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SYNTHESIS AND EVALUATION OF BENZIMIDAZOLE DERIVATIVES AS POTENTIAL ANTIBACTERIAL AGENTS AGAINST *CHLAMYDIA PNEUMONIAE*

SINTEZA IN VREDNOTENJE DERIVATOV BENZIMIDAZOLA KOT POTENCIALNIH UČINKOVIN PROTI BAKTERIJI *CHLAMYDIA PNEUMONIAE*

MASTER'S THESIS
DIPLOMSKA NALOGA

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The master's thesis was conducted at the Division of Pharmaceutical chemistry, Faculty of Pharmacy at the University of Helsinki, Finland under the supervision of co-mentor Paula Kiuru, Ph.D. and Assist. Prof. Matej Sova, Ph.D., as a supervisor on behalf of the Faculty of pharmacy, University of Ljubljana.

In vitro biological tests were made in Pharmaceutical Sciences, Department of Biosciences, Åbo Akademi, Turku, Finland in Prof. Pia Vuorela's group.

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Izjava

Izjavljam, da sem diplomsko nalogo izdelala samostojno pod mentorstvom doc. dr. Mateja Sove in somentorstvom dr. Paule Kiuru.

Statement

I hereby declare that I have conducted the research and written this master's thesis by myself under the mentorship of Assist. Prof. Matej Sova, Ph.D. and co-mentorship Paula Kiuru, Ph.D.

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1 ABSTRACT

Bacterial infections are the main cause of death for more than 12% of the world population. Due to the increasing resistance of pathogenic bacteria to antibacterial drugs, there is an urgent need for the development of novel antibacterials. One of the bacteria that can cause life-threatening infections is *Chlamydia pneumoniae* (*C. pneumoniae*), an obligate intracellular Gram-negative bacteria and a worldwide common pathogen. It can cause acute and chronic respiratory diseases and is related to many other chronic diseases, such as atherosclerosis, myocarditis, vasculitis, lung cancer etc. Due to the lack of chlamydial antibiotic drugs and developed vaccines for preventing chronic diseases, it is necessary to develop novel compounds against *C. pneumoniae*. Benzimidazoles and propyl, octyl, and dodecyl gallates were presented in recent publications as potential antichlamydial agents. We decided to combine benzimidazoles and gallates into a novel class of hybrid molecules to increase antibacterial activity against *C. pneumoniae.* A series of reactions were applied to connect benzimidazole derivatives with propanol chain. After the synthesis of 2-benzimidazoylpropanole several reaction procedures were used to link benzimidazoles and gallic acid, but all of them were unsuccessful. Therefore, we decided to protect hydroxyl groups of gallic acid; besides, we managed to isolate triacetyl gallic acid. However, after the amidation we obtained only traces of deprotected final compound.

Due to the problems with amide and ester bond formations using either protected or unprotected gallic acid, we decided to replace it with an appropriate surrogate 3-methoxybenzoic acid which was found to be one of the most effective fragments in the previous studies of benzimidazole derivatives against *C. pneumoniae.* The surrogate was attached to 2-benzimidazoylpropanole resulting in the final compound 3-(1H-benzo[d]imidazol-2-yl)propyl 3-methoxybenzoate (compound **21**) which was assayed for the inhibition of growth of *C. pneumoniae* strain CWL-029 at concentrations of 200, 150, 100 and 50 μM. It was shown that the compound **21** inhibits the growth of bacteria in a micromolar range with 25% inhibition at 50 μM. Furthermore, the effects of the compound on the host cell viability were also evaluated at the same concentrations and have shown no toxicity against human cells.

To conclude, compound **21** represents an important starting point for the development of novel antibacterial agents against *C. pneumoniae;* however, further optimization and studies are needed to improve the antichlamydial activity.

KEYWORDS

Antibacterial agents, *Chlamydia pneumoniae,* benzimidazole derivatives, gallic acid

2 RAZŠIRJENI POVZETEK

Uvod: Bakterijske infekcije so vsako leto vzrok za smrt več kot 12% svetovne populacije, največ v nerazvitih državah oziroma deželah v razvoju. Tam jih za posledicami bakterijskih infekcij kot so okužbe spodnjih dihal, diareja, malarija, tuberkuloza in neonatalne infekcije umre več kot 21%. Razvoj zdravil za zdravljenje teh bolezni ni prioriteta farmacevtske industrije, ki daje večjo prednost zdravljenju kardiovaskularnih bolezni, ki so glavni vzrok smrti v visoko razvitih državah. V obširno skupino bakterij, ki so pogosti razlog smrti pri bolnikih z bakterijskimi infekcijami, sodi tudi *Chlamydia pneumoniae* (*C. pneumoniae*). Po Gramu negativna, obligatna intracelularna bakterija od svojega gostitelja prejema hranila, strukturne sestavne dele in celo ATP. Njena zgradba je precej preprosta in ima enega izmed najmanjših genomov, ki kodira le 1072 proteinov. Klamidije imajo edinstven in značilen življenjski krog z morfološko jasno infektivno in reproduktivno obliko: elementarno telesce (EB) in retikularno telesce (RB). Metabolno neaktivno zunajcelično EB vstopi v gostiteljsko celico in se v času šestih ur pretvori v metabolno aktivno obliko imenovano RB. Ta se začne razmnoževati in pretvarjati nazaj v EB, ali pa stopi v "perzistentno fazo", kjer se ne razmnožuje, lahko pa povzroči kronično infekcijo. Ravno zaradi kompleksnosti razvoja in različnih oblik klamidije je diagnostika toliko večji izziv. Največkrat se prenaša preko respiratornih izločkov in poleg akutnih in kroničnih pljučnih bolezni (kašlja, pljučnice, bronhitisa, kronične obstruktivne pljučne bolezni, astme) po zadnjih raziskavah povzroča tudi vrsto drugih kroničnih bolezni. Povezujejo jo s kardiovaskularnimi boleznimi – miokarditisom, možgansko kapjo, akutnim miokardnim infarktom in podobnimi. Med slednje spada tudi ateroskleroza, katere pomemben dejavnik za razvoj vnetja je prav *C. pneumoniae*. Za zdravljenje respiratornih bolezni so najbolj učinkoviti tetraciklini, makrolidi in fluorokinoloni; primerni antibiotiki za zdravljenje preostalih kroničnih bolezni še niso odkriti. Velik napredek se kaže pri odkrivanju cepiv proti klamidijam, vendar do danes še niso uspeli odkriti učinkovitega cepiva, zato je nujen razvoj novih učinkovin proti *C. pneumoniae*. Benzimidazoli, ki so sestavni del naših končnih učinkovin, so biciklične aromatske strukture, kjer je benzenu na mestih 4 in 5 prikondenziran imidazol. Benzimidazol kot fragment lahko najdemo v naravi pri vitaminu B_{12} in med številnimi zdravilnimi učinkovinami, med katerimi so tudi antibiotiki. Galati so estri med galno kislino in različnimi alkoholi. Tako kot galna kislina tudi propil, oktil in dodecil galati izkazujejo antioksidativne lastnosti, saj preprečujejo

oksidacijo pri nenasičenih maščobah. Znana pa je tudi njihova protibakterijska, fungicidna in protivirusna aktivnost. Alvesalo J. in sodelavci ter Keurulainen L. in sodelavci so odkrili in objavili spodbudno *in vitro* inhibitorno aktivnost galatov in benzimidazola proti bakteriji *C. pneumoniae*.

Namen dela: V diplomski nalogi bomo sintetizirali nove spojine, ki bodo vsebovale oba omenjena fragmenta, galno kislino in benzimidazolni obroč. Na ta način bomo skušali povečati protibakterijsko aktivnost in tako odpreti nove možnosti pri odkrivanju spojin s protibakterijskim delovanjem proti bakteriji *C. pneumoniae*. Končne spojine bomo ovrednotili z biološkimi testi, kjer bomo spremljali zaviranje rasti *C. pneumoniae.*

Materiali in metode: Celotno praktično delo z analizami je bilo izvedeno na Fakulteti za farmacijo Univerze v Helsinkih, analiza in predstavitev rezultatov v pisni obliki pa na Fakulteti za farmacijo Univerze v Ljubljani. Pri delu smo uporabili komercialno dostopne reagente in učinkovine, kromatografske metode (tankoplastno kromatografijo, »flash« kromatografijo), spektroskopske metode (IR, NMR, HRMS) in mikrovalovni reaktor; končnim spojinam pa smo določili tudi tališče. Z uporabo *in vitro* bioloških testov proti bakteriji *C. pneumoniae* je bila ovrednotena inhibitorna aktivnost sintetiziranih spojin, katerim smo določili tudi toksičnost proti humanim celicam.

Rezultati in razprava: Iz 1,2-diaminobenzena in 3-nitrobenzoil klorida smo preko diamida (**1**) sintetizirali benzimidazolni obroč (**2**), na katerem smo s katalitskim hidrogeniranjem reducirali nitro do amino skupine in tako pripravili izhodno spojino (**3**) za nadaljnje reakcije. 2-benzimidazoilpropanol (**7**) smo pripravili z reakcijo med 1,2-diaminobenzenom in gama-butirolaktonom v raztopini s koncentrirano HCl. Enako reakcijo smo ponovili tudi s 3-kloro in 3-metil derivatoma 1,2-diaminobenzena in dobili dve novi spojini 3-(6-metil-1H-benzo[d]imidazol-2-il)propan-1-ol (**8**) in 3-(6-kloro-1H-benzo[d]imidazol-2-il)propan-1-ol (**9**). Tvorba amida med **3** in nezaščiteno galno kislino z uporabo TEA kot katalizatorja in tvorba estra med **3** in nezaščiteno galno kislino z DCC metodo ni bila uspešna, zato smo se odločili zaščititi galno kislino. Po neuspešnem uvajanju trimetilbutilsililne in benzilne zaščitne skupine nam je galno kislino uspelo zaščititi z acetilnimi skupinami. Triacetil galno kislino (**10**) smo uporabili le za neuspeli poskus tvorbe amidne vezi s **3**, saj bi bila kasnejša odstranitev zaščitne skupine estra na galni kislini ob prisotni estrski vezi med benzimidazolom in galno kislino problematična. Nadaljnjih reakcij smo se zato lotili s fragmentom spojine, ki je v preteklih raziskavah izkazovala aktivnost – 3-metoksibenzojsko kislino (obdržali smo eno zaščiteno hidroksilno

skupino iz galne kisline) in 2-benzimidazoilpropanolom (**7**) s trietilaminom in uspeli sintetizirati končno spojino 3-(1H-benzo[d]imidazol-2-il) propil 3-metoksibenzoat (**21**). V *in vitro* bioloških testih je ta spojina pri koncentraciji 200 μM povzročila popolno odsotnost *C. pneumoniae*, pri 150, 100 in 50 μM pa 93, 49 in 25% inhibicijo rasti *C. pneumoniae*. Pri 150 μM in nižjih testiranih koncentracijah spojina ni pokazala več kot 15% toksičnosti na človeških celicah.

Sklep: Kljub pomanjkanju dostopnih literaturnih virov za sintezo 2-benzimidazoilpropanola smo uspeli najti ustrezno sintezno pot do ključne spojine. Iz začetnih spojin gama-butirolaktona in 1,2-diaminobenzena smo postavili in optimizirali sintezni postopek za pripravo benzimidazolov, ga uspešno uporabili še na dveh derivatih (3-metil in 3-kloro) ter pridobili dve novi spojini **8** in **9**. Od končnih spojin je spojina **21** izkazovala spodbudno aktivnost v *in vitro* testih, zato ta spojina predstavlja pomembno izhodiščno spojino pri načrtovanju novih učinkovin proti *C. pneumoniae.*

3 LIST OF ABBREVIATIONS

C. pneumoniae	*Chlamydia pneumoniae*
Ac	Acetyl
AMI	Acute myocardial infarction
Ar	Aryl
ATP	Adenosine-5'-triphosphate
Bn	Benzyl
DCC	*N,N'*-dicyclohexylcarbodiimide
DCM	Dichloromethane
DIPEA	*N,N*-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	Dimethylformamide
DMSO-d_6	Deuterated dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EB	Elementary body
EGCG	Epigallocatechin gallate
eq	Equivalent
ESI	Electrospray ionization
Et	Ethyl
HIV	Human immunodeficiency virus
HL-cells	Human line cells
HRMS	High resolution mass spectra
Hsp	Heat shock proteins
IF	Immunofluorescence
IFN-γ	Interferon-gamma
Inc	Inclusion membrane proteins
LC-MS	Liquid chromatography–Mass spectrometry
Me	Methyl
MIF	Microimmunofluorescence
MOMP	Major outer membrane protein
MW	Microwave
NMR	Nuclear magnetic resonance

Omp	Outer membrane proteins
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Protection group
Pmp	Polymorphic membrane proteins
ppm	Parts per million
p-TsOH	*Para*-toluenesulfonic acid
RB	Reticulate body
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SEM	Standard error of the mean
TBS	Tert-butyldimethylsilyl
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TMS	Tetramethylsilane
TWAR	Taiwan acute respiratory agent
UV	Ultraviolet

4 INTRODUCTION

4.1 Bacterial infections

Humankind has been dealing with bacterial infections since before the dawn of the human history (1). Even if it is not understandable, human body has more than 10 times more bacterial than body cells (2). They are part of our environment, body surfaces, and cavities with rich and characteristic microbial flora. A healthy patient with an active immune system can recover without a medical treatment against mild diseases caused by microorganisms. An appropriate therapeutic intervention is indicated in the case of an insufficient body response to infection (1).

World Health Organization (WHO) published data on mortality in year 2011 and classified them in broad income groups. 5 out of 10 main causes of death (low respiratory tract infections, diarrheal diseases, malaria, tuberculosis and neonatal infections) in low-income countries are a consequence of bacterial infections, which makes altogether 21.6% of all deaths in these areas. Even though 24.3% of high-income population death causes are results of cardiovascular diseases (ischemic heart disease, stroke or other cerebrovascular disease), there is still more than 12% of the whole world population that would die due to direct outcome of bacterial infections (3). In last 15 years, Food and Drug Administration (FDA) has approved 15 new antibiotic drugs (4, 5), however due to the increasing resistance of pathogenic bacteria to antibacterial drugs, there is an urgent need for the development of novel antibacterials.

