudomonas aeruginosa* CCM 1961, *Staphylococcus aureus* H 5996/08 – methicilin rezistentní (MRSA), *Staphylococcus epidermidis* H 6966/08, *Enterococcus sp.* J 14365/08, *Klebsiella pneumoniae* D 11750/08 a *Klebsiella pneumoniae* J 14368/08 – ESBL pozitivní. Všechny kmeny byly kultivovány na Mueller Hinton agaru při 35 °C.

Sloučeniny byly rozpuštěny v DMSO a poté naředěny Mueller Hinton bujónem s pH upraveným na hodnotu 7,0. Inokulum bylo roztřepáno ve sterilním 0,85% fyziologickém roztoku, denzita byla upravena na hodnotu 0,5 dle McFarlanda. Testovaná koncentrace látek byla v rozmezí 500-0,49 μmol/l, zároveň koncentrace DMSO nepřesáhla 1 % (V/V) a neovlivnila růst mikroorganismů. Jako standardy pro testování byly použity penicilin G (PEN), bacitracin (BAC), neomycin (NEOM), ciprofloxacin (CPX) a fenoxymethylpenicilin (PHE). MIC se stanovila vizuálně po 24 a 48 hodinách inkubace při 35 °C jako nejnižší koncentrace inhibující viditelný růst v porovnání s kontrolou.[103]

4.3.4. Hodnocení antifungální aktivity

Antifungální aktivita byla také hodnocena na katedře Biologických a lékařských věd Farmaceutické fakulty v Hradci Králové. Pro testování bylo použito standardní mikrodiluční bujonové metody v 96-ti jamkových mikrotitračních deskách. Mezi

testované kmeny byly zařazeny *Candida albicans* ATCC 44859, *Candida tropicalis* 156, *Candida krusei* E28, *Candida glabrata* 20/I, *Trichosporon asahii* 1188, *Aspergillus fumigatus* 231, *Lichtheimia corymbifera* 272 a *Trichophyton mentagrophytes* 445. Kultivace probíhala na Sabouraudově glukózovém agaru při 35 °C ve tmě a humidní atmosféře.

Sloučeniny se rozpustily v DMSO a naředily RPMI 1640 růstovým médiem s glutaminem a pH 7,0. Kolonie z agaru byla roztřepána ve sterilní vodě. Hustota inokula se určuje Bürkerovou komůrkou a hodnota vhodná pro testování je 1,0-2,5x10^{-5} CFU/ml (u vláknitých hub 100 konidií na 100 čtverců). Testovaná koncentrace látek byla v rozmezí 500-0,49 μmol/l, zároveň koncentrace DMSO nepřesáhla 1 % (V/V) a neovlivnila růst kvasinek a hub. Jako standardy pro testování byly použity flukonazol (FLU), amfotericin B (AMB), vorikonazol (VOR) a nystatin (NYS). MIC se stanovila vizuálně po 24 a 48 hodinách inkubace při 35 °C jako nejnižší koncentrace inhibující růst v porovnání s kontrolou resp. po 72 a 120 hodinách u vláknitých hub.[104]

4.3.5. Hodnocení antivirové aktivity

Antivirová aktivita byla hodnocena ve spolupráci s prof. Lieve Neasens z Rega Institute for Medical Research – KU Leuven. Použité viry byly ze skupiny RNA i DNA virů a zároveň byla stanovena cytotoxicita na vybraných buněčných liniích. Metodika je obsáhlá a přesahuje rámec této disertační práce.[106-107]

4.3.6. Hodnocení herbicidní aktivity

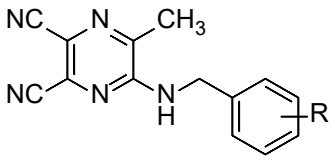
Toto stanovení probíhalo ve spolupráci s fakultou Přírodních věd Komenského Univerzity v Bratislavě pod vedením doc. Katariny Kráľové. Herbicidní aktivita byla měřena jako inhibice fotosyntetického elektronového transportu v chloroplastech rostliny *Spinacea oleracea*. Míra inhibice byla měřena pomocí fotoredukce 2,6-dichlorfenol-indofenolu (elektronový akceptor). Sloučeniny byly rozpuštěny v DMSO, jehož finální koncentrace nepřesáhla 4 % a neovlivnila tak fotochemickou aktivitu chloroplastů. Účinnost látek byla vyjádřena jako 50% inhibiční koncentrace IC_{50} v porovnání s kontrolou.[108,109]

5. KOMENTÁŘ PUBLIKOVANÝCH VÝSLEDKŮ

Výsledky uvedené v této disertační práci byly publikovány formou čtyř článků v odborných vědeckých časopisech, jednoho článku po úpravě odeslaného do redakce (čeká se na konečné vyjádření), dvou příspěvků na elektronických konferencích a také formou jednoho českého patentu, který byl následně uznán i jako evropský, a prošly náročným recenzním řízením. Proto nebudou uvedeny klasicky ve formě monografie, ale jako komentář a diskuze těchto výsledků. Budou komentovány případné vztahy struktura – účinek (SAR) připravených sloučenin s odkazem na danou publikaci. Detaily syntetické a analytické jsou v plném znění uvedeny ve článcích tvořících přílohy této disertační práce. Ostatní sdělení (plakátová sdělení a přednášky na odborných vědeckých konferencích) jsou uvedeny v seznamu všech výstupů. Pro účely komentáře byly připravené sloučeniny rozděleny do skupin dle struktury výchozí látky.

5.1. DERIVÁTY VYCHÁZEJÍCÍ Z 5-CHLOR-6-METHYLPYRAZIN-2,3-DIKARBONITRILU

5.1.1. 5-Chlor-6-methylpyrazin-2,3-dikarbonitril substituovaný benzylaminy (komentář k příloze č. 1)



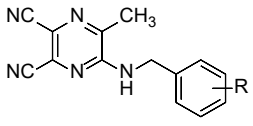
*Obrázek **9** – Struktura benzylamino derivátů odvozených od 5-chlor-6-methylpyrazin-2,3-dikarbonitrilu*

Jedná se o sérii 15 sloučenin, které jsou tvořeny dvěma aromatickými jádry spojenými aminomethylenovým můstkem (-$NHCH_2$-) (Obrázek **9**). Pyrazinové jádro je substituováno dvěma karbonitrilovými skupinami, které lze považovat za analoga karboxamidové funkce PZA.

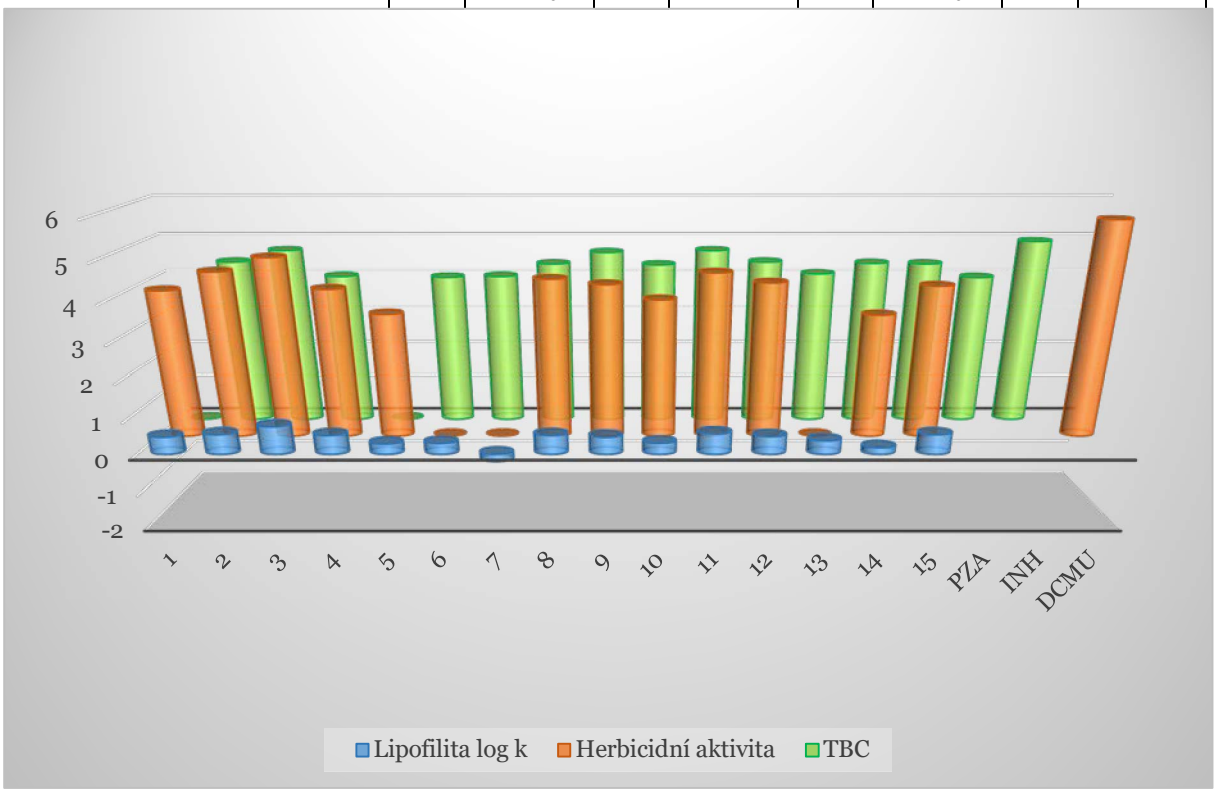
Syntéza finálních produktů byla prováděna v mikrovlnném reaktoru s fokusovaným polem. Výchozí látka byla rozpuštěna v methanolu, byl přidán benzylamin v dvojnásobném molárním množství a ekvimolární množství pyridinu. Celá směs byla zahřívána ve speciální silnostěnné skleněné zkumavce uzavřené septem určené pro mikrovlnný reaktor. Podmínky byly stanoveny experimentálně a jsou následující: teplota zahřívání 150 °C, doba reakce 30 minut, výkon 120 W. Produkty byly následně přečištěny pomocí preparativní chromatografie a analyticky

charakterizovány. Výtěžky se pohybovaly v rozmezí 17 až 62 %. 14 produktů bylo syntetizováno nově, jeden byl již dříve charakterizován v literatuře.

Většina látek z této série vykázala antimykobakteriální aktivitu proti kmeni *M. tbc* H37Rv. Ta se pohybovala v rozmezí 6,25-25 μg/ml resp. 19,6-100,3 μM, z čehož nejúčinnější sloučeniny byly substituovány na benzylaminové části molekuly elektron-akceptorovými skupinami (3,4-Cl; 2-Cl; 4-CF_3). Ostatní sloučeniny vykazující aktivitu také obsahovaly ve své molekule elektron-akceptorové skupiny v podobě halogenu či trifluormethylové skupiny. Naopak sloučeniny s methylovou či methoxy substitucí vykázaly aktivitu minimální až nulovou. Pro porovnání biologické aktivity byly zvoleny dva standardy – pyrazinamid (12,5 μg/ml, resp. 101,5 μM) a isoniazid (1,56 μg/ml, resp. 11,4 μM). Výsledky jsou pro porovnání znázorněny v Obrázku **10**. Pouze dvě sloučeniny vykázaly aktivitu proti atypickým mykobakteriím (*M. kansasii* a *M. avium*), ale její hodnota byla zanedbatelná.



	R		R		R		R
1	2-CH_3	**5**	4-OCH_3	**9**	2-Cl	**13**	2,4-OCH_3
2	3-CF_3	**6**	H	**10**	2-F	**14**	3-NO_2
3	3,4-Cl	**7**	4-NH_2	**11**	4-CF_3	**15**	4-Cl
4	4-CH_3	**8**	3-Cl	**12**	2-CF_3		



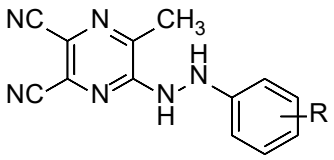
*Obrázek **10** – Aktivita sloučenin vyjádřená jako log (1/MIC) resp. log (1/IC_{50}) [M] ze série benzylamino derivátů odvozených od 5-chlor-6-methylpyrazin-2,3-dikarbonitrilu a porovnání s experimentálně stanovenou lipofilitou (TBC – M. tuberculosis H37Rv)*

Nebyla pozorována aktivita vůči rychle rostoucím mikroorganismům, např. proti *M. smegmatis* či testovaným kmenům bakterií a hub.

V této sérii byla zkoumána i herbicidní aktivita připravených sloučenin, která byla měřena jako míra inhibice fotosyntetického elektronového transportu. Většina látek vykázala aktivitu, která však byla ve srovnání s průmyslově používaným standardem Diurone® (DCMU; 3-(3,4-dichlorfenyl)-1,1-dimethylmočovina) nižší. Nejlepší aktivitu ukázala sloučenina 5-[(3,4-dichlorbenzyl)amino]-6-methylpyrazin-2,3-dikarbonitril a to přibližně devětkrát nižší než zmiňovaný standard (IC_{50}=16,4 μM). Nezůstala ale bez povšimnutí podobnost s DCMU, tedy 3,4-dichlor substituce na fenylovém jádře, která může být pro účinek velice vhodná.

V rámci této série byla zjištěna antimykobakteriální i herbicidní aktivita, která byla v případě té herbicidní závislá na lipofilitě připravených sloučenin. Tento vztah lze vyjádřit jako přímou lineární závislost. Zároveň byly určeny i vhodné substituenty ovlivňující pozitivně aktivitu. Jako nejvhodnější se jevil 3,4-dichlorovaný derivát (sloučenina **3**), který vykázal nejlepší aktivitu v obou biologických hodnoceních. Další skupinou s pozitivním efektem byla trifluormethylové skupina v poloze 4- fenylového jádra, případně v poloze 3- fenylové jádra.

5.1.2. 5-Chlor-6-methylpyrazin-2,3-dikarbonitril substituovaný aromatickými fenylhydraziny (komentář k příloze č. 2)



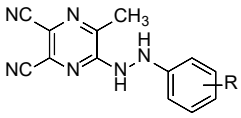
*Obrázek **11** – Struktura aromatických fenylhydrazino derivátů odvozených od 5-chlor-6-methylpyrazin-2,3-dikarbonitrilu*

Jedná se o sérii pěti sloučenin, které jsou tvořeny dvěma aromatickými jádry spojenými hydrazinovým můstkem (-NHNH-), s obecnou strukturou znázorněnou na Obrázku **11**.

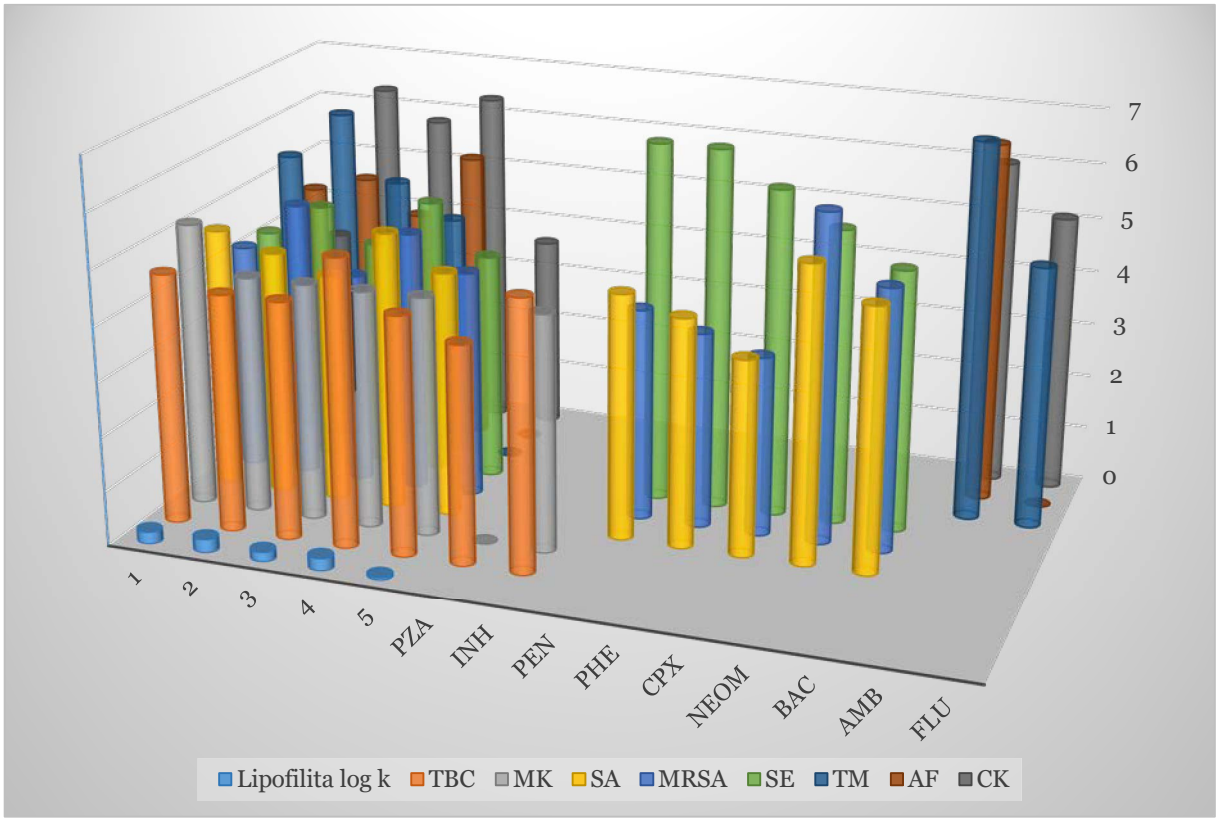
Syntéza finálních produktů byla opět prováděna v mikrovlnném reaktoru s fokusovaným polem. Výchozí látka byla rozpuštěna v methanolu, byl přidán odpovídající fenylhydrazino derivát v trojnásobném molárním množství a pyridin v ekvimolárním množství. Celá směs byla zahřívána ve speciální silnostěnné zkumavce uzavřené septem za podmínek stanovených experimentálně: teplota zahřívání 140 °C,

doba reakce 30 minut, výkon 120 W. Produkty byly následně přečištěny pomocí preparativního chromatografu a analyticky charakterizovány. Výtěžky se pohybovaly v rozmezí 21 až 67 %.

Všechny sloučeniny z této série vykázaly antimykobakteriální aktivitu, ať už proti kmeni *M. tbc* H37Rv či proti ostatním atypickým mykobakteriím. Ta se pohybovala v rozmezí 1,56-100 μg/ml (5,5-338,7 μM). Nejúčinnější sloučenina **4** (1,56 μg/ml) proti kmeni *M. tbc* byla substituována v poloze 4- fenylového jádra chlorem. Takto vysoká aktivita byla zjištěna i u sloučeniny nesubstituované a to vůči kmeni *M. kansasii*. To je velice výhodné s ohledem na to, že PZA je proti tomuto kmeni neúčinný a INH má aktivitu sníženou (6,25-12,5 μg/ml).



	R		R		R
1	H	**3**	2-Cl	**5**	2-NO_2
2	3-Cl	**4**	4-Cl		



*Obrázek **12** – Aktivita sloučenin vyjádřená jako log (1/MIC) [M] ze série fenylhydrazino derivátů odvozených od 5-chlor-6-methylpyrazin-2,3-dikarbonitrilu v porovnání s experimentálně zjištěnou lipofilitou (TBC – M. tuberculosis H37Rv; MK – M. kansasii; SA – Staphylococcus aureus; MRSA – methicilin rezistentní Staphylococcus aureus; SE – Staphylococcus epidermidis; TM – Trichophyton mentagrophytes; AF – Aspergillus fumigatus; CK – Candida krusei)*

V rámci této série byla pozorována i aktivita antibakteriální a antifungální. V souvislosti s antibakteriálním skríningem byla zjištěna zajímavá selektivita vůči grampozitivním mikroorganismům a to především u rodu *Staphylococcus*. Aktivita

byla zjištěna u všech testovaných sloučenin a nejzajímavějším zjištěním byl fakt, že připravené látky inhibují růst methicilin rezistentního kmene *Staphylococcus aureus* (MRSA). Sloučenina **2** substituovaná chlorem v poloze 3- a sloučenina **4** substituovaná chlorem v poloze 4- fenylového jádra svou aktivitou předčily i použité standardy.

Antimykotickou aktivitu prokázaly opět všechny testované látky, ale tato se lišila v rámci různých kmenů. Někdy bylo dosaženo i hodnot lepších než představovaly antifungální standardy. Nejvýraznější inhibice bylo patrná u kmenů *Candida krusei*, *Aspergillus fumigatus* a *Trichophyton mentagrophytes*.

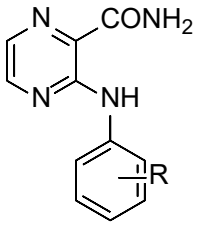
Tato série byla testována i na antivirovou aktivitu. Ta však byla zjištěna pouze u jedné sloučeniny, a to 2-nitro derivátu proti chřipkovému viru A/H1N1, viru vezikulární stomatitidy a kočičímu herpesviru v rozmezí 2,4-9,2 μM.

Problémem těchto sloučenin může být jejich cytotoxicita. Nasvědčují tomu i hodnoty IC_{50} pro lidské hepatocyty HepG2 (21,9 a 27,8 μM pro nejúčinnější látky). Při výpočtu indexu selektivity (SI), který se využívá pro určení bezpečnosti léčiva (hodnota poměru cytotoxicity a MIC by měla být vyšší než 10), však byl pro nejúčinnější sloučeninu stanoven jako SI = 14. Z čehož vyplývá, že je tato sloučenina použitelná.

Připravená série se ukázala být velice slibnou, proto jsme přistoupili k patentové ochraně připravených a charakterizovaných sloučenin a jejich zjištěných biologických aktivit. Byla prokázána antimykobakteriální, antibakteriální a antifungální aktivita, která byla v několika případech lepší než použité standardy (viz Obr. **12**). Jedna ze sloučenin vykázala i antivirovou aktivitu. V rámci této skupiny látek není možné definovat vztah mezi lipofilitou a aktivitou. Částečně je to způsobeno menším množstvím připravených sloučenin oproti ostatním sériím. Nejvyšší aktivita byla pozorována u sloučenin substituovaných chlorem, a to především v poloze 3- a 4- fenylhydrazinového jádra. Jistou nevýhodou těchto sloučenin může být a je jejich toxicita zjištěná na buňkách HepG2. Na druhou stranu nezůstává bez povšimnutí selektivita těchto sloučenin proti grampozitivním bakteriím. To by mohlo svědčit pro mechanismus účinku spojený s ovlivněním konkrétního aparátu v bakteriální buňce a ne pro obecný účinek závislý na zmíněné cytotoxicitě.

5.2. DERIVÁTY VYCHÁZEJÍCÍ Z 3-CHLORPYRAZIN-2-KARBOXAMIDU

5.2.1. 3-Chlorpyrazin-2-karboxamid substituovaný aromatickými aminy (komentář k příloze č. 3)



*Obrázek **13** – Struktura aromatických amino derivátů odvozených od 3-chlorpyrazin-2-karboxamidu*

Série 16 sloučenin, které tvoří pyrazinové a fenylové jádro spojené sekundárním aminovým dusíkem. 15 produktů bylo syntetizováno nově, jeden již byl popsán v literatuře. Na pyrazinovém jádře se dále vyskytuje volná karboxamidová funkce.

Tato série (obecná struktura na Obrázku **13**) byla prvotně publikována s cílem vyzdvihnout výhody mikrovlnné syntézy a optimalizovat podmínky pro aminodehalogenační reakci. Výsledky biologického skríningu budou po dokončení všech testů publikovány v další práci.

Byly porovnány metody konvenčního zahřívání v rámci klasické organické chemie při 110 °C v toluenu a metody mikrovlnné syntézy. Bylo použito několik proměnných. Kromě již zmíněného typu zahřívání to byla také různá rozpouštědla (a na jejich základě i teploty), různé báze, různý reakční čas a nakonec i použití ochlazovací funkce mikrovlnného reaktoru s fokusovaným polem. Tlak zde sloužil jako indikátor stability systému. Pokud by došlo k nadměrnému zvýšení, reakce by byla předčasně ukončena z bezpečnostních důvodů.

Porovnáním dvou základních metod ohřevu, tedy konvenčního a mikrovlnného (MW), bylo zjištěno, že mikrovlnami asistované reakce jsou výrazně výhodnější, a to z hlediska úspory času, úspory rozpouštědel, zvýšení výtěžků a možnosti využití přetlakového systému. Tento uzavřený systém umožňuje využívat nízkovroucí rozpouštědla při teplotách mnohonásobně přesahujících teplotu varu.

Použitá rozpouštědla pro MW syntézu byla tetrahydrofuran a methanol. Obě tato rozpouštědla mají za atmosférického tlaku podobný bod varu (66,0 °C pro THF; 64,7 °C pro MeOH). V rámci reaktoru však byl rozdíl v dosažených teplotách značný. U tetrahydrofuranu nebylo možné dosáhnout teplot vyšších jak 80 °C, jelikož kvůli vznikajícímu přetlaku systém z bezpečnostních důvodů reakci ukončil. Methanol byl

při 140 °C stále použitelný. Zároveň je vhodnější pro použití z důvodu své polarity. Připravené sloučeniny vykázaly lepší rozpustnost v polárnějším rozpouštědle. Ohřev pomocí mikrovln je také efektivnější díky existujícímu dipólu v molekule methanolu.

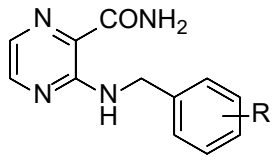
Triethylamin a pyridin, použité jako báze, při konvenčních metodách nevykazovaly prakticky žádný rozdíl ve výtěžcích. Při použití těchto bází v mikrovlnném reaktoru však rozdíl vznikal. Triethylamin je v tomto prostředí nestabilní a dochází k rozkladné reakci.[110] Vzniklé reaktanty (především diethylamin) mohou dále reagovat s výchozími sloučeninami a poskytovat tak nechtěné vedlejší produkty. V tomto případě by se mohlo jednat o 3-diethylaminopyrazin-2-karboxamid. Po preparativní flash chromatografii bohužel nebyl vedlejší produkt izolován a nemohl tak být charakterizován. Pyridin je naopak i při vyšších teplotách stabilní a umožňuje provádět reakce při 140 °C. Z reakční směsi je navíc snadno odstranitelný.

Vliv času na výtěžnost reakcí neměl už takový vliv v rámci porovnávaných hodnot (20, 30 a 45 min). Avšak rozdíly tu byly a nejlepších výsledků bylo dosaženo u reakcí probíhajících 30 minut.

Použití speciální funkce PowerMAX mikrovlnného reaktoru CEM Discover, která zajistí průběžné chlazení celé směsi proudem vzduchu po dobu reakce, a tím zajistí i vyšší výkon dodávaných MW bez nežádoucího nárůstu reakční teploty nad stanovený limit, prokázalo značnou výhodu. Výtěžky těchto reakcí se ve většině případů téměř zdvojnásobily.

Celkově došlo tedy k optimalizaci reakčních podmínek pro aminodehalogenační reakci, které vyšly takto: methanol jako rozpouštědlo, pyridin jako báze, teplota 140 °C, reakční doba 30 minut, 120 W výkon s použitím průběžného chlazení (dle záznamu z reaktoru). Bez průběžného chlazení byl výkon po zahřátí na stanovenou teplotu snížen a pohyboval se v rozmezí 10 až 20 W.

5.2.2. 3-Chlorpyrazin-2-karboxamid substituovaný benzylaminy (komentář k příloze č. 4)



*Obrázek **14** – Struktura benzylamino derivátů odvozených od 3-chlorpyrazin-2-karboxamidu*

Jedná se o sérii 15 sloučenin, které jsou tvořeny opět dvěma aromatickými jádry spojenými aminomethylenovým můstkem (-$NHCH_2$-). Část těchto látek (šest sloučenin) byla připravena v rámci diplomové práce Deriváty pyrazinu jako potenciální antituberkulotika II.[111] Pyrazinové jádro obsahuje volnou karboxamidovou skupinu, vůči které je benzylaminový zbytek situován v poloze 3-. Je zde tak na první pohled patrný motiv pyrazinamidu (viz Obr. **14**).

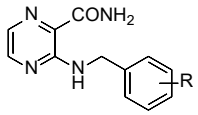
Syntéza finálních produktů byla tedy rozdělena na dvě části. Šest derivátů bylo připraveno pomocí metod organické syntézy s konvenčním ohřevem. Výchozí látka byla rozpuštěna v THF, byl přidán TEA (báze) v ekvimolárním množství a dvojnásobné molární množství benzylaminu. Reakce probíhala za refluxní teploty (70 °C) po dobu 15 hodin. Po přečištění se výtěžky pohybovaly v rozmezí 24 až 50 %. Druhá část sloučenin byla syntetizována pomocí mikrovlnného reaktoru s fokusovaným polem. Výchozí látka byla rozpuštěna ve speciální silnostěnné zkumavce v methanolu, byl přidán benzylamin v dvojnásobném molárním množství a pyridin v ekvimolárním množství. Podmínky byly stanoveny experimentálně: teplota reakce 140 °C, reakční doba 30 minut, výkon reaktoru 120 W. Produkty byly podrobeny preparativnímu chromatografickému přečištění a analyticky charakterizovány. Výtěžky se pohybovaly v rozmezí 26 až 80 % s tím, že nejnižší výtěžky byly dosaženy u substituce nitroskupinou v poloze *meta* fenylového jádra, což je časté a obvyklé. Důvodem je deaktivace jádra záporným mezomerním efektem této skupiny.

Pouze čtyři sloučeniny byly aktivní v testování proti kmeni *Mycobacterium tuberculosis* H37Rv. Aktivita se pohybovala v rozmezí 1,56-12,5 μg/ml resp. 6,4-42,1 μM (viz Obr. **15**). Dvě sloučeniny byly substituovány na benzylaminové části molekuly trifluormethylovou skupinou, která se již ukázala jako vhodný substituent pro aktivitu ve skupině 5-chlor-6-methylpyrazin-2,3-dikarbonitrilu substituovaného benzylaminy, ale zároveň i pro zvýšení cytotoxicity. Zbylé aktivní látky obsahovaly substituenty, jež účinnost ne vždy zvyšují (viz série 5-chlor-6-methylpyrazin-2,3-dikarbonitrilu substituovaného benzylaminy, kap. 5.1.1.), v tomto případě však výjimka potvrzuje pravidlo. V kontrastu s trifluormethylovou substitucí, methylová a aminoskupina výrazně snížily toxicitu. Standardy použité pro testování – pyrazinamid (12,5 μg/ml, resp. 101,5 μM) a isoniazid (0,39 μg/ml, resp. 2,8 μM). Nejúčinnější látka (4-CH_3 derivát) byla svou aktivitou srovnatelná s isoniazidem a terapeutický index (SI), vypočítaný na základě hodnot IC_{50} získaných testováním cytotoxicity na buňkách HepG2 a hodnotě MIC, splnil požadavky na bezpečné léčivo, tedy hodnota SI byla vyšší

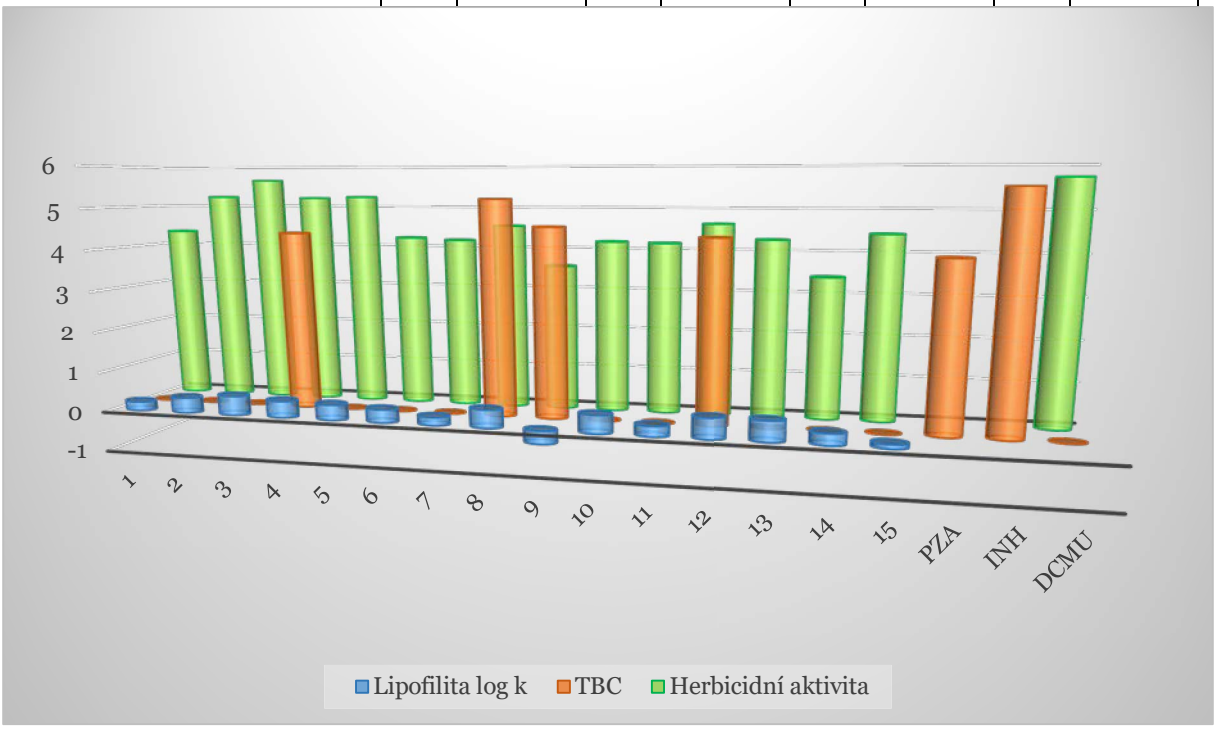
než 10 (konkrétně >250, z důvodu horší rozpustnosti nebylo možné určit přesnou hodnotu). Sloučenina obsahující trifluormethylovou skupinu poloze 3- účinkovala i proti *M. kansasii* (25 μg/ml).

Aktivita vůči kmenům *M. avium* a *M. smegmatis* nebyla pozorována. Taktéž antifungální aktivita nebyla zaznamenána.

Některé deriváty byly účinné proti dvěma kmenům bakterií (*Staphylococcus aureus*, *Enterococcus faecalis*). Společným jmenovatelem byla substituce elektron-akceptorovými skupinami s výjimkou sloučeniny, kde bylo fenylové jádro substituováno dvěma methoxylovými skupinami, které jsou řazeny mezi elektron-donorové. Aktivita byla však ve srovnání se standardy nízká s hodnotami nepřesahujícími 31,25 μM.



	R		R		R		R
1	H	**5**	4-Cl	**9**	4-NH_2	**13**	2-CF_3
2	3-Cl	**6**	2-CH_3	**10**	2-Cl	**14**	2,4-OCH_3
3	3,4-Cl	**7**	4-OCH_3	**11**	2-F	**15**	3-NO_2
4	3-CF_3	**8**	4-CH_3	**12**	4-CF_3		

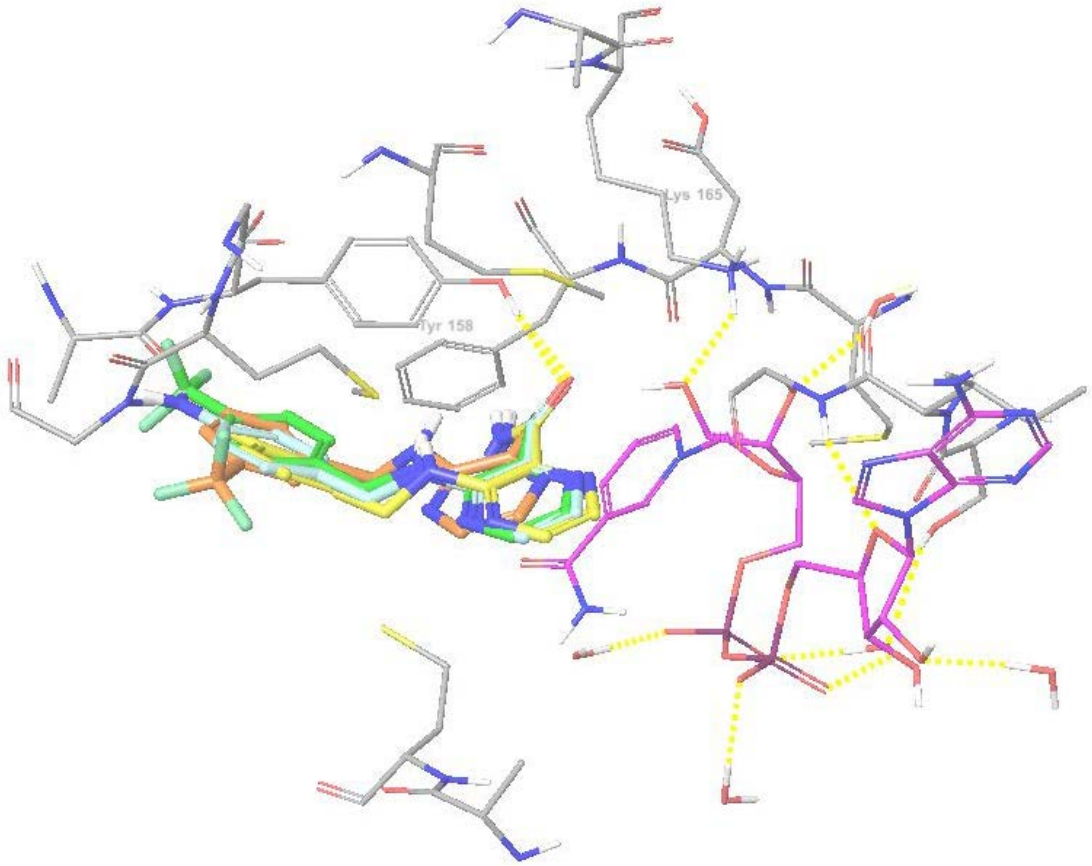


*Obrázek **15** – Aktivita sloučenin vyjádřená jako log (1/MIC) resp. log (1/IC_{50})[M] ze série benzylamino derivátů odvozených od 3-chlorpyrazin-2-karboxamidu v porovnání s experimentálně zjištěnou lipofilitou (TBC – M. tuberculosis H37Rv)*

Prakticky všechny sloučeniny inhibovaly fotosyntetický elektronový transport v chloroplastech rostliny *Spinacea oleracea*. Míra této inhibice byla až na výjimky závislá na lipofilitě. Aktivita některých látek se přiblížila aktivitě standardu Diurone®.

Nejlepší aktivitu ukázala látka **3** 3-[(3,4-dichlorbenzyl)amino]-pyrazin-2-karboxamid, a to srovnatelnou se standardem. Je zde patrná i podobnost s DCMU, tedy 3,4-dichlor substituce na fenylovém jádře, která se pro herbicidní účinek ukázala být velice vhodná. Tento fakt byl zaznamenán i u série benzylamino derivátů odvozených od 5-chlor-6-methylpyrazin-2,3-dikarbonitrilu (kap. 5.1.1.).

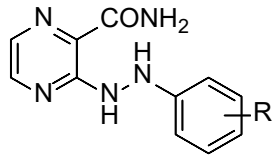
Látky z této série byly podrobeny *in silico* testování formou dokování do mykobakteriálního enzymu enoyl-ACP reduktasy za účelem zjištění možného místa zásahu potenciálních léčiv (viz Obr. **16**). Pro nejúčinnější deriváty byla vypočítána vazebná afinita do aktivního místa enzymu. Ta se svou hodnotou blížila známým inhibitorům. Byly zjištěny stejné vazebné interakce, které jsou pro tento mechanismus účinku nezbytné. Avšak není možné prohlásit, zda jsou tyto sloučeniny inhibitorem či ne. Doposud získané výsledky tuto hypotézu nemohou potvrdit ani vyvrátit. Proto jsou nutné další experimenty, nejlépe inhibiční studie na izolovaném enzymu.



*Obrázek **16** – Kavita enoyl-ACP reduktasy s nadokovanými nejúčinnějšími sloučeninami (oranžová – 3-CF_3 derivát; žlutá – 4-CH_3 derivát; tyrkysová – 4-NH_2 derivát; zelená – 4-CF_3 derivát)*
Orientace benzylaminového jádra směřuje ven z kavity namísto dovnitř aktivního místa
Dokováno pomocí Schrödinger Suite 2014-2, vizualizace pomocí programu Maestro 9.8

Antituberkulotické *in vitro* testování potvrdilo aktivitu u několika látek, jejichž souhrn je uveden na Obrázku **15**. 3-[(4-Methylbenzyl)amino]-pyrazin-2-karboxamid byl svou aktivitou srovnatelný s isoniazidem a zároveň neprojevil *in vitro* cytotoxický efekt na buňkách HepG2. Zároveň se nepotvrdila účinnost vůči ostatním mikroorganismům, z čehož lze vyvodit specifický mechanismus účinku. Herbicidní aktivita se projevila napříč celou sérií a kulminovala opět u derivátů s elektron-akceptorovou substitucí. Hodnoty srovnatelné se standardem dosáhla látka 3-[(3,4-dichlorbenzyl)amino]-pyrazin-2-karboxamid.

5.2.3. 3-Chlorpyrazin-2-karboxamid substituovaný aromatickými fenylhydraziny (komentář k příloze č. 2)



*Obrázek **17** – Struktura aromatických fenylhydrazino derivátů odvozených od 3-chlorpyrazin-2-karboxamidu*

Jedná se opět o sérii pěti sloučenin s obecnou strukturou znázorněnou na Obr. **17**, která obsahují dvě aromatická jádra a spojovací hydrazinový můstek (-NHNH-).

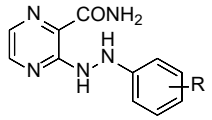
Syntéza těchto látek, urychlená v mikrovlnném reaktoru, vycházela z 3-chlorpyrazin-2-karboxamidu. Ten byl rozpuštěn v methanolu, byl přidán odpovídající aromatický fenylhydrazin v trojnásobném molárním množství a ekvimolární množství pyridinu. Celá směs byla zahřívána s použitím podmínek stanovených experimentálně: reakční teplota 140 °C, reakční doba 30 minut, výkon 120 W. Purifikované produkty byly charakterizovány analytickými metodami. Výtěžky chromatograficky čistého produktu se pohybovaly v rozmezí 17 až 42 %.

Celá skupina připravených látek působila na kmen *M. tbc* H37Rv. Vůči ostatním testovaným kmenům mykobakterií byly tyto sloučeniny neúčinné. Aktivita se pohybovala v rozmezí 6,25-12,5 μg/ml, resp. 23,7-54,5 μM. Tyto hodnoty jsou při srovnání se standardy povzbudivé (INH – 2,8 μM; PZA – 203,1 μM) a zároveň je zde patrná i jistá selektivita. Nejúčinnější sloučenina obsahovala na jádře chlor v poloze 2-. Tedy opět pozitivní vliv elektron-akceptorového substituentu.

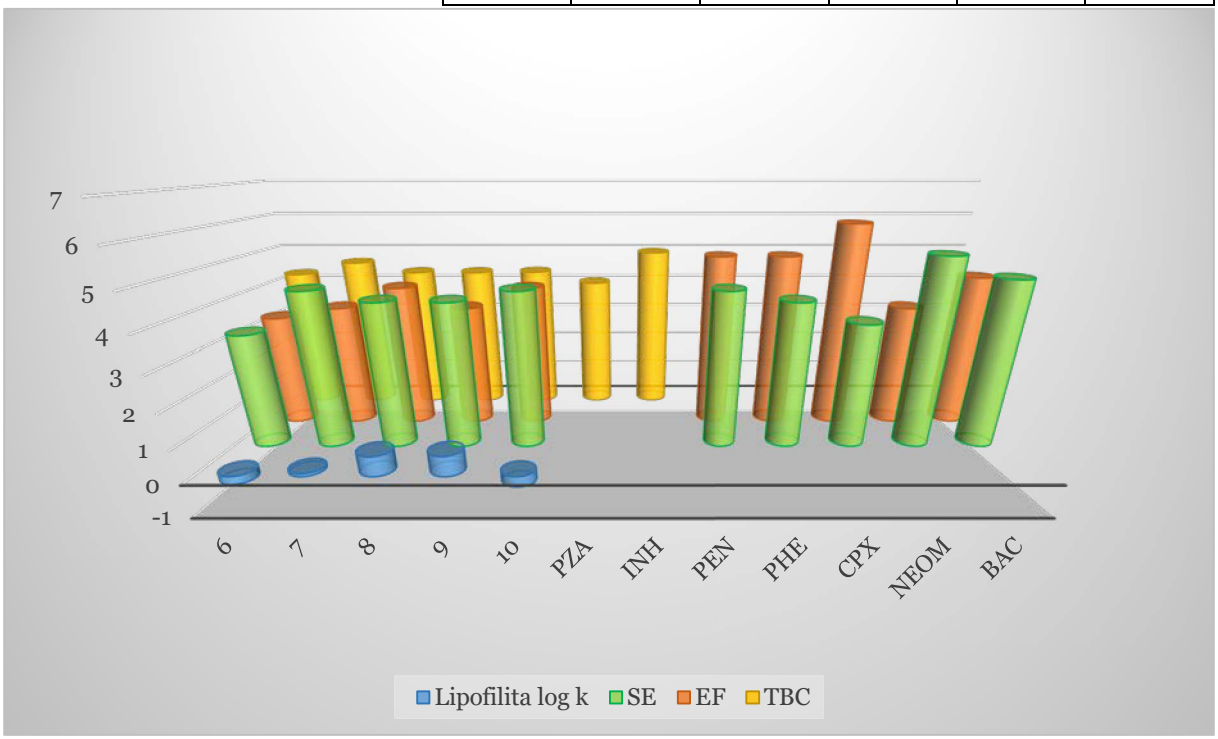
Antimykotická aktivita nebyla na rozdíl od antibakteriální pozorována prakticky žádná. Antibakteriální účinek byl zaznamenán pouze u vybraných kmenů. To je do jisté míry znak selektivity a pravděpodobně i specifického mechanismu účinku. Ovlivněné

kmeny (*Staphylococcus epidermidis* a *Enterococcus faecalis*) jsou řazeny do skupiny grampozitivních mikroorganismů. Podobný trend byl zaznamenán i ve skupině dikarbonitrilových derivátů (viz kapitola 5.1.2.), zde je však aktivita výrazně nižší (31,25-500 μM).

Na druhou stranu tyto sloučeniny vykázaly oproti látkám obsahujícím karbonitrilovou skupinu výrazně nižší cytotoxicitu. Hodnota IC_{50} pro nejúčinnější sloučeniny byla až o dva řády lepší a index selektivity pro antituberkulotickou aktivitu překročil požadovanou minimální hodnotu 10 pro potenciálně bezpečná léčiva (69 pro nesubstituovaný derivát a 77 pro 2-Cl derivát).



	R		R		R
6	H	**8**	3-Cl	**10**	2-NO_2
7	2-Cl	**9**	4-Cl		

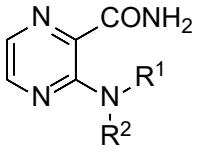


*Obrázek **18** – Aktivita sloučenin vyjádřená jako log (1/MIC) [M] ze série fenylhydrazino derivátů odvozených od 3-chlorpyrazin-2-karboxamidu v porovnání s experimentálně zjištěnou lipofilitou (SE – Staphylococcus epidermidis; EF – Enterococcus faecalis; TBC – M. tuberculosis H37Rv)*

Zjištěná selektivní antimykobakteriální aktivita vůči kmenu *M. tbc* H37Rv, která byla lepší než u standardu PZA, posunula tuto sérii mezi látky s vyšším potenciálem. Zároveň i stanovená toxicita svědčí pro možné použití těchto látek v dalším vývoji. Dalším pozitivem je selektivita vůči grampozitivním bakteriím (a to pouze některým), což znovu nastiňuje možnost konkrétního mechanismu účinku a ne pouze nespecifického účinku založeného na toxicitě látek. Rozdíl mezi touto sérií a sérií se substituovaným pyrazindikarbonitrilem je poloha substituentu, kdy se jako nejlepší

kandidát projevil chlor v poloze 2- fenylhydrazinového jádra, kdežto u série odvozené od dikarbonitrilu to byl chlor v poloze 3- a 4- fenylhydrazinového jádra (viz Obr. **18**).

5.2.4. 3-Chlorpyrazin-2-karboxamid substituovaný alifatickými a alicyklickými aminy (komentář k příloze č. 5)



*Obrázek **19** – Struktura alkylamino derivátů odvozených od 3-chlorpyrazin-2-karboxamidu*

Série 18 sloučenin, které jsou typické volnou karboxamidovou funkcí v poloze 2- pyrazinového jádra a alifatickým či alicyklickým řetězcem navázaným přes sekundární, příp. terciární amino skupinu v poloze 3- (Obr. **19**).

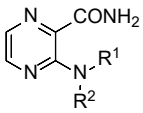
Příprava těchto látek spočívala v aminodehalogenační reakci výchozí látky s alifatickými aminy v prostředí methanolu. Aminy byly do reakce přidávány ve dvojnásobném molárním množství. I přes předpoklad toho, že samotné aminy mohou fungovat jako báze, tak byl do reakční směsi přidáván pyridin v ekvimolárním množství. Výsledné hodnoty výtěžků byly vyšší než bez použití pyridinu. Samotná reakce probíhala za účasti mikrovln a podmínky byly stanoveny experimentálně (teplota 140 °C, reakční čas 30 min, výkon reaktoru 120 W). Výtěžnost reakcí se pohybovala v rozmezí 50 až 96 %. 16 produktů bylo syntetizováno *de novo* a dvě látky byly již předtím popsány v literatuře.

Biologická aktivita těchto sloučenin byla omezena především na herbicidní působení a částečně pak na antifungální účinnost. Antimykobakteriální skríning prokázal pouze slabou aktivitu u čtyř sloučenin s nejdelším alkylovým zbytkem (MIC = 50 μg/ml). Polohové izomery těchto látek substituované v poloze 5- pyrazinového jádra a v poloze 6- pyrazinového jádra vykázaly zpočátku podobný trend. U kratších alifatických řetězců byla aktivita nulová (MIC ≥ 100 μg/ml). S prodlužujícím se řetězcem však aktivita stoupala a deriváty s oktylovým zbytkem aktivitou předčily PZA a v případě 6-oktyl derivátu (MIC = 1,56 μg/ml; 6 μM) i INH.[85]

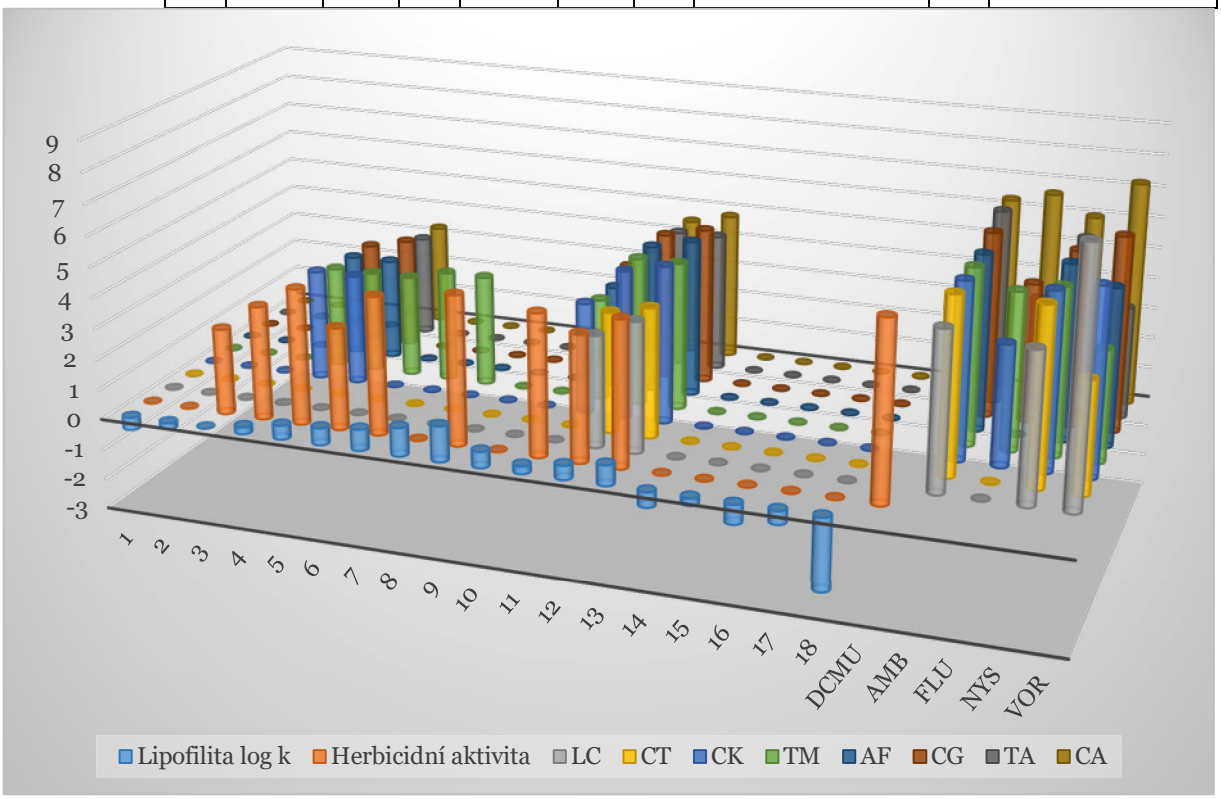
Podobný trend zvyšování aktivity byl pozorován i v rámci antibakteriálního testování, ale dosažená hodnota je zanedbatelná (MIC ≥ 250 μM).

Antimykotické testy ukázaly zajímavý fakt, že pro účinek je velice vhodná substituce nasyceným cyklickým aminem. Látky s dlouhým alkylovým řetězcem (4-7 uhlíků) byly prakticky neúčinné (MIC ≥ 250 μM). Sloučeniny se 6-ti a 7-členným nasyceným cyklem inhibovaly celé spektrum testovaných hub a kvasinek v řádech jednotek až desítek μM. Rozšíření cyklu o jeden uhlík zvýšilo aktivitu až na výjimky dvojnásobně. V porovnání s některými standardy byla aktivita výrazně vyšší.

Herbicidní hodnocení bylo co do počtu aktivních sloučenin úspěšnější. Většina látek inhibovala elektronový transport v chloroplastech. Míra inhibice byla přímo závislá na lipofilitě potažmo na délce alkylového řetězce. S rostoucí lipofilitou rostla i aktivita. Bylo zjištěno, že pro účinek je nezbytná sekundární aminoskupina, přes kterou je navázaný alkylový zbytek. Pokud je ve struktuře nahrazena terciární aminoskupinou, aktivita se vytrácí. Při srovnání s průmyslově používaným DCMU aktivita lepší nebyla.



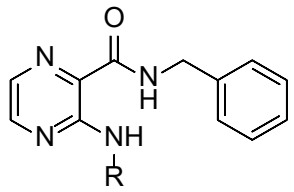
	R¹	R²		R¹	R²		R¹	R²		R¹	R²
1	CH_3	H	**6**	*t*-C_5H_{11}	H	**11**	Cyclopentyl	H	**16**	-$(CH_2)_2O(CH_2)_2$-	
2	C_2H_5	H	**7**	C_6H_{13}	H	**12**	Cyclohexyl	H	**17**	-$(CH_2)_2NCH_3(CH_2)_2$-	
3	C_3H_7	H	**8**	C_7H_{15}	H	**13**	Cycloheptyl	H	**18**	-$(CH_2)_2NH(CH_2)_2$-	
4	C_4H_9	H	**9**	C_8H_{17}	H	**14**	-$(CH_2)_4$-				
5	C_5H_{11}	H	**10**	C_4H_9	C_4H_9	**15**	-$(CH_2)_5$-				



*Obrázek **20** – Aktivita sloučenin vyjádřená jako log (1/MIC), resp. log (1/IC_{50}) [M] ze série alkylamino derivátů odvozených od 3-chlorpyrazin-2-karboxamidu v porovnání s experimentálně zjištěnou lipofilitou (LC – Lichtheimia corymbifera; CT – Candida tropicalis; CK – Candida krusei; TM – Trichophyton mentagrophytes; AF – Aspergillus fumigatus; CG – Candida glabrata; TA – Trichosporon asahii; CA – Candida albicans)*

Tato série látek se zařadila mezi sloučeniny s herbicidní a antifungální účinností (viz Obr. **20**). Bylo zjištěno, že pro antimykotické působení je nezbytný alicyklický řetězec. S rozšiřováním kruhu roste i aktivita, což může být spojeno i s rostoucí lipofilitou. Herbicidní využití by našly látky této série, které ve své struktuře obsahují fragment se sekundární aminoskupinou. Potenciál těchto látek se zvyšuje s rostoucí lipofilitou. Antimykobakteriální aktivita byla pozorována spíše u derivátů substituovaných v poloze 5- a především v poloze 6- pyrazinového jádra. Toto zjištění může vést k závěru, že uspořádání dvou skupin v těsné blízkosti je pro účinek nevýhodné a neumožňuje dostatečnou interakci s cílovou strukturou v mykobakteriální buňce. Cytotoxicita nebyla pro tyto sloučeniny zatím stanovena z důvodu relativně nízké antimykobakteriální účinnosti. Pro potenciální antimykotika je v budoucnosti třeba tento parametr stanovit.

5.2.5. 3-Chlorpyrazin-2-karboxamid substituovaný benzylaminem na karboxamidové funkci a alifatickými aminy v poloze 3- pyrazinového jádra (komentář k příloze č. 6)



*Obrázek **21** – Struktura alkylamino derivátů odvozených od N-benzyl-3-chlorpyrazin-2-karboxamidu*

Tato série s obecnou strukturou znázorněnou na Obr. **21,** složená z pěti látek byla, dokončena jako komplementární pro sloučeniny substituované alkylovým řetězcem v polohách 5- a 6- pyrazinového jádra připravené B. Servusovou-Vaňáskovou.

Deriváty byly připraveny mikrovlnami asistovanou syntézou za podmínek experimentálně stanovených (rozpouštědlo MeOH, báze pyridin, teplota 140 °C, doba reakce 30 minut, výkon 120 W). Výtěžky reakce se pohybovaly v rozmezí 51 až 75 %.

Biologické hodnocení těchto sloučenin nepřineslo žádné zajímavé výsledky. Nebyla pozorována antimykobakteriální, antibakteriální ani antifungální aktivita. Pouze výchozí látka (*N*-benzyl-3-chlorpyrazin-2-karboxamid) vykázala aktivitu proti kmeni *M. tbc* H37Rv s hodnotou 12,5 μg/ml (50 μM), která je však pouze srovnatelná s PZA.

Substituce volné karboxamidové skupiny se zde projevila jako neúčelná a nepřinesla žádné pozitivní efekty. Při porovnání substituovaného a nesubstituovaného derivátu došlo k vymizení existující aktivity. Sloučeniny s alkylovým řetězcem situovaným do poloh 5- a 6- pyrazinového jádra naopak svou aktivitu dále zvyšovaly a to v závislosti na délce řetězce resp. lipofilitě (MIC = 1,56-25 μg/ml resp. 4,6-88 μM). Bližší porovnání vlivu umístění alkylového řetězce bude diskutováno v rámci práce B. Servusové-Vaňáskové.

5.3. SHRNUTÍ A DISKUZE

Základem syntetické práce je použití mikrovlnami asistované syntézy. Podmínky pro tento způsob přípravy sloučenin byly porovnávány a optimalizovány na základě získaných výtěžků. Pro tento přístup oproti metodám s konvenčním ohřevem hovoří minimálně zdvojnásobení výtěžků. Zároveň tato metoda patří mezi tzv. metody „zelené“ chemie – je ekologicky příznivější a dnes je stále více upřednostňována. Reakční podmínky pro mikrovlnný reaktor byly také porovnány a jako ideální bylo zvoleno rozpouštědlo methanol, báze pyridin, teplota 140 °C, počáteční výkon 120 W a čas 30 minut za použití průběžného chlazení.

Porovnáním výsledků biologických hodnocení můžeme konstatovat, že výchozí látka 5-chlor-6-methylpyrazin-2,3-dikarbonitril a její deriváty vykázaly lepší aktivitu než deriváty 3-chlorpyrazin-2-karboxamidu. Toto zjištění může být podpořeno skutečností, že zavedením karbonitrilových skupin se často zvyšuje kromě účinku i cytotoxicita. Ta se pak může podílet na výsledné aktivitě. Na druhou stranu, tento předpoklad nelze uplatnit pokaždé. V sérii benzylamino derivátů odvozených od 3-chlorpyrazin-2-karboxamidu nejlepší sloučenina předčila svou aktivitou i stejně substituovaný derivát druhé výše zmíněné výchozí látky (kap. 5.1.1. a 5.2.2). V rámci této publikace byly porovnány i aktivity již dříve publikovaných derivátů s karbonitrilovou substitucí a opět sloučenina bez karbonitrilové skupiny měla lepší účinnost a zároveň velmi nízkou toxicitu.

U derivátů s druhým aromatickým jádrem lze vysledovat i vliv substituentů na aktivitu daných sloučenin. Jako nejvhodnější se až na výjimky ukázaly elektron-akceptorové skupiny (halogeny, trifluormethylové skupiny, a další). Opět tu vyvstává otázka vyšší toxicity u trifluormethyl derivátů, která je nežádoucí. Byly ale nalezeny sloučeniny aktivnější s potenciálně méně toxickými skupinami. Mezi tyto zástupce můžeme zařadit chlor deriváty (3,4-dichlor substituovaná látka vykázala vynikající

herbicidní aktivitu v obou sériích benzylamino derivátů, viz kap. 5.1.1. a 5.2.2.), amino deriváty či methyl deriváty.

Vliv spojovacího řetězce také není zanedbatelný. Sloučeniny s jádry spojenými aminomethylenovým spojovacím můstkem jsou oproti hydrazinovým derivátům méně účinné. Nabízí se zde vyšší toxicita spojená s hydraziny, ale výsledky stanovení cytotoxicity tomu neodpovídají. Toxicita není výrazně zvýšena tak, aby tyto látky nebyly vhodné pro další testování. Hydrazino deriváty jsou poměrně selektivní. U obou sérií (viz kap. 5.1.2. a 5.2.3.) je patrná selektivita vůči grampozitivním bakteriím. Látky s volnou karboxamidovou skupinou jsou také selektivní vůči kmeni *M. tbc* H37Rv. Dikarbonitrily účinkují i na celé spektrum hub, takže zde se selektivita poněkud vytrácí a vrací se do úvahy spíše cytotoxický efekt. Pro herbicidní působení lépe vystupuje uskupení -NH-CH-. U těchto látek je míra inhibice elektronového transportu měřitelná a závislá na lipofilitě (viz kap. 5.1.1. a 5.2.2.). U derivátů se spojovacím řetězcem typu -NH-NH- nebylo možné vůbec herbicidní aktivitu stanovit, jelikož tyto deriváty odbarvovaly suspenzi chloroplastů.

Vliv alkylového řetězce je také patrný. Ačkoliv tyto látky nevykázaly antimykobakteriální účinnost, vliv délky alkylového řetězce byl pozorovatelný u stanovení herbicidního efektu (viz kap. 5.2.4.). S prodlužujícím se řetězcem stoupala i aktivita. Efekt tedy může být spojován s vyšší lipofilitou, ale i s délkou řetězce, kdy se delší substituenty mohou začlenit do tylakoidní membrány a narušit tak její funkci. Cykloalkylový zbytek se naopak projevil jako velice vhodná substituce pro antimykotickou aktivitu. S rozšiřujícím se cykloalkylovým kruhem roste i antifungální aktivita. V této sérii byl také pozorován důležitý fakt, že výskyt sekundárního aminového dusíku je pro účinek esenciální. Sloučeniny obsahující terciární aminoskupinu ve formě nasycených dusíkatých heterocyklů neukázaly žádnou aktivitu.

Substituce volné karboxamidové skupiny nevedla ke sloučeninám s lepšími biologickými vlastnostmi (viz kap. 5.2.5.). Látky mající alespoň nízkou aktivitu se po substituci benzylaminem na karboxamidové funkci staly neaktivními. Vysvětlení se nabízí dvojí. Pro účinek je nezbytný volný karboxamid, který je dále v mykobakteriální buňce aktivován pyrazinamidasou. Molekula substituovaná v sousedních polohách 2- a 3- pyrazinového jádra objemnějšími substituenty nemá šanci proniknout do specifických kavit potencionálních míst zásahu. Tento předpoklad byl demonstrován na enzymu InhA, který obsahuje úzkou, specifickou kavitu, u které poziční změna substituentů znemožní vstup do aktivního místa.

6. ZÁVĚR

V úvodu disertační práce byl shrnut současný stav tuberkulózy jako onemocnění, její epidemiologie a současná farmakoterapie. Byla popsána rezistence a diskutována jako důvod pro vývoj nových antituberkuloticky působících léčiv. V návaznosti na tuto problematiku byl nastíněn přehled nových molekul zastoupených v některé z fází klinického zkoušení a nejnovější léčiva předschválená pro použití při terapii rezistentní TBC. Pro doplnění byly zmíněny i léčebné režimy používané v současnosti a doporučované WHO.

Další část byla věnována pyrazinamidu jako stěžejní molekule této práce. Byly popsány chemické vlastnosti pyrazinového jádra. Mechanismus účinku PZA je stále pod drobnohledem vědeckých skupin po celém světě. Teorií a názorů existuje již několik. PZA, potažmo POA, vykazují nespecifický, ale i specifický mechanismus účinku. Ten nespecifický je založen na fyzikálně-chemických vlastnostech těchto molekul. Specifický účinek je cílen na konkrétní, nejčastěji enzymatické, místo zásahu. Na závěr byly shrnuty již připravené deriváty PZA, které vykázaly antituberkulotickou aktivitu. Byly vyzdvihnuty vztahy mezi strukturou a zjištěnou aktivitou, které by mohli ovlivnit další směr výzkumu.

Jelikož většina sloučenin byla připravena mikrovlnami asistovanou syntézou, byl v závěru úvodní části shrnut tento moderní trend spolu s faktory, které vedly k jeho využití.

Praktická část se zaměřila na přípravu tří výchozích látek (5-chlor-6-methylpyrazin-2,3-dikarbonitrilu; 3-chlorpyrazin-2-karboxamidu; *N*-benzyl-3-chlorpyrazin-2-karboxamidu). Ty byly podrobeny aminodehalogenační reakci v mikrovlnném reaktoru za experimentálně stanovených podmínek. Bylo připraveno 79 derivátů, z čehož 75 bylo v literatuře dosud nepopsaných. Jednalo se o deriváty substituovaných aromatických aminů, benzylaminů, aromatických hydrazinů a alifatických či alicyklických aminů. Připravené sloučeniny byly podrobeny *in vitro* biologickému testování na antimykobakteriální aktivitu proti *M. tuberculosis* H37Rv, *M. kansasii*, *M. avium* a *M. smegmatis*. V každé sérii připravených látek byla nalezena alespoň jedna aktivní sloučenina proti *M. tbc*. Svou aktivitou část předčila i standard PZA. Ty nejslibnější sloučeniny se svou účinností přiblížily i aktivitě isoniazidu. Souhrn je uveden v Tabulce **9**. Byla zaznamenána i aktivita proti atypickým mykobakteriím, a to především proti *M. kansasii*, které jsou přirozeně rezistentní vůči PZA. U aktivních

substancí v jednotlivých sériích, a poté i v rámci celé práce, byly diskutovány vztahy mezi strukturou a účinkem.

