Charles University in Prague

Faculty of Pharmacy in Hradec Kralove

Department of Organic and Inorganic Chemistry

Synthesis of 5-nitrosalicylanilide derivatives with potential antibacterial activity

Syntéza 5-nitrosalicylanilidových derivátů s potenciální antibakteriální aktivitou

(Diploma Thesis)

Mentor of Diploma thesis — Prof. RNDr. Jarmila Vinšová, CSc.

Hradec Králové 2013 — Kourtidou Maria

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I declare that this thesis is my original copyrighted work. All literature and other resources I used while processing are listed in the bibliography and properly cited.

Hradec Kralove 2013 _____.

Abstract

Kourtidou Maria

Synthesis of 5-nitrosalicylanilide derivatives with potential antibacterial activity

Diploma thesis

Charles University in Prague, Faculty of Pharmacy in Hradec Kralove

Pharmacy

Background: The aim of this diploma thesis was the synthesis of 5-nitrosalicylanilide derivatives as a potential anti-tuberculosis agent.

Methods: The reactions were monitored, and the purity of products was verified by thin layer chromatography in which the plates were coated with silica gel 60 F_{254} and seen by using UV irradiation. Column chromatography was done using silica gel 60.

The melting points of produced compounds were shown on a Melting Point apparatus, IR spectra were recorded using the technique of ART and the NMR spectra were measured in $CDCl_3$ or DMSO-d_6

Results: The starting compounds were generally reacted with success under the presence of chlorobenzene, trimethylamine and phosphorus trichloride and the microwave reactor.

Conclusion: Unfortunately the final products of these experiments had impurities of phenolate salts.

Abstrakt

Kourtidou Maria

Syntéza 5-nitrosalicylanilidových derivátů s potenciální antibakteriální aktivitou

Diplomová práce

Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové

Cíl: Cílem této diplomové práce byla syntéza několika 5-nitrosalicylanilidových derivátů s potenciální antituberkulózní aktivitou.

Metody: Průběh reakcí a čistota produktů byly monitorovány chromatografií na tenké vrstvě na deskách potažených silikagelem 60 F_{254} za použití detekce UV záření. Chromatografie na koloně byla provedena na silikagelu 60.

Teploty tání připravených sloučenin byly měřeny na bodotávku Büchi a jsou uvedeny bez korekce, IČ spektra byla měřena technikou ART a NMR spektra byla měřena v $CDCl_3$ nebo DMSO-d_6.

Výsledky: Kondenzační reakce byly prováděny v mikrovlnném reaktoru. Výchozí sloučeniny se ponechaly reagovat v chlorbenzenu za přítomnosti triethylaminu a chloridu fosforitého.

Závěr: Podle interpretace NMR spekter jsou některé konečné produkty patrně ve formě triethylamoniových solí.

Abbreviations

TB	Tuberculosis
WHO	World Health Organization
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immunodeficiency Disease Syndrome
LTBI	Latent Tuberculosis infection
IGRA	Interferon-gamma release assays
FDA	Food and Drug Administration
BCG	Bacille Calmette–Guérin
AII	Annual Infection Incidence
ARI	Annual Risk of Infection
MDR-TB	Multidrug resistant M. tuberculosis
XDR-TB	Extensively drug-resistant tuberculosis
ClogP	calculated logarithmus of partition coefficient
MetAP	Methionyl Aminopeptidase (Enzyme)
MetAP1	Methionyl Aminopeptidase 1 (Enzyme)
IC50	Half maximal inhibitory concentration
Hep G2	Heptocellular carcinoma
hMetAP1	humanized Methionyl Aminopeptidase 1 (Enzyme)
MtMetAp1c	Mycobacterium tuberculosis Methionyl Aminopeptidase 1
HUVEC	Human Umbilical Vein Endothelial Cell
KinA-Spo0F	Two-component sensor Kinase - phosphotransferase of the sporulation initiation phosphorelay
KinA	Two-component sensor Kinase
NRII-NRI	Nitrogen regulator II-I
MIC	Minumum Inhibitory Concentration
TCS	Two-component regulatory systems
UV	Ultra Violet

IR	Infra Red
ATR	Attenuated Total Reflectance
NMR	Nuclear Magnetic Resonance
DMSO-d_6	Dimethyl sulfoxide-d_6
$CDCl_3$	Deuterated chloroform
PCl_3	Phosphorus trichloride
$NaHCO_3$	Sodium hydrocarbonate

Content

1. General aspects of Tuberculosis

1.1 Introduction

Speaking about the disease of Tuberculosis (TB), it could be said that this is actually a disease caused by the bacteria called *Mycobacterium tuberculosis*[1]. Specific bacteria usually attack the lungs, but they can also damage other parts of the body. It should also be mentioned that the TB spreads through the air when a person with TB of the lungs or throat coughs, sneezes, or talks. If patients have been exposed, they should go to their doctor for tests. Patients have to be more likely to get TB if they have a weak immune system[2].

About the symptoms of TB in the lungs, those may include the following:

- A bad cough that lasts 3 weeks or longer
- Weight loss
- Loss of appetite
- Coughing up blood or mucus
- Weakness or fatigue
- Fever
- Night sweats

But it could also be said that except from the above, there are also skin tests, blood tests, x-rays, and other tests can tell if people have TB. If not treated properly, TB can be deadly for them. They can also usually cure active TB by taking several medicines for a long period of time[1].

1.2 Signs and Symptoms of TB Disease

As to the signs and symptoms of the TB disease, people could not notice any symptoms of illness until the disease is quite advanced. Even then the symptoms loss of weight, loss of energy, poor appetite, fever, a productive cough, and night sweats -- might easily be blamed on another disease[3]. It is

noticeable that only about 10% of people infected with M. tuberculosis ever develop tuberculosis disease. Many of those who suffer TB do so in the first few years following infection, but the bacillus may lie dormant in the body for decades[2].

Although most initial infections have no symptoms and people overcome them, they may develop fever, dry cough, and abnormalities that may be seen on a chest X-ray. This is called primary pulmonary tuberculosis. Pulmonary tuberculosis frequently goes away by itself, but in 50%-60% of cases, the disease can return[4]. Tuberculous pleuritis may occur in 10% of people who have the lung disease from tuberculosis. The pleural disease occurs from the rupture of a diseased area into the pleural space, the space between the lung and the lining of the abdominal cavity[3].

Those people have a nonproductive cough, chest pain, and fever. The disease may go away and then come back at a later date. In a minority of people with weakened immune systems, TB bacteria may spread through their blood to various parts of the body. This is called military tuberculosis and produces fever, weakness, loss of appetite, and weight loss. Cough and difficulty breathing are less common[1].

Generally, return of dormant tuberculosis infection occurs in the upper lungs. Symptoms include common cough with a progressive increase in production of mucus and coughing up blood. Other symptoms could include the following[2]:

- fever
- loss of appetite
- weight loss, and
- night sweats

Except from the above, it is also mentioned that about 15% of people may develop tuberculosis in an organ other than their lungs. In advance,

about 25% of these people usually had known TB with inadequate treatment. The most common sites include the following[2]:

- lymph nodes,
- genitourinary tract,
- bone and joint sites,
- meninges, and
- lining covering the outside of the gastrointestinal tract

1.3 The Bacteria of TB

Speaking about the bacteria of the Tuberculosis (TB), it is mentioned actually that TB is an infectious disease that is caused by a bacterium called *Mycobacterium tuberculosis*[4]. TB primarily affects the lungs, but it can also affect organs in the central nervous system, lymphatic system, and circulatory system among others. The disease was called "consumption" in the past because of the way it would consume from within anyone who became infected[5].

According to Medilexicon`s medical dictionary, tuberculosis is "*A specific disease caused by infection with Mycobacterium tuberculosis, the tubercle bacillus, which can affect almost any tissue or organ of the body, the most common site of the disease being the lungs*"[4].

When a person becomes infected with tuberculosis, the bacteria in the lungs multiply and cause pneumonia along with chest pain, coughing up blood, and a prolonged cough. In addition, lymph nodes near the heart and lungs become enlarged[4]. As the TB tries to spread to other parts of the body, it is often interrupted by the body's immune system[3].

The immune system forms scar tissue or fibrosis around the TB bacteria and this helps fight the infection and prevents the disease from spreading throughout the body and to other people. If the body's immune

system is unable to fight TB or if the bacteria break through the scar tissue, the disease returns to an active state with pneumonia and damage to kidneys, bones, and the meninges that line the spinal cord and brain[4].

