KATHOLIEKE UNIVERSITEIT LEUVEN

FACULTY OF PHARMACEUTICAL SCIENCES

SYNTHESIS OF TETRAHYDROCARBAZOLES
IN SEARCH FOR NEW ANTI-TB CHEMOTHERAPY

Laboratory for Medicinal Chemistry
Prof. Dr. P.Herdewijn (Promotor)

Supervisor: Dr. E.Groaz

Master Thesis handed in
to achieve the
Master's Diploma
In Drug Development by

Henri-Philippe MATTELAER

Academic Year 2011 – 2012

KATHOLIEKE UNIVERSITEIT LEUVEN

FACULTY OF PHARMACEUTICAL SCIENCES

SYNTHESIS OF TETRAHYDROCARBAZOLES
IN SEARCH FOR NEW ANTI-TB CHEMOTHERAPY

Laboratory for Medicinal Chemistry
Prof. Dr. P.Herdewijn (Promotor)

Supervisor: Dr. E.Groaz

Master Thesis handed in
to achieve the
Master's Diploma
In Drug Development by

Henri-Philippe MATTELAER

Academic Year 2011 – 2012

2de herziene druk, 2012

"De auteur en de promotor(en) geven de toelating deze scriptie voor consultatie beschikbaar te stellen en delen ervan te kopiëren voor persoonlijk gebruik. Elk ander gebruik valt onder de beperkingen van het auteursrecht, in het bijzonder met betrekking tot de verplichting uitdrukkelijk de bron te vermelden bij het aanhalen van de resultaten uit deze scriptie. De auteurs en de promotor(en) behouden zich het recht delen van deze scriptie aan te wenden voor wetenschappelijke publicaties."

Datum:	*Auteur,*	*Promotor,*
	Mattelaer Henri-Philippe	*Prof.Dr.P.Herdewijn*

Acknowledgements

First of all I want to thank **prof. dr. R. Busson**, his passion for organic chemistry awoke mine: leading to my search for new insights by broadening my knowledge on chemistry.

But I've learned that being a chemist doesn't make you a scientist.

For that I owe, first of all, my promoter **prof. dr. P. Herdewijn** for giving me the chance to pursue both my medical and chemical interests in my thesis. In our conversations on science I found a kindred spirit, motivating and inciting me to develop my research skills.

Also I want to express an enormous amount of gratitude towards my supervisor, **dr. E. Groaz**; she taught me to act like a scientist in practising chemistry. Literature research, experimenting, failing or succeeding and learning from your observations and results. An experiment is only a failure if you don't learn anything from it.

In practice, theory comes to life.

Many thanks goes towards the fellow **researchers at the Rega institute** for welcoming me in their midst and for all their help with my experiments both in theory and practice.

Also I'm grateful for the help I received from **prof. dr. E. Lescrinnier** with my introduction and figure 1.4. And I would like to thank her and **prof. dr. J. Rozenski** for all their help on the analysis of my compounds by respectively NMR and MS. Not only did they help me with my thesis, they teached me the fundamentals of spectroscopic analysis. I express my gratitude towards **Luc Baudemprez** for all the practical help with recording and interpreting NMR-spectra.

There are many people in my life who I would like to thank for their support and believe in me, but choosing is losing and paper scarce so I will keep it short by thanking the most dearest to me. But nonetheless the importance of their influences in my life is deeply recognised and my apologies go out to the persons offended by the lack of mentioning them.

My **friends** for carrying me through rough times and keeping my feet on the ground. For the period of this thesis special thanks goes out to Bart VDB; from colleague and lab mate to friend, he taught me the thin line between hope and despair in performing chemistry; and Annemie J. The sister I never had, she can rationalise things even I can't and in doing so helped me through some difficult moments.

Last but not least thanks goes out to my **parents** and **my younger brother**, my family for the logistic and mental support. In giving me the chance and motivating me to develop myself, for that I'm eternally and truly grateful.

Dedicated to W. Caers (°1945-†2008)
who taught me that
life's adversities are a test of faith
not in a higher order, but in yourself
"In faith, all is possible"

Table of contents

LIST OF ABBREVIATIONS

TB	Tuberculosis
WHO	World Health Organization
Mtb	*Mycobacterium tuberculosis*
MDR-TB	Multi-Drug-Resistant Tuberculosis
XDR-TB	eXtensively Drug-Resistant Tuberculosis
HIV	Human Immunodeficiency Virus
AIDS	Acquired ImmunoDefiency Syndrome
CD	Clusters of Differentation
BCG	Bacille Calmette-Guérin
Classical TS	Classical Thymidylate Synthase
FDTS	Flavin-Dependent Thymidylate Synthase
H_2folate	7,8-dihydrofolate
H_4folate	5,6,7,8-tetrahydrofolate
CH_2H_4folate	5,10-methylene-5,6,7,8-tetrahydrofolate
NADP(H)	(reduced) Nicotinamide Adenine Dinucleotide Phosphate
FAD(H/H_2)	(reduced) Flavine Adenine Dinucleotide
dUMP	2'-deoxyuridine-5'-monophosphate
dTMP	2'-deoxythymidine-5'-monophosphate
PK	Pharmacokinetics
PD	Pharmacodynamics
BI synthesis	Bischler Indole synthesis
FI synthesis	Fischer Indole synthesis
NMR	Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography
PLC	Preparative thin Layer Chromatography
Rf	Ratio-to-front
MS	Mass Spectrometry
$EtO(CH_2)_2OH$	2-ethoxyethanol
MeOH	methanol
THF	tetrahydrofuran
aq.	aqueous
Et_2O	diethylether
EtOAc	ethyl acetate
DCM	dichloromethane
PE	petroleum ether
DMSO	dimethylsulfoxide
SAR	Structure Activity Relationship
HPLC	High-Performance Liquid Chromatography
NH_2NHBOC	*t*-butylcarbazate
BOC	*t*-butoxycarbonyl
p-TsOH	*p*-tosylic acid

MASTER THESIS PART I:
Research project

'There is a dread disease which so prepares its victim, as it were, for death…a disease in which death takes the glow and hue of life, and life the gaunt and grisly form of death; a disease which medicine never cured, wealth warded off, or poverty could boast exemption from; which sometimes moves in giant strides, and sometimes at tardy pace; but, slow or quick, is ever sure and certain'

Charles Dickens, 1870, in Nicholas Nickleby,
Wiendenfeld and Nicholson, London p.243

1 INTRODUCTION

1.1 Tubercolosis: the epidemic rages on

1.1.1 Global consumption

Tuberculosis (TB) is currently more prevalent in the world than ever before, especially in developing countries and disadvantaged layers of society of developed ones. With 9.8 million new cases estimated this year, the number of infections reached unprecedented heights. The rapid spreading of infection by inhalation of saliva droplets and the incomplete eradication of bacilli in a third of the world's population, despite decades of anti-TB chemotherapy, has made TB a serious burden on public health.[1-4]

The first description of TB dates back to old Indian texts, whilst the ancient Greeks knew TB as phthisis ('wasting away'). Several Egyptian mummies show signs of Pott's disease, a particularly destructive TB manifestation in bone tissue. In the Middle Ages a rare complication in the cervical lymph nodes was known as scrofula. Later on, in the XVII century, TB was called the 'white plague', reaching high death rates and becoming the principal cause of death untill the beginning of the XX century. Affecting every social class, TB was romanticized in the XIX century: thin and pale became the new beauty standards and influenced several artworks. Consumption, named after the dreadful clinical manifestation, took the lives of many famous historical figures e.g. St Francis of Assisi, Chopin, George Orwell, Eleanor Roosevelt and many others.[2]

Decades of scientific research during the XIX and XX centuries led to a greater understanding of the pathogen and the discovery of suitable chemotherapy. Nevertheless TB stroke back from a slumber which was created by the impression of being under control, especially in developed countries. As an example, in 1969 the Medic Chief of the National Institute of Health said "it was time to close the books about infectious diseases". But despite chemotherapy and prophylactic measures, significantly reducing incidence rates in developed nations, there has not been a great effect on the global problem.[2]

As the world's deadliest single infectious agent, TB counts for 7% of all deaths and every year nearly 10 million new cases occur, affecting mostly the young and productive adults. The burden lies predominantly with the developing countries, counting for 98% of TB fatalities and 95% of new cases.[2, 4] One third of the world's population (WHO estimated by epidemiological models) is infected with TB, as an asymptomatically dormant or latent form, acting as a reservoir and therefore rendering the prospect of eradicating *M.tuberculosis* (*Mtb*) seemingly impossible.[4, 5] Recently, the TB epidemic has been aggravated by the rise of multi- and extensively-drug-resistant strains (MDR-TB and XDR-TB).[1]

M.tuberculosis has inherently been a very resilient and persistent pathogen, but the recent resumption of the disease is in fact a consequence of anthropic factors such as drug resistance due to inappropriate treatment and patient non-compliance, the HIV/AIDS pandemic, the increase of injectable drug users, immigration from high prevalence nations to developed ones, the world population aging, human accumulation in prisons, hospitals and homeless shelters. Social inequality is the most important factor leading to malnutrition, lack of access to health care and overall poor living conditions.[2, 4]

1.1.2 *M.Tuberculosis*: centuries of genetic adaptation

TB is an infectious disease caused by some members of the Mycobacteriaceae, grouped as the "*M. tuberculosis complex",* including *M. bovis*, *M. africanum* and most importantly *M. tuberculosis. Mtb* infects new hosts by the means of an airborne aerosol expelled by the coughing of active TB patients. Only 10% of those infected with *Mtb* develop active TB over a lifetime. Other immunocompetent individuals succeed in eliminating the bacilli through means of cellular immunity established 2 to 6 weeks after infection. The only reminiscence left of such previous infection is a positive skin test or the localized lung lesions on X-ray called tubercles, caused by the intracellular growth of *Mtb*. However, in the majority of cases, a passive coexistive state with 'dormant' bacteria is established.[2]

Particles containing 1 to 10 bacilli , enough to cause an infection, are able to remain in suspension for hours and thus contributing to the highly contagious nature of TB.[1, 2] Inhaled bacilli are ingested by alveolar macrophages, starting the inflammatory cascade where both CD4 and CD8 responses are necessary for creating lasting immunity and producing cytokines to control *Mtb* infection.[6] After phagocytic ingestion by the macrophages, most of the bacterial load is immediately eliminated, but also a lot of phagocytes and lung parenchymal cells are killed in the process, creating a caseous necrotic core where extracellular bacilli, hiding from the immune system, can live and divide. Those typical necrotic structures known as granulomas, are essential in containing the infection. Surrounded by cells of the host's immune system, a contained TB infection only inflicts localized lung damage, but only when the necrotic reaction expands, the dreadful nature of the disease is shown. The breaking of a granuloma into a bronchus, known as open-lung TB, allows spreading of the infection in other parts of the lungs and massive expulsion of bacteria into the air by coughing. About 15% of active TB patients develop extra-pulmonary TB. Bacterial overgrowth in granulomas followed by invasion of the circulatory system, gives rise to dissemination of bacilli throughout the body.[2]

The word 'consumption' is derived from the typical symptoms caused by TB. The disease, as it were, consumes the host at a slow pace with multiple symptoms such as fever, weight loss, night sweat and coughing; leading to gradual debilitation, physical exhaustion and, untreated, death in most cases.[2]

Mtb has had over a thousand years to adapt to human physiology and thus acquiring a remarkable level of resilience and persistence.[5] Its versatile genome encodes mostly enzymes involved in lipolysis (for bacterial survival in host) and lipogenesis (for cell wall synthesis). The unusual cell wall structure of Mycobacteria causes not only relative resistance to drying, alkali and many other chemical disinfectants, but also acts as double permeability barrier for both hydrophilic and lipophilic molecules (Figure 1.1). This inherent resistance against xenobiotics is relatively weaker in *Mtb* than in other Mycobacteriaceae and requires the synergy of additional mechanisms such as the production of β-lactamases to achieve significant resistance levels, especially against lipophylic derivates of chemotherapeutic agents suggesting a lipophylic solute transport pathway.[2, 7]

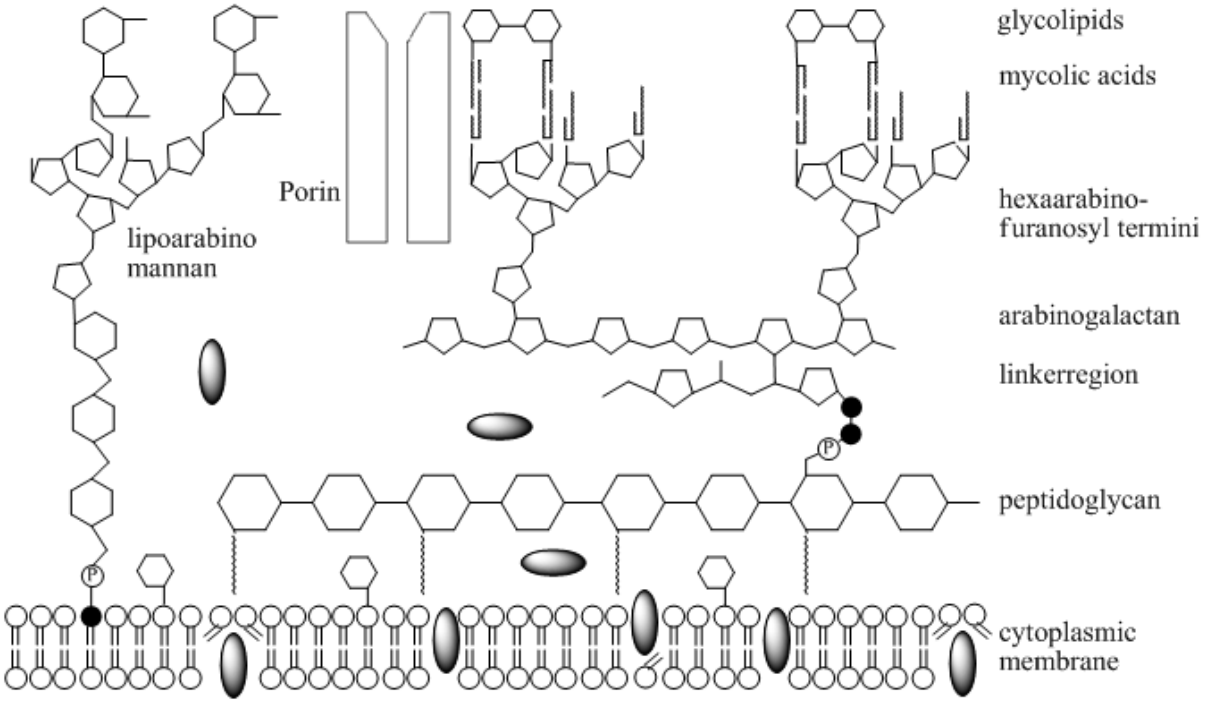


Figure 1.1: the mycobacterial cell wall *adapted from ref [2,7]:* membrane proteins called porins control passage of small hydrophilic molecules and the cell wall uncommon thickness, together with the low fluidity of mycolic acid sheet, makes it more difficult for lypophilic molecules to penetrate. The mycolic acids give rise to the typical mycobacterial 'acid-fast' property. Thus, Mycobacteriae can be separately visualized from Gram-positive or -negative bacteria, by using a different type of staining (Ziehl-Neelsen or acid-fast staining).

Two features are frequently associated with the persistent nature of *Mtb*: dormancy and latency. Due to the incomplete understanding of the importance and mechanism of dormancy, several definitions circulate in the literature. A recent review[6] states the definitions detailed in Box 1.1, thus distinguishing the apparent clinical state of the host (latency) from the metabolic state of the bacteria (dormancy).

Box 1.1: Definitions of dormancy and latency

"**Dormancy**: a state of non-replication that is characterized by long-term viability despite metabolic down-regulation"

"**Latency**: a state of asymptomatic infection characterized by low bacterial counts and a lack of clinical signs of disease"

Latency is difficult to study due to the impossibility to access and visualize the bacilli in humans. Besides, no animal model has been established convincingly reproducing the human disease. Therefore the focus of research has been mostly on dormancy.[6]

Several *in vivo* and *in vitro* models have been developed mimicking infection circumstances to explore new drug targets and possibilities. The best studied *in vitro* model for inducing dormancy is called the Wayne model.[8] This model relies on the gradual depletion of oxygen in sealed cultures of *Mtb* to mimic the hypoxic environment of a granuloma caused by poor perfusion. Hypoxic stress causes metabolic down-regulation and activates a regulon of approximately 50 transcripts called the dormancy survival regulon (dos). This regulon contains genes needed for gaining energy from alternative carbon sources such as fatty acids metabolism and the glyoxylate shunt. Other models aim at different circumstances such as NO to model activated immune cells, slight acidic conditions replicating necrotic environment (Figure 1.2).[6]

Although much uncertainty, dormancy could be contributing to two observations typical to TB. During the course of the disease, a subpopulation of dormant bacilli may be responsible for latency, thus creating an enormous reservoir of chronically infected patients.[4, 6] Reactivation of latent TB in immunocompromised individuals, for instance HIV patients or patients on immunosuppressant therapy, creates a highly contagious and potentially lethal disease.[2]

A dormant subpopulation during active TB could also contribute to the intensive antibiotic treatment needed to eradicate *Mtb*, due to the fact that most anti-TB chemotherapy target actively replicating bacilli.[6, 9]

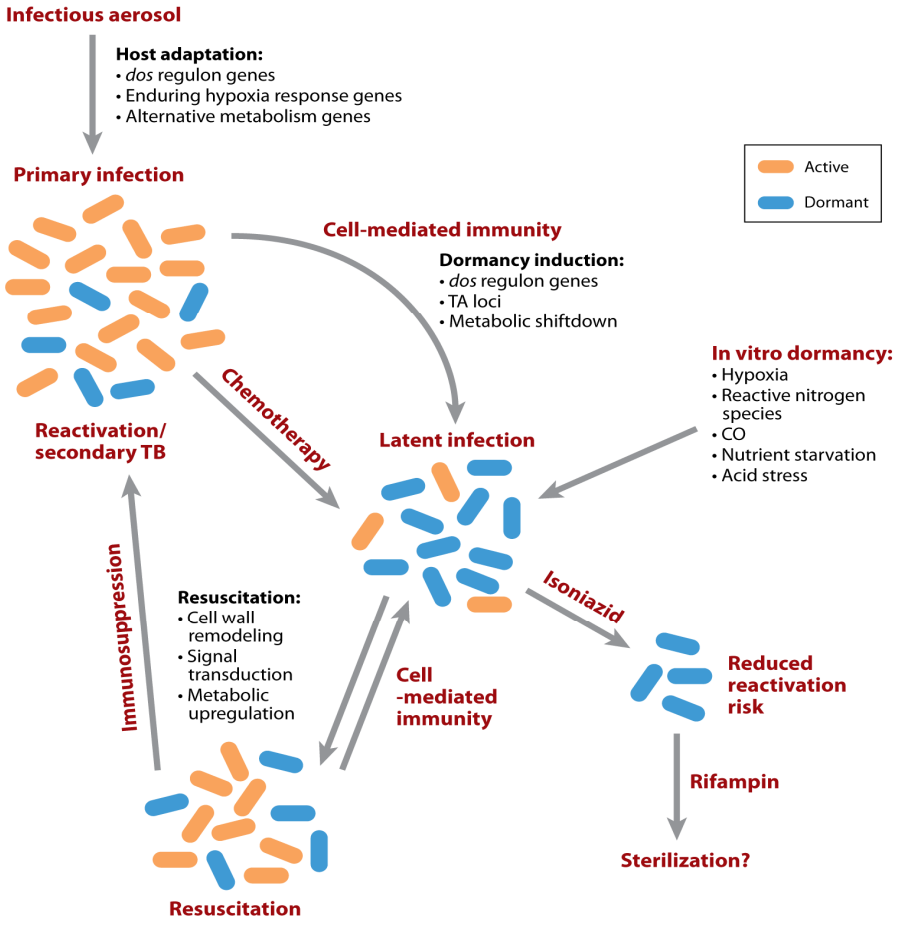


Figure 1.2: stages of TB and environmental factors influencing the progression of the primary infection. *reproduced from ref [6]* Several *in vitro* models were developed for understanding dormancy and to test the potency of antimycobacterials on dormant bacilli.[6] This clinical representation also depicts the synergy of targeting different stages in disease progression. Isoniazid treatment reduces the risk of reactivation by eliminating resuscitating bacilli. Rifampicin complements isoniazid through (slow) bactericidal activity towards the remaining dormant bacteria ('persisters').[8]