Dále se testovala antibakteriální a antifungální účinnost. V několika sériích byla zjištěna aktivita a zároveň i selektivita, ať už k jednotlivým patogenům, nebo i specifickou substitucí na celou paletu mikroorganismů. Zajímavým zjištěním bylo, že cykloalkylamino substituce v poloze 3- pyrazin-2-karboxamidu vyselektovala aktivitu vůči testovaným houbám a kvasinkám.

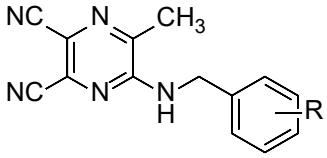
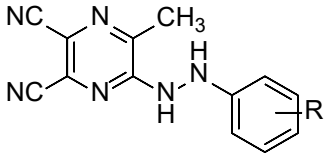
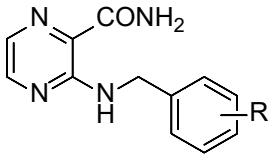
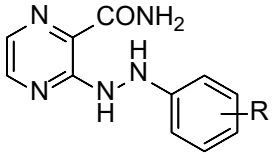
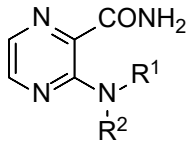
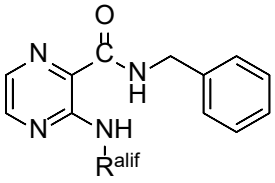
Doplňkově se testovala herbicidní aktivita, která se objevila prakticky v každé sérii, nikdy však nedosáhla hodnoty standardu.

Dalším přínosem této disertační práce je zavedení antimykobakteriálního skríningu proti rychle rostoucí mykobakterii *M. smegmatis*. Metodika byla převzata během výzkumné stáže v Cork Institute of Technology a dále upravena pro potřeby testování na katedře Farmaceutické chemie a Farmaceutické analýzy. Byly optimalizovány podmínky, pipetované objemy a další parametry. Nyní je toto hodnocení součástí standardního biologického testování na této katedře.

Dosažené experimentální výsledky byly průběžně zveřejňovány formou přednášek či plakátových sdělení na národních a mezinárodních konferencích a jako původní články v uznávaných (impaktovaných) časopisech zaměřených na farmaceutickou/medicinální chemii. Dvě série připravených a biologicky zajímavě aktivních sloučenin byly předmětem národní a mezinárodní patentové přihlášky.

Celá skupina připravených sloučenin se tak zařadila mezi látky připravené na Farmaceutické fakultě v Hradci Králové a především na katedře Farmaceutické chemie a Farmaceutické analýzy, na jejíž výzkumný záměr navázala. Získané poznatky je možno dále zúročit v přípravě nových, účinnějších a bezpečnějších derivátů.

*Tabulka **9** – Souhrn nejúčinnějších substitucí v každé připravené sérii, hodnota minimální inhibiční koncentrace MIC u kmene M. tuberculosis H37Rv, cytotoxicita IC_{50} u HepG2 buněk a index selektivity pro antimykobakteriální účinnost v porovnání se standardy*

	R	**MIC *M. tbc***	**IC_{50}/SI**
	3,4-Cl; 2-Cl; 4-CF_3	6,25 μg/ml (19,6-22,0 μM)	Nestanoveno
	4-Cl	1,56 μg/ml (5,5 μM)	26,4 μM / 17
	4-CH_3	1,56 μg/ml (6,4 μM)	> 250 μM / > 160
	2-Cl	6,25 μg/ml (23,7 μM)	481,4 μM / 77
	R^1=C_5H_{11}-C_8H_{17} R^2=H	50 μg/ml (199,7-240,1 μM)	Nestanoveno
	C_4-C_8	>100 μg/ml	Nestanoveno
PZA	pH 5,5-5,6	12,5 μg/ml (101,5 μM)	79,1 mM / 6328[112]
	pH 6,8[113]	400 μg/ml (3249,0 μM)	Nestanoveno
INH		0,39-1,56 μg/ml (2,8-11,4 μM)	30 mM / 19231-76923[114]

7. ABSTRAKT

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové

Katedra	Farmaceutické chemie a Farmaceutické analýzy
Kandidát	Mgr. Ondřej Janďourek
Školitel	Prof. PharmDr. Martin Doležal, Ph.D.
Konzultant	PharmDr. Jan Zitko, Ph.D.
Název disertační práce	Deriváty pyrazinu jako potenciální antituberkulotika (příprava a studium biologických vlastností)

Práce se zabývá deriváty pyrazinu se strukturálním vztahem k pyrazinamidu (PZA), u kterých je předpokládán antituberkulotický účinek. V úvodu této práce jsou shrnuty teoretické poznatky o tuberkulóze (TBC), její epidemiologii a především pak vznik rezistence k současně užívaným léčivům. Je nastíněn stručný přehled aktuálních antituberkulotik, dále nových sloučenin zavedených nedávno do terapie, a také nadějných molekul ve vývoji. Šířeji je popisován PZA řazený mezi léčiva 1. linie, který je stěžejní molekulou této práce. Jsou popsány možné mechanismy účinku a částečně také farmakologický profil. Na závěr je uveden stručný přehled v minulosti již připravených derivátů PZA.

Praktická část této disertační práce je zaměřena na syntézu tří výchozích sloučenin (5-chlor-6-methylpyrazin-2,3-dikarbonitril; 3-chlorpyrazin-2-karboxamid; *N*-benzyl-3-chlorpyrazin-2-karboxamid), které byly substituovány pomocí aminodehalogenační reakce v mikrovlnném reaktoru. Jedná se o 79 derivátů, z nichž je 75 nových, v literatuře ještě nepopsaných. Aminodehalogenace tří výchozích látek probíhala v přítomnosti aromatických aminů, benzylaminů, aromatických fenylhydrazinů a alifatických či alicyklických aminů. Připravené sloučeniny byly podrobeny *in vitro* antimykobakteriálnímu skríningu (*Mycobacterium tuberculosis* H37Rv, *M. kansasii*, *M. avium* a *M. smegmatis*). Některé deriváty v rámci jednotlivých sérií vykázaly aktivitu proti *M. tuberculosis* srovnatelnou nebo i lepší nežli standard PZA (MIC v rozmezí 6-24 μM). Ty nejnadějnější sloučeniny se svou aktivitou blížily i hodnotám isoniazidu. Našly se i deriváty účinkující na atypické mykobakteriální kmeny. V rámci sérií byl diskutován vztah mezi strukturou a účinkem. Většina látek

byla také doplňkově otestována na antibakteriální, antifungální, antivirovou a herbicidní aktivitu. I zde byly nalezeny sloučeniny vykazující zajímavé aktivity, především ve spojitosti s antimykotickou účinností.

Výsledky disertační práce navazují na dlouhodobý výzkumný záměr katedry Farmaceutické chemie a Farmaceutické analýzy v rámci Farmaceutické fakulty v Hradci Králové. Především pak na výsledky výzkumné skupiny prof. PharmDr. Martina Doležala, Ph.D., zabývající se studiem nových potenciálních antituberkulotik odvozených od PZA.

8. ABSTRACT

Charles University, Faculty of Pharmacy in Hradec Králové

Department of	Pharmaceutical chemistry and Pharmaceutical analysis
Candidate	Mgr. Ondřej Janďourek
Supervisor	Prof. PharmDr. Martin Doležal, Ph.D.
Consultant	PharmDr. Jan Zitko, Ph.D.
Title of Doctoral Thesis	Derivatives of pyrazine as potential antituberculars (preparation and study of biological properties)

This work is focused on pyrazine derivatives with the structural relationship to pyrazinamide (PZA) and with the potential antitubercular effect. In the introduction, there are summarized theoretical findings about tuberculosis (TBC), its epidemiology and primarily the development of resistance to current pharmacotherapy. Brief overview of known antituberculars, novel compounds and auspicious molecules in clinical trials is outlined. PZA, which is counted among the first line antitubercular agents, is widely described due to its cardinal role in this thesis. PZA possible mechanisms of action are described together with its pharmacological profile. Finally, brief summary of already prepared derivatives of PZA is stated.

Practical part of this thesis is focused on synthesis of three starting compounds (5-chloro-6-methylpyrazine-2,3-dicarbonitrile; 3-chloropyrazine-2-carboxamide; *N*-benzyl-3-chloropyrazine-2-carboxamide) that were substituted by aminodehalogenation reaction using microwave reactor. There were 79 compounds of which 75 were novel and not described in literature. Aminodehalogenation was accomplished by addition of aromatic amines, benzylamines, aromatic phenylhydrazines, and aliphatic or alicyclic amines. Prepared compounds were *in vitro* evaluated for their antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv, *M. kansasii*, *M. avium* a *M. smegmatis*. Some derivatives from prepared series demonstrated activity against *M. tuberculosis* comparable to or better than standard PZA (MIC ranging between 6 and 24 μM). The most auspicious compounds activities were approximating to the activity of isoniazid. Derivatives active against other mycobacterial species were identified. The structure-activity relationships were discussed. Most of compounds were additionally tested for their antibacterial,

antifungal, antiviral, and herbicidal activities finding compounds showing remarkable results especially in connection with antimycotic efficacy.

The results of this thesis are following the long-standing research intention of the Pharmaceutical chemistry and Pharmaceutical analysis department, Faculty of Pharmacy, Charles University. Mainly, it is connected with research group of prof. PharmDr. Martin Doležal, Ph.D. that is dealing with the study of novel potentially active antitubercular agents derived from PZA.

9. SEZNAM PUBLIKOVANÝCH VĚDECKÝCH A ODBORNÝCH PRACÍ

9.1. ČLÁNKY V IMPAKTOVANÝCH ČASOPISECH A PATENT

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<u>Příloha č. 6</u> SERVUSOVA-VANASKOVA, B.; **JANDOUREK, O.**; PATEROVA, P.; KORDULAKOVA, J.; PLEVAKOVA, M.; KUBICEK, V.; KUCERA, R.; GARAJ, V.; NEASENS, L.; KUNES, J.; DOLEZAL, M.; ZITKO, J. Alkylamino derivatives of *N*-benzylpyrazine-2-carboxamide: Synthesis and antimycobacterial evaluation. *Med Chem Comm*. **2015**, *6*, 1311-1317. doi: 10.1039/c5md00178a. IF_{2015} = 2,495.

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N-Substituted 5-Amino-6-methylpyrazine-2,3-dicarbonitriles: Microwave-Assisted Synthesis and Biological Properties

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Abstract: In this work a series of 15 *N*-benzylamine substituted 5-amino-6-methyl-pyrazine-2,3-dicarbonitriles was prepared by the aminodehalogenation reactions using microwave assisted synthesis with experimentally set and proven conditions. This approach for the aminodehalogenation reaction was chosen due to its higher yields and shorter reaction times. The products of this reaction were characterized by IR, NMR and other analytical data. The compounds were evaluated for their antibacterial, antifungal and herbicidal activity. Compounds **3** (R = 3,4-Cl), **9** (R = 2-Cl) and **11** (R = 4-CF_3) showed good antimycobacterial activity against *Mycobacterium tuberculosis* (MIC = 6.25 μg/mL). It was found that the lipophilicity is important for antimycobacterial activity and the best

substitution on the benzyl moiety of the compounds is a halogen or trifluoromethyl group according to Craig's plot. The activities against bacteria or fungi were insignificant. The presented compounds also inhibited photosynthetic electron transport in spinach chloroplasts and the IC_{50} values of the active compounds varied in the range from 16.4 to 487.0 μmol/L. The most active substances were **2** (R = 3-CF_3), **3** (R = 3,4-Cl) and **11** (R = 4-CF_3). A linear dependence between lipophilicity and herbicidal activity was observed.

Keywords: pyrazinamide derivatives; *in vitro* biological evaluation; microwave-assisted synthesis; tuberculosis; lipophilicity

1. Introduction

Mycobacterium tuberculosis is counted with justification among the most dangerous and successful microorganisms in today's world, especially in developing countries which have become the reservoir of resistant strains (the most burdened countries are in South Africa and East Asia) [1]. These strains are causing the biggest number of problems connected to tuberculosis (TB) treatment [2]. The main problem is resistance. It can be divided into three groups. The first one is called multidrug-resistant TB (MDR-TB) and these microbes are resistant to all first-line antituberculotic drugs (pyrazinamide - PZA, isoniazid - INH, rifampicin - RIF, ethambutol - ETH, streptomycin - STR). The second group is more treacherous. This type of resistance is called extensively or extremely drug-resistant TB (XDR-TB) with the resistance to the first-line anti-TB agents isoniazid and rifampicin together with the resistance to any fluoroquinolone used in the therapy and to at least one of three injectable second-line antituberculotic drugs (amikacin, kanamycin or capreomycin) [3]. The last and the latest category was the most dreaded and was called totally drug-resistant TB (TDR-TB) and the first case was recorded in India [4]. These mycobacterial strains were resistant to all current known therapy. Nowadays, this group has disappeared with the approval of bedaquiline for therapy of resistant forms of tuberculosis. Another problem is connected with the HIV pandemic. These two infectious diseases are influencing each other in a synergic way so this has resulted in efforts to develop new anti-tubercular agents.

This work deals with a microwave-assisted synthesis of pyrazinamide analogues with potential antimycobacterial activity. It is caused by the fact that pyrazinamide is counted among the first-line anti-tuberculosis drugs used in current therapy. Its unique ability to kill the dormant forms of *Mycobacterium tuberculosis* is crucial in shortening the time needed for the treatment, so PZA has sterilizing activity especially in combination with rifampicin [5].

PZA itself has multiple mechanisms of action. The first described was the activation of this prodrug *via* the enzyme pyrazinamidase (EC 3.5.1.19) to form pyrazinoic acid (POA). This metabolite causes a lowering of the inner compartment pH in mycobacterial cells. This leads to inhibition of membrane transport and then to cellular death [6–8]. The gene encoding this enzyme is called *pncA* gene and its mutation is responsible for the origin of mycobacterial resistance to PZA [9].

The second mechanism of action is connected with fatty acid synthase I (FAS I) (EC 2.3.1.85). It is suggested that the disruption of metabolism can be caused by inhibition of the cell membrane

synthesis, which is essential for the survival of *Mycobacteria*, but this mechanism was mainly rejected for PZA itself by Boshoff because there is only low inhibition [10]. On the other hand, the PZA analogues such as 5-chloropyrazinamide, esters of pyrazinoic acid and esters of 5-chloropyrazinoic acid were proven to act in this way [11–13].

Recent research has suggested a novel mechanism of action of PZA - inhibition of *trans*-translation. This process is vital for survival and virulence of *Mycobacteria* and its inhibition leads to blockage of the proteosynthetic apparatus in ribosomes and to cellular death. These assumptions were proven by Zhang *et al.* [14].

Although PZA is the first-line antituberculotic drug, it was found that this molecule has exhibited other interesting biological activities such as antifungal, antibacterial, antiviral and antineoplastic effects [15–20].

There is another application of PZA derivatives that can be used in agriculture. The most successful pyrazine derivative diquat-dibromide (6,7-dihydrodipyrido[1,2-a:2',1'-c]pyrazinediium-dibromide), a non-selective, contact herbicide, which has been used to control many submerged and floating aquatic macrophytes, was found to interfere with the photosynthetic process by releasing strong oxidizers that rapidly disrupt and inactivate cells and cellular functions (at present banned in many EU countries) [21]. Many structural variations of pyrazine compounds with herbicidal properties can be found in the patent literature [22–25]. However, several pyrazine derivatives were also described as inhibitors of Hill reaction which inhibit photosynthetic electron transport (PET) in photosystem (PS) 2 [18,26]. The site of action of these PET inhibitors in the photosynthetic apparatus was situated predominantly on the donor side of PS2, in the section between oxygen evolving complex and intermediate $D^{\bullet}$, *i.e.*, tyrosine radical ($Tyr_D\bullet$) occurring on the 161st position in D_2 protein. Consequently, these compounds can be considered as PS2 herbicides which could have ultimately adverse effect on plant growth. In general, the PET-inhibiting effectiveness of pyrazine derivatives depends on compound lipophilicity and σ Hammett constants of individual substituents. Hosseini *et al.* studied the electronic and structural descriptors, which are the main factors for the cytotoxicity in the series of substituted *N*-phenylpyrazine-2-carboxamides [27].

This study is focused on preparation of *N*-substituted structural and functional derivative of PZA (5-chloro-6-methylpyrazine-2,3-dicarbonitrile) that was treated with ring-substituted benzylamines using the advantages of a microwave reactor. It should be stressed that this type of syntheses has become popular due to its higher yields, shorter reaction times or solvent savings in comparison with conventional organic syntheses [28]. One of the main advantages is the heating. It is uniform through the volume of the sample and the microwaves usually interact with molecules themselves not vessel sides. Another benefit is connected with the temperature reached by the solvent used. The final temperature is usually far higher than the standard boiling point of the solvent when using over-pressurized systems. It is reached and bypassed in seconds. Improved heating usually leads to higher yields and shorter reaction times. There is one limitation for choosing the conditions. It is the polarity of the solvent when the non-polar solvents cannot be used in the way the polar ones can be. If the polar solvent is used in the reaction, there is a direct coupling of microwaves with molecules. More polar solvents have greater ability to interact with microwave radiation. Using the solvents with low polarity (low absorbers) leads to longer times of heating and reaction. On the contrary, if the reagents themselves are polar it could lower the disadvantages of non-polar solvents. Finally there are new

approaches to microwave accelerated methods using ionic liquids or solid phase reactions (adsorption on mineral oxides, phase transfer catalysis, neat reactions) [29]. Microwave assisted condensation in polar solvent is used in this work to accelerate the aminodehalogenation reaction. The conditions for the synthesis were proven experimentally. Antimycobacterial activity of the all prepared compounds was determined and compounds were evaluated also in relation to inhibition of photosynthetic electron transport (PET) in spinach (*Spinacia oleracea* L.) chloroplasts. The structure-activity relationships between the chemical structure and *in vitro* biological activities of evaluated compounds are discussed.

2. Results and Discussion

2.1. Chemistry

The starting compound 5-chloro-6-methylpyrazine-2,3-dicarbonitrile and the final compounds **1**–**15** were synthesized according to the general procedure shown in Scheme 1. The aminodehalogenation reaction of this starting compound and ring-substituted benzylamines yielded a series of 15 secondary amines of which 14 were novel. 5-(Benzylamino)-6-methylpyrazine-2,3-dicarbonitrile (**6**) was previously synthesised by Takematsu *et al.* and the reported melting point was 118–119 °C [30]. The compound we obtained melted at 128.7–130.7 °C. This difference can be caused by the mode of crystallization. All reactions were done using microwave reactor with focused field and yields were in the range between 17% and 62%. Lower yields were caused by the purification using preparative chromatography and recrystallization. It is also known that 3-nitro substitution, for which the yield was the lowest, is counted among the electron-withdrawing groups reducing the basicity of the amine nitrogen. Obtained analytical data were fully consistent with the proposed structures. Table 1 shows the substituents and other data of the synthesized compounds.

Scheme 1. Synthesis of starting compound [30] and microwave assisted aminodehalogenation reaction resulting in a series of compounds **1**–**15**.

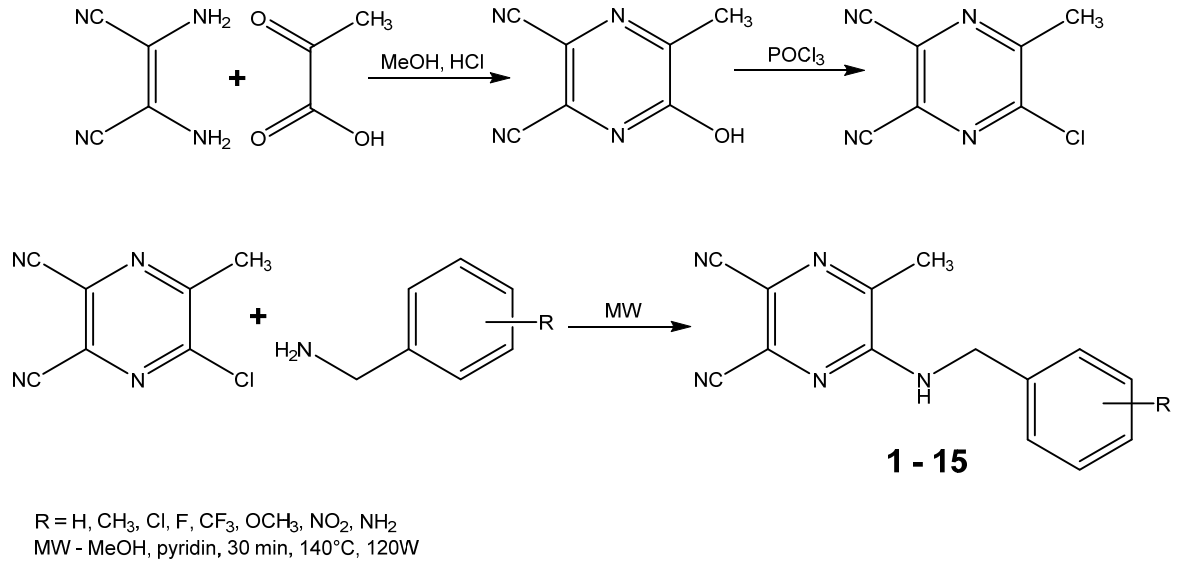


Table 1. Experimentally determined values of lipophilicity log *k*, calculated values of log *P*, electronic Hammett's σ parameters and π parameters, 50% inhibition concentration IC_{50} [μmol/L] values related to PET inhibition in spinach chloroplasts in comparison with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) standard and *in vitro* antimycobacterial activity against *M. tuberculosis* H37Rv (minimal inhibition concentration (MIC) [μg/mL]) of compounds **1**–**15** compared to pyrazinamide (PZA) and isoniazid (INH) standards.

Compound	R	log *P*	log *k*	σ	π	IC_{50} [μmol/L]	MIC *M.tuberculosis* H37Rv [μg/mL]
1	2-CH_3	3.41	0.4668	−0.17	0.1674	114.0	>100
2	3-CF_3	3.84	0.5369	0.43	0.2375	37.7	12.5
3	3,4-Cl	4.04	0.7538	0.60	0.4544	16.4	6.25
4	4-CH_3	3.41	0.5157	−0.17	0.2163	104.7	25
5	4-OCH_3	2.8	0.2820	−0.27	−0.0174	464.6	>100
6	H	2.92	0.2994	0	0	-	25
7	4-NH_2	2.12	−0.2014	−0.15	−0.5008	-	25
8	3-Cl	3.48	0.5172	0.37	0.2178	57.4	12.5
9	2-Cl	3.48	0.4663	0.22	0.1669	79.0	6.25
10	2-F	3.08	0.3042	0.06	0.0048	195.6	12.5
11	4-CF_3	3.84	0.5626	0.51	0.2632	39.6	6.25
12	2-CF_3	3.84	0.4865	0.51	0.1871	71.6	12.5
13	2,4-OCH_3	2.67	0.3699	−0.55	0.0705	-	25
14	3-NO_2	2.71	0.1808	0.71	−0.1186	487.4	12.5
15	4-Cl	3.48	0.5384	0.23	0.2390	86.5	12.5
PZA						-	12.5
INH						-	1.5625
DCMU						1.9	-

$\pi = \log k_{(substituted)} - \log k_{(unsubstituted)}$.

2.2. Calculated and Experimentally Set Lipophilicity

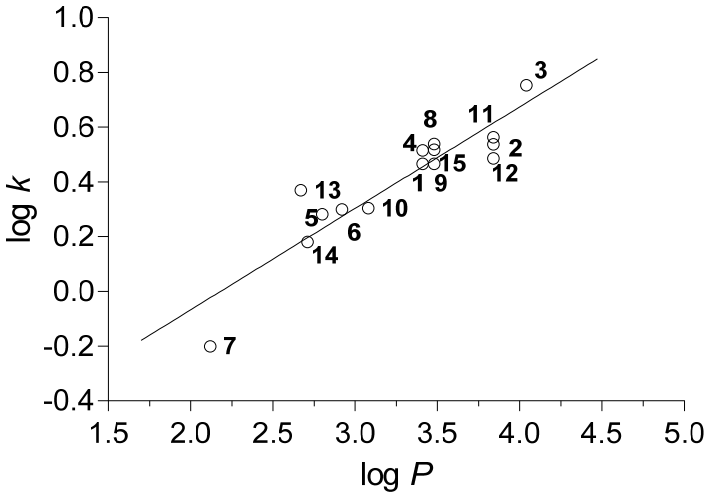
Lipophilicity is one of the most important factors that can affect the biological effect of the compound. It is connected with the membrane transport and other biological processes and it is also connected with solubility in the media. This physical-chemical property can be set experimentally. In this work we have used the Reversed Phase - High Performance Liquid Chromatography (RP-HPLC) methodology for measuring the capacity factors *k* with the calculation of log *k* that can be correlated to calculated values of lipophilicity log *P* (resp. Clog *P*). These calculations were carried out by using PC program CS ChemBioDraw Ultra 13.0. The results of these measurements are shown in the Table 1.

The lowest lipophilicity was shown by compound **7** (R = 4-NH_2) and on the contrary, compound **3** (R = 3,4-Cl) was the most lipophilic compound of this series. Lipophilicity, based on log *k* values, increased for substituents in the benzyl part of the molecule as follows: 4-NH_2 < 3-NO_2 < 4-OCH_3 < H < 2-F < 2,4-OCH_3 < 2-Cl < 2-CH_3 < 2-CF_3 < 4-CH_3 < 3-Cl < 3-CF_3 < 4-Cl < 4-CF_3 < 3,4-Cl

The dependence of the measured log *k* parameters on the calculated log *P* values showed an approximate linearity, which is shown in Figure 1, and the corresponding correlation can be expressed by the following regression equation:

$$\log k = -0.810 \times (\pm 0.150) + 0.371 \times (\pm 0.045) \times \log P$$
$$r = 0.915,\ s = 0.092,\ F = 66.9,\ n = 15 \quad (1)$$

Figure 1. Plot of experimentally measured log *k* parameter on calculated log *P* (CS ChemBioDraw Ultra version 13.0).



2.3. Biological Assays

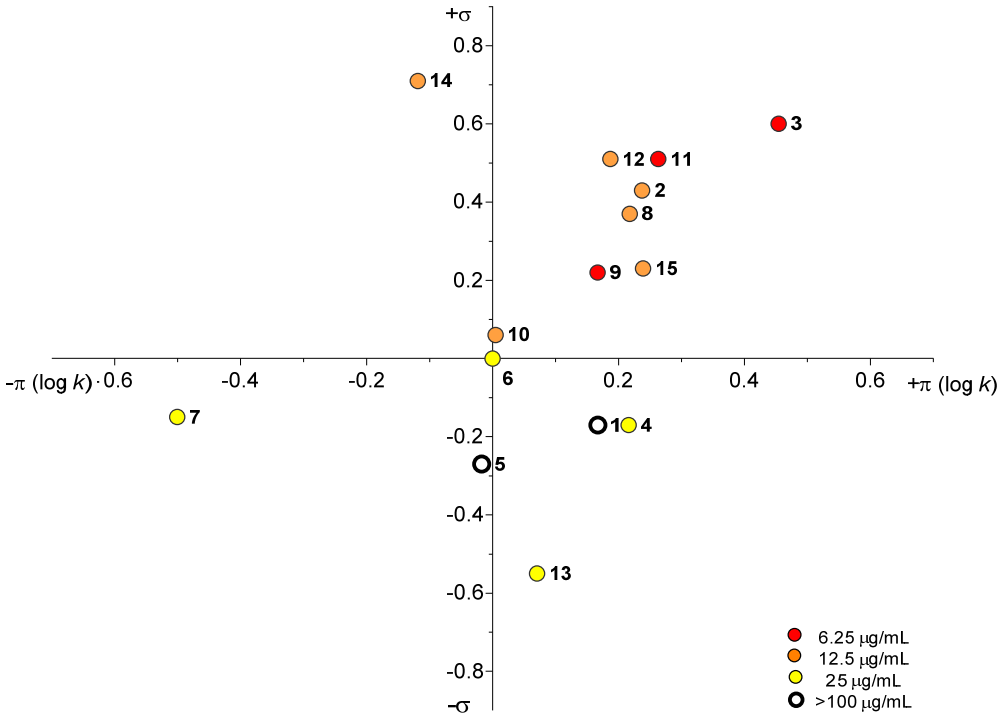
2.3.1. Antimycobacterial *In Vitro* Screening

All prepared compounds were tested against four strains of *Mycobacterium*. These were *M. tuberculosis* and three non-tuberculosis strains. The most active substances against *M. tuberculosis* were compounds **3**, **9** and **11**. Their activity expressed as minimal inhibition concentration (MIC) was 6.25 μg/mL and the activities of standards were 12.5 μg/mL for PZA and 1.5625 μg/mL for INH. Nearly the whole series showed activity against *M. tuberculosis* H37Rv, which was in the range from 25 to 6.25 μg/mL, but compounds **11** and **12** were also active against other strains and both of them were active against *M. avium* 152. These strains are usually resistant or unsusceptible to pyrazinamide. This is the reason why INH was chosen as second standard. Obtained results can be compared with other synthesised compounds and a comparison can be drawn between more mycobacterial strains not only *M. tuberculosis*.

As shown in Figure 2, dependence of antimycobacterial activity on lipophilicity expressed by π constant (log *k*) as well as on the σ constant of the R substituent was observed. The most active compounds (MIC = 6.25 μg/mL) were **3** (R = 3,4-Cl), **9** (R = 2-Cl) and **11** (R = 4-CF_3) and were mentioned above. All of these compounds are situated in the right upper quadrant of the Craig's plot. Further groups are represented by compounds **2** (R = 3-CF_3), **8** (R = 3-Cl), **10** (R = 2-F), **12** (R = 2-CF_3), **14** (R = 3-NO_2) and **15** (R = 4-Cl) showing moderate activity with MIC = 12.5 μg/mL and compounds **4** (R = 4-CH_3), **6** (R = H), **7** (R = 4-NH_2) and **13** (R = 2,4-OCH_3) with MIC = 25 μg/mL. Low antimycobacterial activity with MIC >100 μg/mL was exhibited by compounds **1** (R = 2-CH_3) and **5** (R = 4-OCH_3). It can be stated that the activity is more dependent on π constant than on σ constant. On the other hand this dependence is not unambiguous. However, it is clear that the lipophilicity is

important for antimycobacterial activity and the most suitable ring substituents are electron-withdrawing groups such as halogen or trifluoromethyl moieties.

Figure 2. Dependence of antimycobacterial activity of studied compounds on the π constant (log *k*) as well as on the σ constant of R substituent.



2.3.2. Antimycobacterial *In Vitro* Screening Against *M. smegmatis*

This evaluation was performed against fast-growing *Mycobacterium smegmatis* using isoniazid and ciprofloxacin as standards. None of the compounds showed activity against this mycobacterial strain. This may be caused by the fact that this fast-growing mycobacterium is less susceptible to antibiotic treatment.

2.3.3. Antifungal and Antibacterial *In Vitro* Screening

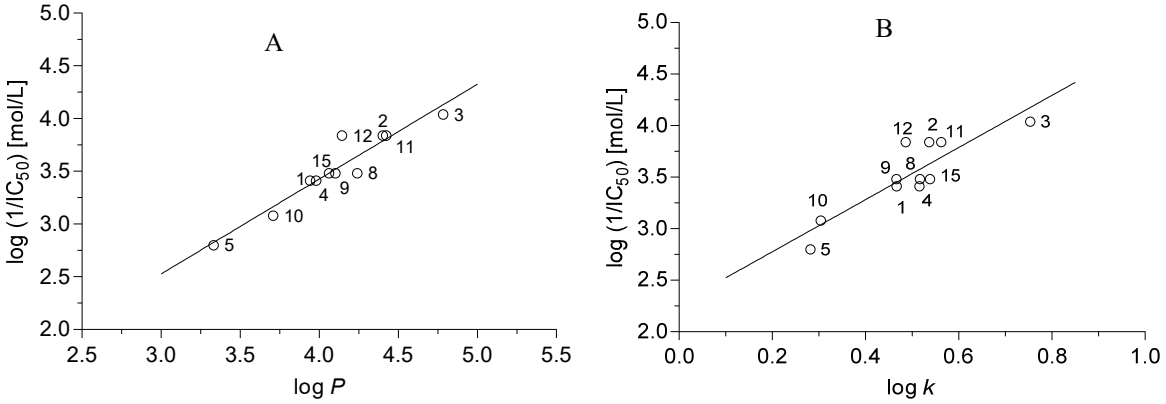
This evaluation was performed in order to obtain results for antifungal and antibacterial activity against eight fungal strains and eight bacterial strains. None of the prepared compounds showed antibacterial activity against the tested strains. On the contrary, compounds **7** (MIC = 500 μmol/L), **10** (MIC = 500 μmol/L) and **12** (MIC = 125 μmol/L) exhibited activity against *Trichophyton mentagrophytes* but in comparison to standards, this activity was negligible. It can be also caused by the bigger susceptibility of this fungal strain being evaluated.

2.3.4. Herbicidal Activity of Prepared Compounds

The studied compounds inhibited photosynthetic electron transport in spinach chloroplasts (Table 1). The PET inhibiting activity of studied compounds **1**–**15** expressed by IC_{50} value varied from 16.4 μmol/L (**3**) to 487.0 μmol/L (**14**). The inhibitory activity of the most active compounds **3** was 8.6 times lower than that of the standard DCMU (IC_{50} = 1.9 μmol/L), a well-known PS2 herbicide.

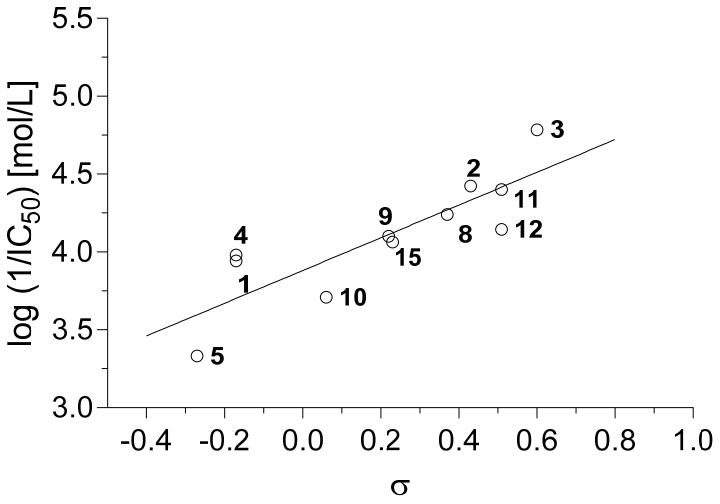
Compounds **6** (R = H), **7** (R = 4-NH_2) and **13** (R = 2,4-OCH_3) were inactive and IC_{50} values related to PET inhibition could not be determined. It can be stated that the inhibitory activity increases linearly with increasing lipophilicity of the compounds based on the dependence of PET inhibiting activity of studied dicarbonitriles **1**–**15** on log *P* (Figure 3A) and log *k* (Figure 3B).

Figure 3. Dependence of PET inhibiting activity on the log *P* (**A**) or log *k* (**B**) of compounds **1**–**15**.



Also if compound **14** (R = 3-NO_2) was excluded, the dependence of PET inhibiting activity on the Hammett constant σ of R substituent showed again linear dependence (Figure 4). A linear increase of PET-inhibiting activity was observed in the range of σ from -0.15 (**7**; R = 4-NH_2) to 0.6 (**3**; R = 3,4-Cl).

Figure 4. Dependence of PET inhibiting activity of compounds **1**–**15** on the σ constant of the R substituent.



The correlation between log (1/IC_{50}) [mol/L] and log *P* or between log (1/IC_{50}) [mol/L] and σ constant of R substituent could be expressed by the following equations:

$$pI_{50} = 0.577 \times (\pm 0.882) + 1.002 \times (\pm 0.250) \log P$$
$$r = 0.950, s = 0.127, F = 82.5, n = 11 \quad (2)$$

$$pI_{50} = 3.881 \times (\pm 0.182) + 1.051 \times (\pm 0.503)\ \sigma$$
$$r = 0.844,\ s = 0.217,\ F = 22.34,\ n = 11 \quad (3)$$

The use of both the above mentioned descriptors (log *P* and σ) in a multilinear correlation did not improve the results of statistical analysis:

$$pI_{50} = 0.949 \times (\pm 1.669) + 0.887 \times (\pm 0.504) \log P + 0.160 \times (\pm 0.594)\ \sigma$$
$$r = 0.952,\ s = 0.131,\ F = 38.6,\ n = 11 \quad (4)$$

Similarly, better results of statistical analysis were obtained for the correlation between log ($1/IC_{50}$) [mol/L] and log *k* than for multilinear correlation using two descriptors (log *k* and σ).

$$pI_{50} = 2.703\ (\pm 0.414) + 2.835\ (\pm 0.816) \log k$$
$$r = 0.934,\ s = 0.144,\ F = 61.8,\ n = 11 \quad (5)$$

$$pI_{50} = 2.997 \times (\pm 0.331) + 2.034 \times (\pm 0.738) \log k + 0.481\ (0.303)\ \sigma$$
$$r = 0.976\ s = 0.093\ F = 80.2\ n = 11 \quad (6)$$

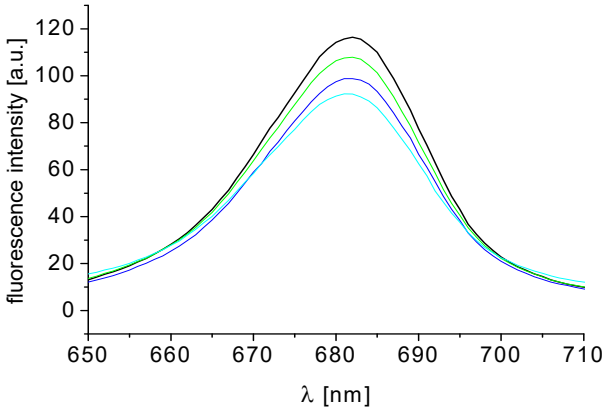
These results indicate that lipophilicity of the compound is determining for PET-inhibiting activity of the studied *N*-substituted 5-amino-6-methylpyrazine-2,3-dicarbonitriles. Lee *et al.* focused on the search for the minimum structural requirements for herbicidal evaluation of 5-(R^1)-6-(R^2)-*N*-(R^3-phenyl)-pyrazine-2-carboxamide analogues as a new class of potent herbicides in a previous paper [31]. Based on IC_{50} values of 19 pyrazine derivatives related to PET inhibition in spinach chloroplasts which were published by Dolezal *et al.* [15], Lee derived and discussed quantitatively 3D-QSARs models between the substituents (R^1–R^3) changes of the analogues and their herbicidal activity using comparative molecular field analysis (CoMFA) and comparative molecular similarity indice analysis (CoMSIA) methods. It was predicted that the herbicidal activity increases when large steric substituents were introduced to one part of the *ortho*- and *meta*- positions on the *N*-phenyl ring as R^3- substituent and small steric substituents on the other part. The same 19 pyrazine derivatives with herbicidal activity were also subjected to the two dimensional quantitative structure activity relationships studies using Vlife Molecular Design 3.0 module which contains various combinations of thermodynamic, electronic, topological and spatial descriptors [32]. It was found that decreasing of number of hydrogen bond acceptor atoms and reduction the any atoms (single, double or triple bonded) separated from any oxygen atom by a seven bond distance in a molecule could be helpful for designing more potent herbicidal agents.

Because the studied *N*-substituted 5-amino-6-methylpyrazine-2,3-dicarbonitriles were found to inhibit the Hill reaction, they can be considered as photosystem 2 (PS2) inhibitors, *i.e.*, PS2 herbicides which ultimately adversely affect growth of weeds as well as agricultural plants. The PS2 inhibitors can act on the donor and/or the acceptor side of PS2. Using EPR spectroscopy it was found that *N*-phenylpyrazine-2-carboxamides interacted with the $D^{\bullet}$ intermediate which is situated at 161st position in D_2 protein occurring on the donor side of PS2. Due to interaction of these pyrazine derivatives with this part of PS2, the photosynthetic electron transport from the oxygen evolving complex to the reaction centre of PS2 was impaired and consequently, the electron transport between PS2 and PS1 was inhibited [33]. Use of an artificial electron-donor, 1,5-diphenylcarbazide (DPC),

acting in the $Z^{\bullet}/D^{\bullet}$ intermediate is suitable to estimate whether the studied PET inhibitor acts only on the donor or also on the acceptor side of PS2. Complete restoration of PET after DPC addition to chloroplasts activity of which was inhibited by an inhibitor, indicates that PET between the core of PS2 (P680) and the secondary quinone acceptor Q_B was not affected by this inhibitor. Consequently, its site of inhibitory action is situated on the donor side of PS2. However, upon addition of DPC to the studied dicarbonitriles only partial restoration of PET was observed (up to 85% of the control) and therefore it could be assumed that the studied compounds block PET not only by interaction with proteins occurring in the section between the oxygen evolving complex (OEC) and the $Z^{\bullet}/D^{\bullet}$ intermediate but to PET inhibition contributes also their interaction with some constituents of photosynthetic apparatus on the acceptor side of PS2. Similar results were obtained previously with 5-*tert*-butyl-*N*-(3-hydroxy-4-chlorophenyl)pyrazine-2-carboxamide and 5-*tert*-butyl-6-chloro-*N*-(3-fluorophenyl)pyrazine-2-carboxamide [33].

Compounds **1**–**15** affected the chlorophyll *a* (Chl*a*) fluorescence in spinach chloroplasts. As shown in Figure 5, the intensity of the Chl*a* emission band at 686 nm belonging to the pigment–protein complexes in photosystem 2 decreased in the presence of compound **3** [34]. This finding indicates a perturbation of the Chl*a*–protein complexes in the thylakoid membrane caused by the tested compound. Similar Chl*a* fluorescence decrease in spinach chloroplasts was observed previously with several PET inhibitors, *i.e.*, *N*-benzylpyrazine-2-carboxamides [35], ring-substituted 3-hydroxynaphthalene-2-carboxanilides [36] and 1-hydroxynaphthalene-2-carboxanilides [37], 2-hydroxynaphthalene-1-carboxanilides [38] and ring-substituted 4-arylamino-7-chloroquinolinium chlorides [39].

Figure 5. Fluorescence emission spectra of Chl*a* of untreated spinach chloroplasts and chloroplasts treated with 0, 0.13, 0.25 and 0.51 mmol/L of compound **3** (R = 3,4-Cl) (the curves from top to bottom); excitation wave length λ = 436 nm; chlorophyll concentration 10 mg/L.



3. Experimental

3.1. General

All the chemicals used for preparation of starting compound and final products were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and were reagent or higher grade of purity. Starting compound was prepared according to proven methodology of conventional organic synthesis.

The final aminodehalogenation reactions were performed in CEM Discover microwave reactor with focused field (CEM Corporation, Matthews, NC, USA) connected to the Explorer 24 autosampler (CEM Corporation) and this equipment was running under CEM's Synergy™ software for monitoring the progress of reactions. The reaction progress was checked by Thin Layer Chromatography (TLC) (Alugram® Sil G/UV254, Machery-Nagel, Postfach, Germany) using 254 nm wavelength UV detection. All the obtained products were purified by crystallization or by preparative flash chromatography (CombiFlash® Rf, Teledyne Isco Inc., Lincoln, NE, USA), using gradient elution with hexane (LacheNer, Neratovice, Czech Republic) and ethyl acetate (Penta, Prague, Czech Republic) as mobile phases. Silica gel (0.040–0.063 nm, Merck, Darmstadt, Germany) was used as the stationary phase. NMR spectra were recorded on Varian Mercury–VxBB 300 (299.95 MHz for ^{1}H and 75.43 MHz for ^{13}C) or Varian VNMR S500 (499.87 MHz for ^{1}H and 125.71 MHz for ^{13}C) spectrometers (Varian Corporation, Palo Alto, CA, USA). Chemical shifts were reported in ppm (δ) and were applied indirectly to tetramethylsilane as a signal of solvent (2.49 for ^{1}H and 39.7 for ^{13}C in DMSO-d_6). Infrared spectra were recorded with spectrometer FT-IR Nicolet 6700 (Thermo Scientific, Waltham, MA, USA) using attenuated total reflectance (ATR) methodology. Melting points were assessed by SMP3 Stuart Scientific (Bibby Scientific Ltd., Staffordshire, UK) and were uncorrected. Elemental analyses were measured with EA 1110 CHNS Analyzer (Fisons Instruments S. p. A., Carlo Erba, Milano, Italy). Calculation of electronic Hammett's σ parameters was carried out on the software ACD/Percepta ver. 2012 (Advanced Chemistry Development, Inc., Toronto, ON, Canada).

3.2. Starting Compound and Final Products Synthesis

The starting compound 5-chloro-6-methylpyrazine-2,3-dicarbonitrile was synthesized in a two-step reaction according to the reported methodology [30]. The first step was a condensation reaction between diaminomaleonitrile (0.025 mol) and pyruvic acid (0.025 mol), which were dissolved in methanol (60 mL), and hydrochloric acid (10 mL, 15%) was added dropwise. It takes two hours to react at room temperature. After the evaporation of two thirds of the solvent, hot water (80 mL) was added and then the rest of methanol was evaporated *in vacuo*. The whole mixture was cooled to 5 °C to initiate the crystallization. The product was collected by suction, dried overnight and subsequently chlorinated with phosphoryl chloride. Product (0.015 mol) was again dissolved in $POCl_3$ (0.060 mol) and cooled to 0 °C. Pyridine (0.020 mol) was added dropwise and after the termination of the exothermic reaction, the mixture was heated to 90 °C for 2 h. Excess $POCl_3$ was evaporated *in vacuo* and the rest of product was extracted into toluene three or four times, the toluene was evaporated and then the crude product was recrystallized from chloroform. The starting compound (1.12 mmol) was finally treated with 15 variously ring-substituted benzylamines (2.24 mmol) and all these aminodehalogenation reactions took place in the microwave reactor. The conditions used for microwave syntheses were as follows—150 °C, 30 min, 120 W, methanol as a solvent, pyridine as a base and were set experimentally. The reaction was monitored by TLC using hexane/ethyl acetate 2:1 mixture as mobile phase. The final compounds were purified using flash column chromatography with gradient elution using a hexane/ethyl acetate system and if necessary recrystallization from a mixture of ethanol and water.

3.3. Analytical Data of the Prepared Compounds

5-Methyl-6-[(2-methylbenzyl)amino]pyrazine-2,3-dicarbonitrile (**1**). Light orange crystalline solid. Yield 47.9%; M.p. 152.1–152.9 °C; IR (ATR-Ge, cm^{-1}): 3381_m (-NH-), 2226_m (-CN), 1570_{vs}, 1521_s, 1403_{vs}, 1349_m (arom.); ^{1}H-NMR (500 MHz) δ 8.61 (1H, bs, NH), 7.25–7.10 (4H, m, H3', H4', H5', H6'), 4.56 (2H, s, NCH_2), 2.47 (3H, s, CH_3), 2.33 (3H, s, CH_3); ^{13}C-NMR (125 MHz) δ 152.9, 147.5, 136.0, 135.6, 130.2, 130.1, 127.5, 127.2, 125.9, 117.4, 115.7, 114.8, 42.5, 21.3, 19.0; Elemental analysis: calc. for $C_{15}H_{13}N_5$ (MW 263.12): 68.42% C, 4.98% H, 26.60% N; found 68.22% C, 5.17% H, 26.44% N.

5-Methyl-6-{[3-(trifluoromethyl)benzyl]amino}pyrazine-2,3-dicarbonitrile (**2**). Yellow-orange crystalline solid. Yield 51.6%; M.p. 158.5–159.7 °C (decomp.); IR (ATR-Ge, cm^{-1}): 3377_m (-NH-), 2227_m (-CN), 1570_{vs}, 1521_s, 1402_{vs}, 1353_m, 1324_{vs} (arom.), 1128_{vs} (-C-F); ^{1}H-NMR (300 MHz) δ 8.75 (1H, bs, NH), 7.77–7.60 (1H, m, H2'), 7.68–7.50 (3H, m, H4', H5', H6'), 4.69 (2H, d, J = 4.6 Hz, NCH_2), 2.46 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 152.9, 147.7, 139.6, 131.8, 130.0, 129.6, 129.2 (q, J = 31.5 Hz), 124.5 (q, J = 4.0 Hz), 124.4 (q, J = 272.3 Hz), 124.0 (q, J = 4.0 Hz), 117.7, 115.6, 114.8, 44.0, 21.2; Elemental analysis: calc. for $C_{15}H_{10}F_3N_5$ (MW 317.27): 56.78% C, 3.18% H, 22.07% N; found 56.59% C, 3.30% H, 21.93% N.

5-[(3,4-Dichlorobenzyl)amino]-6-methylpyrazine-2,3-dicarbonitrile (**3**). Light yellow crystalline solid. Yield 60.9%; M.p. 154.4–156.2 °C; IR (ATR-Ge, cm^{-1}): 3329_m (-NH-), 2227_m (-CN), 1568_{vs}, 1516_s, 1466_m, 1402_{vs} (arom.); ^{1}H-NMR (300 MHz) δ 8.69 (1H, bs, NH), 7.61 (1H, d, J = 1.9 Hz, H2'), 7.57 (1H, d, J = 8.2 Hz, H5'), 7.33 (1H, dd, J = 8.2 Hz, J = 1.9 Hz, H6'), 4.59 (2H, s, NCH_2), 2.46 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 152.8, 147.8, 139.4, 131.1, 130.6, 130.0, 129.8, 129.6, 127.9, 117.8, 115.6, 114.8, 43.3, 21.2; Elemental analysis: calc. for $C_{14}H_9Cl_2N_5$ (MW 318.16): 52.85% C, 2.85% H, 22.01% N; found 52.71% C, 3.05% H, 21.88% N.

5-Methyl-6-[(4-methylbenzyl)amino]pyrazine-2,3-dicarbonitrile (**4**). Light yellow crystalline solid. Yield 60.5%; M.p. 140.8–142.3 °C; IR (ATR-Ge, cm^{-1}): 3370_s (-NH-), 2223_m (-CN), 1576_{vs}, 1519_s, 1438_m, 1400_{vs}, 1351_s (arom.); ^{1}H-NMR (300 MHz) δ 8.68 (1H, bs, NH), 7.24–7.18 (2H, m, AA', BB', H2', H6'), 7.14–7.09 (2H, m, AA', BB', H3', H5'), 4.55 (2H, bs, NCH_2), 2.44 (3H, s, CH_3), 2.26 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 152.8, 147.5, 136.4, 135.0, 130.1, 128.1, 127.5, 117.3, 115.7, 114.9, 44.1, 21.2, 20.8; Elemental analysis: calc. for $C_{15}H_{13}N_5$ (MW 263.30): 68.42% C, 4.98% H, 26.60% N; found 68.30% C, 5.17% H, 26.43% N.

5-[(4-Methoxybenzyl)amino]-6-methylpyrazine-2,3-dicarbonitrile (**5**). Ochre crystalline solid. Yield 61.3%; M.p. 153.0–154.2 °C (decomp.); IR (ATR-Ge, cm^{-1}): 3345_m (-NH-), 2948_w (-OCH_3), 2223_m (-CN), 1585_s, 1570_{vs}, 1511_{vs}, 1462_m, 1403_{vs} (arom.); ^{1}H-NMR (300 MHz) δ 8.66 (1H, bs, NH), 7.29–7.23 (2H, m, AA', BB', H2', H6'), 6.90–6.84 (2H, m, AA', BB', H3', H5'), 4.52 (2H, bs, NCH_2), 3.71 (3H, s, OCH_3), 2.43 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 158.6, 152.8, 147.4, 130.1, 129.9, 129.0, 117.3, 115.7, 114.9, 113.9, 55.2, 43.8, 21.2; Elemental analysis: calc. for $C_{15}H_{13}N_5O$ (MW 279.30): 64.51% C, 4.69% H, 25.07% N; found 64.35% C, 4.81% H, 24.84% N.

5-(Benzylamino)-6-methylpyrazine-2,3-dicarbonitrile (**6**) [30]. Yellow-orange crystalline solid. Yield 53.3%; M.p. 128.7–130.7 °C (described in the literature 118–119 °C); IR (ATR-Ge, cm^{-1}): 3404_m (-NH-), 2228_m (-CN), 1564_{vs}, 1508_{vs}, 1454_m, 1400_{vs}, 1357s (arom.); ^{1}H-NMR (300 MHz) δ 8.72 (1H, bs, NH), 7.37–7.20 (5H, m, H2', H3', H4', H5', H6'), 4.61 (2H, d, *J* = 3.3 Hz, NCH_2), 2.45 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 152.9, 147.5, 138.1, 130.1, 128.5, 127.5, 127.2, 117.4, 115.7, 114.9, 44.3, 21.2; Elemental analysis: calc. for $C_{14}H_{11}N_5$ (MW 249.27): 67.46% C, 4.45% H, 28.10% N; found 67.21% C, 4.63% H, 27.95% N.

5-[(4-Aminobenzyl)amino]-6-methylpyrazine-2,3-dicarbonitrile (**7**). Brown crystalline solid. Yield 37.2%; M.p. 154.9–156.5 °C; IR (ATR-Ge, cm^{-1}): 3413_m (-NH_2), 3376_m (-NH-), 2234_m (-CN), 1572_{vs}, 1519_{vs}, 1440_m, 1402_{vs}, 1352_s (arom.); ^{1}H-NMR (300 MHz) δ 8.54 (1H, bs, NH), 7.04–6.94 (2H, m, AA', BB', H2', H6'), 6.54–6.45 (2H, m, AA', BB', H3', H5'), 4.97 (2H, bs, NH_2), 4.41 (2H, d, *J* = 3.8 Hz, NCH_2), 2.41 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 152.7, 148.0, 147.3, 130.2, 128.7, 124.7, 117.0, 115.8, 114.9, 113.9, 44.2, 21.2; Elemental analysis: calc. for $C_{14}H_{12}N_6$ (MW 264.29): 63.62% C, 4.58% H, 31.80% N; found 63.48% C, 4.45% H, 31.65% N.

5-[(3-Chlorobenzyl)amino]-6-methylpyrazine-2,3-dicarbonitrile (**8**). Yellow crystalline solid. Yield 60.2%; M.p. 151.0–151.8 °C; IR (ATR-Ge, cm^{-1}): 3306_m (-NH-), 2248_m (-CN), 1567_{vs}, 1513_{vs}, 1468_m, 1401_{vs}, 1351_s (arom.); ^{1}H-NMR (300 MHz) δ 8.70 (1H, t, *J* = 5.9 Hz, NH), 7.43–7.39 (1H, m, H2'), 7.38–7.27 (3H, m, H4', H5', H6'), 4.61 (2H, d, *J* = 5.9 Hz, NCH_2), 2.46 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 152.9, 147.7, 140.7, 133.2, 130.4, 130.0, 127.4, 127.2, 126.2, 117.7, 115.6, 114.9, 43.8, 21.2; Elemental analysis: calc. for $C_{14}H_{10}ClN_5$ (MW 283.72): 59.27% C, 3.55% H, 24.68% N; found 59.08% C, 3.71% H, 24.57% N.

5-[(2-Chlorobenzyl)amino]-6-methylpyrazine-2,3-dicarbonitrile (**9**). Dark orange crystalline solid. Yield 24.7%; M.p. decomp.; IR (ATR-Ge, cm^{-1}): 3381_m (-NH-), 2227_m (-CN), 1570_{vs}, 1521_s, 1474_m, 1440_m, 1401_s, 1351_s (arom.); ^{1}H-NMR (300 MHz) δ 8.68 (1H, bs, NH), 7.51–7.24 (4H, m, H3', H4', H5', H6'), 4.66 (2H, s, NCH_2), 2.49 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 153.0, 147.7, 134.9, 132.3, 130.0, 129.4, 129.1, 128.9, 127.4, 117.8, 115.6, 114.8, 42.4, 21.2; Elemental analysis: calc. for $C_{14}H_{10}ClN_5$ (MW 283.72): 59.27% C, 3.55% H, 24.68% N; found 59.08% C, 3.68% H, 24.90% N.

5-[(2-Fluorobenzyl)amino]-6-methylpyrazine-2,3-dicarbonitrile (**10**). Dark yellow crystalline solid. Yield 60.8%; M.p. 134.8–136.3 °C; IR (ATR-Ge, cm^{-1}): 3377_m (-NH-), 2231_m (-CN), 1573_{vs}, 1523_s, 1491_m, 1402_s, 1354_s (arom.); ^{1}H-NMR (300 MHz) δ 8.68 (1H, t, *J* = 5.3 Hz, NH), 7.42–7.10 (4H, m, H3', H4', H5', H6'), 4.63 (2H, d, *J* = 5.3 Hz, NCH_2), 2.46 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 160.0 (d, *J* = 244.8 Hz), 152.9, 147.6, 129.8 (d, *J* = 20.4 Hz), 129.8, 129.4 (d, *J* = 8.3 Hz), 124.6 (d, *J* = 17.8 Hz), 124.5 (d, *J* = 6.6 Hz), 117.7, 115.5 (d, *J* = 9.2 Hz), 115.2, 114.8, 38.3, 21.2; Elemental analysis: calc. for $C_{14}H_{10}FN_5$ (MW 267.26): 62.92% C, 3.77% H, 26.20% N; found 62.71% C, 3.59% H, 26.38% N.

5-Methyl-6-{[4-(trifluoromethyl)benzyl]amino}pyrazine-2,3-dicarbonitrile (**11**). Light yellow crystalline solid. Yield 38.3%; M.p. 148.4–149.6 °C; IR (ATR-Ge, cm^{-1}): 3397_m (-NH-), 2232_m (-CN), 1570_{vs}, 1520_s, 1422_m, 1399_s, 1324_{vs} (arom.), 1114_{vs} (-C-F); ^{1}H-NMR (300 MHz) δ 8.54 (1H, t, *J* = 5.3 Hz,

NH), 7.71–7.64 (2H, m, AA', BB', H2', H6'), 7.38–7.52 (2H, m, AA', BB', H3', H5'), 4.69 (2H, d, J = 5.3 Hz, NCH_2), 2.51 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 152.9, 147.7, 143.1, 130.0, 128.2, 127.9 (q, J = 31.5 Hz), 125.4 (q, J = 3.7 Hz), 124.5 (q, J = 272.0 Hz), 117.8, 115.6, 114.8, 44.0, 21.2; Elemental analysis: calc. for $C_{15}H_{10}F_3N_5$ (MW 317.27): 56.78% C, 3.18% H, 22.07% N; found 56.59% C, 2.99% H, 21.91% N.

5-Methyl-6-{[2-(trifluoromethyl)benzyl]amino}pyrazine-2,3-dicarbonitrile (**12**). Yellow-orange crystalline solid. Yield 49.1%; M.p. 157.8–159.4 °C (decomp.); IR (ATR-Ge, cm^{-1}): 3408_m (-NH-), 2230_m (-CN), 1569_{vs}, 1522_s, 1430_m, 1409_s, 1398_s, 1368_s, 1314_{vs} (arom.), 1114_{vs} (-C-F); ^{1}H-NMR (300 MHz) δ 8.76 (1H, bs, NH), 7.77–7.71 (1H, m, H3'), 7.64–7.43 (3H, m, H4', H5', H6'), 4.77 (2H, bs, NCH_2), 2.51 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 153.0, 147.7, 136.3, 132.9, 130.0, 128.3, 127.8, 126.4 (q, J = 30.3 Hz), 126.1 (q, J = 6.0 Hz), 124.7 (q, J = 274.0 Hz), 118.0, 115.5, 114.7, 41.1, 21.2; Elemental analysis: calc. for $C_{15}H_{10}F_3N_5$ (MW 317.27): 56.78% C, 3.18% H, 22.07% N; found 56.62% C, 3.08% H, 22.00% N.

5-[(2,4-Dimethoxybenzyl)amino]-6-methylpyrazine-2,3-dicarbonitrile (**13**). Ochre crystalline solid. Yield 62.3%; M.p. 151.6–152.9 °C; IR (ATR-Ge, cm^{-1}): 3336_m (-NH-), 2924_w (-OCH_3), 2224_m (-CN), 1609_s, 1574_{vs}, 1509_{vs}, 1467_m, 1440_s, 1405_{vs} (arom.); ^{1}H-NMR (300 MHz) δ 8.45 (1H, t, J = 5.7 Hz, NH), 7.07 (1H, d, J = 8.2 Hz, H6'), 6.55 (1H, d, J = 2.5 Hz, H3'), 6.43 (1H, dd, J = 8.2 Hz, J = 2.5 Hz, H5'), 4.47 (2H, d, J = 5.7 Hz, NCH_2), 3.80 (3H, s, OCH_3), 3.72 (3H, s, OCH_3), 2.44 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 160.1, 158.1, 153.0, 147.4, 130.2, 128.8, 117.3, 117.2, 115.8, 114.9, 104.6, 98.5, 55.7, 55.4, 39.5, 21.3; Elemental analysis: calc. for $C_{16}H_{15}N_5O_2$ (MW 309.32): 62.13% C, 4.89% H, 22.64% N; found 62.36% C, 5.01% H, 22.52% N.

5-Methyl-6-[(3-nitrobenzyl)amino]pyrazine-2,3-dicarbonitrile (**14**). Orange-red crystalline solid. Yield 17.2%; M.p. decomp.; IR (ATR-Ge, cm^{-1}): 3394_m (-NH-), 2231_m (-CN), 1571_{vs}, 1547_m, 1524_{vs}, 1478_m, 1430_m, 1396_s, 1366_s, 1348_{vs} (arom.); ^{1}H-NMR (300 MHz) δ 8.79 (1H, t, J = 5.8 Hz, NH), 8.23 (1H, s, H2'), 8.11 (1H, d, J = 7.8 Hz, H4'), 7.81 (1H, d, J = 7.8 Hz, H6'), 7.62 (1H, t, J = 7.8 Hz, H5'), 4.73 (2H, d, J = 5.8 Hz, NCH_2), 2.47 (3H, s, CH_3); ^{13}C-NMR (75 MHz) δ 152.9, 148.0, 147.8, 140.6, 134.4, 130.0, 130.0, 122.5, 122.3, 117.9, 115.6, 114.8, 43.8, 21.2; Elemental analysis: calc. for $C_{14}H_{10}N_6O_2$ (MW 294.27): 57.14% C, 3.43% H, 28.56% N; found 56.93% C, 3.64% H, 28.56% N.

5-[(4-Chlorobenzyl)amino]-6-methylpyrazine-2,3-dicarbonitrile (**15**). Brown crystalline solid. Yield 30.8%; M.p. 152.8–154.8 °C (decomp.); IR (ATR-Ge, cm^{-1}): 3378_m (-NH-), 2230_m (-CN), 1570_{vs}, 1521_s, 1487_m, 1441_m, 1403_s, 1347_s (arom.), 804_s (-C-Cl); ^{1}H-NMR (500 MHz) δ 8.42 (1H, bs, NH), 7.39–7.33 (4H, m, H2', H3', H5', H6'), 4.59 (2H, s, NCH_2), 2.45 (3H, s, CH_3); ^{13}C-NMR (125 MHz) δ 152.8, 147.6, 137.2, 131.8, 130.0, 129.4, 128.5, 117.6, 115.6, 114.8, 43.7, 21.2; Elemental analysis: calc. for $C_{14}H_{10}ClN_5$ (MW 283.72): 59.27% C, 3.55% H, 24.68% N; found 59.16% C, 3.65% H, 24.62% N.

3.4. Lipophilicity HPLC Determination and Calculations

Experimental lipophilicity parameter log *k* was ascertained using an Agilent Technologies 1200 SL HPLC system with a SL G1315C Diode-Array Detector, ZORBAX XDB-C18 5 μm, 4 × 4 mm, Part No. 7995118-504 chromatographic pre-column and ZORBAX Eclipse XDB-C18 5 μm, 4.6 × 250 mm, Part No. 7995118-585 column (Agilent Technologies Inc., Colorado Springs, CO, USA). The separation process was controlled by Agilent ChemStation, version B.04.02 extended by spectral module (Agilent Technologies Inc.). A solution of MeOH (HPLC grade, 70%) and H_2O (HPLC-Milli-Q Grade, 30%) was used as mobile phase. The total flow of the column was 1.0 mL/min, injection 20 μL, column temperature 30 °C. Detection wavelength λ= 210 nm and monitor wavelength λ= 270 nm were chosen for this measurement. The KI methanol solution was used for the dead time (TD) determination. Retention times (TR) of synthesized compounds were measured in minutes. The capacity factors *k* were calculated using Microsoft Excel according to formula $k = (TR - TD)/TD$, where TR is the retention time of the solute and TD denotes the dead time obtained *via* an unretained analyte. Log *k*, calculated from the capacity factor *k*, is used as the lipophilicity index converted to log *P* scale. Values of log *P* and Clog *P* were calculated with the PC programme CS ChemBioDraw Ultra 13.0 (CambridgeSoft, Cambridge, MA, USA).

3.5. Biological Assays

3.5.1. Antimycobacterial *In Vitro* Screening

Mycobacterial screening was performed against four mycobacterial stems (*M. tuberculosis* H37Rv CNCTC My 331/88, *M. kansasii* CNCTC My 235/80, *M. avium ssp. avium* CNCTC My 80/72, *M. avium* CNCTC My 152/73 (Czech National Collection of Type Cultures, National Institute of Public Health, Prague, Czech Republic)) using isoniazid and pyrazinamide (Sigma-Aldrich) as standards. Culturing medium was Sula's semisynthetic medium (Trios, Prague, Czech Republic) with pH 6.0. Tested compounds were dissolved in dimethylsulfoxide (DMSO) and diluted with medium to final concentrations 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 μg/mL. The method used for this assay was microdilution broth panel method. The final concentration of DMSO did not exceed 1% (v/v) and did not affect the growth of *Mycobacteria*. The cultures were grown in Sula's medium at 37 °C in humid dark atmosphere. The antimycobacterial activity was determined using Alamar Blue colouring after 14 days, resp. 6 days against *M. kansasii,* of incubation as MIC (μg/mL). This evaluation was done in cooperation with Department of Clinical Microbiology, University Hospital in Hradec Kralove, Hradec Kralove, Czech Republic.