TB is generally classified as being either latent or active. Latent TB occurs when the bacteria are present in the body, but this state is inactive and presents no symptoms. Latent TB is also not contagious. Active TB is contagious and is the condition that can make you sick with symptoms[5]. TB is a major cause of illness and death worldwide, especially in Africa and Asia. Each year the disease kills almost 2 million people. The disease is also prevalent among people with HIV/AIDS[2].

Finally, it is mentioned that Tuberculosis is ultimately caused by the Mycobacterium tuberculosis that is spread from person to person through airborne particles. It is not guaranteed, though, that you will become infected with TB if you inhale the infected particles. Some people have strong enough immune systems that quickly destroy the bacteria once they enter the body. Others will develop latent TB infection and will carry the bacteria but will not be contagious and will not present symptoms. Still others will become immediately sick and will also be contagious[3].

1.4 Diagnosis of TB Diseases

As to the way of diagnosis in TB Disease, it is actually mentioned that TB is a disease that is spread through the air from one person to another. There are two kinds of tests that are used to determine if a person has been infected with TB bacteria: the tuberculin skin test and TB blood tests[4]. A positive TB skin test or TB blood test only tells that a person has been infected with TB bacteria. It does not tell whether the person has latent TB infection (LTBI) or has progressed to TB disease. Other tests, such as a chest x-ray and a sample of sputum, are needed to see whether the person has TB disease[3]. Based on the above, the diagnosis of TB disease could be made upon the following ways.

- *Tuberculin skin test*: The TB skin test (also called the Mantoux tuberculin skin test) is performed by injecting a small amount of fluid (called tuberculin) into the skin in the lower part of the arm. A person given the tuberculin skin test must return within 48 to 72 hours to have a trained health care worker look for a reaction on the arm. The health care worker will look for a raised, hard area or swelling, and if present, measure its size using a ruler. Redness by itself is not considered part of the reaction. The skin test result depends on the size of the raised, hard area or swelling. It also depends on the person's risk of being infected with TB bacteria and the progression to TB disease if infected.
- *Positive skin test*: This means the person's body was infected with TB bacteria. Additional tests are needed to determine if the person has latent TB infection or TB disease. A health care worker will then provide treatment as needed.
- Negative skin test: This means the person's body did not react to the test, and that latent TB infection or TB disease is not likely.
- *TB blood tests*: TB blood tests (also called interferon-gamma release assays or IGRAs) measure how the immune system reacts to the bacteria that cause TB. An IGRA measures how strong a person's immune system reacts to TB bacteria by testing the person's blood in a laboratory.

It should be said that two IGRAs are approved by the U.S. Food and Drug Administration (FDA) and are available in the United States as follows[1]:

- QuantiFERON®–TB Gold In-Tube test (QFT-GIT)
- T-SPOT®.TB test (T-Spot)

- *Positive IGRA*: This means that the person has been infected with TB bacteria. Additional tests are needed to determine if the person has latent TB infection or TB disease. A health care worker will then provide treatment as needed.

- *Negative IGRA*: This means that the person's blood did not react to the test and that latent TB infection or TB disease is not likely.

IGRAs are the preferred method of TB infection testing for the following:

- People who have received bacille Calmette–Guérin (BCG). BCG is a vaccine for TB disease.

- People who have a difficult time returning for a second appointment to look for a reaction to the TST.

- *Testing for TB in BCG-Vaccinated Persons* - Many people born outside of the United States have been BCG-vaccinated. People who have had a previous BCG vaccine may receive a TB skin test. In some people, BCG may cause a positive skin test when they are not infected with TB bacteria. If a TB skin test is positive, additional tests are needed. IGRAs, unlike the TB skin tests, are not affected by prior BCG vaccination and are not expected to give a false-positive result in people who have received BCG.

Mentioned to the way of choosing a TB Test, it could be said that the person's health care provider should choose which TB test to use. Factors in selecting which test to use include the reason for testing, test availability, and cost. Generally, it is not recommended to test a person with both a TST and an IGRA[4]. If a person is found to be infected with TB bacteria, other tests are needed to see if the person has TB disease[3].

TB disease can be diagnosed by medical history, physical examination, chest x-ray, and other laboratory tests. TB disease is treated by taking several drugs as recommended by a health care provider. If a person does not have TB disease, but has TB bacteria in the body, then latent TB infection is diagnosed. The decision about treatment for latent TB infection will be based on a person's chances of developing TB disease[2].

In case people suspected of having TB disease should be referred for a medical evaluation, which will include the following details:

- Medical history
- Physical examination
- Test for TB infection (TB skin test or TB blood test)
- Chest radiograph (X-ray)
- Appropriate laboratory tests

1.5 Treatment of TB Disease

The treatment for tuberculosis depends on which type you have, although a long course of antibiotics is most often used. While TB is a serious condition that can be fatal if left untreated, deaths are rare if treatment is completed. For most people, hospital admission during treatment is not necessary[1]. In case of pulmonary TB, if patients are diagnosed with active pulmonary TB (TB that affects their lungs and causes symptoms), they will be referred to a specialist TB treatment team. This is a team of healthcare professionals with experience in treating TB. Patients' TB treatment team may include[3]:

- a respiratory physician - a doctor who specializes in conditions that affect the lungs and breathing
- an infectious disease specialist
- a TB nurse
- a health visitor - a qualified nurse with extra training who helps families with babies and young children to stay healthy
- Patients' GP
- a paediatrician (if necessary) - a doctor who specialises in conditions that affect children

It is also likely that patients will be assigned a key worker. This

is usually a nurse, health visitor or social care support worker who will be the point of contact between patients and the rest of the team and will help co-ordinate the care[6]. Moreover, pulmonary TB is treated using a six-month course of a combination of antibiotics. The usual course of treatment is:

- two antibiotics - isoniazid and rifampicin - every day for six months
- two additional antibiotics - pyrazinamide and ethambutol - every day for the first two months

However, patients may only need to take these antibiotics three times a week if they need supervision[5]. It may be several weeks or months before they start to feel better. The exact length of time will depend on their overall health and the severity of their TB. After taking the medicine for two weeks, most people are no longer infectious and feel much better. However, it is important for patients to continue taking their medicine exactly as prescribed and to complete the whole course of antibiotics[3].

Taking medication for six months is the most effective method of ensuring that the TB bacteria are killed. If patients stop taking antibiotics before they complete the course, or if they skip a dose, the TB infection may become resistant to the antibiotics. This is potentially serious, as it can be difficult to treat and will require a longer course of treatment[3].

If treatment is completed correctly, you should not need any further checks by a TB specialist afterwards. However, they may be given advice about spotting signs that the illness has returned - although this is rare. In rare cases, TB can be fatal even with treatment. Death can occur if the lungs become too damaged to work properly[1].

In the case of extrapulmonary TB (TB that occurs outside the lungs) can be treated using the same combination of antibiotics as those used to treat pulmonary TB. However, they may need to take them for 12 months. If patients have TB that affects your brain, you may also be prescribed a

corticosteroid, such as prednisolone, for several weeks to take at the same time as your antibiotics. This will help reduce any swelling in the affected areas (Ferguson et al, 2006). As with pulmonary TB, it is important for patients to take their medicines exactly as prescribed and to finish the course[4].

In the case of the latent TB, this is where people have been infected with the TB bacteria but do not have any symptoms of active disease. Treatment for latent TB is usually recommended for[2]:

- people 35 years of age or under
- people with HIV, regardless of their age
- healthcare workers, regardless of their age
- people with evidence of scarring caused by TB, as shown on a chest X-ray, but who were never treated

Treatment is not recommended for people who have latent tuberculosis and are over 35 years of age (and do not have HIV and are not healthcare workers). This is because the risk of liver damage increases with age and the risks of treatment outweigh the benefits for some people[6]. Latent TB is also not always treated if it is suspected to be drug-resistant. If this is the case, you may be regularly monitored to check the infection does not become active.

In some cases, treatment for latent TB may be recommended for people requiring immunosuppressant medication. This medication suppresses the immune system (the body's natural defense against illness and infection) and can allow latent TB to develop into an active form of the disease. This may include people taking long-term corticosteroids or people receiving chemotherapy[3]. In these cases, the TB infection should be treated before immunosuppressant medication begins. Treatment for latent TB involves either taking a combination of rifampicin and isoniazid for three months, or isoniazid on its own for six months[1].