1.1.3 TB chemotherapy: past, present and future

Throughout history, before the advent of chemotherapy, treatment of consumption involved fresh air and isolation. In the XIX century mutilating surgery was preformed to tackle the anatomical manifestations of the disease[2]. Ever since Robert Koch discovered the pathogen behind TB in 1882, modern science searches for the means to fight *Mycobacterium tuberculosis.*

By using extracts of Mycobacteriae and developing staining methods, R. Koch also founded TB diagnostics. In 1896 the discovery of *M. bovis* as the cause of bovine TB led to the culture of an avirulent variant known as bacille Calmette-Guérin (BCG). The attenuated BCG vaccine is still widely administered, but the efficacy of this TB vaccine varies, offering only protection to infantile menigeal TB, not pulmonary TB in adults. Therefore, new vaccines are being developed using recombinant or other attenuated strains.[2] Currently seven vaccine candidates are in clinical development.[10]

The real revolution in TB chemotherapy came with the introduction of antibiotics, such as streptomycin (1947), isoniazid (1952) and rifampicin (1963). Due to the drastic reduction of mortality rates and the creation of the impression of control, the search of new TB antibiotics faded resulting in no new first-line drug development since the mid 1980s.[2]

Modern therapy, as recommended by the WHO, consists of the combined oral administration of bactericidal first-line drugs isoniazid, rifampicin, pyrizinamid and ethambutol during the first 2 months. Other drugs combined with isoniazid and rifampicin include fluoroquinoles and parentally administered aminoglycosids such as streptomycin. Nevertheless, due to fast development of resistance and severe side-effects, aminoglycosids are no longer used first-line. This regimen is followed by at least 4 months of isoniazid and rifampicin. Isoniazids highly bactericidal activity towards actively dividing TB bacilli, diminishes over a period of 2-3 weeks remaining bacteriostatic against the remaining 'persisters'. Rifampicin complements isoniazid through (slow) bactericidal activity towards the persistent dormant bacteria (Figure 1.2).[9] Combination therapy is also necessary to prevent developing and spreading of resistant strains such as MDR (resistant to isoniazid and rifampicin) and XDR (also resistant to quinolones and aminoglycosides). Resistance occurs spontaneously due to different genetic mutations ('natural resistance'), whereas the chance of a bacilli being naturally resistant to several drugs at the same time is near to naught. MDR- and XDR-TB are the results of a selection process ('acquired resistance') induced by apparent monotherapy caused by patient non-compliance and inadequate treatment of moderate resistant strains. Treatment for M/XDR relies on the addition and combination of more toxic and bacteriostatic second-line drugs such as *p*-aminosalycilic acid, ethionamide and cycloserine, thus resulting in higher mortality rates due to suboptimal therapy. The total duration of anti-TB therapy can last up to 2 years depending on the *Mtb* strain and the severity of the complications.[2]

Long term administration of even the safer first-line drugs results in several side-effects, diminishing patient-compliance and interfering with other chronic therapies. Common side-effects of anti-TB treatment include peripheral neuritis, hepatotoxicity (isoniazid), impaired vision (ethambutol) and coloration of body fluids (rifampicin). Rifampicin is also a well known inducer of the cytochrome P450 enzyme family in charge of metabolisation of most known drugs.[9] Chronic administration can lead to higher exposure to toxic metabolites and therapeutic failure of other chronic therapies, diabetes and HIV being the most relevant when it comes to TB therapy.[1]

To overcome the renewed challenge posed by TB, the world needs new anti-TB drugs that can target MDR or XDR strains and improve patient compliance (Table 1.1). These high expectations, together with unfavorable economics on TB drug R&D and the fact that very few discovered drugs make it to the developing stage, are the main hurdles slowing down development of new TB chemotherapy.[1]

Table 1.1: desired target product profile for a new TB drug *adapted from ref [1]*

Desired target product profile	Biological characteristics
Shorten treatment duration	Strong bactericidal activity Good activity on latent, dormant or heterogeneous populations More potent and safer regimens of a novel drug or its combination with other drugs
Treat MDR-TB and XDR-TB	Novel chemical class with a new mechanism of action Drugs with low toxicity, especially hepatotoxicity
Reducing pill burden	Combinations of more efficacious drugs to reduce number of pills taken Child-friendly formulation of newer drugs
Lower dosing frequency	Good pharmacokinetics including longer half-life and target tissue levels Retain potency when administered intermittently (for example, 1-3 times a week) Novel fixed-dose formulations and delivery technologies
Drug-drug interactions	No cytochrome P450 induction liabilities Minimal drug-drug induction particularly with anti-retrovirals or oral anti-diabetics

Each target product profile is accompanied by the biological characteristics needed to accomplish the respective feature.

Many new TB drug candidates are derived from old drug classes. For instance old antibiotics with antimycobacterial activity are being tested in TB clinical trials, such as the fluoroquinoles gatifloxacin and moxifloxacin, which are currently in phase III clinical development. On the other hand, known classes of antibiotics are reengineered through chemical modification hoping to yield improved bactericidal activity, better resistance profile, safety or pharmacokinetic (PK) and pharmacodynamic (PD) properties. At the moment, several examples of old reengineered scaffolds are in the TB development pipeline such as the oxazolidones PNU-100480, AZD-5847 and the nitroimidazoles PA-824 and OPC-67683.[1] Even β-lactam antibiotics have been reconsidered possible leads since the discovery of the importance of β-lactamase in TB resistance towards this class of antibiotics (cfr 1.1.2.).[11] The development of specific mycobacterial β-lactamase inhibitors and the testing of new broad spectrum β-lactamase-inhibitors could open the possibility of administering β-lactam antibiotics to TB patients, in particular carbapenems have shown good *in vitro* activity when combined with clavulanic acid (a β-lactam-ase inhibitor).[12]

Discovery of new chemical classes with new mechanisms of action, to tackle resistant strains of *Mtb*, requires screening of pharmaceutical library collections. The limited chemical diversity within these libraries in contrast to the atypical PK requirements posed by anti-TB drugs has been contributing to the poor efficiency of the screening process.[13] Computational analysis of the physicochemical features of first- and second-line TB drugs highlighted the chemical diversity of TB drugs when compared with over a thousand known non-antibacterials. Although antibacterial agents are considered to be generally quite polar, biasing compound libraries towards one physicochemical parameter could be counterproductive towards TB drug development.[1] Nonetheless, a greater understanding of the mycobacterial cell wall and its influence on permeability of (a)polar drugs could lead to the further optimization of existing scaffolds.

1.2 ThyX: A new and promising target

1.2.1 Introducing flavin-dependent thymidylate synthase

Thymidine, one of four DNA building blocks, is essential for all organisms. Until recently, the only known *de novo* pathway for thymidine synthesis was catalyzed by the enzyme 'classical thymidylate synthase' (classical TS), encoded by the gene ThyA. This enzyme has been well studied and is used by drugs, such as 5-fluorouracil, to target human cancer cells. The identification of organisms lacking classical TS led to the discovery of an alternative flavin-dependent thymidylate synthase (FDTS)[14, 15]. Encoded by the ThyX gene, FDTS is present in about 30% of all microorganisms, including several human pathogens such as *S. thyphi, B. anthracis* and *M. tuberculosis*.[16] It has been shown that in the few microorganisms expressing both ThyA and ThyX (e.g. TB), ThyX is essential for optimal growth[17, 18], suggesting expression of both enzymes varies in different stages of development. Selective inhibition of FDTS over human TS seems feasible, because they have a completely different structure and mechanism of action.[19]

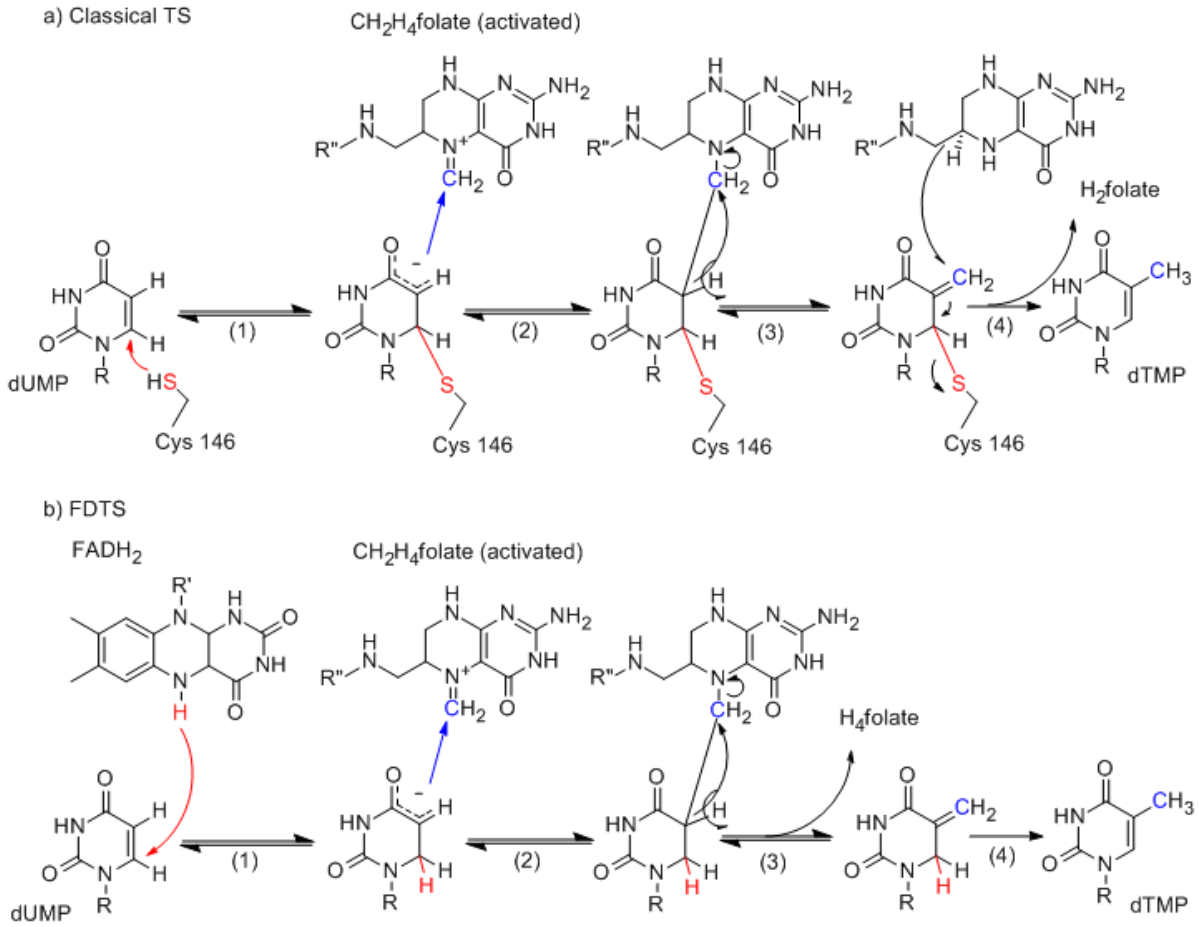


Figure 1.3: Thymidylate synthase mechanisms *adapted from ref [19]*.
(a) The chemical reaction for the classical thymidylate synthase catalyzed reaction. (b) The proposed chemical mechanism for FDTS, not relying on an enzymatic nucleophile. R = 2'-deoxyribose-5'-phosphate; R' = adenosine-5'-pyrophosphate-ribityl; R" = (*p*-aminobenzoyl)-glutamate

1.2.2 FDTS: Catalytic mechanism and structure

In classic TS a conserved cystein residue in the enzyme active site acts as a nucleophile, activating the uracyl moiety through Michael addition on the C6 position of 2'-deoxyuridine-5'-monophosphate (dUMP) (Figure 1.3 (a), step 1). The resulting enolate attacks the activated methylene group of 5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) cofactor (step 2), followed by elimination of tetrahydrofolate (H_4folate). A covalently bound intermediate is formed (step 3), which dissociates from the enzyme after accepting an hydride from H_4folate (step 4), resulting in the formation of 2'-deoxythymidine-5'-monophosphate (dTMP) and dihydrofolate (H_2folate).[16, 19, 20]

In contrast, FDTS posseses no enzymatic nucleophile. Despite the discovery of a conserved serine residue near the substrate binding site, it is considered highly unlikely that this residue would take part in catalyzing thymidylate synthesis due to the absence of basic amino acid residues in the vicinity. The nucleophilic Michael addition is initiated by a hydrid from the $FADH_2$ cofactor, resulting in a non-covalent enolate anion (Figure 1.3 (b), step 1). After attacking CH_2H_4folate (step 2), an isomer of dTMP is supposedly formed (step 3). Due to relative stability of the isomer, it is believed that dTMP is formed by a currently unknown enzymatic intervention (step 4).[19] The FDTS catalytic reaction is preceded by the binding of NADPH to the FDTS-FAD complex, reducing the FAD cofactor to $FADH_2$. The substrate dUMP then binds the FAD-NADPH-FDTS complex. The order of product release is yet to be discovered.[21]

Unlike the dimeric structure of classical TS, FDTS is a tetramer with four catalytic centers, each located at the interface of three subunits. Four FAD molecules are located at the center, the flavine ring interacting with both the enzyme as well as the substrate. Folate and NADP binding sites are yet to be identified [16, 22] (Figure 1.4).

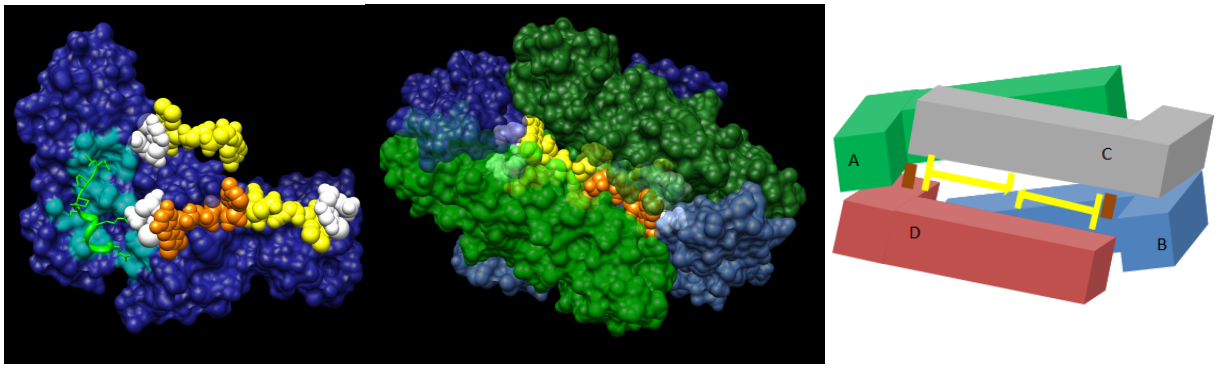


Figure 1.4: Structure of FDTS: left: one subunit, middle: FDTS tetramer, right: simplified representation
Subunits A-D are coloured in blue and green, FAD is represented in orange or yellow and the 5Br-dUMP subrate analogue is shown in white on the left and middle figure. In the simplified representation of subunit interaction, FAD is shown in yellow and the substrate analogue in brown.

2 AIMS

In search for new antimycobacterial agents, the scope of this project is finding novel inhibitors of the enzyme 'Flavin-dependent thymidylate synthase'. By in house screening of over 1500 commercially available compounds, a series of tricyclic compounds with modest inhibitory activity was found. It is believed, through molecular resemblance, that these compounds target either FAD(H_2) or the currently unknown CH_2H_4folate cofactor binding site. The goal of this thesis is to create a library based on the found lead molecule (Figure 2.1 and 4.1; Table 4.2), leading to further structural understanding of FDTS.

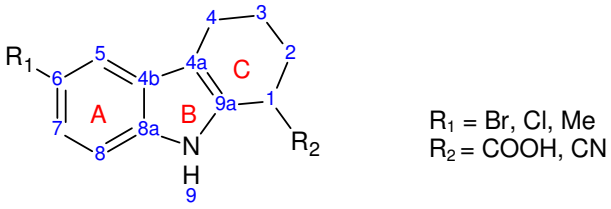


Figure 2.1: Identified tricyclic lead series: 2,3,4,9-Tetrahydro-1H-carbazoles

Substituted tetrahydrocarbazoles are interesting molecules possessing many biological and pharmacological activities, influencing gene expression through histone regulation[23]. In addition more specific targets are reported in the literature such as central nervous system activity[24], antihistamine[25] and anti-inflammatory properties[26].

From the sheer number of ways to synthesize indoles[27], the Bischler[28] (Figure 2.2, a) and Fischer[29] (Figure 2.2, b) indole synthesis will be used throughout this project, due to the wide commercial availability of substituted anilines and phenylhydrazines, respectively. These methods are based upon the disconnection of the N1-C2 and C3-C3a bonds depicted in Figure 2.2. By varying the substitution pattern on the aromatic ring we hope to gain some structural information on the inhibitory binding site located on FDTS.