3.5.2. Antimycobacterial *In Vitro* Screening Against *M. smegmatis*

This assay was focused on the activity against fast growing *Mycobacterium smegmatis* MC2155 (CIT Collection, Cork Institute of Technology, Cork, Ireland). The method used there was also microdilution broth panel method and as the medium was used Middlebrook 7H9 Broth with 10% of OADC supplement (Sigma-Aldrich). Tested compounds were dissolved in DMSO and medium and the final concentrations were set as 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 7.8125 μg/mL. The

final concentration of DMSO did not exceed 2% (v/v) and did not affect the growth of *M. smegmatis*. The standards used for determination of activity were isoniazid and ciprofloxacin. MIC was read after 48 h of incubation at 37 °C, addition of Alamar Blue stain was followed by 12 h of additional incubation with this reagent. This screening was performed under the patronage of the Department of Biological Sciences, Cork Institute of Technology, Cork, Ireland.

3.5.3. Antifungal and Antibacterial *In Vitro* Screenings

Antibacterial evaluation was made using the microdilution broth method in plates M27A-M1 (200+10) against eight bacterial stems from the Czech Collection of Microorganisms (Brno, Czech Republic) or clinical isolates from Department of Clinical Microbiology, University Hospital in Hradec Kralove (Hradec Kralove, Czech Republic) (*Staphylococcus aureus* CCM 4516/08, *Staphylococcus aureus* H 5996/08 methicillin resistant, *Staphylococcus epidermidis* H 6966/08, *Enterococcus* sp. J14365/08, *Escherichia coli* CCM 4517, *Klebsiella pneumoniae* D 11750/08, *Klebsiella pneumoniae* J 14368/05 ESBL positive, *Pseudomonas aeruginosa* CCM 1961). Mueller Hinton broth was used for the cultivation that was done in humid atmosphere at 35 °C. The readings were made after 24 and 48 h and MIC was set as 80% inhibition of control. The standards were neomycin, bacitracin, penicillin G, ciprofloxacin and phenoxymethylpenicillin and tested products were dissolved in DMSO (final concentration of DMSO did not exceed 1% (v/v)) [40].

Antifungal evaluation was also accomplished with microdilution broth method. On the contrary, there was used RPMI 1640 broth with glutamine as medium and conditions were humid and dark atmosphere, pH 7.0 (buffered with 3-morpholinopropane-1-sulfonic acid) and 35 °C. Eight fungal strains were used (*Candida albicans* ATCC 44859, *Candida tropicalis* 156, *Candida krusei* E28, *Candida glabrata* 20/I, *Trichosporon asahii* 1188, *Aspergillus fumigatus* 231, *Absidia corymbifera* 272, *Trichophyton mentagrophytes* 445) together with 4 antimycotic standards amphotericin B, voriconazole, nystatin and fluconazole. The MIC was set as 80% inhibition of control and readings were made after 24 and 48 h (50% IC, 72 and 120 h for fibrous fungi) and tested compounds were also dissolved in DMSO (final concentration of DMSO in medium did not exceed 2.5% (v/v)) [41].

3.5.4. Study of the Inhibition of Oxygen Evolution Rate in Spinach Chloroplasts

Chloroplasts were prepared from spinach (*Spinacia oleracea* L.) according to Masarovicova and Kralova [42]. The inhibition of photosynthetic electron transport (PET) in spinach chloroplasts was determined spectrophotometrically (Genesys 6, Thermo Scientific, Madison, WI, USA) using an artificial electron acceptor 2,6-dichlorophenol-indophenol (DCPIP) according to Kralova *et al.* [43] and the rate of photosynthetic electron transport (PET) was monitored as a photo-reduction of DCPIP. The measurements were carried out in a phosphate buffer (0.02 mol/L, pH 7.2) containing sucrose (0.4 mol/L), $MgCl_2$ (0.005 mol/L) and NaCl (0.015 mol/L). The chlorophyll content was 30 mg/L in these experiments and the samples were irradiated (~100 W/m^2) from a 10 cm distance with halogen lamp (250 W) using a 4 cm water filter to prevent warming of the samples (suspension temperature 4 °C). The studied compounds were dissolved in DMSO due to their limited water solubility. The applied DMSO concentration (up to 4% (v/v)) did not affect the photochemical activity in spinach chloroplasts (PET). The inhibitory efficiency of the studied compounds was expressed as the IC_{50} values, *i.e.*, molar

concentration of the compounds causing 50% decrease in the oxygen evolution relative to the untreated control. The comparable IC_{50} value for a selective herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Diurone®, DCMU) was about 1.9 μmol/L [44].

3.5.5. Study of Fluorescence of Chlorophyll *a* in Spinach Chloroplasts

The fluorescence emission spectra of chlorophyll *a* (Chl*a*) in spinach chloroplasts were recorded on fluorescence spectrophotometer F-2000 (Hitachi, Tokyo, Japan) using excitation wavelength λ_{ex} = 436 nm for monitoring fluorescence of Chl*a*, excitation slit 20 nm and emission slit 10 nm. The samples were kept in the dark 2 min before measuring. The phosphate buffer used for dilution of the chloroplast suspension was the same as described above. Due to low aqueous solubility the compounds were added to a chloroplast suspension in DMSO solution. The DMSO concentration in all samples was the same as in the control (10% (v/v)). The chlorophyll concentration in chloroplast suspension was 10 mg/L.

4. Conclusions

A series of 15 pyrazinamide derivatives (14 of them novel) was synthesized by aminodehalogenation reactions focusing on microwave assisted synthesis. All the final compounds were characterized with IR, NMR and other analytical data and then subjected to *in vitro* evaluation in order to discover their potential antimycobacterial, antifungal, antibacterial and herbicidal activities.

The lipophilicity was measured using RP-HPLC methodology and also calculated or predicted with the PC program CS ChemBioDraw Ultra 13.0. These values were compared and the dependence between log *k* and log *P* was linear.

In antimycobacterial screening compounds **3** (R = 3,4-Cl), **9** (R = 2-Cl) and **11** (R = 4-CF_3) showed good activity against wild strain *M. tuberculosis* H37Rv (MIC = 6.25 μg/mL) compared to the standards pyrazinamide (MIC = 12.5 μg/mL) and isoniazid (MIC = 1.56 μg/mL). Compounds **11** (R = 4-CF_3) and **12** (R = 2-CF_3) were active against the non-tuberculosis strains *M. kansasii* and *M. avium* as well. Although the majority of synthesized compounds were active, there is no clear dependence between lipophilicity and antimycobacterial activity, but it can be stated that the most potent substances were also from the group of most lipophilic compounds and the most favourable substitutions are the electron-withdrawing groups such as chlorine or trifluoromethyl. Activity against fast growing *Mycobacteria* was also determined but no active substances were identified.

No interesting results were observed in antibacterial and antifungal screenings. Three compounds (**7** (R = 4-NH_2), **10** (R = 2-F), and **15** (R = 4-Cl)) showed insignificant activity against the fungus *Trichophyton mentagrophytes*, which was found to be worse compared to the standards. The rest of substances showed no *in vitro* antibacterial or antifungal activity.

On the contrary, *N*-substituted 5-amino-6-methylpyrazine-2,3-dicarbonitriles were found to inhibit the Hill reaction in spinach chloroplasts which indicated that these compounds act as PS2 inhibitors. The IC_{50} values related to PET inhibition varied in the investigated set in the range from 16.4 μmol/L (**3**; R = 3,4-Cl) to 487.0 μmol/L (**14**; R = 3-NO_2). The lipophilicity of the compounds was determinant for PET-inhibiting activity. The site of inhibitory action of studied compounds in the photosynthetic apparatus is situated both on the donor and on the acceptor side of PS2. Perturbation of the

Chl*a*-protein complexes in the thylakoid membranes caused by the tested compounds was documented by a decrease of the Chl*a* emission band intensity at 686 nm belonging to the pigment-protein complexes in photosystem 2.

Based on the obtained results it can be assumed that the antifungal and antibacterial activities of studied compounds are not directly dependent on lipophilicity. This conclusion cannot be applied for the herbicidal activity because there is a linear dependence between activity and lipophilicity. Dependence between antimycobacterial activity and the benzyl substituents was found. This reliance was expressed by the lipophilicity parameter log *k* resp. π constant and showed the importance of the ring-substituted benzyl moiety.

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Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are available from the authors.

PŘÍLOHA Č. 2

DOLEZAL, M.; ZITKO, J.; **JANDOUREK, O.**; SERVUSOVA-VANASKOVA, B. SUBSTITUTED 2-(2-PHENYLHYDRAZINYL) PYRAZINE, PROCESS FOR ITS PREPARATION, ITS USE AND A PHARMACEUTICAL COMPOSITION CONTAINING THE SAME. International Patent WO2016095877. **2016**.

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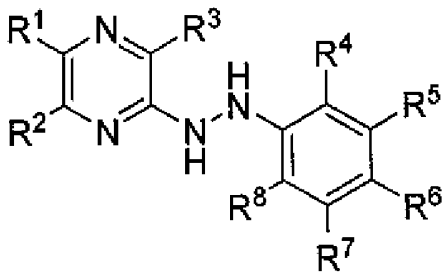

I

(57) Abstract: Substituted 2-(2-phenylhydrazinyl)pyrazine of general formula I, wherein each R^1, R^2 is independently H or CN; R^3 is CH_3 or $CONH_2$; each R^4, R^5, R^6, R^7, R^8 is independently H, CI, or NO_2. The invention further provides a method for preparing such substances, in which the appropriate substituted chloropyrazine is treated with a substituted phenylhydrazine under conditions of microwave synthesis. The compounds of general formula I are characterized by their low toxicity and high activity against mycobacteria. They can also be used in pharmaceutical preparations as antitubercular drugs.

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Substituted 2-(2-phenylhydrazinyl)pyrazine, process for its preparation, its use and a pharmaceutical composition containing the same

Field of the Invention

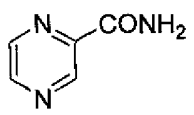
The present invention relates to novel compounds based on substituted phenylhydrazinyl-pyrazines, processes for their preparation, and their use as antituberculous drugs and pharmaceutical preparations containing them.

Background of the Invention

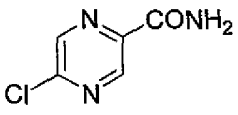
Substituted pyrazine derivatives belong to compounds having significant biological activity, they are used as essential drugs (Miniyar P.B., Murumkar P.R., Patil P.S., et al.: Mini Rev. Med. Chem. **13**, 1607-25, 2013; Ferreira S.B., Kaiser C.R. Exp. Opin. Ther. Patents **22**, 1033-51, 2012).

Tuberculosis (TB), caused by *Mycobacterium tuberculosis complex* (MTB), has for many years belonged to the most widespread infectious diseases in the world. Its incidence and mortality has so far failed to reduce despite existing antituberculotic therapy (World Health Organization, Stop TB Partnership. Tuberculosis Global Facts 2014). The reason is the emergence and development of increasingly resistant forms of the disease agent and co-infection with HIV, the incidence in the Czech Republic is increasing (Ústav zdravotnických informací a statistiky ČR; Tuberkulóza a respirační nemoci 2012, Prha ÚZIS, 2013, 111pp.). Therefore, it is necessary to develop new more efficient low-molecular antituberculotic drugs active against increasingly occurring resistant and/or multiresistant mycobacterial strains.
The most important first line anti-tuberculosis drugs (along with isoniazide, rifampicin and ethambutol) include pyrazinamide (PZA). Only PZA, or rifampicin, are active against latent forms of TB (*i.e.* semidormant mycobacteria occurring in acidic environment tissues). Thanks to PZA is the treatment shortened from 9-12 to 6 months, which is due to the ability to kill mycobacteria persisting in an acidic environment (Zhang H., et al. The FEBS Journal **275**, 753-762, 2008). PZA does not act by a single mechanism, but we recognize still new ways of its action: a) PZA is a prodrug, which is converted with the enzyme nikotinamidase/pyrazinamidase (PncA) to the pyrazinoic acid (POA), as its active form. There is an accumulation of POA and intracellular acidification (Doležal M., Kešetovič D., Zitko J. Curr. Pharm. Design, 2011, vol. 17, no. 32, p. 3506–3514). This disturbance leads to

influencing the activity of enzymatic systems and disrupting the processes that are dependent on the pH gradient at the interface organelle / cytoplasmic compartment (Zhang H., et al. The FEBS Journal **275**, 753-762, 2008). Furthermore, POA acts by inhibiting protein and RNA synthesis, serine uptake, disruption of membrane potential in the acidic pH (Wright H.T., Reynolds K.A. Curr. Opin. Microbiol. **10**, 447-453, 2007); b) PZA influences RpsA - an enzyme system responsible for regulating biological processes, including cell adhesion, migration and differentiation (Zimic M., et al. Microb. Drug Resist. **18**, 372-375, 2012; Sayahi H., et al. Chemistry & Biodiversity **9**, 2582-2596, 2012). Through this process PZA inhibits *trans*-translation, which is a process to prevent the creation of non-functional proteins, and is associated with stress survival, virulence and recovery of nutrient starvation (Shi W., Zhang X., Jiang X., et al. Science **333**, 1630-1632, 2011); c) estimated competition of PZA (and 5-chloro-pyrazine-2-carboxamide) with NADPH for binding to the complex FAS I (Fatty Acid Synthase). The synthesis of mycolic acids, which are part of the cell wall of mycobacteria, is inhibited. Also POA is binding to FAS I, but in different location of the enzyme (Zimic M., et al. Microb. Drug Resist. **18**, 372-375, 2012; Sayahi H., et al. Chemistry & Biodiversity **9**, 2582-2596, 2012). Recently it has been found that FAS I is the primary goal of the pyrazinamide derivatives, including 5-chloropyrazinamide, which also inhibits FAS complex II. FAS I is involved in the synthesis of short chain-mycolic acids, FAS II mediates the synthesis of long chain-mycolic acids. One of the key enzymes of FAD II is the enoyl-ACP reductase (Ngo S.C., et al. Antimicrob. Agents Chemother. **51**, 2430-2435, 2007).



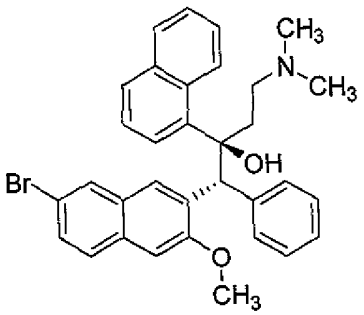
pyrazinamide



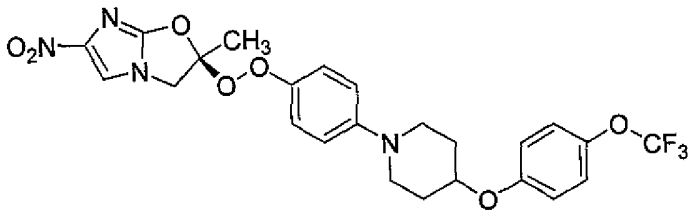
5-chloropyrazine-2-carboxamide

Pharmaceutical research and development has neglected the field of antituberculotics (AT); for almost 40 years there have been no launch of a new drug in this indication group; currently (2015), only 3 new antituberculotics (entirely new structures), bedaquilline, delamanid and pretomanid, are in the third stage of clinical trials worldwide. However, these new ATs must be combined with, *e.g.*, PZA or rifampicin. Due to problems of TB therapy, any new substance is highly expected since resistance develops gradually to actually used

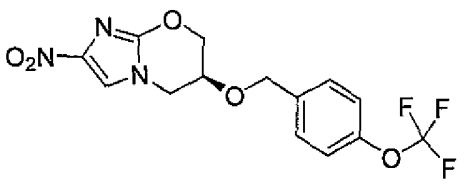
ATs and it is only a matter of time when no effective drugs will be available against TB (Matthias Stehr M., Elamin A.A., Singh M. Curr. Top. Med. Chem. **14**, 110-129, 2014).



bedaquilline

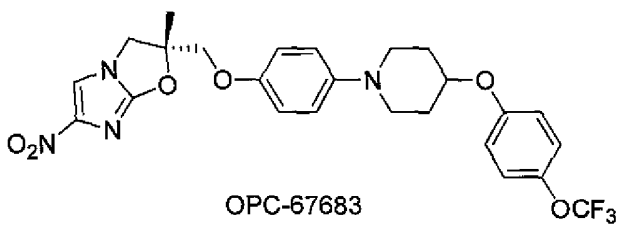


delamanid

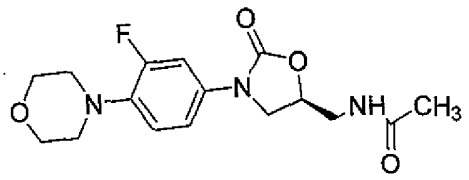


pretomanid

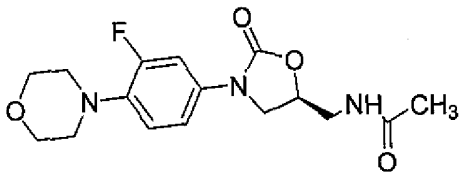
For the reasons mentioned above there persist attempts to find an AT which would act against MDR-TB strains, or against latent forms of TB. They must be structurally novel compounds which act by novel mechanisms other than the currently used AT, or with an improved pharmacokinetic profile. New potential ATs, which are now in preclinical and clinical stage of development, are often substances which contain a nitro moiety in their molecule. Relevant examples include the nitroimidazoles pretomanid, OPC-67683 and delamanid. Another promising group of AT are oxazolidinones, for example linezolid and sutezolid, containing, *inter alia*, the carboxamide functional moiety (Matthias Stehr M., Elamin A.A., Singh M. Curr. Top. Med. Chem. **14**, 110-129, 2014).



OPC-67683

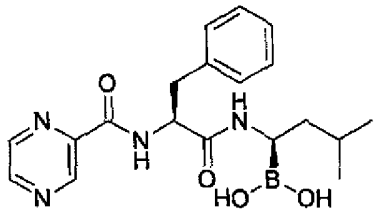


linezolid

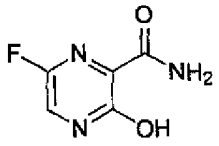


sutezolid

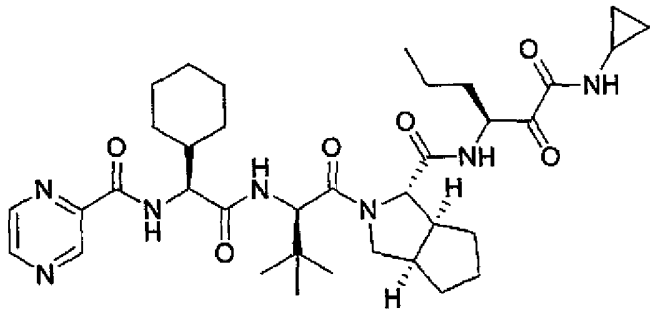
For all these substances, the nitro or carboxamide moieties have proved essential for the antimycobacterial activity, but the mechanisms of action were different for each other (Matthias Stehr M., Elamin A.A., Singh M. Curr. Top. Med. Chem. **14**, 110-129, 2014). The pyrazinecarboxamide motif can be found in a wide range of modern drugs such as the antineoplastic agent bortezomib (Palle Raghavendracharyulu Venkata, Kadaboina Rajasekhar, Murki Veerendeer et al., Bortezomib and proces for producing same. US2010226597 (2010)), the antivirotic favipiravir (Furuta Y, Takahashi K, Shirakib K, et al. T-705 (favipiravir) and related compounds: Novel broad-spectrum inhibitors of RNA viral infections. Antiviral Res. **82**, 95-102, 2009), or telaprevir (Zeuzem S., Andreone P., Pol S., et al. Telaprevir for Retreatment of HCV Infection. New Engl. J. Med. **25**, 2417-28, 2011).



bortezomib



favipiravir



telaprevir

A PZA fragment is also contained in a large number of drugs which are in the clinical trial phase, for example pyrazine derivatives patented as kinase inhibitors (Charrier J.D., Durrant S.J., Kay D., et al. Compounds useful as inhibitors of ATR kinase; US2014113005 (2014);

Song Y., Xu Q., Jia Z., et al. Preparation of pyrazine derivatives for use as Syk kinase activity inhibitors. US20130131040 (2013); Boyle R.G., Boyce R.J. Preparation of 1-(substituted phenyl)-3-(5-cyano-pyrazin-2-yl) urea compounds as Chk-1 kinase inhibitors. WO2013072502 (2013); Collins I., Lainchbury M., Matthews T.P., Reader J.C. Preparation of 5-(pyridin-2-ylamino)pyrazine-2-carbonitrile compounds as CHK1 inhibitors. WO2013068755 (2013); Collins I., Lainchbury M., Matthews T.P., Reader J.C. 5-(pyridine-2-yl-aminopyrazine-2-carbonitrile compounds and their therapeutic use. AU2012335409 (2014)).

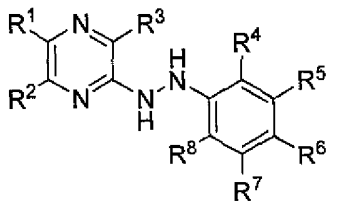
Over the past decades, methods of microwave synthesis methods and their applications suitable for the development of new drugs have got the forefront of interest of pharmaceutical chemists. These reactions usually provide a higher yield, require significantly shorter reaction time, and reduce the consumption of solvents compared to conventional methods of organic synthesis. In some cases, the desired products can be obtained only by using a microwave synthesis. Advantages of these reactions can be explained by the interaction of microwaves and molecules; the possibility of reaching a temperature greater than the boiling point of the solvent at atmospheric pressure (in a sealed system) is also very advantageous (Hayes B.L. Microwave Synthesis: Chemistry at the Speed of Light; CEM Publishing: Matthews, NC, USA, 2002; De la Hoz A., Diaz-Ortiz A., Moreno A. Microwaves in organic synthesis. Thermal and non-thermal microwave effects. Chem. Soc. Rev. **34**, 164–178, 2005). During microwave heating, microwaves act directly on the molecules present in the reaction mixture and heating is hence not dependent on the thermal conductivity of the container, unlike in classical heating. The transmission of energy from the microwave heating to the substance proceeds by two basic mechanisms, either by dipole rotation, or by ion conductivity. Dipole rotation is an interaction of the spin at which polar molecules attempt to align with rapidly changing electric field of the microwaves. This rotational motion of molecules results in energy transfer. The second way is to transmit energy through the ion conduction. If free ions or ionic groups are present in the reaction mixture, these will move in the solution under the influence of an electric field, which leads to energy expenditure due to the increased extent of precipitation and a conversion of kinetic energy into heat occurs. The temperature of the substance also affects the ionic conductivity - as the temperature rises, energy transfer becomes more efficient (Hayes B.L. Microwave Synthesis: Chemistry at the Speed of Light; CEM Publishing: Matthews, NC, USA, 2002; Lidström P., Tierney J., Wathey B., Westman J.

Microwave assisted organic synthesis - a review. Tetrahedron. **57**, 9225-9283, 2001). Also the choice of solvents for microwave-assisted synthesis can be critical to the outcome of the reaction. The important characteristics of used solvents include their polarity, the rule for which is that the more polar solvent, the higher the ability to interact with microwaves will be. Even solvents having a low boiling point can be used in this synthesis because the microwave energy reaches and exceeds the boiling point of most of solvents in a few seconds. Using of pressurized reaction vessels further ensures recovery of solvents with a low boiling point. Another important factor in selecting the solvent is how efficiently the solvent molecules interact with microwaves and convert the microwave energy into thermal energy. The microwave synthesis can be applied even in systems without using any solvent. There are three main types of reactions without solvent: reaction mixtures adsorbed on mineral oxides, reactions of a phase transfer catalyst and a clean reactions (Hayes B.L. Microwave Synthesis: Chemistry at the Speed of Light; CEM Publishing: Matthews, NC, USA, 2002).

The compound 5-chloro-6-methyl-pyrazine-2,3-dicarbonitrile (II) was used in the past for the preparation of a series of substituted 5-benzylamino-6-methylpyrazine-2,3-dicarbonitriles; the obtained products did not show significant biological properties in the tests (Jandourek, O.; Dolezal, M.; Paterova, P.; Kubicek, V.; Pesko, M.; Kunes, J.; Coffey, A.; Guo, J.; Kralova, K. Molecules **19**, 651-671,2014). Also, the compound 3-chloropyrazine-2-carboxamide (III) was used in the past for the preparation of a series of substituted 3-(alkylamino)pyrazine-2-carboxamides; the products obtained did not show significant biological properties in the assays (Jandourek, O.; Dolezal, M.; Kunes, J.; Kubicek, V.; Paterova, P.; Pesko, M.; Buchta, V.; Kralova, K.; Zitko, J. Molecules **19**, 9318-9338, 2014).

Disclosure of the invention

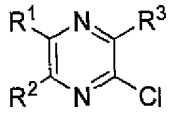
The invention relates to novel substituted 2-(2-phenylhydrazinyl)pyrazine of the general formula I



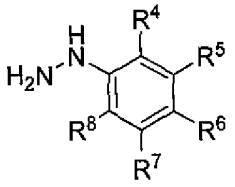
I

wherein each R^1, R^2 is independently H or CN; R^3 is CH_3 or $CONH_2$; each R^4, R^5, R^6, R^7, R^8 is independently H, Cl, or NO_2.

Another object of the invention is a method for preparing of a substituted 2-(2-phenylhydrazinyl)pyrazine of general formula I, consisting in that the substituted chloropyrazine of general formula



wherein each R^1, R^2 is independently H or CN; R^3 is CH_3 or $CONH_2$, is reacted with a substituted phenylhydrazine of general formula



wherein each R^4, R^5, R^6, R^7, R^8 is independently H, Cl, or NO_2, in a polar solvent, under the conditions of microwave synthesis to form a substituted phenylhydrazinylpyrazine of general formula I.

From the above physical conditions of microwave synthesis, we have chosen, as the best, the following reaction conditions: polar solvent: methanol, time 30 min., temperature 140 °C, pressure 15 kPa and an output of 120 W.

Another object of the invention relates to the use of the above mentioned substituted 2-(2-fenylhydrazinyl)pyrazine of the formula I according to the invention for use as an antituberculotic against *Mycobacterium tuberculosis* and against its atypical strains (mycobacteriosis pathogens), including pathogenic strains isolated from the sick patients.

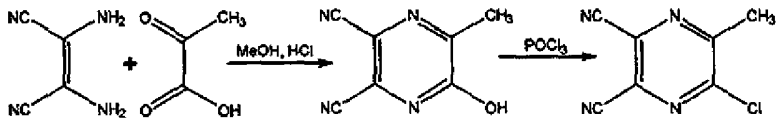
All compounds of the general formula I contain, in their molecule, the hydrazino group, but their toxicity in the tests carried out is very low. These substances are exceptional with by their low toxicity since the hydrazine group in the molecule of a drug usually bears higher toxicity or irritation. Based on current knowledge of pharmaceutical chemistry such

substitution in the field of synthesis of new promising drugs previously has always been considered undesirable.

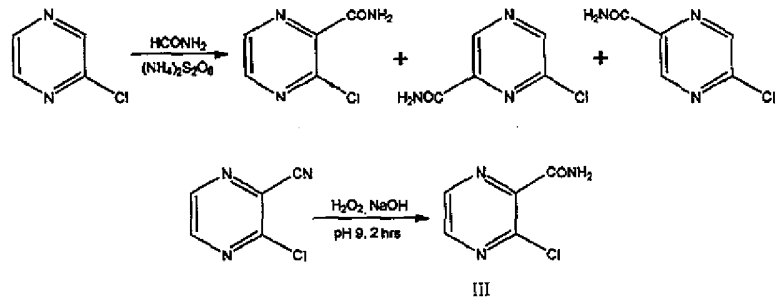
An indispensable role for the effect is also played by theselected a six-membered heterocyclic pyrazine ring, or the pyrazinecarboxamide or pyrazinecarbonitrile fragment, because such moiety represents the aza analog of nicotinamide, which plays a crucial role in the metabolism of mycobacteria.

The essence of the invention consists is a combination of the six-membered heterocycle pyrazine and an aromatic part, linked each other with a linking hydrazine bridge. The heteroaromatic ring is substituted with a carboxamide or carbonitrile moieties, while the aromatic ring is unsubstituted, or substituted with nitro groups or chlorine atoms in various positions.

The starting compounds II and III are accessible by conventional methods of organic synthesis (Takematsu T., Segawa H., Miura, T. et al. A. 2,3-Dicyanopyrazines. USP4259489, 1981; Dlabal K., Palat K., Lycka A., Odlerova Z. Synthesis and ^{1}H- and ^{13}C-NMR spectra of sulfur derivatives of pyrazine derived from amidation product of 2-chloropyrazine and 6-chloro-2-pyrazinecarbonitrile. Tuberculostatic activity. Collect. Czechoslov. Chem. Commun. **55**, 2493–2500, 1990).



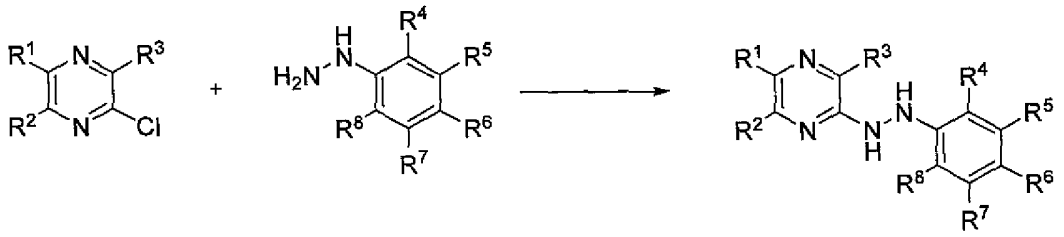
II



III

The final products of general formula I were in turn obtained by amino(hydrazino)dehalogenation of the corresponding substituted chloropyrazine by the reaction with an appropriately substituted phenylhydrazine (Scheme 1), wherein R^1 a R^2 is H

(hydrogen atom) or -CN (carbonitrile group), R^3 is $-CH_3$ (methyl) or $-CONH_2$ (carboxamide) group and R^4, R^5, R^6, R^7, R^8 are H (hydrogen atom), Cl (chlorine) or $-NO_2$ (nitro moiety), under the conditions of microwave synthesis. Their preparation is not synthetically difficult, and the raw material from which they are prepared is readily accessible and inexpensive.



Scheme 1

The synthesis of the products of general formula I was carried out in the focused microwave field apparatus CEM Discover (CEM Corporation, Matthews, NC, USA). Using of the technology of microwaves, which is a part of the electromagnetic waves with wavelengths of 1 cm to 1 m (mainly used frequency of 2450 MHz), greatly facilitates and accelerates all reactions. In fact, microwaves easily and quickly change the polarity of the electromagnetic field used. The resulting oscillation act on the polar compound and, by oscillating the reaction material, give it energy. This might lead to the erosion of bonds and increasing of the kinetic energy of the molecules. This in fact increases the temperature and it is just the thermic effect which is of significant benefit of the methodology used (Hayes B.L. Microwave Synthesis: Chemistry at the Speed of Light; CEM Publishing: Matthews, NC, USA, 2002; De la Hoz A., Diaz-Ortiz A., Moreno A. Microwaves in organic synthesis. Thermal and non-thermal microwave effects. Chem. Soc. Rev. **34**, 164–178, 2005). The apparatus used is equipped with a so-called focused field, which is advantageous since the microwaves are aimed directly to the reaction mixture placed in a waveguide. Another positive aspect of the method used is also time, energy and solvents saving.

The prepared compounds of general formula I have been evaluated *in vitro* against the strains of *M. kansasii* 235/80, *M. avium* 80/72, *M. avium* 152/73 and *M. tuberculosis* H37Rv using a fluid Sula's semisynthetic medium (Trios, Prague, Czech Republic) by the microdilution method in comparison with pyrazinamide (PZA) at pH 5.6. The tested compounds were dissolved in dimethylsulfoxide (DMSO) and diluted with the medium to final concentrations

of 100, 50, 25, 12,5, 6,25, 3,125 and 1,563 μg/mL. The results were read after two or three weeks. Pyrazinamide (PZA) was used as the standard. The results are shown in Table 1. Results indicate high antimycobacterial activity, for all tested compounds of formula I it was 2-16x higher than that of the used standard PZA. High activity was discovered against *M. kansasii* for compounds **1-5** of general formula I and against *M. avium* in the case of compounds **2-4** of general formula I.

Table 1 Antimycobacterial activity of compounds of general formula I

Strain	Compound Evaluated (code) MIC ($\mu g.L^{-1}$)										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	PZA
M. tuberculosis H37Rv	**6.25**	**12.5**	**12.5**	**1.56**	**12.5**	**12.5**	**6.25**	**12.5**	**12.5**	**12.5**	25
M. kansasii My 235/80	**1.56**	**12.5**	**12.5**	**12.5**	**12.5**	>100	>100	>100	>100	>100	>100
M. avium 80/72	>100	**50**	**50**	**50**	**100**	>100	>100	>100	>100	>100	>100
M. avium 152/73	>100	**25**	**12.5**	**12.5**	**100**	>100	>100	>100	>100	>100	>100

All compounds corresponding to general formula I were also tested for their antifungal activity *in vitro* using the microdilution broth assay.

Medium used: RPMI 1640 with glutamine

Incubation duration: 24 – 48 hrs. (for TM: 48 – 72 hrs.)

Incubation method: static, in the dark, humid atmosphere

Reading: visual / photometric (OD 540 nm); MIC = IC_{80}, for filamentous fungi: IC_{50} (inhibition of the control)

pH / buffer: 7.0 / MOPS (0,165 M)

Temperature: 35 °C

Strains evaluated (code, number):

CA – *Candida albicans* ATCC 44859

CT – *Candida tropicalis* 156

CK – *Candida krusei* E28

CG – *Candida glabrata* 20/I

TA – *Trichosporon asahii* 1188

AF – *Aspergillus fumigatus* 231

AC – *Absidia corymbifera* 272

TM - *Trichophyton mentagrophytes* 445

Standards: FLZ – Fluconazole, AMB – Amphotericin B

The results are shown in Table 2. The results show antifungal activity in virtually most of the tested compounds of general formula I. High antifungal activity was discovered of for the compounds **1**, **2** and **4** of general formula I against yeasts and filamentous fungi.

Tab. 2 Antimycotic activity of compounds with general formula I

strain (code)		Compound Evaluated (code) MIC/IC_{80} /IC_{50} (μmol.L^{-1})											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**FLZ**	**AMB**
CA	24h	**15.62**	**125**	>500	**125**	>500	>500	**500**	**500**	>250	>125	0.25	0.016
	48h	**31.25**	**500**	>500	**250**	>500	>500	>500	>500	>250	>125	0.5	0.063
CT	24h	**62.5**	**250**	>500	**250**	>500	>500	>500	>500	>250	>125	0.5	0.063
	48h	**250**	>500	>500	**500**	>500	>500	>500	>500	>250	>125	>128	0.063
CK	24h	**3.9**	**0.49**	**7.81**	**31.25**	>500	>500	**500**	**500**	>250	>125	16	0.125
	48h	**7.81**	**3.9**	**15.62**	**250**	>500	>500	>500	>500	>250	>125	32	0.125
CG	24h	**62.5**	**250**	>500	**15.62**	>500	>500	**500**	>500	>250	>125	4	0.031
	48h	**62.5**	**250**	>500	**31.25**	>500	>500	>500	>500	>250	>125	16	0.125
TA	24h	**62.5**	**250**	>500	**15.62**	>500	**500**	**500**	>500	>250	>125	0.25	1
	48h	**125**	**250**	>500	**62.5**	>500	**500**	>500	>500	>250	>125	0.5	2
AF	24h	**31.25**	**15.62**	**62.5**	**3.9**	>500	>500	**500**	**250**	>250	>125	>128	0.25
	48h	**62.5**	**250**	>500	**15.62**	>500	>500	>500	>500	>250	>125	>128	0.125
AC	24h	**31,25**	**15,62**	**125**	**7,81**	>500	**500**	**500**	**125**	>250	>125	>128	1
	48h	**31.25**	**15.62**	>500	**15.62**	>500	>500	**500**	**250**	>250	>125	>128	2
TM	72h	**500**	**0.49**	**1.65**	**0.49**	**250**	**500**	**500**	**500**	>250	>125	8	1
	120h	**500**	**7.81**	**7.81**	**3.9**	**250**	**500**	**500**	**500**	>250	>125	16	1

The prepared derivatives corresponding to general formula I were tested for their antibacterial activity *in vitro*, using the microdilution broth test.

Medium used: Müeller Hinton broth

Incubation duration: 24 – 48 hrs.

Incubation method: static, in the dark, humid atmosphere

Reading: visual / photometric (OD 540 nm); MIC = IC_{80} (80 % of inhibition of the control)

Temperature: 35 °C

Strains evaluated (code, number):

SA – *Staphylococcus aureus* CCM 4516/08

MRSA - *Staphylococcus aureus* (methicilin resistant) H 5996/08

SE – *Staphylococcus epidermis* H 6966/08

EF – *Enterococcus sp.* J 14365/08

EC – *Escherichia coli* CCM4517

KP – *Klebsiella pneumoniae* D 11750/08

KP-E - *Klebsiella pneumoniae* (ESBL positive) J 14368/08

PA – *Pseudomonas aeruginosa* CCM 1961

Standards: NEOM – Neomycin sulphate, BAC – bacitracin, PEN – penicillin G, CIPR – ciprofloxacin, PHEN – phenoxymethylpenicillin.

The results are shown in Table 3. The results show that selective antibacterial activity was discovered for virtually most of the compounds of general formula I tested against staphylococci and enterococci.

Tab. 3 Antibacterial activity of compounds with general formula I

Strain (code)		Compound Evaluated (code) MIC/IC_{80} ($\mu mol.L^{-1}$)														
		1	2	3	4	5	6	7	8	9	10	NEOM	BA	PEN	CIPR	PHEN
SA	24h	62.5	15.62	62.5	7.81	62.5	>500	500	500	500	>250	3.9	15.3	0.24	0.98	0.24
	48h	62.5	62.5	125	15.62	250	>500	500	500	500	>250	3.9	31.2	0.24	0.98	0.24
MRSA	24h	62.5	7.81	125	15.62	62.5	>500	500	500	500	>250	0.98	15.3	125	500	250
	48h	62.5	62.5	125	15.62	250	>500	>500	>500	500	>250	0.98	31.2	125	500	500
SE	24h	15.62	31.25	62.5	7.81	31.25	500	31.25	62.5	62.5	31.25	3.9	15.6	31.25	250	62.5
	48h	15.62	62.5	62.5	15.62	31.25	500	31.25	62.5	62.5	31.25	7.81	31.2	125	250	250
EF	24h	250	250	125	500	500	500	250	62.5	250	62.5	250	31.2	7.81	0.98	7.81
	48h	250	>500	125	500	500	>500	250	62.5	500	62.5	250	31.2	15.62	0.98	7.81
EC	24h	>500	>500	>500	>500	>500	>500	>500	>500	>500	>250	0.98	>500	125	0.06	>500
	48h	>500	>500	>500	>500	>500	>500	>500	>500	>500	>250	0.98	>500	125	0.06	>500
KP	24h	>500	>500	>500	>500	>500	>500	>500	>500	>500	>250	0.98	>500	250	0.12	>500
	48h	>500	>500	>500	>500	>500	>500	>500	>500	>500	>250	0.98	>500	500	0.12	>500
KP-E	24h	>500	>500	>500	>500	>500	>500	>500	>500	>500	>250	0.98	>500	>500	>500	>500
	48h	>500	>500	>500	>500	>500	>500	>500	>500	>500	>250	0.98	>500	>500	>500	>500
PA	72h	>500	>500	>500	500	>500	>500	>500	>500	>500	>250	7.81	>500	>500	3.9	>500
	120h	>500	>500	>500	500	>500	>500	>500	>500	>500	>250	15.62	>500	>500	7.81	>500

The prepared derivatives corresponding to the general formula I were also tested for antiviral activity at Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium (Prof. Dr. Lieve Naesens and co-workers). The viral infections against which efficacy was evaluated included: Influenza virus A (H1N1; H3N2) and B, Herpes simplex virus-1 and -2, Vesicular stomatitis virus, Vaccinia virus, Parainfluenza-3 virus, Reovirus-1, Sindbisvirus, Coxsackie virus B4, Punta Toro virus, Respiratory syncytial virus, Feline corona virus, Feline herpes virus. Zanamivir, ribavirin, amantadin, rimantadin, ribavirin, and ganciclovir, inter alia, were used as standards. The results are shown in Table 4, wherein the given EC_{50} values [μM] is the effective concentration at which a 50% inhibition of the studied virus induced cytopathic effect occurs. Only for compounds **5** and **6** corresponding to the general formula I antiviral effects have been discovered.

Tab. 4 Antiviral activity of compounds with general formula I

Compd. Code / Standard	EC_{50} [μM]			
	Influenza A/H1N1		Vesicular stomatitis virus	Feline herpes virus
	visual CPE score	MTS		
5	**4.0**	**2.4**	**8.9 / 8.9**	**5.0 / 9,2**
6	-	-	-	**4.0 / 8.2**
Zanamivir	4.0	1.5	-	-
Ribavirin	12	10	-	-
Amantadin	100	201	-	-
Rimantadin	20	7.3	-	-
Ribavirin	-	-	**25.0 / 112.0**	-
Ganciclovir	-	-		**0.9 / 3.8**

The most active compounds of general formula I were subjected to basic toxicity MTT tests on human hepatocytes, or on a CRFK cell culture (Crandel-Ress feline cells). The results are shown in Tables 5 and 6. The results show that even at the highest concentration used (20 μM) of the evaluated potential antitubercular compounds corresponding to the general formula I hepatocyte viability is not affected. Standard methodology was used (Owen T.C. USP5185450, 1993; Crandell R.A., Fabricant C.G., Nelson-Rees W.A. In Vitro **9**, 176-185, 1973).

Table 5 Cytotoxicity of selected compounds of general formula I evaluated on human hepatocytes Hep G2 (data are sorted from highest to lowest toxicity)

Compound	Cytotoxicity IC_{50} (μM)
3	21.9
5	27.8
7	481.4
6	863.7

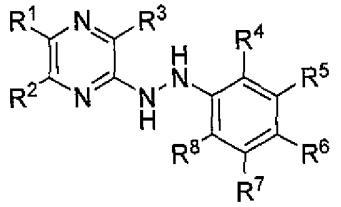
Table 6 Cytotoxicity of selected compounds of general formula I evaluated on CRFK cell culture (cell line derived from kidney cells of domestic cats)

Compound	Cytotoxicity CC_{50} (μM)
1	37
3	54
4	33
5	48

6	24
10	>100

Examples

The following examples illustrate the process of the present invention relating to the preparation and use of substituted 2-(2-fenylhydrazinyl)pyrazines of general formula I



I

wherein the symbols R^1, R^2, R^3, R^4, R^5, R^6, R^7, and R^8 are as defined above.

Thin Layer Chromatography (TLC) was used for monitoring and controlling thesynthetic reactions and for verifying the purity of the products. Hexane and ethyl acetate (1:1) were used as mobile phases. The reaction progress was checked with TLC plates (Merck, Darmstadt, Germany), silica gel 60 F_{254}, using 254 nm wavelenght UV detection.

For chemicals used for the following reactions the basic physical constants have been validated. The solvents used for the preparation of starting compounds II and III (acetone, toluene, and chloroform), were thoroughly purified and dried. A conventional, commercially available, anhydrous methanol was used for the preparation of the final synthesized products.

Newly synthesized products were separated and purified by using of preparative chromatograph CombiFlash® Rf (Teledyne Isco, Inc. Lincoln, Nebraska, USA). Melting point confirmation of pure product was determined with open capillary apparatus Stuart Scientific, SMP30 (Bibby Scientific Ltd., Staffordshire, UK) and was uncorrected.

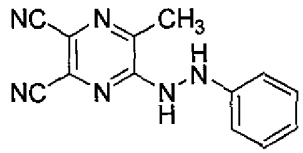
Identity of compounds prepared was recorded and checked by means of ^{1}H-NMR and ^{13}C-NMR spectra on Varian Mercury VX-BB 300 and Varian VNMR S500 spectrometers (Varian Corp., Palo Alto, CA, USA). The spectra were recorded in deuterated $CDCl_3$ or DMSO at the correct room temperature and the frequency of 300 MHz for ^{1}H and 75 MHz for ^{13}C; and 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively. Chemical shifts were reported in ppm (δ) in ppm units and were applied indirectly to tetramethylsilane as a signal of solvent (2.49 for ^{1}H and 39.7 for ^{13}C in DMSO-*d*6).

Infrared spectra were recorded in deuterated $CDCl_3$ or DMSO with spectrometer FT-IR Nicolet 6700 (Thermo Scientific, Waltham, MA, USA) using attenuated total reflectance (ATR) methodology.

Elemental analyses were measured with an EA 1110 CHNS Analyzer (Fisons Instruments S. p. A., Carlo Erba, Milano, Italy). All measured values are given in percent.

Experimental lipophilicity parameter log *k* was ascertained using an Agilent Technologies 1200 SL HPLC system with a SL G1315C Diode-Array Detector, ZORBAX XDB-C18 5 μm, 4 × 4 mm, Part No. 7995118-504 chromatographic pre-column and ZORBAX Eclipse XDB-C18 5 μm, 4.6 × 250 mm, Part No. 7995118-585 column (Agilent Technologies Inc., Colorado Springs, CO, USA). The separation process was controlled by Agilent ChemStation, version B.04.02 extended by spectral module (Agilent Technologies Inc.). A solution of MeOH (HPLC grade, 70%) and H_2O (HPLC-Milli-Q Grade, 30%) was used as mobile phase. The total flow of the column was 1.0 mL/min, injection 20 μL, column temperature 30 °C. Detection wavelength λ= 210 nm and monitor wavelength λ= 270 nm were chosen for this measurement. The KI methanol solution was used for the dead time (TD) determination. Retention times (TR) of synthesized compounds were measured in minutes. The capacity factors *k* were calculated using Microsoft Excel according to the formula $k = (TR - TD)/TD$, where TR is the retention time of the solute and TD denotes the dead time obtained *via* an unretained analyte. Log *k*, calculated from the capacity factor *k*, is used as the lipophilicity index converted to log *P* scale. Values of log *P* and Clog *P* were calculated with the PC programme CS ChemBioDraw Ultra 13.0 (CambridgeSoft, Cambridge, MA, USA).

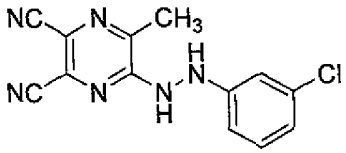
Example 1: 5-Methyl-6-(2-phenylhydrazinyl)pyrazine-2,3-dicarbonitrile (**1**)



Compound **1** is prepared by reaction of phenylhydrazine (3 mmol) with 5-chloro-6-methylpyrazine-2,3-dicarbonitrile (II, 1 mmol) in 3 mL of methanol and pyridine (1 mmol). The reaction is performed in a microwave reactor at the temperature 140°C, pressure 15 kPa and an output of 120 W during 30 min. After completing the reaction, product **1** was isolated and purified by column chromatography on silica gel (mobile phase: hexane / ethyl acetate 1:1), yield 29%. Analytical data for compound **1**: Brown-red crystalline solid; Mp. = 158.1-159.7°C; Elemental analysis calculated for $C_{13}H_{10}N_6$ (m.w. 250.26): 62.39 % C, 4.03 % H, 33.58 % N; found 62.49 % C, 4.30 % H, 33.31 % N; IR (ATR-Ge, cm^{-1}): 3398 (-NH-), 3297 (-NH-), 2238 (-CN), 1603, 1554, 1485, 1445, 1393 (pyr); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.03

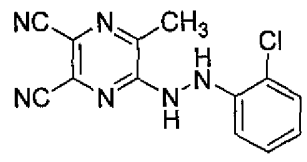
(1H, bs, NH), 8.16 (1H, bs, NH), 7.21-7.06 (2H, m, H2′, H6′), 6.84-6.65 (3H, m, H3′, H4′, H5′), 2.50 (3H, s, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 153.8, 148.2, 146.7, 130.1, 129.1, 119.3, 119.0, 115.5, 114.8, 112.6, 21.2; Lipophilicity: calc. value log *P* = 2.07; experimental determined value log *k* = 0.2218.

Example 2: 5-(2-(3-chlorophenyl)hydrazinyl)-6-methylpyrazine-2,3-dicarbonitrile (**2**)



Compound **2** is prepared by reaction of 3-chlorophenylhydrazine (3 mmol) with 5-chloro-6-methylpyrazine-2,3-dicarbonitrile (II, 1 mmol) in 3 mL of methanol and pyridine (1 mmol). The reaction is performed in a microwave reactor at the temperature 140°C, pressure 15 kPa and an output of 120 W during 30 min. After completing the reaction, product **2** was isolated and purified by column chromatography on silica gel (mobile phase: hexane / ethyl acetate 1:1), yield 65%. Analytical data for compound **2**: Green-brown crystalline solid; Mp. = 177.0-177.8°C; Elemental analysis calculated for $C_{13}H_9ClN_6$ (m.w. 284.70): 54.84% C, 3.19% H, 29.52% N; found 54.95% C, 2.95% H, 29.54% N; IR (ATR-Ge, cm^{-1}): 3314 (-NH-), 3285 (-NH-), 2231 (-CN), 1599, 1549, 1485, 1430, 1398 (pyr); ^1H-NMR (300 MHz, $CDCl_3$) δ 10.05 (1H, bs, NH), 8.46 (1H, bs, NH), 7.16 (1H, t, *J* = 8.0 Hz, H5′), 6.89-6.71 (3H, m, H2′, H4′, H6′), 2.52 (3H, s, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 153.6, 149.9, 147.0, 133.9, 130.7, 130.0, 119.3, 118.8, 115.5, 114.8, 111.8, 111.2, 21.3; Lipophilicity: calc. value log *P* = 2.63; experimental determined value log *k* = 0.2499.

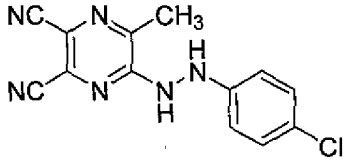
Example 3: 5-(2-(2-chlorophenyl)hydrazinyl)-6-methylpyrazine-2,3-dicarbonitrile (**3**)



Compound **3** is prepared by reaction of 2-chlorophenylhydrazine (3 mmol) with 5-chloro-6-methylpyrazine-2,3-dicarbonitrile (II, 1 mmol) in 3 mL of methanol and pyridine (1 mmol). The reaction is performed in a microwave reactor at the temperature 140°C, pressure 15 kPa and an output of 120 W during 30 min. After completing the reaction, product **3** was isolated and purified by column chromatography on silica gel (mobile phase: hexane / ethyl acetate

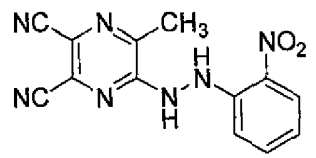
1:1), yield 67%. Analytical data for compound **3**: Green-brown crystalline solid; Mp. > 250.0°C (decomp.); Elemental analysis calculated for $C_{13}H_9ClN_6$ (m.w. 284.70): 54.84% C, 3.19% H, 29.52% N; found 54.78% C, 3.22% H, 29.34% N; IR (ATR-Ge, cm^{-1}): 3289 (-NH-), 2231 (-CN), 1594, 1564, 1488, 1442, 1398 (pyr); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.17 (1H, bs, NH), 7.90 (1H, bs, NH), 7.34 (1H, dd, J = 7.8 Hz, J = 1.1 Hz, H3′), 7.12 (1H, t, J = 7.8 Hz, H5′), 6.88 (1H, dd, J = 7.8 Hz, J = 1.1 Hz, H6′), 6.83-6.75 (1H, m, H4′), 2.54 (3H, s, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 153.6, 146.8, 143.7, 130.0, 129.5, 128.0, 120.3, 119.4, 117.7, 115.4, 114.8, 113.4, 21.2; Lipophilicity: calc. value log P = 2.63; experimental determined value log k = 0.1793.

Example 4: 5-(2-(4-chlorophenyl)hydrazinyl)-6-methylpyrazine-2,3-dicarbonitrile (**4**)



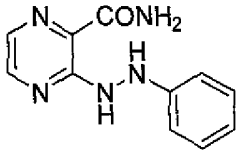
Compound **4** is prepared by reaction of 4-chlorophenylhydrazine (3 mmol) with 5-chloro-6-methylpyrazine-2,3-dicarbonitrile (II, 1 mmol) in 3 mL of methanol and pyridine (1 mmol). The reaction is performed in a microwave reactor at the temperature 140°C, pressure 15 kPa and an output of 120 W during 30 min. After completing the reaction, product **4** was isolated and purified by column chromatography on silica gel (mobile phase: hexane / ethyl acetate 1:1), yield 21%. Analytical data for compound **4**: Light-brown crystalline solid; Mp. = 152.0-152.8°C; Elemental analysis calculated for $C_{13}H_9ClN_6$ (m.w. 284.70): 54.84% C, 3.19% H, 29.52% N; found 55.11% C, 2.89% H, 29.32% N; IR (ATR-Ge, cm^{-1}): 3355 (-NH-), 3306 (-NH-), 2225 (-CN), 1598, 1557, 1491, 1437, 1397 (pyr); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.06 (1H, bs, NH), 8.36 (1H, bs, NH), 7.22-7.13 (2H, m, AA′, BB′, H2′, H6′), 6.86-6.77 (2H, m, AA′, BB′, H3′, H5′), 2.51 (3H, s, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 153.7, 147.3, 146.9, 130.0, 128.9, 122.6, 119.2, 115.5, 114.8, 114.2, 21.2; Lipophilicity: calc. value log P = 2,63; experimental determined value log k = 0.2333.

Example 5: 5-(2-(2-nitrophenyl)hydrazinyl)-6-methylpyrazine-2,3-dicarbonitrile (**5**)



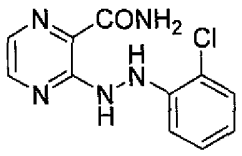
Compound **5** is prepared by reaction of 2-nitrophenylhydrazine (3 mmol) with 5-chloro-6-methylpyrazine-2,3-dicarbonitrile (II, 1 mmol) in 3 mL of methanol and pyridine (1 mmol). The reaction is performed in a microwave reactor at the temperature 140°C, pressure 15 kPa and an output of 120 W during 30 min. After completing the reaction, product **5** was isolated and purified by column chromatography on silica gel (mobile phase: hexane / ethyl acetate 1:1), yield 38%. Analytical data for compound **5**: Dark-brown crystalline solid; Mp. = 189.9-190.6°C; Elemental analysis calculated for $C_{13}H_7N_7O_2$ (m.w. 295.26): 52.88% C, 3.07% H, 33.21% N; found 52.99% C, 2.90% H, 33.21% N; IR (ATR-Ge, cm^{-1}): 3410 (-NH-), 3327 (-NH-), 2232 (-CN), 1614, 1558, 1489, 1445, 1401 (pyr), 1516, 1341 ($-NO_2$); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.40 (1H, bs, NH), 9.58 (1H, bs, NH), 8.22-8.06 (1H, m, Ar), 7.62-7.47 (1H, m, Ar), 7.32-7.19 (1H, m, Ar), 6.98-6.84 (1H, m, Ar), 2.36 (3H, s, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 154.8; 146.4; 144.6; 136.7; 132.5; 130.5; 126.1; 118.6; 117.4; 115.7; 115.3; 114.7; 21.2; Lipophilicity: calc. value log *P* = 1.15; experimental determined value log *k* = -0.0512.

Example 6: 3-(2-phenylhydrazinyl)pyrazine-2-carboxamide (**6**)



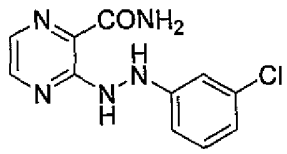
Compound **6** is prepared by reaction of phenylhydrazine (3 mmol) with 3-chloropyrazine-2-carboxamide (III, 1 mmol) in 3 mL methanol and pyridine (1 mmol). The reaction is performed in a microwave reactor at the temperature 140°C, pressure 15 kPa and an output of 120 W during 30 min. After completing the reaction, product **6** was isolated and purified by column chromatography on silica gel (mobile phase: hexane / ethyl acetate 1:1), yield 42%. Analytical data for compound **6**: Orange crystalline solid; Mp. = 161.3-162.0°C; Elemental analysis for $C_{11}H_{11}N_5O$ (m.w. 229,24): 57.63% C, 4.84% H, 30.55% N; found 57.88% C, 4.94% H, 30.52% N; IR (ATR-Ge, cm^{-1}): 3444 (-NH-), 3292 ($-CONH_2$), 1669 (-C=O), 1604, 1533, 1482, 1413 (pyr); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.01 (1H, bs, NH), 8.30 (1H, bs, NH), 8.24 (1H, d, *J* = 2.3 Hz, H5), 7.95-7.90 (2H, H6, NH_2), 7.87 (1H, bs, NH_2), 7.16-7.04 (2H, m, H2′, H6′), 6.76-6.62 (3H, m, H3′, H4′, H5′); ^{13}C NMR (75 MHz, DMSO) δ 168.7, 155.7, 149.6, 146.7, 132.2, 129.0, 127.2, 118.7, 112.2; Lipophilicity: calc. value log *P* = -0.22; experimental determined value log *k* = -0.2382.

Example 7: 3-(2-(2-chlorophenyl)hydrazinyl)pyrazine-2-carboxamide (**7**)



Compound **7** is prepared by reaction of 2-chlorophenylhydrazine (3 mmol) with 3-chloropyrazine-2-carboxamide (III, 1 mmol) in 3 mL methanol and pyridine (1 mmol). The reaction is performed in a microwave reactor at the temperature 140°C, pressure 15 kPa and an output of 120 W during 30 min. After completing the reaction, product **7** was isolated and purified by column chromatography on silica gel (mobile phase: hexane / ethyl acetate 1:1), yield 17%. Analytical data for compound **7**: Brown crystalline solid; Mp. = 193.6-194.7°C; Elemental analysis calculated for $C_{11}H_{10}ClN_5O$ (m.w. 263.68): 50.10% C, 3.82% H, 26.56% N; found 50.36% C, 3.94% H, 26.68% N; IR (ATR-Ge, cm^{-1}): 3448 (-NH-), 3315 (-$CONH_2$), 1679 (-C=O), 1595, 1536, 1490, 1412 (pyr); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.06 (1H, bs, NH), 8.31 (1H, bs, NH), 8.26 (1H, *J* = 2.4 Hz, H5), 7.96 (1H, d, *J* = 2.4 Hz, H6), 7.88 (1H, bs, NH_2), 7.67 (1H, bs, NH_2), 7.28 (1H, dd, *J* = 7.8 Hz, *J* = 1.5 Hz, H3′), 7.10-7.05 (1H, m, H5′), 6.78 (1H, dd, *J* = 7.8 Hz, *J* = 1,5 Hz, H6′), 6.72 (1H, dt, *J* = 7.8 Hz, *J* = 1.5 Hz, H4′); ^{13}C NMR (75 MHz, DMSO) δ 168.5, 155.3, 146.4, 145.1, 132.6, 129.4, 128.0, 127.5, 119.5, 117.4, 113.0; Lipophilicity: calc. value log *P* = 0,34; experimental determined value log *k* = 0.0872.

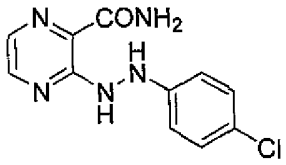
Example 8: 3-(2-(3-chlorophenyl)hydrazinyl)pyrazine-2-carboxamide (**8**)



Compound **8** is prepared by reaction of 3-chlorophenylhydrazine (3 mmol) with 3-chloropyrazine-2-carboxamide (III, 1 mmol) in 3 mL methanol and pyridine (1 mmol). The reaction is performed in a microwave reactor at the temperature 140°C, pressure 15 kPa and an output of 120 W during 30 min. After completing the reaction, product **8** was isolated and purified by column chromatography on silica gel (mobile phase: hexane / ethyl acetate 1:1), yield 24%. Analytical data for compound **8**: Dark brown crystalline solid; Mp. = 119.5-120.9°C; Elemental analysis calculated for $C_{11}H_{10}ClN_5O$ (m.w. 263.68): 50.10% C, 3.82% H,

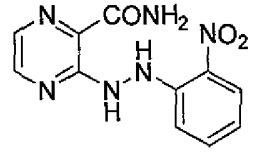
26.56% N; found 50.31% C, 3.71% H, 26,55% N; IR (ATR-Ge, cm^{-1}): 3445 (-NH-), 3253 (-$CONH_2$), 1671 (-C=O), 1598, 1522, 1476, 1413 (pyr); ^{1}H-NMR (300 MHz, $CDCl_3$) δ ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.34 (2H, bs, NH2), 7.92 (2H, bs, H5, H6), 7.63 – 6.82 (4H, m, H2′, H4′, H5′, H6′), 5.71 (2H, bs, NH); ^{13}C NMR (75 MHz, DMSO) δ 168.89, 152.20, 146.20, 140.24, 134.40, 132.36, 129.72, 126.57, 122.94, 120.26, 118.52; Lipophilicity: calc. values log P = 0.34; experimental determined values log k = 0.5898.

Example 9: 3-(2-(4-chlorophenyl)hydrazinyl)pyrazin-2-carboxamide (**9**)



Compound **9** is prepared by reaction of 4-chlorophenylhydrazine (3 mmol) with 3-chloropyrazine-2-carboxamide (III, 1 mmol) in 3 mL methanol and pyridine (1 mmol). The reaction is performed in a microwave reactor at the temperature 140 °C, pressure 15 kPa and an output of 120 W during 30 min. After completing the reaction, product **9** was isolated and purified by column chromatography on silica gel (mobile phase: hexane / ethyl acetate 1:1), yield 23%. Analytical data for compound **9**: Brown crystalline solid; Mp. = 155.3-156.2°C; Elemental analysis calculated for $C_{11}H_{10}ClN_5O$ (m.w. 263.68): 50.10% C, 3.82% H, 26.56% N; found 50.27% C, 3.72% H, 26.35% N; IR (ATR-Ge, cm^{-1}): 3453 (-NH-), 3202 (-$CONH_2$), 1686 (-C=O), 1596, 1527, 1491, 1405 (pyr); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 11.40 (1H, bs, NH), 8.45 (1H, bs, NH), 8.40 (1H, d, J = 2.3 Hz, H5), 8.05 (1H, d, J = 2.3 Hz, H6), 8.01 (1H, bs, NH), 7.75-7.67 (2H, m, AA′, BB′, H2′, H6′), 7.40-7.32 (2H, m, AA′, BB′, H3′, H5′); ^{13}C NMR (75 MHz, DMSO) δ 168.9, 151.7, 145.8, 138.3, 132.9, 128.9, 127.7, 126.1, 121.4; Lipophilicity: calc. values log P = 0.34; experimental determined values log k = 0.5996.

Example 10: 3-(2-(2-nitrophenyl)hydrazinyl)pyrazine-2-carboxamide (**10**)



Compound **10** is prepared by reaction of 2-nitrophenylhydrazine (3 mmol) with 3-chloropyrazine-2-carboxamide (III, 1 mmol) in 3 mL methanol and pyridine (1 mmol). The reaction is performed in a microwave reactor at the temperature 140 °C, pressure 15 kPa and

an output of 120 W during 30 min. After completing the reaction, product **10** was isolated and purified by column chromatography on silica gel (mobile phase: hexane / ethyl acetate 1:1), yield 17%. Analytical data for compound **10**: Red-braun crystalline solid; Mp. = 237.2-238.2°C; Elemental analysis calculated for $C_{11}H_{10}N_6O_3$ (m.w. 274.24): 48.18% C, 3.68% H, 30.65% N; found 48.27% C, 3.73% H, 3.73% N; IR (ATR-Ge, cm^{-1}): 3455 (-NH-), 3279 (-$CONH_2$), 1683 (-C=O), 1615, 1575, 1498, 1439 (pyr); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.24 (1H, bs, NH), 9.53 (1H, bs, NH), 8.35 (1H, bs, NH2), 8.29 (1H, *J* = 1.8 Hz, H5), 8.10 (1H, dd, *J* = 8.1 Hz, H3′), 8.03 (1H, d, *J* = 1.8 Hz, H6), 7.92 (1H, bs, NH2), 7.50 (1H, t, *J* = 8.1 Hz, H5′), 7.17 (1H, d, *J* = 8.1 Hz, H6′), 6.83 (1H, t, *J* = 8.1 Hz, H4′); ^{13}C NMR (75 MHz, DMSO) δ 168.3, 155.0, 146.5, 146.4, 136.7, 133.3, 131.7, 128.0, 126.0, 117.7, 115.2; Lipophilicity: calc. value log *P* = 0.35; experimental determined value log *k* = -0.3118.

Examples of pharmaceutical formulations – tablets

In the manufacture of solid dosage forms, the procedure technologies conventional in this art, a dry or wet granulation, is followed, which is well-known to a person skilled in the art. Commonly employed and recognized excipients providing the dosage form with the desired physical properties are used.

Examples for dry granulation:

Example 11 (content of the active ingredient 100 mg):

Active ingredient of the general formula I (**1** or **6**)	100.0 mg
Microcrystalline cellulose	75.0 mg
Carboxymethyl starch sodium	3.5 mg
Magnesium stearate	0.5 mg
Colloidal silica gel	0.5 mg

Example 12 (content of the active ingredient 200 mg):

Active ingredient of the general formula I (**1** nebo **6**)	200.0 mg
Microcrystalline cellulose	95.0 mg
Carboxymethyl starch sodium	7.0 mg
Magnesium stearate	1.0 mg

Colloidal silica gel	1.0 mg-

Example 13 (content of the active ingredient 300 mg):

Active ingredient of the general formula I (**1** or **6**)	300.0 mg
Microcrystalline cellulose	115.0 mg
Carboxymethyl starch sodium	10.5 mg
Magnesium stearate	1.5 mg
Colloidal silica gel	1.5 mg

Example 14 (content of the active ingredient 400 mg):

Active ingredient of the general formula I (**1** or **6**)	400.0 mg
Microcrystalline cellulose	130.0 mg
Carboxymethyl starch sodium	14.5 mg
Magnesium stearate	2.0 mg
Colloidal silica gel	2.0 mg

Example 15 (content of the active ingredient 500 mg):

Active ingredient of the general formula I (**1** or **6**)	500.0 mg
Microcrystalline cellulose	140.0 mg
Carboxymethyl starch sodium	17.5 mg
Magnesium stearate	2.5 mg
Colloidal silica gel	2.5 mg

The active ingredient is mixed with the individual ingredients and the tableting blend is compressed in a tablet machine in the usual manner.