4.1.1 Bacterial cell

A bacterial cell is less complex as opposed to an animal cell. The bacterial cell does not have a defined nucleus, its internal structures are simpler and some of its biochemical reactions are essential for cell survival as they produce important elements, while the animal cell can acquire them directly from food. The animal cell has a cell membrane, which is not as important for survival as the cell wall in the bacterial cell. Surrounded by a wide range of environments and osmotic pressure, the bacterial cell wall is crucial for bacteria to survive. The cell wall prevents water to enter the cell and consecutively prevents the cell from swelling. If that happened, it would lead to lysis, which describes a breaking down a cell (1).

Two basic types of bacteria can be defined according to Gram staining. So-called Gram positive bacteria have a thick 20-40 nm cell wall and more than 100 layers of peptidoglycan on the membrane. Only a small amount of dye remains in Gram negative bacteria because of its thin 2-7 nm cell wall with only one or two layers of peptidoglycan between two membranes (1, 6). Regardless of the thin cell wall, Gram negative microorganisms contain an additional outer membrane and lipopolysaccharides, and are similar to the animal eukaryotic cell membrane, which gives them special features and characteristics (6).

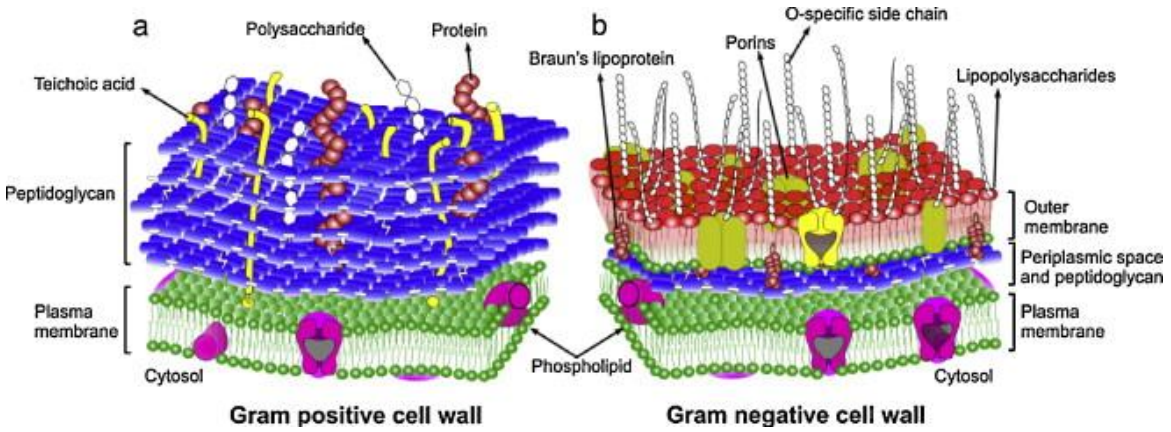


Figure 1: Scheme of Gram positive and Gram negative cell wall (7).

4.1.2 Treatment of bacterial infections

A suitable therapy for preventing bacterial infections has been sought throughout history. Old medical writings record folkloric use of plant and animal preparations for treating various diseases, many of them caused by bacteria (1). Bacteria were first discovered 300 years ago and until the understanding of its role in infection 150 years ago, there was no hope for a rational therapy.

The modern anti-infective era in the scientific approach of developing antibiotic compounds started in 1928, when Alexander Fleming together with colleagues discovered penicillin and its curative effect in various infectious diseases. (8). Many successions of metabolic products from soil led to discovery of several antibiotics and up to the present, more than 300 antibacterial compounds have been launched on the market (9). They have significantly contributed to the fight against bacteria and life expectancy has increased for nearly 15 years (10). The research in antibiotic pharmaceutical area is not that profitable

and not as important for the developed world, as there are other diseases of greater importance. According to the recent situation, new antibacterial drugs are needed to sustain current fight against new bacterial infections.

The word antibiotic is on the one hand used only for natural (produced by bacteria, fungi, etc.) and semisynthetic compounds that heal bacterial infections. On the other hand, it is misused for synthetic compounds which appropriate naming would be chemotherapeutics. Despite this misconstruction, the term antibiotic will be used for drugs performing antibacterial activity. Antibiotic drugs may kill bacteria – act bactericidal or inhibit bacterial growth – perform bacteriostatic (1).

Main mechanisms of antibacterial drugs are inhibition of bacterial cell-wall synthesis (e.g. penicillins), inhibition of protein synthesis (e.g. erythromycin), inhibition of cell metabolism (e.g. sulphonamides), inhibition of nucleic acid transcription and replication (e.g. ciprofloxacin) and alteration of cell membrane permeability (e.g. valinomycin) (11) (6).

Bacteria are able to defend themselves against antibacterial drugs by developing antibiotic resistance. It is ever-increasing public health hazard, which could lead to even more resistant bacteria and head to more deaths and less effective antibacterial drugs. (11).

4.2 *Chlamydia pneumoniae*

4.2.1 Taxonomy

Chlamydia pneumoniae (*C. pneumoniae*) belongs to the order *Chlamydiales*, distinct bacterial section which was separated over 800 million years ago from other bacterial classifications (12). Because of their direct phylogenetic root divergation very early in the evolution of eubacteria, the order *Chlamydiales* represents unique part of the bacterial kingdom (13, 14). The order *Chlamydiales* currently comprises of four families: *Chamydiaceae*, *Parachlamydiaceae*, *Simkaniaceae*, and *Waddliaceae*, while some candidates are proposed but not yet approved for four new families *Piscichlamydiaceae*, *Clavochlamydiaceae*, *Rhabdochlamydiaceae* and *Criblamydiaceae* (Figure 2) (15 – 18).

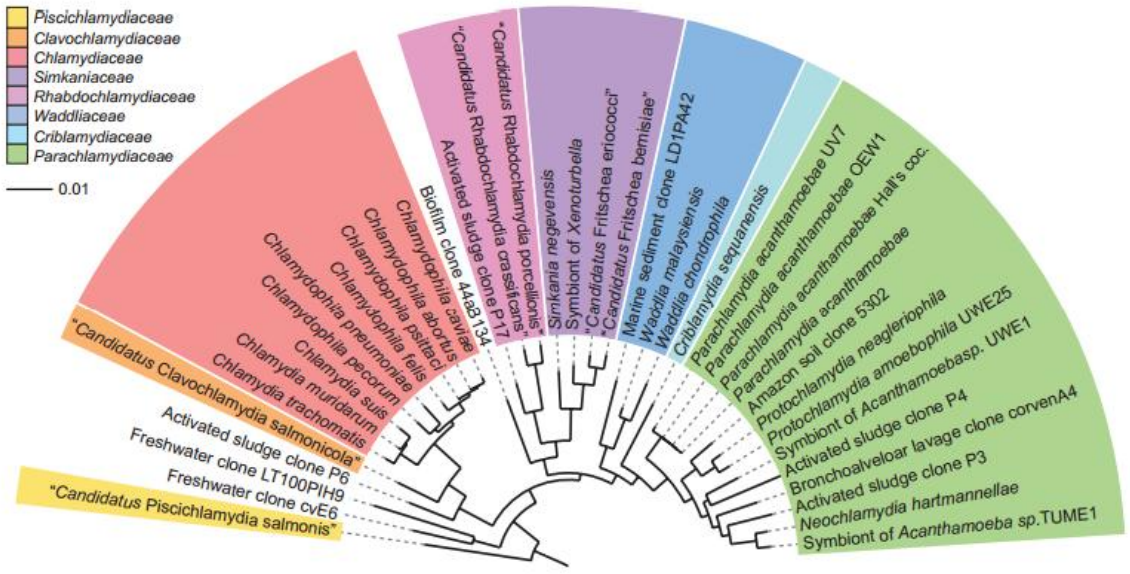


Figure 2: Phylogenetic tree among members of phylum *Chlamydiae* (14).

C. pneumoniae is a species in the family *Chlamydiaceae*. In year 1999, Everett *et al.* classified the family *Chlayidiaceae* on divergence in sequence of at least 80% 16S and 23S rRNA. Author defined *C. pneumoniae* as *Chlamydiophila pneumoniae* as presented in Figure 3 (15).

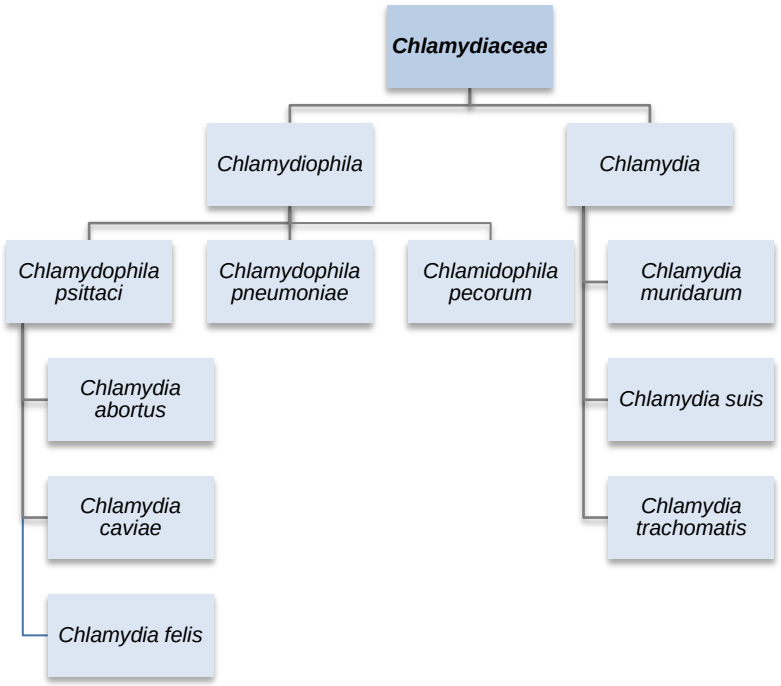


Figure 3: Classification of species in the family *Chlamydiaceae* based by Everett *et al.* (15).

In the thesis, we will refer to the use of former designation of *Chlamydophila* as *Chlamydia* (shortly *C.*) to avoid the controversy surrounding this classification as a result of the lack of extensive support in the field according to minor sequence differences in 16S and 23S rRNA genes (19).

Isolated for the first time from conjunctiva of Taiwanese child in 1965, *C. pneumoniae* was named TW-183. The organism's role as a pathogen was not clear until 1983, when first isolation of AR-39 from a throat swab was taken by a university student with pharyngitis in Seattle. The strain name – serovar TWAR was defined after both isolates and is nowadays the synonym for *C. pneumoniae* (20).

4.2.2 Life cycle

Chlamydiae have a common and unique biphasic development cycle with morphologically clear infectious and reproductive forms: *elementary body* (EB) and *reticulate body* (RB). *C. pneumoniae* life cycle starts with small, around 200 to 400 nm in diameter, pear-shaped EBs that in the first 2 h connect to the sensitive host cell and enter the cell with membrane linked phagosomal fusion. Phagosome with metabolically inactive EB traffic to functional interaction with Golgi apparatus where they start to convert into specialized vacuole called inclusion. In the next 6 h, EB starts to differentiate to large, around 1000 nm in diameter, metabolically active RB. After 10 h, RB undergoes multiple rounds of binary fission and might enter persistent phase or sustain secondary differentiation back into EB. Although 500-1000 new infectious EBs accumulate in host cell, it is interestingly minimally disrupted. In about 2-3 days after the first infection, a release of mature inclusion may occur via cytolysis, a process of exocytosis or extrusion and initiate new development cycle of chronic infection (Reviewed in 13 and 21).

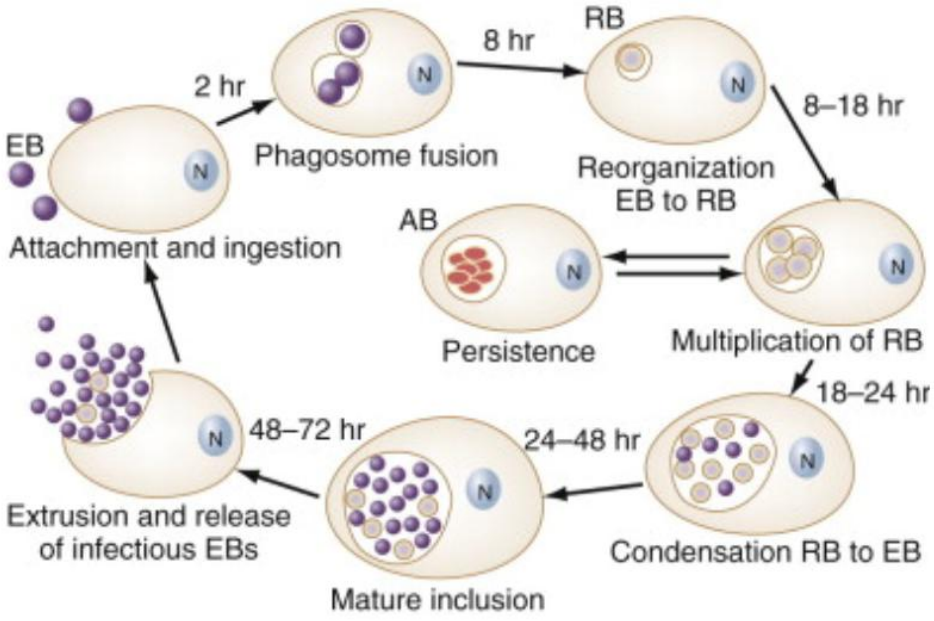


Figure 4: Life cycle of *C. pneumoniae* (13).

Many various theories about chronic infections were proposed to explain the mechanism of persistent *C. pneumoniae*. During persistent state, small inclusions are formed of fever EB and RB and formation of abnormal RBs, called aberrant bodies (ABs). Metabolic activity is reduced and the organism is often resistant to antibiotic treatment (22). Possible reasons for bodies staying in persistent state might be in restriction of certain

nutrients (iron, glucose, and amino acids), IFN-γ and penicillin treatment of *C. pneumoniae* (23), first-choice antibiotics at subinhibitory concentrations (22), long-term continuous infection (24), and inhibition of apoptosis (13, 25).