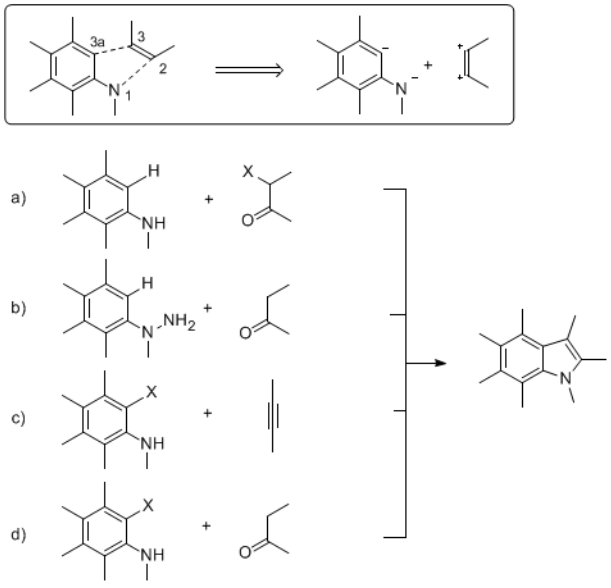


Figure 2.2: Main indole synthesis based on the disconnection of the N1-C2 and C3-C3a *adapted from ref [27]* Substituents at the various positions are unspecified unless indicated.

The compounds showing inhibition (expressed as IC50) in the nM range will then be submitted for structural determination of the enzyme-inhibitor complex by X-ray crystallography. The most interesting compounds, in terms of pharmacokinetics, are tetrahydrocarbazoles derivatives with a carboxylic acid , amide or cyano group located at R_2 (Figure 2.1). Unfortunately amides along with tetrazoles (bioisosters of carboxylic acids) did not show any activity in previous screenings. Nevertheless, in one synthetic cycle the ester, acid, amide and cyano-derivative will be generated and tested.

3 EXPERIMENTAL SECTION

3.1 Materials and methods

For all reactions, chemicals of analytical or synthetic grade were obtained from commercial sources and were used without further purification with the exception of ethyl 2-oxocyclohexanecarbonitrille **5**, previously synthesized by dr. E.Groaz according to a method previously described in the literature.[30]
Technical solvents were obtained from Nyssens Graphics NV (St Job in 't Goor, Belgium). Microwave reactions were done in 10 mL vessels, using a CEM Focused Microwave™ Synthesis Sestem, Model Discover. Column chromatography was performed with silicagel 0.060–0.03mm, 60Å (Across; Geel, Belgium). Analytical Thin Layer Chromatography was performed on Alugram® silica gel UV254 mesh 60, 0.20mm (Macherey-Nagel). For Preparitive Thin Layer Chromatography, MN SilicaGel P / UV 254 (Macherey-Nagel) was used. NMR Spectra were recorded on a Brucker Avance 300 MHz (with 5mm BBO probe) or on a Brucker Avance II 500 MHz (with 5mm TXI probe) NMR spectrometer. Chemical shifts are expressed as δ units (part per million) down field from TMS (tetramethyl silane) for ^{1}H and ^{13}C-spectra. Electrospray ionization mass spectra were acquired on a quadrupole ion trap mass spectrometer (LCQ, ThermoFinnigan, San Jose, CA). Samples were dissolved in a acetonitrile:water (50:50 v/v) mixture and infused at 10 µL/min. Spectra were recorded in positive or negative ionization mode. Electrospray capillary voltage was set to 5 kV and the transfer capillary temperature was 175 °C.

3.2 Synthetic procedures

General method for γ-bromination of cyclohexanonderivatives **1** and **5**

Cyclohexanonderivative **1** or **5** (11.8 mmol, 1eq) was dissolved in dry Et_2O and cooled with stirring to 0 °C under Ar. Bromine (4 mL, 11.8 mmol, 1eq) was then added dropwise over 15 min and the reaction mixture was allowed to warm to room temperature over 90 min. Ice-cold saturated aq. Na_2CO_3 was then added, followed by extraction with EtOAc (3 x 50 mL). The combined organic layers were dried over anhydrous Na_2CO_3 and evaporated in vacuo. The remaining crude residue was purified by column chromatography unless stated otherwise.

Ethyl 3-Bromo-2-oxocyclohexanecarboxylate (2). Purification by column chromatography (hexane/EtOAc 95:5) to yield 2.76 g (94%) as a pale yellow oil. ^{1}H NMR (δ, MeOD): 1.24-1.40 (m, 3H, OCH_2CH_3), 1.70-2.40 (m, 6H, $(CH_2)_3$), 2.40-2.53 (m, 1H, COCHCOO), 4.18-4.33 (m, 2H, OCH_2CH_3), 4.73-4.79 (m, 1H, CHBr); ^{13}C NMR (δ, MeOD): 12.78, 17.17, 21.68, 31.62, 45.20, 60.32, 98.97, 166.29, 171.91; *m/z* = 251 (100), 253 (100) [MH^+]

Ethyl 3-Bromo-2-oxocyclohexanecarbonitrile (6) Purification by column chromatography (hexane/EtOAc 95:5) to yield a transparent oil. After scratching the wall of the vessel with a glass rod, a white solid crystallized. Washing the crystals with small amounts of ice-cold DCM resulted in 39.69% yield of pure compound **6**. ^{1}H NMR (δ, MeOD): 1.70-1.81 (m, 1H, CH_2), 1.83-2.02 (m, 2H, CH_2), 2.09-2.20 (m, 2H, CH_2), 2.30-2.40 (m, 2H, COC*H*CN), 4.74-4.78 (m, 1H, C*H*Br); ^{13}C NMR (δ, MeOD): 16.45, 24.38, 31.48, 44.91, 83.00, 117.62, 163.25; *m/z* = 200.9 (100), 202.9 (100) [MH^+]

General methods for Bischler Indole synthesis: synthesis of esters **3** and nitriles **7**

Method A. Bromo keto ester **2** (4.08 mmol, 1eq) was added to corresponding the aniline (10.2 mmol, 2.5eq) and the mixture was heated at 150°C for 3h. The reaction mixture was allowed to cool to room temperature and was then taken up in DCM. It was then washed with 1N HCL (3 x 50 mL), followed by saturated aq. $NaHCO_3$. The combined organic layers where dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo*. The remaining crude residue was purified by column chromatography using various solvent systems.

Method B. Bromo keto ester **2** or Bromo keto carbonitrile **6** (0.8 mmol, 1 eq) and suitably substituted aniline (2 mmol, 2.5 eq) were dissolved in $EtO(CH_2)_2OH$ and irradiated by microwaves (150W) at 150 °C for 15min. The solvent was evaporated in vacuo and the residue taken up in DCM and then washed with 1N HCL (3 x 50 mL), followed by saturated aq. $NaHCO_3$. The combined organic layers where dried over anhydrous Na_2SO_4, filtered and evaporated in vacuo. The remaining crude residue was purified by column chromatography using various solvent systems.

Method C. Bromo keto ester 2 (4.02 mmol, 1eq) was refluxed in xylene or $EtO(CH_2)_2OH$ together with the corresponding substituted aniline (10.1 mmol, 2.5eq) at 150 °C for 3h. The solvent was then evaporated in vacuo and the residue was taken up in DCM and then washed with 1N HCL (3 x 50 mL), followed by saturated aq. $NaHCO_3$. The combined organic layers where dried over anhydrous Na_2SO_4, filtered and evaporated in vacuo. The remaining crude residue was purified by column chromatography using various solvent systems.

Ethyl 6-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylate (3a) Purification by column chromatography (PE/DCM (2:8)) resulted in 71.7 % yield (method B) as an off-white solid after trituration of the recovered oil with hexane; ^{1}H NMR (δ, $CDCl_3$): 1.31 (t, *J* = 7.1 Hz, 3H, OCH_2CH_3), 1.75-1.90 (m, 1H, -CH_2), 1.99-2.12 (m, 1H, CH_2), 2.12-2.27 (m, 2H, CH_2), 2.68 (t, *J* = 5 Hz, 2H, ArCH_2), 3.83 (t, *J* = 5.7 Hz, 1H, C*H*COO), 4.25 (q, *J* = 7 Hz, 2H, OCH_2CH_3), 7.09 (dd, *J* = 8.5 Hz, 1.7 Hz, 1H, C*H*), 7.18-7.27 (m, 1H, C*H*), 7.43 (bd, *J* = 1.4 Hz, 1H, C*H*), 8.43 (s, 1H, N*H*); ^{13}C (δ, $CDCl_3$): 13.75, 20.45, 21.35, 27.18, 45.97, 60.63, 110.63, 111.68, 117.29, 121.01, 124.55, 129.58, 130.16, 137.15, 174.25; *m/z* = 277.9 (100), 279.9 (33) [MH^+]

Ethyl 6-Bromo-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylate (3b) Purification by column chromatography (PE/DCM (2:8)) resulted in 33 % yield (method A) as a yellow-brown solid after trituration of the recovered oil with hexane; ^{1}H NMR (δ, $CDCl_3$): 1.31 (t, J = 7.2 Hz, 3H, OCH_2CH_3), 1.73-1.88 (m, 1H, CH_2), 1.98-2.12 (m, 1H, CH_2), 2.12-2.24 (m, 2H, CH_2), 2.66 (t, J = 6.2 Hz, 2H, $ArCH_2$), 3.82 (t, J = 6.7 Hz, 1H, CHCOO), 4.24 (q, J = 7.2 Hz, 2H, OCH_2CH_3), 7.15-7.26 (m, 2H, CH, CH), 7.59 (bd, J = 0.5Hz, 1H, CH), 8.44 (s, 1H, NH); ^{13}C NMR (δ, $CDCl_3$): 13.94, 20.25, 21.34, 27.17, 45.99, 60.67, 110.64, 111.29, 115.01, 119.55, 122.78, 129.29, 130.15, 139.15, 174.25, *m/z* = 320.1 (50), 321.9 (50) [M-H$^+$]

Ethyl 6-Fluoro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylate (3c) Purification by column chromatography (PE/DCM (2:8)) resulted in 78.6 % yield (method B) as a brown oil; ^{1}H NMR (δ, $CDCl_3$): 1.30 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 1.72-1.89 (m, 1H, CH_2), 1.98-2.11 (m, 1H, CH_2), 2.11-2.22 (m, 2H, -CH_2), 2.63-2.69 (m, 2H, $ArCH_2$), 3.81 (t, J = 6,7 Hz, 1H, CHCOO), 4.22 (q, J = 7.1 Hz, 2H, OCH_2CH_3), 6.87 (td, J = 9.2 Hz, 2.7 Hz, 1H, CH), 7.09 (dd, J = 9.5 Hz, 2.4Hz, 1H, CH), 7.16-7.23 (m, 1H, CH), 8.43 (s, 1H, NH); ^{13}C NMR (δ, $CDCl_3$): 13.95, 20.35, 21.44, 25.65, 39.82, 61.00, 103.97 (d, $^2J_{CF}$ = 23.3 Hz), 109.52 (d, $^2J_{CF}$ = 26.2 Hz), 111.00 (d, $^3J_{CF}$ = 9.7 Hz), 111.74 (d, $^4J_{CF}$ = 4.9 Hz), 127.33 (d, $^3J_{CF}$ = 9.8 Hz), 131.28, 132.14, 157.41 (d, $^1J_{CF}$ = 234 Hz), 172.22; *m/z* = 259.8 (100) [M-H$^+$]

Ethyl 6-Methyl-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylate (3d) Purification by column chromatography (PE/DCM (2:8)) resulted in 91.4 % yield (method B) as a yellow solid. ^{1}H NMR (δ, $CDCl_3$): 1.29 (t, J = 7.2 Hz, 3H, OCH_2CH_3), 1.72-1.89 (m, 1H, CH_2), 1.97-2.11 (m, 1H, CH_2), 2.11-2.21 (m, 2H, -CH_2), 2.43 (s, 3H, CH_3Ar), 2.65-2.73 (m, 2H, $ArCH_2$), 3.80 (t, J = 6,6 Hz, 1H, CHCOO), 4.21 (q, J = 7.2 Hz, 2H, OCH_2CH_3), 6.94-7.02 (m, 1H, CH), 7.16-7.22 (m, 1H, CH), 7.26 (bs, 1H, CH), 8.29 (s, 1H, NH); ^{13}C NMR (δ, $CDCl_3$): 13.99, 20.45, 21.18, 21.52, 25.77, 39.83, 60.88, 110.16, 111.12, 117.69, 122.98, 127.25, 128.06, 129.39, 134.03, 172.47; *m/z* = 258.0 (100) [MH$^+$]

Ethyl 6-Methoxy-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylate (3e) Purification by column chromatography (EtOAc/n-hexane (3:7)) resulted in 87.9 % (method B) yield as a yellow-brown solid; ^{1}H NMR (δ, $CDCl_3$): 1.27 (t, J = 7.2 Hz, 3H, OCH_2CH_3), 1.72-1.87 (m, 1H, CH_2), 1.95-2.09 (m, 1H, CH_2), 2.09-2.20 (m, 2H, CH_2), 2.63-2.71 (m, 2H, $ArCH_2$), 3.74-3.81 (m, 1H, CHCOO), 3.82 (s, 3H, CH_3OAr), 4.19 (q, J = 7 Hz, 2H, OCH_2CH_3), 6.79 (dd, J = 8.7 Hz, J = 2.4 Hz, 1H, CH), 6.92 (bd, J = 2.4 Hz, 1H, CH), 7.15 (d, J = 8.7 Hz, 1H, CH), 8.33 (s, 1H, NH); ^{13}C NMR (δ, $CDCl_3$): 14.00, 20.53, 21.51, 25.81, 39.87, 55.65, 60.90, 100,20, 111.24, 111.30, 111.32, 127.36, 130.23, 130.89, 153.61, 172.40; *m/z* = 274.4 [MH$^+$]

Ethyl 7-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylate (3f) Purification by PLC (EtOAc/n-hexane (3:7)) resulted in 33.16 % yield (method B) as a yellow solid; ^{1}H NMR (δ, $CDCl_3$): 1.30 (t, J = 7.4 Hz, 3H, OCH_2CH_3), 1.75-1.85 (m, 1H, CH_2), 1.98-2.07 (m, 1H, CH_2), 2.13-2.18 (m, 2H, CH_2), 2.64-2.69 (m, 2H, $ArCH_2$), 3.76-3.8 (m, 1H, CHCOO), 4.22 (q, J = 7.2 Hz, 2H, OCH_2CH_3), 7.09 (dq, J = 8.4 Hz, 1.5 Hz, 1H, CH), 7.24-7.26 (m, 1H, CH), 7.34 (dd, J = 8.4 Hz, 2.7 Hz, 1H, CH), 8.42 (s, 1H, NH); ^{13}C NMR (δ, $CDCl_3$): 14.20, 20.52, 21.61, 25.84, 39.93, 61.26, 110.67, 111.90, 118.96, 119.71, 125.83, 127.38, 130.30, 136.22, 172.46; *m/z* = 277.8 (100), 279.8 (33) [MH$^+$]

Ethyl 5-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylate (3g) Purification by PLC (EtOAc/n-hexane (3:7)) resulted in 23.75 % yield (method B) as a yellow oil; ^{1}H NMR (δ, $CDCl_3$): 1.30 (t, J = 7.2 Hz, 3H, OCH_2CH_3), 1.75-1.85 (m, 1H, CH_2), 1.98-2.07 (m, 1H, CH_2), 2.11-2.17 (m, 2H, CH_2), 2.98-3.16 (m, 2H, $ArCH_2$), 3.77-3.83 (m, 1H, CHCOO), 4.23 (q, J = 7 Hz, 2H, OCH_2CH_3), 6.97-7.04 (m, 2H, CH, CH), 7.15-7.19 (m, 1H, CH), 7.24 (s, 1H, NH); ^{13}C NMR (δ, $CDCl_3$): 14.21, 21.93, 22.64, 255.46, 40.09, 61.32, 109.43, 111.98, 119.84, 122.12, 124.56, 126.15, 130.48, 137.08, 172.53 *m/z* = 277.9 (100), 279.9 (33) [MH^+]

6-Fluoro-2,3,4,9-tetrahydro-1H-carbazole-1-carbonitrile (7c) Purification by column chromatography (PE/DCM (2:8)) resulted in 44.80% yield (method B) as a yellow-brown solid. ^{1}H NMR (δ, $CDCl_3$): 1.77-1.90 (m, 1H, CH_2), 1.96-2.07 (m, 1H, CH_2), 2.09-2.22 (m, 2H, CH_2), 2.53-2.72 (m, 2H, CH_2), 3.92 (t, J = 5.9 Hz, 1H, CHCN), 6.89 (td, J = 9 Hz, J = 2.5 Hz, 1H, CH), 7.08 (dd, J = 9.2 Hz, J = 2.4 Hz, 1H, CH), 7.13-7.20 (m, 1H, CH), 8.53 (bs, 1H, NH); ^{13}C NMR (δ, $CDCl_3$): 19.86, 20.75, 25.59, 27.34, 103.27 (d, $^{2}J_{CF}$ = 24.8 Hz), 110.53 (d, $^{2}J_{CF}$ = 25.6 Hz), 111.61 (d, $^{3}J_{CF}$ = 9.7 Hz), 112.14 (d, $^{4}J_{CF}$ = 4.4 Hz), 119.93, 126.91 (d, $^{3}J_{CF}$ = 9.8 Hz), 127.05, 132.42, 157.55 (d, $^{1}J_{CF}$ = 235.5 Hz) ; *m/z* = 213.2 (100) [$M-H^+$]

6-Methoxy-2,3,4,9-tetrahydro-1H-carbazole-1-carbonitrile (7e) Purification by column chromatography (PE/DCM (2:8)) resulted in 33.93% yield (method B) as a white solid. ^{1}H NMR (δ, $CDCl_3$): 1.84-2.01 (m, 1H, CH_2), 2.01-2.17 (m, 1H, CH_2), 2.17-2.34 (m, 2H, CH_2), 2.63-2.84 (m, 2H, CH_2), 3.89 (s, 3H, OCH_3) 4.01 (t, J = 6.1 Hz, 1H, CHCN), 6.86-7.10 (m, 2H, CH, CH), 7.21-7.35 (m, 1H, CH), 8.22 (bs, 1H, NH); ^{13}C NMR (δ, $CDCl_3$): 19.99, 20.90, 25.70, 27.46, 55.63, 100.39, 111.60, 112.40, 118.41, 119.80, 125.89, 126.99, 130.98, 153.91; *m/z* = 225.0 (100) [$M-H^+$]

7-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carbonitrile (7f) Purification by PLC (EtOAc/n-hexane (3:7)) resulted in 24.33% yield (method B) as a yellow solid. ^{1}H NMR (δ, $CDCl_3$): 1.81-1.99 (m, 1H, CH_2), 1.99-2.14 (m, 1H, CH_2), 2.14-2.29 (m, 2H, CH_2), 2.62-2.82 (m, 2H, CH_2), 3.96-4.02 (m, 1H, CHCN), 7.04-7.12 (m, 1H, CH), 7.18-7.41 (m, 2H, CH, CH), 8.70 (bs, 1H, NH); ^{13}C NMR (δ, $CDCl_3$): 19.80, 20.72, 25.50, 27.28, 109.60, 110.84, 112.13, 119.11, 119.99,122.74, 125.15, 128.03, 136.24; *m/z* = 229.2 (100) 231.2 (33)[$M-H^+$]

5-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carbonitrile (7g) Purification by PLC (EtOAc/n-hexane (3:7)) resulted in 22.52% yield (method B) as a yellow oil. ^{1}H NMR (δ, $CDCl_3$): 1.83-2.02 (m, 1H, CH_2), 2.02-2.17 (m, 1H, CH_2), 2.17-2.32 (m, 2H, CH_2), 2.61-2.80 (m, 2H, CH_2), 3.98-4.03 (m, 1H, CHCN), 7.05-7.11 (m, 1H, CH), 7.20-7.42 (m, 2H, CH, CH), 7.79 (bs, 1H, NH); ^{13}C NMR (δ, $CDCl_3$): 19.80, 20.72, 25.50, 27.28, 109.60, 110.84, 112.13, 119.11, 119.99,122.74, 125.15, 128.03, 136.24; *m/z* = 229.2 (100) 231.2 (33)[$M-H^+$]

General method for hydrolysis of substituted tetrahydrocarbazole esters to obtain the acids **4**

Tetrahydrocarbazole ester **3** (0.5 mmol, 1 eq) was dissolved in 4 mL of MeOH/THF (1:1) and 2 mL of a 0.5 M aqueous solution of LiOH (1 mmol, 2eq). The reaction mixture was stirred at room temperature and monitored by TLC. After completion, the reaction was quenched by adding 1 mL of 2M solution of acetic acid in THF (2 mmol, 4eq). Organic solvents were evaporated *in vacuo* and to the remaining oil, brine was added; followed by extraction with EtOAc (3 x 50 mL) and drying over anhydrous sodium sulphate. After evaporation, the crude residue was purified by column chromatography and/or preparative TLC to afford compounds **4**.