Examples of wet granulation:

Example 16 (content of the active ingredient 100 mg):

Active ingredient of the general formula I (**1** or **6**)	100.0 mg
Potato starch	48.0 mg
Lactose	27.0 mg
Povidone	3.0 mg

Carboxymethyl starch sodium	4.0 mg
Magnesium stearate	0.2 mg
Talc	1.8 mg

Example 17 (content of the active ingredient 200 mg):

Active ingredient of the general formula I (**1** or **6**)	200.0 mg
Potato starch	60.8 mg
Lactose	34.2 mg
Povidone	6.0 mg
Carboxymethyl starch sodium	8.0 mg
Magnesium stearate	0.4 mg
Talc	3.6 mg

Example 18 (content of the active ingredient 300 mg):

Active ingredient of the general formula I (**1** or **6**)	300.0 mg
Potato starch	73.6 mg
Lactose	41.4 mg
Povidone	9.0 mg
Carboxymethyl starch sodium	12.0 mg
Magnesium stearate	0.6 mg
Talc	5.4 mg

Example 19 (content of the active ingredient 400 mg):

Active ingredient of the general formula I (**1** or **6**)	400.0 mg
Potato starch	82.3 mg
Lactose	46.8 mg
Povidone	12.0 mg
Carboxymethyl starch sodium	16.0 mg
Magnesium stearate	0.8 mg
Talc	7.2 mg

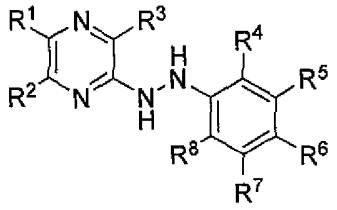
Example 16 (content of the active ingredient 500 mg):

Active ingredient of the general formula I (**1** or **6**)	500.0 mg
Potato starch	96.0 mg
Lactose	54.0 mg
Povidone	15.0 mg
Carboxymethyl starch sodium	20.0 mg
Magnesium stearate	1.0 mg
Talc	9.0 mg

The active ingredient is gradually mixed with lactose, potato starch, the mixture is granulated in an aqueous solution of povidone, the dried granulate is mixed with sodium carboxymethyl starch, magnesium stearate and talc and the resulting blend is compressed in a tablet machine in the usual way.

CLAIMS

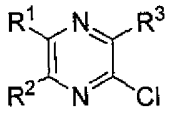
1. Substituted 2-(2-phenylhydrazinyl)pyrazines of the general formula I



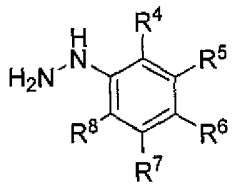
I

wherein each R^1, R^2 is independently H or CN; R^3 is CH_3 or $CONH_2$; each R^4, R^5, R^6, R^7, R^8 is independently H, Cl, or NO_2.

2. A process for preparing 2-(2-phenylhydrazinyl)pyrazines of general formula I according to claim 1, characterized in that a substituted chloropyrazine of general formula



wherein each R^1, R^2 is independently H or CN; R^3 is CH_3 or $CONH_2$, is reacted with a substituted phenylhydrazine of general structure



wherein each R^4, R^5, R^6, R^7, R^8 is independently H, Cl, or NO_2, in a polar solvent, under the conditions of microwave synthesis to form a substituted 2-(2-phenylhydrazinyl)pyrazine of general formula I.

3. The process according to claim 2, characterized in that the microwave synthesis is carried out in a polar solvent preferably in methanol, at 140°C and 15 kPa pressure and power of 120 W for 30 minutes.

4. Substituted 2-(2-phenylhydrazinyl)pyrazines of general formula I according to claim 1 for use as a medicament.

5. Substituted 2-(2-phenylhydrazinyl)pyrazines of general formula I according to claim 1 for use as an antituberculotic drug.

6. Substituted 2-(2-phenylhydrazinyl)pyrazines of general formula I according to claim 1 for use as an antibacterial drug for the treatment of mycobacteriosis caused by *M. kansasii* and/or *M. avium*.

7. Substituted 2-(2-phenylhydrazinyl)pyrazines of general formula I according to claim 1 for use as an antifungal drug for the treatment of infections caused by yeasts and filamentous fungi.

8. Substituted 2-(2-phenylhydrazinyl)pyrazines of general formula I according to claim 1 for use as an antibacterial drug for the treatment of infections caused by staphylococci and enterococci.

9. Substituted 2-(2-phenylhydrazinyl)pyrazines of general formula I according to claim 1 for use as an antiviral drug for the treatment of viral infections.

10. Use of substituted 2-(2-phenylhydrazinyl)pyrazines of general formula I according to claim 1 for the formulation of a drug for the treatment of tuberculosis.

11. Use of substituted 2-(2-phenylhydrazinyl)pyrazines of general formula I according to claim 1 for the formulation of a drug for the treatment of mycobacteriosis caused by *M. kansasii* and/or *M. avium*.

12. Use of substituted 2-(2-phenylhydrazinyl)pyrazines of general formula I according to claim 1 for the formulation of a drug for the treatment of fungal infections caused by yeasts and filamentous fungi.

13. Use of substituted 2-(2-phenylhydrazinyl)pyrazines of general formula I according to claim 1 for the formulation of a drug for the treatment of bacterial infections caused by staphylococci and enterococci.

14. Use of substituted 2-(2-phenylhydrazinyl)pyrazines of general formula I according to claim 1 for the formulation of drug for the treatment of viral infections.

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2015/000127

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07D241/04 A61K31/4965 A61P31/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07D A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SILVANA C NGO ET AL: "Inhibition of isolated mycobacterium tuberculosis fatty acid synthase I by pyrazinamide analogs", ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, AMERICAN SOCIETY FOR MICROBIOLOGY, US , vol. 51, no. 7 1 July 2007 (2007-07-01), pages 2430-2435, XP002698058, ISSN: 0066-4804, DOI: 10.1128/AAC.01458-06 Retrieved from the Internet: URL:http://aac.asm.org/content/51/7/2430 [retrieved on 2007-05-07] p. 2433, Table 2; ----- -/--	1-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
18 December 2015	24/03/2016

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MAKAM PARAMESHWAR ET AL: "2-(2-Hydrazinyl)thiazole derivatives: Design, synthesis andin vitroantimycobacterial studies", EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY, vol. 69, 1 July 2007 (2007-07-01), pages 564-576, XP028762830, ISSN: 0223-5234, DOI: 10.1016/J.EJMECH.2013.08.054 p. 566, Table 1; p. 568, table 2; -----	1-14

1

PŘÍLOHA Č. 3

JANDOUREK, O.; KUNES, J.; DOLEZAL, M. Microwave-Assisted Synthesis of Pyrazinamide Derivatives: the Coupling Reaction of 3-Chloropyrazine-2-Carboxamide and Ring-Substituted Anilines. *Curr Org Synthesis*. **2015**, *12*, 189-196. doi: 10.2174/1570179411999141106 101501. IF_{2015} = 2,117.

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Current Organic Synthesis, 2015, *12*, 189-196

Microwave-Assisted Synthesis of Pyrazinamide Derivatives: The Coupling Reaction of 3-Chloropyrazine-2-Carboxamide and Ring-Substituted Anilines

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Ondrej Jandourek

Abstract: A new approach for aminodehalogenation reaction between 3-chloropyrazine-2-carboxamide and ring-substituted anilines is described. A series of 16 compounds (15 of them novel) derived from pyrazinamide have been synthesized successfully using the advantage of microwave-assisted reaction. The conditions for this aminodehalogenation reaction have been put to investigation to optimize the conversion, yield and time. The final methodology reduces the time needed for reaction from 24 hours using conventional heating to 30 minutes under microwave irradiation and increases the yield. The synthetic procedure together with analytical data of all compounds is presented. Lipophilicity properties were calculated and experimentally determined.

Keywords: Aminodehalogenation, aniline, carboxamide, heterocycles, microwave synthesis, pyrazinamide, tuberculosis.

1. INTRODUCTION

Tuberculosis (TB) is one of the most dangerous and unpredictable infectious diseases. It is caused by *Mycobacterium tuberculosis* (MTB). The number of new cases has been falling slowly since 2006. During the year 2010, there were 8.8 million cases of TB worldwide and one eighth of these patients died. One reason could be complications which have appeared in the last decades – resistance to current first-line antituberculotic drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol) and HIV co-infection [1]. There are a few stages of resistance: MDR-TB (multidrug resistant TB) when the pathogen is resistant to the first line antitubercular drugs, XDR-TB (extreme drug resistant TB) when there exists the resistance to the first line antitubercular drugs together with at least three main intravenous second line antitubercular drugs, and the newest one - TDR-TB (totally drug resistant TB) when resistance to whole current known therapy occurs. The number of resistant strains is still rising up, especially in South Africa or East Asia, where the most burdened countries are situated [2, 3]. HIV co-infection leads to seventy times higher risk of contagion between positive patients. When AIDS manifests itself, the risk of TB transmission is 170 times higher compared to HIV negative people. 10% of TB infected people are also HIV positive. So these facts result in an effort to find new active compounds. And the microwave assisted reaction is one of the ways how the novel active compounds can be found.

Microwave-assisted synthesis is very popular and the application of this methodology has rapidly risen during the last years. Using microwaves instead of conventional heating for organic reactions lead to higher yields, better conversions, solvent savings, volumetric heating (uniform throughout the sample) and last but not least shortening of the reaction times. The main disadvantage of emerging side-products can be eliminated by using preparative chromatography or other suitable separating methods [4]. The microwave-accelerated heating (direct interactions with molecules themselves, not solvent or vessel, higher temperatures than conventional heating) leads to new technologies for drug discovery and development. The wavelengths of microwave radiation are located in the range from 1 millimetre to 1 metre (frequencies 0.3 - 300 GHz). The microwave device we used works with wavelength 12.2 centimetres (frequency 2.455 GHz). The effect of microwave irradiation consists of thermal and non-thermal effects. The ability of some compounds or solvents to transform the electromagnetic energy into heat is one of the thermal effects. It is necessary to have a dipole moment in the molecule of solvent or another used compound because it causes sensitivity to the external electric field which provides the energy for rotation of the molecule itself. The phase difference between the oscillating electric field of microwave apparatus and dipolar structures causes energy loss that gives rise to dielectric heating. Another parameter of solvent important for better interaction with the external electric field is loss tangent which expresses the extent of ability to convert the energy into heat. This results in rapid temperature increase. Non-thermal effects are not so clear and are a controversial matter with a few theories and models postulated. They would appear to be less important than stated in earlier publications. Also using over pressurized system leads to the possibility of reaching much higher temperature above the boiling point of solvents (in this case the temperature of methanol was more than two times higher than its boiling point at atmospheric pressure) [5-18].

Compounds bearing the pyrazine ring possess remarkable biological activities and pharmaceutical importance, some derivatives occur as natural products and/or drugs. PZA itself is a very important anti-tuberculosis drug because it shortens the time needed for therapy up to two thirds (18 months -> 6 months) due to its activity against dormant forms of MTB. Its mechanism of action was not clearly explained for a long time. One of the first theses claims that PZA itself needs the activation *via* enzyme pyrazinamidase (nicotinamidase) (EC 3.5.1.19). Its active form is pyrazinecarboxylic acid which is suspected to cause the acidification of inner compartment of mycobacterial cells that leads to cellular death [19, 20]. The theory which suggested the inhibiting mechanism of fatty acid synthase I (FAS I) (EC 2.3.1.85) followed after that. This enzyme is essential for the composition of mycobacterial cell wall. This mechanism is specific for 5-chloropyra-zinamide derivative rather than for PZA itself [21]. Zhang and his co-workers have come with a new theory that has suggested the inhibition of *trans*-translation as the main mode of action (a mechanism which is critical for the survival and virulence of bacteria). Then they have proved this

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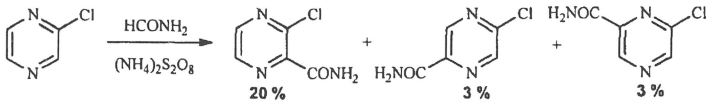


Fig. (1). Synthesis of starting compound by homolytic amidation of 2-chloropyrazine.

theory *in vitro* [22]. Some pyrazine derivatives have proven activity in biological screenings (antifungal, antibacterial, herbicidal, antituberculotic, antineoplastic, *etc.*) [23-29]. As a first-line antituberculotic agent, the small molecule of pyrazinamide (PZA) is very suitable for chemical modifications using a microwave reactor, especially for a few reactions (Buchwald-Hartwig amination or aminodehalogenation reactions) which can be accomplished in minutes, instead of hours or days. We have chosen the modifications for new PZA derivatives on the behalf of these results. Substituents were also chosen according to our previous experiences and gathered results of our research. Microwave-assisted reactions were used because of many positive aspects *e.g.* lower costs (smaller amounts of solvents and starting compounds...), speed and safety.

2. MATERIALS AND METHODS

2.1. General

All chemicals used were reagent or higher grade of purity. They were purchased from Sigma-Aldrich or Fluka (Sigma-Aldrich, Co., Steinheim, Germany). Plates for thin layer chromatography (TLC) were made by Merck (Silica gel 60 F254) (Merck KGaA, Darmstadt, Germany) and the detection wavelength was 254 nm (UV light). Microwave reactions were performed by microwave reactor with focused field (CEM Discover, CEM Corporation, Matthews, North Carolina, USA) equipped with autosampler (Explorer 24, CEM Corporation). The reaction course was monitored with CEM's Synergy™ software. All compounds were purified using preparative chromatography (CombiFlash® RF, Teledyne Isco, Inc., Lincoln, NE, USA). Silica gel was used as the stationary phase 0.040 - 0.063 mm (Merck KGaA, Darmstadt, Germany) and it was made use of hexane as the mobile phase (Lach-Ner, s.r.o., Neratovice, Czech Republic) and ethyl acetate (Penta, Prague, Czech Republic) in gradient elution. NMR spectra were measured with Varian Mercury-VxBB 300 spectrometer (Varian corp., Palo Alto, California, USA, 299.95 MHz for ^{1}H, 75.43 MHz for ^{13}C) or Varian VNMR S500 spectrometer (Varian corp., Palo Alto, California, USA, 499.87 MHz for ^{1}H, 125.71 MHz for ^{13}C). Chemical shifts are reported in ppm (δ) and are indirectly related to TMS (tetramethylsilane) *via* a signal of solvent (2.49 for ^{1}H and 39.7 for ^{13}C in DMSO-d_6 or 7.28 for ^{1}H and 77.0 for ^{13}C in $CDCl_3$). Melting points were determined by SMP3 Stuart Scientific apparatus (Bibby Sterling LTD, Staffordshire, UK) and are uncorrected. Infrared spectra was recorded by ATR methodology (Attenuated Total Reflectance) using spectrometer FT-IR Nicolet 6700 (Nicolet - Thermo Scientific, Waltham, MA, USA) on germanium crystal, and elemental analysis was performed on EA 1110 CHNS Analyzer (Fisons Instruments S. p. A., Carlo Erba, Milano, Italy). Lipophilicity parameters log *P* and Clog *P* were calculated with programme CS ChemBioOffice Ultra 13.0 (CambridgeSoft, Cambridge, MA, USA). Experimental lipophilicity parameter log *k* was ascertained using HPLC system. Agilent Technologies 1200 SL liquid chromatography with Diode-array Detector SL G1315C, chromatographic pre-column ZORBAX XDB-C18 5 μm, 4 x 4 mm, Part No. 7995118-504 and column ZORBAX Eclipse XDB-C18 5 μm, 4.6 x 250 mm, Part No. 7995118-585 (Agilent Technologies Inc., Colorado Springs, CO, USA) were used. The separation process was controlled by Agilent ChemStation, version B.04.02 extended by spectral module (Agilent Technologies Inc.). A solution of MeOH (HPLC grade, 70 %) and H_2O (HPLC-Milli-Q grade, 30 %) was "used as mobile phase. The total flow of the column was 1.0 mL/min, injection 20 μL, column temperature 30 °C. 210 nm as detection wavelength and 270 nm as monitor wavelength were chosen. The KI methanol solution was used for the dead time (T_D) determination. Retention times (T_R) of synthesized compounds were measured in minutes. The capacity factors *k* were calculated using Microsoft Excel according to formula $k = (T_R - T_D)/T_D$, where T_R is the retention time of the solute and T_D denotes the dead time obtained *via* an unretained analyte. Log *k*, calculated from the capacity factor *k*, is used as the lipophilicity index converted to log *P* scale.

2.2. Chemistry

The starting compound, 3-chloropyrazine-2-carboxamide, was synthesized by two different methods. The first one (Fig. **1**) proceeded from 2-chloropyrazine (170 mmol) which was dissolved in formamide (146.5 mL). This mixture heated to 90 °C was treated with ammonium peroxodisulfate (180 mmol) in small amounts and stirred for 3 hours at the same temperature. It was left to stand for 24 hours at laboratory temperature and then diluted with water. The filtrate collected by suction was extracted continuously with chloroform for 16 hours [30, 31]. The mixture of three isomers was separated by preparative chromatography with gradient elution in the system hexane - ethyl acetate using silica gel filled columns. The separation started with 100% of hexane and ethyl acetate was added gradually. 3-Chloropyrazine-2-carboxamide was eluted when the ratio between these two solvents was approximately 20 : 80. After this purification, the crude product was recrystallized from ethanol. This method was not so efficacious because the yields from this reaction are usually about 15-20% and the time for preparation is two days, so another procedure was necessary to be found.

The second method was partial hydrolysis of nitrile group (Fig. **2**) of 3-chloropyrazine-2-carbonitrile (Fluorochem, Co., UK). This compound (104 mmol) was added portion-wise into the mixture of concentrated (35%) hydrogen peroxide (29 mL) and water (195 mL), which was alkalized with 8% (w/v) solution of sodium hydroxide to pH 9 and heated to 50 °C. The mixture was stirred for 2 hours and 30 minutes at 55 °C and pH 9 after addition of the whole amount of carbonitrile. Then it was necessary to cool the whole mixture in the fridge to initiate the crystallization. The crude product was also recrystallized from ethanol [30]. The yield of this reaction was approximately 80% and it was not necessary to purify the product using the preparative chromatography.

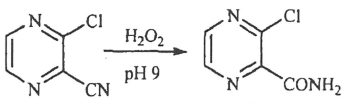


Fig. (2). Synthesis of starting compound *via* partial hydrolysis.

The final products, *N*-substituted 3-aminopyrazine-2-carboxamides, were prepared from the starting compound 3-chloropyrazine-2-carboxamide that was treated with a range of 16 various ring-substituted anilines (Fig. **3**). All reactions were carried out using the microwave reactor with focused field. However, the first three products were synthesised under various reaction conditions in order to increase the yield and to compare the conventional and microwave heating outcomes. The parameters of reactions which were modified were temperature, time, solvent or base including classic reflux method. These steps were proceeded in order to optimize the conditions for the aminodehalogenation reaction. All these new compounds were purified using preparative flash chromatography with gradient elution (hexane - ethyl acetate) and

recrystallized from ethanol or ethanol and water if necessary. All final products were chemically characterized (^{1}H NMR, ^{13}C NMR, IR, melting point and elemental analysis).

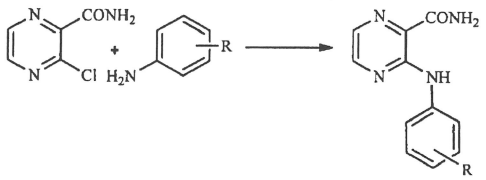


Fig. (3). General scheme of aminodehalogenation reaction.

2.3. Process of Optimization

We have chosen different reaction conditions (see Table **1**). The first condition, that was necessary to choose, was a solvent. Toluene was used for reflux method with conventional heating because of its higher boiling point. This solvent was also used due to better preparation of sample for preparative chromatography (faster evaporation) and experiences with this solvent in our working group. Tetrahydrofuran (THF) and methanol (MeOH) were chosen for microwave-assisted reactions due to their differences in polarity. The second variable was the base added for neutralization of emerging hydrogen chloride. Two compounds were used - pyridine and triethylamine. Next parameters to be set were time, temperature and the usage of PowerMAX function that was used to reach higher microwave power. It is a special function of Discover reactor that could ensure the higher power output during whole reaction times. The problem arises when the temperature reaches the set point, the microwave output would be lowered to not overheat mixture. If the mixture is cooled during the whole reaction then a lower temperature would lead to endeavour to get the desired temperature by higher power output. The power output decreases to hold the set temperature after reaching it when using non-PowerMAX methods. The starting compound (3-chloropyrazine-2-carboxamide, 200 mg, 1.27 mmol) was added into 50ml round bottom flask together with toluene (10 ml). This mixture was treated with the aromatic amine (2.54 mmol), pyridine or triethylamine (1.27 mmol) and preheated to 110 °C. The reaction mixture reacted for 24 hours with whole time stirring. The result of the reaction was monitored by TLC. The developing system hexane and ethyl acetate (3:2) was found to show the best results. This method was used for conventional heating. For microwave procedure, 3-chloropyrazine-2-carboxamide (200 mg, 1.27 mmol) and aromatic amine (2.54 mmol) were put into special 10ml vial with septum cap. Then 3 ml of solvent (THF or MeOH) and a base (pyridine or triethylamine, 1.27 mmol) were added. The sealed vial was put into the reactor. The microwave output was set to 200W with high stirring using PowerMAX function or not. The temperature and reaction times were specified according to Table **1**. Reaction monitoring was carried out at the end of procedure using TLC chromatography. The developing conditions were the same as they were for the conventional reaction. Results are presented in Table **1**. The lower temperature used for THF is caused by the fact that using higher temperatures leads to spurting the mixture from the vial even if using the pressurized system. Afterwards TLC followed with the mixture absorbed on silica gel. Flash chromatography with gradient elution was used for purification. The recrystallization was carried out using ethanol or ethanol and water if necessary.

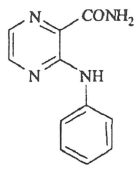
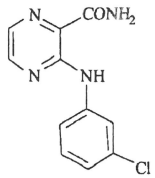
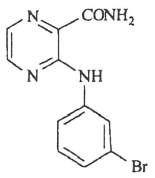
3. RESULTS AND DISCUSSION

Many ways for preparation of the amines using aminodehalogenation reaction are known and used. But on the other hand, poor results of these techniques on the synthesis of proposed phenylaminopyrazines led to attempts to modify and improve them along with taking the advantage of microwave technology. The trials, which were focused on solvent, showed that methanol was the best solvent. It is caused by the fact that toluene and tetrahydrofuran are not so useful for the microwave reactor. They belong to non-polar solvents so the thermal effect of microwaves does not affect them in sufficient manner. Another problem was associated with THF. When the temperature was higher than 80 °C, the system got probably over-pressurized and the mixture squirted out of the vial. But the highest pressure indicated by the system's sensor before the leakage was only 172.4 kPa, so it is not absolutely clear why this happened. The base was chosen on the ground of the stability. Triethylamine is probably unstable under microwave conditions and is decomposing quickly. A few studies focused on the stability of this tertiary amine, but not exactly under microwave conditions, were published. One of them showed that triethylamine which is exposed to the acid catalysis of hydrochloric acid on alumina decomposed very quickly to two main products - ethylene and diethylamine [32]. In view of the fact that hydrochloric acid is formed during the aminodehalogenation reaction, it could be one of the alternatives to occur. The emerging diethylamine could react with 3-chloropyrazine-2-carboxamide to yield 3-diethylaminopyrazine-2-carboxamide as a side product. However the products of the aminodehalogenation using triethylamine as a base were isolated only in insufficient amounts to be characterised and also it was not a subject of interest of this study. On the contrary, pyridine is more stable and its basicity is sufficient to be used as a base in this work. The temperature also varied in wide range and the highest one showed the best results. However, when the temperature exceeded 140 °C, the product started to decompose. The last parameter was time. The optimal value was found to be approximately 30 minutes. Shortening the duration of the reaction led to lower yields. Longer time did not show better results than 30 minutes by contrast. And sometimes the yield was also lower. PowerMAX function was used in order to reach higher power output that was guaranteed by continuous cooling during whole reaction time. These reaction mixtures were more irradiated and the results showed interesting consequences leading to higher yields compared to syntheses performed without PowerMAX function. It cannot be concluded if this is a proof of non-thermal effects. There are many theories and postulates but it is still a unknown field of microwave assisted chemistry. On the other hand few aspects showed the positive effect of irradiation using higher power output compared to lower one *i.e.* yields were approx. two times higher when using PowerMAX function. We obtained 16 compounds with 15 of them were novel and it was necessary to characterize them. Compound **1** (3-(phenylamino) pyrazine-2-carboxamide) had been synthesised in recent work by Taylor *et* al. [33]. The melting point was given as 170-172 °C and elemental analysis was measured with these results: 61.9% C, 4.7% H and 26.7% N. Our measurements were as follows: M. p. 169.9-171.2 °C; 61.69% C, 4.58% H and 26.14% N. These results are comparable to each other. All phenyl-ring substituted 3-phenylaminopyrazine-2-carboxamides **1-16** are presented in Table **2**. The yields that we obtained from reactions were in the range from 12% to 85%. They were affected by the electronic properties of the substituents and/or their steric effects. The lowest yields were observed with electron withdrawing substituents such as -CF_3; 3,4-Cl_2; 3-Br or *ortho* substituted anilines predisposed for steric hindrance, *e.g.* 2,5-$(CH_3)_2$.

3.1. Optimized Procedure of the Aminodehalogenational Reaction

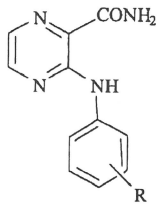
3-Chloropyrazine-2-carboxamide (200 mg, 1.27 mmol) and substituted aniline were put into special thick-walled glass 10 mL vial together with a magnetic stirrer. Methanol (3 ml) and pyridine (100 mg, 1.27 mmol) were added. The sealed tube was put into the microwave reactor. The conditions were set according to previous optimization. They were: power output 200W, temperature 140 °C, reaction time 30 minutes using PowerMAX function (Fig. 4).

Table 1. Experimental conditions used for the optimization and calculated yields as a result of this process.

Compound	Solv. + Temp.	Base	Pressure	Time	Yield
	Toluene 110 °C	Pyridine	AP	Overnight	41.6%
		Triethylamine	AP	Overnight	43.2%
	Tetrahydrofuran 80 °C	Pyridine	N/A	20 min	3.6%
			N/A	30 min	3.7%
			N/A	45 min	2.6%
		Triethylamine	N/A	20 min	0%
			N/A	30 min	0%
			N/A	45 min	0%
	Methanol 140 °C	Pyridine	730.8 kPa	20 min	11.8%
			751.5 kPa	30 min	51.7%
			599.8 kPa	45 min	48.9%
			820.5 kPa	**PowerMAX, 30 min**	**84.9%**
		Triethylamine	N/A	20 min	0%
			668.8 kPa	30 min	0%
			N/A	45 min	0%
	Toluene 110 °C	Pyridine	AP	Overnight	4.4%
		Triethylamine	AP	Overnight	2.3%
	Tetrahydrofuran 80 °C	Pyridine	N/A	20 min	0%
			N/A	30 min	0%
			N/A	45 min	0%
		Triethylamine	N/A	20 min	0%
			N/A	30 min	0%
			N/A	45 min	0%
	Methanol 140 °C	Pyridine	730.8 kPa	20 min	0%
			586.1 kPa	30 min	18.0%
			599.8 kPa	45 min	8.6%
			668.8 kPa	**PowerMAX, 30 min**	**36.8%**
		Triethylamine	N/A	20 min	0%
			703.3 kPa	30 min	0%
			N/A	45 min	0%
	Toluene 110 °C	Pyridine	AP	Overnight	8.4%
		Triethylamine	AP	Overnight	3.9%
	Tetrahydrofuran 80 °C	Pyridine	N/A	20 min	0%
			N/A	30 min	0%
			N/A	45 min	0%
		Triethylamine	N/A	20 min	0%
			N/A	30 min	0%
			N/A	45 min	0%
	Methanol 140 °C	Pyridine	717.1 kPa	20 min	13.1%
			703.3 kPa	30 min	19.9%
			834.3 kPa	45 min	17.1%
			820.5 kPa	**PowerMAX, 30 min**	**25.5%**
		Triethylamine	N/A	20 min	0%
			572.3 kPa	30 min	0%
			N/A	45 min	0%

N/A - not available (due to the leakage of the mixture out of the vial or unsuccessful experiment with TEA);
AP - atmospheric pressure.

Table 2. List of prepared compounds, their appearance and lipophilic properties.



No.	R	Appearance	log *P* / Clog *P*	log *k*
1	H	bright yellow crystals	0.87 / 2.18	0.270
2	2,5-$(CH_3)_2$	bright yellow crystals	1.84 / 3.17	0.528
3	4-iPro	bright yellow crystals	2.10 / 3.60	0.864
4	3-F	ochre crystals	1.03 / 2.33	0.386
5	3-Cl	yellow crystals	1.43 / 2.90	0.599
6	3-Br	yellow crystals	1.70 / 3.05	0.661
7	3,4-Cl_2	yellow crystals	1.99 / 3.50	0.892
8	3-CF_3	light yellow crystals	1.79 / 3.10	0.636
9	4-CF_3	dark yellow crystals	1.79 / 3.10	0.697
10	3,5-$(CF_3)_2$	light ochre crystals	2.71 / 3.99	1.100
11	2-OH	dark yellow to orange crystals	0.48 / 1.51	-0.010
12	3-OH	yellow crystals	0.48 / 1.51	-0.273
13	4-OH	light brown crystals	0.48 / 1.51	-0.365
14	3-OCH_3	golden crystals	0.74 / 2.10	0.251
15	4-OCH_3	dark yellow crystals	0.74 / 2.10	0.159
16	3,5-$(OCH_3)_2$	yellow crystals	0.62 / 2.08	0.262

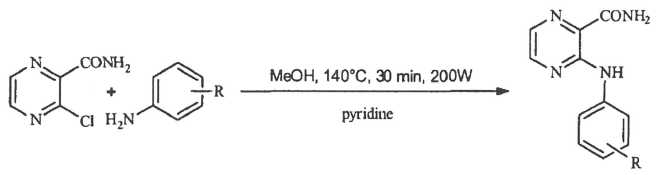


Fig. (4). Optimized conditions for microwave-assisted aminodehalogenation reaction.

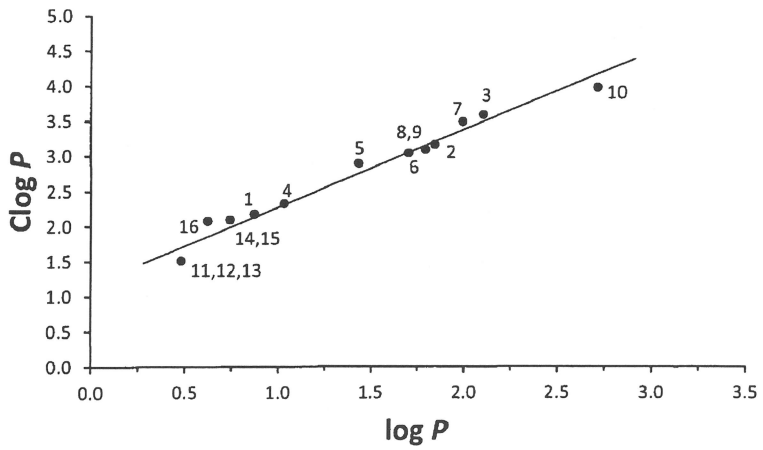


Fig. (5). Dependence of calculated values Clog *P* on calculated values log *P*.

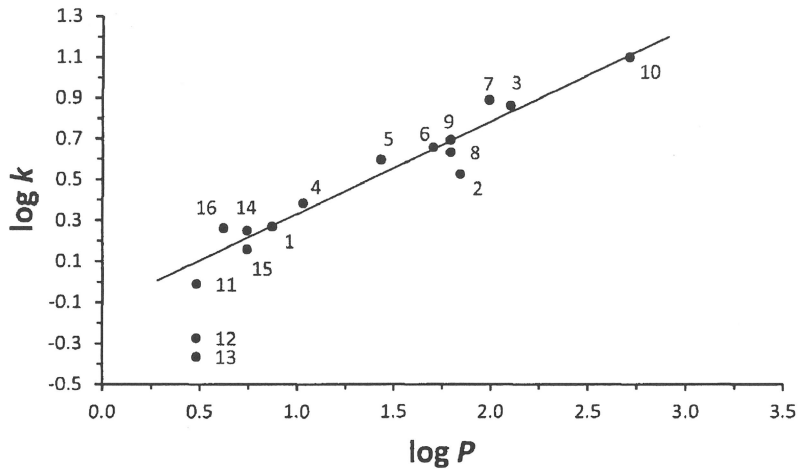


Fig. (6). Dependence of experimentally determined log *k* on calculated log *P* values of tested compounds.

3.2. Lipophilicity

There were acquired two calculated parameters of lipophilicity. The difference between log *P* and Clog *P* is based on various ways of calculations. Different algorithm is used to get both values. But there should be the linear dependence between log *P* and Clog *P* that is shown in Fig. **5**. Disadvantage of this method is the impossibility of distinguishing the positional isomers of compounds.

The dependence of experimentally determined lipophilicity parameter log *k* on calculated log *P* is shown in Fig. **6**. While for compounds **1-11** and **14-16** linear dependence of log *k* on log *P* was determined (r = 0.967), the estimated log *k* values for 3-[(3-hydroxyphenyl)amino]pyrazine-2-carboxamide (**12**) and 3-[(4-hydroxyphenyl)amino]pyrazine-2-carboxamide (**13**) were considerably lower than expected. This could be connected with the presence of hydroxyl group in *m*- resp. *p*- position that can be easily ionized.

3.3. Analytical Data of Prepared Compounds

3-(Phenylamino)pyrazine-2-carboxamide (**1**) [33]. Yield 84.9%; M.p. 169.9-171.2 °C; IR (ATR-Ge, cm^{-1}): 3435m (-NH-), 3162w (-$CONH_2$), 1674s (-C=O), 1600s, 1574m, 1520s, 1492m, 1444s, 1416m, 1183s (arom.); ^{1}H NMR (300 MHz, DMSO) δ 11.32 (1H, bs, NH), 8.42 (1H, bs, NH_2), 8.38 (1H, d, *J*=2.5 Hz, H5), 8.01 (1H, d, *J*=2.5 Hz, H6), 7.97 (1H, bs, NH_2), 7.66 (2H, t, *J*=7.7 Hz, H2′, H6′), 7.32 (2H, t, *J*=7.7 Hz, H3′, H5′), 7.02 (1H, t, *J*=7.7 Hz, H4′); ^{13}C NMR (75 MHz, DMSO) δ 169.0, 152.0, 145.9, 139.3, 132.5, 128.1, 127.5, 122.7, 120.0; Elemental analysis calculated for $C_{11}H_{10}N_4O$ (MW 214.22): 61.67% C, 4.71% H, 26.15% N; found 61.69% C, 4.58% H, 26.14% N.

3-[(2,5-Dimethylphenyl)amino]pyrazine-2-carboxamide (**2**). Yield 28.0%; M.p. 155.1-156.6 °C; IR (ATR-Ge, cm^{-1}): 3404m (-NH-), 3265m, 3208m (-$CONH_2$), 2921m (-CH_3), 1662s (-C=O), 1585s, 1515s, 1483m, 1455m, 1412m, 1328m, 1232m, 1191s (arom.); ^{1}H NMR (500 MHz, DMSO) δ 11.10 (1H, bs, NH), 8.41 (1H, bs, NH_2), 8.37 (1H, d, *J*=2.4 Hz, H5), 8.02-8.00 (1H, m, H6′), 7.99 (1H, d, *J*=2.4 Hz, H6), 7.93 (1H, bs, NH_2), 7.10 (1H, d, *J*=7.5 Hz, H3′), 6.79 (1H, d, *J*=7.5 Hz, H4′), 2.27 (3H, s, CH_3), 2.24 (3H, s, CH_3); ^{13}C NMR (125 MHz, DMSO) δ 169.1, 152.3, 146.0, 137.6, 135.4, 132.2, 130.3, 127.5, 125.2, 123.8, 121.4, 21.2, 17.8; Elemental analysis calculated for $C_{13}H_{14}N_4O$ (MW 242.28): 64.45% C, 5.82% H, 23.13% N; found 64.54% C, 5.78% H, 23.15% N.

3-{[4-(Propan-2-yl)phenyl]amino}pyrazine-2-carboxamide (**3**). Yield 73.9%; M.p. 199.8-201.6 °C; IR (ATR-Ge, cm^{-1}): 3447m (-NH-), 3188w (-$CONH_2$), 2958m (-CH_3), 2925w (-CH-), 1664s (-C=O), 1607s, 1568m, 1515s, 1419s, 1327m, 1237m, 1181s (arom.); ^{1}H NMR (500 MHz, DMSO) δ 11.20 (1H, bs, NH), 8.39 (1H, bs, NH_2), 8.35 (1H, d, *J*=2.4 Hz, H5), 7.98 (1H, d, *J*=2.4 Hz, H6), 7.94 (1H, bs, NH_2), 7.58-7.53 (2H, m, AA′, BB′, H2′, H6′), 7.21-7.17 (2H, m, AA′, BB′, H3′, H5′), 2.90-2.79 (1H, m, CH), 1.18 (6H, d, *J*=6.8 Hz, CH_3); ^{13}C NMR (125 MHz, DMSO) δ 169.0, 152.1, 146.0, 143.0, 137.0, 132.2, 127.3, 126.7, 120.3, 33.0, 24.2; Elemental analysis calculated for $C_{14}H_{16}N_4O$ (MW 256.30): 65.61% C, 6.29% H, 21.86% N; found 65.77% C, 6.37% H, 20.73% N.

3-[(3-Fluorophenyl)amino]pyrazine-2-carboxamide (**4**). Yield 31.1%; M.p. 196.6-197.7 °C; IR (ATR-Ge, cm^{-1}): 3421m (-NH-), 3207w (-$CONH_2$), 1690s (-C=O), 1611s, 1581s, 1525s, 1492m, 1452s, 1411m, 1226s, 1188s, 1143s (arom.); ^{1}H NMR (300 MHz, DMSO) δ 11.50 (1H, bs, NH), 8.46 (1H, bs, NH_2), 8.43 (1H, d, *J*=2.5 Hz, H5), 8.07 (1H, d, *J*=2.5 Hz, H6), 8.02 (1H, bs, NH_2), 7.87-7.77 (1H, m, H2′), 7.39-7.23 (2H, m, H5′, H6′), 6.87-6.77 (1H, m, H4′); ^{13}C NMR (75 MHz, DMSO) δ 168.8, 162.6 (d, *J*=241.1 Hz), 151.6, 145.8, 141.1 (d, *J*=11.2 Hz), 133.1, 130.5 (d, *J*=10.0 Hz), 127.9, 115.7 (d, *J*=2.6 Hz), 108.9 (d, *J*=21.2 Hz), 106.4 (d, *J*=26.3 Hz); Elemental analysis calculated for $C_{11}H_9FN_4O$ (MW 232.21): 56.89% C, 3.91% H, 24.13% N; found 57.03% C, 3.78% H, 24.25% N.

3-[(3-Chlorophenyl)amino]pyrazine-2-carboxamide (**5**). Yield 36.8%; M.p. 196.2-197.3 °C; IR (ATR-Ge, cm^{-1}): 3418m (-NH-), 3160w (-$CONH_2$), 1687s (-C=O), 1613m, 1598s, 1568m, 1522s, 1430s (arom.), 1076m (-C-Cl); ^{1}H NMR (300 MHz, DMSO) δ 11.48 (1H, bs, NH), 8.53-8.38 (2H, m, H5, NH_2), 8.14-7.95 (3H, m, H6, H2′, NH_2), 7.49-7.39 (1H, m, H6′), 7.33 (1H, t, *J*=7.8 Hz, H5′), 7.10-7.02 (1H, m, H4′); ^{13}C NMR (75 MHz, DMSO) δ 168.8, 151.6, 145.8, 140.9, 133.4, 133.2, 130.7, 127.9, 122.2, 119.0, 118.4; Elemental analysis calculated for $C_{11}H_9ClN_4O$ (MW 248.67): 53.13% C, 3.65% H, 22,53% N; found 53.05% C, 3.63% H, 22.66% N.

3-[(3-Bromophenyl)amino]pyrazine-2-carboxamide (**6**). Yield 25.5%; M.p. 183.3-184.4 °C; IR (ATR-Ge, cm^{-1}): 3421m (-NH-), 3216w (-$CONH_2$), 1684s (-C=O), 1617m, 1595s, 1563m, 1517s, 1474m, 1425m, 1349m (arom.), 1071m (-C-Br); ^{1}H NMR (300 MHz, DMSO) δ 11.47 (1H, bs, NH), 8.46 (1H, bs, NH_2), 8.44 (1H, d, *J*=2.4 Hz, H5), 8.14 (1H, t, *J*=1.7 Hz, H2′), 8.07 (1H, d, *J*=2.4 Hz, H6), 8.02 (1H, bs, NH_2), 7.48 (1H, d, *J*=8.2 Hz, H6′), 7.27 (1H, t, *J*=8.2 Hz, H5′), 7.18 (1H, d, *J*=8.2 Hz, H4′); ^{13}C NMR (75 MHz, DMSO) δ 168.8, 151.6, 145.8, 141.0, 133.2, 131.0, 127.9, 125.1, 121.9, 121.8, 118.8; Elemental analysis calculated for $C_{11}H_9BrN_4O$ (MW 293.12): 45.07% C, 3.09% H, 19,11% N; found 45.22% C, 2.99% H, 19.01% N.

3-[(3,4-Dichlorophenyl)amino]pyrazine-2-carboxamide (**7**). Yield 12.3%; M.p. 241.2-242.2 °C; IR (ATR-Ge, cm^{-1}): 3439m (-NH-), 3212w, 3075w (-$CONH_2$), 1686s (-C=O), 1621s, 1593s, 1561m,

1520s, 1476m, 1415m, 1397m (arom.), 806vs (C-Cl); ^{1}H NMR (300 MHz, DMSO) δ 11.52 (1H, bs, NH), 8.57-8.38 (2H, m, NH_2, H5), 8.28-7.97 (3H, m, NH_2, H6, H6′), 7.62-7.44 (2H, m, H2′, H5′); ^{13}C NMR (75 MHz, DMSO) δ 168.7, 151.4, 145.7, 139.5, 133.5, 131.3, 130.8, 128.1, 123.8, 120.7, 120.1; Elemental analysis calculated for $C_{11}H_8Cl_2N_4O$ (MW 283.11): 46.67% C, 2.85% H, 19,79% N; found 46.71% C, 2.77% H, 19.65% N.

3-{[3-(Trifluoromethyl)phenyl]amino}pyrazine-2-carboxamide (**8**). Yield 23.4%; M.p. 200,1-200.6 °C; IR (ATR-Ge, cm^{-1}): 3440m (-NH-), 3232w, 3152w (-$CONH_2$), 1675m, 1652m (-C=O), 1604s, 1525s, 1450s, 1413m, 1341s (arom.), 1111vs (-CF_3); ^{1}H NMR (300 MHz, DMSO) δ 11.58 (1H, bs, NH), 8.60-8.35 (2H, m, H5, NH_2), 8.31-7.94 (3H, m, H6, H2′, NH_2), 7.88-7.69 (1H, m, H6′), 7.64-7.24 (2H, m, H4′, H5′); ^{13}C NMR (75 MHz, DMSO) δ 168.8, 151.6, 145.8, 140.2, 133.4, 130.2, 129.8 (q, *J*=31.5 Hz), 128.0, 124.4 (q, *J*=272.3 Hz), 123.6, 118.8 (q, *J*=3.8 Hz), 115.6 (q, *J*=4.2 Hz); Elemental analysis calculated for $C_{12}H_9F_3N_4O$ (MW 282.22): 51.07% C, 3.21% H, 19.85% N; found 51.00% C, 3.03% H, 19.92% N.

3-{[4-(Trifluoromethyl)phenyl]amino}pyrazine-2-carboxamide (**9**). Yield 12.6%; M.p. 169.3-170.3 °C; IR (ATR-Ge, cm^{-1}): 3445m (-NH-), 3154w (-$CONH_2$), 1689s (-C=O), 1606s, 1575m, 1526s, 1416s (arom.), 1320s (-CF_3), 1244m, 1182s, 1155m, 1109s, 1066s (arom.); ^{1}H NMR (500 MHz, DMSO) δ 11.69 (1H, bs, NH), 8.50 (1H, bs, NH_2), 8.46 (1H, d, *J*=2.4 Hz, H6), 8.12 (1H, d, *J*=2.4 Hz, H5), 8.07 (1H, bs, NH_2), 7-92-7.87 (2H, m, AA′, BB′, H3′, H5′), 7.68-7.63 (2H, m, AA′, BB′, H2′, H6′); ^{13}C NMR (125 MHz, DMSO) δ 168.8, 151.5, 145.7, 143.0, 133.7, 128.2, 126.3 (q, *J*=2.9 Hz), 124.7 (q, *J*=270.9 Hz), 122.3 (q, *J*=31.6 Hz), 119.4; Elemental analysis calculated for $C_{12}H_9F_3N_4O$ (MW 282.22): 51.07% C, 3.21% H, 19.85% N; found 50.93% C, 3.39% H, 20.02% N.

3-{[3,5-bis(Trifluoromethyl)phenyl]amino}pyrazine-2-carboxamide (**10**). Yield 29.8%; M.p. 201.1-203.1 °C; IR (ATR-Ge, cm^{-1}): 3470m (-NH-), 3212m (-$CONH_2$), 1675s (-C=O), 1620s, 1603s, 1525s, 1474m, 1444m, 1385s (arom.), 1325m (-CF_3), 1272s, 1178s, 1158s, 1120s (arom.); ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.38 (1H, bs, NH), 8.42-8.37 (1H, m, H6), 8.24 (2H, s, H2′, H6′), 8.04-8.00 (1H, m, H5), 7.88 (1H, bs, NH_2), 7.53 (1H, s, H4′), 5.73 (1H, bs, NH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.8, 152.0, 146.1, 140.8, 133.5, 132.3 (q, *J*=32.3 Hz), 127.3, 123.4 (q, *J*=272.8 Hz), 119.8 (q, *J*=2.9 Hz), 115.9 (q, *J*=3.8 Hz); Elemental analysis calculated for $C_{13}H_8F_6N_4O$ (MW 350.22): 44.58% C, 2.30% H, 16.00% N; found 44.44% C, 2.28% H, 16.15% N.

3-[(2-Hydroxyphenyl)amino]pyrazine-2-carboxamide (**11**). Yield 52.4%; M.p. 169.9-171.4 °C; IR (ATR-Ge, cm^{-1}): 3452m (-OH), 3349m (-NH-), 3229m (-$CONH_2$), 1647s (-C=O), 1603s, 1573s, 1519s, 1455s, 1424s, 1348m, 1231s, 1195s (arom.); ^{1}H NMR (300 MHz, DMSO) δ 11.32 (1H, bs, NH), 9.88 (1H, bs, OH), 8.42-8.39 (1H, m, H3′), 8.38 (1H, d, *J*=2.0 Hz, H5), 8.30 (1H, bs, NH_2), 7.97 (1H, d, *J*=2.0 Hz, H6), 7.80 (1H, bs, NH_2), 6.92-6.73 (3H, m, H4′, H5′, H6′); ^{13}C NMR (75 MHz, DMSO) δ 168.5, 151.8, 147.0, 145.7, 131.7, 127.9, 122.4, 119.7, 119.0, 114.5; Elemental analysis calculated for $C_{11}H_{10}N_4O_2$ (MW 230.22): 57.39% C, 4.38% H, 24.34% N; found 57.53% C, 4.39% H, 24.42% N.

3-[(3-Hydroxyphenyl)amino]pyrazine-2-carboxamide (**12**). Yield 62.7%; M.p. 202.8-204.2 °C (decomposition); IR (ATR-Ge, cm^{-1}): 3649m (-OH), 3431m (-NH-), 3212m (-$CONH_2$), 1684m (-C=O), 1636m, 1609s, 1577s, 1521s, 1458m, 1230m, 1195m (arom.), 1161s (-OH); ^{1}H NMR (300 MHz, DMSO) δ 11.29 (1H, bs, NH), 9.39 (1H, bs, OH), 8.42 (1H, bs, NH_2), 8.38 (1H, d, *J*=2.6 Hz, H5), 8.00 (1H, d, *J*=2.6 Hz, H6), 7.96 (1H, bs, NH_2), 7.29-7.24 (1H, m, H2′), 7.10 (1H, t, *J*=8.0 Hz, H5′), 7.09-6.96 (1H, m, H4′), 7.47-6.40 (1H, m, H6′); ^{13}C NMR (75 MHz, DMSO) δ 169.0, 158.0, 152.0, 145.9, 140.4, 132.4, 129.8, 127.5, 110.7, 109.9, 106.8; Elemental analysis calculated for $C_{11}H_{10}N_4O_2$ (MW 230.22): 57.39% C, 4.38% H, 24.34% N; found 57.52% C, 4.52% H, 24.41% N.

3-[(4-Hydroxyphenyl)amino]pyrazine-2-carboxamide (**13**). Yield 70.8%; M.p. 188.5-190.0 °C; IR (ATR-Ge, cm^{-1}): 3427m (-OH), 3281m (-NH-), 3172w (-$CONH_2$), 1673s (-C=O), 1584m, 1513s, 1470m, 1437m, 1422m, 1228s, 1185s (arom.); ^{1}H NMR (300 MHz, DMSO) δ 10.92 (1H, bs, NH), 9.20 (1H, bs, OH), 8.34 (1H, bs, NH_2), 8.27 (1H, d, *J*=2.4 Hz, H5), 7.90 (1H, d, *J*=2.4 Hz, H6), 7.87 (1H, bs, NH_2), 7.43-7.35 (2H, m, AA′, BB′, H2′, H6′), 6.77-6.68 (2H, m, AA′, BB′, H3′, H5′); ^{13}C NMR (75 MHz, DMSO) δ 169.1, 155.5, 152.4, 146.1, 131.6, 130.7, 126.9, 122.5, 115.5; Elemental analysis calculated for $C_{11}H_{10}N_4O_2$ (MW 230.22): 57.39% C, 4.38% H, 24.34% N; found 57.48% C, 4.19% H, 24.50% N.

3-[(3-Methoxyphenyl)amino]pyrazine-2-carboxamide (**14**). Yield 49.2%; M.p. 177.3-179.2 °C; IR (ATR-Ge, cm^{-1}): 3394m (-NH-), 3252w, 3214w (-$CONH_2$), 2843w (-OCH_3), 1663s (-C=O), 1614s, 1601s, 1580s, 1520s, 1493s, 1423s, 1256m, 1191s, 1181s, 1159s, 1045s (arom.); ^{1}H NMR (500 MHz, DMSO) δ 11.34 (1H, bs, NH), 8.42 (1H, bs, NH_2), 8.40 (1H, d, *J*=2.5 Hz, H5), 8.02 (1H, d, *J*=2.5 Hz, H6), 7.97 (1H, bs, NH_2), 7.40 (1H, t, *J*=2.1 Hz, H2′), 7.23 (1H, t, *J*=8.3 Hz, H5′), 7.15 (1H, dd, *J*=8.3 Hz, *J*=2.1 Hz, H4′), 6.61 (1H, dd, *J*=8.3 Hz, *J*=2.1 Hz, H6′), 3.75 (3H, s, OCH_3); ^{13}C NMR (125 MHz, DMSO) δ 168.9, 159.9, 151.9, 145.9, 140.5, 132.6, 129.8, 127.6, 112.4, 108.0, 105.8, 55.2; Elemental analysis calculated for $C_{12}H_{12}N_4O_2$ (MW 244.25): 59.01% C, 4.95% H, 22.94% N; found 58.96% C, 4.93% H, 22.82% N.

3-[(4-Methoxyphenyl)amino]pyrazine-2-carboxamide (**15**). Yield 74.2%; M.p. 211.8-213.8 °C; IR (ATR-Ge, cm^{-1}): 3424m (-NH-), 3162w (-$CONH_2$), 2849w (-OCH_3), 1679s (-C=O), 1606s, 1578s, 1514s, 1505s, 1416m, 1243s, 1180s (arom.); ^{1}H NMR (300 MHz, DMSO) δ 11.04 (1H, bs, NH), 8.36 (1H, bs, NH_2), 8.30 (1H, d, *J*=1.8 Hz, H5), 7.94 (1H, d, *J*=1.8 Hz, H6), 7.90 (1H, bs, NH_2), 7.59-7.48 (2H, m, AA′, BB′, H2′, H6′), 6.95-6.85 (2H, m, AA′, BB′, H3′, H5′), 3.71 (3H, s, OCH_3); ^{13}C NMR (75 MHz, DMSO) δ 169.0, 155.3, 152.2, 146.1, 132.2, 131.9, 127.1, 122.1, 114.3, 55.4; Elemental analysis calculated for $C_{12}H_{12}N_4O_2$ (MW 244.25): 59.01% C, 4.95% H, 22.94% N; found 59.03% C, 4.97% H, 22.80% N.

3-[(3,5-Dimethoxyphenyl)amino]pyrazine-2-carboxamide (**16**). Yield 33.7%; M.p. 206.4-207.9 °C; IR (ATR-Ge, cm^{-1}): 3397m (-NH-), 3213w (-$CONH_2$), 2844w (-OCH_3), 1662s (-C=O), 1584s, 1517s, 1453m, 1416s, 1229s, 1206s, 1192s, 1165s, 1152s, 1065s (arom.); ^{1}H NMR (300 MHz, DMSO) δ 11.33 (1H, bs, NH), 8.43 (1H, bs, NH_2), 8.40 (1H, d, *J*=2.5 Hz, H5), 8.02 (1H, d, *J*=2.5 Hz, H6), 7.98 (1H, bs, NH_2), 6.90 (2H, d, *J*=2.1 Hz, H2′, H6′), 6.19 (1H, t, *J*=2.1 Hz, H4′), 3.73 (3H, s, OCH_3); ^{13}C NMR (75 MHz, DMSO) δ 168.9, 160.9, 151.9, 145.9, 141.0, 132.6, 127.6, 98.3, 94.7, 55.3; Elemental analysis calculated for $C_{13}H_{14}N_4O_3$ (MW 274.11): 56.93% C, 5.14% H, 20.43% N; found 57.06% C, 5.04% H, 20.27% N.

CONCLUSION

Better yields of starting compound 3-chloropyrazine-2-carboxamide were achieved by using the procedure of partial nitrile group hydrolysis of 3-chloropyrazine-2-carbonitrile. This technique was three to four times more effective than the method of homolytic amidation of 2-chloropyrazine and other advantage was the purity when there were no emerging positional isomers of the starting compound [28, 29]. Novel conditions for aminodehalogenation reaction between 3-chloropyrazine-2-carboxamide and substituted anilines were developed. The conditions were experimentally proved changing solvent, base, time and temperature. Results of these experiments were also compared to conventional heating methods. The best combination of conditions were as follows: methanol used as a solvent, pyridine used as a base, 140 °C, 30

minutes using PowerMAX function. A series of 16 compounds (15 of them novel) was synthesized *via* microwave-assisted synthesis successfully according to NMR and other analytical measurements. These carboxamides were prepared in higher yields and during shorter reaction times than using conventional methods. Fewer products were obtained only by using microwave-assisted reaction. Yields were at least two times higher compared to conventional heating methods or MW assisted reactions without PowerMAX function. Lipophilicity of synthesised compounds was calculated as well as experimentally determined. Results showed linear dependence between these values excluding compounds with easily ionisable groups such as hydroxyl. All these products were designed as potentially active compounds against *Mycobacterium* species. Further studies including biological activity testing and expansion to more derivatives are currently in progress in our laboratory.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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PŘÍLOHA Č. 4

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Aminodehalogenation of 3-chloropyrazine-2-carboxamide with variously substituted benzylamines yielded a series of fifteen 3-benzylaminopyrazine-2-carboxamides. Four compounds possessed *in vitro* whole cell activity against *Mycobacterium tuberculosis* H37R$_v$ that was at least the same as standard pyrazinamide. MIC ranged from 6 to 42 μM. The best MIC (6 μM) was reached by 3-((4-methylbenzyl)amino)pyrazine-2-carboxamide (**8**) that also exerted low cytotoxicity in HepG2 cell line (IC_{50} = >250 μM). Only moderate activity against *Enterococcus faecalis* and *Staphylococcus aureus* was observed. No activity was detected against any tested fungal strains. Molecular docking with mycobacterial enoyl-ACP-reductase (InhA) was performed to investigate a possible target of prepared compounds. Active compounds shared the common binding interactions of known InhA inhibitors. Antimycobacterial activity of title compounds was compared to previously published benzylamino substituted pyrazines with differing substitution on pyrazine core (carbonitrile moiety). The title series possessed comparable activity and lower cytotoxicity than molecules containing carbonitrile group on pyrazine ring.

Introduction

Tuberculosis (TB) still remains global health problem in spite of decreasing total incidence of new cases. It is the second leading cause of death in the group of infectious diseases.[1] It is mainly caused by *Mycobacterium tuberculosis* (*M. tbc*). Estimated 9.6 million people developed TB and 1.5 million people died from TB in 2014.[1] Although huge progress was noted in diagnostics and treatment, the major problem has arisen with the resistance of mycobacterial strains to current therapy. These multidrug-resistant (MDR-TB) or extensively drug-resistant (XDR-TB) strains are immense threat that is necessary to focus on. The partial headway was made by discovering bedaquiline, a novel drug active against resistant mycobacterial strains.[2]

The current common therapy of non-resistant TB is based on administering a cocktail of first-line antitubercular agents (rifampicin/rifabutin, isoniazid, ethambutol and pyrazinamide) within the period lasting from six to twelve months. The last mentioned drug – pyrazinamide (PZA) – is successfully used to shorten the time needed for treatment up to two thirds. It is entrained by its unique ability to kill the dormant forms of mycobacteria.[3] PZA is endowed with multiple mechanisms of action based on its parent form or its metabolite pyrazinoic acid (POA). One of the first theories associated with PZA mechanism of action is based on active transport of PZA into mycobacterial cell and its subsequent activation *via* nicotinamidase (pyrazinamidase; EC 3.5.1.19) to POA. Upon intracellular accumulation, POA acidifies the inner compartment of the cell causing the disruption of membrane potentials and dysfunction of pH sensitive enzymes.[4] This principle was disproved last year by Peterson *et al*.[5] Both PZA and POA also inhibit specific mycobacterial enzyme Fatty Acid Synthase I (FAS I; EC 2.3.1.85). Inhibition of FAS I leads to the depletion of mycolic acids and to the disruption of mycobacterial cell wall function.[6] Further research refers to the fact that POA also inhibits *trans*-translation. This process is also vital for the cell survival and its blockade induces cellular death.[7] One of the latest investigations reveals the fact that PZA inhibits aspartate decarboxylase (PanD; EC 4.1.1.11). This enzyme is responsible for conversion of *L*-aspartic acid to β-alanine, which is necessary for CoA synthesis. Blockade of this biochemical pathway usually leads to energy depletion and incapability to survive.[8]

PZA itself evinces unique physical and chemical properties and its small molecule is exceedingly suitable for modifications. The alkylamino substitution of pyrazine ring in positions 5- and 6- is considerably well known quite often showing good antimycobacterial activities.[9-12] This work deals with benzylamino derivatives since compounds containing benzylamino moiety were previously reported as anti-infective substances. *N*-Benzylpyrazine-2-carboxamides (*i.e.* containing benzylamino moiety attached to pyrazine nucleus *via* carbonyl linker) were reported by Servusova *et al.* showing moderate antitubercular activities.[13-15] Benzylamino derivatives of pyrazine (with benzylamino moiety attached directly to the pyrazine core) were reported by Zitko *et al.* and Jandourek *et al.* showing moderate activities as well.[16,17] In contrast to compounds proposed in this article, the previously reported benzylamino derivatives contained at least one carbonitrile moiety (see **Figure 1**). Such modifications did not lead to improved antitubercular effect. These results confirmed our

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Electronic Supplementary Information (ESI) available: General methods, complete synthetic procedures, analytical data of prepared compounds as well as screening methodology. See DOI: 10.1039/x0xx00000x

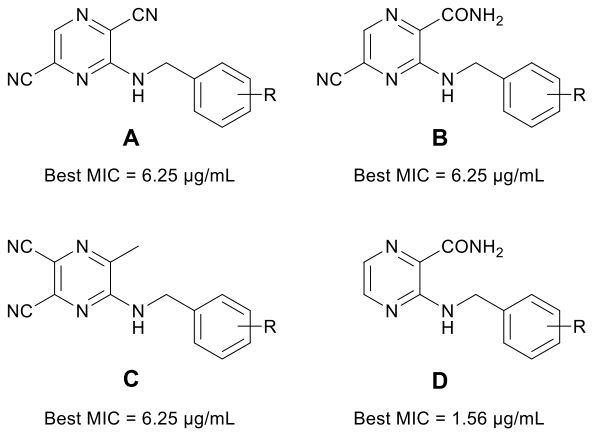


Figure 1 – General structures of discussed compounds (A,B – Zitko et al.[16]; C – Jandourek et al.[17]; D – this work) and best MICs against *M. tbc* $H37R_v$

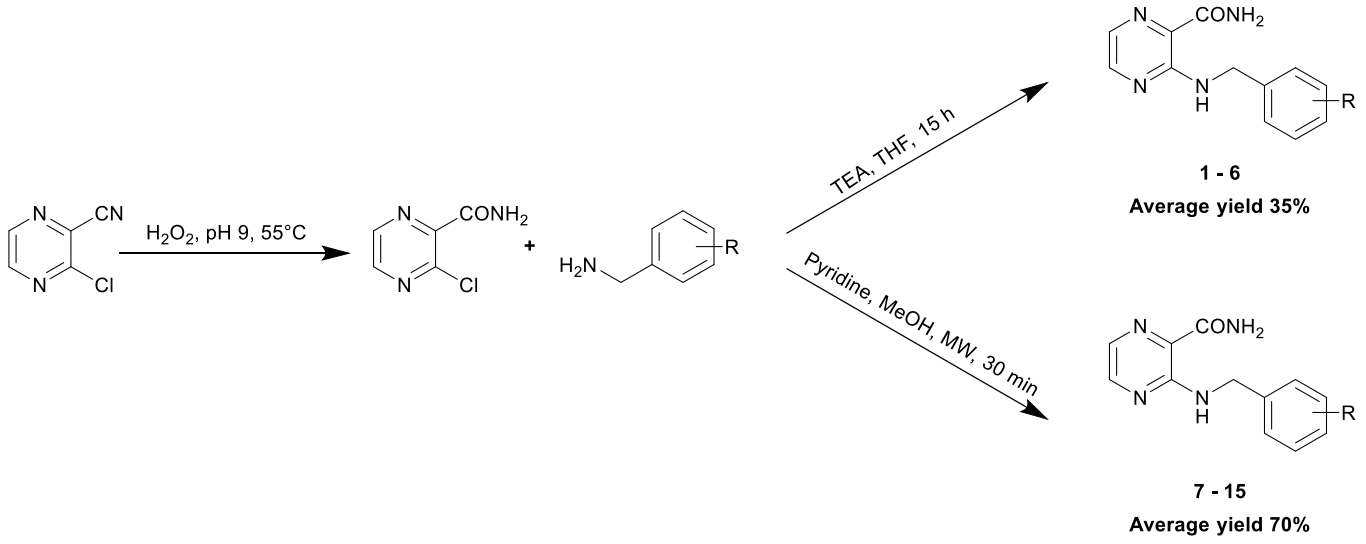
intentions to prepare novel derivatives with different substitutions to improve the efficacy and safety.

We have chosen pyrazine ring without carbonitrile moieties (lower cytotoxicity), free carboxamide group (possibility of activation to POA) and benzylamino substitution attached through amino group directly to pyrazine core. These compounds were investigated whether this combination made on the basis of previous publications would demonstrate better biological properties.

3-Chloropyrazine-2-carboxamide was let to react with variously substituted benzylamines by means of nucleophilic substitution of chlorine. Some of intended reactions were carried out using microwave-assisted synthesis, which can save the time and used materials according to literature. In addition, the yields tend to be higher than usually.[18] We have investigated differences between conventional organic synthesis (conventional heating methods) and microwave-assisted reaction. The comparison is stated.