4.2.3 Structure

C. pneumoniae is an obligatory intracellular parasite with a double cell membrane, which is a typical characteristic of Gram-negative bacteria. All *Chlamydiae* species appear to lack the classical peptidoglycan between membranes, even though they contain the genes, which are necessary for peptidoglycan synthesis and also penicillin-binding proteins (13, 26, 27). Outer membrane contains cysteine-rich proteins (Omp), which are functional equivalents of peptidoglycan, formed from sulphur bridges and support the cell to stay rigid and osmotically stabile. The main and most abundant Omp is the major outer membrane protein (MOMP) next to several polymorphic membrane proteins (Pmp) (28). In the envelope, there are also other proteins such as heat shock proteins (Hsp) and inclusion membrane (Inc) proteins and lipopolysaccharides (26).

C. pneumoniae has one of the smallest bacterial genome, consisting of only 1072 likely protein codes within only 1.2 million bases. A quite evident reason for that is intracellular lifestyle, which helps the bacteria to easily get all nutrients, structural components and even ATP from hosting organism (29, 30).

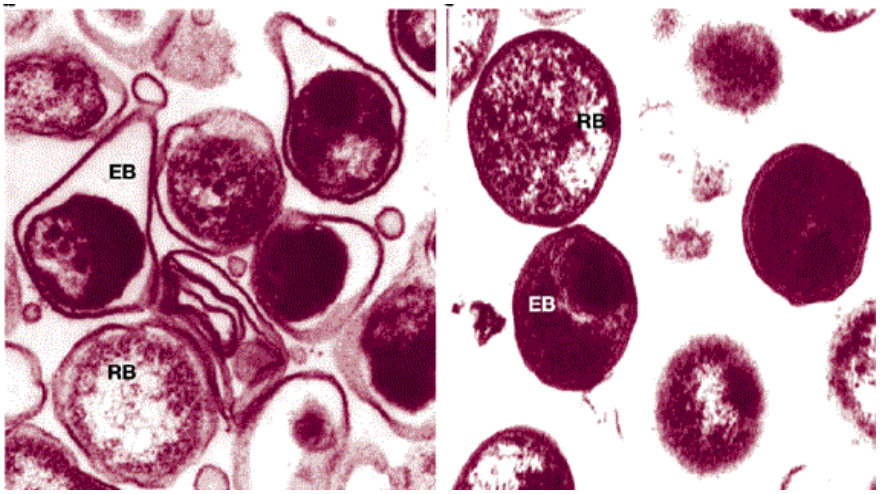


Figure 5: Structure of *C. pneumoniae* EB and RB (31).

4.2.4 Laboratory diagnosis

Many methods have been applied in order to find the appropriate and most reliable diagnosis detection of *C. pneumoniae* in clinical samples. Differentiation between acute primarily infection, reinfection, a chronic persistent state infection, or past infection is very difficult. It is very demanding to detect *C. pneumoniae* infections primarily because of the lack of the standardized well-validated methods; additionally, those techniques require experienced specialized laboratories.

Different methods for detecting *C. pneumoniae* infections can be used. *C. pneumoniae* cell cultures can be isolated from a cell culture of the respiratory tract, lungs, adenoids or sputum. However this method is not frequently used due to problems concerning bacterial slow-growing sensitivity and tough isolation (13).

Detection of specific *C. pneumoniae* monoclonal antibodies can be performed on tissues with EB in various samples (normal artery, brain, lung or other tissues), using immunohistochemistry method (32). In addition, polymerase chain reaction (PCR) with amplifying specific nucleic acid can be performed to detect *C. pneumoniae* in as few as 50 to 300 μL of sample (33). This test turned out to have high false-negative or false-positive results so recently PCR has been developed as real-time PCR-based protocols (34).

The microimmunofluorescence (MIF) test is one of the serologic tests for identifying antibodies against *C. pneumoniae* using EB as antigens. It is the best and most frequently used method for a laboratory diagnosis of acute *C. pneumoniae* infection. The MIF test can distinguish recent from past infection and reinfection from primarily infection determining IgG and IgM serum fractions in a certain period. It seems to be the most sensitive measure of infection (20). MIF is a technically demanding method which requires operators with a lot of experience with fluorescence microscopy; for the interpretation of the result to be subjective, at least two operators are necessary to decide about antibodies titers (35).

4.2.5 Transmission, epidemiology and clinical manifestation

Primary, quite inefficient mode of spreading *C. pneumoniae* from person to person is via respiratory secretions by droplets and hands but there has been also evidence of other effective carriers (tissue paper, countertops). Incubation period of *C. pneumoniae* is several weeks which is longer than for other pneumoniae-causing infections. Even though infection spreads slowly, reinfection appears to be frequent, suggesting that infection-induced immunity is incompletely protective, short-lived or both (13, 20, 25).

C. pneumoniae is a worldwide common human pathogen. Based on many studies in the US and other countries, the most common infection with *C. pneumoniae* seems to be among school-aged children up to 20 years old when seroprevalence is about 50%. The percentage increases to 75% in senior age. It is known that antibodies can protect us only 3-5 years, resulting in common appearance of infections and reinfections. In addition, seroprevalence among male adults is more frequent than that among adult women (20).

To date, it is known that only three of *Chlamydia*e are causing human diseases: *C. psittaci*, *C. trachomatis* and *C. pneumoniae*, whereas the others occur in animals. *C. psittaci* infects primarily bird species but causes also psittacosis in human. *C. trachomatis* is associated with oculogenital diseases - conjunctivitis, blindness, genital and rectal infections (13).

Initial acute infections are often limited to mucosal epithelium and generally producing minimal or no symptoms. Many of them might resolve naturally, but others appear to persist in asymptomatic state (13). Nevertheless, *C. pneumoniae* can cause acute and chronic diseases.

4.2.5.1 Respiratory diseases

As a common respiratory pathogen, it has been isolated from serum of patients with mild acute upper respiratory tract diseases: persistent cough, common cold, sinusitis, otitis media and acute pharyngitis (Reviewed in (36)). TWAR antibodies are also found in lower respiratory tract diseases like acute bronchitis, as well as sarcoidosis. Acute infections with *C. pneumoniae* are clinically rare and appear to be mild or asymptomatic (13). According to series of studies, *C. pneumoniae* appears to be the reason for around 5-20% of community-acquired pneumonia worldwide and approximately 5% of bronchitis and

sinusitis cases (20, 37). In Slovenia, based on a study from year 2003, *C. pneumoniae* infection appears to be the causative agent for 21.1% mild community-acquired pneumonias (38).

Among chronic respiratory diseases, there exists evidence of the association between *C. pneumoniae* infection and chronic bronchitis, chronic obstructive pulmonary disease, lung cancer, sarkoidosis, and asthma (13).

4.2.5.2 Other chronic diseases

Persistent infection with *C. pneumoniae* seems to be more important for developing other than respiratory tract infections. Since the first report in The Lancet by Saikku *et al.* (39) in year 1988, many cardiovascular diseases such as subacute inflammatory endocarditis, myocarditis, vasculitis, coronary heart disease (CHD), stroke, transient ischemic attack, abdominal aortic aneurism and acute myocardial infarction (AMI) have been reported to follow chronic *C. pneumoniae* infection (Reviewed in 36 and 40). Furthermore, infection might be the causative agent in development of atherosclerosis, a dangerous modern disease. Besides some bacteria and viruses such as *Helicobacter pylori,* cytomegalovirus, and herpes simplex virus, *C. pneumoniae* connection with atherosclerosis have been most comprehensively studied (41). To date, up to 900 different articles (899 hits: Pub Med, 06.11.2012 "*Chlamydia pneumoniae*" atherosclerosis) were published about the association of atherosclerosis and *C. pneumoniae* infection.

Bacteria may have an important role in the initiation of the inflammatory activation producing cytokines. (42). In one of the studies it was found that cytokines enhance matrix metalloproteinase 1 and 9 which was followed by the stimulation with tumor necrosis factor alpha and granulocyte monocyte-colony stimulating factor which is an important causative agent for atherosclerosis (43).

In addition to that, *Chlamydia* infection is associated with central nervous system diseases such as meningitis, encephalitis, meningoencephalitis (44), Guillain-Barre syndrome (45), Alzheimer disease (46), and multiple sclerosis (47). Other severe diseases linked to *C. pneumoniae* are small and cell lung cancer (48), rare Szezary's syndrome as well as erythema nodosum, reactive arthritis, Sweet syndrome and eye diseases (Reviewed in 36).

4.2.6 Treatment

Recent directions in treating *C. pneumoniae* respiratory infections, if adequately detected, are summarized in

Table 1.

Table 1: Regimens used for respiratory infection caused by *C. pneumoniae* (13).

Chemical antibiotic group	**Substance**	**Dosage**	**Interval**
Adults			
Tetracyclines	Doxycycline	100 mg orally; twice daily	14-21 days
	Tetracycline	250 mg orally; four times daily	14-21 days
Macrolides	Azithromycin	1.5 g orally	Over 5 days
	Clarithromycin	500 mg orally; twice daily	10 days
Fluoroquinolones	Levofloxacin	500 mg intravenously or orally; once a day	7-14 days
	Moxifloxacin	400 mg orally; once a day	10 days
Children			
Macrolides	Erythromycin	Suspension; 50 mg/kg per day	10-14 days
	Clarithromycin	Suspension; 15 mg/kg per day	10 days
	Azithromycin	Suspension; 10 mg/kg once a day, followed by 5 mg/kg once daily	4 days

Tetracyclines, macrolides and fluoroquinolones are the most effective, while *C. pneumoniae* is not susceptible to penicillin, ampicillin or sulpha drugs (20).

Only little is known about the treatment for other than respiratory *C. pneumoniae* diseases. Following the connection between coronary artery disease (CAD) and *C. pneumoniae*, many trials have tried to find the most appropriate treatment against persistent chlamydial infection. Clinical trials individually performed with azithromycin, roxithromycin or clarithromycin might show some activity against *C. pneumoniae* chronic infection in connection with CAD, yet the results with late established atherosclerosis are disappointing (41).

The insidious nature of *C. pneumoniae* infection makes prevention very difficult, and the development of antichlamydial vaccines remains an important goal for researchers. To date, there is no acceptable human chlamydial vaccine due to development challenges such as safety considerations, suboptimal and inadequate immunogenicity of vaccine candidates, lack of effective delivery systems and potent adjuvants and knowledge gaps on how to induce long-term immunity. Both, prophylactic and therapeutic vaccines are urgently needed to prevent population and stop spreading *C. pneumoniae* infection.

Contemporary trends for designing efficacious *Chlamydia* vaccine are:

- Induction of both T-cell and antibody responses;
- Judicious selection of a safe, nontoxic immunogen;
- Subunit(s) vaccine candidate with broad serovar specificity;
- Effective delivery that induces appropriate targeting of vaccine antigen to the relevant mucosal inductive site;
- The use of potent, safe adjuvants and other immunomodulatory strategies to boost immune efforts and foster mucosal immunity (49).

5 BENZIMIDAZOLES AND GALLATES AS PRIVILEGED STRUCTURES IN DRUGS

5.1 Benzimidazoles

Benzimidazoles are bicyclic aromatic organic compounds in which benzene is fused with imidazole on positions 4 and 5. They can possess acidic or basic properties since the NH group in benzimidazole can be strongly acidic or weakly basic. Benzimidazoles with unsubstituted NH group can form prototropic tautomerism, which leads to equilibrium mixtures of asymmetrically substituted compounds (50).

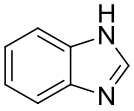


Figure 6: Benzimidazole.

Benzimidazoles attracted drug researchers' attention after they discovered that the most similar compound in the nature consisting of benzimidazole is vitamin B_{12}, also called cobalamin (Figure 7). After they determined that vitamin B_{12} is capable of stimulating the bacterial growth, they noticed that single benzimidazole fragment suppresses bacterial growth. This discovery was the initiative in development of the antibacterial compounds with benzimidazole moiety (51).

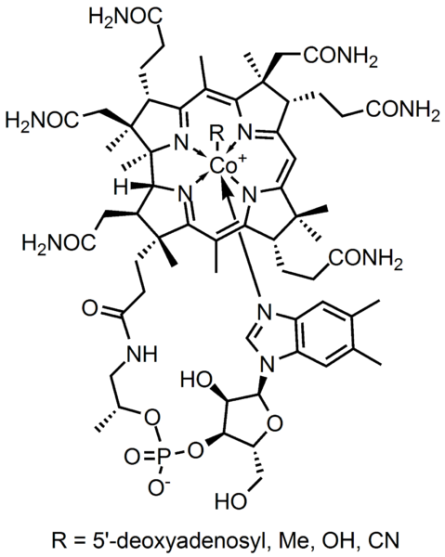


Figure 7: Vitamin B_{12}.

As drugs, benzimidazoles exhibit many important pharmacotherapeutic effects, possessing activities such as antibacterial, antiviral (HIV inhibitors), antiparasitic,

antihypertensive, antiulcer, antiproliferative, antitumor, anti-inflammatory, antioxidant, antiprotozoal, androgen receptor antagonist, anticancer, and anticonvulsant (52).

High throughput screening within smaller targeted library of compounds was made on 3D structure of RNA-methyltransferase. It has highly similar amino acid sequence to dimethyladenosine transferase, which was found to be crucial for bacterial survival and is closely related to nonhuman *C. pneumoniae* target protein. In the database with more than 300,000 molecules, 33 were purchased and tested on *C. pneumoniae* inhibition, cytotoxicity and viability. Two benzimidazole based derivatives showed good antichlamydal activity (53). Furthermore, Keurulainen L *et al*. synthesized 33 2-arylbenzimidazole derivatives, described bioactivity testing and investigated that those compounds might be important hits for further investigations (54).

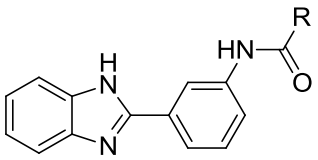
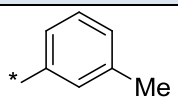
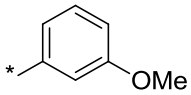
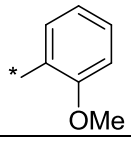


Figure 8: 2-Arylbenzimidazole scaffold.

Table 2: Chemical structures of some benzimidazole derivatives and their inhibitory activity and human cell viability at 10 μM concentration of compounds.