By mentioning the side effects of treatment, it could be said that the Rifampicin can reduce the effectiveness of some types of contraception, such as the combined contraceptive pill. It could be said that patients should make use of an alternative method of contraception, such as condoms, while taking rifampicin[2]. In rare cases, these antibiotics can cause damage to the liver or the eyes, which can be serious. Therefore, their liver function may be tested before they begin treatment. If they are going to be treated with ethambutol, their vision should also be tested at the beginning of the course of treatment.

Patients have to contact their TB treatment team immediately if they have any of the following symptoms[1]:

- feeling sick or being sick
- yellowing of your skin (jaundice) and darkening of your urine
- unexplained fever - a temperature of 38C (100.4F) or above
- tingling or numbness in your hands or feet
- skin rash or itchy skin
- changes to your vision, such as blurred vision or color blindness

For the above reasons, patients have to see medicines information for tuberculosis for more information about the side effects of their medication. Sometimes people find it difficult to take their medication every day. If this affects them, their treatment team can work with them to find a solution. Usually, they will be asked to join a programme of "directly observed therapy". This can include supervised treatment, which will involve regular contact with their treatment team (daily or three times a week) to support them taking their medication. This can take place in their home, the treatment clinic or somewhere else more convenient[5].

As to the antibiotic-resistant tuberculosis (TB), it is mentioned that like

most bacteria, bacteria that cause TB can develop a resistance to antibiotics. This means the medicines can no longer kill the bacteria they are meant to fight[5]. Tuberculosis (TB) that develops a resistance to one type of antibiotic is not usually a concern because alternative antibiotics are available. In 2011, more than 8 out of 100 cases of TB were resistant to at least one type of antibiotic normally used to treat the condition. Although, in a number of cases:

- TB develops a resistance to two antibiotics - this is known as multi-drug resistant tuberculosis (MDR-TB)
- TB develops a resistance to three or more antibiotics - this is known as extensively drug-resistant tuberculosis (XDR-TB)

In 2011, almost 2 of every 100 TB cases were resistant to at least two antibiotics[6]. Both MDR-TB and XDR-TB will usually require treatment for at least 18 months using a combination of different antibiotics. As these conditions are difficult to treat, patients may be referred to a specialist TB clinic for treatment and monitoring[2].

1.6 Prevention

The TB prevention consists of two main parts. The first part of TB prevention is to stop the transmission of TB from one person to another one. This is done through firstly, identifying people with active TB, and then curing them through the provision of drug treatment.[3] With proper TB treatment someone with active TB disease will very quickly not be infectious and so can no longer spread the disease to others. The second main part of TB prevention is to prevent people with latent TB from developing active, and infectious, TB disease[1].

Anything which increases the number of infectious people, such as the presence of TB and HIV infection together, or which increases the number of people infected by each infectious person, such as ineffective treatment

because of drug resistant TB, reduces the overall effect of the main TB prevention efforts. As a result it is then more likely that the number of people globally developing active TB will increase rather than decrease. There is a vaccine for TB, but it makes only a small contribution to TB prevention, as it does little to interrupt the transmission of TB among adults[2].

About the TB prevention - the BCG vaccine, it is mentioned that the TB vaccine called Bacillus Calmette-Guerin (BCG) was first developed in the 1920s. It is one of the most widely used of all current vaccines, and it reaches more than 80% of all new born children and infants in countries where it is part of the national childhood immunization program. However, it is also one of the most variable vaccines in routine use[5].

The BCG vaccine has been shown to provide children with excellent protection against the disseminated forms of TB, however protection against pulmonary TB in adults is variable. Since most transmission originates from adult cases of pulmonary TB, the BCG vaccine is generally used to protect children, rather than to interrupt transmission amongst adults. The BCG vaccine will often result in the person vaccinated having a positive result to a TB skin test[2].

In case of a TB Treatment as TB prevention, it is mentioned that TB drug treatment for the prevention of TB, also known as chemoprophylaxis, can reduce the risk of a first episode of active TB occurring in people either exposed to infection, or with latent TB. It can also reduce the risk of a recurrent TB episode[3]. For TB prevention the World Health Organization (WHO) recommends the drug isoniazid should be taken daily for at least six months and preferably nine months.

The main "target" groups for TB treatment for prevention, are those most at risk of progressing from latent to active TB. These include:

- Infants and children aged less than 4 years old;
- People infected within the previous two years;
- People infected with both TB and HIV;

- People who have certain clinical conditions or conditions which compromise their immune system, such as people with diabetes, and people with chronic renal failure.

Isoniazid is a cheap drug, but in a similar way to the use of the BCG vaccine, it is mainly used to protect individuals rather than to interrupt transmission between adults. This is because children rarely have infectious TB, and it is hard to administer isoniazid on a large scale to adults who do not have any symptoms[4]. Taking isoniazid daily for six months is difficult in respect of adherence, and as a result many individuals who could benefit from the treatment, stop taking the drug before the end of the six month period[5].

At a Pediatric HIV/AIDS conference in Kampala, doctors were unable to agree as to whether children infected with HIV should be given Isoniazid as preventative treatment for TB. Those arguing for the drug treatment as prevention, claimed that in children co-infected with HIV and TB, up to 50% of exposed children ended up developing the disease[2].

There have also been concerns about the possible impact of TB treatment for prevention programs on the emergence of drug resistance. However, a review of the scientific evidence has now shown that there is no need for this to be a concern. The benefit of isoniazid preventative therapy for people living with HIV, and who have, or may have had latent TB, has also recently been emphasized[1].

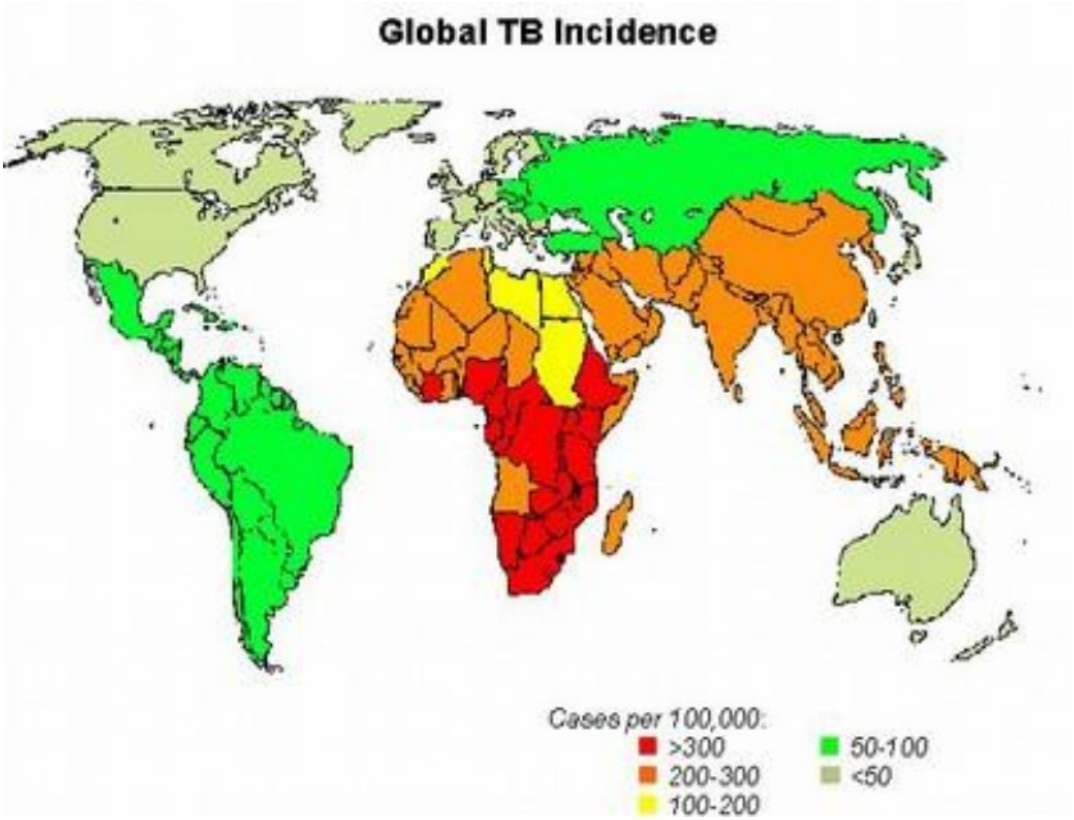
1.7 Epidemiology

As to the epidemiology of TB, the most relevant indicators include actually the TB incidence, the decrease of this incidence over time, and TB meningitis incidence among children between 0 and 4 years of age. It is also important to use TB infection indicators such as infection prevalence at a specific age and annual infection incidence (AII). In a cohort of tuberculin skin test negative children, a second test must be performed after one year to

determine AII[2].