Additional biological assays (antimycobacterial, antibacterial, antifungal) were performed to complete the scope of activity of these novel pyrazinamide derivatives that were preliminarily published under the terms of electronic conference contributions.[19,20]

Finally, prepared compounds were docked into active site of *M. tuberculosis* enoyl-ACP-reductase (InhA; EC 1.3.1.9). InhA has been repeatedly evaluated as an effective antimycobacterial target. It is a crucial enzyme involved in the long chain fatty acids biosynthesis. It is responsible for a reduction of the double bond between C2 and C3 of the enoyl intermediate linked to the acyl carrier protein (ACP). Inhibition of InhA leads to insufficient synthesis of mycolic acids, which are essential for mycobacterial cell wall integrity.[21] A molecular modeling study has been performed to verify if the InhA inhibition is a possible mechanism of action for prepared derivatives due to their structural similarity to known InhA inhibitors such as triclosan or PT70. It is obvious that the requirement of two planar heteroaromatic or aromatic structural fragments connected with a linker, which is specifically represented by -NH-CH_2- formation, is fulfilled. Another resemblance is actually expressed by carbonyl part of carboxamide group serving as a hydrogen bond accepting moiety. These structural features are mutual for the typical InhA inhibitors.[22] Molecular targets of PZA/POA as discussed above were considered for docking simulations. However, there are limitations, which make them inapplicable for our compounds. The complex of POA bound to ribosomal protein S1 (involved in *trans*-translation process) was resolved (pdb: 4NNI). From the complex it is obvious that POA could be substituted in positions 5- and/or 6-, but not in position 3-.[23] Pyrazinamidase (PncA) active site cavity is quite small and from the model based on the X-ray determined structure of PncA (pdb: 3PL1), it is quite obvious that the only position for possible substitutions of PZA is position 6- of the pyrazine ring.[24] Substitution of position 3- by a large moiety (benzylamino in our case) would divert the PZA core from position and orientation needed for the enzymatic conversion by PncA. Crystal structure of aspartate decarboxylase (PanD) was resolved (pdb: 2C45), but there are no complexes with bound PZA/POA. The



Scheme 1 - Synthesis of starting compound 3-chloropyrazine-2-carboxamide and aminodehalogenation reaction with variously substituted benzylamines carried out under different conditions

Table 1 - Summary of prepared compounds, calculated and measured parameters of lipophilicity, results of antimycobacterial and antibacterial evaluation expressed as minimal inhibitory concentration (MIC) compared to standards

No.	R	MW	Log *P*/Clog*P*	Log *k*	Minimal inhibitory concentrations			
					M. tbc H37R$_v$ (μg/mL)	*M. tbc* H37R$_v$ (μM)	*M. smegmatis* (μg/mL)	Bacteria (μM)
1	H	228.25	0.63/1.64	0.215	>100	n.a.	≥500	>500
2	3-Cl	262.69	1.19/2.36	0.373	>100	n.a.	≥500	>500
3	3,4-Cl	298.14	1.75/2.95	0.470	>100	n.a.	≥500	>500
4	3-CF_3	297.14	1.56/2.53	0.403	12.5	42	250	31.25 SA[a]
5	4-Cl	262.69	1.19/2.36	0.373	>100	n.a.	250	250 SA[a]
6	2-CH_3	242.28	1.12/2.09	0.320	>100	n.a.	≥500	>500
7	4-OCH_3	258.25	0.51/1.56	0.194	>100	n.a.	≥500	>500
8	4-CH_3	242.28	1.12/2.14	0.441	1.56	6	≥500	>500
9	4-NH_2	243.26	-0.17/0.42	-0.313	6.25	26	250	>500
10	2-Cl	262.69	1.19/2.36	0.441	>100	n.a.	≥500	>500
11	2-F	246.24	0.79/1.79	0.248	>100	n.a.	≥500	125 EF[b]
12	4-CF_3	296.25	1.56/2.53	0.502	12.5	42	≥500	125 EF[b]
13	2-CF_3	296.25	1.56/2.53	0.495	>100	n.a.	≥500	125 EF[b]
14	2,4-OCH_3	288.30	0.38/1.65	0.274	>100	n.a.	≥500	62.5 EF[b]
15	3-NO_2	273.25	n.d./1.39	0.101	>100	n.a.	≥500	>500
INH	-	137.14	-	-	0.39	3	7.81 – 15.63	-
PZA	-	123.12	-	-	12.5	102	≥500	-
RFM	-	822.94	-	-	n.d.	n.d.	0.78 – 1.56	-
CPX	-	331.37	-	-	n.d.	n.d.	0.10 – 0.20	-

[a] *Staphylococcus aureus* CCM 4516/08; [b] *Enterococcus faecalis*. J 14365/08

Log *P*/Clog*P* – calc. parameter of lipophilicity (ChemDraw 15.0); Log *k* – experim. determined lipophilicity; n.a. – not active; n.d. – not determined

binding site and interaction pattern for PZA/POA is therefore unknown. Simulations of PZA/POA binding to PanD by docking and docking followed by molecular dynamics simulation of the complex gave uncertain results.[25] FAS I enzyme complex is very extensive so the X-ray resolved crystal structure of sufficient quality is not yet available.[26]

Results and discussion

Chemistry

Starting compound 3-chloropyrazine-2-carboxamide was synthesized from 3-chloropyrazine-2-carbonitrile through partial hydrolysis of nitrile group under controlled conditions with specific pH and temperature.[27] This procedure was chosen on the basis of higher yields compared to direct amidation of the pyrazine ring.[27,28]

This initial compound was then let to react with variously substituted benzylamines (see **Scheme 1**) (see ESI for full detailed description of synthetic procedure). Six final compounds (**1**-**6**) were prepared by conventional heating methods utilizing tetrahydrofuran (THF) as a solvent, triethylamine as a base and two equivalents of corresponding benzylamine. Reaction was stopped after 15 hours and its progress was checked using TLC in the system hexane:ethyl acetate (1:1). Products were purified by preparative flash chromatography and final yields were ranged between 24% and 50%. These lower gains were taken as an impulse to complete the series under the microwave conditions. So compounds **7**-**15** were prepared successfully in the MW reactor using methanol as a solvent and pyridine as a base. Sealed thick-walled tubes were used as an over-pressurized system to reach higher temperature (140°C) than the standard boiling point of methanol. Time needed for conversion was shortened from 15 hours to 30 minutes. Compounds were also purified making use of flash chromatography. Not surprisingly, the yields were 70% on the average starting at 26% and ending at 80%. The lowest yield (26%) was observed for compound **15** that is substituted with nitro group in *meta* position. In general, this kind of substitution is hard to obtain in higher yields as its electron withdrawing properties decrease the nucleophilicity of benzylamino nitrogen. Difference between these two approaches can highlight advantages of microwave-assisted reactions. Time needed to complete the conversion was shortened ten times and amount of used solvents was

substantially minimized. The variance between yields was also significant. On the other hand, some limitations are emerging with this technology. It can be stated that not every base is usable for microwave-assisted reactions. Triethylamine, which acted as a base for conventional heating reactions, is inapplicable for MW in consideration of the fact that molecule of TEA is being decomposed during the process. Emerging substances (diethylamine) can react with starting compounds giving inadvertent side products.[29,30]

Final products were characterized by ^{1}H and ^{13}C NMR spectra, IR spectroscopy, elemental analysis, and also with their melting points. The acquired analytical data, which are presented in ESI, were fully in accordance with proposed structures.

Biological assays

Antimycobacterial screening

All of prepared compounds were screened for *in vitro* activity against *Mycobacterium tuberculosis* H37R_v, *M. kansasii* My 235/80 and *M. avium* 152/73 (for complete procedure see ESI). Experiments were carried out in liquid broth with pH value adjusted to 5.6. It is known that *in vitro* activity of PZA is strongly dependent on pH (MIC values ranging from units of μg/mL at pH 5.5 to hundreds of μg/mL in neutral conditions).[31-34] Using neutral pH and high concentration is not suitable for our compounds due to their limited solubility in the testing water-based medium. We were aware that acidic pH can negatively affect the growth of *M. tbc*. However, it was proved that *M. tbc* can be cultivated for 14 – 21 days of incubation under these conditions.[35-38] In our experiments, drug-free controls were included to validate the growth of mycobacteria under chosen acidic conditions.

Microtitration plate assay was used to determine activity and results were read using resazurin dye based methodology.

Series contains compounds that show activity against *M. tuberculosis* H37R_v. Such activity was detected for compounds **4**, **8**, **9** and **12** with MIC values in the range 1.56–12.5 μg/mL (resp. 6–42 μM) as seen in **Table 1**. It cannot be stated that there is a dependence between the lipophilicity and the activity. The values of lipophilicity either for active or inactive compounds vary in wide range (see **Figure 2**). Compounds **4** and **12** are substituted with trifluoromethyl group, which is usually used fruitfully to prepare potentially

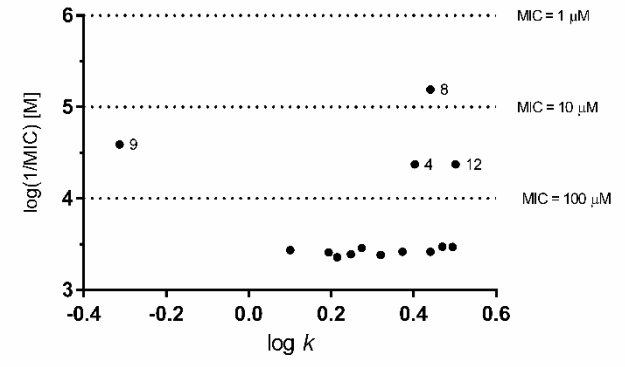
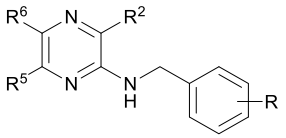


Figure 2 - Dependency of antimycobacterial activity (1/MIC) on experimental parameter of lipophilicity log *k*

Table 2 - Activity of selected title compounds against *M. tuberculosis* H37R_v in comparison with previously prepared derivatives containing carbonitrile moiety[16,17]



MIC against *M. tbc* H37R_v

No.	R	R^2	R^5	R^6	μg/mL	μM
1	H	$CONH_2$	H	H	>100	-
1-A	H	$CONH_2$	CN	H	>100	-
1-B	H	CN	CN	H	12.5	78
1-C	H	CH_3	CN	CN	25	100
4	3-CF_3	$CONH_2$	H	H	12.5	42
4-A	3-CF_3	$CONH_2$	CN	H	25	78
4-B	3-CF_3	CN	CN	H	>100	-
4-C	3-CF_3	CH_3	CN	CN	12.5	39
8	4-CH_3	$CONH_2$	H	H	1.56	6
8-A	4-CH_3	$CONH_2$	CN	H	12.5	47
8-B	4-CH_3	CN	CN	H	6.25	25
8-C	4-CH_3	CH_3	CN	CN	25	95
9	4-NH_2	$CONH_2$	H	H	6.25	26
9-A	4-NH_2	$CONH_2$	CN	H	12.5	47
9-B	4-NH_2	CN	CN	H	25	100
9-C	4-NH_2	CH_3	CN	CN	25	95
INH	-	-	-	-	0.39	3
PZA	-	-	-	-	12.5	102

active substances. On the other hand, these structures often show higher cytotoxicity that is undesirable.[16,39-41] For that reason cytotoxicity screening was performed. Contrarily, compounds **8** and **9** are substituted with less burdening groups and activities demonstrated by them are more auspicious.

Derivative **8** showed the best activity which was close to the activity of standard isoniazid (MIC = 3 μM) (see **Table 1**). Acquired results were compared to corresponding 3-benzylamino-5-cyanopyrazine-2-carboxamides (**Table 2**, A), 3-benzylaminopyrazine-2,5-dicarbonitriles (**Table 2**, B) and 5-benzylamino-6-methylpyrazine-2,3-dicarbonitriles (**Table 2**, C) prepared previously in order to compare the effect of cyano *vs.* carboxamide substitution. The results were mostly comparable. Nevertheless, there are compounds that should be pointed out. Compound **4** (R = 3-CF_3; MIC = 12.5 μg/mL) was more active than matching substances **4-A** (MIC = 25 μg/mL) or **4-B** (MIC = >100 μg/mL) and the activity of derivative **4-C** (MIC = 12.5 μg/mL) was nearly the same.[16,17] Compound **9** (R = 4-NH_2; MIC = 6.25 μg/mL) showed better activity than **9-A** (MIC = 12.5 μg/mL), **9-B** (MIC = 25 μg/mL) or **9-C** (MIC = 25 μg/mL).[16,17] Compound **1** (R = H; MIC = >100 μg/mL) proved to be much less active than corresponding dicarbonitriles **1-B** (MIC = 12.5 μg/mL) and **1-C** (MIC = 25 μg/mL). However, corresponding cyanopyrazine-2-carboxamide (**1-A**; MIC = >100 μg/mL) also showed inactivity.[16,17] In contrast, compound **8** (R = 4-CH_3; MIC = 1.56

μg/mL) was certainly more active than resembling compounds **8-B** (MIC = 6.25 μg/mL), **8-A** (MIC = 12.5 μg/mL) and **8-C** (MIC = 25 μg/mL).[16,17] It can be deduced that carbonitrile group on the pyrazine core is not necessary to obtain better activity and compounds without this modification are equally or more active against *M. tuberculosis* (see **Table 2**).

Just one compound was effective against other mycobacterial strain than *M. tuberculosis* with the activity comparable to standard. Specifically, derivative **4** showed activity against *Mycobacterium kansasii* (MIC = 25 μg/mL, resp. 84 μM) whilst MIC of isoniazid ranged between 1.56 – 12.5 μg/mL (resp. 11 – 91 μM).

***Mycobacterium smegmatis* screening**

The screening was performed again on the basis of adjusted microtitration plate assay with resazurin dye staining (see ESI). This complementary test was completed in order to find any relationships between activities against typical slow growing mycobacterial strains and *Mycobacterium smegmatis*. The behavior of the last named microorganism is alike other strains but it is not a human pathogen. Its generation time is also advantageous because it is disproportionally shorter.

Results of this assay were dissimilar to previous antimycobacterial screening. Poor activity was proven entirely by three compounds, namely compound **4**, **5** and **9** as seen in **Table 1**. The value 250 μg/mL was not absolutely comparable to used standards isoniazid (15.63 μg/mL), rifampicin (0.39 μg/mL) and ciprofloxacin (0.10 μg/mL). It is apparent that prepared substances are active against *M. tuberculosis* and probably act through pathway that is not mutual for both types of mycobacteria.

Antibacterial and antifungal screening

All compounds were tested *in vitro* for their activity against eight common bacterial strains and eight fungal stems with standard methodology (see ESI).

Four from fifteen synthesized substances exerted moderate to low activity against *Enterococcus faecalis* and the values ranged between 62.5 μM and 125 μM (see **Table 1**). Compounds **4** and **5** were active against *Staphylococcus aureus* with the mean activity 31.25 μM. All these results were insignificant compared to standards used in this screening.

No antifungal activity was observed for any of prepared substances.

Cytotoxicity assays

The most active compounds (**4, 8, 9** and **12**) were tested for their cytotoxic effects. Results of these experiments are presented as inhibitory concentration, which is necessary to decrease the viability of the cell population to 50% from the maximal viability (IC_{50}) compared to 100% cell viability control. The cytotoxicity of tested compounds was measured using the standard hepatic cell line HepG2.

Table 3 - Cytotoxicity of active compounds and calculated selectivity index (SI) values

Compound	IC_{50} (μM)	SI (IC_{50}/MIC)
4	76.5	1.8
8	>250	>41.7
9	>750	>28.8
12	57.3	1.4

Used CellTiter 96® Aqueous One Solution Assay is based on the bioreduction of tetrazolium dye MTS by cells into a coloured formazan product, which is then determined colorimetrically. Reduction of this reagent is accomplished by NADPH or NADH produced in metabolically active cells. The quantity of formazan is directly proportional to the number of living cells.

Compounds can be divided into two groups according to their IC_{50} as seen in **Table 3**. The first group is represented by substances **4** and **12** substituted with trifluoromethyl group. The value of IC_{50} is at the order of tens of μM expressing relatively high cytotoxicity. This course was expected according to the character of substitution that was stated above. It can be also connected with higher lipophilicity causing the intracellular accumulation. The second group (**8**, **9**) showed encouraging results within the order of hundreds or thousands of μM. The factual values of IC_{50} for these compounds were not possible to determine due to the low solubility at higher concentrations but it is at least three orders improvement in comparison to compounds **4** and **12**.

Selectivity index (SI) was calculated for antimycobacterial activity (*M. tuberculosis*) and was defined as a ratio between IC_{50} and MIC (μM). Values above 10 are considered to be safe values for a potential novel drug. This condition is met by compounds **8** and **9** (see **Table 3**).

Lipophilicity determination

Calculated lipophilicity parameters log *P* and Clog*P* were predicted using ChemBioDraw Ultra 14 program. These values were compared with experimentally measured parameter of lipophilicity log *k*. These figures were derived from retention times acquired by RP-HPLC (see ESI).

Theoretical (log *P*, Clog*P*) and experimental (log *k*) values were correlated to show the linear dependency, even though the

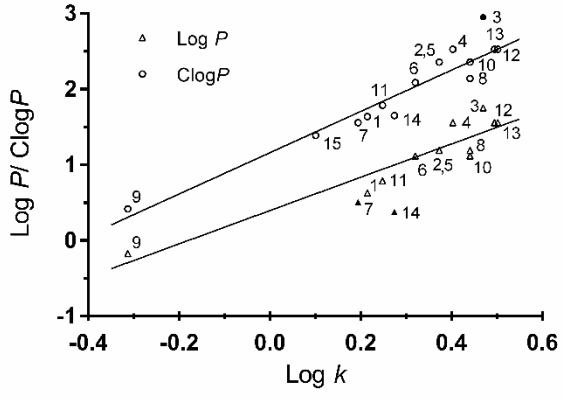


Figure 3 – Linear dependencies of two differently calculated lipophilicity parameters on experimentally measured values

values of log *P* and Clog*P* calculated by ChemBioDraw algorithm did not reflect the influence of substituent's position on the aromatic ring (see **Figure 3**).

Correlation of these parameters can be expressed by the following regression equations. The first equation is showing the dependency of log *P* on log *k*. Compounds **7** and **14** were excluded from calculations due to their distinct value that could be caused by the character of substitution (methoxy group).

$$\log P = 0.397(\pm 0.104) + 2.199(\pm 0.263) \log k$$
$$R^2 = 0.875,\ s = 0.194,\ F = 69.7,\ n = 12$$

The second equation expresses the dependency of Clog*P* on log *k*. This relationship is more predicative showing better parameters of regression.

$$\text{Clog}P = 1.162(\pm 0.074) + 2.726(\pm 0.210) \log k$$
$$R^2 = 0.934,\ s = 0.159,\ F = 168.6,\ n = 14$$

Compound **9** differs from the others in both parameters, which can be caused by the character of substitution. Amino group is able to be easily ionized, which can result in distortion of the outcomes of performed measurements.

Docking

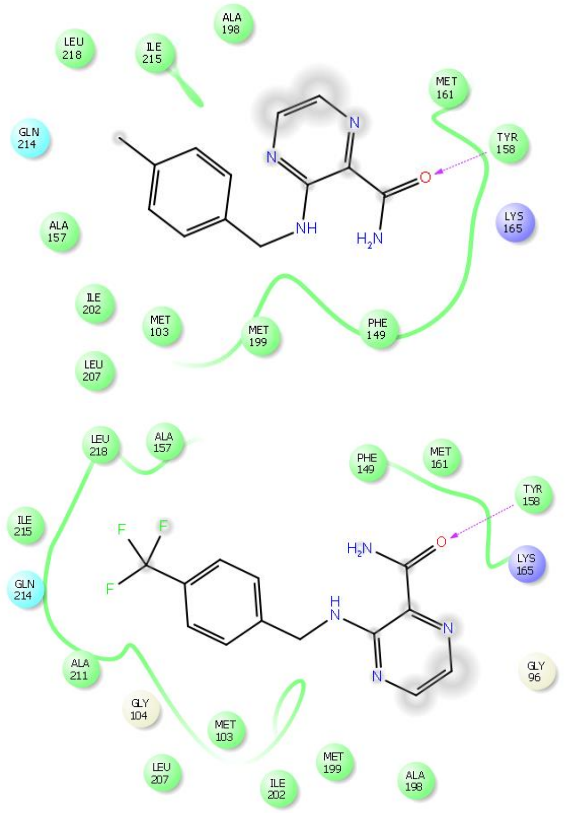


Figure 4 - 2D visualization of compounds **8** and **12** in active site of InhA showing interactions with substrate binding loop

Table 4 - Results of affinity calculations by Glide Scoring function in relation with *in vitro* activity against *M. tuberculosis* H37R$_v$

Compound	Affinity (kcal/mol)	MIC *M. tbc* H37R$_v$ (μM)
4TZK Ligand	-9.82	-
4	-8.47	42
8	-7.40	6
9	-7.82	26
12	-8.49	42

The results of affinity calculations show the important effect of carboxamide group of assessed compounds. The affinity towards InhA is high within the series containing compounds with substituents in 3- position of the pyrazine core. Such arrangement enables close proximity of the carbonyl group to **Tyr 158** and NAD^+ ribose and allows the substituent in the aforementioned 3- position to form additional less specific interactions with substrate binding group residues at the same time (see **Figure 4**). None of the assessed compounds reached the affinity higher or equal to (3*S*)-1-cyclohexyl-*N*-(3,5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide (-9.82 kcal/mol) that is original 4TZK ligand.

The best scored compounds **4** and **12** (affinity -8.47 kcal/mol resp. 8.49 kcal/mol, see **Table 4**) attain similar pose within the active site as most of the direct InhA inhibitors, forming the hydrogen bond network between **Tyr 158**, NAD^+ ribose and pyrazine core. Carboxamide moiety fulfils similar role to the carbonyl moiety of 4TZK original ligand or phenolate of known inhibitors such as triclosan or PT70 (ring A) (see **Figure 5**). The hydrophobic benzyl part of discussed compounds exerts hydrophobic interactions with lipophilic residues of the substrate binding loop (**Met 103**, **Pro 193**, **Met 199**, **Leu 207** and **Ile 215**) (see **Figure 6**). The orientation of this benzyl moiety is in accordance with orientation of hexyl chain of PT70 that is oriented in the hydrophobic entry tunnel, which normally hosts the long alkyl chain of mycolic acid intermediate (see **Figure 5**). The most efficient compound **8** did not achieve such promising score compared to standard ligand. This might happen due to less hydrophobic substitution of non-pyrazine molecule part that results in less potent hydrophobic interactions with substrate binding loop hydrophobic residues.

Conclusions

Four of fifteen prepared compounds showed notable antimycobacterial activity against *M. tuberculosis* H37R$_v$. Compounds **4**, **8**, **9** and **12** exerted activities similar to or better than PZA. 4-Methylbenzylamino derivative (**8**) showed also activity nearly the same as standard isoniazid. Prepared benzylaminopyrazine-2-carboxamides were compared to

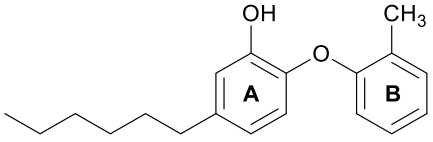


Figure 5 – Chemical structure of PT70

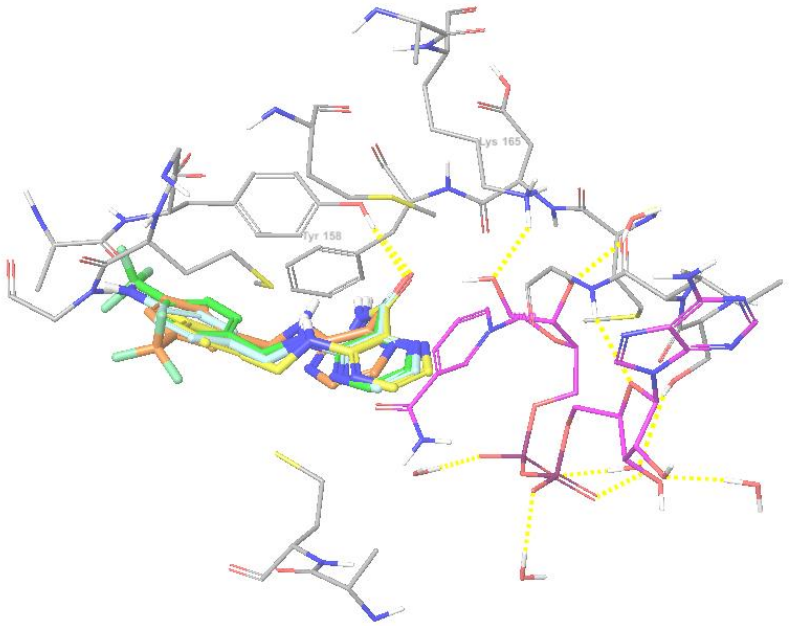


Figure 6 – The most active compounds docked into the active site of InhA (ACP-enoyl reductase) (**4** – orange; **8** – yellow; **9** – aquamarine; **12** – green)

previously synthesized benzylaminopyrazines with different substitution on pyrazine ring. Corresponding compounds were determined to be active, nevertheless the title structures of this research without carbonitrile moiety appeared to exert at least double activity even though the lipophilicity was lower. Cytotoxicity assays performed on HepG2 cells confirmed preceding assumption that molecules containing trifluoromethyl moiety are highly cytotoxic despite the fact they are usually active. On the other hand, compounds **8** and **9** showed very low cytotoxicity and calculated selectivity index was considered to be propitious. Antitubercular activity of these two compounds is rather specific than nonselective based on cytotoxic effect. The docking studies did not exclude that inhibition of InhA might contribute to the mechanism of action of presented derivatives. However, comparison of *in vitro* and *in silico* results suggests that InhA inhibition is probably not the main mechanism of action of these 2,3-disubstituted derivatives, or, the disagreement might be caused by differences in mycobacterial cell penetration among individual compounds. Nevertheless, the predicted binding poses of active derivatives exhibit the key interactions observed with the most of the confirmed InhA inhibitors. The pyrazine carboxamide group plays a crucial role by forming the important interactions with **Tyr 158** and NAD^+. Substitution of the pyrazine core seems to determine the affinity of assessed compounds and further stabilize possible inhibitor within the active site. It enables interactions with various residues within the pocket and therefore anchor the inhibitor in the active site. Compounds **8** and **9** are suitable for further structural modifications that can improve their biological properties (activity, cytotoxicity) and physical properties (solubility, lipophilicity).

Acknowledgements

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Electronic Supplementary Information

Synthesis of novel pyrazinamide derivatives based on 3-chloropyrazine-2-carboxamide and their antimicrobial evaluation

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General methods

All chemicals were of reagent or higher grade of purity. They were mainly purchased from Sigma-Aldrich or Fluka (Sigma-Aldrich, Co., Steinheim, Germany). Plates for thin layer chromatography (TLC) were provided by Merck (Silica gel 60 F_{254}) (Merck KGaA, Darmstadt, Germany). Microwave assisted reactions were performed using microwave reactor with focused field equipped with autosampler Explorer 24 (CEM Discover, CEM Corporation, Matthews, North Carolina, USA). The reaction progress was monitored with CEM´s Synergy™ software. All compounds were purified using techniques of preparative flash chromatography (CombiFlash ® RF, Teledyne Isco, Inc., Lincoln, NE, USA). Silica gel (0.040 – 0.063 mm) was used as the stationary phase (Merck KGaA) and hexane (Lach-Ner, s.r.o., Neratovice, Czech Republic) together with ethyl acetate (Penta, Prague, Czech Republic) as the mobile phase in gradient elution. NMR spectra were acquired at ambient temperature by Varian Mercury-VxBB 300 spectrometer (Varian corp., Palo Alto, California, USA, 299.95 MHz for ^{1}H, 75.43 MHz for ^{13}C) or Varian VNMR S500 spectrometer (Varian corp., Palo Alto, California, USA, 499.87 MHz for ^{1}H, 125.71 MHz for ^{13}C). Chemical shifts were referenced in ppm (δ) and were indirectly related to TMS (tetramethylsilane) *via* a signal of solvent (2.49 for ^{1}H and 39.7 for ^{13}C in DMSO-d_6 or 7.28 for ^{1}H and 77.0 for ^{13}C in $CDCl_3$). Infrared spectra were recorded using ATR methodology (Attenuated Total Reflectance) by spectrometer FT-IR Nicolet 6700 (Nicolet – Thermo Scientific, Waltham, MA, USA) on germanium crystal. Elemental analysis was performed on EA 1110 CHNS Analyser (Fisons Instruments S. p. A., Carlo Erba, Milano, Italy) and the values were given as percentages. Melting points were determined by SMP3 Stuart Scientific apparatus (Bibby Sterling LTD, Staffordshire, UK) in open capillary and are uncorrected. Yields are expressed as percentages and refer to products after all purification steps.

General synthetic procedure

Starting compound was prepared *via* partial hydrolysis of nitrile group of 3-chloropyrazine-2-carbonitrile (Fluorochem, Co., Hadfield, Derbyshire, UK). The mixture of concentrated (30%) hydrogen peroxide (29 mL) and water (195 mL) was prepared and alkalized with 8% (w/v) solution of sodium hydroxide to obtain the solution with pH 9. Carbonitrile (104 mmol) was being added portion-wise into the heated (50 °C) mixture over the period of 30 minutes. The whole mass was stirred for additional 2.5 hours at 55 °C and pH 9 (pH was periodically monitored and adjusted by drops of 8% NaOH solution). Reaction mixture was cooled in the fridge to initiate the crystallization. The crude product was recrystallized from ethanol.[1] The yield of this reaction was approximately 80%.

Compounds **1**–**6** were prepared according to conventional methods of organic synthesis. 1.27 mmol of 3-chloropyrazine-2-carboxamide was dissolved in 20 mL of THF in round bottom flask and then was treated with two equivalents of corresponding benzylamine and equimolar amount of triethylamine. The reaction was conducted with continuous stirring and heating (70 °C) under reflux in oil bath for 15 hours. Compounds **7**–**15** were synthesised using the microwave reactor with focused field. 1.27 mmol of 3-chloropyrazine-2-carboxamide was put into the thick-walled tube together with 2.54 mmol of corresponding benzylamine, 1.27 mmol of pyridine, approx. 5 mL of methanol, magnetic stirrer, and then was sealed with special cap. The parameters of reaction were set according to previously published paper as follows – 140 °C, 30 minutes, 200 W.[2] The progress of reaction was checked by TLC (hexane: ethyl acetate – 1:1). Regardless of the method used for synthesis, all reaction mixtures were adsorbed on silica and subjected to preparative flash chromatography (hexane and ethyl acetate, gradient elution, detection wavelengths 260 nm and 280 nm). Products were recrystallized from ethanol or ethanol and water if necessary. All final substances were chemically characterized (^{1}H NMR, ^{13}C NMR, IR, melting point and elemental analysis).

Antimycobacterial evaluation

Antimycobacterial screening was performed against *M. tuberculosis* $H37R_v$ CNCTC My 331/88, *M. kansasii* Hauduroy CNCTC My 235/80, and *M. avium* CNCTC My 152/73 (Czech National Collection of Type Cultures, National Institute of Public Health, Prague, Czech Republic) using isoniazid and pyrazinamide (Sigma-Aldrich) as standards. Culturing medium used for assays was Sula´s semisynthetic broth with adjusted pH 5.6 (Trios, Prague, Czech Republic). The cultures were grown at 37 °C in dark and humid atmosphere. Tested compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with medium to final concentrations 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 μg/mL except for INH where the dilution continues up to 0.1 μg/mL. The method used for this screening was microdilution broth panel method. Mycobacterial inoculum was suspended in sterile water and the density was adjusted in the range between 0.5 and 1.0 McFarland scale. Suspensions were diluted with broth by 10^{-1}, added to microtitration plates and incubated for 14 days for *M. tuberculosis*, 5-7 days for *M. kansasii* and 5 days for *M. avium*. Drug-free controls were included containing broth with DMSO. The final concentration of DMSO was 0.5% (v/v) and did not affect the growth of mycobacteria. The antimycobacterial activity was determined using resazurin based dye (Alamar blue) and results were read after 24 hours of incubation after addition of 30 μL of stain (0.005% solution of resazurin sodium salt in the 1:1 mixture of water and 10% (v/v) water solution of Tween 80).[3] Minimum inhibitory concentration was defined as the lowest concentration of tested compound which prevented colour change (from blue to pink).

Evaluation of activity against *Mycobacterium smegmatis*

Antimycobacterial assay was performed with fast growing *Mycobacterium smegmatis* CCM 4622 (ATCC 607) from Czech Collection of Microorganisms (Brno, Czech Republic). The technique used for activity determination was microdilution broth panel method using 96-well microtitration plates. Culturing medium was Middlebrook 7H9 broth (Sigma-Aldrich) enriched with 0.4% of glycerol (Sigma-Aldrich) and 10% of Middlebrook OADC growth supplement (Himedia, Mumbai, India). Tested compounds were dissolved in DMSO (Sigma-Aldrich) then broth was added to obtain concentration 2000 μg/mL. Standards used for activity determination were isoniazid (INH), rifampicin (RIF) and ciprofloxacin (CPX) (Sigma-Aldrich). Final concentrations were reached by binary dilution and addition of mycobacterial suspension and were set as 500, 250, 125, 62.5, 31.25, 15.63, 7.81 and 3.91 μg/mL except to standards ciprofloxacin and rifampicin where the final concentrations were 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20 and 0.10 μg/mL. Drug-free controls were included containing Middlebrook 7H9 broth and DMSO. The final concentration of DMSO did not exceeded 2.5% (v/v) and did not affect the growth of *M. smegmatis*. *Mycobacterium smegmatis* was cultured in liquid medium Middlebrook 7H9 enriched with glycerol (0.4%) and OADC growth supplement (10%). Bacteria were always passaged after seven days of incubation. Testing mycobacterial suspension was prepared by adjusting the density to 1.0 McFarland scale and diluting it with broth in a ratio of 1:20. Plates were sealed with polyester adhesive film and incubated in dark at 37°C without agitation. The addition of 0.01% solution of resazurin sodium salt (20 μL) followed after 48 hours of incubation. Stain was prepared by dissolving resazurin sodium salt (Sigma-Aldrich) in deionised water to get 0.02% solution. Then 10% aqueous solution of Tween 80 (Sigma-Aldrich) was prepared. Both liquids were mixed up making use of the same volumes and filtered through syringe membrane filter. After addition of the dye, microtitration panels were then incubated for additional four hours. Antimycobacterial activity was expressed as minimum inhibitory concentration (MIC) and the value was read on the basis of colour change (blue colour – active; pink colour – inactive). MIC values for standards were in ranges 7.81 – 15.63 μg/mL for INH, 0.78 – 1.56 μg/mL for RIF and 0.10 – 0.20 μg/mL for CPX. All experiments were conducted in duplicate.

Antibacterial and antifungal evaluation

Assay was based on microdilution broth panel method in 96-well microtitration plates.[4] The organisms used in experiments included strains *Staphylococcus aureus* CCM 4516/08, *Escherichia coli* CCM 4517, *Pseudomonas aeruginosa* CCM 1961 (Czech Collection of Microorganisms, Brno, Czech Republic) that are recommended as standards for antibacterial screening. Additional strains were acquired from Department of Clinical Microbiology, University Hospital, Hradec Kralove, Czech Republic as clinical isolates - *Staphylococcus aureus* H 5996/08 – methicillin resistant (MRSA), *Staphylococcus epidermidis* H 6966/08, *Enterococcus faecalis* J 14365/08, *Klebsiella pneumoniae* D 11750/08, *Klebsiella pneumoniae* J 14368/08 – ESBL positive. All microorganisms were cultured on Mueller-Hinton agar (MHA) (Difco/Becton Dickinson, Detroit, MI, USA) at 35 °C. Bacterial inoculum was prepared by suspending colony in sterile 0.85% saline. The cell density of the inoculum was adjusted to 0.5 McFarland scale. Compounds were dissolved in DMSO and the antibacterial activity was determined in Mueller-Hinton broth (Difco/Becton Dickinson) buffered to pH 7.0. Negative controls (growth controls) consisted of medium and DMSO. The final concentration of DMSO did not exceed 1% (v/v) of the total solution composition. The minimum inhibitory concentration (MIC) was defined as 95% inhibition of bacterial growth compared to provided control and was determined after 24 and 48 hours of static incubation at 35 °C. Bacitracin, neomycin, ciprofloxacin and phenoxymethylpenicillin were chosen as standards.

Antifungal screening was also carried out using microdilution broth method with tested strains – *Candida albicans* ATCC 44859, *C. tropicalis* 156, *C. krusei* E28, *C. glabrata* 20/I, *Trichosporon asahii* 1188, *Aspergillus fumigatus* 231, *Lichtheimia corymbifera* 272 and *Trichophyton mentagrophytes* 445. Compounds were dissolved in DMSO and diluted using RPMI 1640 medium with glutamine buffered to pH 7.0 (MOPS) in a twofold manner. The final concentration of DMSO in the tested medium did not exceed 2.5 % (v/v). Static incubation was performed in the dark and humid atmosphere at 35 °C for 24 and 48 hours (resp. 72 and 120 hours for *Trichophyton mentagrophytes*). Drug-free controls were included and fluconazole, amphotericin B, voriconazole and nystatin were used as standards.[5]

Cytotoxicity determination

The human hepatocellular liver carcinoma cell line HepG2 (passage 12) purchased from Health Protection Agency Culture Collections (ECACC, Salisbury, UK) was routinely cultured in MEM (Minimum Essentials Eagle Medium) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (PAA), 1% L-Glutamine solution (Sigma-Aldrich) and non-essential amino acid solution (Sigma-Aldrich) in a humid atmosphere containing 5% of CO_2 at 37°C. Cells were harvested after trypsin/EDTA (Sigma-Aldrich) treatment at 37°C for subculturing. Cells treated with tested substances were used as experimental groups. Untreated HepG2 cells were applied to represent control groups. HepG2 cells were seeded in density 10 000 cells per well in a 96-well plates and were treated with tested substances dissolved in DMSO next day. The tested compounds were prepared in seven concentrations (1 – 1000 µM) in triplicates. The controls (100% cell viability, 0% cell viability, control without cells, and vehicle controls) were also prepared in triplicates. The reagent from CellTiter 96 Aqueous One Solution Cell Proliferation Assay (CellTiter 96, Promega Corporation, Madison, WI, USA) was added after incubation in a humidified atmosphere containing 5% of CO_2 at 37°C lasting for 24 hours. Additional incubation at 37 °C followed and absorbance was recorded at 490 nm. A standard toxicological parameter IC_{50} was calculated using GraphPad Prism software 6.02 (GraphPad Software, La Jolla, CA, USA).

Lipophilicity determination

Theoretical lipophilicity parameters log *P* and Clog*P* were calculated by algorithms of program CS ChemBioOffice Ultra 14.0 (CambridgeSoft, Cambridge, MA, USA).

Experimental lipophilicity parameter log *k* was pinpointed using HPLC methods. Agilent Technologies 1200 SL liquid chromatography with Diode-array Detector SL G1315C, chromatographic pre-column ZORBAX XDB-C18 5 µm, 4 x 4 mm, Part No. 7995118-504 and column ZORBAX Eclipse XDB-C18 5 µm, 4.6 x 250 mm, Part No. 7995118-585 (Agilent Technologies Inc., Colorado Springs, CO, USA) were used in HPLC system. The separation process was handled by Agilent ChemStation, version B.04.02 extended with spectral module (Agilent Technologies Inc.). A mixture of methanol (HPLC grade, 70 %) and water (HPLC-Milli-Q Grade, 30 %) was used as mobile phase. The total flow rate of the column was set to 1.0 mL/min, injection 20 µL, column temperature 30 °C. 210 nm as detection wavelength and 270 nm as monitor wavelength were chosen. The methanolic solution of KI was used for the determination of dead time (TD). Retention times (TR) of synthesized compounds were measured in minutes. The capacity factors *k* were calculated according to formula $k = (TR - TD)/TD$, where TR is the retention time of the solute and TD denotes the dead time obtained *via* an unretained analyte (KI). Log *k*, which was calculated from the capacity factor *k*, is used as the lipophilicity index.

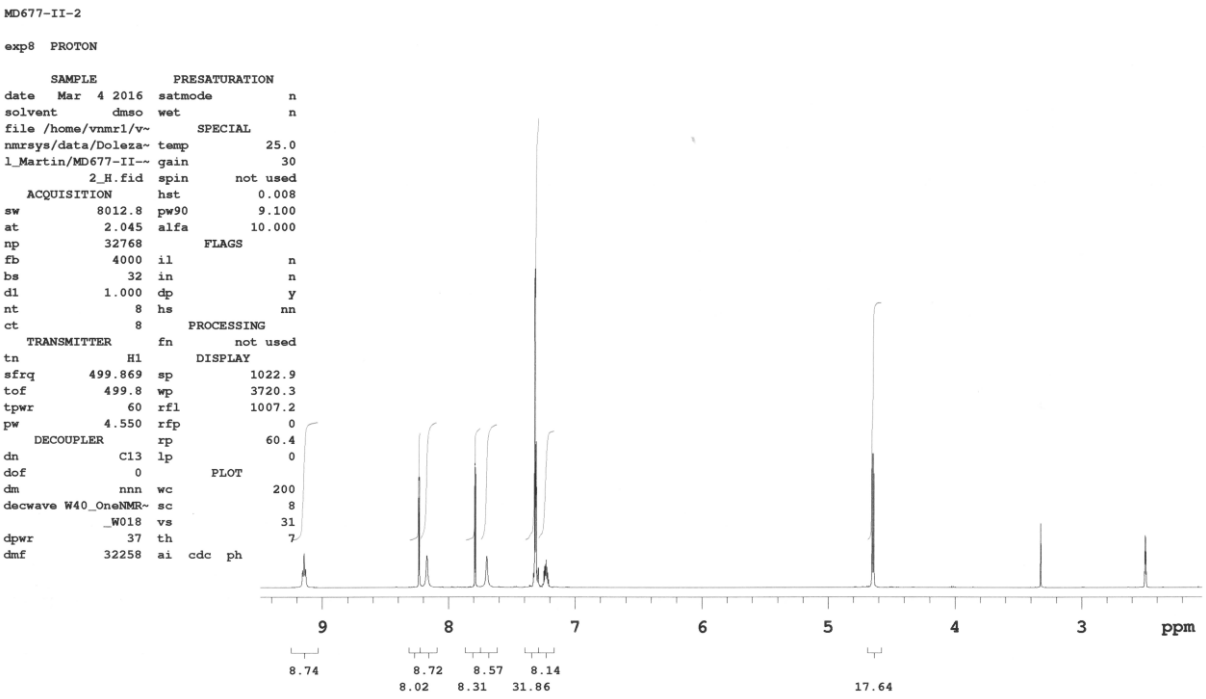
Docking studies

All molecular modelling was done using Schrödinger Suite (Release 2014-2) and visualizations were prepared in Maestro 9.8 (Schrödinger, LLC, New York, NY, USA.). Docking was carried out using software Glide (Schrödinger). The crystal structure of enzyme enoyl-ACP-reductase was prepared using PDB structure 4TZK as starting geometry, using Maestro Protein Preparation Wizard with default settings. The ligand and the non-bonding water molecules were removed. Restrain energy minimization was performed using OPLS-2005 force field (gradient - 0.001 RMS kcal/mol/A^2). The original ligand was used to determine the binding site using the grid generation tool. The ligand structures were prepared in Maestro 2D sketcher. The compounds were subsequently docked using the standard precision (SP) protocol with flexible sampling of ligands and without any constraints. Top-scored compounds were subsequently docked using the extra precision (XP) protocol. The docking protocol was validated by re-docking the original co-crystalized ligand. Average RMSD for five best-scored poses after redocking of the original ligand was 0.85 Å.

Analytical data of prepared compounds

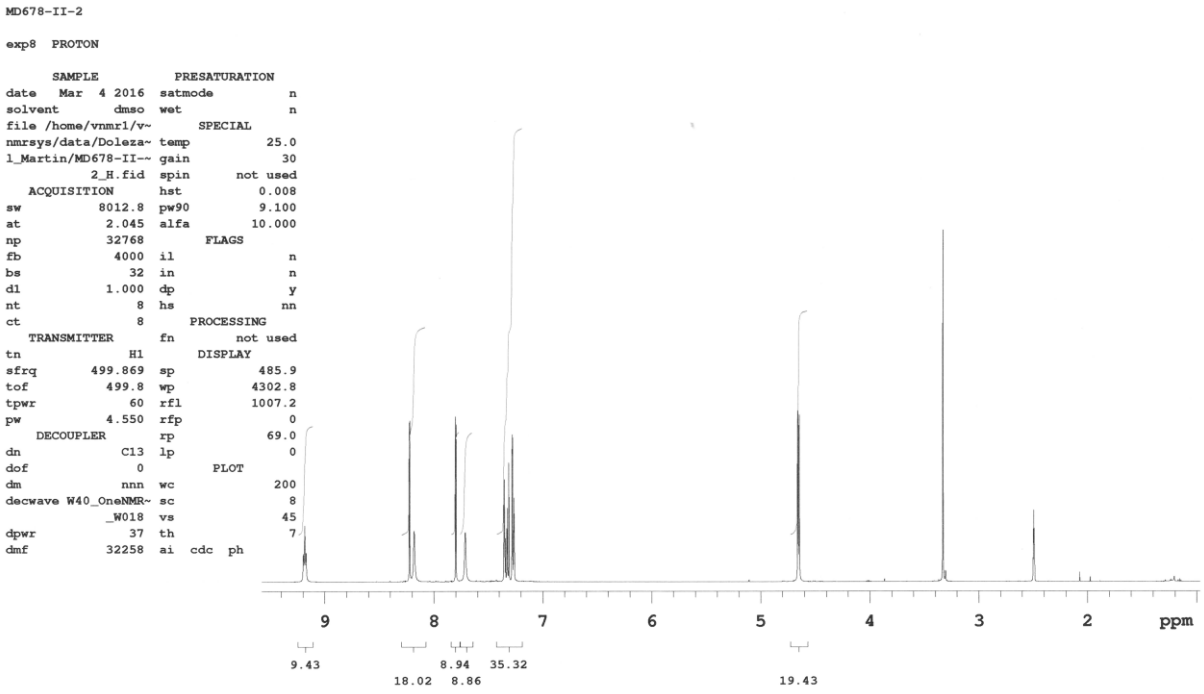
*3-(Benzylamino)pyrazine-2-carboxamide (**1**)*

Yellow solid. Yield 35%; M.p. 121.3-123.5 °C; IR (ATR-Ge, cm^{-1}): 3452 (-NH-), 3126 (-$CONH_2$), 2869 (-CH_2-), 1689 (-C=O), 1584, 1529, 1508, 1473, 1369, 1236, 1180 (arom.); ^{1}H NMR (500 MHz, DMSO) δ 9.14 (1H, t, J = 5.7 Hz, NH), 8.23 (1H, d, J = 2.4 Hz, H6), 8.18 (1H, bs, NH_2), 7.79 (1H, d, J = 2,4 Hz, H5), 7.70 (1H, bs, NH_2), 7.33-7.28 (4H, m, H2´, H3´, H5´, H6´), 7.25-7.20 (1H, m, H4´), 4.63 (2H, d, J = 5.7 Hz, CH_2). ^{13}C NMR (125 MHz, DMSO) δ 169.0, 154.3, 146.5, 139.7, 130.2, 128.6, 127.4, 127.0, 126.7, 43.5. Elemental analysis found: C, 62.88%; H, 5.50%; N, 24.16%. Calculated for $C_{12}H_{12}N_4O$ (MW 228.25): C, 63.15%; H, 5.30%; N, 24.55%.



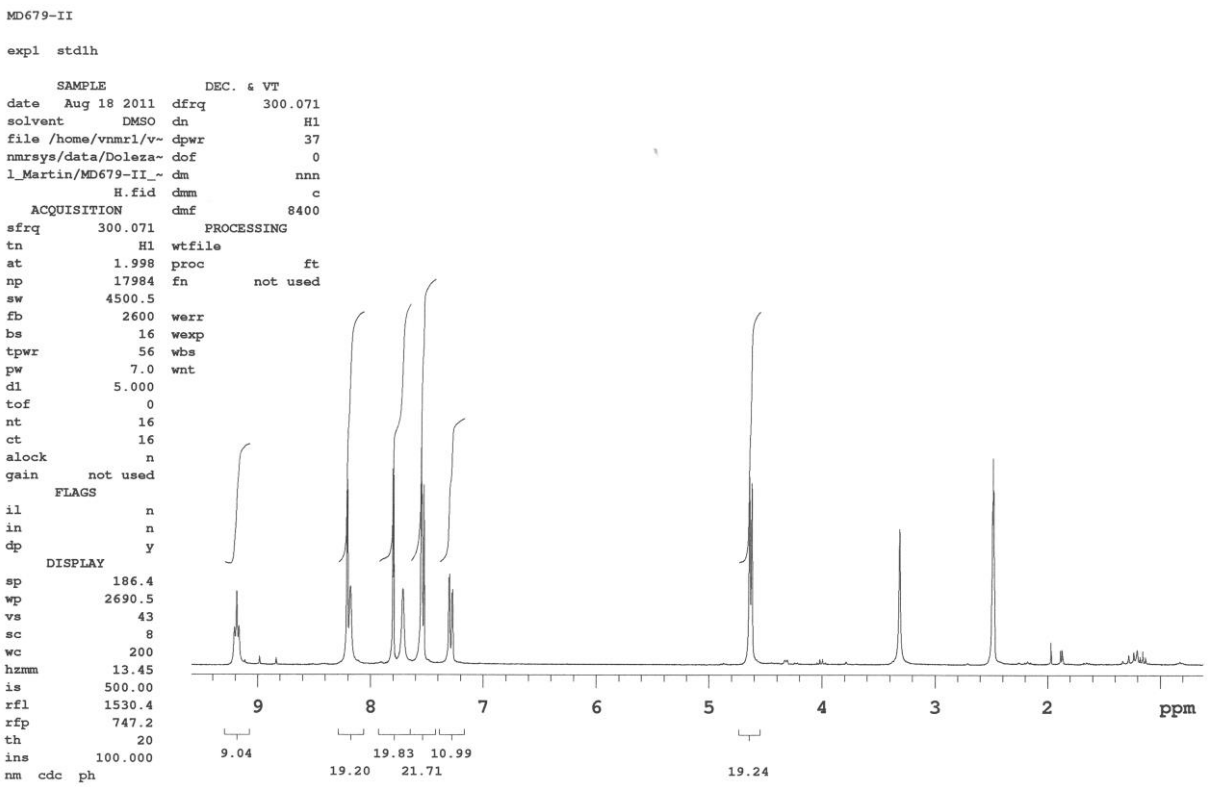
*3-((3-Chlorobenzyl)amino)pyrazine-2-carboxamide (**2**)*

Dark yellow solid. Yield 32%; M.p. 146.1-148.9 °C; IR (ATR-Ge, cm^{-1}): 3435 (-NH-), 3196 (-$CONH_2$), 2926 (-CH_2-), 1666 (-C=O), 1578, 1534, 1511, 1408, 1338, 1262, 1231, 1189 (arom.); ^{1}H NMR (500 MHz, DMSO) δ 9.17 (1H, t, J = 6.0 Hz, NH), 8.22 (1H, d, J = 2.4 Hz, H6), 8.18 (1H, bs, NH_2), 7.79 (1H, d, J = 2.4 Hz, H5), 7.71 (1H, bs, NH_2), 7.39-7.23 (4H, m, H2´, H4´, H5´, H6´), 4.65 (2H, d, J = 6.0 Hz, CH_2). ^{13}C NMR (125 MHz, DMSO) δ 168.9, 154.2, 146.5, 142.6, 133.2, 130.5, 130.4, 127.1, 126.9, 126.9, 126.0, 43.0. Elemental analysis found: C, 55.18%; H, 4.51%; N, 20.94%. Calculated for $C_{12}H_{11}ClN_4O$ (MW 262.69): C, 54.87%; H, 4.22%; N, 21.33%.



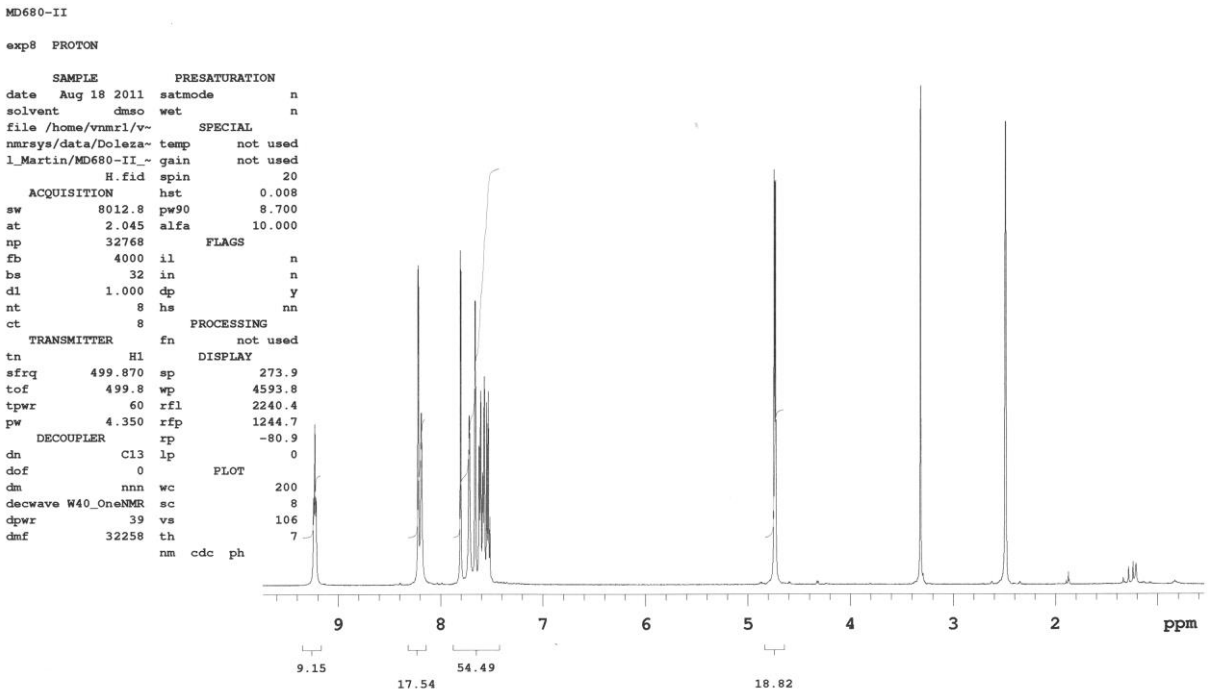
*3-((3,4-Dichlorobenzyl)amino)pyrazine-2-carboxamide (**3**)*

Yellow solid. Yield 24%; M.p. 169.5-172.1 °C; IR (ATR-Ge, cm^{-1}): 3472 (-NH-), 3174 (-$CONH_2$), 2924 (-CH_2-), 1677 (-C=O), 1576, 1529, 1500, 1465, 1416, 1338, 1233, 1187 (arom.); ^{1}H NMR (300 MHz, DMSO) δ 9.19 (1H, t, J = 6.2 Hz, NH), 8.24-8.20 (1H, m, H6), 8.18 (1H, bs, NH_2), 7.82-7.79 (1H, m, H5), 7.72 (1H, bs, NH_2), 7.58-7.53 (1H, m, H5´), 7.53 (1H, d, J = 1.7 Hz, H6´), 4.64 (2H, d, J = 6.2 Hz, CH_2). ^{13}C NMR (75 MHz, DMSO) δ 168.8, 154.1, 146.4, 141.4, 131.0, 130.7, 130.6, 129.3, 129.3, 127.7, 127.0, 42.5. Elemental analysis found: C, 49.09%; H, 3.76%; N, 18.69%. Calculated for $C_{12}H_{10}Cl_2N_4O$ (MW 298.14): C, 48.86%; H, 3.39%; N, 18.86%.



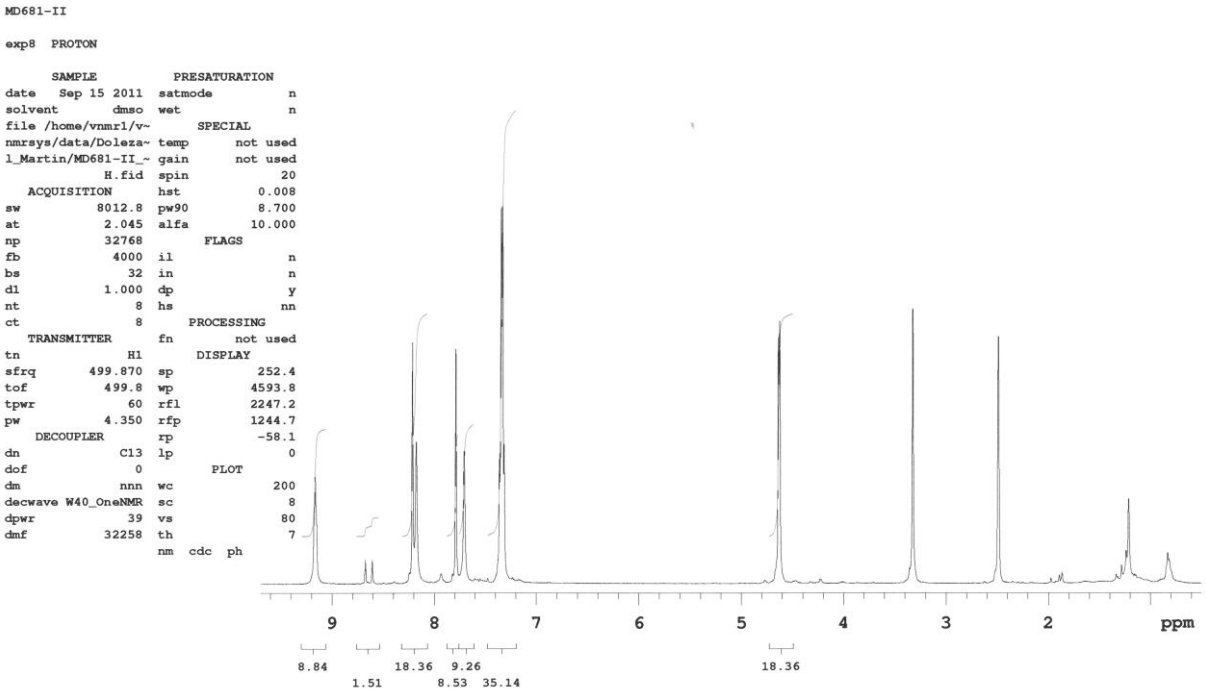
*3-((3-Trifluoromethylbenzyl)amino)pyrazine-2-carboxamide (**4**)*

Dark yellow solid. Yield 31%; M.p. 98.1-100.3 °C; IR (ATR-Ge, cm^{-1}): 3468 (-NH-), 3201 (-$CONH_2$), 2937 (-CH_2-), 1674 (-C=O), 1581, 1530, 1507, 1453, 1416, 1236, 1185, 1164 (arom.), 1331 (-CF_3); ^1H NMR (500 MHz, DMSO) δ 9.23 (1H, t, J = 6.1 Hz, NH), 8.22 (1H, d, J = 2.2 Hz, H6), 8.19 (1H, bs, NH_2), 7.80 (1H, d, J = 2.2 Hz, H5), 7.72 (1H, bs, NH_2), 7.66 (1H, s, H2´), 7.64-7.50 (3H, m, H4´, H5´, H6´), 4.74 (2H, d, J = 6.1 Hz, CH_2). ^{13}C NMR (125 MHz, DMSO) δ 168.9, 154.2, 146.4, 141.6, 131.4, 130.5, 129.6, 129.2 (q, J = 31.3 Hz), 127.0, 124.4 (q, J = 272.0 Hz), 123.8 (q, J = 3.9 Hz), 123.7 (q, J = 3.9 Hz), 43.1. Elemental analysis found: C, 52.48%; H, 3.40%; N, 18.76%. Calculated for $C_{13}H_{11}F_3N_4O$ (MW 297.14): C, 52.71%; H, 3.74%; N, 18.91%.



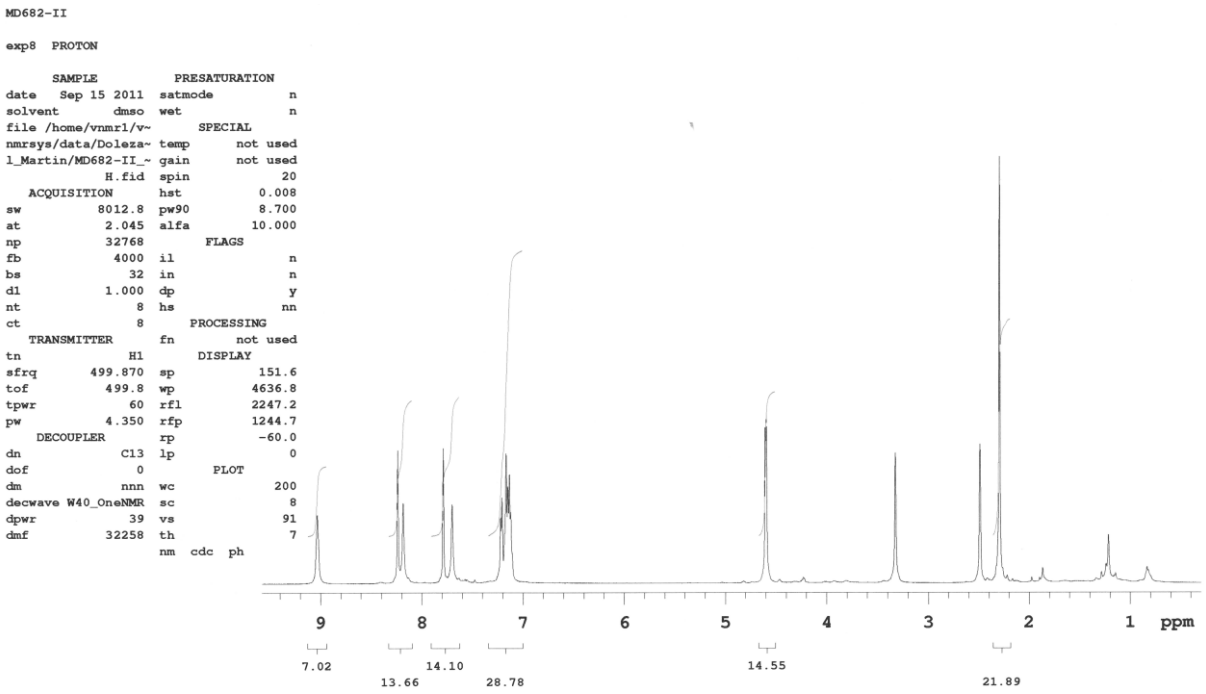
*3-((4-Chlorobenzyl)amino)pyrazine-2-carboxamide (**5**)*

Yellow solid. Yield 59 %; M.p. 141.0-142.0 °C; IR (ATR-Ge, cm^{-1}): 3408 (-NH-), 3290 (-$CONH_2$), 2930 (-CH_2-), 1682 (-C=O), 1589, 1530, 1510, 1468, 1413, 1339, 1232, 1188 (arom.); ^{1}H NMR (500 MHz, DMSO) δ 9.17 (1H, bs, NH), 8.23-8.20 (1H, m, H6), 8.18 (1H, bs, NH_2), 7.81-7.77 (1H, m, H5), 7.71 (1H, bs, NH_2), 7.38-7.30 (4H, m, H2´, H3´, H5´, H6´), 4.63 (2H, d, J = 5.9 Hz, CH_2). ^{13}C NMR (125 MHz, DMSO) δ 168.9, 154.2, 146.5, 139.0, 131.4, 130.4, 129.2, 128.5, 126.9, 42.8. Elemental analysis found: C, 55.08%; H, 4.64%; N, 21.58%. Calculated for $C_{12}H_{11}ClN_4O$ (MW 262.69): C, 54.87%; H, 4.22%; N, 21.33%.



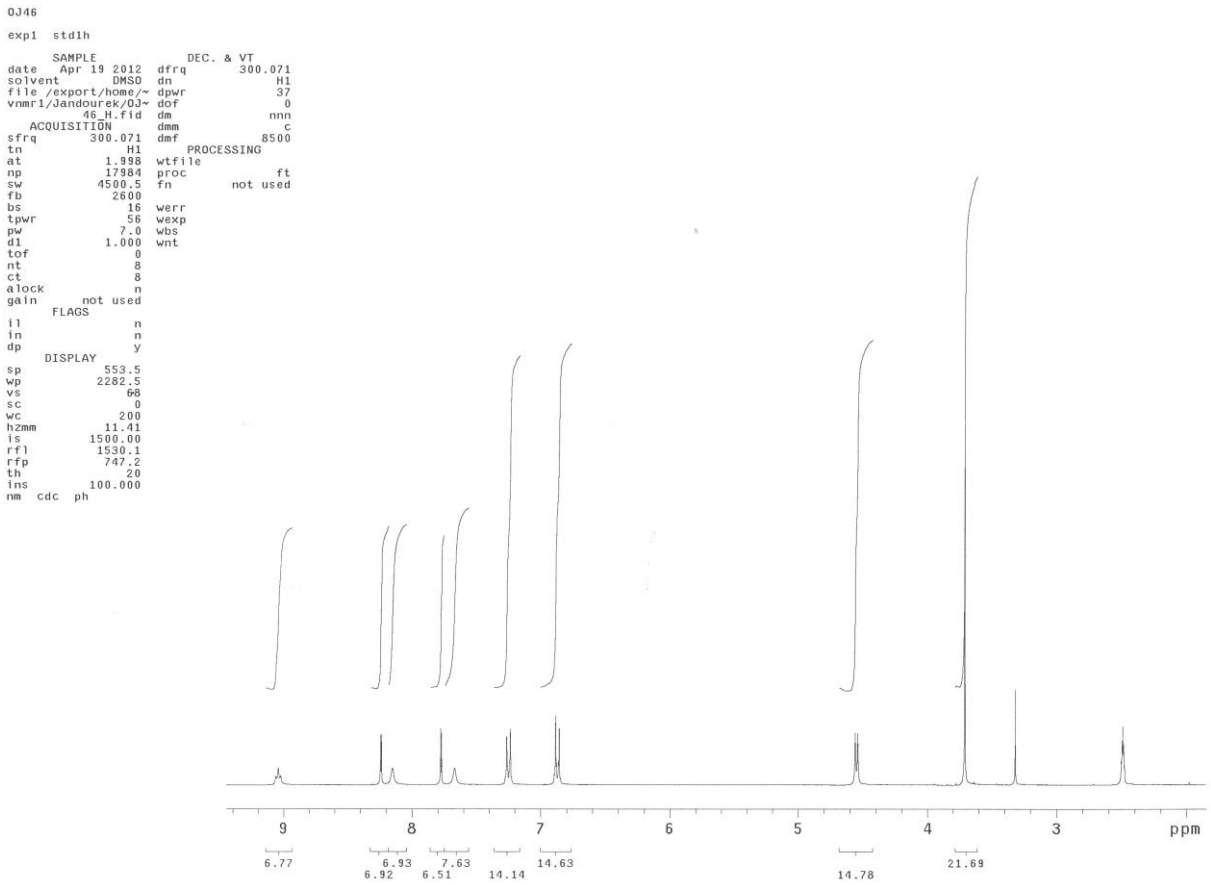
*3-((2-Methylbenzyl)amino)pyrazine-2-carboxamide (**6**)*

Yellow solid. Yield 38%; M.p. 148.3-150.5 °C; IR (ATR-Ge, cm^{-1}): 3435 (-NH-), 3201 (-$CONH_2$), 2925 (-CH_2-), 1665 (-C=O), 1575, 1536, 1512, 1465, 1411, 1339, 1229, 1192 (arom.); ^{1}H NMR (500 MHz, DMSO) δ 9.03 (1H, bs, NH), 8.24 (1H, s, H6), 8.19 (1H, bs, NH_2), 7.79 (1H, s, H5), 7.70 (1H, bs, NH_2), 7.27-7.06 (4H, m, H3´, H4´, H5´, H6´), 4.60 (2H, d, J = 4.9 Hz, CH_2), 2.30 (3H, s, CH_3). ^{13}C NMR (125 MHz, DMSO) δ 169.0, 154.4, 146.4, 137.2, 135.9, 130.3, 130.2, 127.5, 127.2, 126.7, 126.1, 41.8, 18.8. Elemental analysis found: C, 64.28%; H, 6.07%; N, 22.90%. Calculated for $C_{13}H_{14}N_4O$ (MW 242.28): C, 64.45%; H, 5.82%; N, 23.13%.