6-Bromo-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylic acid (4b) Purification by column chromatography (MeOH/DCM (1:9)) resulted in 87.60 % yield as an off-white solid; ^{1}H NMR (*δ,* $CDCl_3$): 1.75-1.91 (m, 1H, CH_2), 1.97-2.11 (m, 1H, CH_2), 2.14-2.24 (m, 2H, CH_2), 2.63-2.74 (m, 2H, ArCH_2), 3.89 (t, *J* = 6.5 Hz, 1H, C*H*COO), 7.14-7.26 (m, 2H, C*H*, C*H*), 7.59 (bd, *J* = 1.4 Hz, 1H, C*H*), 8.37 (s, 1H, N*H*); ^{13}C NMR (*δ,* $CDCl_3$): 20.17, 21.17, 25.33, 39.36, 111.83, 111.90, 112.23, 120.72, 124.45, 128.63, 129.46, 134.29, 177.97; *m/z* = 291.8 (100) 293.8 (95) [M-H$^+$]

6-Fluoro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylic acid (4c) Purification by column chromatography (MeOH/DCM (1:9)), followed by PLC (MeOH/DCM (1:9)) resulted in 14.00 % yield as a yellow oil; ^{1}H NMR (*δ,* MeOH): 1.76-1.90 (m, 1H, CH_2), 1.95-2.10 (m, 1H, CH_2), 2.10-2.25 (m, 2H, CH_2), 2.62-2.74 (m, 2H, ArCH_2), 3.81 (t, *J* = 6 Hz, 1H, C*H*COO), 6.79 (td, *J* = 9.2 Hz, *J* = 2,5Hz, 1H, C*H*), 7,03 (dd, *J* = 9.7 Hz, 2.5 Hz, 1H, C*H*), 7.21-7.26 (m, 1H, C*H*); ^{13}C NMR (*δ,* MeOH): 16.63, 20.04, 26.45, 40.03, 101.58 (d, $^{2}J_{CF}$ = 23.1 Hz), 107.97 (d, $^{2}J_{CF}$ = 25.4 Hz), 109.98 (d, $^{4}J_{CF}$ = 4.3 Hz), 110.62 (d, $^{3}J_{CF}$ = 9.7 Hz), 127.0182 (d, $^{3}J_{CF}$ = 10.5 Hz), 132.54 (d, $^{3}J_{CF}$ = 9.8 Hz), 157.06 (d, $^{1}J_{CF}$ = 230.9 Hz); *m/z* = 231.9 (100) [M-H$^+$]

6-Methyl-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylic acid (4d) Purification by column chromatography (MeOH/DCM (1:9)) resulted in 86.00 % yield as a yellow-brown solid; ^{1}H NMR (*δ,* MeOH): 1.76-1.91 (m, 1H, CH_2), 1.91-2.06 (m, 1H, CH_2), 2.06-2.25 (m, 2H), 2.38 (s, 3H, ArCH_3), 2.63-2.71 (m, 2H, ArCH_2), 3.81 (t, *J* = 6 Hz, 1H, C*H*COO), 6.84-6.90 (m, 1H, C*H*), 7.14-7.19 (m, 2H, C*H*; C*H*) ^{13}C NMR (*δ,* MeOH): 20.11, 20.92, 26.37, 39.47, 81.24, 108.41, 109.16, 116.69, 121.89, 122.41, 126.65, 127.29, 134.47, 175.16; *m/z* =228.3 (100) [M-H$^+$]

6-Methoxy-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylic acid (4e) Purification by PLC (MeOH/DCM (1:9)) resulted in 75.69 % yield as a brown oil; ^{1}H NMR (*δ,* MeOH): 1.66-1.84 (m, 1H, CH_2), 1.84-2.20 (m, 3H, CH_2), 2.55-2.69 (m, 2H, ArCH_2), 3.71-3.83 (m, 4H, CH_3O, C*H*COO), 6.70 (dd, *J* = 8.7 Hz, *J* = 2.5Hz, 1H, C*H*), 6.88 (d, *J* = 2.4 Hz, 1H, C*H*), 7.15 (d, *J* = 8.7 Hz, C*H*); ^{13}C NMR (*δ,* MeOH): 20.20, 21.01, 26.38, 39.79, 54.72, 99.62, 109.89, 110.21, 110.80, 127.11, 130.86, 131.35, 153.08, 175.60; *m/z* = 244.2 (100) [M-H$^+$]

7-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylic acid (4f) Purification by PLC (MeOH/DCM (1:9)) resulted in 38.93% yield as a pale yellow oil; ^{1}H NMR (*δ,* MeOH): 1.75-1.89 (m, 1H, CH_2), 1.91-2.21 (m, 3H, CH_2), 2.63-3.70 (m, 2H, ArCH_2), 3.76-3.82 (m, 1H, C*H*COO), 6.92 (dd, *J* = 8.3Hz, *J* = 1.9Hz, 1H, C*H*), 7.28-7.33 (m, 2H, C*H*, C*H*); ^{13}C NMR (*δ,* MeOH): 19.99, 20.93, 26.42, 29.03, 109.90, 109.96, 117.84, 118.07, 125.47, 126.02, 131.54, 136.42, 175,90; *m/z* = 247.9 (100) 249.9 (33) [M-H$^+$]

5-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylic acid (4g) Purification by PLC (MeOH/DCM (1:9)) resulted in 43.10 % yield as a pale yellow oil; ^{1}H NMR (*δ,* MeOH): 1.73-1.89 (m, 1H, CH_2), 1.94-2.12 (m, 3H, CH_2), 3.00-3.09 (m, 2H; ArCH_2), 3.73-3.80 (m, 1H, C*H*COO), 6.84-6.95 (m, 2H, C*H*, C*H*), 7.19 (dd, *J* = 7.8 Hz, J = 1.3 Hz, 1H, C*H*); ^{13}C NMR (*δ,* MeOH): 21.23, 22.38, 26.24, 40.76, 108.92, 109.58, 118.13, 120.54, 123.90, 124.86, 132.33, 137.27, 176.58; *m/z* = 248.0 (100) 250.0 (33) [M-H$^+$]

General method for aminolyse of substituted tetrahydrocarbazole esters to obtain the amides **5**

Tetrahydrocarbazole ester 3 (0.5 mmol, 1 eq) was dissolved in 10 mL of MeOH saturated with ammonia in a sealed tube. The reaction was stirred at 60 °C while monitored by TLC. After completion, the solvent was evaporated *in vacuo* and the crude residue was purified by column chromatography using different solvent systems to afford the compounds **5**.

6-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide (5a) Purification by column chromatography (MeOH/DCM (1:9)) resulted in 80.16 % yield as a yellow-brown oil; ^{1}H NMR (*δ,* MeOH): 1.71-1.87 (m, 1H, CH_2), 1.94-2.06 (m, 1H, CH_2), 2.06-2.20 (m, 2H, CH_2), 2.57-2.75 (m, 2H, ArCH_2), 3.68-3.81 (m, 1H, C*H*COO), 6.99 (dd, *J* = 8.7 Hz, *J* = 2.2Hz, 1H, C*H*), 7.21 (d, *J* = 8.7 Hz, 1H, C*H*), 7.34 (d, *J* = 2.2 Hz, 1H, C*H*); ^{13}C NMR (*δ,* MeOH): 19.90, 20.76, 27.59, 41.15, 110.47, 111.29, 116.55, 120.55, 123.55, 127.90, 132.23, 134.61, 177.21; *m/z* = 248.1 (100) 250.1 (33) [MH$^+$]

3.3 Screening of ThyX inhibitors in a radioactive tritium-release assay

Cloning of the ThyX gene, protein expression and purification was done as previously published.[31]

Determining inhibition level at 50µM 5µL 10mM of tested compound was added to 10µL of an aqueous solution containing 0.5 nmol mTHF; 1.5 µg ThyX; 0.25 nmol FAD; 12.5 nmol NADPH in 96well plate. To initiate the reaction 10 µL of 75 µM 5-^{3}H dUMP was added and the reaction mixture was incubated for 10 min at room temperature. The reaction was terminated by addition of 20 µL of stop solution (2 N trichloroacetic acid/4.3mM dUMP (3:1)), followed by the removing of unreacted substances through activated charcoal (150 µL 10% (w/v) suspension). The plate was kept on ice for 15 min and then centrifuged at 4500 rpm at 4 °C for 10 min. 100 µL of supernatant was transferred into a new plate and after addition of 150 µL of microscint 40, assayed by liquid scintillation counting to determine the amount of tritriated water produced by the reaction.

4 RESULTS AND DISCUSSION

4.1 Synthesis of tetrahydrocarbazoles

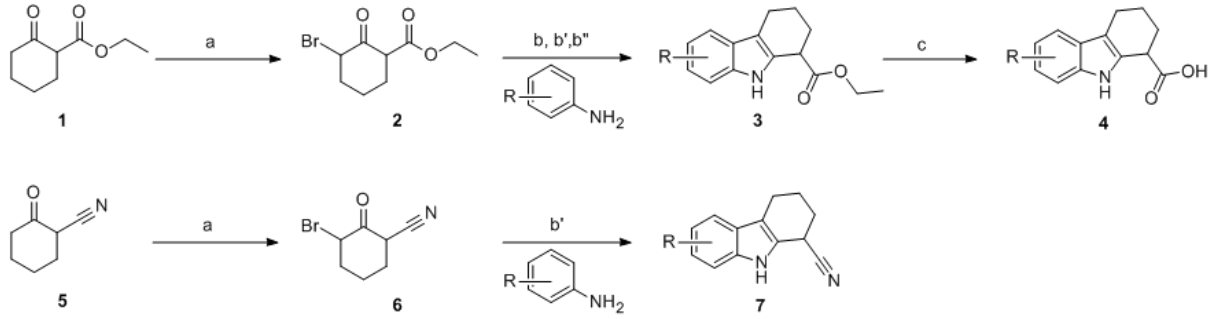
4.1.1 Introduction

Substituted tetrahydrocarbazoles were synthesized following a method known as the Bischler Indole (BI) synthesis, based on the reaction of readily available ethyl 3-bromo-2-oxocyclohexanecarboxylate **2** with variously substituted anilines (Scheme 4.1, b). Basic hydrolysis of the so formed esters **3a-j** gave the corresponding carboxylic acids **4a-j** (Scheme 4.1, c).

The first description of the cyclization of *ortho*-unsubstituted anilines and α-halogenated arylketone by Möhlau and Bischler dates back to 1881 and 1892 respectively.[32] In a patent by Ott in 1923, the use of α-halocyclohexanones was mentioned to yield tetrahydrocarbazoles in a two-step synthesis *via* isolation of the intermediate arylaminocyclohexanone.[28, 32] Despite the availability and stability of the reagents and the lack of need for excess reagents (such as metalylic catalysts), the development of the BI synthesis has been slowed down by the relative low yields and the unpredictable regiochemistry.[32, 33]

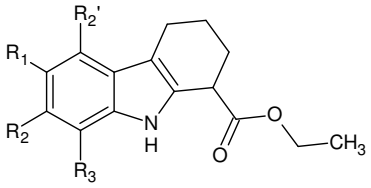
Different BI synthetic protocols were compared to define the influence of thermal heating (in the presence or absence of solvent) against dielectric heating, along with the influence of different solvents (xylene, $EtO(CH_2)_2OH$) and addition of catalytic agents (e.g. N,N-dimethylaniline).

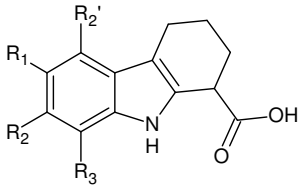
Scheme 4.1[a]

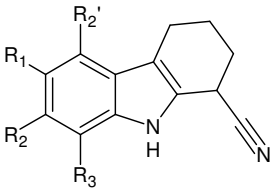


[a]Reagents and conditions: (a) Br_2, Et_2O ; (b) BI method A: 150°C 3h; (b') BI method B: 150°C 15 min 150W/$EtO(CH_2)_2OH$; (b") BI method C: 150°C 3h reflux; (c) LiOH, H_2O/MeOH/THF

Figure 4.1: Proposed compound library based upon the original lead series







3a: R_1= Cl	;R_2=R_2'=R_3=H	**4a:** R_1= Cl	;R_2=R_2'=R_3=H	**7a:** R_1= Cl	;R_2=R_2'=R_3=H
3b: R_1= Br	;R_2=R_2'=R_3=H	**4b:** R_1= Br	;R_2=R_2'=R_3=H	**7b:** R_1= Br	;R_2=R_2'=R_3=H
3c: R_1= F	;R_2=R_2'=R_3=H	**4c:** R_1= F	;R_2=R_2'=R_3=H	**7c:** R_1= F	;R_2=R_2'=R_3=H
3d: R_1= Me	;R_2=R_2'=R_3=H	**4d:** R_1= Me	;R_2=R_2'=R_3=H	**7d:** R_1= Me	;R_2=R_2'=R_3=H
3e: R_1= MeO	;R_2=R_2'=R_3=H	**4e:** R_1= MeO	;R_2=R_2'=R_3=H	**7e:** R_1= MeO	;R_2=R_2'=R_3=H
3f: R_2= Cl	;R_1=R_2'=R_3=H	**4f:** R_2= Cl	;R_1=R_2'=R_3=H	**7f:** R_2= Cl	;R_1=R_2'=R_3=H
3g: R_2'= Cl	;R_1=R_2=R_3=H	**4g:** R_2'= Cl	;R_1=R_2=R_3=H	**7g:** R_2'= Cl	;R_1=R_2=R_3=H
3h: R_2= MeO	;R_1=R_2'=R_3=H	**4h:** R_2= MeO	;R_1=R_2'=R_3=H	**7h:** R_2= MeO	;R_1=R_2'=R_3=H
3i: R_2'= MeO	;R_1=R_2=R_3=H	**4i:** R_2'= MeO	;R_1=R_2=R_3=H	**7i:** R_2'= MeO	;R_1=R_2=R_3=H
3j: R_3= Cl	;R_1=R_2=R_2'=H	**4j:** R_3= Cl	;R_1=R_2=R_2'=H	**7j:** R_3= Cl	;R_1=R_2=R_2'=H

4.1.2 Results

Synthesis of tetrahydrocarbazole esters and acids

The γ-bromination of ethyl 2-oxocyclohexanecarboxylate **1** was carried out according to a procedure described in the literature.[23] The reaction was carried out several times in different quantities, scaling up progressively from 1 to 10 g of starting material **1**. The use of technical diethylether (Et_2O) instead of dry Et_2O failed to gave **2**. The use of larger amounts of bromine led to a dibrominated compound as analyzed by mass spectrometry, although no NMR experiments were performed to determine the site of the addition of the second bromine atom. Purification of **2** by column chromatography has been found to be necessary in order to ease the following purification of the tetrahydrocarbazole esters **3**, formed by BI synthesis.

Various substituted tetrahydrocarbazole esters **3** (Figure 6.1, 6.2) were obtained through BI synthesis according to three different methods. Method A, consisted in performing a neat reaction[23], heating the suitable substituted aniline together with **2** in the absence of solvent. The reaction was clearly influenced by the substitution pattern of the aromatic ring, as shown in Table 4.1. Electron-withdrawing substituents afforded lower amounts of the desired product than electron-donating groups. TLC analysis of crude reaction mixtures pointed out that neat reactions performed with *p*-halogen substituted anilines gave rise to several side-reactions in contrast to reactions carried out with a *p*-methyl or *p*-methoxy aniline.

The first attempts using *p*-fluoroaniline, *p*-toluidine and *p*-anisidine resulted in the isolation of a side product which was hypothesized to be an regio-isomer (substituted 2,3,4,9-tetrahydro-1H-carbazole-4-carboxylate esters) in 1-4% yield, as shown by ^{1}H NMR (Figure 6.3) and mass spectroscopy (Figure 4.2) [32, 33] Extensive 2D NMR experiments should be able to give more evidence towards confirming this finding.

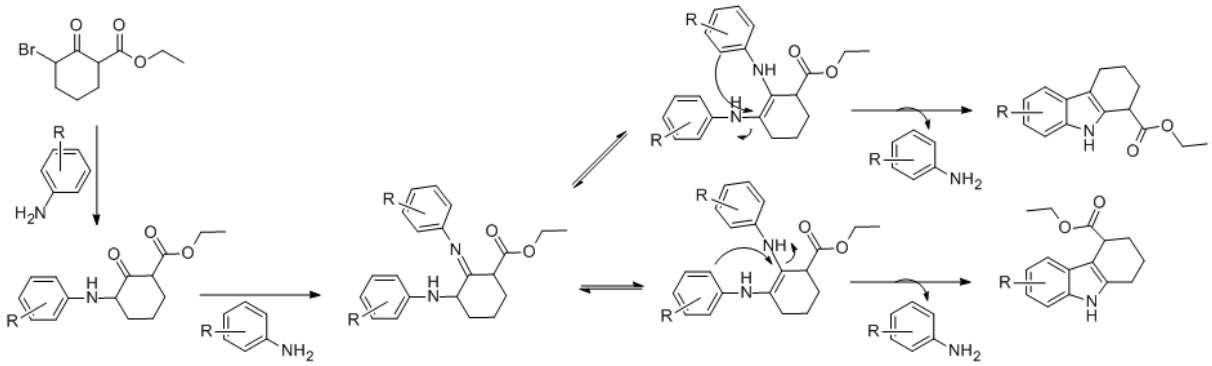


Figure 4.2: Plausible reaction mechanisms in the BI synthesis of tetrahydrocarbazoles *adapted from ref. [32].* Also other mechanisms are cited in the literature[33] in which the reaction does not take place through an imine intermediate. Instead, the second equivalent of aniline acts as a catalytic base.