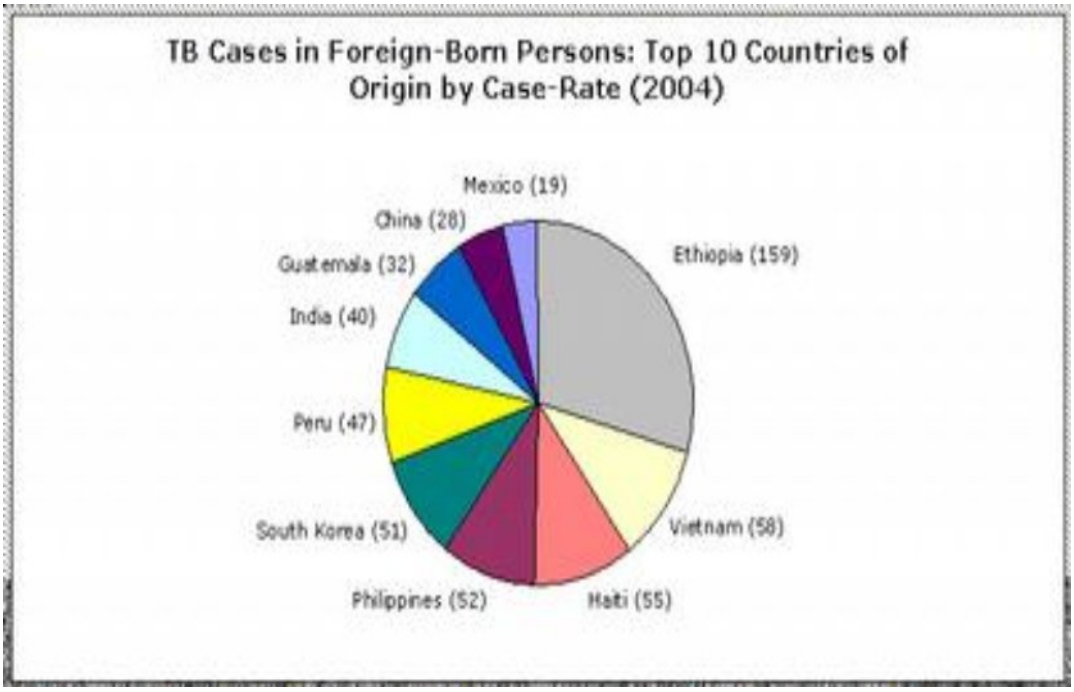
Although, this measurement can be difficult to perform and a bias exists for those who are BCG vaccinated, which produces more positive results and hence an inflated AII. To avoid these complications, Styblo defines annual risk of infection (ARI) as the prevalence of infection in two cohorts of the same age during consecutive years. ARI is determined by calculating the decline and prevalence of the last year, which should be the same as the AII. Styblo also estimated that 8-12 infected cases would arise from one smear-positive TB case, even though a recent study reduced this approximation to 2.6-5.8 because of improved TB control[5].

It is mentioned that ninety-five percent of all tuberculosis cases in the world occur in developing countries. Tuberculosis is prevalent in Russia, India, Southeast Asia, sub-Saharan Africa, and parts of Latin America. Countries with a high prevalence of HIV infection have the greatest tuberculosis burden[4].

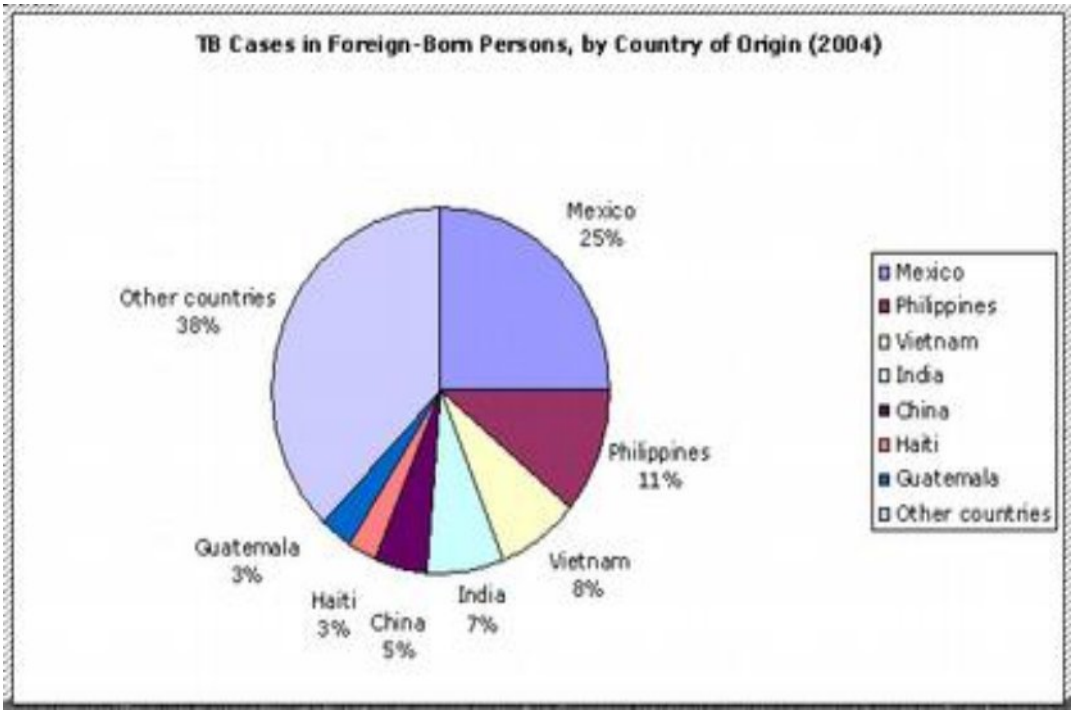


The following ten countries of origin account for the largest number of TB cases among immigrants[6]:

- Mexico
- Philippines
- Vietnam
- India
- China
- Haiti
- South Korea
- Guatemala
- Ethiopia
- Peru

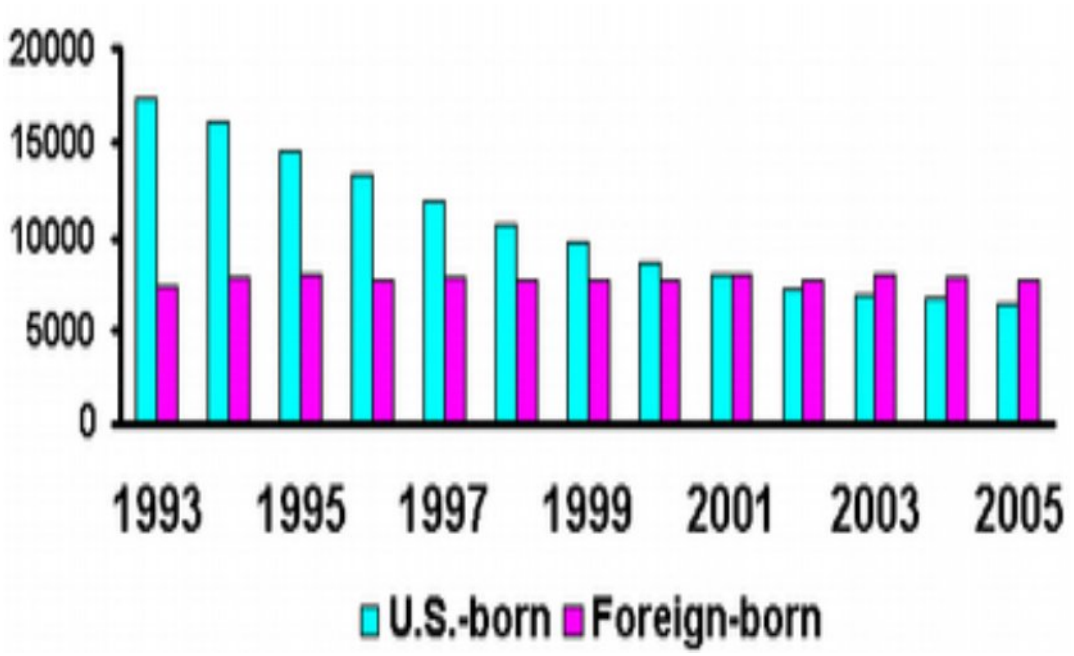


(Number of cases per 100,000 population)