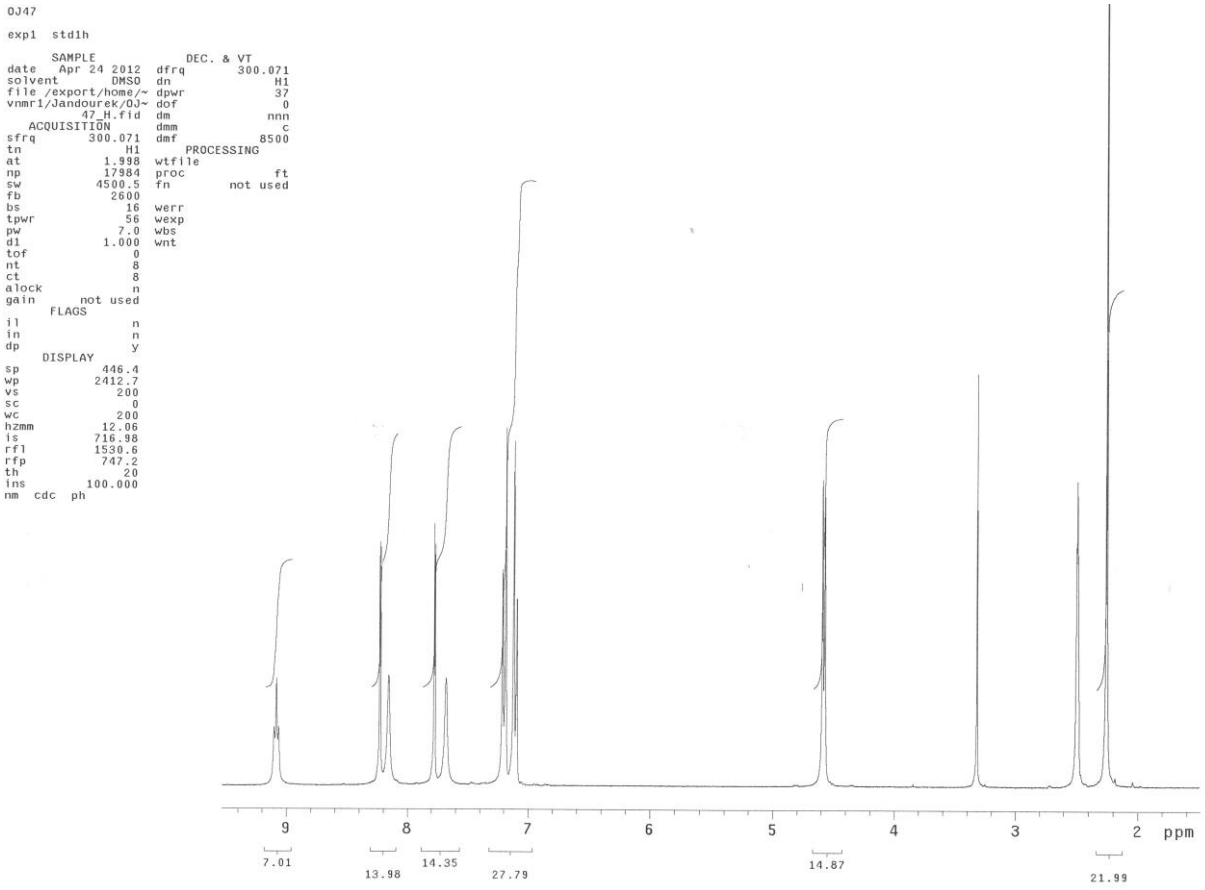
*3-((4-Methoxybenzyl)amino)pyrazine-2-carboxamide (**7**)*

Yellow solid. Yield 73%; M.p. 148.5-151.3 °C; IR (ATR-Ge, cm^{-1}): 3464 (-NH-), 3126 (-$CONH_2$), 2952 (-OCH_3), 2910 (-CH_2-), 1690 (-C=O), 1612, 1582, 1530, 1509, 1469, 1413, 1245, 1173 (arom.); ^{1}H NMR (300 MHz, DMSO) δ 9.04 (1H, t, J = 5.7 Hz, NH), 8.24 (1H, d, J = 2,4 Hz, H6), 8.15 (1H, bs, NH_2), 7.78 (1H, d, J = 2,5 Hz, H5), 7.67 (1H, bs, NH_2), 7.28-7.23 (2H, m, H2´,H6´), 6.90-6.85 (2H, m, H3´, H5´), 4.55 (2H, d, J = 5.7 Hz, CH_2), 3.71 (3H, s, OCH_3). ^{13}C NMR (75 MHz, DMSO) δ 168.88, 158.44, 154.25, 146.52, 131.45, 130.10, 128.82, 126.65, 114.00, 55.20, 43.05. Elemental analysis found: C, 60.74%; H, 5.93%; N, 21.71%. Calculated for $C_{13}H_{14}N_4O_2$ (MW 258.25): C, 60.45%; H, 5.46%; N, 21.69%.



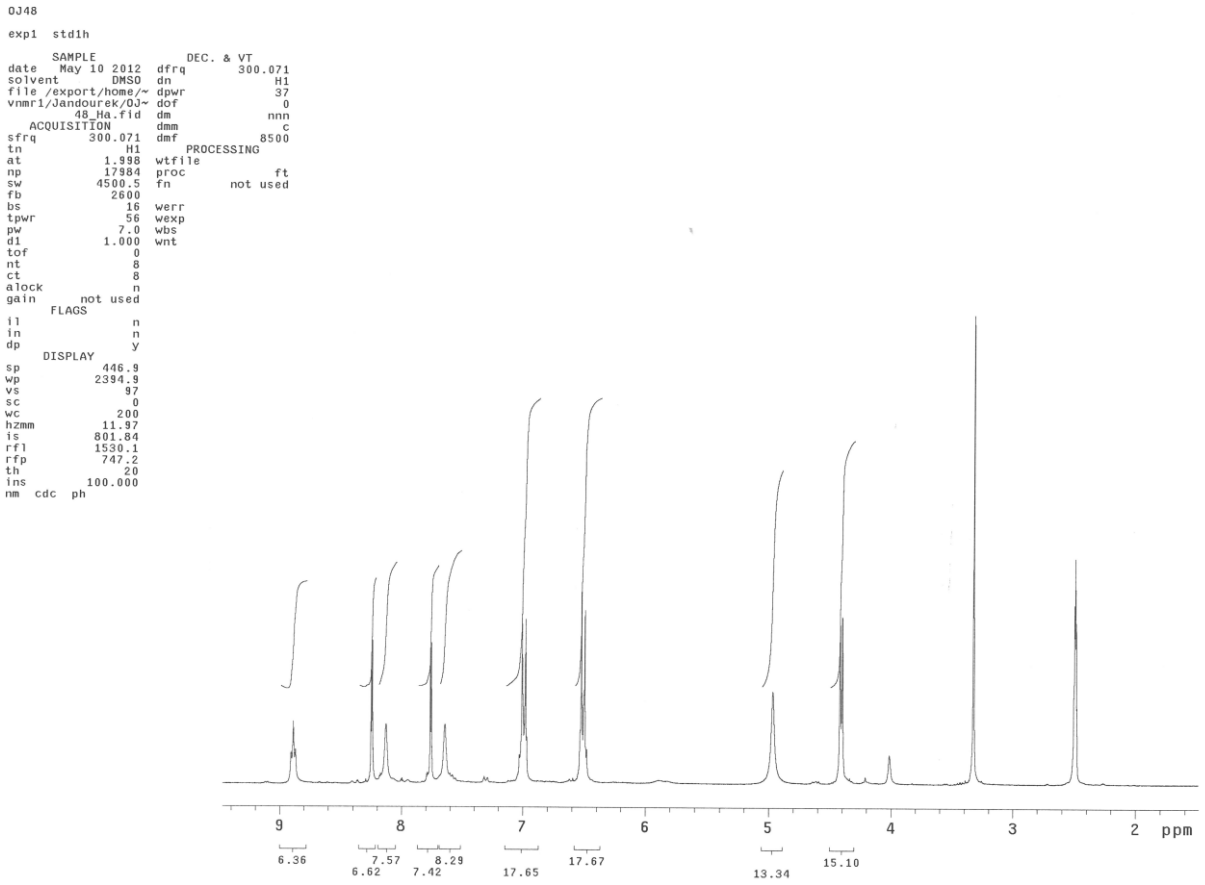
*3-((4-Methylbenzyl)amino)pyrazine-2-carboxamide (**8**)*

Yellow solid. Yield 72 %; M.p. 145.0-146.9 °C; IR (ATR-Ge, cm^{-1}): 3427 (-NH-), 3195 (-$CONH_2$), 2926 (-CH_2-), 1669 (-C=O), 1579, 1531, 1503, 1419, 1333, 1232, 1188 (arom.); ^{1}H NMR (300 MHz, DMSO) δ 9.08 (1H, t, J = 6.0 Hz, NH), 8.23 (1H, d, J = 2.4 Hz, H6), 8.16 (1H, s, NH_2), 7.78 (1H, d, J = 2.5 Hz, H5), 7.68 (1H, s, NH_2), 7.21-7.10 (4H, dd, H2´, H3´, H5´, H6´), 4.58 (2H, d, J = 5.8 Hz, CH_2), 2.25 (3H, s, CH_3). ^{13}C NMR (75 MHz, DMSO) δ 168.95, 154.30, 146.52, 136.54, 136.08, 130.15, 129.13, 127.40, 126.68, 43.32, 20.84. Elemental analysis found: C, 64.98%; H, 6.32%; N, 22.84%. Calculated for $C_{13}H_{14}N_4O$ (MW 242.28): C, 64.45%; H, 5.82%; N, 23.13%.



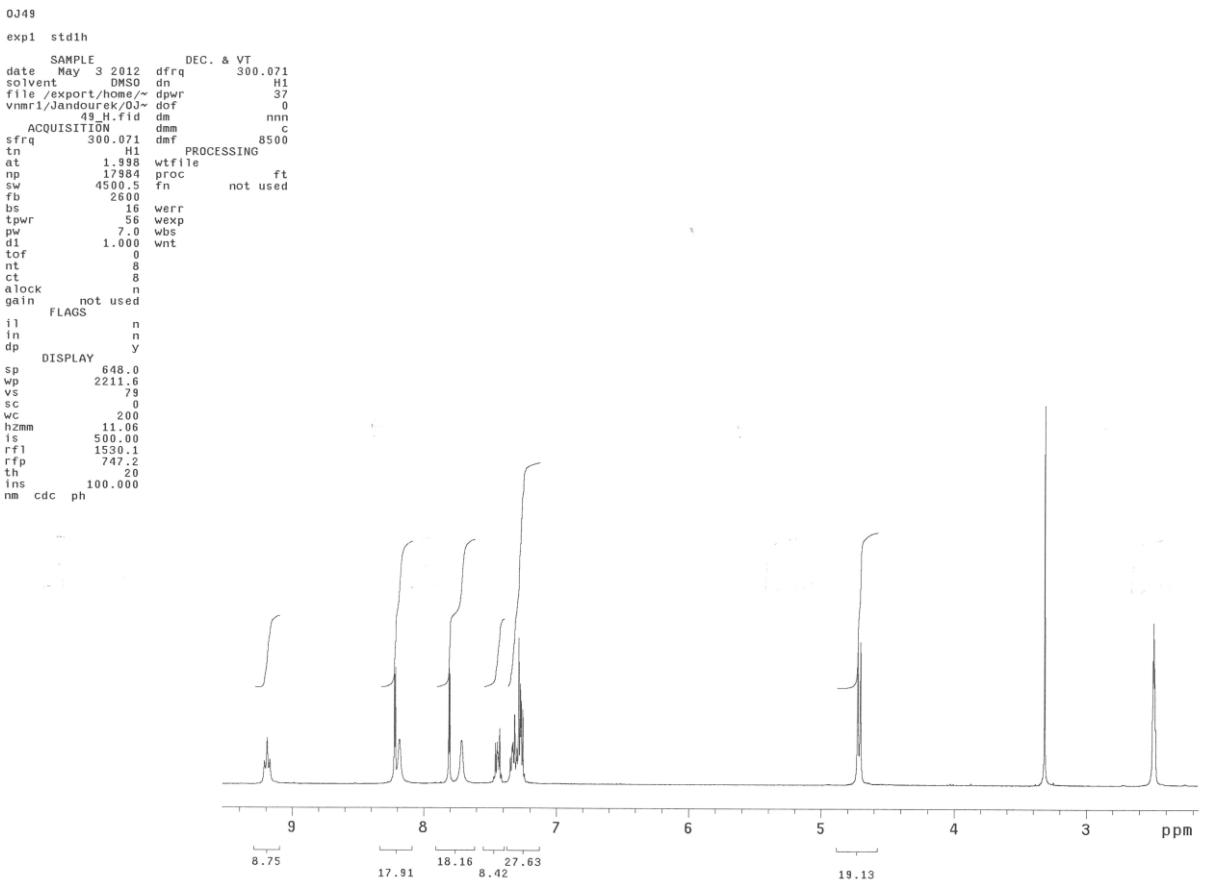
*3-((4-Aminobenzyl)amino)pyrazine-2-carboxamide (**9**)*

Ochre solid. Yield 46%; M.p. 140.8-142.3 °C; IR (ATR-Ge, cm^{-1}): 3426 (-NH-), 3324 ($-NH_2$), 3194 ($-CONH_2$), 1670 (-C=O), 1614, 1578, 1514, 1464, 1413, 1231, 1191 (arom.); ^1H NMR (300 MHz, DMSO) δ 8.89 (1H, t, J = 5.5 Hz, NH), 8.24 (1H, d, J = 2.3 Hz, H6), 8.13 (1H, s, NH_2), 7.76 (1H, d, J = 2.3 Hz, H5), 7.65 (1H, s, NH_2), 7.03-6.97 (2H, m, H2´, H6´), 6.54 – 6.48 (2H, m, H3´, H5´), 4.97 (2H, s, NH_2´), 4.41 (2H, d, J = 5.5 Hz, CH_2). ^{13}C NMR (75 MHz, DMSO) δ 168.97, 154.24, 147.91, 146.58, 129.87, 128.63, 126.49, 126.04, 114.03, 43.56. Elemental analysis found: C, 58.78%; H, 5.79%; N, 29.04%. Calculated for $C_{12}H_{13}N_5O$ (MW 243.26): C, 59.25%; H, 5.39%; N, 28.79%.



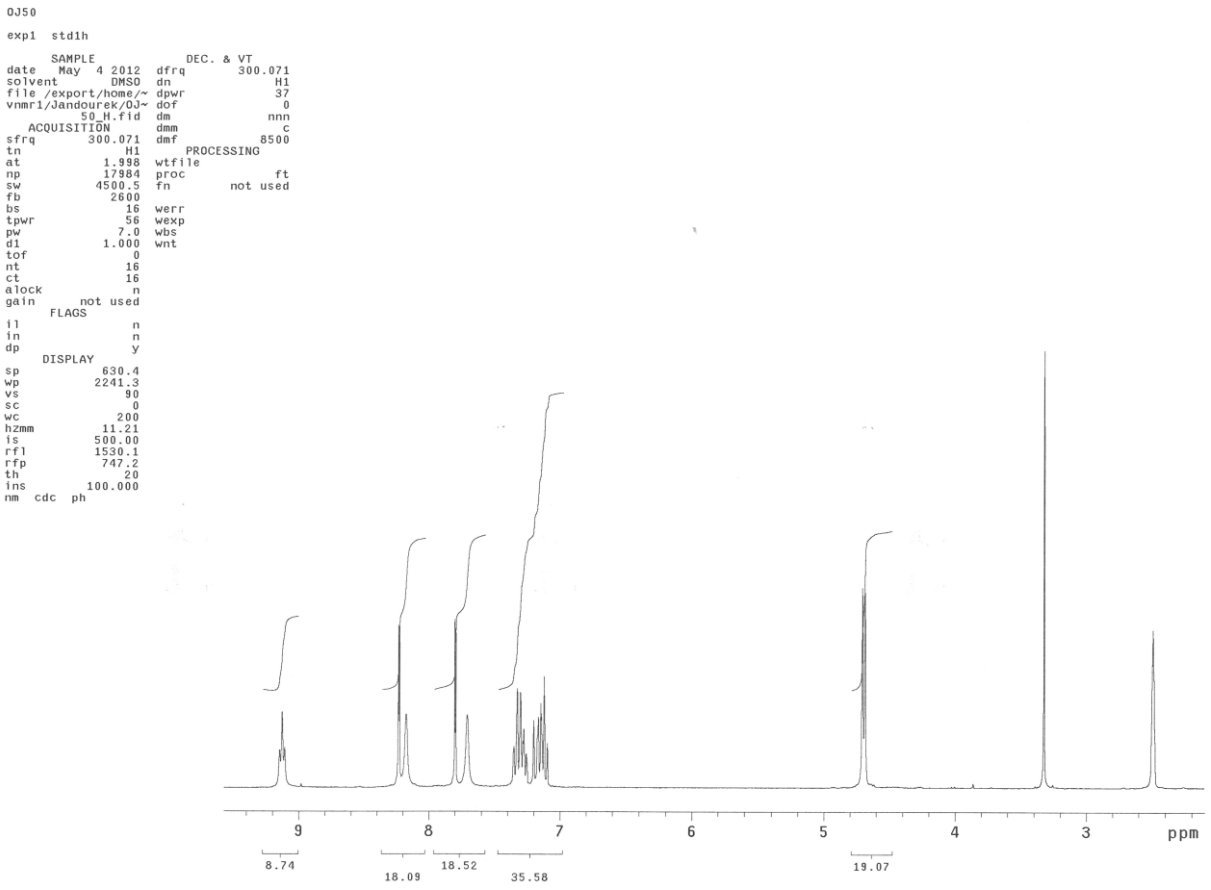
*3-((2-Chlorobenzyl)amino)pyrazine-2-carboxamide (**10**)*

Ochre solid. Yield 80%; M.p. 182.4-184.3 °C; IR (ATR-Ge, cm^{-1}): 3456 (-NH-), 3291 (-$CONH_2$), 2921 (-CH_2-), 1661 (-C=O), 1583, 1569, 1509, 1471, 1409, 1231, 1187 (arom.); ^{1}H NMR (300 MHz, DMSO) δ 9.19 (1H, t, J = 6.1 Hz, NH), 8.22 (1H, d, J = 2.4 Hz, H6), 8.19 (1H, s, NH_2), 7.81 (1H, d, J = 2.3 Hz, H5), 7.71 (1H, s, NH_2), 7.47-7.41 (1H, m, H5´), 7.36 – 7.22 (3H, m, H2´, H3´, H4´), 4.71 (2H, d, J = 6.1 Hz, CH_2). ^{13}C NMR (75 MHz, DMSO) δ 168.86, 154.17, 146.44, 136.69, 132.43, 130.51, 129.40, 128.90, 128.77, 127.37, 126.96, 41.64. Elemental analysis found: C, 55.11%; H, 4.65%; N, 21.02%. Calculated for $C_{12}H_{11}ClN_4O$ (MW 262.69): C, 54.87%; H, 4.22%; N, 21.33%.



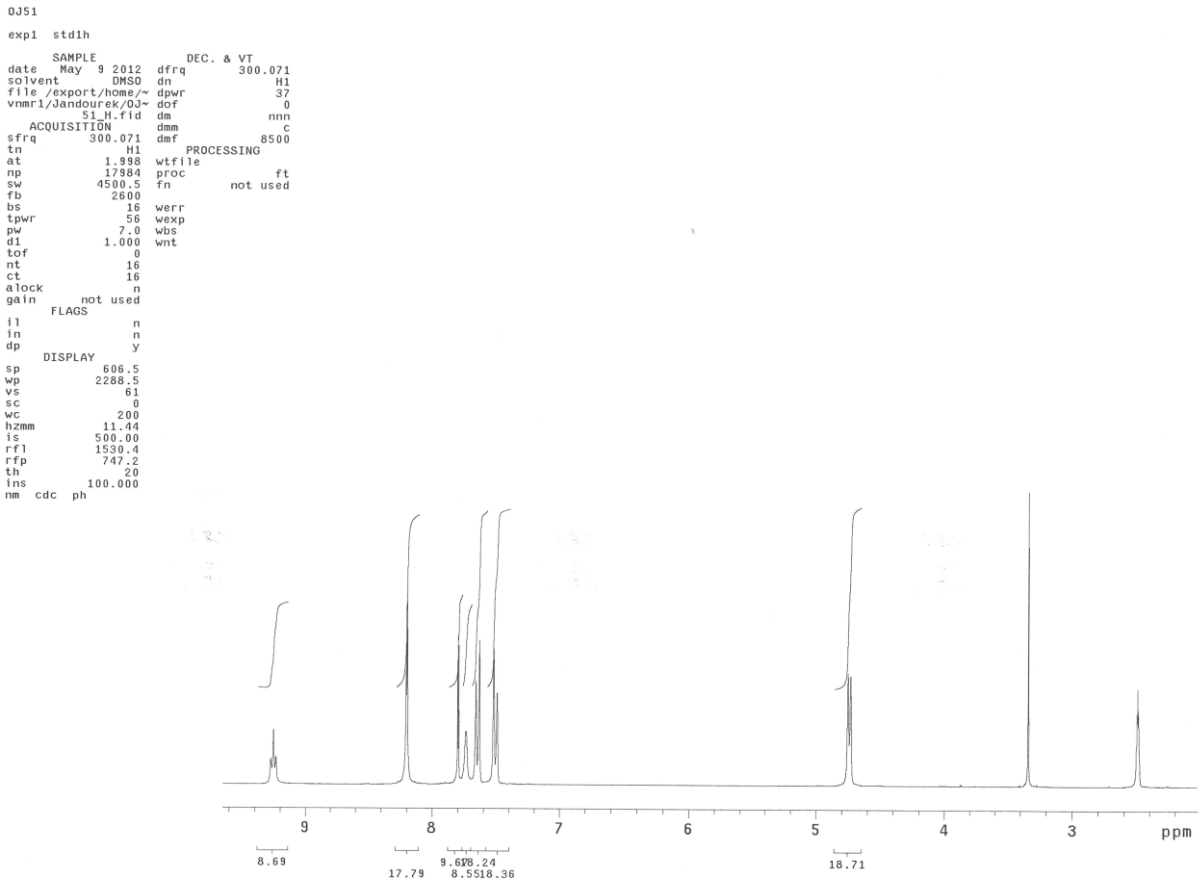
*3-((2-Fluorobenzyl)amino)pyrazine-2-carboxamide (**11**)*

Dark yellow solid. Yield 66%; M.p. 171.3-174.3 °C; IR (ATR-Ge, cm^{-1}): 3446 (-NH-), 3277 (-$CONH_2$), 2921 (-CH_2-), 1666 (-C=O), 1572, 1534, 1514, 1486, 1420, 1225, 1190 (arom.); ^{1}H NMR (300 MHz, DMSO) δ 9.13 (1H, t, J = 5.9 Hz, NH), 8.23 (1H, d, J = 1.8 Hz, H6), 8.18 (1H, s, NH_2), 7.80 (1H, d, J = 1.7 Hz, H5), 7.71 (1H, s, NH_2), 7.40 – 7.08 (4H, m, H2´, H3´, H4´, H5´), 4.70 (2H, d, J = 6.0 Hz, CH_2). ^{13}C NMR (75 MHz, DMSO) δ 168.88, 162.12, 158.88, 154.21, 146.45, 130.44, 129.57-128.54 (m), 126.91, 126.36 (d, J = 14.7 Hz), 124.53 (d, J = 3.4 Hz), 115.33 (d, J = 21.0 Hz), 37.62 (d, J = 4.4 Hz). Elemental analysis found: C, 58.73%; H, 4.19%; N, 22.32%. Calculated for $C_{12}H_{11}FN_4O$ (MW 246.24): C, 58.53%; H, 4.50%; N, 22.75%.



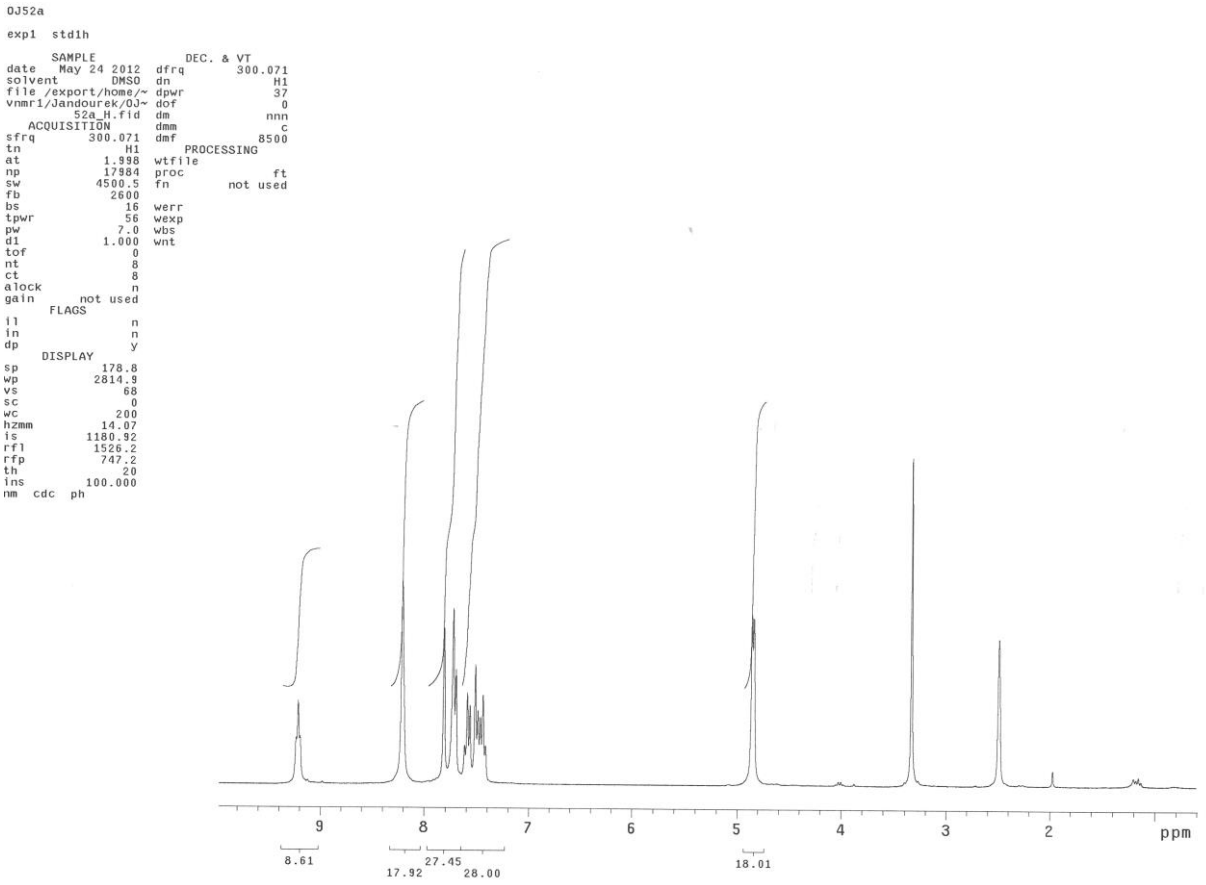
*3-((4-Trifluoromethylbenzyl)amino)pyrazine-2-carboxamide (**12**)*

Yellow solid. Yield 46%; M.p. 136.8-138.3 °C; IR (ATR-Ge, cm^{-1}): 3423 (-NH-), 3257 ($-CONH_2$), 1678 (-C=O), 1584, 1532, 1510, 1414, 1231, 1189 (arom.), 1322 ($-CF_3$); ^1H NMR (300 MHz, DMSO) δ 9.25 (1H, t, J = 6.1 Hz, NH), 8.20 (1H, d, J = 2.4 Hz, H6), 8.18 (1H, s, NH_2), 7.80 (1H, d, J = 2.4 Hz, H5), 7.74 (1H, s, NH_2), 7.65 (2H, d, J = 8.0 Hz, H2´, H6´), 7.51 (2H, d, J = 7.9 Hz, H3´. H5´), 4.74 (2H, d, J = 6.1 Hz, CH_2). ^{13}C NMR (75 MHz, DMSO) δ 168.91, 154.23, 146.44, 145.00 (d, J = 1.7 Hz), 130.52, 127.86, 127.34, 126.97, 126.35, 125.38 (q, J = 3.8 Hz), 122.74, 43.14, 40.53. Elemental analysis found: C, 52.83%; H, 3.87%; N, 18.73%. Calculated for $C_{13}H_{11}F_3N_4O$ (MW 296.25): C, 52.71%; H, 3.74%; N, 18.91%.



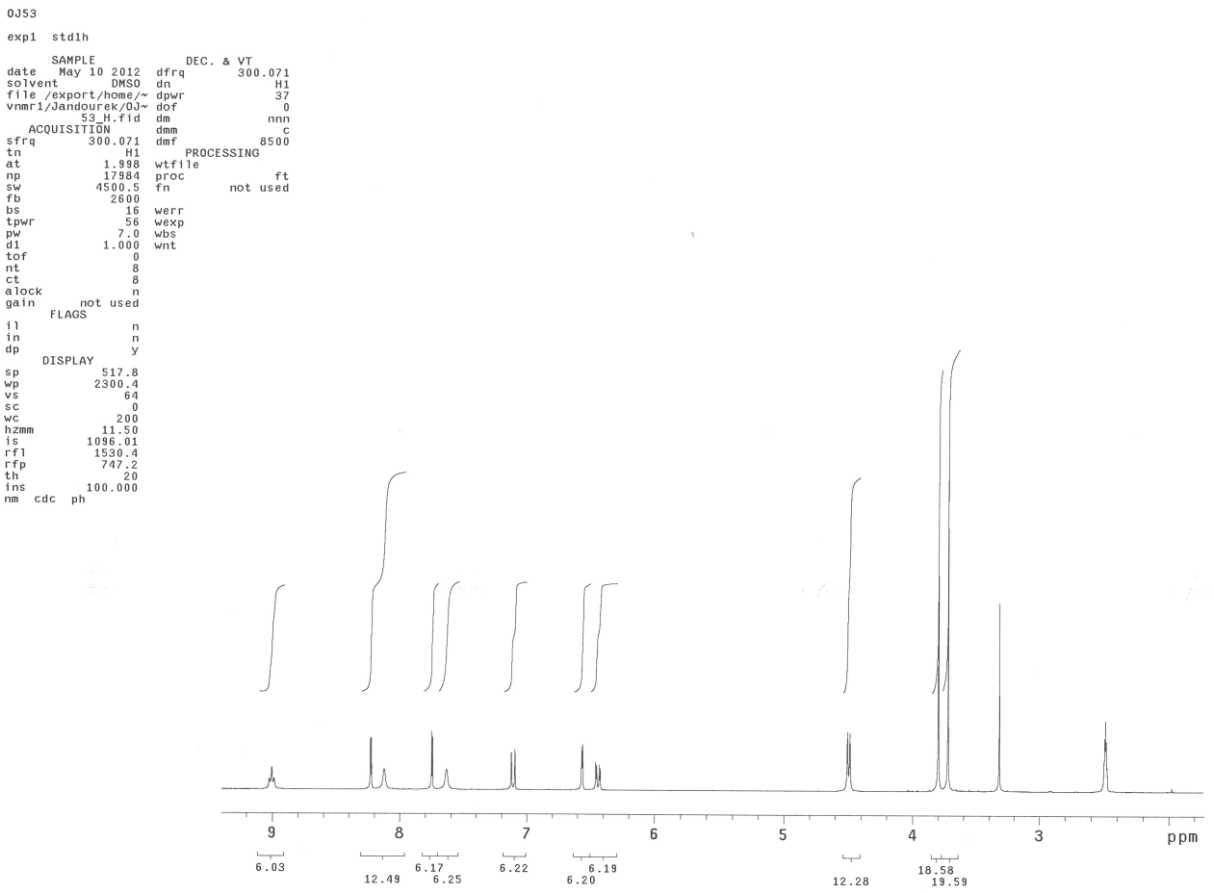
*3-((2-Trifluoromethylbenzyl)amino)pyrazine-2-carboxamide (**13**)*

Light yellow solid. Yield 55%; M.p. 173.7-175.3 °C; IR (ATR-Ge, cm^{-1}): 3456 (-NH-), 3254 ($-CONH_2$), 1672 (-C=O), 1586, 1578, 1516, 1414, 1232, 1189 (arom.), 1339 ($-CF_3$); ^1H NMR (300 MHz, DMSO) δ 9.22 (1H, t, J = 5.9 Hz, NH), 8.21 (1H, d, J = 1.8 Hz, H6), 8.20 (1H, s, NH_2), 7.82 (1H, d, J = 2.1 Hz, H5), 7.81 (1H, s, NH_2), 7.72 (1H, J = 8.5 Hz, H5´), 7.64 – 7.39 (3H, m, H2´, H3´, H4´), 4.85 (2H, d, J = 5.9 Hz, CH_2). ^{13}C NMR (75 MHz, DMSO) δ 168.88, 154.13, 146.46, 138.07 (d, J = 1.9 Hz), 132.90, 130.64, 128.78, 127.53, 127.04, 126.88 – 125.82 (m), 122.94, 40.36, 40.31. Elemental analysis found: C, 53.05%; H, 3.86%; N, 18.74%. Calculated for $C_{13}H_{11}F_3N_4O$ (MW 296.25): C, 52.71%; H, 3.74%; N, 18.91%.



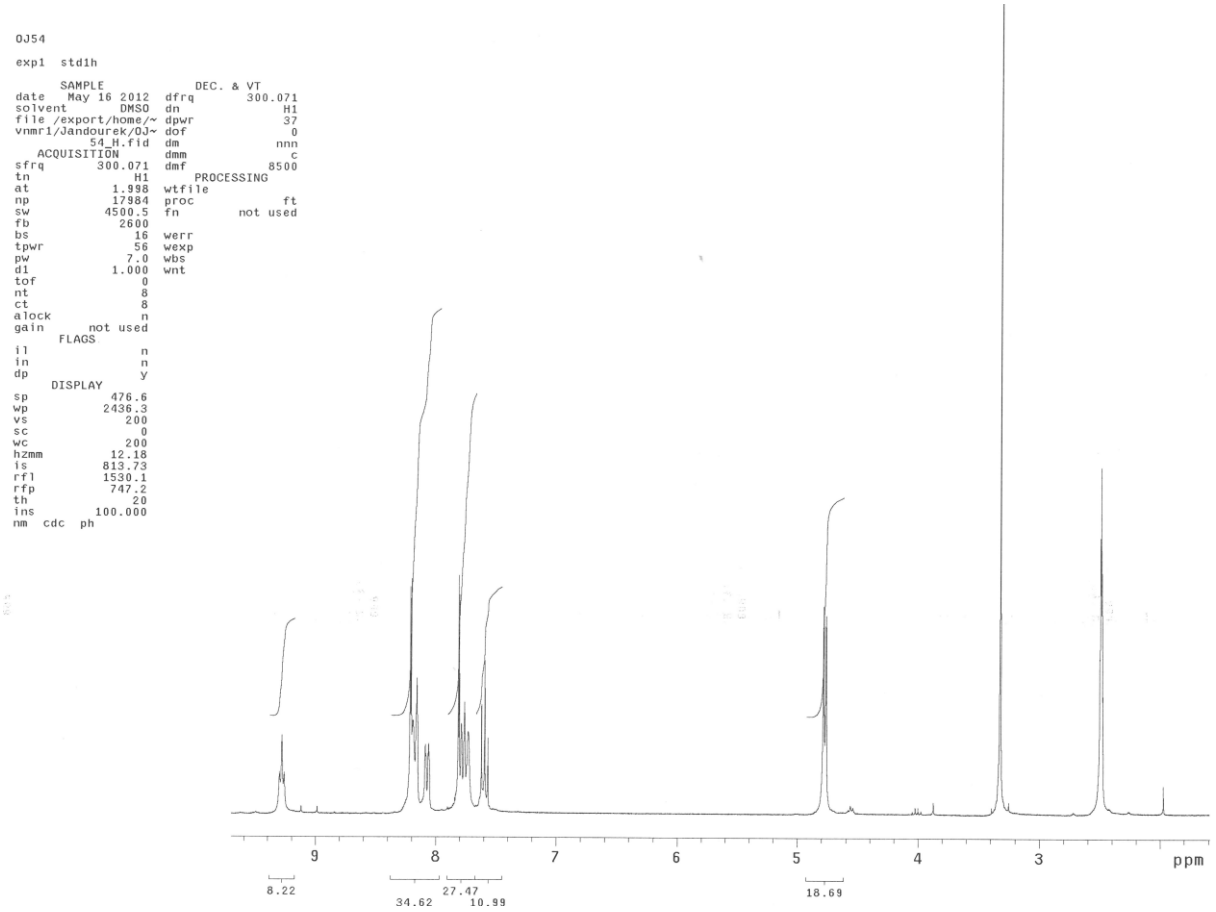
*3-((2,4-Dimethoxybenzyl)amino)pyrazine-2-carboxamide (**14**)*

Yellow solid. Yield 66%; M.p. 147.9-149.6 °C; IR (ATR-Ge, cm^{-1}): 3403 (-NH-), 3215 (-$CONH_2$), 2951 (-OCH_3), 2832 (-CH_2-), 1677 (-C=O), 1650, 1617, 1578, 1535, 1506, 1468, 1427, 1250, 1190, 1158 (arom.); ^{1}H NMR (300 MHz, DMSO) δ 9.00 (1H, t, J = 5.7 Hz, NH), 8.23 (1H, d, J = 2.4 Hz, H6), 8.12 (1H, s, NH_2), 7.75 (1H, d, J = 2.4 Hz, H5), 7.64 (1H, s, NH_2), 7.11 (1H, d, J = 8.3 Hz, H2´), 6.57 (1H, d, J = 2.4 Hz, H3´), 6.44 (1H, dd, J = 8.3, 2.4 Hz, H5´), 4.49 (2H, d, J = 5.7 Hz, CH_2), 3.80 (3H, s, OCH_3), 3.72 (3H, s, OCH_3). ^{13}C NMR (75 MHz, DMSO) δ 189.90, 168.94, 160.05, 158.31, 154.34, 146.53, 129.85, 129.28, 126.64, 119.01, 104.51, 98.66, 55.61, 55.35. Elemental analysis found: C, 58.52%; H, 6.08%; N, 19.38%. Calculated for $C_{14}H_{16}N_4O_3$ (MW 288.30): C, 58.32%; H, 5.59%; N, 19.43%.



*3-((3-Nitrobenzyl)amino)pyrazine-2-carboxamide (**15**)*

Light brown solid. Yield 26%; M.p. 165.6-168.3 °C; IR (ATR-Ge, cm^{-1}): 3423 (-NH-), 3193 (-$CONH_2$), 2923 (-CH_2-), 1668 (-C=O), 1578, 1511, 1461, 1412, 1232, 1185 (arom.), 1522, 1347 (-NO_2); ^{1}H NMR (300 MHz, DMSO) δ 9.28 (1H, t, J = 6.2 Hz, NH), 8.21 (1H, d, J = 2.5 Hz, H6), 8.19 (1H, s, NH_2), 8.16 (1H, t, J = 2.0 Hz, H6´), 8.07 (1H, m, H4´), 7.81 (1H, d, J = 2.4 Hz, H5), 7.79 (1H, s, NH_2), 7.77 – 7.70 (1H, m, H2´), 7.59 (1H, t, J = 7.9 Hz, H3´), 4.78 (2H, d, J = 6.2 Hz, CH_2). ^{13}C NMR (75 MHz, DMSO) δ 168.82, 154.10, 147.99, 146.39, 142.62, 134.10, 130.64, 130.00, 127.05, 121.88, 121.84, 42.90. Elemental analysis found: C, 53.02%; H, 4.45%; N, 25.64%. Calculated for $C_{12}H_{11}N_5O_3$ (MW 273.25): C, 52.75%; H, 4.06%; N, 25.63%.



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PŘÍLOHA Č. 5

JANDOUREK, O.; DOLEZAL, M.; KUNES, J.; KUBICEK, V.; PATEROVA, P.; PESKO, M.; BUCHTA, V.; KRALOVA, K.; ZITKO, J. New potentially active pyrazinamide derivatives synthesized under microwave conditions. *Molecules*. **2014**, *19*, 9318-9338. doi: 10.3390/molecules19079318. IF_{2014} = 2,416.

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Article

New Potentially Active Pyrazinamide Derivatives Synthesized Under Microwave Conditions

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Abstract: A series of 18 *N*-alkyl substituted 3-aminopyrazine-2-carboxamides was prepared in this work according to previously experimentally set and proven conditions using microwave assisted synthesis methodology. This approach for the aminodehalogenation reaction was chosen due to higher yields and shorter reaction times compared to organic reactions with conventional heating. Antimycobacterial, antibacterial, antifungal and photosynthetic electron transport (PET) inhibiting *in vitro* activities of these compounds were investigated. Experiments for the determination of lipophilicity were also performed. Only a small number of substances with alicyclic side chain showed activity against fungi which was the same or higher than standards and the biological efficacy of the compounds increased with rising lipophilicity. Nine pyrazinamide derivatives also inhibited PET in

spinach chloroplasts and the IC_{50} values of these compounds varied in the range from 14.3 to 1590.0 μmol/L. The inhibitory activity was connected not only with the lipophilicity, but also with the presence of secondary amine fragment bounded to the pyrazine ring. Structure-activity relationships are discussed as well.

Keywords: aminodehalogenation reaction; antifungal activity; inhibition of photosynthetic electron transport; pyrazinamide; microwave-assisted synthesis; structure-activity relationships

1. Introduction

Pyrazinamide (PZA) is considered to be one of the most important drugs in the World. It has numerous uses and the pyrazine ring is also contained in many natural compounds. Nevertheless, its main field of use is still the treatment of tuberculosis (TB). The reason is clear—*Mycobacterium tuberculosis* is the most dreadful microorganisms in the World. Although the incidence of new TB cases has been falling slowly throughout the last decade, the danger is posed by the group of resistant strains [1]. There are currently two types of resistance. The first group consists of multidrug-resistant mycobacteria, which are resistant to all first-line antituberculotic agents (rifampicin/rifabutin, isoniazid, pyrazinamide, ethambutol, streptomycin). The second group is more treacherous because of its resistance to isoniazid and rifampicin combined with resistance to any fluoroquinolone used in the current therapy and to at least one of the three parenteral second-line antituberculotic drugs (amikacin, kanamycin, capreomycin) [2]. There was another category in the past called totally drug-resistant TB that was first observed in India. These strains were resistant to all known first- and second-line antituberculosis agents and posed a threat to epidemiologists all over the World. But they have practically disappeared with the discovery and approval of the new anti-tuberculosis agent bedaquiline for their therapy because it is active against the resistant *Mycobacterium tuberculosis* [3]. PZA is classified as the first-line antituberculotic agent that is unique for its therapeutic properties. It acts in acidic environment as a bactericidal sterilising drug that is able to kill the dormant forms of tubercle bacilli, especially in combination with rifampicin. These so called persistors are just situated *in nidus* where an acidic pH is typical [4].

On the contrary, the mechanism of action of PZA is still under the investigation by researchers all over the World. One of the first theories suggested was that it is necessary to activate PZA via the enzyme pyrazinamidase (nicotinamidase) (EC 3.5.1.19) to the active form pyrazinecarboxylic acid (POA). This acid molecule gets into the mycobacterial cell and causes the inner cell compartment acidification during the ionic efflux. These consequences lead to cellular death [5–7]. The gene encoding pyrazinamidase is known as *pncA* gene and its mutation leads to the resistance of mycobacteria to pyrazinamide [8].

Another theory propounded the inhibition of fatty acid synthase I (FAS I) (EC 2.3.1.85). This is the enzyme essential for synthesis of mycolic acids, which are vital for the mycobacterial cell wall integrity. Despite the fact that PZA is effective in this aspect, this mechanism was rejected by Boshoff who has proven that PZA caused only low inhibition [9]. PZA analogues that act as FAS I inhibitors are from the group of 5-chloropyrazinamide, esters of 5-chloropyrazinoic acid or POA and their derivatives [10–12].

A recent theory introduced by Zhang *et al.* proposed the inhibition of *trans*-translation as the main way of PZA action. This cellular process is essential for survival and virulence of mycobacteria and its interruption leads to fatal blockade of proteosynthesis. All these hypotheses were supported by *in vitro* experiments [13].

PZA and its derivatives have also many other useful applications, as they show antiviral, antifungal, antibacterial and as well as antineoplastic activities [14–20]. Several pyrazine derivatives were also found to inhibit photosynthetic electron transport (PET) in plant chloroplasts [21–23]. Using EPR spectroscopy it was found that the site of action of pyrazine-derived PET inhibitors is the intermediate $D^{\bullet}$, *i.e.*, tyrosine radical ($Tyr_D^{\bullet}$) situated in the 161st position in D_2 protein on the donor side of photosystem (PS) 2 [24,25]. Similar site of action in the photosynthetic apparatus of spinach chloroplasts was confirmed for 2-alkylsulfanylpyridine-4-carbothioamides [26] and substituted benzanilides and thiobenzanilides [27]. Further experiments with artificial electron donor 1,5-diphenylcarbazide (DPC) confirmed that PET between PS2 and PS1 was interrupted by 2,6-disubstituted pyridine-4-thiocarboxamides exclusively on the donor side of PS2 in the section between the primary electron donor of PS2 (H_2O) and $Z^{\bullet}/D^{\bullet}$ intermediate and the photosynthetic transport chain from P 680 to plastoquinone Q_B occurring on the acceptor side of PS2 was not damaged [25], while PET on the acceptor side of PS2 was partially damaged by 5-*tert*-butyl-6-chloro-*N*-(3-fluorophenyl)pyrazine-2-carboxamide and 5-*tert*-butyl-*N*-(3-hydroxy-4-chlorophenyl)pyrazine-2-carboxamide [25] or by *N*-benzylpyrazine-2-carboxamides [22]. Many structural variations of pyrazine compounds with herbicidal properties can be found in the patent literature focused on compounds with herbicidal activity that are useful for the control of unwanted vegetation [28–30].

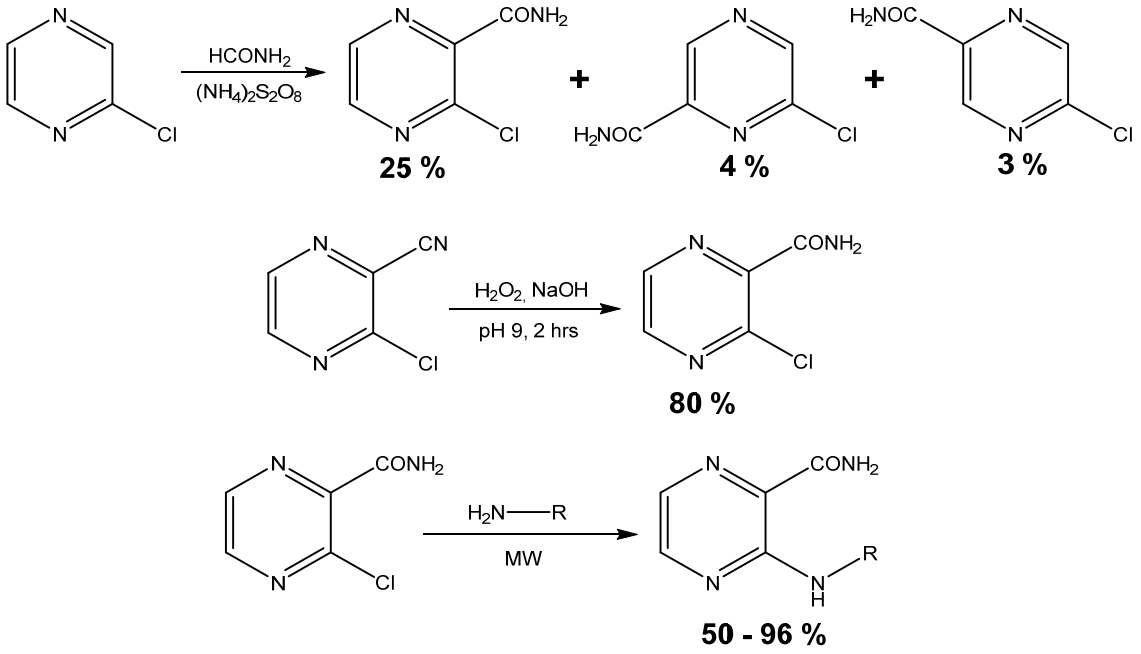
A further aspect of this work is the use of microwave-assisted reactions. It is known that microwave-assisted syntheses have become into general awareness during the last decades and their applications are still in the focus of many researchers. These reactions usually provide higher yields, shorter reaction times and better chemical conversions in comparison with conventional heating methods. Moreover, sometimes the desired products can be obtained only by using the microwaves accelerated reactions [31]. If emerging side-products are detected, they can be eliminated by many separation methods such as chromatography or crystallization. The advantages of these reactions are explained by the interaction between microwaves and molecules themselves, neither vessel sides nor any solvents used. There is also the possibility of reaching temperatures higher than the boiling point of solvent, especially in over-pressurized systems. This has opened a new way for drug discovery and development [32]. We focus herein on the synthesis of pyrazine derivatives with aliphatic or alicyclic side chains and their antimycobacterial, antibacterial, antifungal and PET-inhibiting activities.

2. Results and Discussion

2.1. Chemistry

Compounds synthesized in this work were prepared according to the general procedures shown in Scheme 1. The identity and purity were proven by NMR spectra, elemental analysis and melting point.

Scheme 1. Synthesis of starting compound and microwave assisted synthesis of final compounds.



R - aliphatic, alicyclic amines and saturated heterocycles containing nitrogen
MW - microwaves; 150 °C, 30 min, 120 W, methanol, pyridine

The second method of starting compound preparation was approximately three times more efficacious than the first one comparing the yields of the reactions (80% *vs.* 25%). The melting point of 3-chloropyrazine-2-carboxamide was compared to the value stated in a previously published paper and the results were practically the same, as our measured value was in the 187.1–189.2 °C range and the reported values were 186.0–186.5 °C [33] and 188.0–190.0 °C [34]. 3-Chloropyrazine-2-carboxamide was then treated with 18 aliphatic or alicyclic amines and saturated heterocycles containing nitrogen. A series of experiments was done at the beginning to compare the efficiency of both methods—conventional and microwave-assisted heating (Table 1).

The remaining reactions were performed in microwave reactor and the conditions, which were proven experimentally, were as follows: 140 °C, 30 min, 120 W, methanol as a solvent and pyridine as a base (Scheme 1). Yields obtained from the aminodehalogenation were in a range between 50.0% and 95.8% and their variability can be explained by the steric effects of some substituents (dibutyl-, *tert*-pentyl-) that lead to worse outcomes.

This reaction yielded *N*-substituted 3-aminopyrazine-2-carboxamides (Table 2). We prepared a series consisted of 18 compounds of which 16 were novel. They were analytically characterized (NMR and IR spectra, melting point, elemental analysis) and submitted to biological screening. The obtained analytical data were fully consistent with the proposed structures. 3-(Methylamino)pyrazine-2-carboxamide was previously synthesized by Osden *et al.* (reported melting point was 200–201 °C) and by Albert *et al.* (reported melting point 198–199 °C) [33,35]. The light yellow crystalline compound we obtained melted at 200.0–201.4 °C. 3-(Cyclohexylamino)pyrazine-2-carboxamide was previously synthesized by Keir *et al.* and reported melting point was 128–129 °C [36]. Our light yellow crystalline compound melted at 129.0–130.3 °C.

Table 1. Table comparing the efficiency of conventional and microwave-assisted methodology showing the yields, reaction times and conditions.

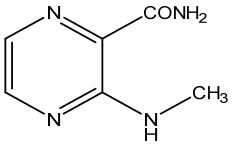
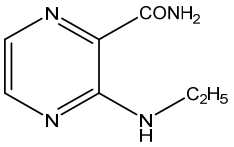
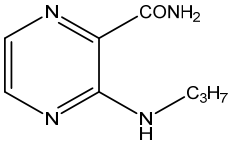
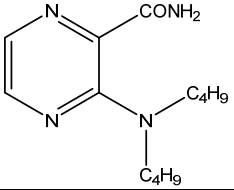
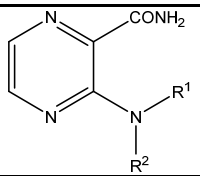
Compound		Type of Synthesis	Time	Conditions	Yield
1		MW	30 min	140 °C, 120 W, MeOH, overpressure, 1 eq. pyridine, 2 eq. amine	61.4%
		Conventional	60 min	110 °C, toluene, 1 eq. pyridine, 2 eq. amine	53.0%
2		MW	30 min	140 °C, 120 W, MeOH, overpressure, 1 eq. pyridine, 2 eq. amine	78.7%
		Conventional	60 min	110 °C, toluene, 1 eq. pyridine, 2 eq. amine	46.3%
3		MW	30 min	140 °C, 120 W, MeOH, overpressure, 1 eq. pyridine, 2 eq. amine	87.0%
		Conventional	60 min	110 °C, toluene, 1 eq. pyridine, 2 eq. amine	56.1%
10		MW	30 min	140 °C, 120 W, MeOH, overpressure, 1 eq. pyridine, 2 eq. amine	67.2%
		Conventional	60 min	110 °C, toluene, 1 eq. pyridine, 2 eq. amine	0%

Table 2. List of prepared compounds and their lipophilicity.

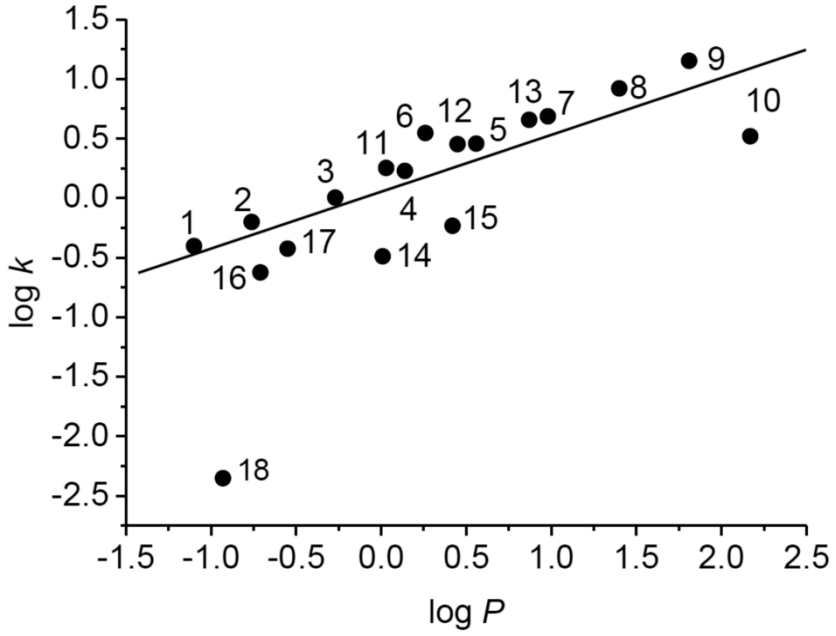


Nr.	R^1	R^2	log*P*/Clog*P*	log*k*
1	CH_3	H	−1.10/0.20	−0.4028
2	C_2H_5	H	−0.76/0.73	−0.2014
3	C_3H_7	H	−0.27/1.25	0.0038
4	C_4H_9	H	0.14/1.78	0.2287
5	C_5H_{11}	H	0.56/2.31	0.4563
6	*tert*-C_5H_{11}	H	0.26/1.96	0.5468
7	C_6H_{13}	H	0.98/2.84	0.6860
8	C_7H_{15}	H	1.40/3.37	0.9198
9	C_8H_{17}	H	1.81/3.90	1.1533
10	C_4H_9	C_4H_9	2.17/3.40	0.5197
11	cyclopentyl	H	0.03/1.67	0.2537
12	cyclohexyl	H	0.45/2.23	0.4523
13	cycloheptyl	H	0.87/2.79	0.6575
14	$-(CH_2)_4-$		0.01/0.34	−0.4889
15	$-(CH_2)_5-$		0.42/0.90	−0.2333
16	$-(CH_2)_2O(CH_2)_2-$		−0.71/−0.49	−0.6238
17	$-(CH_2)_2NCH_3(CH_2)_2-$		−0.55/0.08	−0.4253
18	$-(CH_2)_2NH(CH_2)_2-$		−0.93/−0.50	−2.3522

2.2. Calculated and Experimentally Determined Lipophilicity

The dependence of experimentally determined log*k* on calculated log*P* values of tested compounds is shown in Figure 1. While for compounds **1**–**17** linear dependence of log*k* on log*P* was determined (r = 0.816), the estimated log*k* value for 3-(piperazin-1-yl)pyrazine-2-carboxamide (**18**) was considerably lower than expected. This could be connected with the presence of a secondary cyclic amine that can be very easily ionized.

Figure 1. Dependence of experimentally determined log*k* on calculated log*P* values of tested compounds.



2.3. Biological Assays

2.3.1. Antimycobacterial *in Vitro* Screening

All prepared compounds were tested against the wild strain *Mycobacterium tuberculosis* H37Rv. Four substances (compounds **6**–**9**) showed low activity against this strain with corresponding minimal inhibition concentration (MIC) values equal to 50 μg/mL. However, this activity was negligible comparing to the activity of the standard isoniazid (0.39 μg/mL) and pyrazinamide (12.5 μg/mL).

2.3.2. Antifungal and Antibacterial *in Vitro* Screening

The results of antibacterial screening have shown only low activities against *Staphylococcus epidermidis*. The antibacterial activity of compounds **5**, **7** and **13** expressed by 80% inhibition concentration (IC_{80}) values was lower than 250 μmol/L and did not reach the values of antibiotic standards.

Antifungal evaluation showed more interesting results. There were seven active substances and two of them (compounds **12** and **13**) showed better activity than two standards (voriconazole, fluconazole) (Table 3). The alicyclic side chain with seven carbons was more active than the 6-membered ring. The 80% inhibition of control was observed in the range from 15.62 to 31.25 μmol/L for compound **12**

and in the range from 7.81 to 31.25 μmol/L for compound **13** against nearly a whole spectrum of fungi tested after 24 h of incubation. Five substances (compounds **4**, **5**, **7**, **8** and **11**) were less active than the standards and their IC_{80} values were not lower than 250 μmol/L. The activity spectrum of these compounds was also wider and did not include only one fungal strain.

Table 3. Results of antifungal evaluation for the compounds with alicyclic side chains and corresponding standards.

		IC_{80} [μmol/L]						
		11	**12**	**13**	**AMP**	**VOR**	**NYS**	**FLU**
CA	24 h	500	31.25	15.62	0.12	0.008	0.98	0.24
	48 h	>500	62.5	31.25	0.49	0.008	1.95	0.24
CT	24 h	>500	125	62.5	1.95	250	1.95	>500
	48 h	>500	500	500	1.95	250	3.9	>500
CK	24 h	250	15.62	7.81	1.95	0.98	1.95	125
	48 h	500	62.5	31.25	1.95	1.95	3.9	250
CG	24 h	250	15.62	7.81	0.98	250	1.95	31.25
	48 h	>500	31.25	15.62	1.95	250	3.9	250
TA	24 h	500	31.25	31.25	0.49	7.81	1.95	250
	48 h	>500	250	125	0.98	31.25	1.95	500
AF	24 h	500	15.62	7.81	1.95	0.49	1.95	>500
	48 h	>500	62,5	7.81	1.95	0.98	3.9	>500
LC	24 h	>500	250	62.5	7.81	250	15.62	>500
	48 h	>500	500	250	7.81	250	31.25	>500
TM	72 h	500	15.62	15.62	1.95	0.06	3.9	7.81
	120 h	500	15.62	15.62	1.95	0.12	7.81	125

CA: *Candida albicans*; CT: *C. tropicalis*; CK: *C. krusei*; CG: *C. glabrata*; TA: *Trichosporon asahii*; AF: *Aspergillus fumigatus*; LC: *Lichtheimia corymbifera*; TM: *Trichophyton mentagrophytes*; AMP: amphotericin B; VOR: voriconazole; NYS: nystatin; FLU: fluconazole.

It is not possible to predict structure-activity relationships for antibacterial and antifungal efficacy of studied compounds in the whole tested group due to small number of active substances and a lack of IC_{80} values better than standards. However, a saturated ring is probably important for antifungal activity and the activity also increases with increasing lipophilicity of the compounds. Unfortunately, the series is too small (only three compounds) to prove this hypothesis.

2.3.3. Photosynthetic Electron Transport Inhibiting Activity Evaluation

The 3-substituted pyrazine-2-carboxamide derivatives were tested for their PET inhibiting activity. However, the PET-inhibiting activity, expressed by IC_{50} value (compound concentration in mol/L causing 50% inhibition of PET) in the investigated set, could be determined only for nine compounds. The values varied in a wide concentration range from 14.3 μmol/L (compound **9**) to 1590.0 μmol/L (compound **3**) (Table 4). The rest of compounds **1**, **2**, **10**, **14**, **15**, **16**, **17**, and **18** did not inhibit PET in spinach chloroplasts. Because all tested compounds containing heterocyclic amines as substituent in position 3 on the pyrazine ring, *i.e.*, **14**, **15**, **16**, **17**, and **18**, were found to be inactive it could be concluded that increased basicity of these compounds caused by the introduction of nitrogen atom(s)

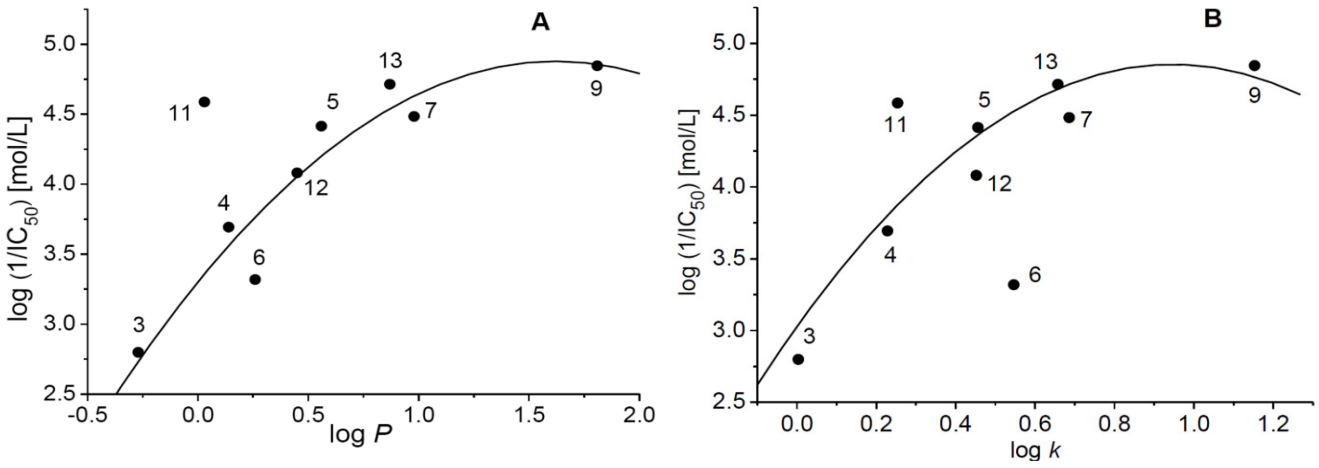
into the ring is connected with activity loss. Moreover, these substituents ultimately create a trisubstituted amine while by introduction of amino alkyl or amino cycloalkyl substituents to pyrazine-2-carboxamide disubstituted amine is formed. However, it could be stressed that trisubstituted amine is present also in compound with R = $(C_4H_9)_2$ (**10**) which did not show PET inhibiting activity as well.

Table 4. IC_{50} values [μmol/L] of tested compounds related to PET inhibition in spinach chloroplasts in comparison with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) standard.

Compound	IC_{50} [μmol/L]
3	1590
4	203
5	38.6
6	480
7	32.9
9	14.3
11	25.8
12	83.0
13	19.3
DCMU	1.9

The PET-inhibiting activity was strongly affected by lipophilic properties of the compounds (Figure 2) expressed as log*P* or log*k*. The PET-inhibiting activity increased with increasing lipophilicity of the compound and the dependence of log (1/IC_{50}) *vs.* log*P* showed quasi-parabolic course (Figure 2A). The only exception was compound **11** (R = cyclopentyl with log*P* = 0.03) activity of which was considerably higher (IC_{50} = 25.8 μmol/L) than that of compound **4** with log*P* = 0.14 (IC_{50} = 203 μmol/L). Similarly, quasi-parabolic course was also observed for the dependence of PET inhibiting activity on log*k*. However, with respect to the quasi-parabolic course of this dependence, compound **11** (log*k* = 0.2537) showed higher and compound **6** (log*k* = 0.5468) lower PET inhibiting activity (Figure 2B).

Figure 2. Dependence of PET-inhibiting activity on lipophilicity of studied compounds expressed as log*P* (**A**) or log*k* (**B**).



The dependence of log ($1/IC_{50}$) on log*P* can be expressed by following equation after excluding the 3-(cyclopentylamino)pyrazine-2-carboxamide (**11**):

$$\log(1/IC_{50}) = 3.302\ (\pm 0.137) + 1.943\ (\pm 0.366)\ \log P - 0.599\ (\pm 0.211)\ (\log P)^2$$
$$r = 0.955,\ s = 0.253,\ F = 25.79,\ n = 8 \quad (1)$$

On the other hand, after excluding the 3-(*tert*-pentylamino)pyrazine-2-carboxamide (6) the dependence of log ($1/IC_{50}$) on log*k* can be expressed by following equation:

$$\log (1/IC_{50}) = 3.028\ (\pm 0.329) + 0.386\ (\pm 0.126)\ \log k - 2.040\ (\pm 1.028)\ (\log k)^2$$
$$r = 0.881,\ s = 0.378,\ F = 8.68,\ n = 8 \quad (2)$$

The obtained results indicated that IC_{50} could not be determined for compounds with too low lipophilicity, namely **1** (log*P* = −1.3), **2** (log*P* = −0.76).

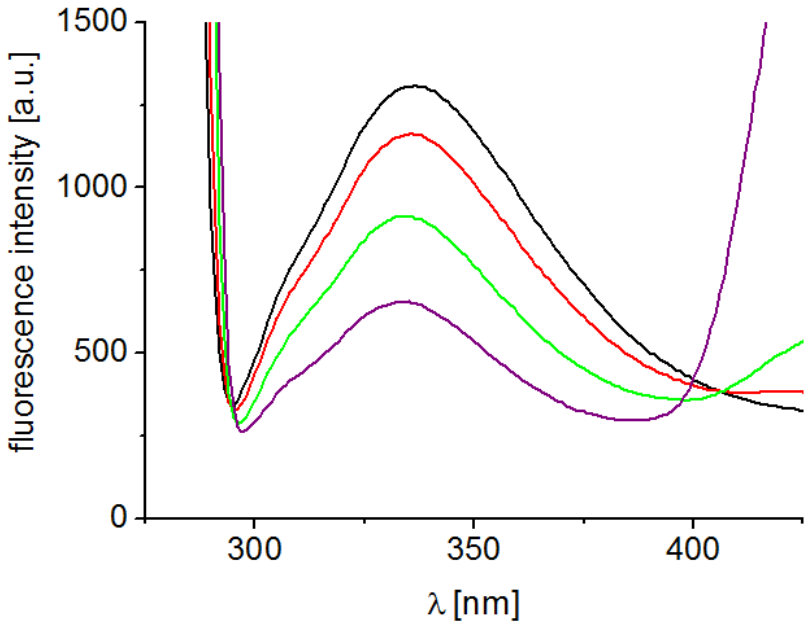
As mentioned above, the ineffectiveness of compound **10** (R = $(C_4H_9)_2$) as well as compounds **14**–**18** with heterocyclic substituents could be connected mainly with the absence of a secondary amine fragment (-NH-) bounded to the pyrazine ring. Consequently, possible interactions of these compounds with constituents of the photosynthetic apparatus are limited. On the other side, a 2-carboxamino substituent $CONH_2$ on the pyrazine ring plays an important role in the interactions of the tested pyrazine derivatives with constituents of the photosynthetic apparatus. Moreover, increasing length of the alkyl substituent contributes not only leads to better transport of the compound to its site of action but incorporation of longer alkyl chains into the thylakoid membrane that results in membrane damage and consecutive increase of PET inhibiting activity. Similar results were also obtained with 2-alkylthio-4-pyridinecarbothioamides [26].