Method B utilized microwave irradiation.[33] No reaction was observed using xylene as solvent for anilines with electron withdrawing as well for those with electron donating substituents, confirming previous published results[32, 34], stating the importance of using solvents with a strong dipole moment such as ethoxyethanol or dimethylformamide, as a energy transfer agent in microwave assisted reactions. The use of ethoxyethanol gave satisfying results; an overall increase in yield was observed compared with the solvent-free method A, especially when using *p*-chloro- and *p*-fluoroaniline. Addition of extra catalytic base (e.g. N,N-dimethylaniline), as suggested in the proposed reaction mechanism[32, 33], gave no significant increase in yield.

Table 4.1: Yields of Bischler Indole synthesis carried out with *p*-anilines
resulting in the tetrahydrocarbazole esters **3a-e**

R=	-Cl	-Br	-F	-Me	-MeO
Method A	26.00%	33.00%	7.81%	78.00%	81.02%
Method B	71.70%	17.46%	78.60%	91.40%	87.90%

Method C as adapted from a procedure already described[33, 35] using an overnight reflux gave similar results in compared to the solvent-free (and less time consuming) method A and was therefore not further explored.

With the purpose of broadening our compound library other substitutions patterns were tried, to obtain tetrahydrocarbazole esters **3** with substituents on the 5,7 and 8 positions. The use of *m*-chloroaniline and *m*-anisidine led to mixtures of respectively **3f, 3g** and **3h, 3i**. Several attempts using different solvent systems led to the separation of **3f** and **3g**, but not their methoxy-substituted counterparts (**3h**, **3i)**, by preparative TLC. By comparing the amount of **3a** versus the amount of **3f + 3g** formed in the microwave, we can hypothesize on the importance of the position of substituent on the deactivation of the phenyl ring. But the differences where too small (71.7% vs 67.21%) and the experiments were not repeated in such extent to draw any conclusions on this matter. By repeating this condensation with method A, confirmed the previous conclusion namely the benefit of microwave irradiation in the BI synthesis. The use of *o*-chloroaniline did not lead to the formation of the desired product, nor any isomers as analyzed by MS. Several attempts using 2,4-disubstituted (Br, Me) anilines also did not yield the desired product while using our current developed methods.

Hydrolysis of the esters **3** according to a method described in the literature [36], gave satisfactory results by TLC monitoring, but after quenching the reaction and evaporation of the solvent, a mixture of several compounds was obtained, from whom the desired carboxylic acid was not detected by MS. The use of milder conditions to quench the reaction gave the corresponding carboxylic acids **4** after purification (Figure 6.4).

Synthesis of tetrahydrocarbazole carbonitrilles

After obtaining the previously mentioned tetrahydrocarbazole derivatives, the developed methods were implemented to expand our library with the analogous carbonitrilles **7a-j**. Our focus was limited to the compounds that were not commercially obtained and previously tested.

The same method of γ-bromination[23] was used, using ethyl 2-oxocyclohexanecarbonitrille **5**, to obtain ethyl 3-bromo-2-oxocyclohexanecarbonitrille **6**. The first noticeable difference was the formation of a white solid during the reaction. The previously developed work-up required dissolving the deposit in order to extract and purify the compound. Different solvents where tried for the purification of the compound by column chromatography, but showed to be ineffective. A white solid was found crystallizing from the transparent oil after keeping it overnight in a refrigerator. After optimizing the crystallisation process and washing the crystals, the white solid was found to be the desired starting material for the BI indole synthesis of the tetrahydrocarbazole nitrille derivatives. Post-hoc literature study confirmed the previous findings in synthesizing compound **6** and proposed the possibility of shortening the purification process by only performing a recrystallisation, although this route was not further explored. [37]

Only method B (microwave) was used to synthesize tetrahydrocarbazole carbonitrilles **7** due to the previous investigation of the BI synthesis. The reactions performed using *p*-fluoro- and *p*-methoxyaniline where not preformed in such extend that a conclusion could be drawn from the small differences in yield. (44.80% vs 33.93% + 4,01%). In the previously mentioned yield comparison the fraction of isomer was included. The formation of substituted 2,3,4,9-tetrahydro-1H-carbazole-4-carboxylate nitriles was only observed using *p*-methoxyaniline. As with the tetrahydrocarbazole esters **3** the BI synthesis yielded no

results using disubstituded and *o*-substituted anilines. Also the use of *m*-substituted aniline gave mixtures of **7 f+g** and **7 h+i** of which the methoxy substituted were not separable using various methods of column chromatography.

4.2 Inhibitory activity on ThyX

The inhibition of ThyX-catalyzed dTMP-synthesis was measured in a standard radioactive assay using purified ThyX-protein and [5-^{3}H]-dUMP as a radiolabeled substrate.[20, 38] The amount of tritiated water released during the reaction was measured by liquid scintillation counting. A positive and negative inhibitory control was carried out, respectively 5-F-dUMP and DMSO, and the amount of inhibition of synthesized compounds was expressed as a percentage relative to these controls.

All isolated isomers of the desired tetrahydrocarbazoles showed no activity towards inhibiting FDTS. (data not shown). Although not definitive, it gives some evidence towards excluding further investigation of other substitution patterns on the C-ring (Figure 2.1).

Table 4.2: Inhibition of ThyX by the respective *p*-substituted tetrahydrocarbazole (50µM)

		R_1=					
		-NO_2	-Cl	-Br	-F	-Me	-MeO
R_2=	COOEt	-	52%[a]	26.4%[b]	NA[b,c]	NA[b,c]	NA[b,c]
	COOH	-	49%[a]	72%[a]	30.9%[b]	67%[a]	14.4%[b]
	CN	15%[a]	59%[a]	65%[a]	28.6%[b]	49%[a]	15.6%[b]

[a]Results from previous in house screenings, [b]Results from the synthesized compounds
[c]NA: No inhibitory activity shown. All values are means of three independent experiments.

The results shown in Table 4.2 and Table 4.3 confirm the interest in cyano derivatives. Earlier results on commercially available compounds already pointed this out along with considering the most useful pharmacological properties. Esters gave little to none inhibition and carboxylic acids gave better results, but were less consistent than the cyano derivatives. Inhibitory activity is decreased in strong electronically deactivated systems, in other words tetrahydrocarbazoles containing a nitrogroup or fluorine substituent show less activity than those containing other halogens. Also the methoxy substituted tetrahydrocarbazoles showed lower levels of activity towards inhibiting FDTS, indicating that a strongly activated aromatic system also isn't favorable for optimal interaction with the currently still unkown binding site.

By looking at Table 4.3 no conclusions can be drawn on the most favorable position of the substituent on the aromatic part of the target lead. Although the fact that this conclusion can only be drawn for the cyano derivatives, could lead to some interesting ideas on how the binding site can be visualized. Both 5-Cl and 7-Cl derivatives gave similar results, maybe indicating the presence of multiple interaction possibilities in the region of the binding site accommodating the aromatic part of the tetrahydrocarbazole. 6-Cl showed overall the same results no matter what substituent on R_2, this could point towards a middle ground in which every type of R_2 substituent is easily accommodated while the other 2 isomers can only dock as or even because of the cyano substituent.

Table 4.3: Inhibition of ThyX by the respective chlorotetrahydrocarbazole (50μM)

		R=		
		5-Cl	6-Cl	7-Cl
R_2=	COOEt	16.4%[b]	52%[a]	9.3%[b]
	COOH	19%[b]	49%[a]	25.9%[b]
	CN	49.7%[b]	59%[a]	54.5%[b]

[a]Results from previous in house screenings, [b]Results from the synthesized compounds
All values are means of three independent experiments.

5 CONCLUSION

A series of tetrahydrocarbazoles was found to show modest inhibitory activity towards Flavine-Dependent Thymidylate Synthase, a potential new target in the search for new anti-TB chemotherapeutics to oppose the recent worldwide resumption of TB. To broaden our knowledge on this recently discovered target, the goal of this project is to expand the current lead hoping to gain structural information on the binding site located on FDTS. Substituted tetrahydrocarbazoles were synthesized following a method known as the Bischler Indole synthesis. Despite the commercial availability of starting materials and simple reaction procedures, the wide application of the BI synthesis has been hampered by poor yield and variable regiochemistry. But the recent trend towards green chemistry has fueled the search for undemanding procedures (e.g. diminishing the use of solvents, metallic catalysts).[34]

Different BI synthesic procedures were compared leading to several conclusions. The reaction is influenced by the substitution pattern on the aromatic ring, with electron-donating groups leading to higher yields than electron-withdrawing groups. The use of microwave irradiation led to higher yields, an effect that significantly improved reactions carried out with electronically deactivated anilines. The use of ethyl 3-bromo-2-oxocyclohexanecarboxylate as α-haloketone led to the formation of only small amounts of isomers indiscriminately which substituted aniline was used in contrast to earlier findings exploring the regioselectivity of the BI synthesis using α-halogenated arylketones.[33]

Besides various substitution patterns on the aromatic ring of tetrahydrocarbazoles ethyl esters, also the activity of their corresponding carboxylic acid and nitrile derivatives are of interest. The desired ester derivatives and their isomers (as well as nitrile derivative isomers) did not show any significant activity when tested as inhibitors of FDTS. Carboxylic acids showed some improvement, but the best results where found among the nitrile derivatives. Although strongly deactivating as well as strongly activating substituents showed remarkable decrease in activity towards inhibiting the target enzyme. Other positions of substituents didn't gave any definitive answers towards excluding possibilities on further developing the discovered lead series, but can lead to ideas towards visualizing the binding site e.g. the equal activity of isomers **7f** and **7g** (Table 4.3) could lead towards hypothesizing multiple interaction possibilities in the binding site.

Further research is needed to examine the specific nature of this application of the BI synthesis, expand the current developed library and in foremost improve the current core structure in order to increase inhibitory potency towards FDTS by creating additional interaction possibilities between this newly found lead and his promising, selective target.

6 APPENDIX

Figure 6.1: ^{1}H-NMR spectrum of Ethyl 6-Fluoro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylate (3c) A) structure of **3c**; B) ^{1}H-NMR spectrum with the dedication of peaks.

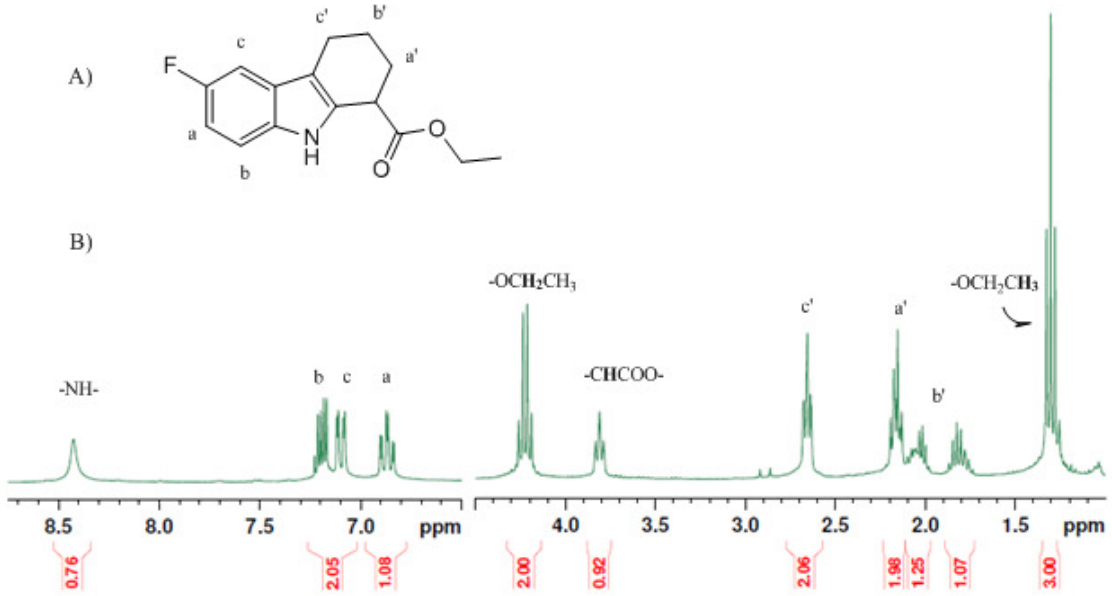


Figure 6.2: ^{1}H-NMR Overlay of compounds 3b, 3d, 3e illustrating the electrostatic effect of the ring substituent on the aromatic protons. Arrows, in their respective colour, indicate the methyl/methoxygroup.

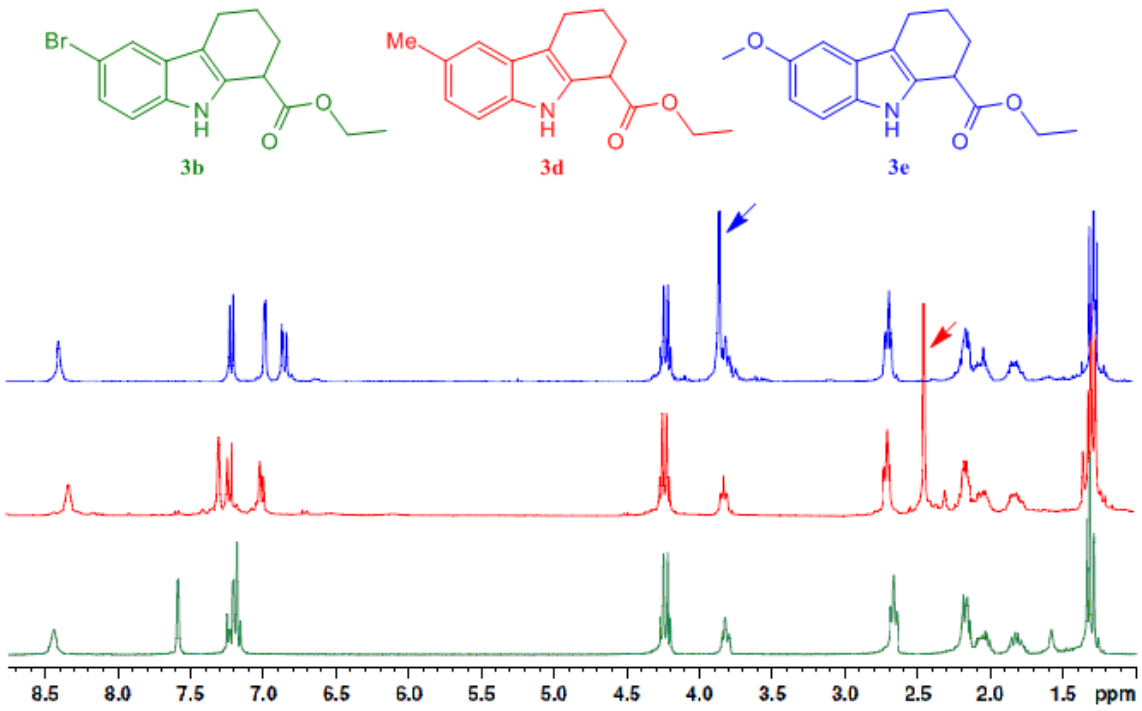


Figure 6.3: structure and ^{1}H-NMR spectrum of Ethyl 6-Methyl-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylate (A) and his suspected 4-carboxylate isomer (B). A small difference in the aliphatic ring signal pattern is noticeable (I). Also in absence of the spatial effects from the carbonyl acting as a hydrogen acceptor, a noticeable decrease in chemical shift of the NH-signal is observed (II).

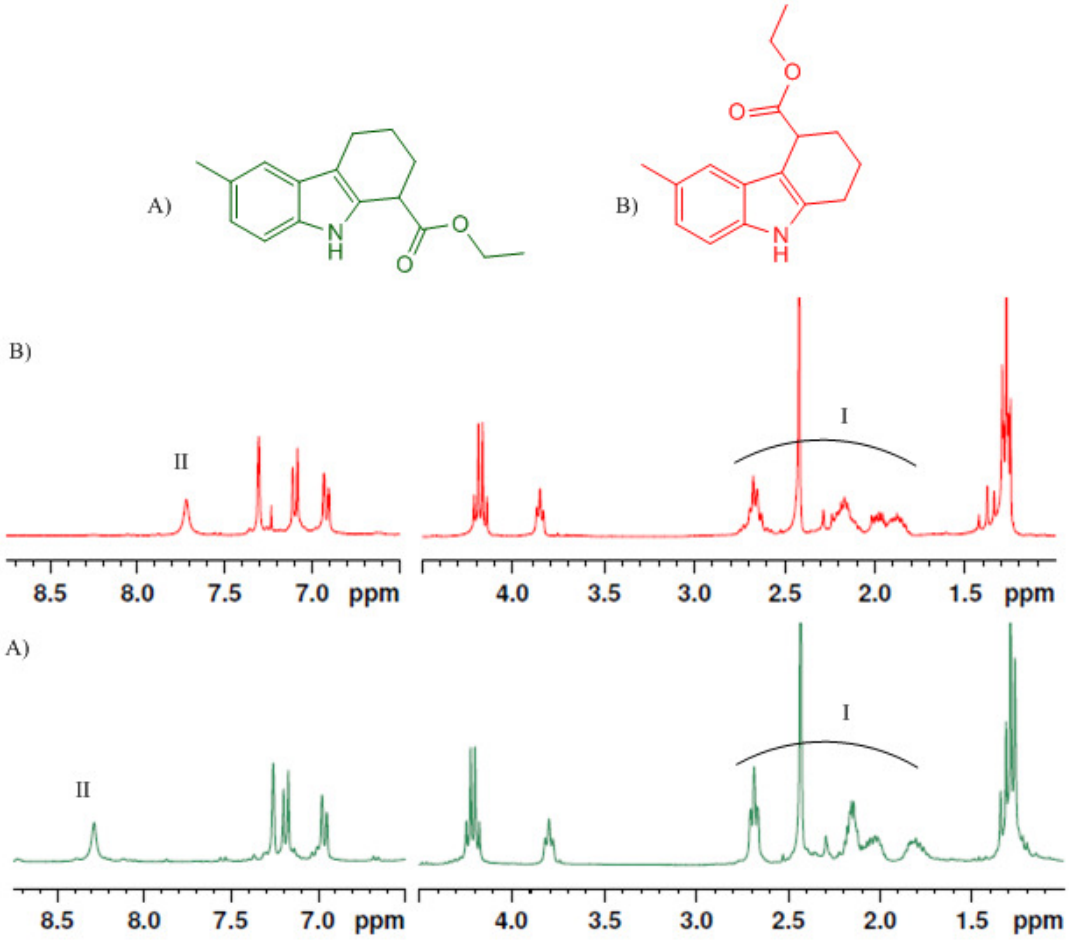


Figure 6.4: ^{1}H-NMR overlay (4.5-1ppm) of compounds 3b and 4b illustrating the hydrolysis reaction. The ethyl esters proton signals are no longer visible in the spectrum of **4b** (I). Also a slight change in the proton signal in α to the carbonyl can be noticed (II).