Compound	R	Inhibition (%) ± SEM	Viability (%)
1)		98 ± 0.7	99
2)		98 ± 7.8	97
3)		0	107
4)	-Me	0	100

The majority of compounds were shown to have good antichlamydial inhibition; however, the highest inhibition was obtained for those compounds with substituted phenyl on position 3 (as compound 1). High human cell viability for some compounds in Table 2 (only two out of 33 tested in study had lower viability than 90%) shows that compounds are non-toxic to human cells.

Recently, an extensive conformation study was made in order to explain the mechanism of 2-arylbenzimidazole activity as inhibitors of *C. pneumoniae* growth. The

main discovery was that the compounds, which can more easily adopt a non-planar conformation show higher bioactivity (55).

5.2 Gallates

Gallic acid (Figure 9 a)) is 3,4,5-trihydroxybenzoic acid that can be found in many plants in free state or as a part of polyphenolic tannins (56). Esters of gallic acid and alkyl alcohols are called alkyl gallates (gallates). Since 1947, the cosmetics, pharmaceutical (topical drugs) and food industry have used gallates as antioxidants and preservatives. As excipients they prevent oxidation of unsaturated fats in the products and consequently unpleasant smell and appearance. Currently registered antioxidant gallates are propyl (C_3), octyl (C_8) and dodecyl (C_{12}) gallates (Figure 9 b)) (Reviewed in 57). In general, their activity spectrum is antibacterial and antifungal, while gallic acid and gallates also showed some inhibition of DNA and RNA viruses (58, 59).

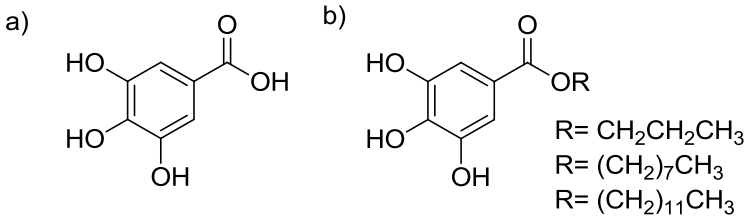


Figure 9: a) Gallic acid b) Propyl-, octyl-, and dodecil gallates.

One of the gallic acid consisting molecules is also epigallocatechin gallate – EGCG (Figure 10) which is a major catechin found in green tea (*Camellia sinensis*). It was discovered to have a strong antioxidative, antiangiogenic, and also cancer chemopreventive effect. Other activities that are worth mentioning are beneficial effects in diabetes, Parkinson's disease, Alzheimer's disease, stroke, and obesity (Reviewed in 60).

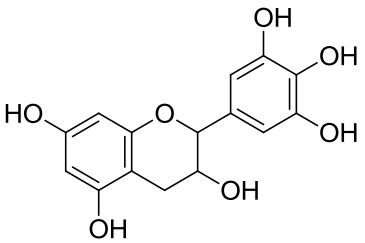


Figure 10: Epigallocatechin gallate.

Alvesalo J *et al*. screened antimicrobial activity of natural flavonoids and other natural and structurally similar synthetic compounds against *C. pneumoniae* in human cell line.

High *in vitro* inhibition against *C. pneumoniae* was found for flavones, flavonols, synthetic flavonoids, natural coumarins, and synthetic coumarins as well as for gallates using immunofuorescence (IF) staining method after incubation of cells with compounds. The tested methyl-, propyl-, octyl-, and dodecyl gallates and EGCG are considered as highly active compounds. Furthermore, four most effective compounds were tested for the effect of pre-treatment and continuous treatment on formation of chlamydial inclusions and yield of infectious chlamydial particles. Octyl gallate resulted to be the best, as it showed a 100% inhibition at 50 μm concentration in all tests (61).

6 AIMS AND OBJECTIVES

We established our work plan based on the published studies by Alvesalo J *et al*. (61) and Keurulainen L *et al*. (54) where high potency of antichlamydial effects of benzimidazoles (Figure 6) and gallates (Figure 9) are presented. We will combine those two fragments into a novel class of compounds to increase antibacterial activity against *C. pneumoniae.*

Our work plan will include:

1. Synthesis of novel compounds, which will link two important fragments for antichlamydial activity – benzimidazole and gallates. Our goal is to develop and optimize synthetic procedures for the preparation of compounds with general formula presented in Figure 11. The chain length between gallic acid and benzimidazole is planned to be three carbons to slightly resemble the octyl gallate in length. In addition, shorter or longer linkers will also be synthesized.

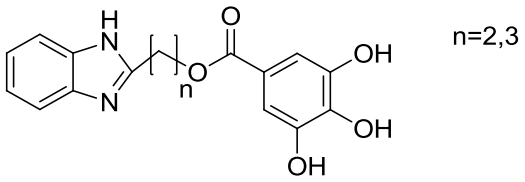


Figure 11: Hybrid molecules composed of two important fragments that showed antichlamydial activity.

2. Evaluation of antibacterial activity of synthesized compounds against *C. pneumoniae* by determination of growth inhibition.
3. Toxicity determination of active compounds on human cell lines.

7 MATERIALS AND METHODS

7.1 Materials

All reagents were commercially available and were acquired from Fluka, Sigma Aldrich, Mallinckrodt Baker, Merck, Alfa Aesar, Acros Organics, and Riedel-de-Haën. THF was distilled over sodium/benzophenone.

7.2 Methods

Chromatographic methods

Thin-layer chromatography (TLC)

The progress of the chemical reaction was monitored by thin-layer chromatography on Merck (Darmstadt, Germany) silica gel 60-F_{254} plates; 0.2 mm thin with added fluorescence indicator. Plates were conducted at 254 nm wavelength with UV lamp to detect spots on the plate.

Flash SiO_2 column chromatography (Flash)

To separate and purify compounds from reaction mixture, column chromatography was performed with a Merck silica gel 60 (230 – 400 mesh) using different eluents.

Biotage flash chromatography

Flash chromatography was mostly performed on Biotage SP1 purification system (Uppsala, Sweden) using 25+ M or 12+ M cartridges (25 or 12 mL/min flow rate, detection 254 nm).

Spectroscopic method

Nuclear magnetic resonance (NMR)

Reaction mixtures and compounds were primarily tested with NMR on a Varian Mercury 300 MHz spectrometer (Varian, Palo Alto, CA). ^{1}H and ^{13}C NMR were recorded in solutions in DMSO-d_6 or $CDCl_3$ purchased from Aldrich (Schnelldorf, Germany). Chemical shifts (δ) are given in parts per million (ppm) relative to the NMR solvent signals (DMSO-d_6 2.50 and 39.51 ppm, $CDCl_3$ 7.26 ppm) or TMS internal standard (0.00 ppm).

Combination of chromatographic and spectroscopic method

LC-MS

Liquid chromatography–mass spectrometry analyses were performed by the use of an HP1100 instrument with a UV detector (210 nm) and an Esquire LC spectrometer (Bruker Daltonik, Bremen, Germany) with ESI ion source. Signal separation was carried out by the use of an XBridge C18 column (2.1 x 500 mm, 2.1 µm). The eluent consisted of water (+ 0.1% HCO_2H) and acetonitrile (+ 0.1% HCO_2H) (gradient run 80: 20 -> 5: 95). Purity of all tested compounds was >95%.

High resolution mass spectra (HRMS)

HRMS were measured on a Synapt G2 HDMS Q-TOF-instrument (Milford, MA, USA) with positive ESI.

Determination of melting points

Melting points were measured using an IA9100 digital melting point apparatus (Electrothermal Engineering, Essex, UK).

Microwave assisted synthesis

Microwave assisted synthesis was conducted by Biotage SP1MW instrument (Uppsala, Sweden).

Nomenclature and drawing chemical structures

Structural formulas were drawn and named in the program ChemDraw version 12.0 by CambridgeSoft.

Biological testing

Immunofluorometric – IF Measurement of *C. pneumoniae* Strain CWL-029

The dried coverslips were stained with Pathfinder Chlamydia Culture Confirmation System (Bio-Rad, Hercules, CA, USA) containing isothiocyanate conjugated monoclonal antibody. The Pathfinder solution was distributed to a marked slide, and coverslips were placed on the slide cells facing down and incubated for 30 min at 37˚C in a humid environment. The

excess label was removed by dipping the coverslips twice in 6.7 mM PBS (pH 7.4) and once in purified water (Milli-Q). Coverslips were mounted to objective glasses, and the chlamydial inclusions were counted under a fluorescence microscope (Nikon Eclipse TE300, Tokyo, Japan) in a magnification of 200. Four eye-fields from every coverslips were counted, totaling 12 measurements per compound.

Resazurin test

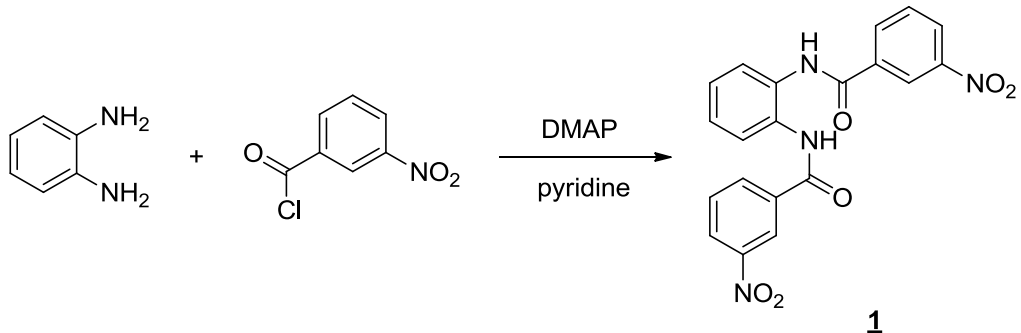
A solution containing $4x10^5$ mammalian cells/ml (HL-cells) is prepared ($1x10^5$ cells/well). The cellular solution is added in 96-well plates 200 µl/well. Incubation for 24 hours at 37°C. 20 µl of culture media replaced with compound 20 µl/well. Incubation for 72 hours at 37°C. Culture media is replaced with 90% PBS and 10% resazurin. 2 hours incubation at 37°C followed by fluorescence measurement.

8 EXPERIMENTAL PART

8.1 Synthesis of benzimidazoles

8.1.1 Synthesis of 3-(1H-benzo[d]imidazol-2-yl)anilines, according to lit. (54)

8.1.1.1 Synthesis of bisamide



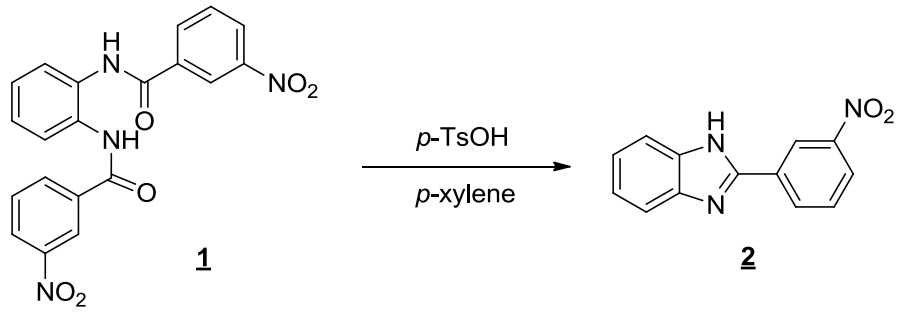
1

1,2-diaminobenzene (1.08 g; 10.00 mmol; 1.0 eq) was dissolved in 20 ml pyridine in 20 ml tube and cooled down in an ice bath. To the obtained solution 3-nitrobenzoyl chloride (3.90 g; 21.00 mmol; 2.1 eq) and DMAP (61.00 mg; 0.50 mmol; 0.05 eq) were added and the reaction mixture was stirred under microwave irradiation (15 min; 60˚C; sealed tube). After irradiation, the reaction mixture was poured into 2 M HCl solution in an ice bath. The precipitate was collected and washed with 4 M HCl solution (2 x 30 ml), H_2O (1 x 30 ml), 1 M NaOH solution (1 x 30 ml), H_2O (1 x 30 ml) and Et_2O (1 x 20 ml) to give a crude white solid **1**, which was used for the next step without purification.

***N,N'*-(1,2-phenylene)bis(3-nitrobenzamide) (1)**	
Molecular weight	406.35 g/mol
Yield	4.08 g (101%)
Appearance	(Crude) white solid (lit. (54): off-white powder)

Analysis	
1H NMR (300 MHz, DMSO-d6)	δ 7.32–7.35 (m, 2H, 2Ar-H), 7.70 (dd, J = 6.4 Hz, J = 3.9 Hz, 2H, 2Ar-H), 7.82 (t, J = 7.8 Hz, 2H, 2Ar-H), 8.39–8.44 (m, 4H, 4Ar-H), 8.79 (s, 2H, 2Ar-H), 10.38 (s, 2H, 2NH) ppm
13C NMR (75 MHz, DMSO-d6)	δ 122.6, 125.8, 126.2, 126,2, 130.2, 131.4, 134.2, 136.1, 147.7, 163.6 ppm

8.1.1.2 Cyclisation

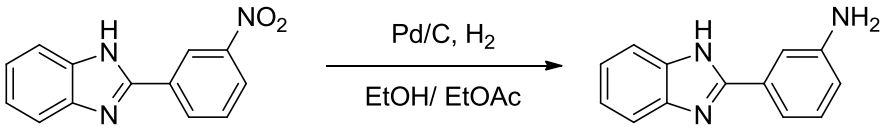


N,*N*'-(1,2-phenylene)bis(3-nitrobenzamide) (**1**) (3.66 g; 9.00 mmol; 1 eq) and *p*-TsOH (2.74 g; 14.40 mmol; 1.6 eq) were refluxed in 70 ml *p*-xylene at 140˚C for 18 h. After cooling, mixture was portioned between 120 ml EtOAc and 160 ml 1 M NaOH solution. The aqueous layer was furthermore extracted with EtOAc (2 x 80 ml). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Crude product was recrystalized from EtOH and distilled water to give yellow crystals (**2**).