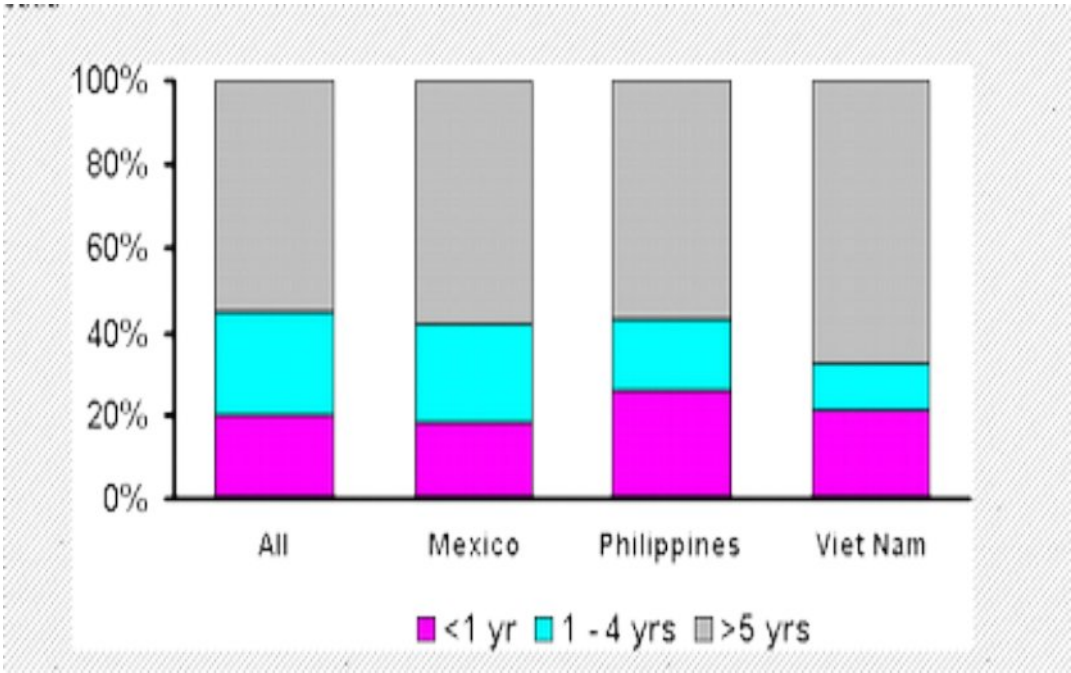


In the US, over half of all active TB cases occur in immigrants. The reported cases of active TB in foreign-born persons has remained at 7000-8000 per year, while the number of cases in US-born people has dropped from 17,000 in 1993 to 6,500 in 2005. As a result, the percentage of active TB cases in immigrants has increased steadily (from 29% of all cases in 1993 to 54% in 2005).

Cases of TB in US-born vs. Foreign-born persons:

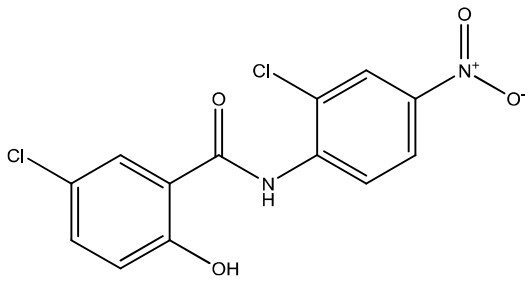


The risk of latent disease progressing to active tuberculosis is highest in the first few years after immigration, which is why newly-arrived immigrants (within five years) are a priority for testing and treatment. However, half of all TB reactivations in immigrants occur after their first five years in the US, so testing this group should be encouraged as well. TB Cases in Foreign-born Persons, by Duration of US residence (2005).

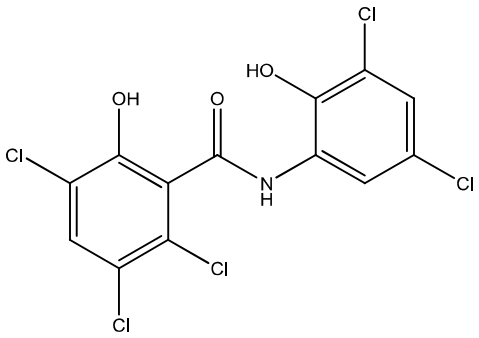


1.8 Characteristics of Salicylanilides

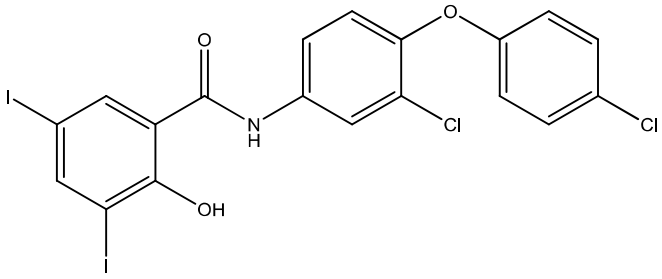
Salicylanilide is a chemical compound which is the amide of salicylic acid and aniline. It is classified as both a salicylamide and an anilide. Derivatives of salicylanilide have a variety of pharmacological uses. Chlorinated derivatives including niclosamide, oxyclozanide, and rafoxanide are used as anthelmintics, especially as flukicides[7]. Brominated derivatives including dibromsalan, metabromsalan, and tribromsalan are used as disinfectants with antibacterial and antifungal activities[8].



Niclosamide



Oxyclozanide

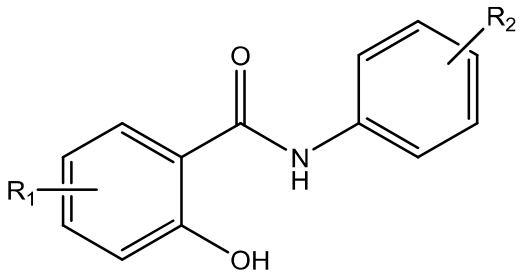


Rafoxanide

1.9 Effect of Salicylanilides in Anti-tuberculosis Drugs

It is obvious that salicylanilide esters are generally more active against *M. tuberculosis* than the parent salicylanilides. Similarly, the halogenation of the unsubstituted salicylanilide 1a resulted in derivatives with an improved

antimycobacterial activity (1a versus 1b, 1c, 1d). Esters with *N*-acetyl-L-phenylalanine exhibited the highest *in vitro* potency, followed by pyrazinoates, benzoates and then benzenesulfonates. Intriguingly, MIC values do not clearly correlate with the enzyme inhibition values corresponding to either of the enzymes MtMetAP1c or ICL[9].



1

Salicylanilides 1	R1	R2
1a	H	H
1b	5-Cl	4-CF_3
1c	5-Cl	3,4-diCl
1d	5-Br	4-CF_3

When MIC and the lipophilicity (calculated log*P*) were compared, there is no clear dependence; however the physico- chemical properties seem to be one of the factors influencing the antimycobacterial activity. The esterification led to the derivatives with higher lipophilicity and higher activity, but the highly active pyrazinoates showed lower Clog*P* than the parent salicylanilides. Similarly, the most lipophilic benzoates do not excel the MIC of esters with *N*-acetyl-L-phenylalanine. Presented molecules share certain cytotoxicity, although the esterification of the parent salicylanilides by all organic acids attenuated this property[9].

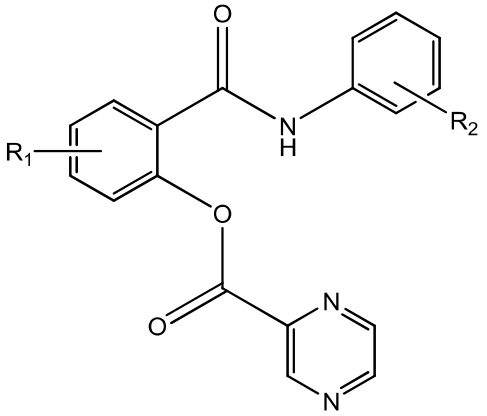
The hypothesis that salicylanilides and their esters act as pro- drugs for

the parent acids and anilines may be disproved by the fact that free acids (5-chlorosalicylic, 4-chlorosalicylic, 5-bromosalicylic, benzoic, pyrazine-2-carboxylic, benzenesulfonic acids and *N*-acetyl-L-phenylalanine) as well as anilines (3,4- dichloroaniline, 3-(trifluormethyl)aniline, 4-(trifluormethyl) aniline and 3-bromoaniline) showed only a mild antimycobacterial activity and a cytotoxicity significantly lower than their amides and esters. MICs of evaluated organic acids against *M. tuberculosis* were 500 mmol/L and cytotoxicities 718.1 mmol/L; MIC of anilines are mostly >1000 mmol/L, only for 3,4-dichloroaniline 250 mmol/L and their IC50 ranges within 413.5-1038.5 mmol/L.