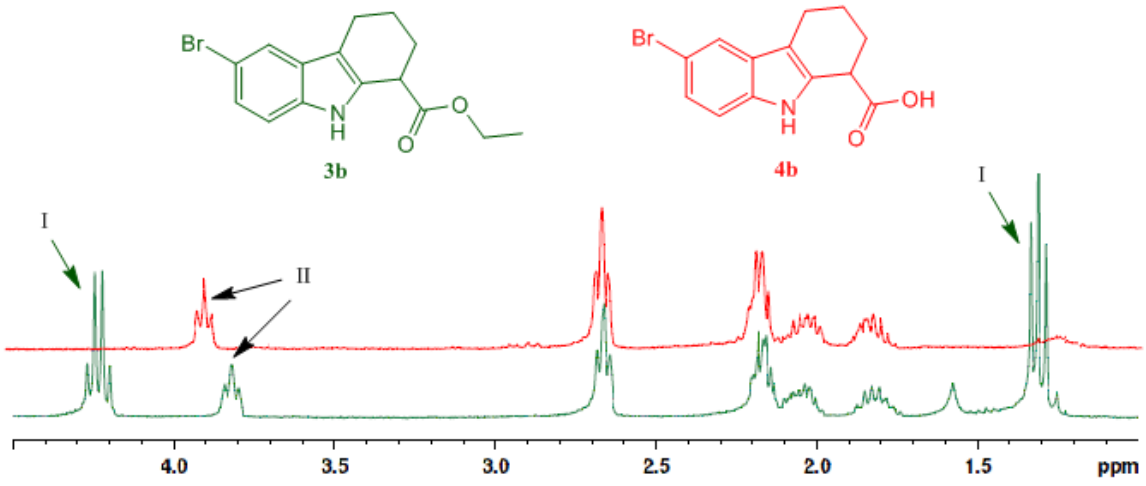


Figure 6.5: Comparison of 13C-NMR of compounds 3c and 7c. This comparison illustrates the difference in effect in chemical shift on the aliphatic region under 30 ppm (C-ring) derived from ester- and nitrile-substituents. Both spectra show the 13C-19F coupling (J_{CF}) in the aromatic region above 100 ppm (see also the NMR data in 3.2). The difference between ester and nitrile signals is also noticeable but more transparent in the following figures (Figure 6.6 and 6.7) including the dedication of all signals.

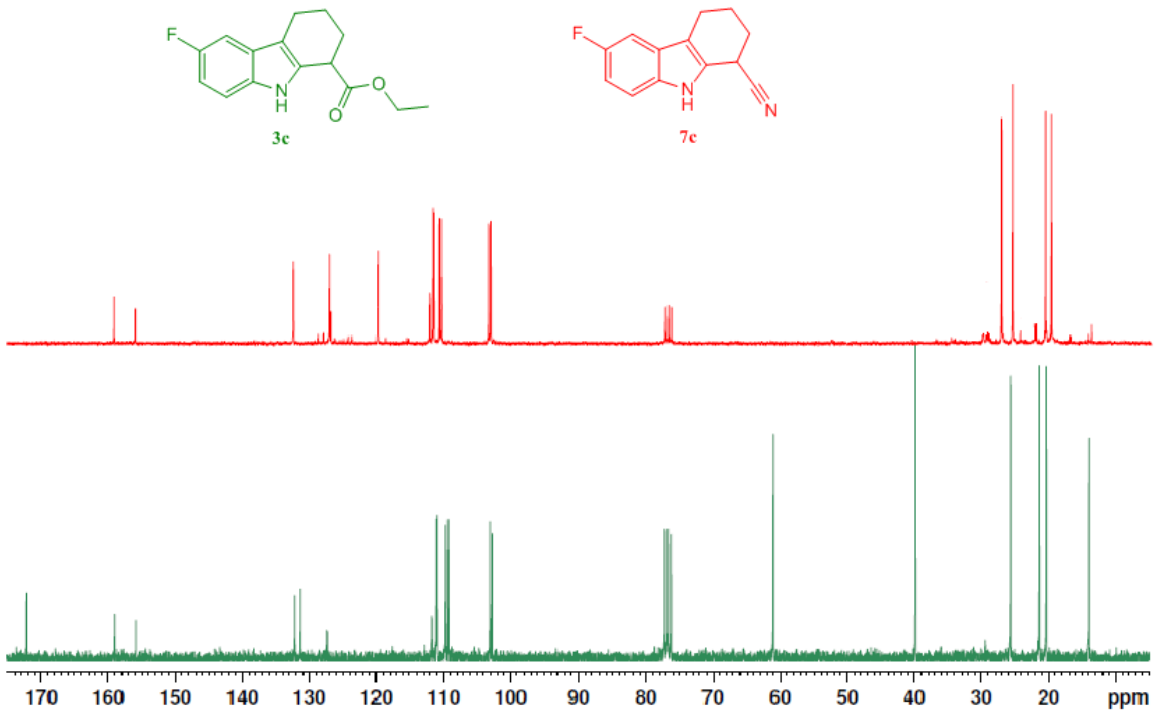


Figure 6.6: ^{13}C-NMR of ethyl 6-fluoro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxylate (3c) together with dedication of peaks and shift signals are shown in Hz to illustrate the J_{CF} written out in section 3.2

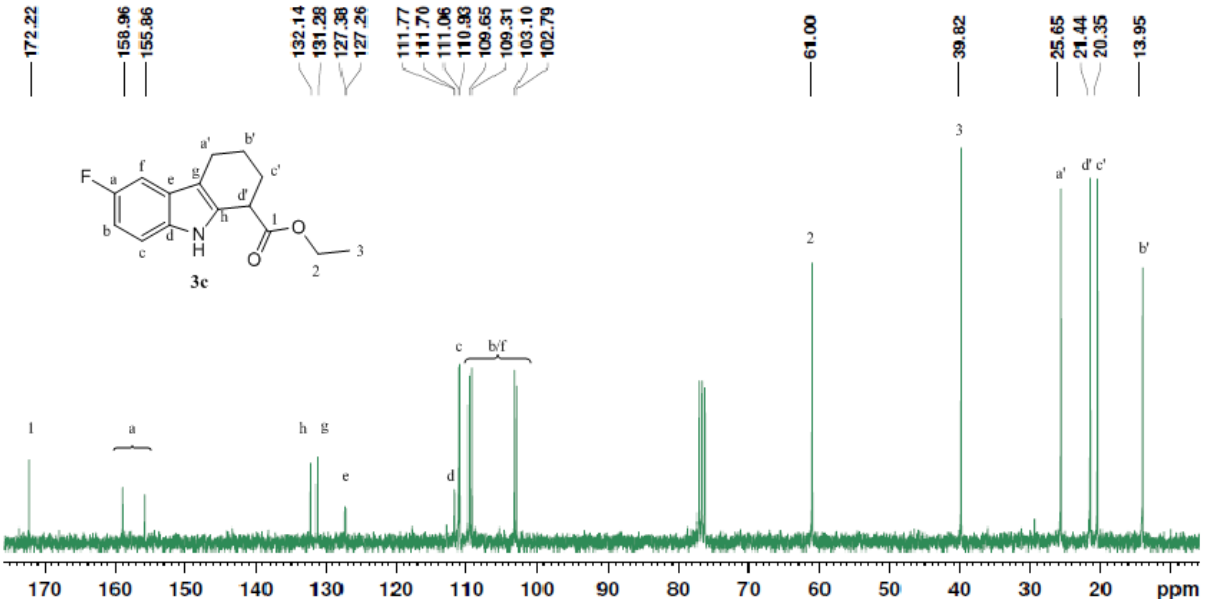
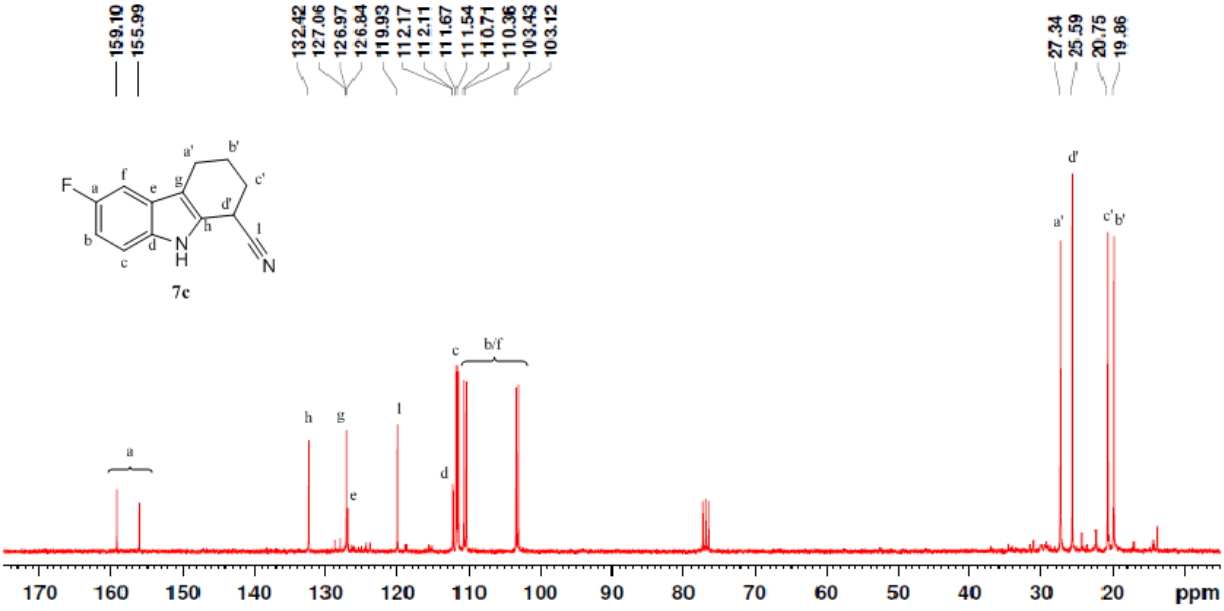


Figure 6.7: ^{13}C-NMR of 6-fluoro-2,3,4,9-tetrahydro-1H-carbazole-1-carbonitrile (7c) together with dedication of peaks and shift signals are shown in Hz to illustrate the J_{CF} written out in section 3.2



7 REFERENCES

1. Koul, A., Arnoult, E., Lounis, N., Guillemont, J. & Andries, K. The challenge of new drug discovery for tuberculosis. Nature 469, 483-490 (2011).
2. Ducati, R. G., Ruffino-Netto, A., Basso, L. A. & Santos, D. S. The resumption of consumption -- a review on tuberculosis. Mem Inst Oswaldo Cruz 101, 697-714 (2006).
3. Dye, C. & Williams, B. G. The population dynamics and control of tuberculosis. Science 328, 856-61.
4. WHO. The Global Plan to Stop TB 2011-2015: Transforming the Fight Towards Elimination of Tuberculosis (World Health Organisation, 2010).
5. Zumla, A. et al. Viewpoint: Scientific dogmas, paradoxes and mysteries of latent Mycobacterium tuberculosis infection. Trop Med Int Health 16, 79-83.
6. Chao, M. C. & Rubin, E. J. Letting sleeping dos lie: does dormancy play a role in tuberculosis? Annu Rev Microbiol 64, 293-311.
7. Cole, S. T. (ed.) Tuberculosis and the tubercle bacillus (ASM press, 2005).
8. Wayne, L. G. & Sohaskey, C. D. Nonreplicating persistence of mycobacterium tuberculosis. Annu Rev Microbiol 55, 139-63 (2001).
9. Handbook of anti-tuberculosis agents. Tuberculosis 88, 85-170 (2008).
10. Beresford, B. & Sadoff, J. C. Update on research and development pipeline: tuberculosis vaccines. Clin Infect Dis 50 Suppl 3, S178-83.
11. Chambers, H. F. et al. Can penicillins and other beta-lactam antibiotics be used to treat tuberculosis? Antimicrob Agents Chemother 39, 2620-4 (1995).
12. Hugonnet, J. E., Tremblay, L. W., Boshoff, H. I., Barry, C. E., 3rd & Blanchard, J. S. Meropenem-clavulanate is effective against extensively drug-resistant Mycobacterium tuberculosis. Science 323, 1215-8 (2009).
13. Payne, D. J., Gwynn, M. N., Holmes, D. J. & Pompliano, D. L. Drugs for bad bugs: confronting the challenges of antibacterial discovery. Nat Rev Drug Discov 6, 29-40 (2007).
14. Murzin, A. G. Biochemistry. DNA building block reinvented. Science 297, 61-2 (2002).
15. Myllykallio, H. et al. An alternative flavin-dependent mechanism for thymidylate synthesis. Science 297, 105-7 (2002).
16. Koehn, E. M. & Kohen, A. Flavin-dependent thymidylate synthase: a novel pathway towards thymine. Arch Biochem Biophys 493, 96-102.
17. Sassetti, C. M. & Rubin, E. J. Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci U S A 100, 12989-94 (2003).
18. Sassetti, C. M., Boyd, D. H. & Rubin, E. J. Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol 48, 77-84 (2003).
19. Koehn, E. M. et al. An unusual mechanism of thymidylate biosynthesis in organisms containing the thyX gene. Nature 458, 919-23 (2009).
20. Hunter, J. H., Gujjar, R., Pang, C. K. & Rathod, P. K. Kinetics and ligand-binding preferences of Mycobacterium tuberculosis thymidylate synthases, ThyA and ThyX. PLoS One 3, e2237 (2008).
21. Wang, Z. et al. Oxidase activity of a flavin-dependent thymidylate synthase. Febs J 276, 2801-10 (2009).
22. Sampathkumar, P., Turley, S., Sibley, C. H. & Hol, W. G. NADP+ expels both the co-factor and a substrate analog from the Mycobacterium tuberculosis ThyX active site: opportunities for anti-bacterial drug design. J Mol Biol 360, 1-6 (2006).
23. Napper, A. D. et al. Discovery of indoles as potent and selective inhibitors of the deacetylase SIRT1. Journal of Medicinal Chemistry 48, 8045-8054 (2005).
24. Mooradian, A., Dupont, P. E., Hlavec, A. G., Aceto, M. D. & Pearl, J. 3-Aminotetrahydrocarbazoles as a new series of central nervous system agents. J Med Chem 20, 487-92 (1977).
25. Chang-Fong, J. et al. 1,2,3,4-tetrahydrocarbazoles as 5-HT6 serotonin receptor ligands. Bioorg Med Chem Lett 14, 1961-4 (2004).
26. Trujillo, J. I. et al. Novel tetrahydro-beta-carboline-1-carboxylic acids as inhibitors of mitogen activated protein kinase-activated protein kinase 2 (MK-2). Bioorg Med Chem Lett 17, 4657-63 (2007).
27. Gribble, G. W. Recent developments in indole ring synthesis-methodology and applications. Journal of the Chemical Society-Perkin Transactions 1, 1045-1075 (2000).
28. Campaigne, E. & Lake, R. D. Synthesis of Tetrahydrocarbazoles and Carbazoles by the Bischler Reaction. Journal of Organic Chemistry 24, 478-487 (1959).
29. Robinson, B. The Fisher Indole Synthesis (Wiley-Interscience, New York, 1982).
30. Liu, H. J. et al. A modified Robinson annulation process to alpha,alpha-disubstituted-beta,gamma-unsaturated cyclohexanone system. Application to the total synthesis of nanaimoal. Tetrahedron 59, 1209-1226 (2003).
31. Sampathkumar, P. et al. Structure of the Mycobacterium tuberculosis flavin dependent thymidylate synthase (MtbThyX) at 2.0A resolution. J Mol Biol 352, 1091-104 (2005).

32. Chen, J. & Hu, Y. Z. Microwave-assisted one-pot synthesis of 1,2,3,4-tetrahydrocarbazoles. Synthetic Communications 36, 1485-1494 (2006).
33. Vara, Y. et al. Regiochemistry of the microwave-assisted reaction between aromatic amines and alpha-bromoketones to yield substituted 1H-indoles. Organic & Biomolecular Chemistry 6, 1763-1772 (2008).
34. Sridharan, V., Perumal, S., Avendano, C. & Menendez, J. C. Microwave-assisted, solvent-free bischler indole synthesis. Synlett, 91-95 (2006).
35. Trujillo, J. I. et al. Novel tetrahydro-beta-carboline-1-carboxylic acids as inhibitors of mitogen activated protein kinase-activated protein kinase 2 (MK-2). Bioorganic & Medicinal Chemistry Letters 17, 4657-4663 (2007).
36. Sanchez-Larios, E., Holmes, J. M., Daschner, C. L. & Gravel, M. NHC-Catalyzed Spiro Bis-Indane Formation via Domino Stetter-Aldol-Michael and Stetter-Aldol-Aldol Reactions. Organic Letters 12, 5772-5775 (2010).
37. Baldur Föhlisch, R. H., Elisabeth Wolf, John J. Stezowski, Emil Eckle. Synthese von 3-Oxo-8-oxabicyclo[3.2.1]oct-6-en-2-carbonitrilen aus γ-Brom-β-oxonitrillen und Furan via [4+3]-cycloaddition von 1-Cyanallylium-2-olaten. Chem. Ber. 115, 355-380 (1982).
38. Lomax, M. I. & Greenberg, G. R. A new assay of thymidylate synthetase activity based on the release of tritium from deoxyuridylate-5-3-H. J Biol Chem 242, 109-13 (1967).

MASTER THESIS PART II:
Research proposal

1 INTRODUCTION

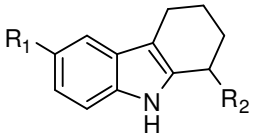
The discovery of a new synthetic pathway towards thymidine, one of the four DNA building blocks, has led to new possibilities in drug design.[1,2] Flavine dependent thymdylate synthase (FDTS), the central enzyme in the newly found pathway, is found in several microorganisms including some human pathogens[3] and was proven to be essential even in those expressing both FDTS and the classical thymidylate synthase (classical TS).[4, 5] Although the mechanism of action of FDTS is rather unclear, kinetic studies[6] and structural analysis led to several hypotheses on how FDTS converts thymine to thymidine.[3,7] The differences, in structure and mechanism of action, between FDTS and classical TS, set the ground for developing new and selective antibiotics especially against *Mycobacterium tuberculosis* (*Mtb*).

Recently, TB has been striking back from our self-induced slumber. The development of new anti-TB antibiotics halted since the mid 1980's because of the establishment of effective therapies. But now mankind made a new problem out of one of his oldest diseases.[8] An inherently already resilient germ became a serious burden for public health due to inappropriate treatment and patient non-compliance causing the rise of multi- and extensively resistant strains (MDR- and XDR-TB), which became epidemic due to other factors such as the HIV/AIDS pandemic, increase of injectable drug users, immigration from high prevalence nations to developed ones. Social inequality is the most important factor leading to malnutrition, lack of access to health care and overall poor living conditions.[8, 9]

The need for new anti-TB chemotherapy has risen to cope with the new challenges *Mtb* opposes to global health. Antimycobacterial treatment as WHO prescribes is a difficult one, combining several drugs and maintaining this treatment during several months despite many side-effects.[8] Although several new therapies against *Mtb* are being tested, most of them are improved antibiotics derived from old drug families.[10] Thus, FDTS seems to be a promising target to treat new resistant strains of TB and to develop a shorter treatment with significantly less side-effects.