2-(3-nitrophenyl)-1H-benzo[d]imidazole (2)	
Molecular weight	239.23 g/mol
Yield	1.82 g (84%)
Appearance	Yellow crystals (lit. (54): yellow crystals)

Analysis	
^{1}H NMR (300 MHz, DMSO-d_6)	δ 7.23–7.28 (m, 2H, 2Ar-H), 7.65 (dd, *J* = 6.0 Hz, *J* = 3.3 Hz, 2H, 2Ar-H), 7.85 (t, *J* = 8.1, 1H, Ar-H), 8.30–8.34 (m, 1H, Ar-H), 8.59–8.62 (m, 1H, Ar-H), 9.01 (t, *J* = 2.1, 1H, Ar-H), 13.14 (s, 1H, NH) ppm
^{13}C NMR (75 MHz, DMSO-d_6)	δ 120.8, 122.7, 124.2, 130.6, 131.7, 132.5, 148.4, 149.0 ppm
Melting point	208˚C (lit. (54): 208˚C)

8.1.1.3 Reduction of NO_2



2 **3**

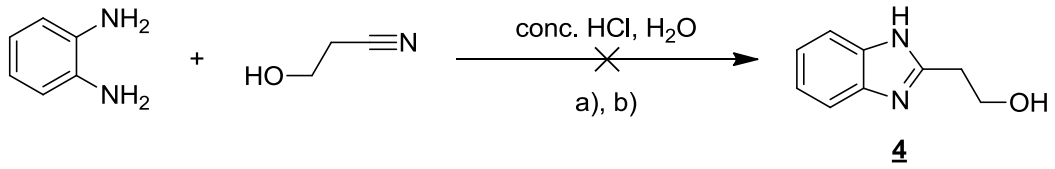
To the solution of 2-(3-nitrophenyl)-1H-benzo[d]imidazole (**2**) (1.76 g; 7.37 mmol; 1 eq) in 150 ml EtOH/EtOAc (2:1), 10% Pd/C (0.147 g – 0.02 g/mmol starting compound) was added in small portions and the solution was hydrogenated at room temperature for 5.5 h. The reaction mixture was filtered through a small pad of Celite, rinsed with EtOH, and the filtrate was evaporated *in vacuo* to give a crude green-brown solid (**3**).

3-(1H-benzo[d]imidazol-2-yl)aniline (3)	
Molecular weight	209.25 g/mol
Yield	1.49 g (96%)
Appearance	(Crude) green-brown solid (lit. (54): beige)

Analysis	
^{1}H NMR (300 MHz, DMSO-d_6)	δ 5.30 (s, 2H, NH_2), 6.68 (dt, J = 8.1 Hz, J = 0.9 Hz, 1H, Ar-H), 7.15–7.20 (m, 3H, 3Ar-H), 7.28 (d, J = 7.5 Hz, 1H, Ar-H), 7.43 (t, J = 1.8 Hz, 1H, Ar-H), 7.55 (s, 2H, 2Ar-H), 12.70 (s, 1H, NH) ppm
Melting point	264˚C

8.1.2 Synthesis of 2-(1H-benzo[d]imidazol-2-yl)ethanoles

8.1.2.1 According to lit. (62)



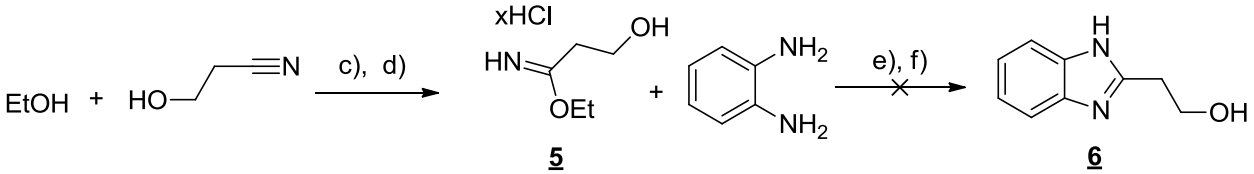
4

a) reflux at 120˚C for 21 h; b) microwave irradiated for 2 h at 100˚C, sealed tube

1,2-diaminobenzene (0.61 g; 5.62 mmol; 0.8 eq) and 3-hydroxypropionitrile (0.48 ml; 7.03 mmol; 1 eq) were dissolved in 5 ml water containing concentrated HCl (37%, 1.14 ml, 13.78 mmol; 1.9 eq) and exposed to reaction contitions a) or b).

To the aqueous solution of **4** mixture, 2 ml of 25% NH_3 solution was added till pH neutral and extracted with EtOAc (3 x 8 ml). Organic layers were collected and washed with brine, dried over Na_2SO_4, filtered and evaporated under reduced pressure to give a crude black solid. ^{1}H NMR indicated that no product – **4** was obtained.

8.1.2.2 According to lit. (63)



5 **6**

c) in aqueous solution: To the solution of 3-hydroxypropionitrile (0.48 ml; 7.03 mmol; 1 eq) and EtOH (0.48 ml; 8.36 mmol; 1.2 eq) in 10 ml Et_2O concentrated HCl (37%; 1.14 ml; 13.78 mmol; 1.9 eq) was added and stirred at room temperature for 2 h to give a solution of **5**.

d) under dry condition: To the solution of 3-hydroxypropionitrile (0.48 ml; 7.03 mmol; 1 eq) and 2 M HCl in Et_2O (7.03 ml; 14.06 mmol; 2 eq) dry EtOH (0.48 ml; 8.36 mmol; 1.2 eq) was added and stirred under argon atmosphere at room temperature for 2 h to give **5**.

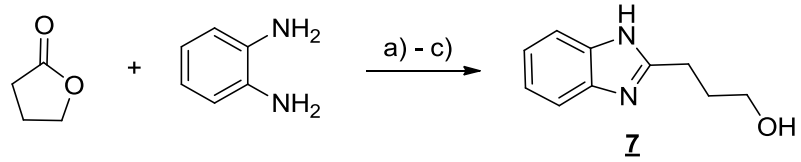
e) To a solution of **5** (procedure c), 1,2-diaminobenzene (0.61 g; 5.62 mmol; 0.8 eq) was added and refluxed at 45˚C for 3 h. After 3 h, 15 drops of NH_3 solution and 10 ml water was added and extracted with EtOAc (3 x 15 ml). Organic layer was washed with brine, dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure to give brown oil as crude product. More solid material was in water layer, which was collected as pink solid. ^{1}H NMR indicated that no product – **6** was obtained.

f) To a solution of **5** (procedure d), 1,2-diaminobenzene (0.61 g; 5.62 mmol; 0.8 eq) and 5 ml dry EtOH were added and refluxed at 85˚C for 20 h. The reaction mixture was poured into 15 ml saturated $NaHCO_3$ and extracted with DCM (3 x 15 ml). Organic layers were combined and dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure to give black oil. ^{1}H NMR indicated that no product – **6** was obtained.

8.1.3 Synthesis of 3-(1H-benzo[d]imidazol-2-yl)propan-1-oles

8.1.3.1 Gamma-butyrolactone as starting material according to lit. (64), (65) and (66)

8.1.3.1.1 With 1,2-diaminobenzene



7

a) Under dry conditions: To 1,2-diaminobenzene (0.31 g; 2.90 mmol; 1 eq) in 15 ml of dry Et_2O, gamma-butyrolactone (0.22 ml; 2.90 mmol; 1 eq) and 2 M HCl in Et_2O (2.90 ml; 5.81 mmol; 2 eq) was added. The obtained solution was refluxed at 45˚C for 4 h. The reaction mixture was washed with 10% Na_2CO_3 solution (1 x 20 ml) and aqueous layer was extracted with Et_2O (1 x 20 ml). Combined organic layers were washed with brine, dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure to give brown solid. ^{1}H NMR indicated that no product – **7** was obtained.

b) In aqueous conditions at reflux: To 1,2-diaminobenzene (0.63 g; 5.81 mmol; 1 eq) in 10 ml water, gamma-butyrolactone (0.45 ml; 5.81 mmol; 1 eg) was added. After stirring, concentrated HCl (37%; 1.44 ml; 17.42 mmol; 3 eq) was added and reaction mixture was

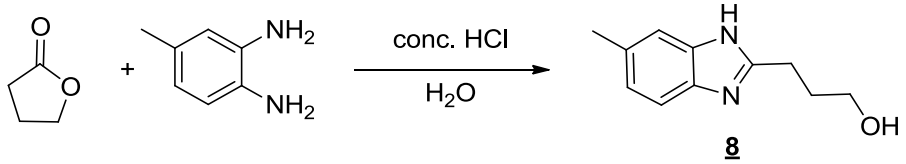
stirred at 120˚C for 24 h. According to TLC, it seemed like 1,2-diaminobenzene did not react yet, so more gamma-butyrolactone (0.089 ml; 1.16 mmol; 0.2 eq) and concentrated HCl (37%; 0.30 ml; 3.50 mmol; 0.5 eq) was added. After 48 h, 10 ml 2 M NaOH was added to the reaction mixture. Aqueous layer was extracted with EtOAc (3 x 20 ml) and combined organic layers were washed with brine, dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure. Crude product was recrystallized from acetonitrile: isopropanole (5:1) to give light green-grey crystals (7).

c) Into a microwave tube, the mixture of 1,2-diaminobenzene (0.14 g; 1.25 mmol; 1 eq), gamma-butyrolactone (0.12 ml; 1.50 mmol; 1.2 eq), and concentrated HCl (37%; 0.31 ml; 3.75 mmol; 3 eq) in 3 ml of water was stirred under microwave irradiation at 120˚C for 4 h, 4-5 bar. 2 ml of 2 M NaOH was added to the mixture and extracted with EtOAc (3 x 10 ml). Combined organic layers were washed with water, brine, dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure to give a crude yellow solid (7).

3-(1H-benzo[d]imidazol-2-yl)propan-1-oles (7)	
Molecular weight	176.22 g/mol
Yield	proc b): 0.58 g (57%); proc c): 0.09 g (40%)
Appearance	proc b): Light green-grey crystals; proc c): (Crude) yellow solid (lit (67): fine white crystals)

Analysis	
^{1}H NMR (300 MHz, DMSO-d_6)	δ 1.91 (q, J = 7.5 Hz, 2H, CH_2), 2.84 (t, J = 7.2 Hz, 2H, Ar-CH_2), 3.48 (t, J = 6.3 Hz, 2H, CH_2-OH), 4.63 (s, 1H, OH), 7.07–7.12 (m, 2H, 2Ar-H), 7.44 (s, 2H, 2Ar-H), 12.16 (s, 1H, NH) ppm
^{13}C NMR (75 MHz, DMSO-d_6)	δ 25.3, 30.8, 60.2, 121.0, 155.1 ppm
Melting point	164–165˚C (lit. (67): 165–167˚C)
ESI-HRMS	$C_{10}H_{13}N_2O$ (MH^+): Calculated (m/z): 177.1028; Result (m/z): 177.1036

8.1.3.1.2 In aqueous conditions refluxing with 4-methylbenzene-1,2-diamine



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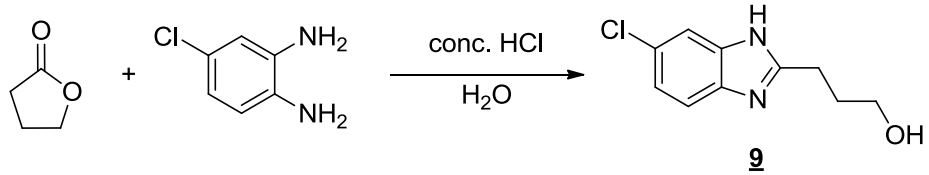
To 4-methylbenzene-1,2-diamine (1.70 g; 13.92 mmol; 1 eq) in 10 ml water, gamma-butyrolactone (1.07 ml; 13.92 mmol; 1 eq) was added. After stirring, concentrated HCl (37%; 2.89 ml; 34.84 mmol; 3 eq) was added and reaction mixture was stirred at 120˚C for 67 h. Into a beaker with 20 ml 2 M NaOH, reaction mixture was added drop wise and extracted with EtOAc (4 x 20 ml). Combined organic layers were washed with brine, dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure. Crude product was recrystallized from acetonitrile: isopropanole (3:1) to give grey-black crystals (**8**).

3-(6-methyl-1H-benzo[d]imidazol-2-yl)propan-1-ol (8)	
Molecular weight	190.24 g/mol
Yield	1.37 g (52%)
Appearance	Grey-black crystals

Analysis	
^{1}H NMR (300 MHz, DMSO-d_6)	δ 1.89 (q, J = 7.8 Hz, 2H, CH_2), 2.38 (s, 3H, CH_3), 2.81 (t, J = 7.2, 2H, Ar-CH_2), 3.47 (q, J = 6 Hz, 2H, CH_2-OH), 4.62 (t, J = 4.8 Hz, 1H, OH), 6.91 (d, J = 7.5 Hz, 1H, Ar-H), 7.18–7.38 (m, 2H, 2Ar-H), 11.99 (s, 1H, NH) ppm
^{13}C NMR (75 MHz, DMSO-d_6)	δ 21.2, 25.4, 30.8, 60.2, 122.4, 154.7 ppm
Melting point	133-134˚C
ESI- HRMS	$C_{11}H_{15}N_2O$ (MH^+): Calculated (m/z): 191.1184; Result (m/z): 191.1190

8.1.3.1.3 In aqueous conditions refluxing with 4-chlorobenzene-1,2-diamine



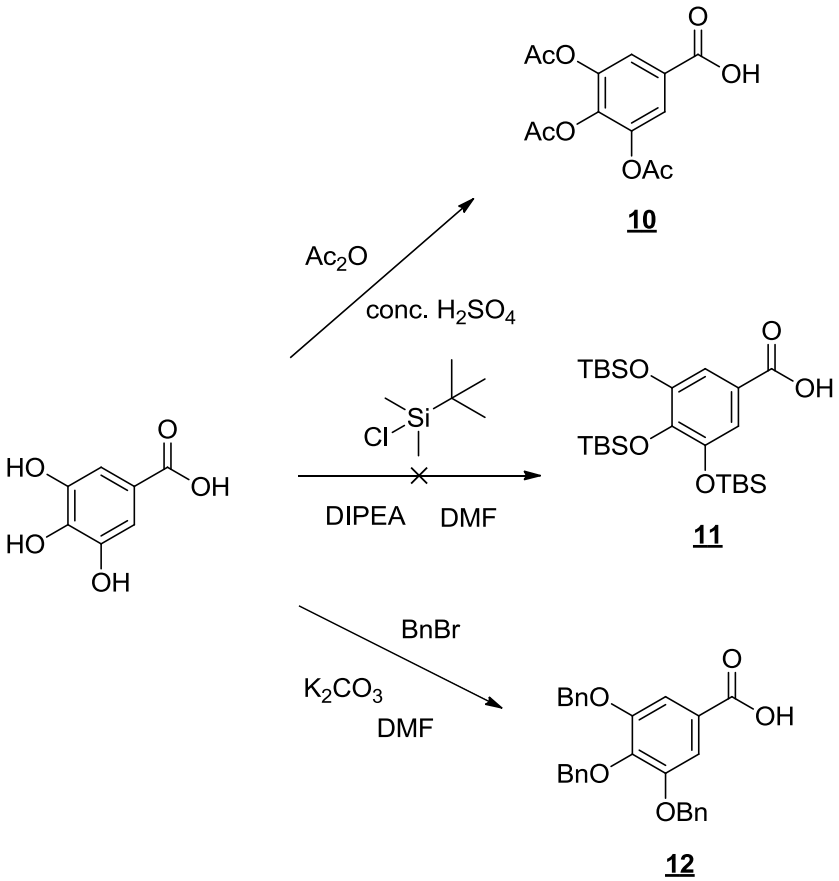
9

To 4-chlorobenzene-1,2-diamine (0.83 g; 5.81 mmol; 1 eq) in 15 ml water, gamma-butyrolactone (0.45 ml; 5.81 mmol; 1 eq) was added. After stirring, concentrated HCl (37%; 1.44 ml; 17.42 mmol; 3 eq) was added and reaction mixture was stirred at 100˚C for 72 h. Into a beaker with 20 ml 2 M NaOH, reaction mixture was added dropwise and extracted with EtOAc (3 x 20 ml). Combined organic layers were washed with water, brine, dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure. The crude product was recrystalized from acetonitrile: isopropylamine = 5:1 to give dark red crystals (**9**).