Also the equimolar mixtures of appropriate salicylanilides with acids used for the esterification have not reached the antimycobacterial potencies of esters. It seems that both antimycobacterial and cytotoxic properties are predominantly associated with salicylanilide (2-hydroxy-N- phenylbenzamide) core in particular[9].

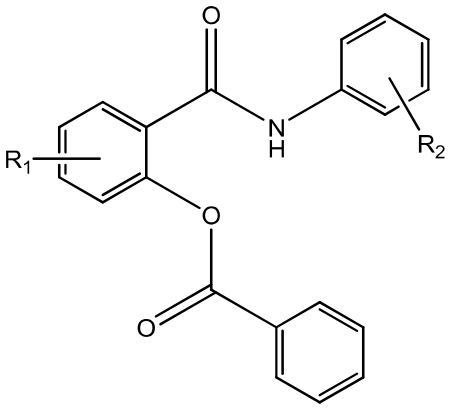
While investigated compounds have a certain activity against methionine aminopeptidase, their potencies are only moderate. All salicylanilides 1 and their esters inhibited both human and mycobacterial forms of MetAPs at 10 mM in the range from 5 to 49 %. The benzoylation of salicylanilides led to the derivatives with decreased MetAP inhibitory activity, and other acyl groups did not improve the activity any further[9].

Regrettably, the majority of the molecules inhibited the human enzyme more strongly than the MtMetAPs. This action may contribute to an undesirable cytotoxicity against human cells. Two derivatives 3c (the most favorable evaluated molecule) and 3d showed significantly higher specificity for the inhibition of myco- bacterial than human MetAP; four esters (3b, 3e, 4a, 4d) expressed the equal or almost equal inhibition values for both the enzymes. The esterification by hydrophilic pyrazine-2-carboxylic acid provided the most interesting salicylanilide derivatives where the activity is retained or in one case certainly improved substantially towards mycobacterial enzyme with a desirable selectivity over the human MetAP[9].



3

Salicylanilides 3	R1	R2
3b	4-Cl	3,4-diCl
3c	4-Br	4-CF_3
3d	4-Cl	3-CF_3
3e	4-Cl	3-Br



4

Salicylanilides 4	R1	R2
4a	4-Cl	4-CF_3
4d	5-Cl	3,4-diCl

While the inhibition of human MetAP1 may contribute to the cytotoxicity (similarly to antituberculosis property), the IC50 values of Hep G2 (~0.4-8.0 μmol/L for substituted salicylanilides) do not correlate sharply with this activity, when described salicylanilides showed lower than 50% inhibition of hMetAP1 at 10 μM. Additionally, hMetAP1 inhibition alone does not translate into strong cytotoxicity. A case in point, SB9OO64, MtMetAP1c inhibitor used as a positive control in this work, has an IC50 of 4.29 μM for MtMetAp1c and 0.12 μM for hMetAP1; but 19.4 μM for HUVEC inhibition (only human cell type tested).

Although salicylanilide derivatives moderately affect the function of mycobacterial MetAP, their concentration in the assay was much higher than the MIC towards M. tuberculosis including drug- resistant strains. The explanation for their potent in vitro efficacy exclusively due to inhibition of MetAP is plainly inadequate; however it may contribute to the antimycobacterial activity[9].

1.10 Mechanisms of Salicylanilide Antimicrobial Action

Salicylanilides (2-hydroxy-N-phenylbenzamides) have been used in medicine since the 40s. Before the inhibition of two- component regulatory systems (TCS) was discovered salicylanilides were considered phenols due to the presence of free aromatic hydroxyl group and their mechanism of action. The toxicity of salicylanilides to both mammalian and bacterial cells has largely been ascribed to their general effect on energy metabolism[10].

The mechanism of action of halogenated salicylanilides, specifically tetrachlorosalicylanilide (3,5-dichloro-*N*-(3,4-dichloro- phenyl)-2-hydroxybenzamide; was intensively studied during the 1960s. This compound has been found to uncouple oxidative phosphorylation in mitochondria at 0.35 μg/cm^3 and its MIC for *Staphylococcus aureus* was determined to be 0.15 μg/cm^3. Studies found that for a bacteriostatic effect for related compounds the necessary absorption is about 105 molecules per cell, and that salicylanilides cause leakage and membrane damage. After binding they

disrupt their function as the semi-permeable barrier; furthermore, several other effects have also been noted, e.g. terminal output domain, resulting in the activation or repression of genes under its control[10].

Additional levels of control and fine-tuning are achieved by specific phosphatase activities of the sensor kinase or aspartyl phosphatases that serve to limit the activation of the response regulator transcription-factor by the removal of phosphoryl groups in response to antithetical signal. TCS are often integrated into complex regulatory networks in the form of phosphorelays, which allow greater levels of control and facilitate the interpretation of multiple signals. These processes are in sharp contrast to eukaryotic signal transduction systems because the activation or repression of gene transcription is achieved through the transfer of phosphate from ATP to the response regulator *via* a high-energy phosphohistidine intermediate of the cognate kinase. On the other hand eukaryots employ serine/threonine and tyrosine kinases[10].

Chemical library screening against KinA-Spo0F, a TCS which regulates sporulation in *Bacillus subtilis,* revealed that the anthelmintic closantel and the topical antibacterial tetrachlorosalicylanilide (inhibited autophosphorylation of the KinA kinase. In another study of TCS inhibitors, there was only a slight correlation between the inhibition of KinA-Spo0F and other enzymatic systems in *E. coli* NRII-NRI. When the molecular mechanism and the site of action of TCS inhibitors tetrachlorosalicylanilide and closantel were investigated in detail, some differences were described in the molecular base of their action and in the efficacy. Closantel binds to the car- boxyl-terminal catalytic domain of the sensor kinase and causes structural changes leading to the aggregation[10].

Tetrachlorosalicylanilide in a concentration of 0.2 μg/cm^3 inhibited the energy-dependent cellular uptake of inorganic phosphate and amino acids, but not of glucose. Moreover it blocks glucose and amino acid incorporation into biomolecules and releases amino acids from the cell pool. In the concentration of 1.25 μg/cm^3, tetrachlorosalicylanilide completely inhibited

respiration. Succine oxidase was more sensitive than was glucose oxidation and glucose fermentation, which were relatively indifferent to the action. Resistance to this class of drugs results from the inhibition of penetration through the cell wall. The mechanism of low activity on Gram-negative bacteria (MIC for *Escherichia coli* 30 μg/cm^3) acts in the same way[10].

In 1998 it was found that the sites of salicylanilide disinfectant action were most likely biomembranes, where they penetrate or portion into the phospholipid bilayer, possibly displacing phospholipide molecules. Salicylanilides abolish the transmembrane pH gradient and membrane integrity, resulting in leakage as well as the disruption of transport, respiratory and energy coupling processes.

The mechanism of action of salicylanilides based on the inhibition of two-component regulatory systems (TCS) was proposed in 1998. In all bacterial species there are multiple two-component systems, each controlling the transcription of several genes. The ubiquitous TCS are intimately involved in the maintenance of bacterial cell homeostasis and the expression of virulence factors, including resistance to antibiotics, in response to external and internal environmental stimuli. In many different organisms TCS regulate diverse responses, exceptions being virulence, further nutrient acquisition, energy metabolism, adaptation to physical or chemical aspects of the environment and complex developmental pathways. In *Caulobacter crescentus* TCS play a key role in the regulation of DNA replication and in the cell cycle[10].

2.The aim of the thesis

The aim of this diploma thesis was the synthesis of 5-nitrosalicylanilide derivatives from substituted salicylic acid and substituted aniline as potent anti-tuberculous drugs.