Our lab has taken an interest in developing new antibiotics based on the inhibition of FDTS. Several projects are running simultaneously aiming for the further elucidation of the structure of FDTS and its catalytic mechanism while developing compounds with potential antimycobacterial activity. Two routes are explored in developing such inhibitors. The first starts from 5-fluorouridine-5'-monophosphate (5F-UMP), a known inhibitor of both classical TS and FDTS, and strives for the development of an inhibitor docking into the catalytic site. The second route aims to find inhibitors targeting other sites e.g. for cofactors needed for FDTS activity such as CH_2H_4folate, FAD(H_2) and NAD(H).

In house screening of over 1500 commercially available compounds resulted in the discovery of a series of tetrahydrocarbazoles (Figure 1.1), moderately towards inhibiting FDTS. In the first part of this thesis, synthesis of the known scaffold was explored together with its expansion in order to create a library of compounds. These compounds where then tested to gather more structural information on FDTS, whose CH_2H_4folate cofactor binding site is still unkown. Due to molecular resemblance the tetrahydrocarbazoles are believed to bind the previously mentioned location or the already known FAD(H_2) binding site.



R_1 = Br, Cl, Me, NO_2
R_2 = COOEt, COOH, CN

R_2=	R_1= -NO_2	-Cl	-Br	-Me
COOEt	-	52%	-	-
COOH	-	49%	72%	67%
CN	15%	59%	65%	49%

Figure 1.1: Identified tricyclic lead series: 2,3,4,9-Tetrahydro-1H-carbazoles tested by in house screening (results shown in the added table)

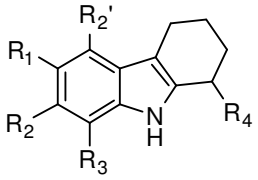
2 AIMS OF THIS STUDY

After preparing various compounds using the Bischler Indole (BI) synthesis as described in the first part of this thesis, the scope of this research proposal is to suggest possible methods to improve the newly found tetrahydrocarbazole lead. Increasing inhibitory activity of these compounds could lead to obtaining successful (co-crystallisation) X-ray diffraction experiments or NMR experiments. By filling in the blanks on the structural knowledge of FDTS, molecular modelling could be used to speed up the development of new anti-TB chemotherapy based on the inhibition of FDTS.

The first step in expanding the compound library proposed at the start of this research project (Table 2.1) is to synthesize the compounds which could not be obtained using the BI method. Improving the original procedures while exploring new types of reactions such as the Fischer Indole (FI) synthesis should allow to attain multisubstituted tetrathydrocarbazoles which could confirm the multiple binding site hypothesis proposed in the first part of this thesis.

Due to the lack of significant increase in activity observed in the already synthesized compounds, exploring other more hindered substituents to gain additional interaction possibilities seems to be the next step towards developing the tetrahydrocarbazole core. After obtaining better inhibitors of FDTS and the derived structural information as mentioned earlier on, the variation of these newly introduced functional groups will be implemented to manipulate various important drug properties such as permeability and solubility.

Table 2.1: Proposed compound library based upon the original lead series



		COOEt	COOH	CN
R_2=R_2'=R_3=H				
R_1=	Cl	**3a**	**4a**	**7a**
	Br	**3b**	**4b**	**7b**
	F	**3c**	**4c**	**7c**
	Me	**3d**	**4d**	**7d**
	MeO	**3e**	**4e**	**7e**
R_1=R_2'=R_3=H				
R_2=	Cl	**3f**	**4f**	**7f**
	MeO	**3h**	**4h**	**7h**
R_1=R_2=R_3=H				
R_2'=	Cl	**3g**	**4g**	**7g**
	MeO	**3i**	**4i**	**7i**
R_1=R_2=R_2'=H				
R_3=	Cl	**3j**	**4j**	**7j**

3 PRELIMINARY RESULTS

3.1 Inhibitors targeting the active centre of FDTS

Known inhibitors of FDTS were found to be 5-fluoro- and 5-bromo-uridine-5'-monophosphate (respectively 5F-dUMP and 5Br-dUMP). Co-crystallisation experiments with FAD and 5Br-dUMP[11] revealed critical interactions between the oxygen atoms of the 5'-monophosphate group with amino acid residues of the catalytic site of FDTS. In addition to this interaction, the pyrimidine ring is close enough to the isoalloxazine ring of FAD for π-stacking and hydrogen bonds. (Figure 3.1) The information derived from these X-ray crystallography studies led our lab to conduct a Structure Activity Relationship (SAR) study on position 5 of the natural dUMP substrate.[12]

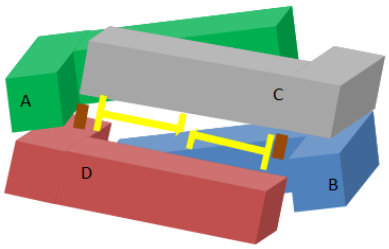


Figure 3.1: Simplified representation of the FDTS structure
The identical subunits A-D are coloured in a different colour, FAD is shown in yellow and the 5Br-dUMP substrate analogue in brown.

The SAR study showed potent activity when small substituents such as fluorine and bromine were introduced at the 5 position, while sterically more demanding groups such as 4-fluorofenyl or a long-chain propargylamide moiety resulted in strong and selective mycobacterial FDTS inhibition (Table 5.1).[12]

The results found in this previous study led to the exploration of a series of 6-aza dUMP analogues due to tolerability of the catalytic site towards structural variation. The 5'-monophosphate moiety was kept for evaluation due to the important interaction with the binding site as previously mentioned. A SAR study was conducted on the same series of dUMP analogues by systematically changing the 5 position, but placing a nitrogen at the 6-position. Unpublished results revealed significantly decreased inhibitory activity towards FDTS with 6-aza dUMP analogues derived from promising compounds of the first SAR study mentioned.[13]

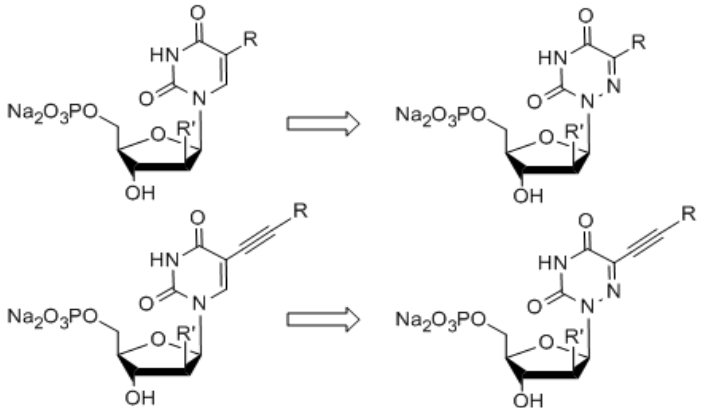


Figure 3.2: Exploring 6-aza-dUMP derivatives based on results earlier published[12] R-groups were systematically changed according to table 5.1

3.2 The new tetrahydrocarbazole lead

3.2.1 Introduction

The scope of the research project preceding this literature study was developing suitable synthethic protocols for synthetizing various tetrahydrocarbazoles according to Table 2.1. These compounds were then tested for their inhibitory potential towards FDTS.

3.2.2 Synthesis according to established BI protocols

Three methods of Bischler Indole synthesis were used, improved and compared. (Scheme 3.1) [14-16] The solvent-free and the reflux protocols gave similar results and therefore the overnight reflux was not explored further in favour of the faster synthesis methods (neat 3h, microwave 15 min). Performing the microwave reaction without solvent had no effect as with the addition of xylene and N,N-dimethylaniline. Careful consideration and literature research led to the high boiling ethoxyethanol as ideal dipolar solvent.[17]

Although both the neat and microwave method (respectively method A and B) were influenced by the electron withdrawing or donating properties of the substituent on the aniline, the microwave method resulted in overall higher yields especially with strong deactivated starting materials such as *p*-fluoroaniline. (Table3.1)

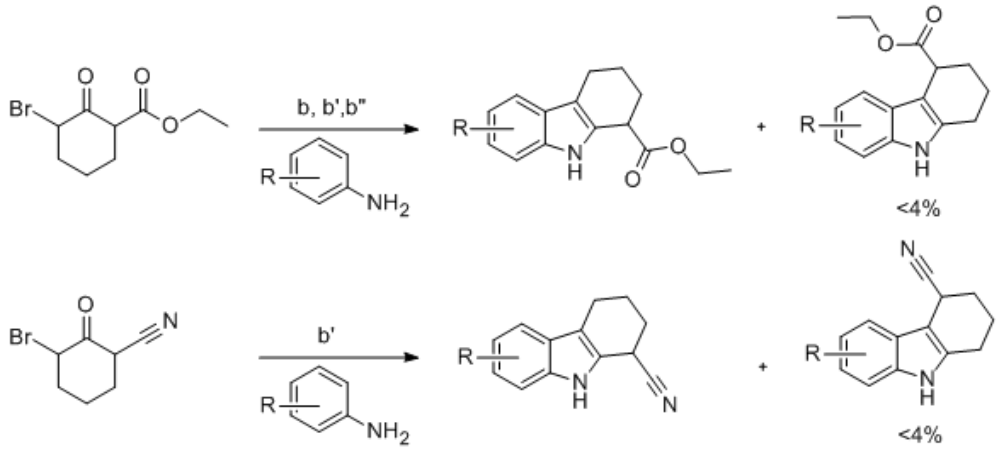
Table 3.1: Yields of Bischler Indole synthesis carried out with *p*-anilines
resulting in the tetrahydrocarbazole esters **3a-e**

R=	-Cl	-Br	-F	-Me	-MeO
Method A	26.00%	33.00%	7.81%	78.00%	81.02%
Method B	71.70%	17.46%	78.60%	91.40%	87.90%

The use of *m*-anilines gave a mixture of two compounds which were only separable by preparative thin layer chromatography (PTLC) when using *m*-chloroaniline, not while using *m*-methoxyaniline. The use of *o*-substituted anilines and multisubstituted anilines did not afford any product.

The developed Bischler Indole synthesis protocols confirmed earlier published results reporting the formation of isomers (Scheme 3.1).[15] Due to the small yields of products (< 4%), the influence of the substituents on the isomeric ratio was not reliably determinable. The possible mechanisms of BI synthesis resulting in different isomers were previously elucidated in the first part of this thesis.

Scheme 3.1[a]



[a]Reagents and conditions: (b) BI method A: 150°C 3h; (b') BI method B: 150°C 15 min 150W/EtO$(CH_2)_2$OH; (b") BI method C: 150°C 3h reflux

3.2.3 Inhibitory activity of the obtained tetrahydrocarbazoles

Inhibition of FDTS was tested according to previously published methods[12], both for tetrahydrocarbazole derivatives and for the dUMP analogues mentioned in section 3.1 and table 5.1. The level of inhibition was determined relatively to 5-F-dUMP as a positive control.

Table 3.1: Inhibition of ThyX by the respective *p*-substituted tetrahydrocarbazole (50µM)

		R_1=					
		-NO_2	-Cl	-Br	-F	-Me	-MeO
R_2=	COOEt	-	52%[a]	26.4%[b]	NA[b,c]	NA[b,c]	NA[b,c]
	COOH	-	49%[a]	72%[a]	30.9%[b]	67%[a]	14.4%[b]
	CN	15%[a]	59%[a]	65%[a]	28.6%[b]	49%[a]	15.6%[b]

[a]Results from previous in house screenings, [b]Results from the synthesized compounds
[c]NA: No inhibitory activity shown. All values are means of three independent experiments

The isolated isomers from Scheme 3.1 did not show any inhibitory activity (data not shown), which could exclude further exploring the substitution pattern on the aliphatic ring especially the 4 position of the tetrahydrocarbazole core. Esters showed overall less activity than carboxylic acids and carbonitriles, the laters showing the best results (Table 3.1 and 3.2) and also having the most promising drug properties in terms of pharmacokinetics.

Table 3.2: Inhibition of ThyX by the respective chlorotetrahydrocarbazole (50µM)

		R=		
		5-Cl	6-Cl	7-Cl
R_2=	COOEt	16.4%[b]	52%[a]	9.3%[b]
	COOH	19%[b]	49%[a]	25.9%[b]
	CN	49.7%[b]	59%[a]	54.5%[b]

[a]Results from previous in house screenings, [b]Results from the synthesized compounds
All values are means of three independent experiments

Table 3.2 shows the inhibitory activity produced by the variation of position of the substituent located on the aromatic ring. The equal activity of compounds **7f** and **7g** could direct towards the presence of multiple interaction sites around the aromatic ring. A possible way to test this hypothesis is to develop a successful method using disubstituted anilines.

4 EXPERIMENTAL APPROACH

4.1 Improving and expanding the obtained tetrahydrocarbazole-core library

4.1.1 General improvements of the current protocols

More information on the isomeric ratio formed during the BI synthesis reaction could be obtained by carefully scaling up the microwave protocol. The microwave used in this research project (CEM Focused Microwave™ Synthesis System, Model Discover) can be outfitted with a container holding reaction vessels up to 100 mL, 10 times the volume used earlier on. In addition, this model can be upgraded with modifications for better temperature and pressure control. The experiments performed in the first part of this thesis used a standard elevation of pressure without any monitoring or feedback mechanism. Increasing the pressure could ease up work-up through the use of lower boiling bipolar solvents.[18]

A patent by Deppe *et al*[19] mentions the synthesis of multi- and *m*-substituted tetrahydrocarbazoles in reactions scaled up to 1g. These reactions were continuously monitored by TLC or HPLC until completion. By applying this concept, degradations or uncompleted reactions resulting in the previously negative results could be avoided. Preparative HPLC was used to separate tetrahydrocarbazoles synthesized from *m*-anilines. The utilised separation protocols went from ordinary reversed-phase chromatography (column: Waters XTerra MS C18, 3.5 µm, 2.1x100mm; mobile phase: acetonitrile/water gradients with formic acid (0.1%)) to chiral separation on carbamate substituted amylase columns (Figure 4.1) (CHIRALPAK AD-H various dimensions; mobile phase: isocratic ethanol/heptane (2:8)). The chiral separation led also to the isolations of enantiomeres differing at the chiral centre located at the C1 position. (Figure 4.2) Testing the tetrahydrocarbazole carbonitriles **7** as separate enantiomers could lead to additional information towards the spatial orientation of the enzyme-substrate interaction involving the aliphatic nitrile-group. In addition, if the spatial orientation around this chiral centre would be proven to be significant, chiral separation would greatly improve the inhibitory activity of these compounds towards FDTS. But as with all chiral drugs, the time and cost of the purification should be compensated by the benefit of obtaining pure enantiomeres such as higher activity and less toxicity.[20]

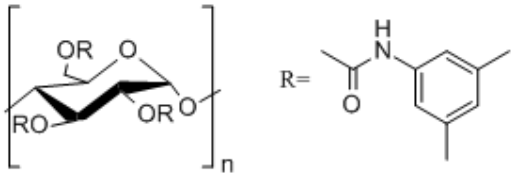


Figure 4.1: Stationary phase of the chiral colums used for separation tetrahydrocarbazoles

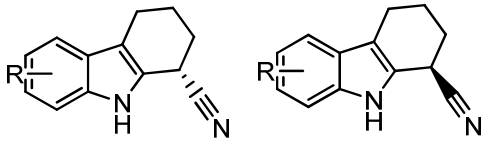


Figure 4.2: Illustrating both enantiomers of the tetrahydrocarbazole carbonitrile lead

4.1.2 Exploring other reaction possibilities: the Fischer indole synthesis

One of the most used methods to synthethize indoles and their derivatives is the Fischler Indole (FI) synthesis.[21] The development of this reaction has benefitted from the enormous amount of biologically active indole alkaloids and derivatives found in nature and now by screening.[22, 23] Based on the N1-C2 and C3-C3a disconnection of the indole ring (as discussed in the first part of this thesis), the wide application of the FI reaction has resulted several different protocols worth exploring.

Discovered in 1883[24], this reaction gives an efficient method for the cyclization of enolizable *N*-arylhydrazones into indoles through sigmatropic rearrangement (Figure 4.3, c), followed by nucleophilic addition to an imine intermediate (Figure 4.3, d) and loss of ammonia (Figure 4.3, e). As proposed in Figure 4.4, the reaction can be carried out by simply heating the appropriate ketone with the arylhydrazine in the presence of the appropriate acid.[22] Various possibilities can be found throughout literature mostly using acetic acid or hydrochloric acid in refluxing ethanol.[25-28] The use of $ZnCl_2$ (10 mol%) and microwave irradiation has been shown to be beneficial in obtaining indole derivatives through means of the FI reaction.[29]

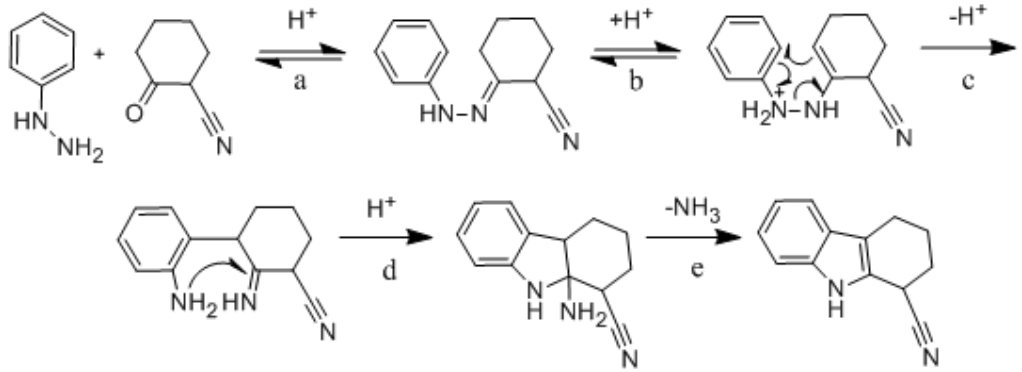


Figure 4.3: FI synthesis reaction mechanism *based on ref [22]* with startingmaterials in order to obtain 2,3,4,9-tetrahydro-1H-carbazole-1-carbonitriles. Due to the unsymmetrical keton, isomers can be formed when the cyclization occurs at the other α-positions in relation to the carbonyl function. Although this is considered to be unlikely, due to the impossibility of continuing the reaction by loss of ammonia in lack of a β-proton relative to the free amine function of the intermediate (isomer reaction pathway not shown)

In this context, a method using iodoanilines has been shown to deliver multisubstituted tetrahydrocarbazoles.[30] Despite the successful adaptation of this method from an earlier published Pd-catalyzed mechanism,[31,32] more research should be done on the importance of the catalyst mentioned (CuI, $CsCO_3$). The reaction depicted in Figure 4.4 can be carried out by 2 procedures only different in the number of steps carried out. The difference lies in whether the aryl hydrazine intermediate is isolated or not. Although no comparison of both procedures was made, several multisubstituted indole derivatives were obtained in yields over 50%.[30] As a last note on this synthetic approach towards multisubstituted tetrahydrocarbazoles, it is worth mentioning that the starting material namely iodoanilines, are commercially available with various substitution patterns and readily prepared.[33, 34]

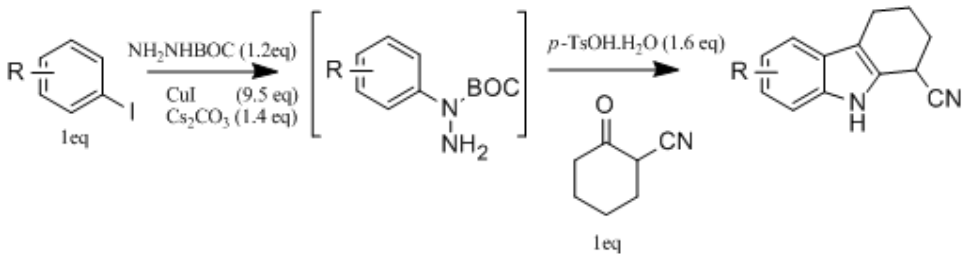


Figure 4.4: Proposed protocol for the synthesis of tetrahydrocarbazoles *via* the FI synthesis reaction *based on ref [30]* the arylhydrazine intermediate can be isolated or not depending on which procedure mentioned in the earlier cited reference. Abbreviations: NH_2NHBOC = *t*-butylcarbazate; *p*-TsOH = *p*-tosylic acid

4.2 Further modifications along the aromatic ring

In order to enhance the inhibitory activity of the already obtained compounds, it would be interesting to explore more hindered substituents than simple halogens or methyl/methoxy-groups. An example of systematic exploration of the addition of more hindered structures can be found in the SAR at the 5-position of dUMP, mentioned earlier on in Chapter 3.1, Appendix 5.1. Introducing such groups not only could give spacial information, but also the addition of new functional groups should lead to find the additional interactions needed to increase inhibitory potency of the tetrahydrocarbazole compounds.