Artificial electron donors could be used to specify the site of inhibitory action of PET inhibitors in the photosynthetic apparatus because they can restore PET activity in chloroplasts of which was previously inhibited. For example, 2,5-diphenylcarbazide (DPC) can supply electrons in the site of $Z^{\bullet}/D^{\bullet}$ intermediate which is situated on the donor side of PS2 [37]. Consequently, if PET was inhibited in the section between the oxygen evolving complex and $Z^{\bullet}/D^{\bullet}$ intermediate, DPC can restore PET between the core of PS2 (P680) and secondary quinone acceptor Q_B which is situated on the acceptor side of PS2 and 2,6-dichlorophenol-indophenol (DCPIP) photoreduction occurs again. However, if the site of PET inhibitor is located between P680 and Q_B, photoreduction of DCPIP (an artificial electron acceptor used for monitoring the PET through PS2 from H_2O to Q_B) remains inhibited. Activity of chloroplasts, which was restricted by studied pyrazine-2-carboxamides, was practically completely restored after DPC addition. Thus, it can be concluded that the site of action of tested compounds in the electron transport chain is situated exclusively on the donor side of PS2, between oxygen evolving complex and $Z^{\bullet}/D^{\bullet}$ intermediate, while the section of the electron transport chain on the acceptor side of PS2 between P680 and Q_B is not damaged. Similar results were also obtained with *N*-substituted 5-amino-6-methylpyrazine-2,3-dicarbonitriles [23], 2-benzylsulphanylbenzimidazoles [38] and ring-substituted 1-hydroxynaphthalene-2-carboxanilides [39]. On the other hand, addition of DPC restored PET in chloroplasts, which was inhibited by *N*-benzylpyrazine-2-carboxamides only to 77%–88% indicating that the site of PET inhibition is situated not only on the donor but also on the acceptor side of PS2 [22]. Similar sites of action on both sides of PS2 were determined previously for

5-*tert*-butyl-*N*-(3-hydroxy-4-chlorophenyl)-pyrazine-2-carboxamide and 5-*tert*-butyl-6-chloro-*N*-(3-fluorophenyl)pyrazine-2-carboxamide [24].

Interaction of substituted pyrazine-2-carboxamides with residues of aromatic amino acids (AAA), mainly tryptophan and tyrosine occurring in photosynthetic proteins situated mainly in PS2, which was documented by the quenching of AAA fluorescence at 334 nm contributed to PET inhibition. Figure 3 presents fluorescence emission spectra of AAA of untreated spinach chloroplasts and of chloroplasts treated with increasing concentrations of compound **7**. As shown in Figure 3, the quenching of the fluorescence of aromatic amino acids at 334 nm increased with increasing concentration of pyrazine derivative. This finding is in accordance with the results obtained in the above mentioned study of PET inhibition in spinach chloroplasts by tested pyrazine-2-carboxamides using artificial electron acceptor DCPIP.

Figure 3. Fluorescence emission spectra of aromatic amino acids in suspension of spinach chloroplasts without and with compound **7** (c = 0, 6, 24 and 48 μmol/L; the curves from top to bottom); excitation wave length λ = 275 nm; chlorophyll concentration 10 mg/L.



The quenching of the fluorescence of aromatic amino acids in the presence of 5-bromo- and 3,5-dibromo-2-hydroxy-*N*-phenylbenzamides and ring-substituted 2-hydroxynaphthalene-1-carboxanilides was observed previously [40,41].

3. Experimental

3.1. General

All chemicals were reagent or higher grade of purity and were purchased from Sigma-Aldrich (Steinheim, Germany) or Fluorochem Ltd. (Hadfield, Derbyshire, UK), unless stated otherwise.

The starting compound was prepared according to proven conventional organic synthesis methodology. The final aminodehalogenation reaction was performed in a CEM Discover microwave

reactor with a focused field (CEM Corporation, Matthews, NC, USA) connected to an Explorer 24 autosampler (CEM Corporation) and this equipment was running under CEM's Synergy™ software for setting and monitoring the conditions of reactions. The temperature of the reaction mixture was monitored by internal infrared sensor. The progress of the reaction was checked by Thin Layer Chromatography (TLC) (Alugram® Sil G/UV_{254}, Machery-Nagel, Postfach, Germany) with UV detection using wavelength 254 nm.

All obtained products were purified by crystallization or by preparative flash chromatograph CombiFlash® Rf (Teledyne Isco Inc., Lincoln, NE, USA). The type of elution was gradient, using the mixture of hexane (LachNer, Neratovice, Czech Republic) and ethyl acetate (Penta, Prague, Czech Republic) as mobile phase. Silica gel (0.040–0.063 nm, Merck, Darmstadt, Germany) was used as the stationary phase.

NMR spectra were taken with spectrometers Varian Mercury-VxBB 300 with frequencies 299.95 MHz for ^{1}H and 75.43 MHz for ^{13}C or Varian VNMR S500 (499.87 MHz for ^{1}H and 125.71 MHz for ^{13}C) (Varian Corporation, Palo Alto, CA, USA). Chemical shifts were reported in ppm (δ) and were applied indirectly to tetramethylsilane as a signal of solvent (2.49 for ^{1}H and 39.7 for ^{13}C in DMSO-d_6). Infrared spectra were recorded with spectrometer FT-IR Nicolet 6700 (Thermo Scientific, Waltham, MA, USA) using attenuated total reflectance (ATR) methodology. Elemental analyses were measured with EA 1110 CHNS Analyzer (Fisons Instruments S. p. A., Carlo Erba, Milano, Italy). Melting points were assessed by SMP3 Stuart Scientific (Bibby Sterling Ltd., Staffordshire, UK) and were uncorrected. Log*P* and Clog*P* were calculated with PC program CS ChemBioDraw Ultra 13.0 (CambridgeSoft, Cambridge, MA, USA).

3.2. Synthesis of Starting Compound and Final Products

The starting compound 3-chloropyrazine-2-carboxamide was synthesized using two published procedures. The first method was classified as less effective and was based on the homolytic amidation of 2-chloropyrazine. Thus, 2-chloropyrazine (0.17 mol) was dissolved in formamide (3.7 mol), heated to 90 °C and ammonium peroxodisulphate (0.18 mol) was added portionwise over one hour period. This mixture reacted for another one hour at 90 °C and then it was left to stand for 24 h at laboratory temperature. Dilution with 100 mL of water was followed by filtration and this filtrate was extracted continuously with chloroform for 16 h [34,42]. The mixture of three positional isomers was separated by flash chromatography using silica gel as stationary phase. The second process used 3-chloropyrazine-2-carbonitrile, which was submitted to partial hydrolysis of the nitrile group. The powdered carbonitrile (0.104 mol) was added little by little into the reaction mixture of concentrated hydrogen peroxide (0.95 mol) and water (195 mL) heated to 50 °C. The pH was adjusted and regulated around a value of 9 using an 8% solution of sodium hydroxide and the temperature of the reaction was regulated between 55 and 60 °C. The reaction was stopped after 2.5 h and was cooled to 5 °C. Newly-emerged crystals were removed by suction and recrystallized from ethanol [42].

The starting compound (1.27 mmol) was treated with 18 aliphatic amines, alicyclic amines or saturated heterocycles containing at least one nitrogen atom (2.54 mmol). Four reactions were completed by conventional heating methods. The conditions were 110 °C, toluene as a solvent and pyridine (1.27 mmol) as a base. The reaction time was set to one hour. Then the reactions were completed

using the microwave reactor with focused field and conditions used for syntheses were 140 °C, 30 min, 120 W, methanol used as a solvent and pyridine (1.27 mmol) as a base. They were set experimentally with respect to prior experience. The progress of reaction was monitored with TLC in system hexane/ethyl acetate (1:1). Then the mixture was separated by flash column chromatograph using gradient elution. Mobile phases were hexane and ethyl acetate again.

3.3. Analytical Data of the Prepared Compounds

3-(Methylamino)pyrazine-2-carboxamide (**1**). Light yellow crystalline solid. Yield 61.4%; m.p. 200.0–201.4 °C (described in the literature 200–201 °C [35] or 198–199 °C [33]); IR (ATR-Ge, cm^{-1}): 3413_m (-NH-), 3293_m, 3179_m (-CO**NH_2**), 1686_{vs} (-**CO**NH_2), 1589_s, 1513_s, 1436_m, 1414_s, 1194_{vs} (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.65–8.55 (1H, m, NH), 8.23 (1H, d, J = 2.3 Hz, Ar), 8.11 (1H, bs, NH_2), 7.72 (1H, d, J = 2.3 Hz, Ar), 7.63 (1H, bs, NH_2), 2.90 (3H, d, J = 5.3 Hz, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 168.9, 155.1, 146.6, 129.5, 126.9, 27.3; Elemental analysis: calc. for $C_6H_8N_4O$ (MW 152.15): 47.36% C, 5.30% H, 36.82% N; found 47.43% C, 5.45% H, 36.68% N.

3-(Ethylamino)pyrazine-2-carboxamide (**2**). Light yellow crystalline solid. Yield 78.7%; m.p. 117.4–118.5 °C; IR (ATR-Ge, cm^{-1}): 3381_m (-NH-), 3294_m, 3209_m (-CO**NH_2**), 1683_{vs} (-**CO**NH_2), 1582_s, 1533_m, 1500_{vs}, 1419_m, 1192_{vs} (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.68 (1H, t, J = 4.7 Hz, NH), 8.22 (1H, d, J = 2.3 Hz, Ar), 8.11 (1H, bs, NH_2), 7.72 (1H, d, J = 2.3 Hz, Ar), 7.64 (1H, bs, NH_2), 3.47–3.32 (2H, m, NCH_2), 1.14 (3H, t, J = 7.0 Hz, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 169.0, 154.4, 146.6, 129.6, 126.5, 34.8, 14.8; Elemental analysis: calc. for $C_7H_{10}N_4O$ (MW 166.18): 50.59% C, 6.07% H, 33.71% N; found 50.66% C, 5.98% H, 33.51% N.

3-(Propylamino)pyrazine-2-carboxamide (**3**). Yellow crystalline solid. Yield 87.0%; m.p. 93.9–94.9 °C; IR (ATR-Ge, cm^{-1}): 3402_m (-NH-), 3337_m, 3186_m (-CO**NH_2**), 1671_{vs} (-**CO**NH_2), 1578_s, 1533_m, 1514_{vs}, 1423_s, 1189_{vs} (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.77 (1H, t, J = 6.5 Hz, NH), 8.21 (1H, d, J = 2.3 Hz, Ar), 8.12 (1H, bs, NH_2), 7.72 (1H, d, J = 2.3 Hz, Ar), 7.64 (1H, bs, NH_2), 3.34 (2H, q, J = 6.5 Hz, NCH_2), 1.63–1.46 (2H, m, CH_2), 0.89 (3H, t, J = 6.5 Hz, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 169.1, 154.6, 146.6, 129.6, 126.5, 41.7, 22.2, 11.6; Elemental analysis: calc. for $C_8H_{12}N_4O$ (MW 180.21): 53.32% C, 6.71% H, 31.09% N; found 53.45% C, 6.79% H, 30.93% N.

3-(Butylamino)pyrazine-2-carboxamide (**4**). Yellow crystalline solid. Yield 92.3%; m.p. 83.1–84.4 °C; IR (ATR-Ge, cm^{-1}): 3409_m (-NH-), 3326_m, 3177_m (-CO**NH_2**), 1671_{vs} (-**CO**NH_2), 1578_s, 1533_m, 1518_{vs}, 1423_s, 1187_{vs} (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.75 (1H, t, J = 6.6 Hz, NH), 8.22 (1H, d, J = 2.3 Hz, Ar), 8.12 (1H, bs, NH_2), 7.73 (1H, d, J = 2.3 Hz, Ar), 7.64 (1H, bs, NH_2), 3.38 (2H, q, J = 6.6 Hz, NCH_2), 1.59–1.46 (2H, m, CH_2), 1.41–1.25 (2H, m, CH_2), 0.88 (3H, t, J = 6.6 Hz, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 169.1, 154.6, 146.6, 129.6, 126.5, 40.5, 31.1, 19.9, 13.9; Elemental analysis: calc. for $C_9H_{14}N_4O$ (MW 194.23): 55.65% C, 7.27% H, 28.85% N; found 55.77% C, 7.27% H, 28.93% N.

3-(Pentylamino)pyrazine-2-carboxamide (**5**). Light yellow crystalline solid. Yield 92.8%; m.p. 105.0–106.3 °C; IR (ATR-Ge, cm^{-1}): 3379_m (-NH-), 3321_m, 3145_m (-CO**NH_2**), 1663_{vs} (-**CO**NH_2),

1587_s, 1531_m, 1509_{vs}, 1418_s, 1185_{vs} (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.76 (1H, t, J = 6.5 Hz, NH), 8.22 (1H, d, J = 2.3 Hz, Ar), 8.12 (1H, bs, NH_2), 7.73 (1H, d, J = 2.3 Hz, Ar), 7.64 (1H, bs, NH_2), 3.38 (2H, q, J = 6.5 Hz, NCH_2), 1.62–1.46 (2H, m, CH_2), 1.37–1.19 (4H, m, CH_2), 0.85 (3H, t, J = 6.5 Hz, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 169.1, 154.6, 146.6, 129.6, 126.4, 40.5, 28.9, 28.7, 22.1, 14.1; Elemental analysis: calc. for $C_{10}H_{16}N_4O$ (MW 208.26): 57.67% C, 7.74% H, 26.90% N; found 57.63% C, 7.67% H, 27.06% N.

3-(tert-Pentylamino)pyrazine-2-carboxamide (**6**). Yellow crystalline solid. Yield 51.3%; m.p. 65.0–66.7 °C; IR (ATR-Ge, cm^{-1}): 3396_m (-NH-), 3265_m, 3185_m (-CO**NH_2**), 1668_{vs} (-**CO**NH_2), 1581_{vs}, 1504_{vs}, 1415_s, 1183_{vs} (pyrazine ring); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.65 (1H, bs, NH), 8.13 (1H, d, J = 2.3 Hz, Ar), 7.74 (1H, bs, NH_2), 7.61 (1H, d, J = 2.3 Hz, Ar), 5.56 (1H, bs, NH_2), 1.86 (2H, q, J = 7.6 Hz, CH_2), 1.42 (6H, s, CH_3), 0.87 (3H, t, J = 7.6 Hz, CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 169.7, 154.9, 146.3, 128.9, 125.5, 54.2, 33.0, 26.4, 8.4; Elemental analysis: calc. for $C_{10}H_{16}N_4O$ (MW 208.26): 57.67% C, 7.74% H, 26.90% N; found 57.80% C, 7.89% H, 26.70% N.

3-(Hexylamino)pyrazine-2-carboxamide (**7**). Light yellow crystalline solid. Yield 93.6%; m.p. 64.3–65.6 °C; IR (ATR-Ge, cm^{-1}): 3404_m (-NH-), 3314_m, 3192_m (-CO**NH_2**), 1659_{vs} (-**CO**NH_2), 1587_{vs}, 1536_m, 1509_{vs}, 1424_m, 1190_{vs} (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.76 (1H, t, J = 6.2 Hz, NH), 8.22 (1H, d, J = 2.3 Hz, Ar), 8.12 (1H, bs, NH_2), 7.73 (1H, d, J = 2.3 Hz, Ar), 7.64 (1H, bs, NH_2), 3.38 (2H, q, J = 6.2 Hz, NCH_2), 1.60–1.44 (2H, m, CH_2), 1.39–1.16 (6H, m, CH_2), 0.84 (3H, t, J = 6.2 Hz, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 169.1, 154.6, 146.6, 129.6, 126.4, 40.5, 31.2, 28.9, 26.4, 22.2, 14.1; Elemental analysis: calc. for $C_{11}H_{18}N_4O$ (MW 222.29): 59.44% C, 8.16% H, 25.20% N; found 59.41% C, 8.24% H, 25.10% N.

3-(Heptylamino)pyrazine-2-carboxamide (**8**). Yellow crystalline solid. Yield 91.5%; m.p. 66.0–67.3 °C; IR (ATR-Ge, cm^{-1}): 3407_m (-NH-), 3316_m, 3176_m (-CO**NH_2**), 1674_{vs} (-**CO**NH_2), 1580_{vs}, 1532_m, 1514_{vs}, 1421_m, 1184_s (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.76 (1H, t, J = 6.3 Hz, NH), 8.22 (1H, d, J = 2.3 Hz, Ar), 8.12 (1H, bs, NH_2), 7.72 (1H, d, J = 2.3 Hz, Ar), 7.64 (1H, bs, NH_2), 3.38 (2H, q, J = 6.3 Hz, NCH_2), 1.60–1.45 (2H, m, CH_2), 1.37–1.15 (8H, m, CH_2), 0.83 (3H, t, J = 6.3 Hz, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 169.1, 154.6, 146.6, 129.6, 126.4, 40.5, 31.4, 29.0, 28.6, 26.7, 22.2, 14.1; Elemental analysis: calc. for $C_{12}H_{20}N_4O$ (MW 236.31): 60.99% C, 8.53% H, 23.71% N; found 61.12% C, 8.57% H, 23.64% N.

3-(Octylamino)pyrazine-2-carboxamide (**9**). Light yellow crystalline solid. Yield 95.8%; m.p. 67.2–68.3 °C; IR (ATR-Ge, cm^{-1}): 3407_m (-NH-), 3321_m, 3211_m (-CO**NH_2**), 1664_{vs} (-**CO**NH_2), 1594_{vs}, 1539_m, 1510_{vs}, 1423_m, 1197_{vs} (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.76 (1H, t, J = 6.4 Hz, NH), 8.22 (1H, d, J = 2.3 Hz, Ar), 8.11 (1H, bs, NH_2), 7.73 (1H, d, J = 2.3 Hz, Ar), 7.64 (1H, bs, NH_2), 3.38 (2H, q, J = 6.4 Hz, NCH_2), 1.61–1.45 (2H, m, CH_2), 1.37–1.14 (10H, m, CH_2), 0.83 (3H, t, J = 6.4 Hz, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 169.1, 154.6, 146.6, 129.6, 126.5, 40.5, 31.4, 29.0, 28.9, 28.8, 26.7, 22.3, 14.1; Elemental analysis: calc. for $C_{13}H_{22}N_4O$ (MW 250.34): 62.37% C, 8.86% H, 22.38% N; found 62.42% C, 8.81% H, 22.25% N.

3-(Dibutylamino)pyrazine-2-carboxamide (**10**). Light yellow crystalline solid. Yield 67.2%; m.p. 129.3–130.8 °C; IR (ATR-Ge, cm^{-1}): 3372_m, 3197_m (-CO**N**$\mathbf{H_2}$), 1647_{vs} (-**CO**NH_2), 1616_m, 1558_m, 1507_m, 1457_m, 1179_s (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.08 (1H, d, J = 2.3 Hz, Ar), 7.96 (1H, bs, NH_2), 7.73 (1H, d, J = 2.3 Hz, Ar), 7.52 (1H, bs, NH_2), 3.40 (4H, q, J = 7.5 Hz, NCH_2), 1.56–1.42 (4H, m, CH_2), 1.31–1.13 (4H, m, CH_2), 0.85 (6H, t, J = 7.5 Hz, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 169.5, 151.7, 141.8, 135.5, 129.7, 48.8, 29.3, 19.8, 14.0; Elemental analysis: calc. for $C_{13}H_{22}N_4O$ (MW 250.34): 62.37% C, 8.86% H, 22.38% N; found 62.27% C, 8.66% H, 22.32% N.

3-(Cyclopentylamino)pyrazine-2-carboxamide (**11**). Yellow crystalline solid. Yield 90.5%; m.p. 107.8–108.8 °C; IR (ATR-Ge, cm^{-1}): 3399_m (-NH-), 3285_m (-CO**N**$\mathbf{H_2}$), 1655_{vs} (-**CO**NH_2), 1580_{vs}, 1525_m, 1505_{vs}, 1419_m, 1172_{vs} (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.79 (1H, d, J = 7.0 Hz, NH), 8.23 (1H, d, J = 2.3 Hz, Ar), 8.12 (1H, bs, NH_2), 7.73 (1H, d, J = 2.3 Hz, Ar), 7.64 (1H, bs, NH_2), 4.33–4.12 (1H, m, NCH), 2.05–1.88 (2H, m, CH_2), 1.75–1.49 (4H, m, CH_2), 1.49–1.33 (2H, m, CH_2); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 169.1, 154.2, 146.6, 129.7, 126.4, 51.5, 32.9, 23.5; Elemental analysis: calc. for $C_{10}H_{14}N_4O$ (MW 206.24): 58.24% C, 6.84% H, 27.17% N; found 58.23% C, 6.85% H, 26.98% N.

3-(Cyclohexylamino)pyrazine-2-carboxamide (**12**). Light yellow crystalline solid. Yield 92.5%; m.p. 129.0–130.3 °C (described in the literature 128–129 °C [36]); IR (ATR-Ge, cm^{-1}): 3455_m (-NH-), 3254_m (-CO**N**$\mathbf{H_2}$), 1683_{vs} (-**CO**NH_2), 1583_s, 1518_{vs}, 1473_m, 1418_m, 1182_s (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.78 (1H, d, J = 7.6 Hz, NH), 8.21 (1H, d, J = 2.3 Hz, Ar), 8.12 (1H, bs, NH_2), 7.72 (1H, d, J = 2.3 Hz, Ar), 7.64 (1H, bs, NH_2), 3.96–3.80 (1H, m, NCH), 1.96–1.82 (2H, m, CH_2), 1.73–1.60 (2H, m, CH_2), 1.60–1.47 (1H, m, CH_2), 1.43–1.14 (5H, m, CH_2); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 169.1, 153.8, 146.6, 129.6, 126.2, 48.0, 32.4, 25.5, 24.4; Elemental analysis: calc. for $C_{11}H_{16}N_4O$ (MW 220.27): 59.98% C, 7.32% H, 25.44% N; found 59.78% C, 7.38% H, 25.33% N.

3-(Cycloheptylamino)pyrazine-2-carboxamide (**13**). Light yellow crystalline solid. Yield 87.4%; m.p. 102.7–104.1 °C; IR (ATR-Ge, cm^{-1}): 3450_m (-NH-), 3293_m, 3180_m (-CO**N**$\mathbf{H_2}$), 1674_{vs} (-**CO**NH_2), 1581_{vs}, 1506_{vs}, 1465_m, 1416_s, 1177_{vs} (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.84 (1H, d, J = 7.6 Hz, NH), 8.22 (1H, d, J = 2.3 Hz, Ar), 8.12 (1H, bs, NH_2), 7.71 (1H, d, J = 2.3 Hz, Ar), 7.64 (1H, bs, NH_2), 4.15–3.99 (1H, m, NCH), 1.95–1.79 (2H, m, CH_2), 1.65–1.37 (10H, m, CH_2); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 169.1, 153.7, 146.6, 129.5, 126.3, 50.2, 34.2, 27.7, 23.7; Elemental analysis: calc. for $C_{12}H_{18}N_4O$ (MW 234.30): 61.52% C, 7.74% H, 23.91% N; found 61.50% C, 7.94% H, 24.05% N.

3-(Pyrrolidin-1-ylamino)pyrazine-2-carboxamide (**14**). Yellow crystalline solid. Yield 82.7%; m.p. 179.6–180.0 °C; IR (ATR-Ge, cm^{-1}): 3384_m, 3189_m (-CO**N**$\mathbf{H_2}$), 1638_{vs} (-**CO**NH_2), 1551_s, 1517_s, 1462_s, 1437_s, 1191_s (pyrazine ring); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.14 (1H, d, J = 2.3 Hz, Ar), 7.75 (1H, d, J = 2.3 Hz, Ar), 7.18 (1H, bs, NH_2), 5.84 (1H, bs, NH_2), 3.54–3.39 (4H, m, NCH_2), 2.00–1.85 (4H, m, CH_2); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 168.8, 151.5, 143.6, 129.4, 129.2, 49.5, 25.4; Elemental analysis: calc. for $C_9H_{12}N_4O$ (MW 192.22): 56.24% C, 6.29% H, 29.15% N; found 56.16% C, 6.23% H, 29.11% N.

3-(Piperidin-1-ylamino)pyrazine-2-carboxamide (**15**). Light yellow crystalline solid. Yield 93.0%; m.p. 126.4–127.7 °C; IR (ATR-Ge, cm^{-1}): 3385_m, 3183_m (-CO**NH$_2$**), 1646_{vs} (-**CO**NH$_2$), 1552_s, 1518_s, 1484_{vs}, 1440_{vs}, 1183_{vs} (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.13 (1H, d, J = 2.1 Hz, Ar), 7.92 (1H, bs, NH_2), 7.82 (1H, d, J = 2.3 Hz, Ar), 7.51 (1H, bs, NH_2), 3.45–3.33 (4H, m, NCH_2), 1.64–1.42 (6H, m, CH_2); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 168.9, 153.0, 142.2, 135.9, 131.1, 48.2, 25.4, 24.1; Elemental analysis: calc. for $C_{10}H_{14}N_4O$ (MW 206.24): 58.24% C, 6.84% H, 27.17% N; found 58.29% C, 6.77% H, 27.37% N.

3-(Morpholin-4-yl)pyrazine-2-carboxamide (**16**). Light yellow crystalline solid. Yield 91.3%; m.p. 146.1–147.5 °C; IR (ATR-Ge, cm^{-1}): 3330_m, 3184_m (-CO**NH$_2$**), 1655_{vs} (-**CO**NH$_2$), 1609_m, 1554_s, 1519_m, 1484_{vs}, 1436_s, 1109_{vs} (pyrazine ring); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.20 (1H, d, J = 2.3 Hz, Ar), 7.91 (1H, d, J = 2.3 Hz, Ar), 7.49 (1H, bs, NH_2), 5.87 (1H, bs, NH_2), 3.82 (4H, t, J = 4.7 Hz, OCH_2), 3.54 (4H, t, J = 4.7 Hz, NCH_2); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 167.8, 154.4, 143.6, 132.3, 131.3, 66.7, 49.0; Elemental analysis: calc. for $C_9H_{12}N_4O_2$ (MW 208.22): 51.92% C, 5.81% H, 26.91% N; found 51.80% C, 5.91% H, 26.83% N.

3-(4-Methylpiperazin-1-yl)pyrazine-2-carboxamide (**17**). Sandy yellow crystalline solid. Yield 50.0%; m.p. 168.8–170.5 °C (decomp.); IR (ATR-Ge, cm^{-1}): 3374_m, 3190_m (-CO**NH$_2$**), 1647_{vs} (-**CO**NH$_2$), 1552_s, 1519_m, 1492_{vs}, 1440_s, 1186_s (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.16 (1H, d, J = 2.3 Hz, Ar), 7.97 (1H, bs, NH_2), 7.88 (1H, d, J = 2.3 Hz, Ar), 7.56 (1H, bs, NH_2), 3.42 (4H, t, J = 5.0 Hz, NCH_2), 2.35 (4H, t, J = 5.0 Hz, NCH_2), 2.18 (3H, s, CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 168.8, 152.8, 142.3, 136.1, 131.8, 54.5, 47.0, 46.0; Elemental analysis: calc. for $C_{10}H_{15}N_5O$ (MW 221.26): 54.28% C, 6.83% H, 31.65% N; found 54.19% C, 6.84% H, 31.48% N.

3-(Piperazin-1-yl)pyrazine-2-carboxamide (**18**). Yellow crystalline solid. Yield 65.8%; m.p. decomp.; IR (ATR-Ge, cm^{-1}): 3389_m (-NH-), 3293_m, 3179_m (-CO**NH$_2$**), 1678_{vs} (-**CO**NH$_2$), 1593_s, 1559_s, 1527_m, 1464_s, 1433_s, 1159_s (pyrazine ring); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.75 (1H, bs, NH), 8.26 (1H, bs, Ar), 8.08 (1H, bs, NH_2), 8.02 (1H, bs, Ar), 7.67 (1H, bs, NH_2), 3.68–3.59 (4H, m, NCH_2), 3.16–3.07 (4H, m, NCH_2); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 168.2, 152.6, 142.8, 136.0, 133.4, 44.5, 42.5; Elemental analysis: calc. for $C_9H_{13}N_5O$ (MW 207.23): 52.16% C, 6.32% H, 33.79% N; found 52.19% C, 6.31% H, 33.59% N.

3.4. Lipophilicity HPLC Determination and Calculations

Experimental lipophilicity parameter log$k$ was ascertained using an Agilent Technologies 1200 SL liquid chromatography HPLC system with a SL G1315C Diode-array Detector, chromatographic pre-column ZORBAX XDB-C18 5 μm, 4 × 4 mm, Part No. 7995118-504 and column ZORBAX Eclipse XDB-C18 5 μm, 4.6 × 250 mm, Part No. 7995118-585 (Agilent Technologies Inc., Colorado Springs, CO, USA) were used. The separation process was controlled by Agilent ChemStation, version B.04.02 extended by spectral module (Agilent Technologies Inc.). A solution of MeOH (HPLC grade, 70%) and H_2O (HPLC-Milli-Q Grade, 30%) was used as mobile phase. The total flow of the column was 1.0 mL/min, injection 20 μL, column temperature 30 °C. 210 nm as detection wavelength and 270 nm as monitor wavelength were chosen. The KI methanol solution was used for the dead time (T_D)

determination. Retention times (T_R) of synthesized compounds were measured in minutes. The capacity factors *k* were calculated using Microsoft Excel according to formula $k = (T_R - T_D)/T_D$, where T_R is the retention time of the solute and T_D denotes the dead time obtained via an unretained analyte. Log*k*, calculated from the capacity factor *k*, is used as the lipophilicity index converted to log*P* scale.

3.5. Biological Assays

3.5.1. Antimycobacterial *in Vitro* Screening

Mycobacterial screening was performed against *M. tuberculosis* H37Rv CNCTC My 331/88 (Czech National Collection of Type Cultures, National Institute of Public Health, Prague, Czech Republic) using isoniazid and pyrazinamide (Sigma-Aldrich) as standards. Culturing medium was Middlebrook 7H9 broth (Sigma-Aldrich) with the addition of glycerol (Sigma-Aldrich) and OADC supplement (Himedia, Mumbai, India). Tested compounds were dissolved in dimethylsulfoxide (DMSO) and diluted with medium to final concentrations 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 μg/mL. The method used for this assay was microdilution broth panel method. The final concentration of DMSO did not exceed 1% (v/v) and did not affect the growth of mycobacteria. The cultures were grown in Middlebrook 7H9 medium at 37 °C in humid dark atmosphere. The antimycobacterial activity was determined using Alamar Blue colouring after 14 days of incubation as MIC (μg/mL). This evaluation was done in cooperation with Department of Clinical Microbiology, University Hospital in Hradec Kralove (Hradec Kralove, Czech Republic).

3.5.2. Antifungal and Antibacterial *in Vitro* Screenings

Antibacterial evaluation was made using the microdilution broth method in plates M27A-M1 (200 + 10) against 8 bacterial stems from Czech Collection of Microorganisms (Brno, Czech Republic) or clinical isolates from Department of Clinical Microbiology, University Hospital in Hradec Kralove (*Staphylococcus aureus* CCM 4516/08, *Staphylococcus aureus* H 5996/08 methicillin resistant, *Staphylococcus epidermidis* H 6966/08, *Enterococcus* sp. J 14365/08, *Escherichia coli* CCM 4517, *Klebsiella pneumoniae* D 11750/08, *Klebsiella pneumoniae* J 14368/05 ESBL positive, *Pseudomonas aeruginosa* CCM 1961). Mueller Hinton broth was used for the cultivation that was done in humid atmosphere and by 35 °C. The readings were made after 24 and 48 h and MIC was set as 80% inhibition of control. The standards were neomycin, bacitracin, penicillin G, ciprofloxacin and phenoxymethylpenicillin [43].

Antifungal evaluation was also accomplished with microdilution broth method. On the contrary, there was used RPMI 1640 broth with glutamine as medium and conditions were humid and dark atmosphere, pH 7.0 (adjusted with 0.165 M morpholinepropanesulfonic acid, MOPS) and 35 °C. 8 fungal strains were used (*Candida albicans* ATCC 44859, *Candida tropicalis* 156, *Candida krusei* E28, *Candida glabrata* 20/I, *Trichosporon asahii* 1188, *Aspergillus fumigatus* 231, *Lichtheimia corymbifera* 272, *Trichophyton mentagrophytes* 445) together with four antimycotic standards amphotericin B, voriconazole, nystatin and fluconazole. The MIC was set as 80% inhibition of control and readings were made after 24 and 48 h (50% IC, 72 and 120 h for filament fungi) [44].

3.5.3. Study of Photosynthetic Electron Transport Inhibition

The inhibition of photosynthetic electron transport (PET) in spinach chloroplasts was determined spectrophotometrically (Genesys 6, Thermo Scientific, Madison, WI, USA) using an artificial electron acceptor 2,6-dichlorophenol-indophenol (DCPIP) [45]. The rate of photosynthetic electron transport was monitored as a photoreduction of DCPIP. Chloroplasts were prepared from *Spinacia oleracea* L. [46]. The measurements were carried out in phosphate buffer (0.02 mol/L, pH 7.2) containing sucrose (0.4 mol/L), $MgCl_2$ (0.005 mol/L) and NaCl (0.015 mol/L). The chlorophyll concentration was 30 mg/L and the samples were irradiated (~100 W/m^2 with 10 cm distance) with a halogen lamp (250 W). The 4 cm wide water filter was used to prevent warming of the suspension so the temperature did not exceed 22 °C. The studied compound were dissolved in DMSO due to their limited water solubility. The applied DMSO concentration (up to 4%) did not affect the photochemical activity in spinach chloroplasts. The efficiency of studied compounds was expressed as 50% inhibition concentration relative to the untreated control. The standard for these measurements was DCMU (Diurone®).

The emission fluorescence spectra of aromatic amino acids were recorded on the fluorescence spectrophotometer F-2000 (Hitachi, Tokyo, Japan). The samples of chloroplast suspensions with and without studied inhibitor were excited at wavelength of 275 nm, excitation slit 20 nm and emission slit 10 nm. The samples were kept in the dark for 2 min prior to the measurement. The phosphate buffer used for dilution of the chloroplast suspension was the same as described above. The compounds were added to chloroplast suspension in DMSO solution due to low aqueous solubility. The DMSO concentration in all samples was the same as in the control (10%). The chlorophyll concentration in chloroplast suspension was 10 mg/L.

4. Conclusions

Eighteen *N*-substituted 3-aminopyrazine-2-carboxamides were synthesized in this work with the application of focused field microwave technology. The reaction conditions, which were set experimentally, lead to higher yields and shorter reaction times compared to the conventional heating method. The difference between these conditions is obvious. The temperature reached during the reactions is much higher than the boiling point of methanol and toluene under normal conditions. Also the time is at least two times shorter. All the compounds were purified and then their structure was confirmed by NMR and IR spectra and they were then tested for their potential biological activities.

The experimentally measured lipophilicity was compared to the CS ChemBioDraw Ultra 13.0 program predicted values. The dependence between log*k* and log*P* was linear with one exception where the effect of ionization is appreciable.

The majority of compounds did not show antimycobacterial activity against *M. tuberculosis*. There were only four compounds (**6**–**9**) with very low activity that were characterized by long or ramified side aliphatic side chain which is probably better for penetration into mycobacterial cell. But the values were significantly lower than the values of standards so it is not possible to predict any structure-activity relationships.

Quite interesting results were found in antifungal assays. Three compounds (**11**–**13**) showed activities against whole tested fungal spectrum and the activity of compounds **12** and **13** was also

better than that of a fluconazole standard. Although there were not enough active compounds in the antibacterial and antifungal screenings, we can propose a hypothesis that the side alicyclic chain is necessary for antifungal activity and that the efficacy rises with increasing number of carbons in the alicyclic ring, *i.e.*, increasing lipophilicity.

PET inhibition screening showed some interesting consequences. There were nine compounds active and activity of the most active substance (compound **9**) expressed by IC_{50} value was 14.3 μmol/L. A quasi-parabolic course was observed for the dependence of PET inhibiting activity on the lipophilic properties of substituents and side chain of the most effective compounds had seven or eight carbons. The active compounds were found to be PS2 inhibitors acting on the donor side of photosystem 2.

Based on the obtained results it can be concluded that the biological activity of the compounds is affected by the presence of secondary amine group bounded to pyrazine ring. If this -NH- fragment is missing, the efficacy would also disappear. This phenomenon can be seen in both evaluations.

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Author Contributions

Ondrej Jandourek, Jan Zitko and Martin Dolezal designed the compounds and the overall study. Ondrej Jandourek carried out the syntheses of final products. Vladimir Kubicek measured the lipophilicity of compounds. Jiri Kunes recorded and interpreted the NMR spectra. Pavla Paterova and Vladimir Buchta carried out the antimycobacterial evaluation. Matus Pesko and Katarina Kralova tested compounds for PET inhibition activity and interpreted the results.

Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are available from the authors.

PŘÍLOHA Č. 6

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Alkylamino derivatives of *N*-benzylpyrazine-2-carboxamide: synthesis and antimycobacterial evaluation†

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A series of alkylamino derivatives of *N*-benzylpyrazine-2-carboxamide was designed, synthesized and assayed *in vitro* for their antimycobacterial, antibacterial, antifungal as well as antiviral activities. Final structures were prepared from 6-chloro (**1**), 5-chloro (**2**) or 3-chloro (**3**) derivatives of *N*-benzylpyrazine-2-carboxamide by nucleophilic substitution of chlorine with *n*-alkylamines in the range from butylamine to octylamine (labelled **a**–**e**). Series **1a**–**e** and **2a**–**e** exerted higher activity against *Mycobacterium tuberculosis* H37Rv compared to the corresponding pattern compounds and the reference compound pyrazinamide. The most active derivatives reached an activity MIC = 4.6–10 μM (*M. tbc* H37Rv). More importantly, activity was also observed against other tested mycobacterial strains (including drug-resistant strains). Substitution of 3-chlorine was disadvantageous and led to completely inactive compounds **3a**–**e**. Some compounds showed activity against Gram-positive bacterial strains (including MRSA) or influenza virus, but no antifungal activity was observed.

Introduction

Even in the 21st century, tuberculosis (TB) still remains a serious and global health threat. The absolute number of TB cases per year has been slightly decreasing since the beginning of this millennium,[1,2] nevertheless in 2013 about 9 million new cases of TB were reported and associated with 1.5 million deaths.[3] The alarming increase of drug-resistant strains underlines the need for new antituberculosis drugs.

One strategy to design potential new drugs is by structural-modification of known and therapeutically used drugs. Pyrazinamide (PZA) is a first-line antituberculotic drug[4] with multiple mechanisms of action. It acts under its parent form or as a prodrug metabolized to pyrazinoic acid (POA).[5–8] One of the confirmed mechanisms of action for both PZA and POA is the inhibition of mycobacterial fatty acid synthase I (FAS I), which leads to depletion of mycolic acid-essential components of the mycobacterial cell wall.[9] Due to its simple structure, the PZA scaffold is, in theory, amenable to many diverse structural modifications.

Recently we reported that the antimycobacterial activity is enhanced by *n*-alkylamino substitution of the pyrazine.[10–13] As Zitko *et al.* stated, this type of substitution also led to less toxic compounds compared to pattern chloropyrazine derivatives.[13] To confirm this hypothesis, a series of 6-alkylamino-*N*-benzylpyrazine-2-carboxamides (**1a**–**e**) and 5-alkylamino-*N*-benzylpyrazine-2-carboxamides (**2a**–**e**) were designed and synthesized from their respective chloro-*N*-benzylpyrazine-2-carboxamides (**1**, **2**),[14] which in our previous study exerted moderate antimycobacterial activity (MIC = 50–100 μM) against *Mycobacterium tuberculosis* H37Rv.[14] To fully understand the relationship between the position of the alkylamino chain and antimycobacterial activity, a series of 3-alkylamino-*N*-benzylpyrazine-2-carboxamides (**3a**–**e**) was prepared as well.

All compounds were evaluated for activity against four standard mycobacterial strains and seven drug-resistant strains of *Mycobacterium tuberculosis*. Additionally, their antibacterial, antifungal and antiviral activities were determined.

Triclosan (TCL) and its derivatives are known inhibitors of mycobacterial enoyl-ACP-reductase (InhA).[15–17] InhA belongs to the complex of fatty acid synthase II (FAS II) and is one of

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† Electronic supplementary information (ESI) available: Detailed synthetic procedures, the analytical data of presented compounds as well as the biological methods and the docking procedure are available in the ESI. See DOI: 10.1039/c5md00178a

the crucial enzymes involved in the biosynthesis of mycolic acids (modification of fatty acids produced by FAS I).[18,19] With respect to the alkylamino derivatives presented in this letter, it is very interesting that the 5-alkyl diphenyl ethers (Fig. 1) with a C_4 to C_8 alkyl chain possessed significantly lower IC_{50} values in the InhA *in vitro* enzyme inhibition assay. Their inhibitory activity increased with growing alkyl chain and 5-octyl-2-phenoxyphenol possessed an IC_{50} of 5 nM, which is a major improvement over TCL (IC_{50} = 1000 nM).[20] The structural similarity between the 5-alkyl diphenyl ethers (as derivatives of TCL) and the 5-alkylamino-*N*-benzylpyrazine-2-carboxamides (**2a–e**) presented in this article raised the question whether our compounds could possess the same mechanism of action as TCL and its alkyl derivatives, *i.e.* based on inhibition of InhA. To test this hypothesis, we performed molecular docking of selected compounds into mycobacterial InhA.

Results and discussion

Chemistry

The pattern compounds **1** and **2** were described previously,[14] nevertheless the synthesis of compound **1** was modified. To increase the reaction yield, Schotten–Bauman conditions were applied. Excess of benzylamine was dissolved in water and added portionwise to 6-chloropyrazine-2-carbonyl chloride in dichloromethane. The reaction mixture was stirred for about 4 hours at RT. A portion of originating *N*-benzyl-6-chloropyrazine-2-carboxamide (**1**) reacted further with excess of benzylamine and yielded *N*-benzyl-6-benzylaminopyrazine-2-carboxamide (**1′**, Scheme 1) as an unintended side-product. Its structure was confirmed by NMR and MS analysis. Formation of this type of side-product was also observed during the synthesis of pattern compound **3**, for which the synthetic procedure is thoroughly explained in the ESI.†

The syntheses of compounds **1a–e** and **2a–e** were performed *via* a aminodehalogenation reaction, where corresponding pattern compound **1** or **2** was treated with 5 equivalents of the respective amine using triethylamine as a base (Scheme 2A). The reaction mixture was refluxed in a small amount of ethanol up to 8 hours as indicated by TLC (silica, hexane–EtOAc 2 : 1). The synthesis of final compounds **3a–e** was accelerated by microwave irradiation. Microwave conditions were determined experimentally in previous research,[21] (Scheme 2B).

The crude products were absorbed on silica gel and purified by flash-chromatography (gradient elution, hexane–EtOAc). To remove residual non-aromatic amine, derivatives **1a–2e** were recrystallized from ethanol. Compounds **1a–e** and **2a–e** were isolated as white solids, compounds **3a–e** as yellow liquids. The analytical data, which were fully consistent with the proposed structures, are included in the ESI.† The yields (chromatographically pure product) ranged from 37% to 80%.

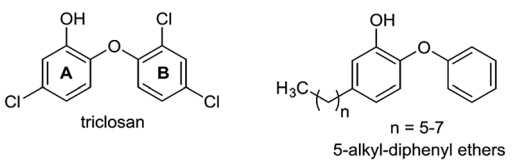
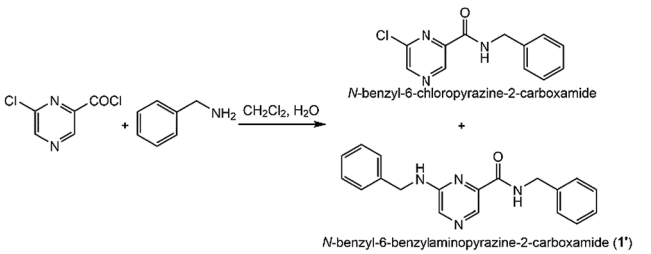


Fig. 1 Structures of triclosan (TCL) and its 5-alkyl derivatives with enoyl-ACP-reductase inhibitory activity.

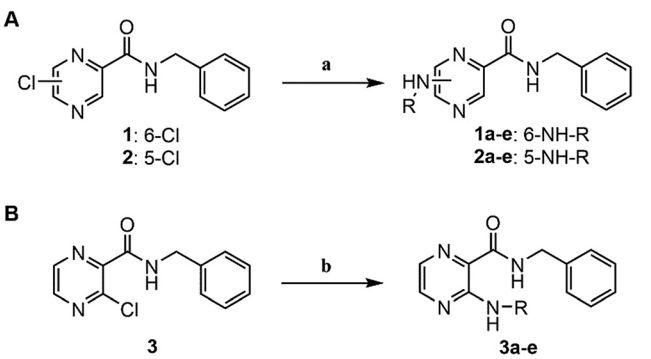


Scheme 1 Synthesis of pattern compound **1** and formation of side-product **1′**. Reaction proceeded under mild conditions – RT, 4 h. Side-product originated in the ratio 1 : 5 by substitution of chlorine on the pyrazine core with benzylamine.

Antimycobacterial activity

All the prepared compounds (including compounds **1**, **2** and **3**) as well as the clinically used standards PZA and isoniazid (INH) were evaluated using the microplate alamar blue assay (MABA)[22] for activity against *Mycobacterium tuberculosis* H37Rv (*Mtb*), *M. kansasii* and two strains of *M. avium*. The results were expressed as minimal inhibitory concentration (MIC) in $\mu g\ mL^{-1}$ or μM (data in parentheses), Table 1. Both tested strains of *Mycobacterium avium* were completely resistant to the tested compounds (MIC >100 $\mu g\ mL^{-1}$). Comparing the results in μM, alkylamino derivatives **1a–e** and **2a–e** showed higher activity against *Mtb* H37Rv than the corresponding pattern compounds **1** (MIC = 50 μM) and **2** (MIC = 100 μM). For the most active derivatives **1d**, **1e** and **2c-e** (MIC = 4.6–10 μM), the activity was up to 20-times better compared to the clinically used drug PZA (MIC = 102 μM).

More importantly, compounds **1a–e** derived from *N*-benzyl-6-chloropyrazine-2-carboxamide (**1**) exhibited activity against *Mycobacterium kansasii* (naturally resistant to PZA,



Scheme 2 Syntheses of target structures. (A): Pattern compound **1** or **2** refluxed in EtOH with corresponding *n*-alkylamine up to 8 h, triethylamine (TEA) was used as a base (conditions **a**). (B): Microwave assisted syntheses of final compounds **3a–e**. Conditions **b**: 140 °C, 30 minutes, 120 W, MeOH, pyridine.

Table 1 Summary of prepared compounds. *In vitro* antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv (*Mtb*) and *Mycobacterium kansasii*, MIC in μg mL^{-1} or μM (data in parentheses). Cytotoxicity provided on different types of cells, expressed as CC_{50} or MCC in μM. Anti-influenza virus activity and cytotoxicity provided on MDCK cells, values in μM

			MIC μg mL^{-1} (μM)		Cytotoxicity (μM)				Antiviral activity (μM)			
									MDCK[e] cytotoxicity		Antiviral EC_{50}[f] Influenza A/H1N1 (A/PR/8)	
No.	Log *k*	R	*Mtb* H37Rv	*M. kansasii*	CRFK[a] CC_{50}	HEL[b] MCC	HeLa[c] MCC	Vero[d] MCC	CC_{50}	MCC	Visual CPE score	MTS
1	0.200	—	12.5 (50)	100 (404)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1′	0.483	6-Benzyl	50 (157)	>100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1a	0.549	6-C_4H_9	12.5 (44)	50 (176)	>100	>100	>100	100	66	100	>100	>100
1b	0.768	6-C_5H_{11}	12.5 (42)	25 (84)	>100	>100	>100	>100	33	20	>100	>100
1c	0.986	6-C_6H_{13}	6.25 (20)	12.5 (40)	>100	>100	>100	100	9.5	20	>100	>100
1d	1.207	6-C_7H_{15}	3.13 (10)	12.5 (38)	>100	>100	>100	>100	2.4	4.0	>100	>100
1e	1.431	6-C_8H_{17}	3.13 (9.2)	6.25 (18)	>100	>100	>100	>100	1.3	4.0	>100	>100
2	0.186	—	25 (100)	100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2a	0.424	5-C_4H_9	25 (88)	>100	>100	>100	>100	>100	57	100	>100	>100
2b	0.638	5-C_5H_{11}	6.25 (21)	>100	>100	>100	>100	>100	>100	>100	37	3.3
2c	0.859	5-C_6H_{13}	3.13 (10)	>100	>100	>100	>100	≥100	>100	>100	52	62
2d	1.089	5-C_7H_{15}	3.13 (10)	>100	>100	>100	>100	≥100	>100	>100	21	47
2e	1.312	5-C_8H_{17}	1.56 (4.6)	>100	28	100	100	20	62	≥20	1.1	>100
3	n.d.	—	12.5 (50)	100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3′	1.040	3-Benzyl	>100	>100	>100	>100	>100	>100	>100	20	>100	>100
3a	1.081	3-C_4H_9	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
3b	1.321	3-C_5H_{11}	>100	>100	>100	≥100	>100	>100	>100	≥20	>100	>100
3c	1.564	3-C_6H_{13}	>100	>100	>100	100	>100	>100	>100	100	>100	>100
3d	1.809	3-C_7H_{15}	>100	>100	>100	100	>100	>100	>100	100	>100	>100
3e	2.054	3-C_8H_{17}	>100	>100	>100	>100	>100	>100	>100	100	>100	>100
PZA	−0.687		12.5 (102)	>100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
INH	−0.743		0.39 (2.8)	1.56 (11)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Log *k* stated as average of $n = 3$, SD values were negligible, relatively ranging from 0.01 to 0.19%.[a] Crandell feline kidney cells. [b] Human embryonic lung fibroblasts. [c] Human cervix epithelial cells. [d] African green monkey kidney cells. [e] Madin canine kidney cells. [f] EC_{50} – concentration producing 50% inhibition of virus-induced cytopathic effect (CPE), as determined by visual scoring of the CPE or by measuring the cell viability with colorimetric formazan-based MTS assay. n.d. not done. MCC – compound concentration producing minimal changes in cell morphology estimated by the MTS cell viability assay.

MIC > 100 μg mL^{-1}), while 5-alkylamino isomers (**2a–e**) as well as 3-alkylamino isomers (**3a–e**) were completely inactive (MIC > 100 μg mL^{-1}). Actually, compounds **3a–e** derived from *N*-benzyl-3-chloropyrazine-2-carboxamide (**3**) did not exhibit activity against any tested mycobacterial strain.

In concordance with previously published studies on alkylamino derivatives of PZA,[10–13] the activity of 6-alkylamino (**1a–e**) and 5-alkylamino (**2a–e**) isomers depended on the length of the alkyl chain and culminated in compounds with hexyl- to octylamino substitution. Chart 1 shows the correlation between lipophilicity (expressed as log *k*) and antimycobacterial activity against *Mtb* (for compounds **1a–e** and **2a–e**) and *M. kansasii* (for compounds **1a–e**).

All alkylamino derivatives **1a–e**, **2a–e** and **3a–e** were evaluated for activity against resistant strains of *Mycobacterium tuberculosis*. As shown in Table 2, the 6- and 5-alkylamino derivatives exhibited activity, which was again dependent on the length of the alkyl chain, *i.e.* compounds with a C_6–C_8 chain (labelled **c–e**) exhibited the highest activity. Examples of correlation between lipophilicity and activity against resistant strains are included in the ESI.† The 5-isomers (**2a–e**) showed higher activity compared to their respective 6-isomers (**1a–e**). Poor activity was observed for compound **3a** (*N*-benzyl-3-butylpyrazine-2-carboxamide), while the rest of the 3-alkylamino derivatives were inactive (MIC ≥ 1000 μM).

Simoes *et al.*[23] reported a series of amides of pyrazinoic acid, which exhibited very slow hydrolysis in the plasma, rat

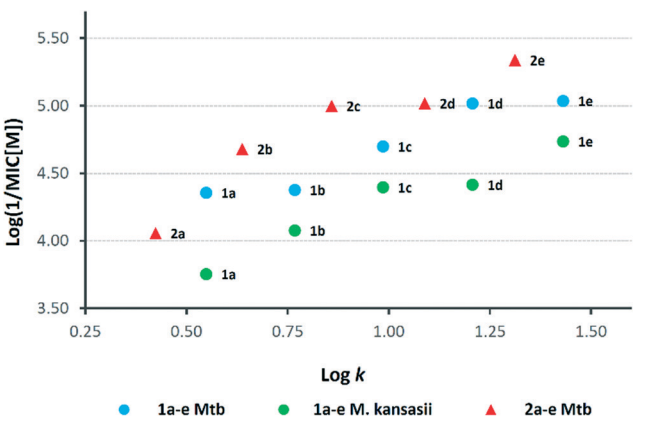


Chart 1 Correlation between antimycobacterial activity and lipophilicity expressed as log *k*.

Table 2 Antimycobacterial activity against drug-resistant strains, minimal inhibitory concentrations in μM

MIC (μM)

		Mtb 7357/1998		*Mtb* 234/2005		*Mtb* 9449/2007		*Mtb* 8666/2010		*Mtb* Praha 1		*Mtb* Praha 4		*Mtb* Praha131	
No.	R	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d
1a	6-C_4H_9	62.5	62.5	62.5	62.5	62.5	125	62.5	62.5	62.5	62.5	32	62.5	32	62.5
1b	6-C_5H_{11}	32	62.5	62.5	62.5	32	62.5	62.5	62.5	62.5	62.5	32	62.5	32	62.5
1c	6-C_6H_{13}	32	32	16	32	32	32	16	32	16	32	32	32	16	32
1d	6-C_7H_{15}	16	16	16	16	16	16	16	16	16	16	8	16	8	16
1e	6-C_8H_{17}	16	16	16	16	16	16	16	16	16	16	16	16	16	16
2a	5-C_4H_9	125	125	125	250	62.5	125	125	250	125	250	62.5	125	62.5	125
2b	5-C_5H_{11}	16	32	16	32	16	32	16	32	16	32	16	32	16	16
2c	5-C_6H_{13}	8	8	4	8	8	8	4	8	4	8	8	8	4	4
2d	5-C_7H_{15}	4	8	4	4	4	8	4	4	4	4	4	8	2	4
2e	5-C_8H_{17}	8	8	4	8	8	8	4	8	4	8	8	8	4	4
3a	3-C_4H_9	125	250	125	250	125	250	125	250	125	125	125	125	125	250
3b	3-C_5H_{11}	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$
3c	3-C_6H_{13}	10^3	$>10^3$	10^3	$>10^3$	10^3	10^3	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$
3d	3-C_7H_{15}	10^3	10^3	10^3	$>10^3$	$>10^3$	$>10^3$	10^3	$>10^3$	10^3	$>10^3$	$>10^3$	$>10^3$	10^3	$>10^3$
3e	3-C_8H_{17}	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$
INH		16	32	16	16	16	16	16	32	16	16	16	16	16	16

MDR-TB strains: 234/2005 and 7357/1998 both resistant to INH, rifampicin (RIF), rifabutin, streptomycin, ethambutol and ofloxacin; Praha 1 resistant to INH, RIF, rifabutin, streptomycin, ethambutol and clofazimine; 8666/2010 resistant to INH, RIF, rifabutin; 9449/2007 and Praha 4 both resistant to INH, RIF, rifabutin, ethambutol and streptomycin. XDR-TB strain: Praha 131 resistant to INH, RIF, rifabutin, streptomycin, ethambutol, ofloxacin, gentamicin and amikacin.

liver homogenate and were even stable in *M. smegmatis* homogenate. These derivatives failed in antimycobacterial testing and Simoes *et al.*[23] assumed that the lack of activity is caused by insufficient rate of hydrolysis to POA. Our derivatives are sterically more demanding (large substituent on the carboxamide moiety) than the simple amides tested by Simoes *et al.*,[23] therefore their stability (resistance to amidases) is expected to be even higher. According to the results[23] and previously published studies,[12,24] we do not expect that the presented derivatives are hydrolyzed to their corresponding pyrazinecarboxylic acids.

Antibacterial and antifungal activities

The studied compounds were evaluated against eight bacterial and eight fungal strains (see the ESI† for the complete list of tested strains). All fungal strains as well as Gram-negative strains were completely insensitive to the tested compounds (MIC > 500 μM). Compounds **1a–e** exhibited moderate or weak activity against Gram-positive strains including methicillin-resistant *Staphylococcus aureus*. Notably, compound **1e** (*N*-benzyl-6-octylpyrazine-2-carboxamide) displayed activity against *S. aureus* (MIC = 3.9 μM) comparable to the reference compounds (see the ESI†).

Toxicity assay

In vitro cytotoxicity[25–27] assays on several cell lines were performed for compounds **1a–e**, **2a–e** and **3a–e**. The results (Table 1) were expressed as the concentration causing minimal changes in cell morphology (MCC) or as 50% cytotoxic concentration (CC_{50}) – a concentration reducing cell viability by 50% as assessed by a colorimetric formazan assay. Except for compound **2e**, the tested compounds were not cytotoxic in CRFK, HEL, HeLa and Vero cell lines at the highest concentration tested, *i.e.* 100 μM.

Antiviral activity

In addition, we determined whether any of the studied compounds has potential activity against diverse DNA and RNA viruses. The virus panel (see the ESI† for the full list) included pathogens of medical importance such as herpesviruses, HIV and influenza virus. Most compounds did not produce any visible antiviral activity. The notable exception was series **2b–e** which was moderately active against the influenza virus, with **2e** being the most potent one (Table 1). The latter compound also inhibited the replication of respiratory syncytial virus (data not shown) with an antiviral EC_{50} value of 8.9 μM. The basis for the antiviral effect of **2b–e** remains to be identified.

Docking

To perform the docking studies, we selected the derivatives with the highest antimycobacterial activity in the whole cell assay, *i.e.* hexylamino to octylamino derivatives (**1c-e**, **2c–e**). We included their corresponding 3-alkylamino isomers (**3c–3e**) to test the influence of the position of the alkylamino chain on the docking results. Out of the large number of crystal structures of InhA available from the Protein Data Bank, we chose PDB entry: 2X23. This structure is a closed form of the enzyme co-crystalized with inhibitor PT70, which is a

slow and tight-binding inhibitor with an alkyl diphenyl ether structure.[28]

To verify the docking procedure, the co-crystallized ligand (PT70) from the PDB structure was removed and redocked using Glide XP mode docking protocol with flexible sampling of ligand. The RMSD of the core atoms of the ligand (omitting the atoms of the flexible alkyl chain) was approx. 0.26 Å. According to the results of the molecular docking (Fig. 2A), the 6-alkylamino-*N*-benzylpyrazine-2-carboxamides (**1c–e**) and 5-alkylamino-*N*-benzylpyrazine-2-carboxamides (**2c–e**) may exert interactions typical for TCL-based inhibitors of mycobacterial enoyl-ACP-reductase InhA.[20,28] The carboxamide oxygen of the presented compounds plays the same role as the phenolic oxygen of TCL, *i.e.* to act as an H-bond acceptor, forming interactions with the –OH group of Tyr158 and the 2′-hydroxyl of the ribose of NAD^+. The pyrazine core of the title compounds is oriented similarly to the phenol aromatic ring of the TCL derivatives (the so called A-ring) and shows a π–π stacking interactions with the nicotinamide core of NAD^+, and π–π edge-to-face interactions with Phe149. The benzyl core of the discussed derivatives occupies the same hydrophobic cavity as the B-ring of the TCL derivatives. The planes of the (hetero)aromatic rings of the title compounds are almost identical to the corresponding planes of PT70 (Fig. 2B). The alkylamino chain is placed in the tunnel leading to the enzyme's surface, in the same manner as the alkyl chain of PT70 and similar TCL derivatives. This hydrophobic entry tunnel hosts the lipophilic chain of the mycolic acid intermediate, which is the substrate of the InhA enzyme. The docking scores of the presented compounds (see the ESI†) were close to the score of the co-crystallized ligand PT70 (the best score was predicted for **1e**, XP GScore = −9.705; the XP GScore of PT70 was −10.543).

On the contrary, the 3-alkylamino derivatives **3c–e** were not able to orient inside the cavity of the active site in a manner similar to PT70, and had low docking scores (XP GScore from −3.692 for **3c** to −6.175 for **3e**). Apparently, the presence of two large substituents (benzyl and alkylamino chain) on neighbouring atoms C2 and C3 of the pyrazine core leads to a molecular shape that is not compatible with the cavity.

To summarize, compounds **1c–e** and **2c–e** showed all important ligand–enzyme interactions of triclosan and therefore could be potential inhibitors of InhA. This hypothesis was tested *via* analysis of mycolic acid production in the strain of *M. tuberculosis* H37Ra treated with compounds **1d** and **2e** as described in the following paragraph. These derivatives were chosen according to their MIC values for *M. tuberculosis* H37Rv and selectivity to the mycobacterial strains. The XP GScore was not taken as the main criterion for selection, having in mind that derivatives with longer alkyl chain will have a higher score caused mainly by non-specific hydrophobic interactions with the enzyme. Compound **1e** (reaching the highest XP GScore) was excluded from the screening due to low selectivity (antibacterial and antiviral activities).

Effect on mycolic acid production

The effect of compounds **1d** and **2e** on mycolic acid synthesis was evaluated by metabolic labelling of *Mycobacterium tuberculosis* H37Ra with ^{14}C acetate. Derivatized radiolabeled fatty/mycolic acids were separated by thin layer chromatography (TLC) and visualized by autoradiography. Isoniazid (INH)-inhibiting mycobacterial enoyl-ACP-reductase (InhA)[29] was used as a control drug. As expected, the treatment of *Mtb* with INH led to the inhibition of the synthesis of all types of mycolic acids and the production of short chain fatty acids was not affected. The compounds **1d** and **2e** did not affect mycolic acid synthesis (Fig. 3).

Lipophilicity

Lipophilicity parameter $C\log P$ was calculated by CS ChemBioDraw Ultra version 14.0. (CambridgeSoft, Cambridge, MA, USA). Additionally, the lipophilicity was measured experimentally by RP-HPLC and expressed as $\log k$ derived from the retention times of individual compounds. Correlation between the calculated $C\log P$ and the experimentally determined $\log k$ values showed linearity inside the series of positional isomers. As discussed in the ESI,† the $C\log P$ algorithm did not correctly reflect the influence of the position of the alkylamino substituent on a compound's lipophilicity. Therefore $\log k$ is more useful for interseries comparisons and is used as the main lipophilicity parameter in this manuscript.

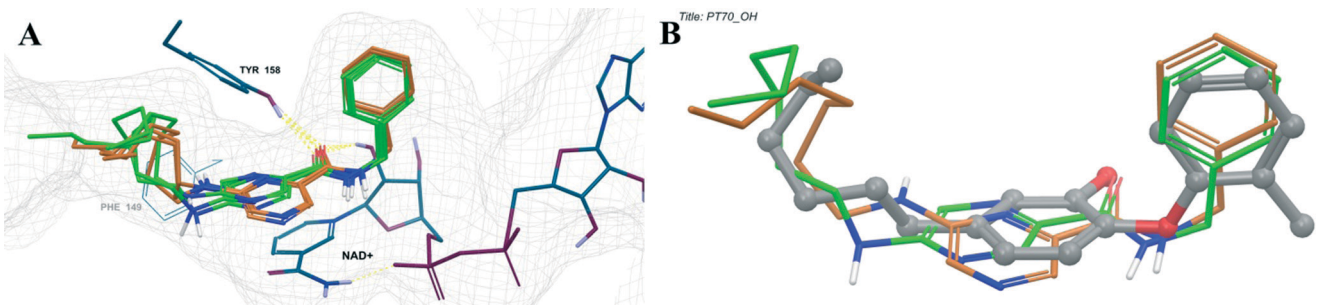


Fig. 2 (A) Compounds **1c–e** (orange carbons) and **2c–e** (green carbons) docked into the active site of mycobacterial enoyl-ACP-reductase (InhA, PDB: 2X23). (B) Predicted poses of **1e** (orange carbons) and **2e** (green carbons) in comparison with the co-crystallized inhibitor PT70 (grey balls and sticks).

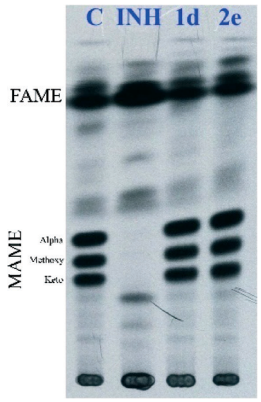


Fig. 3 Analysis of the effects of **1d** and **2e** on mycolic acid synthesis. The autoradiograph of TLC with separated fatty acid methyl esters (FAME) and mycolic acid methyl esters (MAME) obtained from [1,2-^{14}C]-acetate-labelled *Mtb* H37Ra strain treated with **1d**, **2e** or INH in final concentration of 5.0 μg mL^{-1}. Line C – untreated control.

Conclusions

To conclude, substitution of 6-chlorine or 5-chlorine with *n*-alkylamino substituent yielded derivatives with comparable or increased activity against *Mycobacterium tuberculosis* H37Rv compared to the corresponding pattern compounds **1** and **2**. No significant differences between 6-alkylamino (**1a–e**) and 5-alkylamino (**2a–e**) isomers were observed. Generally, activity increased with prolongation of the alkyl chain (corresponding to the increase in lipophilicity) and culminated in compounds with heptylamino (**1d**, **2d**) and octylamino (**1e**, **2e**) substitution. The series of 6-alkylamino-*N*-benzylpyrazine-2-carboxamides (**1a–e**) also exerted activity against *Mycobacterium kansasii*, which is naturally resistant to pyrazinamide.

The 6-alkylamino and 5-alkylamino isomers also showed activity against drug-resistant strains of *Mycobacterium tuberculosis* culminating in heptyl/octylamino derivatives, where the 5-alkylamino isomers exhibited marginally higher activity compared to the 6-isomers.

On the contrary to series **1** and **2**, substitution of chlorine with an alkylamino substituent in *N*-benzyl-3-chloropyrazine-2-carboxamide (**3**, MIC = 12.5 μg mL^{-1} for *Mtb*), led to inactive 3-alkylamino derivatives (**3a–e**).

In vitro activity of PZA is strongly dependent on pH and decreases with the increase in pH. The MIC value for PZA was in accordance with the literature.[23,30,31]

The studied compounds exhibited no antifungal activity and mostly no significant antibacterial activity. The only exception was compound **1e** (*N*-benzyl-6-octylpyrazine-2-carboxamide), showing activity against *Staphylococcus aureus* (MIC = 3.9 μM).