3.Experimental part

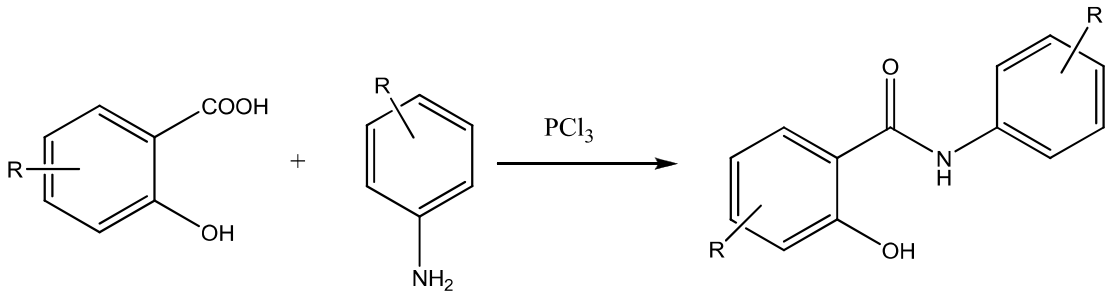
General methods

The reactions were monitored, and the purity of products was verified by thin layer chromatography in which the plates were coated with 0.2 mm silica gel 60 F_{254} (Merck, Prague, Czech Republic) and visualized using UV irradiation (254 and 366 nm). Column chromatography was performed using silica gel 60 with a particle size of 0.063-0.2 mm (Fluka, Prague, Czech Republic).

The melting points were determined on a Melting Point B-540 apparatus (Bűchi Labortechnik AG, Flawil, Switzerland) in open capillaries and are uncorrected. IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) over the range of 400-4000 cm^{-1} using ATR technique. The NMR spectra were measured in DMSO-d_6 or $CDCl_3$ solutions at ambient temperature on a Varian Mercury Vxbb 300 (300 MHz for ^{1}H and 75.5 MHz for ^{13}C, Varian Comp. Palo Alto, CA, USA) and Varian Mercury (500 MHz for ^{1}H and 125 MHz for ^{13}C, Varian Comp. Palo Alto, CA, USA).

Salicylanilide preparation

Reaction scheme:



General procedure:

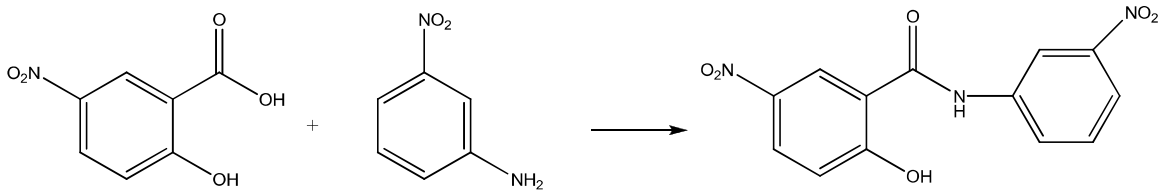
Substituted salicylic acid (0.01mol) was dissolved in 50 ml of chlorobenzene, 0.01 mol of substituted aniline was added. The mixture was stirred 10 minutes and after that 0.435 ml (0,005 mol) of PCl_3 was added drop by drop.

The reaction mixture was boiled under the reflux condenser in the microwave reactor for 20 minutes at 120 ^{o}C.

After one day of standing, the precipitate was filtered off and recrystallized from ethanol.

2-Hydroxy-5-nitro-N-(3-nitrophenyl)benzamide

Reaction scheme:



Contents:

- 1.83 g of 2-hydroxy-5-nitrobenzoic acid
- 1.3932 g of 3-nitroaniline
- 50 ml of chlorobenzene
- 0.435 ml of PCl_3
- 1.2 ml of triethylamine

Procedure:

The mixture of the above contents was boiled in the microwave reactor at 120 ^{o}C and 400 watt for 20 minutes. After that, 30 ml of $NaHCO_3$ were added in order to form precipitation and this precipitate was filtrated.

This precipitate, which was named Ma 2(1), was mixed with diluted HCl (5ml of concentrated HCl +10 ml of distilled H_2O) and let it stirred for 15 minutes.

Then, it was filtered off and washed with distilled water. Moreover, it was washed with 5 ml of $NaHCO_3$ and after that with water once more.

This solution was dried, recrystallized from ethanol and filtered off three times to obtain the product.

Weight of product: 0.2042 g

Yield: 6,33%

Melting point: 235-237 ^{o}C

Chemical Formula: $C_{13}H_9N_3O_6$

Molecular Weight: 303,23

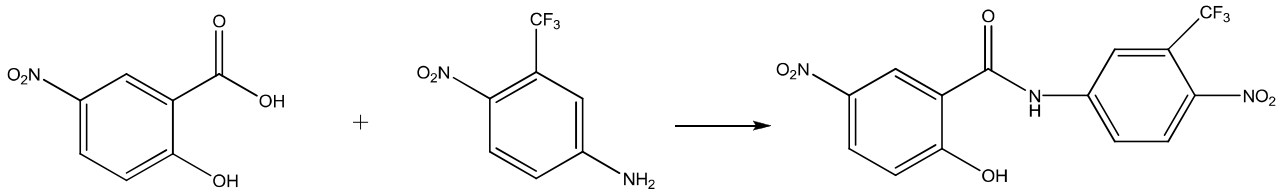
IR ν(CONH-) 1626 cm^{-1}

^{1}H NMR (500 MHz, DMSO) δ 10.91 (1H, bs, NH), 8.76-8.73 (1H, m, H2´), 8.69 (1H, d, *J*=2.4 Hz, H6), 8.29 (1H, dd, *J*=9.3 Hz, *J*=2.4 Hz, H4), 8.07 (1H, d, *J*=8.3 Hz, H6´), 8.01-7.96 (1H, m, H4´), 7.66 (1H, t, *J*=8.3 Hz, H5´),), 7.16 (1H, d, *J*=9.3 Hz, H3)

^{13}C NMR (125 MHz, DMSO) δ 164.6, 163.1, 148.1, 139.5, 139.5, 130.4, 128.7, 126.7, 126.1, 120.3, 118.9, 118.1, 114.8

2-Hydroxy-5-nitro-N-(4-nitro-3- (trifluoromethyl)phenyl)-benzamide

Reaction scheme:



Contents:

- 1.8391 g of 2-hydroxy-5-nitrobenzoic acid
- 2.0616 g of 3-trifluoromethyl-4-nitroaniline
- 50 ml of chlorobenzene
- 0.435 ml of PCl_3
- 1.2 ml of triethylamine

Procedure:

The mixture of the above contents was boiled in the microwave reactor at 120^{o}C and 400 watt for 20 minutes. After that, 30 ml of $NaHCO_3$ were added in order to form precipitation and this precipitate was filtrated.

This precipitate, which was named Ma 3(1), was mixed with ethyl acetate and heated until completely dissolved. Then HCl was added and the two layers, that were created, were separated.

The ethyl acetate layer (the upper part) was kept, evaporated and recrystallized to obtain the final product.

Weight of product: 0.3392 g

Yield: 8,69 %

Melting point: 247-249.5 ^{o}C

Chemical Formula: $C_{14}H_8F_3N_3O_6$

Molecular Weight: 371,23

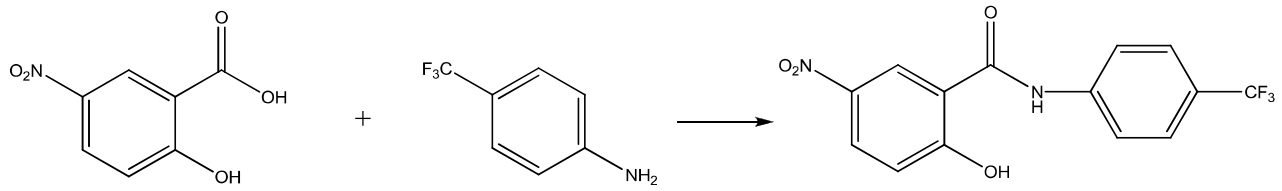
IR ν(CONH-) 1629 cm^{-1}

^{1}H NMR (300 MHz, DMSO) δ 11.17 (1H, bs, NH), 8.62 (1H, d, *J*=2.9 Hz, H6), 8.44-8.38 (1H, m, H2´), 8.28 (1H, dd, *J*=9.1 Hz, *J*=2.9 Hz, H4), 8.25-8.19 (2H, m, H5´, H6´), 7.16 (1H, d, *J*=9.1 Hz, H3)

^{13}C NMR (75 MHz, DMSO) δ 164.7, 162.9, 143.2, 142.2, 139.4, 128.8, 127.8, 126.3, 123.8, 123.0 (q, *J*=32.2 Hz), 122.2 (q, *J*=272.9 Hz), 120.7, 118.7 (q, *J*=5.7 Hz), 118.1

2-Hydroxy-5-nitro-N-(4-(trifluoromethyl)phenyl)benzamide

Reaction scheme:



Contents:

- 1.8326 g of 2-hydroxy-5-nitrobenzoic acid
- 1.6187 g of 4-trifluoromethylaniline
- 50 ml of chlorobenzene
- 0.435 ml of PCl_3
- 1.2 ml of triethylamine

Procedure:

The mixture of the above contents was boiled in the microwave reactor at 120^{o}C and 400 watt for 20 minutes. After that, 20 ml of $NaHCO_3$ were added in order to form precipitation and this precipitate was filtrated.