Of the various methods for the modification of indole-like structures, coupling reactions with iodo-indoles seems to be the most versatile and widely used application.[35] By applying the current developed protocols for BI synthesis to obtain iodo-substituted tetrahydrocarbazoles, a valuable intermediate will be generated for coupling new moieties on to the aromatic ring. Combining the different methods depicted in Figure 4.5 in obtaining a library of compounds has one extra advantage: all these methods use a (different) Pd-catalyst and thus the influence of various Pd-complexes can be examined while synthezing these compounds.

According to a method described by Bhanage *et al*[36] it is possible to replace an aromatic iodide moiety by a cyano group without using toxic cyanide sources such as CuCN (Figure 4.5, a), but further modifying this group through means of hydrolysis will prove to be difficult without losing the aliphatic cyano group located at the 1 position of the tetrahydrocarbazole core. This can be circumvented by introducing an amide directly (Figure 4.5, b).[37] The size and number of substituents located on the amine used as starting material in the previously mentioned reaction, can be modified to conduct a SAR. This also applies to the other reactions with unspecified R-groups mentioned in Figure 4.5. Other coupling reactions using iodo substituted indole derivatives are described using arylboronic acids and alkynes, respectively the Suzuki[38] and Sonogashira[39, 40] coupling reaction. Exploring various reactions not only yields slightly different substituents, but it also allows to obtain larger compound libraries based on easily available commercial compounds.

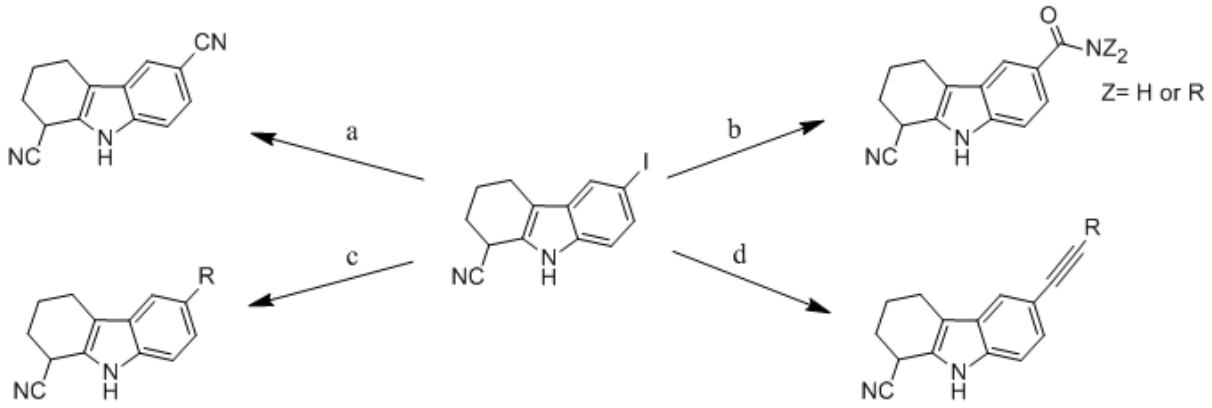


Figure 4.5: Illustrating further modification starting from 6-iodo-2,3,4,9-tetrahydro-1H-carbazole-1-carbonitrille

a) formaldehyde, $Pd(OAc)_2$/Xantphos, $POCl_3$ 140°C,48h; b) CO, NH_2R or NHR'R", $Pd(P^tBu_3)_2$, toluene, 100°C; c) $ArB(OH)_2$, $Pd(PPh_3)_4$, aq. Na_2CO_3, toluene:EtOH (1:1) reflux; d) HC≡CR, $PdCl_2(PPh_3)$/CuI, Et_3N, rt, 8h; Reaction conditions mentioned are of the most interesting procedures derived from various published articles[36-40]

5 CONCLUSION

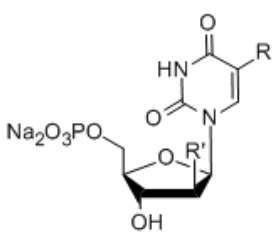
In search for new ways to cope with the newly reemerged (resistant) TB epidemic, our lab has taken an interest in FDTS, a recently discovered enzyme involved in a new pathway for synthezing thymidine. The global approach that targets this enzyme consists of simultaneous development of inhibitors and structure determination by NMR en X-Ray crystallography.

High throughput screening afforded a series of tetrahydrocarbazoles with moderate inhibitory activity towards FDTS. The results of this thesis confirmed earlier findings from in house screening along with the elimination of several possible routes in lead development. Further modifications to introduce new moieties in the lead seems to be necessary to increase the inhibitory potency of these compounds. The combination of the BI synthetic method, developed in the first part of this thesis, with the versatility of Pd-catalyzed chemistry should lead to the discovery of more complex molecules with more possibilities of interaction with the targeted enzyme (Fragment Growing). The tetrahydrocarbazole lead can also be subjected to coupling reactions in order to link it to another moderately active lead to be found by screening (Fragment Linking).[41] Both FG and FL are part of fragment-based drug discovery, a strategy of increasing importance in which smaller molecules are used in screening libraries leaving more room for manipulation before molecular weight becomes to high.[42]

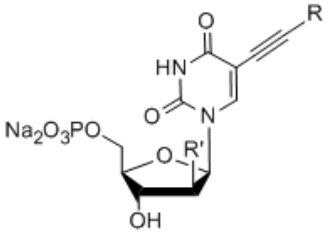
On a final note it is worth mentioning that more potent inhibitors will be subjected to X-Ray co-crystallographic experiments with the target enzyme. This along with the detailed NMR studies will be needed to identify and map the interaction sites of the newly developed inhibitors. After processing this data, further research can be accelerated by molecular modelling experiments. When reached this point, the lead can be reconsidered using scaffold hopping.[43, 44] Identifying key features of the core structure will allow to interchange this tetrahydrocarbazole structure with analogues scaffold such as (iso)quinolines.[45] This will not only yield bigger and more versatile virtual compound libraries, it also allows for further manipulation to improve pharmacokinetic, pharmacodynamic and toxicologic properties in order to reach the ultimate goal of developing effective and safer antimycobacterial drugs.

6 APPENDIX

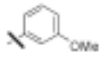
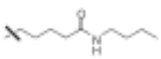
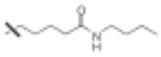
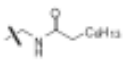
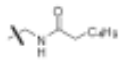
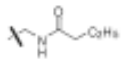
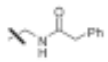
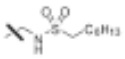
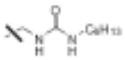
Table 5.1: Inhibition of FDTS and classical TS from *Mtb* *adapted from ref [12]*



1-2
5-F-dUMP



3-6

			FDTS-inhibition[a]		Clasical TS-inhibition[a]	
cmpd	R	R'	% inhibition (50 µM)	IC_{50} (µM)	% inhibition (50 µM)	IC_{50} (µM)
5-F-dUMP[b]	F	H	95.1	0.29	100	0.57
1		H	81.3	7.4	8.8	>50
2a[c]		H	74	10	1.1	>50
2b		H	16	>50	0	>50
3a	$C_{10}H_{21}$	H	82.6	27.8	32.5	>50
3b	$C_{10}H_{21}$	OH	19	>50	26.2	>50
4a		H	74.6	8.03	6.4	>50
4b		OH	21	>50	5.5	>50
5a		H	92.8	0.91	15.6	>50
5b		H	89.9	8.61	14	>50
5c[d]		H	72	26.2	14.8	>50
5d		H	86.9	7.3	32.7	>50
6a		H	84.7	6.93	7.2	>50
6b		H	90.9	4.84	17.9	>50

[a]Values are means of three independent experiments. [b]5-F dUMP used as positive control. [c]93% purity. [d]92%purity

7 REFERENCES

1. Murzin, A. G. Biochemistry. DNA building block reinvented. Science 297, 61-2 (2002).
2. Myllykallio, H. et al. An alternative flavin-dependent mechanism for thymidylate synthesis. Science 297, 105-7 (2002).
3. Koehn, E. M. & Kohen, A. Flavin-dependent thymidylate synthase: a novel pathway towards thymine. Arch Biochem Biophys 493, 96-102.
4. Sassetti, C. M., Boyd, D. H. & Rubin, E. J. Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol 48, 77-84 (2003).
5. Sassetti, C. M. & Rubin, E. J. Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci U S A 100, 12989-94 (2003).
6. Hunter, J. H., Gujjar, R., Pang, C. K. & Rathod, P. K. Kinetics and ligand-binding preferences of Mycobacterium tuberculosis thymidylate synthases, ThyA and ThyX. PLoS One 3, e2237 (2008).
7. Koehn, E. M. et al. An unusual mechanism of thymidylate biosynthesis in organisms containing the thyX gene. Nature 458, 919-23 (2009).
8. Ducati, R. G., Ruffino-Netto, A., Basso, L. A. & Santos, D. S. The resumption of consumption -- a review on tuberculosis. Mem Inst Oswaldo Cruz 101, 697-714 (2006).
9. WHO. The Global Plan to Stop TB 2011-2015: Transforming the Fight Towards Elimination of Tuberculosis (World Health Organisation, 2010).
10. Koul, A., Arnoult, E., Lounis, N., Guillemont, J. & Andries, K. The challenge of new drug discovery for tuberculosis. Nature 469, 483-90 (2011).
11. Sampathkumar, P. et al. Structure of the Mycobacterium tuberculosis flavin dependent thymidylate synthase (MtbThyX) at 2.0A resolution. J Mol Biol 352, 1091-104 (2005).
12. Herdewijn, P. et al. Synthesis and Evaluation of 5-Substituted 2 '-deoxyuridine Monophosphate Analogues As Inhibitors of Flavin-Dependent Thymidylate Synthase in Mycobacterium tuberculosis. Journal of Medicinal Chemistry 54, 4847-4862 (2011).
13. Martin Kögler, R. B., Steven De Jonghe, Jef Rozenski, Kristien Van Belle, Thierry Louat, Piet Herdewijn. Synthesis and evaluation of 6-aza-2'-deoxyuridine monophosphate analogues as inhibitors of thymidylate synthases in *Mycobacterium tuberculosis. currently unpublished*.
14. Napper, A. D. et al. Discovery of indoles as potent and selective inhibitors of the deacetylase SIRT1. Journal of Medicinal Chemistry 48, 8045-8054 (2005).
15. Vara, Y. et al. Regiochemistry of the microwave-assisted reaction between aromatic amines and alpha-bromoketones to yield substituted 1H-indoles. Organic & Biomolecular Chemistry 6, 1763-1772 (2008).
16. Trujillo, J. I. et al. Novel tetrahydro-beta-carboline-1-carboxylic acids as inhibitors of mitogen activated protein kinase-activated protein kinase 2 (MK-2). Bioorg Med Chem Lett 17, 4657-63 (2007).
17. Chen, J. & Hu, Y. Z. Microwave-assisted one-pot synthesis of 1,2,3,4-tetrahydrocarbazoles. Synthetic Communications 36, 1485-1494 (2006).
18. CEM Focused MicrowaveTM Synthesis Sestem, Model Discover. *User Manual*.
19. Deppe H., B. A., Metz G., Erb M. (ed. Organisation, W. I. P.) (Santhera Pharmaceuticals (Schweiz) AG (Hammerstrasse 47, 4410 Liestal, CH), 2008).
20. Caner, H., Groner, E., Levy, L. & Agranat, I. Trends in the development of chiral drugs. Drug Discov Today 9, 105-10 (2004).
21. Robinson, B. The Fisher Indole Synthesis (Wiley-Interscience, New York, 1982).
22. Humphrey, G. R. & Kuethe, J. T. Practical methodologies for the synthesis of indoles. Chemical Reviews 106, 2875-2911 (2006).
23. Gribble, G. W. Recent developments in indole ring synthesis-methodology and applications. Journal of the Chemical Society-Perkin Transactions 1, 1045-1075 (2000).
24. Fischer, E. & Jourdan, F. Ueber die Hydrazine der Brenztraubensaüre. Berichte der deutschen chemischen Gesellschaft 16, 2241–2245 (1883).
25. Stadlbauer, W., Van Dang, H. & Berger, B. S. Synthesis and Reactions of 4-Hydroxy-8,9,10,11-tetrahydropyrido[3,2, 1-jk]carbazol-6-ones. Journal of Heterocyclic Chemistry 47, 807-824 (2010).
26. Li, L. H. et al. Potent and highly selective DP1 antagonists with 2,3,4,9-tetrahydro-1H-carbazole as pharmacophore. Bioorganic & Medicinal Chemistry Letters 20, 7462-7465 (2010).
27. Gu, W. & Wang, S. F. Synthesis and antimicrobial activities of novel 1H-dibenzo[a,c]carbazoles from dehydroabietic acid. European Journal of Medicinal Chemistry 45, 4692-4696 (2010).
28. Gudmundsson, K. S. et al. Substituted tetrahydrocarbazoles with potent activity against human papillomaviruses. Bioorganic & Medicinal Chemistry Letters 19, 3489-3492 (2009).
29. Lipinska, T. M. & Czarnocki, S. J. A new approach to difficult Fischer synthesis: The use of zinc chloride catalyst in triethylene glycol under controlled microwave irradiation. Organic Letters 8, 367-370 (2006).

30. Buchwald, S. L. & Chae, J. Y. Palladium-catalyzed regioselective hydrodebromination of dibromoindoles: Application to the enantioselective synthesis of indolodioxane U86192A. Journal of Organic Chemistry 69, 3336-3339 (2004).
31. Buchwald, S. L., Wagaw, S. & Yang, B. H. A palladium-catalyzed strategy for the preparation of indoles: A novel entry into the Fischer indole synthesis. Journal of the American Chemical Society 120, 6621-6622 (1998).
32. Buchwald, S. L., Wagaw, S. & Yang, B. H. A palladium-catalyzed method for the preparation of indoles via the Fischer indole synthesis. Journal of the American Chemical Society 121, 10251-10263 (1999).
33. Diederich, F. & Lustenberger, P. Towards asymmetric catalysis in the major groove of 1,1 '-binaphthalenes. Helvetica Chimica Acta 83, 2865-2883 (2000).
34. Yagi, S., Kitayama, H. & Takagishi, T. Synthesis of a novel tweezers-type host aiming at chiral discrimination by circular dichroism spectroscopy. Journal of the Chemical Society-Perkin Transactions 1, 925-932 (2000).
35. Cacchi, S. & Fabrizi, G. Synthesis and functionalization of Indoles through palladium-catalyzed reactions. Chemical Reviews 105, 2873-2920 (2005).
36. Bhanage, B. M., Sawant, D. N., Wagh, Y. S., Tambade, P. J. & Bhatte, K. D. Cyanides-Free Cyanation of Aryl Halides using Formamide. Advanced Synthesis & Catalysis 353, 781-787 (2011).
37. Vilar, R. et al. Rapid carbonylative coupling reactions using palladium(I) dimers: applications to (11)CO-radiolabelling for the synthesis of PET tracers. Organic & Biomolecular Chemistry 9, 3499-3503 (2011).
38. Lloyd-Williams, P. et al. Arylboronic acids and arylpinacolboronate esters in Suzuki coupling reactions involving indoles. Partner role swapping and heterocycle protection. Journal of Organic Chemistry 69, 6812-6820 (2004).
39. Sonogashira, K., Tohda, Y. & Hagihara, N. Convenient Synthesis of Acetylenes - Catalytic Substitutions of Acetylenic Hydrogen with Bromoalkenes, Iodoarenes, and Bromopyridines. Tetrahedron Letters, 4467-4470 (1975).
40. Sakamoto, T., Nagano, T., Kondo, Y. & Yamanaka, H. Palladium-Catalyzed Coupling Reaction of 3-Iodoindoles and 3-Iodobenzo[B]Thiophene with Terminal Acetylenes. Chemical & Pharmaceutical Bulletin 36, 2248-2252 (1988).
41. de Kloe, G. E., Bailey, D., Leurs, R. & de Esch, I. J. P. Transforming fragments into candidates: small becomes big in medicinal chemistry. Drug Discovery Today 14, 630-646 (2009).
42. Bembenek, S. D., Tounge, B. A. & Reynolds, C. H. Ligand efficiency and fragment-based drug discovery. Drug Discovery Today 14, 278-283 (2009).
43. Zhao, H. Y. Scaffold selection and scaffold hopping in lead generation: a medicinal chemistry perspective. Drug Discovery Today 12, 149-155 (2007).
44. Böhm, H.-J., Flohr, A. & Stahl, M. Scaffold hopping. Drug Discov Today: Technologies 1, 217-224 (2004).
45. Shishido, Y. et al. Synthesis of benzamide derivatives as TRPV1 antagonists. Bioorganic & Medicinal Chemistry Letters 18, 1072-1078 (2008).