3-(6-chloro-1H-benzo[d]imidazol-2-yl)propan-1-ol (9)	
Molecular weight	210.66 g/mol
Yield	0.47 g (38%)
Appearance	Dark red crystals

Analysis	
^{1}H NMR (300 MHz, DMSO-d_6)	δ 1.87–1.96 (m, 2H, CH_2), 2.85 (t, J = 7.5, 2H, Ar-CH_2), 3.48 (t, J = 5.4, 2H, CH_2-OH), 7.12 (dd, J = 1.8 Hz, J = 1.8 Hz, 1H, Ar-H), 7.48 (d, J = 11.1 Hz, 2H, 2Ar-H), 12.33 (s, 1H, NH) ppm
^{13}C NMR (75 MHz, DMSO-d_6)	δ 25.2, 30.6, 60.0, 121.2, 156.8 ppm
Melting point	170˚C
ESI-HRMS	$C_{10}H_{12}N_2OCl$ (MH^+): Calculated (m/z): 211.0638; Result (m/z): 211.0647

8.2 Introduction of protecting groups for gallic acid



a) According to lit. (68): To gallic acid (1.50 g; 8.82 mmol; 1 eq) in 6 ml of Ac_2O, one drop of concentrated H_2SO_4 was added. The clear solution was heated at 80˚C for 0.3 h, cooled, and dropwise added to 20 ml of ice-water and left at room temperature for 3 h. White solid was filtered and dissolved in 70 ml EtOAc to give smoothly yellow liquid. Solvent was evaporated under reduced pressure and dried in Schlenk line to give a white solid (**10**).

3,4,5-triacetoxybenzoic acid (10)	
Molecular weight	296.23 g/mol
Yield	2.03g (78%)
Appearance	White solid

Analysis	
^{1}H NMR (300 MHz, DMSO-d_6)	δ 2.31 (s, 9H, 3CH_3CO), 7.87 (s, 2H, 2Ar-H) ppm
Melting point	163˚C (lit. (68): 163˚C)

b) According to lit. (69): Diisopropylethylamine – DIPEA (5.94 ml; 35.27 mmol; 6 eq) was added syringed into a suspension of gallic acid (1.00 g; 5.88 mmol; 1 eq) and *tert*-butyldimethylsilyl chloride (4.43 g; 29.39 mmol; 5 eq) in 15 ml dry DMF under argon atmosphere at room temperature. The clear solution turned into suspension after 1.6 h and left stirring for 3.5 h. The reaction mixture was added to 50 ml of cold 1 M H_2PO_4 to give white precipitate. The product was extracted into *n*-hexane (3 x 45 ml). The organic extract was washed with saturated solution of $NaHCO_3$ (3 x 45 ml), followed by brine, dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure to give clear yellow oil. The crude tetrasilylated derivative was treated with 5 ml H_2O, 7 ml THF and 15 ml AcOH at room temperature for 1 h. The reaction mixture was poured into 40 ml ice-cold H_2O and extracted with Et_2O:*n*-hexane = 1:1 (3 x 40 ml), washed with water, brine, dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure to give clear yellow oil as crude **11**, which was purified by Biotage using DCM as eluent to give white solid. ^{1}H NMR indicated that no product – **11** was obtained.

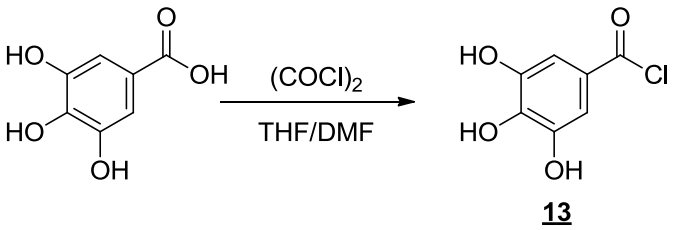
c) According to lit. (70): A mixture of gallic acid (1.00 g; 5.88 mmol; 1 eq) and anhydrous K_2CO_3 (7.71 g; 48.53 mmol; 8.25 eq) in 15 ml dry DMF was stirred at room temperature under argon atmosphere for 1 h. Although after 1 h all solids were not dissolved yet, benzyl bromide (4.4 ml; 36.99 mmol; 6.3 eq) was added dropwise into the solution over 0.5 h at 60˚C. The reaction mixture was stirred at 60˚C for 3 h, cooled and K_2CO_3 was filtered off. The filtrate was added into 20 ml water and extracted with Et_2O (1 x 20 ml). Organic layer was washed with water (3 x 10 ml) to give orange solution, which was dried over Na_2SO_4, filtered and evaporated under reduced pressure to give clear orange oil. The residue was dissolved in aqueous EtOH (50%, 60 ml) containing 20 ml 5 M NaOH and refluxed as purple solution at 102˚C for 3 h. The solution was left to cool down, diluted with 20 ml H_2O, adjusted to pH 2 with concentrated HCl and stirred as yellow solution with some orange-purple solid at room temperature for 30 min. Crude solids were recrystallized from MeOH to afford white crystals as **12**.

3,4,5-tris(benzyloxy)benzoic acid (12)	
Molecular weight	440.49 g/mol
Yield	0,09g (3%)
Appearance	White solid

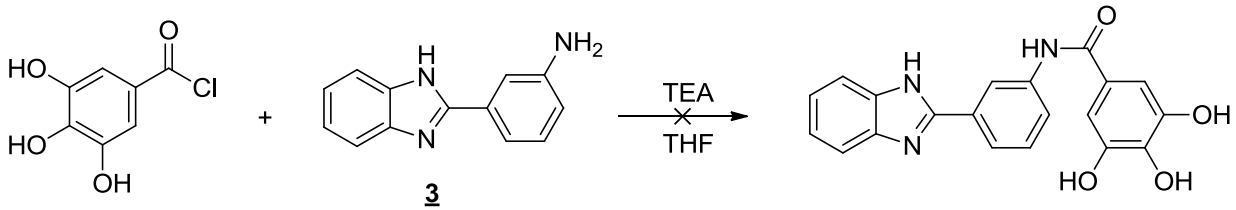
Analysis	
^{1}H NMR (DMSO-d_6)	δ 5,04 (s, 2H, CH_2), 5,13–5,19 (m, 4H, $2CH_2$), 7,27–7,48 (m, 20H, 20Ar-H) ppm

8.3 Amidation of benzimidazoles and gallic acid

8.3.1 With unprotected gallic acid



13

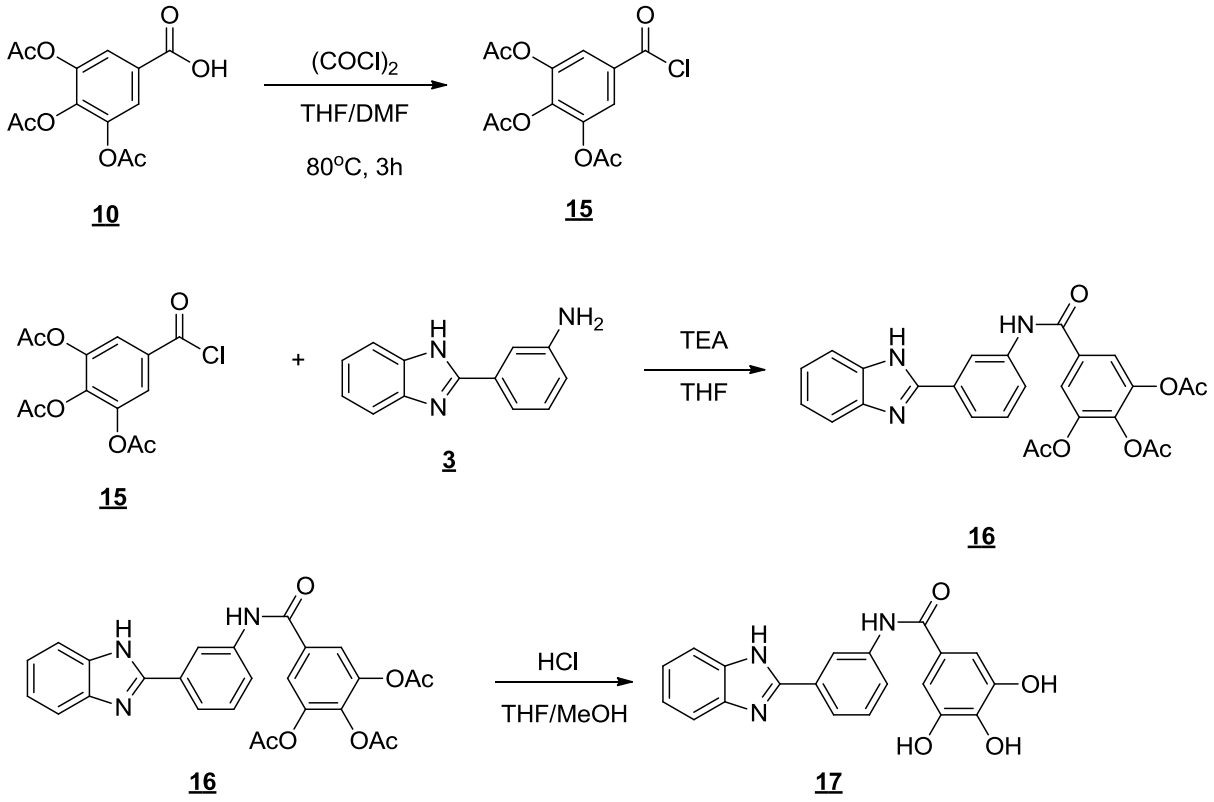


13 **3** **14**

A solution of gallic acid (0.20 g; 1.20 mmol; 1.2 eq), oxalyl chloride (0.10 ml; 1.20 mmol; 1.2 eq) and one drop of DMF in 2 ml anhydrous THF was stirred at room temperature under argon atmosphere for 1.5 h to give **13**.

According to lit. (54): The solution of 3-(1H-benzo[d]imidazol-2-yl)aniline (**3**) (0.21 g; 1.00 mmol; 1 eq) in 10 ml anhydrous THF and TEA (0.21 ml; 1.5 mmol; 1.5 eq) were added to the mixture of **13**. After stirring at room temperature for 20 h, the residue was partitioned between 100 ml water and 50 ml EtOAc. The aqueous layer was separated and extracted with EtOAc (1 x 50 ml) and the combined organic phases were washed with saturated $NaHCO_3$ (1 x 50 ml), brine, dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure to give clear orange-brown solid as crude. ^{1}H NMR indicated that no product – **14** was obtained.

8.3.2 With protected gallic acid



A solution of 3,4,5-triacetoxybenzoic acid (**10**) (0.36 g; 1.20 mmol; 1.2 eq), oxalyl chloride (0.10 ml; 1.20 mmol; 1.2 eq) and one drop of DMF in 4 ml anhydrous THF was stirred at 80˚C under argon atmosphere for 3 h to give **15**. More oxalyl chloride (0.03 ml; 0.3 mmol; 0.3 eq) was added but no difference was noticed on TLC.

The solution of 3-(1H-benzo[d]imidazol-2-yl)aniline (**3**) (0.21 g; 1.00 mmol; 1 eq) in 10 ml anhydrous THF and TEA (0.21 ml; 1.5 mmol; 1.5 eq) were added to the mixture of **15**. The solution was changed from clear orange to a yellow emulsion. After stirring at room temperature for 1.5 h, the residue was partioned between 100 ml water and 50 ml EtOAc. The aqueous layer was separated and extracted with EtOAc (1 x 50 ml) and the combined organic phases were washed with saturated $NaHCO_3$ (1 x 50 ml), brine, dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure to give crude product (**16**) which was used in further reaction without purification.

According to lit. (71): Crude 5-((3-(1H-benzo[d]imidazol-2-yl)phenyl)carbamoyl)benzene-1,2,3-triyl triacetate (**16**) (0.68 g; 1.39 mmol; 1 eq) was added to a solution of 5 ml THF, 5 ml MeOH and 2.5 ml concentrated HCl (37%). The

reaction mixture was stirred at 60˚C for 1 h. Solvent was evaporated under reduced pressure and the residue was purified with flash column chromatography using DCM/MeOH (93:3) as eluent to give some fractions and again purified with flash column chromatography using Acetone/MeOH (95:5) and the traces of the product was isolated as yellow crystals (**17**).