This precipitate, which was named Ma 4(1), was recrystallized and filtrated. The product from this filtration was mixed with ethyl acetate and heated until completely dissolved and then HCl was added. The two layers, that were created, were separated and the ethyl acetate layer (the upper part) was kept.

This solution was evaporated and recrystallized from ethanol to obtain the final product.

Weight of product: 0.2725 g

Yield: 7,89%

Melting point: 260-261 ^{o}C

Chemical Formula: $CH_9F_3N_2O_4$

Molecular Weight: 326,23

IR ν(CONH-) 1659 cm^{-1}

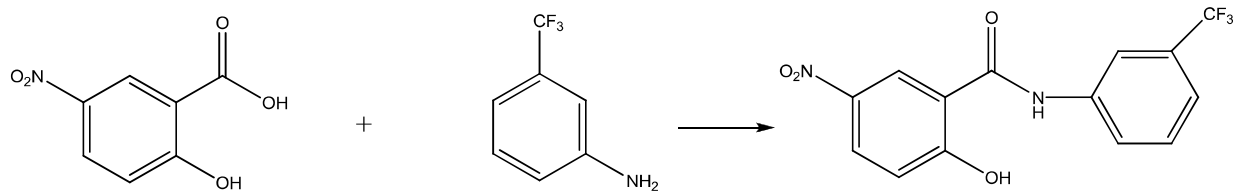
^{1}H NMR (300 MHz, DMSO) δ 10.79 (1H, bs, NH), 8.68 (1H, d, *J*=2.9 Hz, H6), 8.28 (1H, dd, *J*=9.1 Hz, *J*=2.9 Hz, H4), 7.98-7.89 (2H, m, AA´, BB´, H3´, H5´), 7.77-7.68 (2H, m, AA´, BB´, H2´, H6´), 7.16 (1H, d, *J*=9.1 Hz, H3)

^{13}C NMR (75 MHz, DMSO) δ 164.4, 163.0, 142.0, 139.6, 128.6, 126.3 (q, *J*=3.5 Hz), 124.9 (q, *J*=32.2 Hz), 124.5 (q, *J*=271.8 Hz), 122.7, 120.6, 120.5, 118.1

Literature[11] was found in Reaxys, without any details. The journal is not on-line accessible

2-Hydroxy-5-nitro-N-(3-(trifluoromethyl)phenyl)benzamide

Reaction scheme:



Contents:

- 1.8336 g of 2-hydroxy-5-nitrobenzoic acid
- 1.6154 g of 3-trifluoromethylaniline
- 50 ml of chlorobenzene
- 0.435 ml of PCl_3
- 1.2 ml of triethylamine

Procedure:

The mixture of the above contents was boiled in the microwave reactor at 120oC and 400 watt for 20 minutes. After that, 30 ml of $NaHCO_3$ were added in order to form precipitation and this precipitate (Ma 5(1)) was filtrated and recrystallized from ethanol.

The precipitation formed after this recrystallization, was mixed with ethyl acetate and heated until completely dissolved. In addition it was filtrated and evaporated till dryness. Then it was recrystallized from ethanol and ethyl acetate with approximately 25 ml of diluted HCl was added, once more, forming two layers which were separated.

From this separation the ethyl acetate phase (upper layer) was kept and evaporated till dryness. The compound from that evaporation was recrystallized from ethanol.

In this solution a little amount of distilled water was added and evaporated till dryness. Finally, distilled water was added once more and filtrated in order to obtain the product.

Weight of product: 0.1380 g

Yield: 4%

Melting point: 180-182 ^{o}C

Chemical Formula: $C_{14}H_9F_3N_2O_4$

Molecular Weight: 326,23

IR ν(CONH-) 1619 cm^{-1}

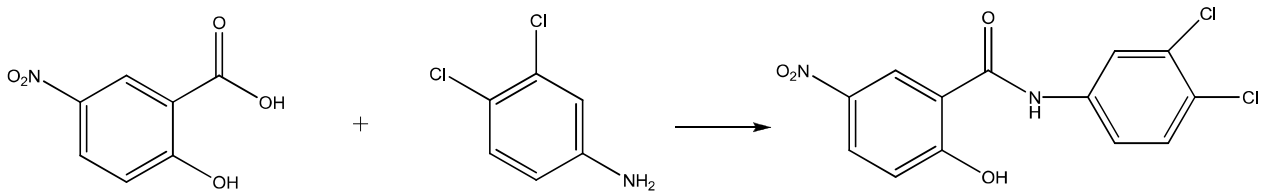
^{1}H NMR (300 MHz, DMSO) δ 10.78 (1H, bs, NH), 8.71 (1H, d, *J*=2.9 Hz, H6), 8.28 (1H, dd, *J*=8.8 Hz, *J*=2.9 Hz, H4), 8.20 (1H, bs, H2´), 7.94 (1H, d, *J*=7.9 Hz, H4´), 7.61 (1H, t, *J*=7.9 Hz, H5´), 7.49 (1H, d, *J*=7.9 Hz, H6´), 7.16 (1H, d, *J*=8.8 Hz, H3)

^{13}C NMR (75 MHz, DMSO) δ 164.6, 163.2, 139.5, 139.1, 130.2, 129.8 (q, *J*=32.2 Hz), 128.6, 126.0, 124.4, 124.3 (q, *J*=272.9 Hz), 120.1, 120.8 (q, *J*=3.5 Hz), 118.1, 116.9 (q, *J*=3.5 Hz)

Literature:[12],[13] found in Reaxys without access to primary journal sources

N-(3,4-Dichlorophenyl)-2-hydroxy-5-nitrobenzamide

Reaction scheme:



Contents:

- 1.8413 g of 2-hydroxy-5-nitrobenzoic acid
- 1.7711 g of 3,4-dichloroaniline
- 50 ml of chlorobenzene
- 0.435 ml of PCl_3
- 1.2 ml of triethylamine

Procedure:

The mixture of the above contents was boiled in the microwave reactor at 120^{o}C and 400 watt for 20 minutes. After that, 30 ml of $NaHCO^3$ were added in order to form precipitation and this precipitate was filtrated.

The product obtained from this filtration, which was named Ma 6(1), was mixed with ethyl acetate until completely dissolved and filtrated.

The filtrate was evaporated till dryness and then recrystallized three times. Moreover, ethyl acetate with approximately 25 ml of diluted HCl were added, forming two layers and separated.

From this separation the water phase (upper layer) was kept and evaporated till dryness. The compound from that evaporation was recrystallized from ethanol and filtrated to obtain the final product.

Weight of product: 0.2185 g

Yield: 6,33%

Melting point: 279-281 ^{o}C

Chemical Formula: $C_{13}H_8Cl_2N_2O_4$

Molecular Weight: 327,12

IR ν(CONH-) 1630 cm^{-1}

^{1}H NMR (300 MHz, DMSO) δ10.68 (1H, bs, NH), 8.66 (1H, d, *J*=2.9 Hz, H6), 8.26 (1H, dd, *J*=8.8 Hz, *J*=2.9 Hz, H4), 8.08 (1H, d, *J*=2.1 Hz, H2´), 7.60 (1H, d, *J*=8.8 Hz, H5´),), 7.65 (1H, dd, *J*=8.8 Hz, *J*=2.1 Hz, H6´), 7.14 (1H, d, *J*=8.8 Hz, H3)

^{13}C NMR (75 MHz, DMSO) δ 164.3, 163.0, 139.5, 138.4, 131.2, 130.8, 128.6, 126.1, 126.0, 121.9, 120.7, 120.1, 118.1

4. Discussion and conclusion

During my experimental work 5 products were prepared. Two of them were already mentioned in previous researches, 2-Hydroxy-5-nitro-*N*-(3-(trifluoromethyl)phenyl)benzamide and 2-Hydroxy-5-nitro-*N*-(4-(trifluoromethyl)phenyl)benzamide, but due to limited access to these documents , not any further information about their characteristics are mentioned in this diploma thesis.

According NMR spectra interpretations all prepared compounds seem to be in a form of phenolate triethylammonium salts.

Unfortunately, the anti-tuberculotic activity of the prepared salicylanilides is not yet illustrated, since the tests are still in progress.

5. Bibliography – References

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