Side-products **1′** and **3′** occurring during the synthesis of pattern compounds **1** and **3** were evaluated for their biological activities and no significant activity was observed.

Based on the results of the *in vitro* cytotoxicity assays, we assume that the presented derivatives are non-toxic.

Molecular docking of compounds **1c–e** and **2c–e** suggested potential inhibition of mycobacterial enoyl-ACP-reductase. In view of these results, an *in vitro* study was performed. However, no effect on mycolic acid synthesis was observed for selected compounds **1d** and **2e**.

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Supplementary information

Alkylamino derivatives of *N*-benzylpyrazine-2-carboxamide: Synthesis and antimycobacterial evaluation.

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General

All chemicals were purchased from Sigma-Aldrich (Höhenkirchen, Germany). All organic solvents used for the synthesis were of analytical grade. The reactions were monitored using Merck Silica 60 F_{254} TLC plates (Merck, Darmstadt, Germany). Compounds were purified using automated chromatograph CombiFlash Rf (Teledyne Isco, Lincoln, NE, USA) using columns filled with Kieselgel 60, 0.040-0.063 mm (Merck, Darmstadt, Germany); gradient elution (hexane/ethyl-acetate), detection wavelength 260 nm, monitor wavelength 280 nm. NMR analysis was performed on Varian Mercury VX-BB 300 (Varian, Palo Alto, CA, USA) at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts were recorded as δ values in parts per million (ppm) and were indirectly referenced to tetramethylsilane (TMS). IR spectra were measured in ATR mode using a Ge crystal-plate on Nicolet Impact 400 (Nicolet, Madison, WI, USA). The mass spectra were recorded in the mixture of MeOH, H_2O, formic acid (80:20:0.02 v/v) using LCQ Advantage Max ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The electrospray ionisation was performed in the positive mode. Melting points were determined on Stuart SMP30 melting point apparatus (Bibby Scientific Limited, Staffordshire, UK) and are uncorrected. Yields refer to chromatographically pure products after all purification operations. The synthesis of comp. **3a-e** took place in microwave reactor with focused field CEM Discover (CEM Corporation, Matthews, NC, USA) connected with autosampler Explorer 24 (CEM Corporation, Matthews, NC, USA) and equipped with CEM's Synergy™ software for monitoring the reaction progress.

General synthetic procedures

*Synthesis of starting compound **1** and **2***

Synthesis of parent compounds **1** and **2** was described previously.[1] Briefly, for synthesis of compound **1**, 20 mmol of 6-chloropyrazine-2-carboxylic acid[2] was dissolved in anhydrous toluene, treated with thionyl chloride (3 eq., 60 mmol) and heated for about 1.5 h under reflux. Then, the excess of thionyl chloride was removed by repeated evaporation with anhydrous toluene *in vacuo*. Originated acyl chloride was directly used without any purification in the subsequent step. Benzylamine (1.5 eq., 30 mmol) and TEA (1 eq., 20 mmol) were dissolved in water and added portion-wise to acyl chloride in dichloromethane (Schotten-Baumann biphasic conditions). Reaction mixture was than stirred at RT for 4 h and monitored using TLC with hexane/ethyl acetate 2:1. Organic layer was separated, dried over anhydrous Na_2SO_4, adsorbed on silica purified using flash-chromatography. Side-product, later identified as *N*-benzyl-6-benzylaminopyrazine-2-carboxamide (**1'**), occurred during the synthesis of parent compound (**1**).

For compound **2**, 5-hydroxypyrazine-2-carboxylic acid (Sigma-Aldrich) was used as starting material for synthesis of 5-chloropyrazine-2-carbonyl chloride.[3] During the reaction with thionyl chloride, the formation of acyl chloride occurs simultaneously with the nucleophilic substitution of the hydroxyl group for chlorine. *N*,*N*-Dimethylformamide (DMF) was added to the reaction mixture as catalyst.[4] Second step of the synthesis proceeded under mild conditions: 1.5 eq. of benzylamine (MW = 107.15 g/mol) was dissolved in anhydrous acetone with TEA (1 eq.) and added drop-wise to the stirred solution of acyl chloride. The reaction mixture was stirred at RT for about 4 h and monitored using TLC with hexane/ethyl acetate 2:1 mixture as eluent. After this time small amount of silica gel was added and solvents were evaporated under reduced pressure. Adsorbed mixture was addressed to flash column chromatography.

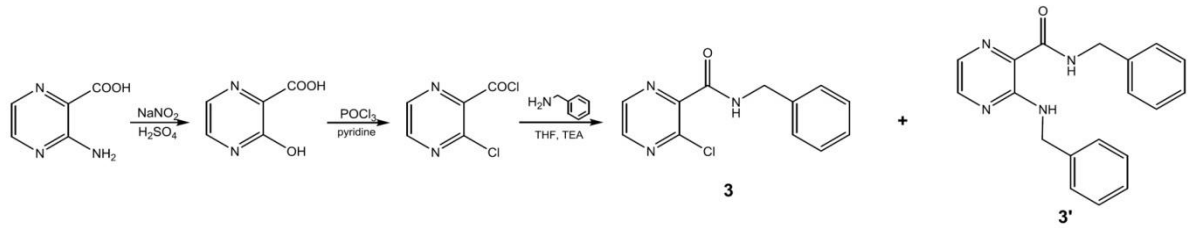
Synthesis of final compounds 1a-e and 2a-e

To synthesize final compounds, 1 mmol of *N*-benzyl-6-chloropyrazine-2-carboxamide (**1**) or *N*-benzyl-5-chloropyrazine-2-carboxamide (**2**) was dissolved in ethanol with TEA (1 eq., 1 mmol). Five molar equivalents of corresponding non-aromatic amine were added to the reaction mixture and refluxed in small amount of ethanol generally for 8 hours. The completion of the reaction was checked by TLC chromatography (eluent: hexane/ethyl acetate, 1:1). The crude product was absorbed on silica gel by solvent

evaporation and purified by flash chromatography (hexane/ethyl acetate gradient elution). To remove residual non-aromatic amine, final compounds were recrystallized from ethanol.

*Synthesis of starting compound **3***

N-benzyl-3-chloropyrazine-2-carboxamide (**3**) was synthesised in three step reaction. The first step was based on diazotation reaction. 3-Aminopyrazine-2-carboxylic acid (50 mmol) was added in small amounts into the cooled concentrated sulphuric acid (30 mL) being stirred. Then the nitrosylsulphuric acid was prepared by cooling the concentrated sulphuric acid (37.5 mL) to 0 °C and adding sodium nitrite (50 mmol) portion-wise over a period of 15 minutes. The nitrosylation mixture (0-2 °C) was added drop-wise to the cold solution of 3-aminopyrazine-2-carboxylic acid and stirred again for 15 minutes. Then mixture was poured portion-wise on cracked ice and stirred until the end of nitrogen evolution. Strongly acidic suspension was filtered by suction and the collected solid was washed with distilled water to become free of acid and then dried.[5] Crude 3-hydroxypyrazine-2-carboxylic acid was purified by recrystallization from water. Next step was the preparation of 3-chloropyrazine-2-carbonyl chloride. 3-Hydroxypyrazine-2-carboxylic acid (7 mmol) was dissolved in phosphoryl chloride (13 mL), few drops of pyridine were added and the mixture was stirred and heated to reflux for 2 hours.[6] The excess of $POCl_3$ was evaporated under reduced pressure to give 3-chloropyrazine-2-carboxylic acid chloride. Crude acyl chloride was dissolved in dry THF, and benzylamine (5 eq., 35 mmol) together with TEA (2 eq., 10 mmol) in small amount of dry THF were added portion-wise. The mixture was stirred at room temperature for 3 hours, then adsorbed on silica gel and purified by preparative flash-chromatography. As described previously for synthesis of compound **1**, side-product (**3'**), emerging during aminodehalogenation reaction where both chlorines were substituted with benzylamine, was observed (**Scheme 1**).



Scheme 1. Synthesis of parent compound **3** and its side-product **3'**.

Synthesis of final compounds 3a-e

N-benzyl-3-chloropyrazine-2-carboxamide (**3**, 1.2 mmol) was treated with respective aliphatic amine (2 eq., 2.4 mmol) to yield 3-alkylamino-*N*-benzylpyrazine-2-carboxamides. All reactions were performed in the microwave reactor with focused field. Conditions (experimentally determined in previous research)[7]: 140°C, 30 minutes, 120 W, methanol (solvent, 3 mL), pyridine (1 eq., 1.2 mmol). The progress of reaction was monitored with TLC using the system hexane/ethyl acetate (1:1). The crude product was purified with flash column chromatography (silica, gradient elution hexane/ethyl acetate).

Analytical data

N-benzyl-6-chlororpyrazine-2-carboxamide (**1**)
Analytical data in accordance with previously published results[1] (NMR spectra measured under different conditions). White solid. Yield: 63 %; m.p. 58.1-58.9 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.33 (s, 1H, H3), 8.74 (s, 1H, H5), 7.97 (bs, 1H, CONH), 7.43–7.20 (m, 5H, ArH), 4.67 (d, J = 6.1 Hz, 2H, CH_2Ar); ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.56, 147.47, 147.25, 143.88, 142.03, 137.36, 128.76, 127.89, 127.74, 43.57. Log k = 0.200; CLog$P$ = 2.385.

N-benzyl-6-benzylaminopyrazine-2-carboxamide (**1'**)
White solid. Identified side-product; m.p. 132.8-134.1 °C; IR (cm^{-1}): 3340 (NH), 3309 (NH), 3032, 2935, 1655 (CO); ^{1}H NMR (300 MHz, DMSO) δ9.02 (t, J = 6.4 Hz, 1H, CONH), 8.23 (s, 1H, H3), 8.13 (s, 1H, H5), 7.89 (t, J = 5.8 Hz, 1H, NH), 7.42 – 7.17 (m, 10H, ArH, Ar'H), 4.63 (d, J = 5.8 Hz, 2H, CH_2), 4.49 (d, J = 6.4 Hz, 2H, CH_2); ^{13}C NMR (75 MHz, DMSO) δ 163.95, 153.48, 141.49, 139.85, 139.64, 136.80, 129.58, 128.47, 128.01, 127.44, 127.35, 127.05, 126.97, 43.78, 42.33. Anal. Calcd. For $C_{19}H_{18}N_4O$ (318.38): 71.68% C, 5.70% H, 17.60% N; Found: 71.61% C, 5.68% H, 17.49% N. MS (ESI, Pos.): m/z 319.39 $(M+H)^+$. Log k = 0.483; CLog$P$ = 3.914.

N-benzyl-6-butylaminopyrazine-2-carboxamide (**1a**)
White solid. Yield: 41%; m.p. 94.6-95.9 °C; IR (cm^{-1}):3320 (NH), 3087, 2960, 2930, 2863, 1655 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.60 (s, 1H, H3), 8.05 (s, 1H, H5), 7.99 (bs, 1H, CONH), 7.37 – 7.27 (m, 5H, ArH), 4.99 (t, J = 5.7 Hz, 1H, NH), 4.65 (d, J = 6.0 Hz, 2H, CH_2Ar), 3.27 – 3.17 (m, 2H, CH_2), 1.66 – 1.54 (m, 2H, CH_2), 1.41 (m, 2H, CH_2), 0.98 – 0.83 (m, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.79, 152.90, 141.44, 138.09, 134.78, 130.97, 128.69, 127.63, 127.48, 43.24, 41.12, 31.24, 20.06, 13.74. Anal. Calcd. For $C_{16}H_{20}N_4O$ (284.36): 67.58% C, 7.09% H, 19.70% N; Found: 67.61% C, 7.08% H, 19.69% N. MS (ESI, Pos.): m/z 285.20 $(M+H)^+$. Log k = 0.549; CLog$P$ = 4.053.

N-benzyl-6-pentylaminopyrazine-2-carboxamide (**1b**)
White solid. Yield: 39%; m.p. 72.5-73.4 °C; IR (cm^{-1}): 3324 (NH), 3063, 2964, 2929, 2859, 1655 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.62 (s, 1H, H3), 8.02 (s, 1H, H5), 8.00 (bs, 1H, CONH), 7.39 – 7.24 (m, 5H, ArH), 4.85 (t, J = 6.7 Hz, 1H, NH), 4.65 (d, J = 6.1 Hz, 2H, CH_2Ar), 3.32 (q, J = 6.7 Hz, 2H, CH_2), 1.68 – 1.55 (m, 2H, CH_2), 1.40 – 1.25 (m, 4H, CH_2), 0.89 (t, J = 6.7 Hz, 3H, CH_3);

^{13}C NMR (75 MHz, $CDCl_3$) δ 163.93, 152.77, 141.14, 138.11, 135.11, 131.56, 128.67, 127.61, 127.45, 43.21, 41.39, 29.04, 28.89, 22.32, 13.92. Anal. Calcd. For $C_{17}H_{22}N_4O$ (298.38): 68.43% C, 7.43% H, 18.78% N; Found: 68.25% C, 7.45% H, 18.90% N. MS (ESI, Pos.): m/z 299.22 $(M+H)^+$. Log *k* = 0.768; CLog*P* = 4.582.

N-benzyl-6-hexylaminopyrazine-2-carboxamide (**1c**)
White solid. Yield: 37%; m.p. 75.7-76.2 °C; IR (cm^{-1}): 3330 (NH), 3054, 2929, 2857, 1656 (CO);^{1}H NMR (300 MHz, $CDCl_3$) δ 8.63 (s, 1H, H3), 8.01 (s, 1H, H5), 8.00 (bs, 1H, CONH), 7.37 – 7.26 (m, 5H, ArH), 4.78 (t, *J* = 5.7 Hz, 1H, NH), 4.65 (d, *J* = 6.2 Hz, 2H, CH_2Ar), 3.32 (td, *J* = 7.1, 5.5 Hz, CH_2), 1.67 – 1.55 (m, 2H, CH_2), 1.43 – 1.22 (m, 6H, CH_2), 0.91 – 0.84 (m, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.93, 152.75, 141.16, 138.15, 135.06, 131.69, 128.68, 127.63, 127.46, 43.22, 41.48, 31.45, 29.20, 26.61, 22.51, 13.96. Anal. Calcd. For $C_{18}H_{24}N_4O$ (312.41): 69.20% C, 7.74% H, 17.93% N; Found: 69.18% C, 7.79% H, 18.01% N. MS (ESI, Pos.): m/z 313.25 $(M+H)^+$. Log *k* = 0.986; CLog*P* = 5.111.

N-benzyl-6-heptylaminopyrazine-2-carboxamide (**1d**)
White solid. Yield: 40%; m.p. 60.9-61.7 °C; IR (cm^{-1}):3399 (NH), 3336 (NH), 3030, 2929, 2858, 1665 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.63 (s, 1H, H3), 8.01 (s, 1H, H5), 8.00 (bs, 1H, CONH), 7.37 – 7.26 (m, 5H, ArH), 4.80 (t, *J* = 5.6 Hz, 1H, NH), 4.65 (d, *J* = 6.1 Hz, 2H, CH_2Ar), 3.32 (td, *J* = 7.1, 5.5 Hz, 2H, CH_2), 1.67 – 1.55 (m, 2H, CH_2), 1.42 – 1.19 (m, 8H, CH_2), 0.92 – 0.83 (m, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.94, 152.76, 141.15, 138.15, 135.08, 131.67, 128.68, 127.63, 127.46, 43.22, 41.47, 31.68, 29.25, 28.96, 26.90, 22.53, 14.03. Anal. Calcd. For $C_{19}H_{26}N_4O$ (326.44): 69.91% C, 8.03% H, 17.16% N; Found: 69.79% C, 7.97% H, 17.09% N. MS (ESI, Pos.): m/z 327.23 $(M+H)^+$. Log *k* = 1.207; CLog*P* = 5.640.

N-benzyl-6-octylaminopyrazine-2-carboxamide (**1e**)
White solid. Yield: 63%; m.p. 62.1-62.7 °C; IR (cm^{-1}):3394 (NH), 3334 (NH), 2929, 2854, 1666 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.61 (s, 1H, H3), 8.05 (s, 1H, H5), 7.99 (bs, 1H, CONH), 7.39 – 7.27 (m, 5H, ArH), 4.95 (bs, 1H, NH), 4.65 (d, *J* = 5.9 Hz, 2H, CH_2Ar), 3.32 (td, *J* = 7.1, 5.5 Hz, 2H, CH_2), 1.68 – 1.54 (m, 2H, CH_2), 1.44 – 1.15 (m, 10H, CH_2), 0.87 (t, *J* = 6.3 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.83, 152.87, 141.40, 138.11, 134.81, 131.09, 128.69, 127.61, 127.47, 43.22, 41.47, 31.72, 29.25, 29.20, 29.15, 26.94, 22.59, 14.05. Anal. Calcd. For $C_{20}H_{28}N_4O$ (340.46): 70.56% C, 8.29% H, 16.46% N; Found: 70.51% C, 8.37% H, 16.39% N. MS (ESI, Pos.): m/z 341.25 $(M+H)^+$. Log *k* = 1.431; CLog*P* = 6.169.

N-benzyl-5-chlororpyrazine-2-carboxamide (**2**)
Analytical data in accordance with previously published results.[1] White solid. Yield: 68 %; m.p. 102.3-103.0 °C.^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.26 (s, 1H, H3), 8.61 (s, 1H, H6), 7.97 (bs, 1H, NH), 7.48–7.23(m, 5H, ArH), 4.65 (d, 2H, J = 6.0 Hz, CH_2Ar); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 162.10, 151.93,144.36, 142.71, 142.39, 137.34, 128.70, 128.01, 127.65, 43.55. Log *k* = 0.186; CLog*P* = 2.385.

N-benzyl-5-butylaminopyrazine-2-carboxamide (**2a**)
White solid. Yield: 78%; m.p. 120.1-121.2 °C; IR (cm^{-1}): 3399 (NH), 3306 (NH), 3027, 2951, 2922, 2865, 1661 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (s, 1H, H3), 7.80 (t, *J* = 6.0 Hz, 1H, CONH), 7.69 (d, *J* = 1.5 Hz, 1H, H6), 7.37 – 7.27 (m, 5H, ArH), 5.21 (t, *J* = 5.5 Hz, 1H, NH), 4.63 (d, *J* = 6.1 Hz, 2H, CH_2Ar), 3.40 (td, *J* = 7.2, 5.7 Hz, 2H, CH_2), 1.68 – 1.56 (m, 2H, CH_2), 1.50 – 1.33 (m, 2H, CH_2), 0.95 (t, *J* = 7.3 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.14, 155.75, 143.07, 138.43, 133.18, 129.12, 128.60, 127.73, 127.32, 43.14, 41.27, 31.33, 20.03, 13.73. Anal. Calcd. For $C_{16}H_{20}N_4O$ (284.36): 67.58% C, 7.09% H, 19.70% N; Found: 67.65% C, 7.00% H, 19.63% N. MS (ESI, Pos.): m/z 285.10 $(M+H)^+$. Log *k* = 0.424; CLog*P* = 4.053.

N-benzyl-5-pentylaminopyrazine-2-carboxamide (**2b**)
White solid. Yield: 79%; m.p. 128.7-129.3 °C; IR (cm^{-1}):3406 (NH), 3300 (NH), 3027, 2930, 2860, 1660 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (s, 1H, H3), 7.80 (t, *J* = 6.1 Hz, 1H, CONH), 7.69 (s, 1H, H6), 7.38 – 7.27 (m, 5H, ArH), 5.21 (t, *J* = 5.8 Hz, 1H, NH), 4.63 (d, *J* = 6.1 Hz, 2H, CH_2Ar), 3.45 – 3.27 (m, 2H, CH_2), 1.71 – 1.53 (m, 2H, CH_2), 1.35 (dp, *J* = 7.3, 4.7, 4.1 Hz, 4H, CH_2), 0.94 – 0.86 (m, 3H CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.14, 155.74, 143.07, 138.43, 133.18, 129.11, 128.60, 127.73, 127.32, 43.14, 41.54, 28.99, 28.94, 22.31, 13.92. Anal. Calcd. For $C_{17}H_{22}N_4O$ (298.38): 68.43% C, 7.43% H, 18.78% N; Found: 68.40% C, 7.49% H, 18.71% N. MS (ESI, Pos.): m/z 299.11 $(M+H)^+$. Log *k* = 0.638; CLog*P* = 4.582.

N-benzyl-5-hexylaminopyrazine-2-carboxamide (**2c**)
White solid. Yield: 75%; m.p. 117.7-118.3 °C; IR (cm^{-1}):3400 (NH), 3303 (NH), 3064, 3027, 2955, 2928, 2866, 1660 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (d, *J* = 1.2 Hz, 1H, H3), 7.80 (t, *J* = 6.0 Hz, 1H, CONH), 7.69 (d, *J* = 1.3 Hz, 1H, H6), 7.39 – 7.26 (m, 5H, ArH), 5.21 (t, *J* = 5.7 Hz, 1H, NH), 4.63 (d, *J* = 6.0 Hz, 2H, CH_2Ar), 3.43 – 3.33 (m, 2H, CH_2), 1.69 – 1.54 (m, 2H, CH_2), 1.45 – 1.23 (m, 6H, CH_2), 0.88 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.14, 155.75, 143.08, 138.44, 133.17, 129.12, 128.60, 127.74, 127.32, 43.14, 41.58, 31.42, 29.22, 26.54, 22.51, 13.96. Anal. Calcd. For $C_{18}H_{24}N_4O$ (312.41): 69.20% C, 7.74% H, 17.93% N; Found: 69.24% C, 7.81% H, 17.85% N. MS (ESI, Pos.): m/z 313.14 $(M+H)^+$. Log *k* = 0.859; CLog*P* = 5.111.

N-benzyl-5-heptylaminopyrazine-2-carboxamide (**2d**)
White solid. Yield: 65%; m.p. 95.8-96.1 °C; IR (cm^{-1}):3388 (NH), 3329 (NH), 2929, 2858, 1641 (CO);^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (s, 1H, H3), 7.80 (t, *J* = 6.1 Hz, 1H, CONH), 7.69 (s, 1H, H6), 7.37 – 7.27 (m, 5H, ArH), 5.19 (t, *J* = 5.8 Hz, 1H, NH), 4.63 (d, *J* = 6.1 Hz, 2H, CH_2Ar), 3.43 – 3.34 (m, 2H, CH_2), 1.69 – 1.56 (m, 2H, CH_2), 1.44 – 1.19 (m, 8H, CH_2), 0.88 (t, *J* = 6.5 Hz, 3H, CH_3);^{13}C NMR (75 MHz, $CDCl_3$) δ 164.13, 155.74, 143.09, 138.44, 133.18, 129.09, 128.60, 127.74, 127.32, 43.14, 41.58, 31.67, 29.27, 28.91, 26.83, 22.52, 14.01. Anal. Calcd. For $C_{19}H_{26}N_4O$ (326.44): 69.91% C, 8.03% H, 17.16% N; Found: 69.97% C, 8.07% H, 17.02% N. MS (ESI, Pos.): m/z 327.13 $(M+H)^+$. Log *k* = 1.089; CLog*P* = 5.640.

N-benzyl-5-octylaminopyrazine-2-carboxamide (**2e**)
White solid. Yield: 80 %; m.p. 97.0-97.9 °C; IR (cm^{-1}): 3389 (NH), 3317 (NH), 2919, 2855, 1660 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (s, 1H, H3), 7.80 (t, *J* = 6.1 Hz, 1H, CONH), 7.69 (s, 1H, H6), 7.36 – 7.26 (m, 5H, ArH), 5.16 (t, *J* = 5.7 Hz, 1H, NH), 4.63 (d, *J* = 6.0 Hz, 2H, CH_2Ar), 3.38 (q, *J* = 6.7 Hz, 2H, CH_2), 1.70 – 1.53 (m, 2H, CH_2), 1.47 – 1.17 (m, 10H, CH_2), 0.87 (t, *J* = 6.0 Hz, 3H, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.14, 155.75, 143.11, 138.46, 133.22, 129.08, 128.61, 127.76, 127.33, 43.16,

41.60, 31.73, 29.28, 29.22, 29.15, 26.88, 22.59, 1405. Anal. Calcd. For $C_{20}H_{28}N_4O$ (340.46): 70.56% C, 8.29% H, 16.46% N; Found: 70.47% C, 8.41% H, 16.41% N. MS (ESI, Pos.): m/z 341.15 $(M+H)^+$. Log *k* = 1.312; CLog*P* = 6.169.

N-benzyl-3-chloropyrazine-2-carboxamide (**3**)
Yellow solid.Yield: 80 %; m.p. 109.3-110.7 °C; IR (cm^{-1}) 3292 (NH), 1660 (CO); ^{1}H NMR (300 MHz, DMSO) δ 9.31 (t, *J* = 6.0 Hz, 1H, CONH), 8.71 (d, *J* = 2.5 Hz, 1H, H5), 8.64 (d, *J* = 2.4 Hz, 1H, H6), 7.35 (d, *J* = 4.4 Hz, 2H,ArH), 7.32 – 7.21 (m, 3H, ArH), 4.49 (d, *J* = 6.1 Hz, 2H, CH_2Ar); ^{13}C NMR (75 MHz, DMSO) δ 164.09, 148.45, 145.80, 145.49, 142.94, 139.07, 128.82, 127.74, 127.46, 42.75.Anal. Calcd. For $C_{12}H_{10}ClN_3O$ (247.68): 58.19% C, 4.07% H, 16.97% N; Found: 58.14% C, 4.10% H, 16.89% N. MS (ESI, pos.): m/z 247.95 $(M+H)^+$. CLog*P* = 247.68.

N-benzyl-3-benzylaminopyrazine-2-carboxamide (**3'**)
Yellow solid. Identified side product of the reaction; m.p. 69.8-70.6 °C; IR (cm^{-1}): 3353 (NH), 3334 (NH), 3061, 3032, 1647 (CO); ^{1}H NMR (300 MHz, DMSO) δ9.39 (t, *J* = 6.4 Hz, 1H, CONH), 9.09 (t, *J* = 5.9 Hz, 1H, NH), 8.26 (d, *J* = 2.4 Hz, 1H, H5), 7.81 (d, *J* = 2.6 Hz, 1H, H6), 7.38 – 7.16 (m, 10H, ArH, Ar'H), 4.63 (d, *J* = 5.9 Hz, 2H, CH_2), 4.44 (d, *J* = 6.4 Hz, 2H, CH_2); ^{13}C NMR (75 MHz, DMSO) δ 166.27, 154.14, 146.58, 139.61, 139.56, 130.22, 128.61, 128.45, 127.54, 127.46, 127.45, 126.97, 126.65, 43.64, 42.36. Anal. Calcd. For $C_{19}H_{18}N_4O$ (318.38): 71.68% C, 5.70% H, 17.60% N; Found: 71.73% C, 5.65% H, 17.68% N. MS not measured. Log *k* = 1.040 ; CLog*P* = 3.964.

N-benzyl-3-(butylamino)pyrazine-2-carboxamide (**3a**)
Yellow liquid.Yield: 75 %; IR (cm^{-1}): 3324 (NH), 2957, 2929, 2871, 1653 (CO); ^{1}H NMR (300 MHz, DMSO) δ 9.35 (t, *J* = 6.4 Hz, 1H, CONH), 8.71 (t, *J* = 5.6 Hz, 1H, NH), 8.25 (d, *J* = 2.4 Hz, 1H, H5), 7.76 (d, *J* = 2.6 Hz, 1H, H6), 7.30 (d, *J* = 4.3 Hz, 2H, ArH), 7.25 – 7.20 (m, 3H, ArH), 4.43 (d, *J* = 6.4 Hz, 2H,CH_2Ar), 3.41 – 3.34 (m, 2H, CH_2), 1.61 – 1.45 (m, 2H, CH_2), 1.40 – 1.26 (m, 2H, CH_2), 0.89 (t, *J* = 7.3 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 166.36, 154.42, 146.65, 139.59, 129.58, 128.44, 127.48, 126.94, 126.34, 42.31, 40.53, 31.07, 19.91, 13.88. MS (ESI, pos.): m/z 285.20 $(M+H)^+$; $C_{16}H_{20}N_4O$ (284.36). Log *k* = 1.081; CLog*P* = 4.103.

N-benzyl-3-(pentylamino)pyrazine-2-carboxamide (**3b**)
Yellow liquid. Yield: 74 %; IR (cm^{-1}): 3322 (NH), 2956, 2929, 2859, 1653 (CO);^{1}H NMR (300 MHz, DMSO) δ 9.34 (t, *J* = 6.4 Hz, 1H, CONH), 8.71 (t, *J* = 5.6 Hz, 1H, NH), 8.25 (d, *J* = 2.4 Hz, 1H, H5), 7.76 (d, *J* = 2.4 Hz, 1H, H6), 7.30 (d, *J* = 4.3 Hz, 2H, ArH), 7.26 – 7.18 (m, 3H, ArH), 4.43 (d, *J* = 6.4 Hz, 2H, CH_2Ar), 3.42 – 3.34 (m, 2H, CH_2), 1.62 – 1.46 (m, 2H, CH_2), 1.35 – 1.23 (m, 4H, CH_2), 0.85 (t, *J* = 7.0 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 166.35, 154.40, 146.64, 139.58, 129.57, 128.43, 127.48, 126.94, 126.34, 42.31, 40.53, 28.94, 28.64, 22.08, 14.10.MS (ESI, pos.): m/z 299.21 $(M+H)^+$; $C_{17}H_{22}N_4O$ (298.39). Log *k* = 1.321; CLog*P* = 4.632.

N-benzyl-3-(hexylamino)pyrazine-2-carboxamide (**3c**)
Yellow liquid. Yield: 71 %; IR (cm^{-1}): 3322 (NH), 2955, 2928, 2857, 1654 (CO); ^{1}H NMR (300 MHz, DMSO) δ 9.34 (t, *J* = 6.4 Hz, 1H, CONH), 8.71 (t, *J* = 5.6 Hz, 1H, NH), 8.24 (d, *J* = 2.3 Hz, 1H, H5), 7.75 (d, *J* = 2.5 Hz, 1H, H6), 7.30 (d, *J* = 4.3 Hz, 2H, ArH), 7.25 – 7.19 (m, 3H, ArH), 4.43 (d, *J* = 6.4 Hz, 2H, CH_2Ar), 3.38 (q, *J* = 6.7 Hz, 2H, CH_2), 1.53 (p, *J* = 7.0 Hz, 2H, CH_2), 1.39 – 1.19 (m, 6H, CH_2), 0.83 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 166.35, 154.41, 146.64, 139.58, 129.56, 128.42, 127.48, 126.93, 126.34, 42.31, 40.53, 31.18, 28.91, 26.40, 22.24, 14.09.MS (ESI, pos.): m/z 313.20 $(M+H)^+$; $C_{18}H_{24}N_4O$ (312.42). Log *k* = 1.564; CLog*P* = 5.161.

N-benzyl-3-(heptylamino)pyrazine-2-carboxamide (**3d**)
Yellow liquid. Yield: 70 %; IR (cm^{-1}): 3320 (NH), 2955, 2926, 2855, 1653 (CO); ^{1}H NMR (300 MHz, DMSO) δ 9.34 (t, *J* = 6.5 Hz, 1H, CONH), 8.71 (t, *J* = 5.6 Hz, 1H, NH), 8.24 (d, *J* = 2.4 Hz, 1H, H5), 7.75 (d, *J* = 2.5 Hz, 1H, H6), 7.29 (d, *J* = 4.3 Hz, 2H, ArH), 7.25 – 7.18 (m, 3H, ArH), 4.43 (d, *J* = 6.4 Hz, 2H, CH_2Ar), 3.37 (q, *J* = 6.5 Hz, 2H, CH_2), 1.53 (p, *J* = 6.9 Hz, 2H, CH_2), 1.36 – 1.16 (m, 8H, CH_2), 0.83 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 166.36, 154.41, 146.65, 139.59, 129.57, 128.43, 127.49, 126.94, 126.35, 42.31, 40.53, 31.41, 28.94, 28.62, 26.69, 22.23, 14.12.MS (ESI, pos.): m/z 327.24 $(M+H)^+$; $C_{19}H_{26}N_4O$ (326.44). Log *k* = 1.809; CLog*P* = 5.690.

N-benzyl-3-(octylamino)pyrazine-2-carboxamide (**3e**)
Yellow liquid. Yield: 51 %; IR (cm^{-1}): 3309 (NH), 2970, 2926, 2855, 1653 (CO); ^{1}H NMR (300 MHz, DMSO) δ 9.34 (t, *J* = 6.4 Hz, 1H, CONH), 8.71 (t, *J* = 5.5 Hz, 1H, NH), 8.24 (d, *J* = 2.5 Hz, 1H, H5), 7.75 (d, *J* = 2.4 Hz, 1H, H6), 7.30 (d, *J* = 4.4 Hz, 2H, ArH), 7.25 – 7.19 (m, 3H, ArH), 4.43 (d, *J* = 6.4 Hz, 2H, CH_2Ar), 3.37 (q, *J* = 6.6 Hz, 2H, CH_2), 1.53 (p, *J* = 6.8 Hz, 2H, CH_2), 1.38 – 1.12 (m, 10H, CH_2), 0.91 – 0.77 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 166.35, 154.41, 146.63, 139.58, 129.55, 128.42, 127.48, 126.93, 126.34, 42.31, 40.53, 31.41, 28.90, 28.82, 26.72, 22.26, 14.13.MS (ESI, pos.): m/z 341.23 $(M+H)^+$; $C_{20}H_{28}N_4O$ (340.47). Log *k* = 2.054; CLog*P* = 6.219.

HPLC lipophilicity determination - capacity factor *k* and calculated log *k*

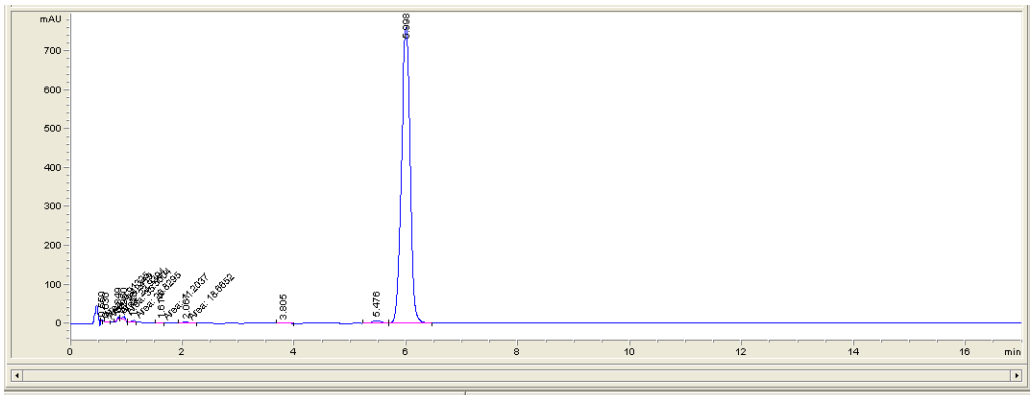
Agilent Technologies 1200 SL liquid chromatograph with Diode-array Detector SL G1315C, chromatographic column ZORBAX XDB-C18 RRHT1.8 μm, 4.6 x 50 mm, Part No. 927975-902 (Agilent Technologies Inc., Colorado Springs, CO, USA) were used. The separation process was controlled by Agilent ChemStation, version B.04.02 extended by spectral module (Agilent Technologies Inc.). A solution of MeOH (HPLC grade, 70 %) with H_2O (HPLC-Milli-Q Grade, 30 %) was used as mobile phase. The total flow of mobile phase was 1.0 mL/min, injection 20 μL, column temperature 30 °C. 210 nm as detection wavelength and 270 nm as monitor wavelength were chosen. The KI methanol solution was used for the dead time (T_D) determination. Retention times (T_R) of synthesized compounds were measured in minutes. The capacity factors *k* were calculated using Microsoft Excel according to formula $k = (T_R - T_D)/T_D$, where T_R is the retention time of the solute and T_D denotes the dead time obtained via an unretained analyte. Log *k*, calculated from the capacity factor *k*, is used as the lipophilicity index converted to log *P* scale. Method was used for

compounds **1'**, **1a-e**, **2a-e**, **3'** and **3a-e** (measured in triplicates). Results were stated as average of n = 3 (SD values were negligible, relatively ranging from 0.01 to 0.19%).

HPLC chromatograms of compounds 3a-3e

Chromatograms of derivatives **3a-3e** are included. Main peak of corresponding compound represents area in the range from 96.8 % to 99.2 % of total peak areas. This can be taken as one of the purity criteria. System peaks, *i.e.* peaks found in the dead time (0.46 min) are not taken into account as the peaks do not represent any substances.

N-benzyl-3-(butylamino)pyrazine-2-carboxamide (**3a**)



File Information

Sample | OJ111

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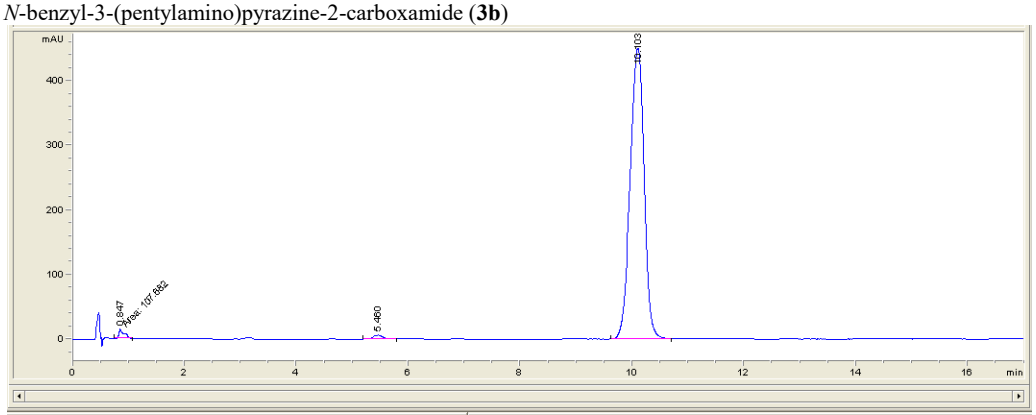
1	0.55	5.9	4	0.0246	0.067	0.472
2	0.63	9.4	1.7	0.0947	0.107	0.285
3	0.849	23.9	7.9	0.0505	0.273	1.481
4	0.96	35.5	9.9	0.0599	0.404	1.324
5	1.126	26.8	5.5	0.0815	0.306	1.643
6	1.614	11.2	1.5	0.1231	0.128	2.117
7	2.067	18.7	2.7	0.1142	0.213	1.49
8	3.805	9.2	1	0.1168	0.104	1.386
9	5.476	81.7	6.7	0.1748	0.931	1.042
10	5.998	8558.9	759.3	0.1785	97.468	1.192

N-benzyl-3-(pentylamino)pyrazine-2-carboxamide (**3b**)

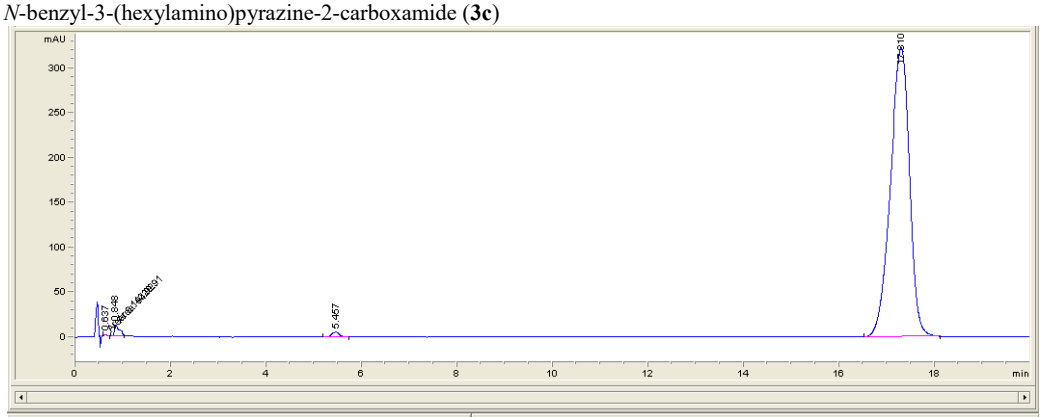


File Information

Sample OJ112

#	Time	Area	Height	Width	Area%	Symmetry
1	0.847	107.7	14.4	0.1244	1.358	0.38
2	5.46	78.8	6.1	0.1933	0.994	1.076
3	10.103	7743.3	450.5	0.2695	97.648	1.204

N-benzyl-3-(hexylamino)pyrazine-2-carboxamide (**3c**)

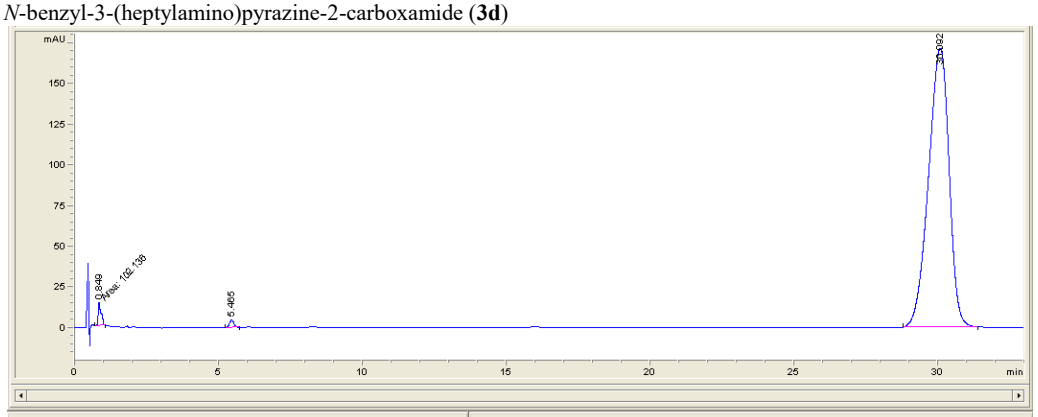


File Information

Sample | OJ113

#	Time	Area	Height	Width	Area%	Symmetry
1	0.637	6.1	1.7	0.0598	0.066	0.557
2	0.848	94.9	12.9	0.1224	1.017	0.377
3	5.457	58	5.3	0.1517	0.621	1.04
4	17.31	9176.4	321.9	0.4396	98.296	1.294

N-benzyl-3-(heptylamino)pyrazine-2-carboxamide (**3d**)

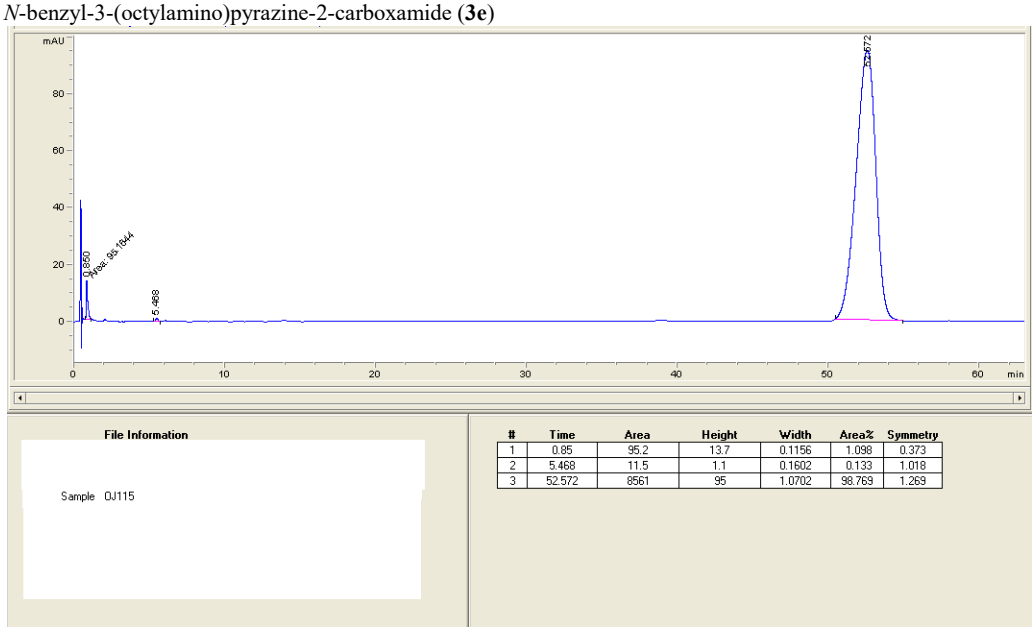


File Information

Sample | OJ114

#	Time	Area	Height	Width	Area%	Symmetry
1	0.849	102.1	14.2	0.1203	1.177	0.402
2	5.465	49.3	4.6	0.1569	0.568	1.108
3	30.092	8526.3	171.4	0.7575	98.255	1.281

N-benzyl-3-(octylamino)pyrazine-2-carboxamide (**3e**)



#	Time	Area	Height	Width	Area%	Symmetry
1	0.85	95.2	13.7	0.1156	1.098	0.373
2	5.468	11.5	1.1	0.1602	0.133	1.018
3	52.572	8561	95	1.0702	98.769	1.269

Lipophilicity calculation and correlation between measured and calculated data.

Clog*P* (the logarithm of n-octanol/water partition coefficient *P* based on established chemical interactions) values were calculated using the program CS ChemBioDraw Ultra ver. 14.0 (CambridgeSoft, Cambridge, MA, USA).

The dependence of the calculated Clog*P* values on the measured log *k* parameters showed a linearity within individual series of compounds (**Fig. 1**) and the corresponding correlations can be expressed by the following regression equations:

1a-e: Clog*P* = 2.4012 log *k* + 2.7381; R^2 = 1.0000; n = 5

2a-e: Clog*P* = 2.3750 log *k* + 3.0580; R^2 = 0.9998; n = 5

3a-e: Clog*P* = 2.1733 log *k* + 1.7580; R^2 = 1.0000; n = 5

As seen from **Fig. 1**, the Clog*P* algorithm did not distinguish between corresponding 6-alkylamino (**1a-e**) and 5-alkylamino (**2a-e**) isomers. For example, the calculated lipophilicity Clog*P* = 4.053 was the same for both isomers **1a** and **2a**. Clog*P* values predicted for 3-alkylamino isomers (**3a-e**) were insignificantly compared to corresponding 5-alkylamino and 6-alkylamino derivatives (Clog*P* for **3a** was 4.103). On contrary, experimentally measured log *k* values indicate different lipophilicity for the positional isomers.

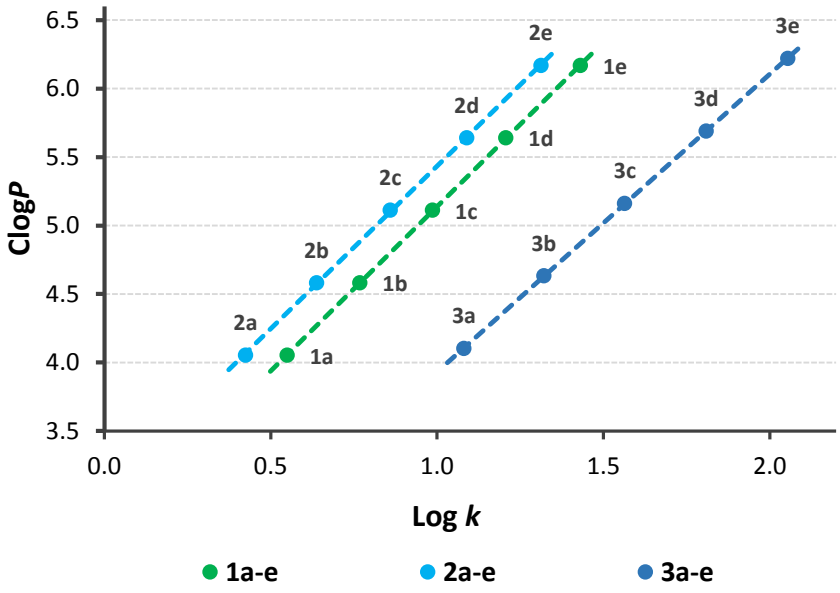


Fig. 1. Plot of calculated Clog*P* values on experimentally determined log *k* parameters.

Strikingly, 3-alkylamino isomers (**3a-e**) are much lipophilic then predicted. This is probably due to the possibility of intramolecular *H*-bond formation as depicted for compound **3d** in **Fig. 2**. Consequently, it can be assumed that log *k* values specify lipophilicity of compounds more precisely than calculated Clog*P* values.

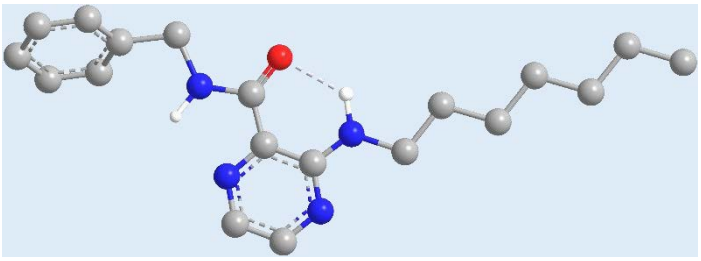


Fig. 2. Predicted conformation of **3d** as generated by CORINA 3D (available online at https://www.molecular-networks.com/online_demos/corina_demo).

MS analysis of side-product *N*-benzyl-6-benzylaminopyrazine-2-carboxamide (1')

The mass spectra were recorded in the mixture of MeOH, H_2O, formic acid (80:20:0.02 v/v) using LCQ Advantage Max ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The electrospray ionisation was performed in the positive mode. According to the fragment analysis, *N*-benzyl-6-benzylaminopyrazine-2-carboxamide was identified as a side-product, **Fig. 3**.

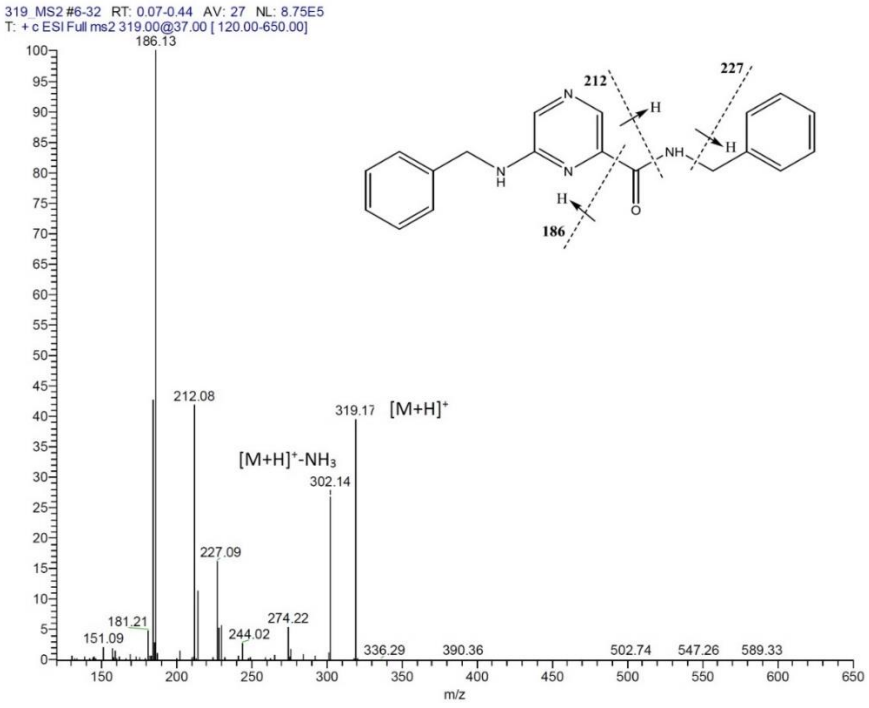


Fig. 3. Mass spectrum of side-product **1'** with labelled fragments.

In vitro antimycobacterial evaluation

Microplate alamar blue assay.[8] Antimycobacterial evaluation was performed at the Department of Clinical Microbiology, University Hospital and Faculty of Medicine in Hradec Králové, Charles University in Prague, Czech Republic. Four mycobacterial strains were used: *Mycobacterium tuberculosis* H37Rv CNCTC My 331/88, *M. avium* CNCTC My 80/72, *M. avium* CNCTC My 152/73 and *M. kansasii* CNCTC My 235/80 (Czech National Collection of Type Cultures, National Institute of Public Health, Prague, Czech Republic). The test compounds were dissolved in DMSO, diluted with Šula's semisynthetic medium (Trios, Prague, Czech Republic) to final concentrations 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 μg/mL and placed into microdilution panel. Tested species were added in the form of suspension in isotonic saline solution. The final concentration of DMSO was 0.5 % (v/v); this concentration of DMSO did not affect the growth of mycobacteria. The cultures were grown in Šula's semisynthetic medium at pH 5.6 and 37 °C. 30 μL of working solution (1:1 mixture of 0.1% resazurin sodium salt (aq. sol.) and 10% Tween 80) was used for visualization of growth. The working solution was usually added after 5 days of incubation for *M. avium*, after 5-7 days for

M. kansasii and 10-14 days for *M. tuberculosis*. Results were then determined after 24 h and interpreted according to Framzblau *et al*.[8] The minimal inhibition concentration (MIC, μg/mL) was determined as the lowest concentration which prevented a colour change from blue to pink.
Resistant strains: clinically isolated *M. tuberculosis* 234/2005, *M. tuberculosis* 9449/2007, *M. tuberculosis* 7357/1998, *M. tuberculosis* 8666/2010, *M. tuberculosis* Praha 1, *M. tuberculosis* Praha 4 and *M. tuberculosis* Praha 131. Microplate dilution method. Tested compounds were dissolved in DMSO, diluted with Šula's semisynthetic medium (Trios, Prague, Czech Republic) to final concentrations from 1 to 1000 μM. INH was used as a standard in a sterile water solution at a concentration range from 0.5 to 250 μM. Suspensions of the mycobacterial strains were adjusted to density of 1.0 on McFarland standard. MIC was determined as the lowest concentration which inhibited the visual growth after incubation at 37 °C for 14/21 days.

Examples of correlation between antimycobacterial activity (selected strains) and lipophilicity log *k* are presented in **Fig. 4** (for compounds **1a-e**) and **Fig. 5** (for compounds **2a-e**). A similar type of correlation was observed of all tested strains – activity culminates in compounds with hexyl- to octylamino substitution (labelled **c-e**).

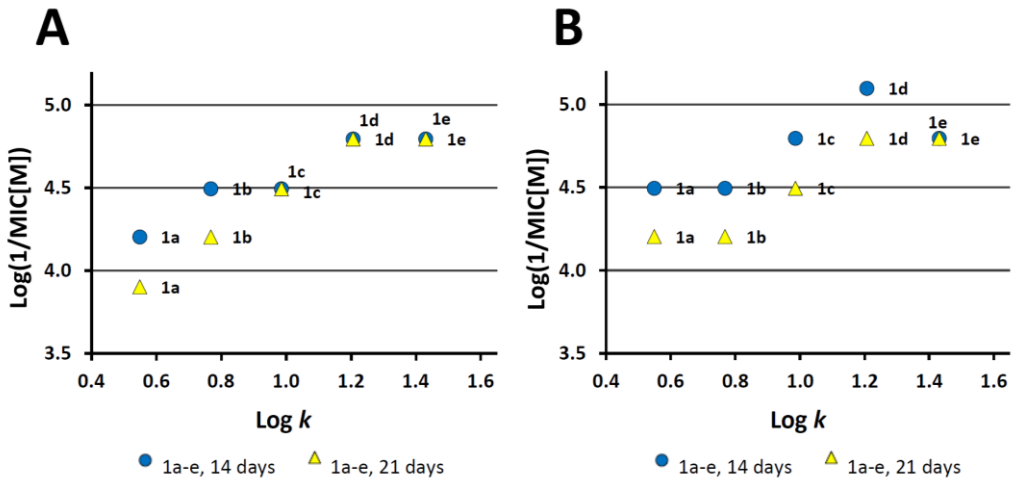


Fig. 4. Correlation between antimycobacterial activity and lipophilicity (Log *k*) for compounds **1a-e**. **A**: Multidrug-resistant strain of *Mycobacterium tuberculosis* 234/2005; **B**: Multidrug-resistant strain of *Mycobacterium tuberculosis* 8666/2010.

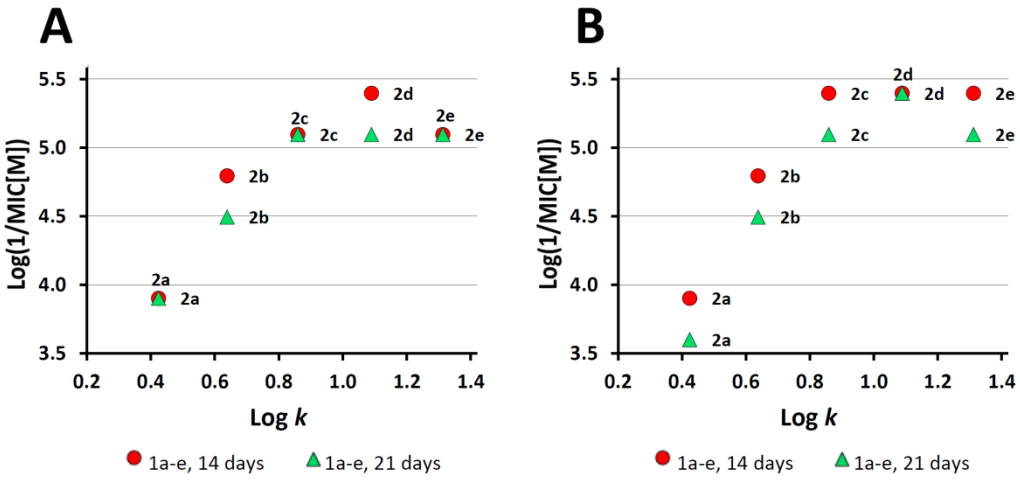


Fig. 5. Correlation between antimycobacterial activity and lipophilicity (Log *k*) for compounds **2a-e**. **A**: Multidrug-resistant strain of *Mycobacterium tuberculosis* 9449/2007; **B**: Multidrug-resistant strain of *Mycobacterium tuberculosis* Praha 1.

In vitro antibacterial evaluation

Microdilution broth method.[9] The organisms examined included strains from Czech Collection of Microorganisms (Brno, Czech Republic): *Staphylococcus aureus* CCM 4516/08, *Escherichia coli* CCM 4517, *Pseudomonas aeruginosa* CCM 1961. These strains are recommended as standards for testing of antibacterial activities. Other strains were clinical isolates (Department of Clinical Microbiology, University Hospital and Faculty of Medicine in Hradec Králové, Charles University in Prague, Czech Republic):

Staphylococcus aureus H 5996/08-methicilin resistant (MRSA), *Staphylococcus epidermidis* H 6966/08, *Enterococcus sp.* J 14365/08, *Klebsiella pneumoniae* D 11750/08, *Klebsiella pneumoniae* J 14368/08-ESBL positive. All strains were subcultured on Mueller-Hinton agar (MHA) (Difco/Becton Dickinson, Detroit, MI) at 35 °C. Bacterial inocula were prepared by suspending in sterile 0.85% saline. The cell density of the inoculum was adjusted to yield suspension of density equivalent 0.5 McFarland scale (1.5×10^8 viable CFU/mL). The compounds were dissolved in DMSO, and the antibacterial activity was determined in Mueller-Hinton liquid broth (Difco/Becton Dickinson, Detroit, MI), buffered to pH 7.0. Controls consisted of medium and DMSO alone. The final concentration of DMSO in the test medium did not exceed 1% (v/v) of the total solution composition. The minimum inhibitory concentration (MIC), defined as 95% inhibition of bacterial growth as compared to control, was determined after 24 and 48 h of static incubation at 35 °C.

Table 1.
Antibacterial activity of the most active derivatives, MIC values defined as 95% inhibition of bacterial growth.

No.	MIC (μM)					
	SA		MRSA		SE	
	24h	48h	24h	48h	24h	48h
1a	62.5	62.5	>500	>500	250	>500
1b	125	>500	125	>500	125	>500
1c	31.3	500	31.3	500	31.3	500
1d	500	>500	250	>500	500	>500
1e	3.9	3.9	31.3	500	62.5	>500
Neomycin	1.95	3.9	3.9	7.81	15.6	15.6
Bacitracin	7.81	7.81	7.81	31.3	15.6	31.3
Penicillin G	0.49	0.98	62.5	125	125	250

SA = *Staphylococcus aureus*
MRSA = methicillin-resistant *S. aureus*
SE = *S. epidermidis*

In vitro antifungal evaluation

The Department of Medical and Biological Sciences at the Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Czech Republic, performed the antifungal susceptibility assays, which was carried out using microdilution broth method. (National Committee for Clinical Laboratory Standards (NCCLS). Method for Antifungal Disc Diffusion Susceptibility Testing of Yeasts: Approved Guideline M44-A; NCCLS: Wayne, PA, USA, 2004.) Compounds were dissolved in DMSO and diluted in a twofold manner with RPMI 1640 medium with glutamine buffered to pH 7.0 (3-morpholinopropane-1-sulfonic acid). The final concentration of DMSO in the tested medium did not exceed 2.5 % (v/v) of the total solution composition. Static incubation was performed in the dark and humid, at 35 °C for 24 and 48 h (respectively 72 and 120 h for *Trichophyton mentagrophytes*). Drug-free controls were included. Fluconazole was used as standard. Tested species: *Candida albicans* ATCC 44859, *C. tropicalis* 156, *C. krusei* E28, *C. glabrata* 20/I, *Trichosporon asahii* 1188, *Aspergillus fumigates* 231, *Lichtheimia corymbifera* 272 and *Trichophyton mentagrophytes* 445.

Cytotoxicity measurement

Cytotoxicity was investigated on Crandell feline kidney (CrFK) cells, human embryonic lung (HEL) fibroblasts, human cervix epithelial (HeLa) and African green monkey kidney (Vero) cells, according to published procedures.[10-12] Briefly, the cells were seeded in 96-well plates and allowed to reach subconfluency. After addition of the test compounds at serial dilutions, the cultures were incubated at 37°C during 4-6 days. Then, the compounds' cytotoxicity was determined by microscopy and expressed as the minimal cytotoxic concentration (MCC) or compound concentration producing minimal changes in cell morphology, or by performing the formazan-based MTS cell viability assay, yielding the 50% cytotoxic concentration (CC_{50})[11-12].

Antiviral evaluation

Antiviral activity in cell culture was assessed by cytopathic effect (CPE) reduction assays with a broad panel of viruses[10-12]. The following viruses were examined on human embryonic lung fibroblast cells: herpes simplex virus type 1 (HSV-1); a thymidine kinase-deficient (TK^-) HSV-1 KOS strain resistant to acyclovir; herpes simplex virus type 2 (HSV-2); vaccinia virus; human adenovirus type 2; and vesicular stomatitis virus (VSV). The viruses examined on human cervix carcinoma HeLa cells were: VSV; Coxsackie B4 virus; and respiratory syncytial virus (RSV). African Green Monkey Vero cells were used to determine the antiviral effect on para-influenza-3 virus; reovirus-1; Sindbis virus; Coxsackie B4 virus and Punta Toro virus. Human influenza A/H1N1, A/H3N2 and B viruses were assessed on Madin-Darby canine kidney (MDCK) cells. Finally, activity against human immunodeficiency virus (HIV) type 1 and type 2 was studied in human MT-4 lymphoblast cells. To perform the tests, the virus was added to semiconfluent cell cultures in 96-well plates and, simultaneously, serial dilutions of the test compounds were added. The plates were incubated until clear CPE was reached (typically 3-6 days). Microscopic scoring was then performed to determine the

antiviral activity [expressed as 50% effective concentration (EC_{50})]. In the case of HIV-1, HIV-2 and influenza virus, virus-induced CPE was determined by the colorimetric formazan-based MTS cell viability assay.

Effect on mycolic acid synthesis

The strain of *Mycobacterium tuberculosis* H37Ra (OD_{600} = 0.185) was grown in the presence of compounds **1d** and **2e** (5 µg/mL) or isoniazid (5 µg/mL) at 37°C for 20 h and then [1,2-^{14}C] acetate (0.5 µCi/mL, specific activity 106 mCi/mmol, Amersham Radiolabeled Chemicals) was added followed by further 24 h cultivation. The cells were harvested and excessively washed with physiological saline solution. Mycolic acid methyl esters were prepared as described previously.[13] Briefly, 1 mL of 15% tetrabutylammonium hydroxide (Sigma-Aldrich) was added to cell pellets and the samples were saponified at 100 °C overnight. Fatty/mycolic acids were subsequently methylated by adding 1.5 mL of dichloromethane, 150 µL of iodomethane and 1 mL of dd H_2O for 4 h at room temperature with mixing. After centrifugation, the upper layer was discarded and the lower organic phase was washed twice with dd H_2O, dried under nitrogen and extracted by 2 mL of diethyl ether. After bath sonication and centrifugation at 1 000 x g diethyl ether extract was transferred to a new glass tube, dried under nitrogen and dissolved in 200 µL of $CHCl_3/CH_3OH$ (2:1, v/v). ^{14}C labelled FAME/MAME were analyzed by TLC. For each sample 10 000 dpm were loaded on Silica Gel 60-precoated F_{254} plates and developed in n-hexane/ethyl acetate (95:5, v/v, 3 x). The FAME/MAME were visualized by exposure of TLC plates to Kodax Biomax MR films for 5 days at -70°C.

Docking procedure

All molecular modelling was done using Schrödinger Suite (Release 2014-2) and visualizations were prepared in Maestro 9.8 (Schrödinger, Inc.). Ligands were drawn manually in Maestro, converted to 3D and prepared as ligands using LigPrep (energy minimization using OPLS_2005 force field, generation of possible states at pH 7.0 ± 2.0, without generation of tautomers). Target protein was downloaded from PDB databank (pdb: 2X23) and prepared using Maestro Protein Preparation Wizard with default settings and as follows. Ionisation states of protein residues and cofactor NAD^+ were calculated by PROPKA with default settings (pH = 7.0 ± 3.0). Water molecules were removed with the exception of HOH2009, HOH2112 and HOH2171, which mediate the interaction of NAD^+ with protein. Grid box for docking box was centered on the co-crystallized inhibitor and had outer size of 22 Å to easily accommodate even large octylamino derivatives. The docking was performed using Glide in XP (extra precision) mode with flexible sampling of ligands and without any constraints. Hydroxyl of Phe158 and 2'-OH of the ribose of NAD^+ were treated as rotatable.

Table 2.
Best XP GScore values for compounds **1c-e**, **2c-e** and **3c-e** docked into the active site of mycobacterial enoyl-ACP-reductase (InhA, pdb: 2X23) in comparison with co-crystallized ligand **PT70**.

Compound	PT70	1c	1d	1e	2c	2d	2e	3c	3d	3e
XP GScore	-10.543	-8.752	-8.566	-9.705	-9.048	-9.208	-8.330	-3.692	-5.545	-6.175

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