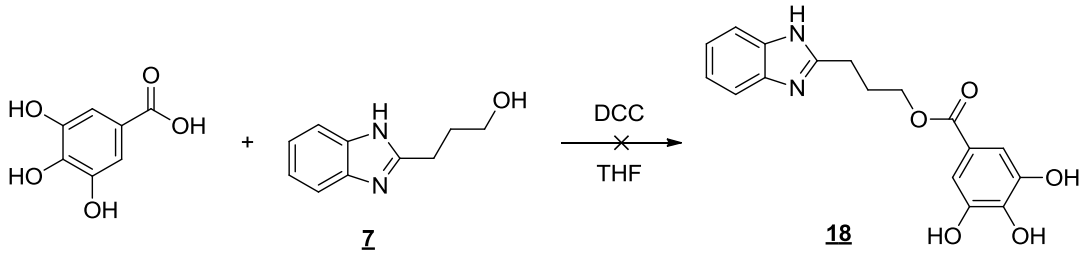
N-(3-(1H-benzo[d]imidazol-2-yl)phenyl)-3,4,5-trihydroxybenzamide (17)	
Molecular weight	361.1 g/mol
Yield	0.05 g (10%)

Analysis	
Appearance	Yellow crystals
LC-MS	0.799 min, MH^+ 362.2 g/mol, 100% pure
^{1}H NMR (300 MHz, CD_3OD)	*

*LC-MS showed the presence of the product but due to bad resolution, peaks in NMR spectra could not be assigned.

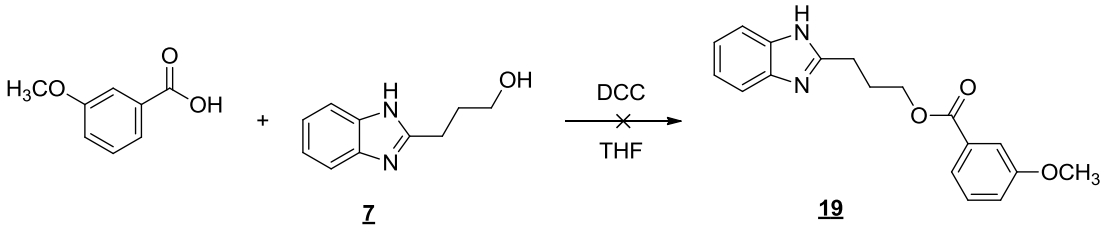
8.4 Esterification of benzimidazoles with (derivatives of) gallic acid

8.4.1 With unprotected gallic acid according to lit. (72)



To a solution of gallic acid (0.06 g; 0.37 mmol; 1 eq) and 3-(1H-benzo[d]imidazol-2-yl)propan-1-ol (**7**) (0.06 g; 0.37 mmol; 1 eq) in 25 ml of THF cooled to 0˚C, DCC (0.16 g; 0.77 mmol; 2.1 eq) solution in 4 ml of THF was added. After stirring at room temperature for 42 h, the solvent was removed under reduced pressure. The residue was extracted with EtOAc (4 x 25 ml) and filtered. The filtrate was washed with 10% citric acid (2 x 45 ml), water (1 x 45 ml), saturated $NaHCO_3$ solution (2 x 45 ml) and water (1 x 30 ml). Organic layer was dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure to give yellow solid as crude product (**18**). ^{1}H NMR indicated that no product – **18** was obtained.

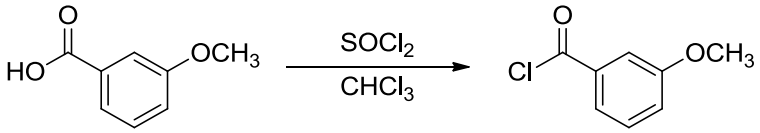
8.4.2 With 3-methoxybenzoic acid according to lit. (72)



To a solution of 3-methoxybenzoic acid (0.15 g; 1.00 mmol; 1 eq) and 3-(1H-benzo[d]imidazol-2-yl)propan-1-ol (**7**) (0.18 g; 1.00 mmol; 1 eq) in 12 ml of THF cooled to 0˚C was added DCC (0.43 g; 2.10 mmol; 2.1 eq) solution of in 6 ml of THF. After stirring at room temperature for 20 h, the solvent was removed under reduced pressure. To the residue, 25 ml of DCM was added and solids were filtered off. The filtrate was washed with saturated $NaHCO_3$ solution (1 x 20 ml), water and brine. Organic layer was dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure to give crude product,

which was purified with Biotage using EtOAc/*n*-hexane (1:4) as an eluent to give white solid. ^{1}H NMR indicated that no product – **19** was obtained.

8.4.3 Synthesis of an ester via acyl chloride according to lit. (73)



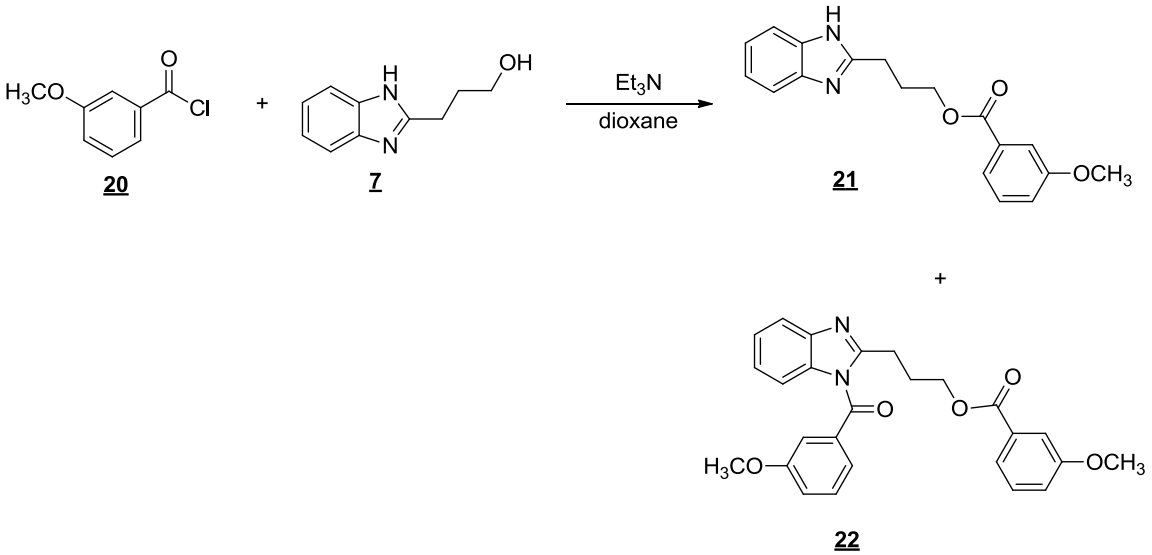
20

3-methoxy benzoic acid (0.15 g; 1.00 mmol; 1 eq) was transformed into acyl chloride by reaction with $SOCl_2$ (0.15 ml; 2.00 mmol; 2 eq) in 5 ml of $CHCl_3$ at 60˚C for 4 h. The reaction mixture was cooled down to room temperature and the solvent was removed under reduced pressure to give white oil (**20**).

3-methoxybenzoyl chloride (20)	
Molecular weight	170.59 g/mol
Yield	0.17g (100%)
Appearance	White oil

Analysis	
^{1}H NMR (300 MHz $CDCl_3$)	δ 3.87 (s, 3H, CH_3), 7.20–7.30 (m, 1H, Ar-H), 7.39–7.49 (m, 1H, Ar-H), 7.59–7.63 (m, 1H, Ar-H), 7.73–7.80 (m, 1H, Ar-H) ppm

8.4.4 Esterification with benzimidazole propanol and acyl chloride (74)



A solution of 3-methoxybenzoyl chloride (**20**) (0.17 g; 1.00 mmol; 1 eq), triethylamine (0.14 ml; 1.00 mmol; 1 eq) and 3-(1H-benzo[d]imidazol-2-yl)propan-1-ol (**7**) (0.18 g; 1.00 mmol; 1 eq) in 10 ml dry dioxane was stirred under argon atmosphere at room temperature for 20 h. Then 20 ml of water was added to obtain cloudy white solution, which was extracted with DCM (3 x 20 ml). Combined organic layers were washed with 1 M HCl and dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure to give white oil which was purified with Biotage using gradient EtOAc/*n*-hexane (1:1) – 5 CV than EtOAc/hex (3:1) – 3 CV to just EtOAc – 4 CV (elution of the product). Fractions 5-8 were recrystallized from *n*-hexane/EtOAc (3:1) to give white needles (**21**) as a pure product. Fractions 2-4 as transparent oil gave also an amide (**22**) at benzimidazole nitrogen.

3-(1H-benzo[d]imidazol-2-yl)propyl 3-methoxybenzoate (21)	
Molecular weight	310.35 g/mol
Yield	0.03 g (11%)
Appearance	White needles

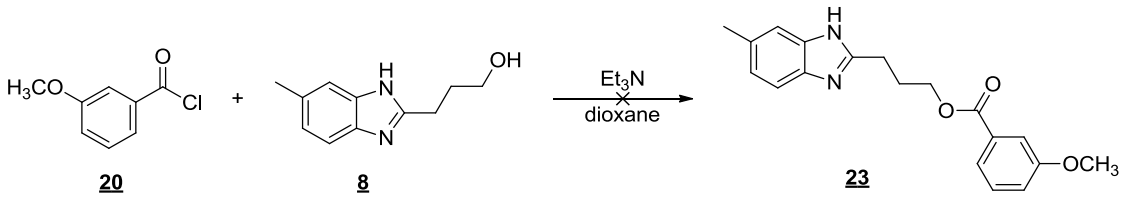
Analysis	
^{1}H NMR (300 MHz, DMSO-d_6)	δ 2.24 (t, J = 7.2 Hz, 2H, CH_2), 2.98 (t, J = 7.8 Hz, 2H, Ar-CH_2), 3.80 (s, 3H, CH_3), 4.37 (t, J = 6.3 Hz, 2H, CH_2OOC), 7.09–7.13 (m, 2H, 2Ar-H), 7.20–7.23 (m, 1H, Ar-H), 7.36–7.44 (m, 3H, 3Ar-H), 7.48–7.50 (m, 2H, 2Ar-H), 12.21 (s, 1H, NH) ppm
^{13}C NMR (75 MHz,	δ 25.2, 26.5, 55.29, 64.20, 110.66, 118.06, 119.10, 120.75, 121.34, 121.37,

DMSO-d_6)	129.81, 131.08, 134.25, 143.30, 154.20, 159.24, 165.51 ppm
LC-MS	1.867 min, MH^+ 311.2 g/mol, 96% pure
Melting point	92–93˚C
ESI-HRMS	$C_{18}H_{19}N_2O_3$ (MH^+): Calculated (m/z): 311.1396; Result (m/z): 311.1394

3-(1-(3-methoxybenzoyl)-1H-benzo[d]imidazol-2-yl)propyl 3-methoxybenzoate (22)	
Molecular weight	444.48 g/mol
Yield	0.03 g (10%)
Appearance	Transparent oil

Analysis	
^{1}H NMR (300 MHz, DMSO-d_6)	δ 2.25 (t, J = 6.9 Hz, 2H, CH_2), 2.98 (t, J = 7.2 Hz, 2H, Ar-CH_2), 3.81 (s, 6H, 2CH_3), 4.38 (t, J = 6.0 Hz, 2H, CH_2OOC), 7.09-7.12 (m, 2H, 2Ar-H), 7.17-7.23 (m, 2H, 2Ar-H), 7.39-7.55 (m, 8H, 4Ar-H) ppm
^{13}C NMR (75 MHz, DMSO-d_6)	δ 25.69, 27.04, 55.75, 55.82, 64.70, 114.49, 119.29, 119.58, 121.55, 121.84, 122.00, 130.10, 130.27, 131.67, 132.76, 154.69, 159,74, 159.80, 166.03, 167.54 ppm

8.4.5 Esterification with 6-methyl benzimidazole propanol



20 **8** **23**

A solution of 3-methoxybenzoyl chloride (**20**) (0.17 g; 1.00 mmol; 1 eq), triethylamine (0.14 ml; 1.00 mmol; 1 eq) and 3-(6-methyl-1H-benzo[d]imidazol-2-yl)propan-1-ol (**8**) (0.19 g; 1.00 mmol; 1 eq) in 10 ml of dry dioxane was stirred under argon atmosphere at room temperature for 48 h. Then 20 ml of water was added to obtain a cloudy yellow-brown solution, which was extracted with DCM (2 x 20 ml). Combined organic layers were washed with 1 M HCl (3 x 20 ml) and dried over Na_2SO_4, filtered and solvent was evaporated under reduced pressure to give brown oil as a crude product **23** which was purified with Biotage using gradient EtOAc/*n*-hexane (1:1). ^{1}H NMR indicated that no pure product – **19** was obtained.

8.5 Biological test results

Table 3: Inhibition of *C. pneumoniae* growth at different concentration of **21** and their effect on the human cell viability.

Concentration of **21**	200 µM	150 µM	100 µM	50 µM
Inhibition of bacterial growth				
Inhibition (%)	nd**	93	49	25
SEM*	nd**	2.42	5.96	5.59
Resazurin test				
Viability (%)***	79	86	89	89
SD	13	19	9	5

*SEM = standard error of the mean

**nd = not detected *C. pneumoniae* cells

***Viability (%) = 100 – Toxicity

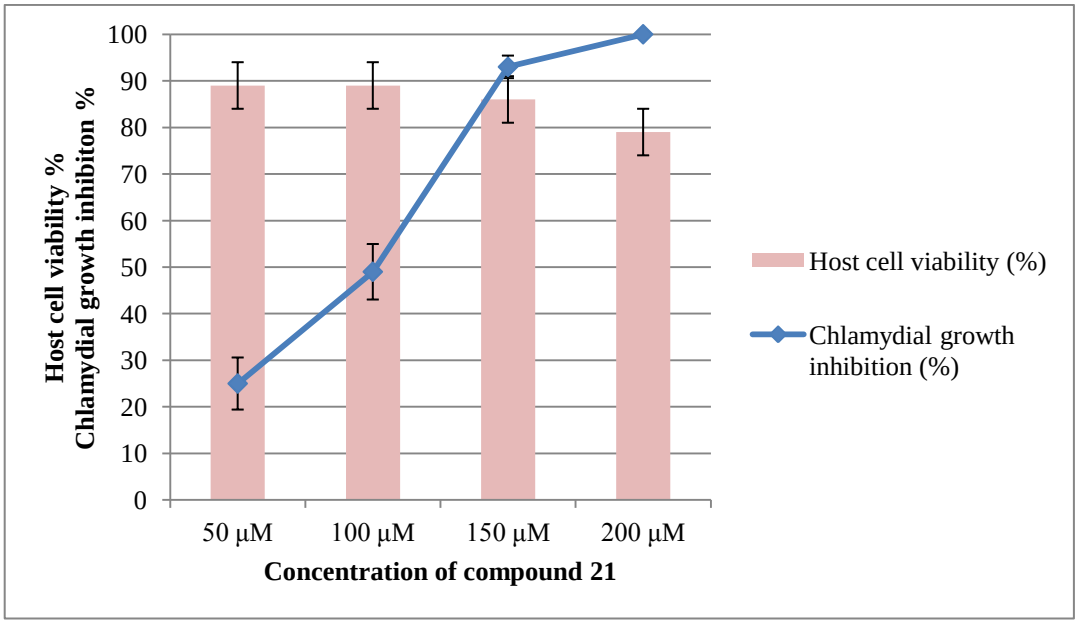


Figure 12: Antichlamydial effect (lines) and host cell viability (bars) of the compound **21**.

9 RESULTS AND DISCUSSION

9.1 Comments of synthetic procedures

In order to synthesize 3-(1H-benzo[d]imidazol-2-yl)aniline (**3**), we started with 1,2-diaminobenzene and 3-nitrobenzoyl chloride, based on the published procedure from Keurulainen *et al.* (54). After the microwave-assisted synthesis of bisamides (**1**) with acyl chloride, amine and DMAP (a catalyst) in pyridine as a solvent, *p*-toluenesulfonic acid in *p*-xylene was used for condensation of bisamide into the 2-(3-nitrophenyl)-1H-benzo[d]imidazole (**2**). Furthermore, the nitro group on phenyl substituent was hydrogenated in the presence of palladium catalyst (Pd/C) to form 3-(1H-benzo[d]imidazol-2-yl)aniline (**3**).

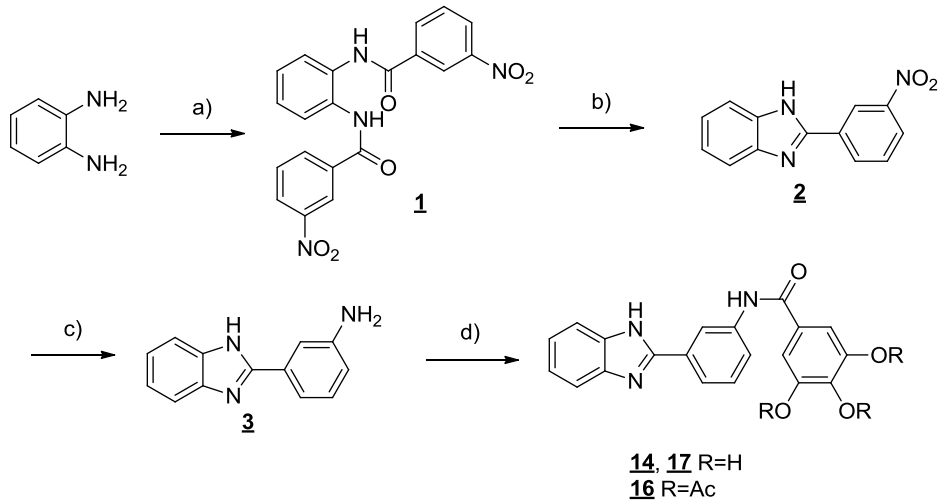


Figure 13: (a) 3-nitrobenzoyl chloride, DMAP, pyridine; (b) *p*-TsOH, *p*-xylene; (c) Pd/C, EtOH/EtOAc; (d) **14**: gallic acid, $(COCl)_2$, THF/DMF, TEA, THF; **16**: **10**, $(COCl)_2$, THF/DMF, TEA, THF; **17**: **16**, HCl, THF/MeOH.

We obtained a slightly better yield of the latest reaction as the one published in the original article – there was 3-(1H-benzo[d]imidazol-2-yl)aniline synthesized with 94% yield while we gained 96%. Further reaction steps were made in the original article from 3-(1H-benzo[d]imidazol-2-yl)anilines with acyl chlorides and TEA in anhydrous THF to obtain the amides that were to be tested furthermore on the inhibition of *C. pneumoniae.*

We tried two possible procedures for the synthesis of 2-benzimidazoylethanoles (**4**) with 1,2-diaminobenzene, but unfortunately we could not prepare the product neither directly with 3-hydroxypropionitrile (based on the published procedure from (62)), nor

with imidoester hydrochloride as the intermediate (based on the published procedure from (63)). A possible reason that the reaction did not work out is probably low nucleophilicity of aromatic amine group in 1,2-diaminobenzene. Therefore, we decided to synthesize 2-benzimidazoylpropanoles but we could not find any suitable procedure. Finally, we tried the reaction with 1,2-diaminobenzene and gamma-butyrolactone. We modified the published procedure from abstracts of Induseragam *et al.* (64), Freedman (65) and Akopyan *et al.* (66). Under dry conditions with 2 M HCl in refluxing Et_2O, there was only starting material after 4 h of reaction time, while on the other hand product was formed in aqueous solutions with concentrated HCl. Due to too low yields for reactions in microwave reactor (40%), we performed the reactions in refluxing solvent and obtained higher yields up to 57%. According to these findings, we used the same reaction procedure for 4-methyl- and 4-chloro substituted 1,2-diaminobenzene to obtain novel compounds 3-(6-methyl-1H-benzo[d]imidazol-2-yl)propan-1-ol (**8**) and 3-(6-chloro-1H-benzo[d]imidazol-2-yl)propan-1-ol (**9**), respectively.

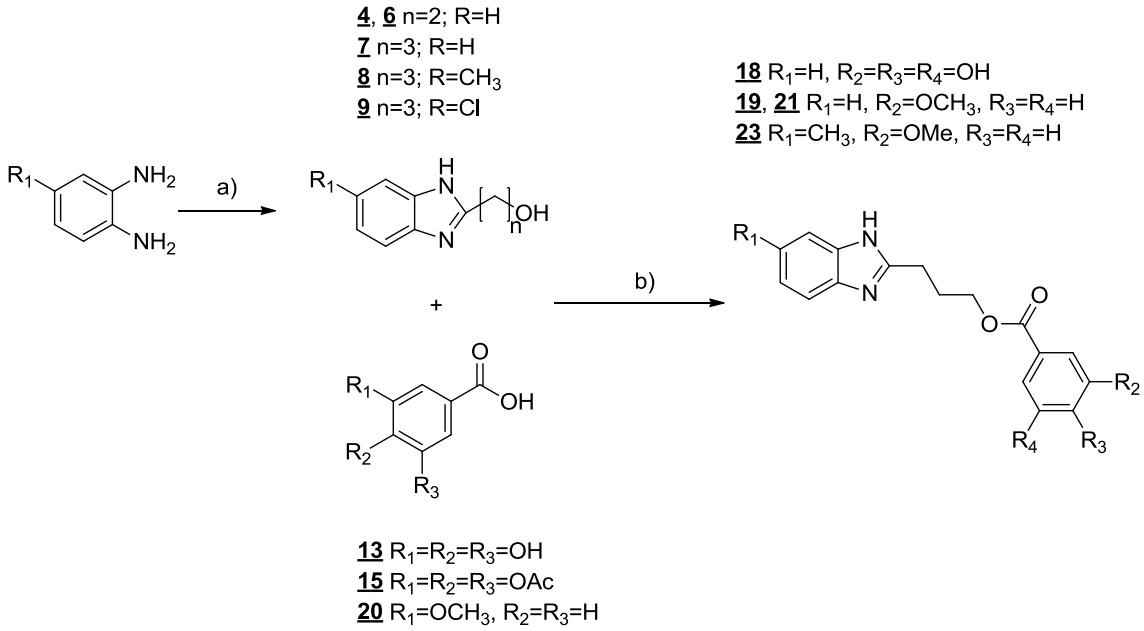
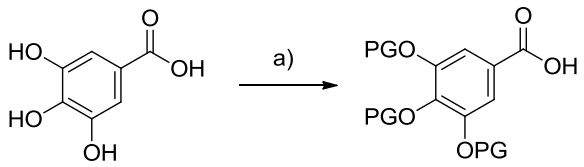


Figure 14: (a) **4**: 3-hydroxypropionitrile, conc. HCl, H_2O; **6**: EtOH, conc. HCl, Et_2O, 3-hydroxypropionitrile; **7**, **8**, **9**: Gamma-butyrolactone, conc. HCl in Et_2O; (b) **18**, **19**: **7**, **20**, DCC, THF; **21**: $SOCl_2$, $CHCl_3$, **7**, Et_3N, dioxane; **23**: **8**, **20**, Et_3N, dioxane.

The next reactions were amide bond formation (**18**) or DCC-mediated esterification (**14**) between (unprotected) gallic acid and benzimidazoles. Both reactions were unsuccessful – compounds were not detected by NMR in the final reaction solution.

Therefore, we decided to protect hydroxyl groups of gallic acid to ensure that only the carboxylic group of gallic acid will react.

Three different methods were used to protect hydroxyl groups of gallic acid. Firstly, triacetyl gallic acid was prepared (**10**). It was only used in amide bond formation, because cleaving of acetyl groups can also affect the other ester bond. Secondly, the long reaction procedure was used for the preparation of TBS protected gallic acid and we tried to purify the crude product several times but we did not succeed in obtaining the pure TBS protected gallic acid. Last but not least, a different method was used to prepare benzyl-protected gallic acid. However, it was unsuccessful due to unknown precipitate coming out of the solution.



10 PG=Ac
11 PG=TBS
12 PG=Bn

Figure 15: (a) **10**: Ac_2O, conc. H_2SO_4; **11**: TBSCl, DIPEA, DMF; **12**: anh. K_2CO_3, DMF, BnBr.

The same method (71) as for amide bond formation with unprotected gallic acid (**14**) was used for the synthesis of amides with protected triacetyl gallic acid. First, we prepared acyl chloride with oxalyl chloride in THF/DMF solution at 80°C. An amide between triacetylgalloyl acid chloride and 3-(1H-benzo[d]imidazol-2-yl)aniline (**3**) was formed after adding TEA in THF to both substances. The final step included deprotection, which was unsuccessful even though many separation procedures were tried.

Due to the synthetic problems with unprotected and protected gallic acid, we decided to replace it with an appropriate surrogate 3-methoxybenzoic acid. DCC mediated esterification did not give any product again, therefore, the acyl chloride method with $SOCl_2$ in order to obtain 3-methoxybenzoyl chloride for further reaction with **7** was used. In presence of triethylamine as a base in dioxane, target compound 3-(1H-benzo[d]imidazol-2-yl)propyl 3-methoxybenzoate (**21**) was obtained and purified with column chromatography on Biotage. The side product due to the additional reaction on benzimidazole NH group was also isolated (**22**). The same method was used for the synthesis of 6-methyl derivative (**23**).

9.2 Results of biological testing

Keurulainen L *et al.* (54) reported the results of their compounds from biological testing with IF measurement method at 10 μM concentration. Altogether 33 final compounds were synthesized and tested, herein are presented only few of them. The majority of the obtained compounds (25 out of 33) exhibited from 39 to 100% inhibition of growth at 10 μM.

Our compound **21** consisting of 2-arylbenzimidazole and 2-methylbenzoyl fragment was structurally similar to the most active of 33 compounds (compound 2) presented in Table 2. It was tested for *C. pneumoniae* inhibition. At 200 μM concentration, there were no formed inclusions of *C. pneumoniae* found in media. At 150 μM there are some inclusions, but inhibition was 93%, while at 100 μM and 50 μM, the inhibition decreased to 49% or 25%, respectively. It has a slightly weaker inhibition than the one tested in the article. However, the same pattern of the inhibition can be observed for both compounds.

The host cell viability percentage was evaluated with the resazurin test, with which we assessed the amount of intracellular ATP that was present in the cells treated with the tested compound and compared to cells treated with the DMSO-adjusted medium. The amount of ATP in a cell is considered to be a delicate indicator for cell viability (54). 21% toxicity to host cells was observed at the highest concentration of the compound **21**, on the other hand, at lower concentrations it became constant – it did not affect host cells more than 20% which is the threshold for toxicity. Therefore, it can be concluded that the compound **21** is well tolerated in host cells.

10 CONCLUSION

Chlamydia pneumoniae is known as an important respiratory pathogen and was found to have an important role in serious diseases and conditions such as atherosclerosis. Due to life-threatening infections with *C. pneumoniae* and the lack of good antichlamydial drugs, we decided to synthesize and evaluate novel compounds as inhibitors of *C. pneumoniae*. Our synthetic pathways included the preparation of compounds that combine two important fragments that were found to have a good antichlamydial activity – gallates and benzimidazole.

According to the published synthetic procedure for 2-benzimidazoylpropanole, we modified and optimized the route to 2-benzimidazoylpropanoles from 1,2-diaminobenzene and gamma-butyrolactone. Furthermore, we developed a synthetic procedure in a microwave reactor. The optimized procedure was used for the synthesis of two compounds: 3-(6-methyl-1H-benzo[d]imidazol-2-yl)propan-1-ol (**8**) and 3-(6-chloro-1H-benzo[d]imidazol-2-yl)propan-1-ol (**9**).

We discovered that gallic acid should be protected in order to obtain final compounds with benzimidazole and gallic acid moieties. However, only acetyl protecting group for gallic acid was found as the appropriate protection. Unfortunately, the attempt to make amides with protected gallic acid and 2-benzimidazoylpropanoles was unsuccessful. Due to synthetic problems with gallic acid we decided to replace it with commercially available 3-methoxybenzoic acid. As a result, we obtained one final compound (**21**) with 3-methoxybenzoic acyl chloride and 2-benzimidazoylpropanole, which inhibited the growth of bacteria in micromolar range (25% inhibition at 50 µM). Furthermore, it was also shown that **21** has low toxicity and below 200 µM is well tolerated in host cells.

Based on the biological test results we can conclude that our target compound **21** represents an important starting compound for the development of novel group of antichlamydial compounds.

Further optimization studies in that research field are needed in finding suitable protecting groups for phenolic groups and benzimidazole NH group, synthesis of appropriate 1,2-diaminobenzene derivatives, further examination of ester bond formation and optimization of reactions in a microwave reactor to decrease reaction time and increase the overall yield. Furthermore, more derivatives of the compound **21** must be synthesized to obtain the structure-antichlamydal activity relationship and improve the potency.

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