# EXPLORING NATURAL PRODUCTS FOR TREATMENT AND PROPHYLAXIS OF MALARIA


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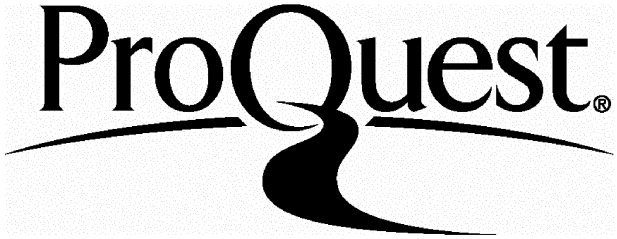
Submitted for the degree of Doctor of Philosophy

The School of Pharmacy

University of London

January 2011



## *DECLARATION*

This thesis describes research conducted in the School of Pharmacy, University of London between May 2007 and May 2010 under the supervision of Dr. Deniz Tasdemir. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature Date 17.01.2011

## *ABSTRACT*

Malaria is the number one parasitic disease worldwide with half of the world's population at risk and nearly one million death annually. Natural products have had an enormous impact in malaria chemotherapy as the majority of current antimalarial agents are natural products or derive from a natural product scaffold isolated from plants traditionally used against malaria. The development of resistance by the deadliest parasite species *Plasmodium falciparum* against many antimalarial agents, including the artemisinin combinations, has become a great concern for global public health. Hence, new antimalarial drugs for chemotherapy and prophylaxis are urgently needed.

Type II fatty acid biosynthesis pathway (FAS-II) has been recently shown to be indispensable for the liver stage parasites. Fatty acid biosynthesis is a crucial pathway for all living organisms as fatty acids are essential for membrane formation and energy production. *Plasmodium* employs type II FAS with fundamental structural and organisational differences versus the type I human FAS. This renders plasmodial FAS-II to be an excellent target for liver stage parasites and causal malaria prophylaxis. Thus, the essential FAS-II enzymes FabI, FabG and FabZ were included in the screening for new malaria prophylactic agents.

One approach in the search for natural antimalarial drugs was the screening of pure natural products. Selected secondary lichen metabolites (evernic acid, vulpic acid, psoromic acid and (+)-usnic acid) were assessed for their potency against *P. falciparum* blood stage parasites and *P. yoelii* liver stage parasites, plasmodial FAS-II enzymes and their cytotoxicity. Evernic acid was identified as first natural product with potential against liver stage parasites ($IC_{50}$ 19.5 μM) and the FAS-II enzyme FabZ as potential target, for which it is a competitive inhibitor.
Another set of natural products tested in this study were 22 selected natural chalcones, a chemical group that acts as precursors for the well-known family of flavonoids. The assessment against *P. falciparum* blood stage parasites identified 2',6'-dihydroy-4,4'-dimethoxydihydrochalcone as chalcone with the best antiplasmodial activity ($IC_{50}$ 3.7 μM), next to the known inhibitor licochalcone A ($IC_{50}$ 1.0 μM). In addition butein, homobutein, eriodictyolchalcone and licochalcone A were identified as promising inhibitors of the FAS-II enzyme FabZ. Structure-activity relationship studies were performed.

Another approach was the screening of selected Turkish plants *Anthemis cretica subsp. anatolica*, *Anthemis pestalozzae* (Asteraceae), *Salvia virgata* (Lamiaceae), *Scrophularia lucida* and

*Scrophularia pinardii* (Scrophulariaceae). Previous studies have shown that members of the genera *Anthemis*, *Salvia* and *Scrophularia* displayed significant antiplasmodial potential. Hence aerial parts and roots were extracted separately with methanol (crude extracts), which was followed by liquid-liquid partitioning and yielded the hexane, chloroform and aqueous methanol subextracts. The crude extracts and subextracts were screened for their potential against *P. falciparum* blood stage parasites, FAS-II enzymes and for cytotoxicity. All species showed good to moderate antiplasmodial activity and inhibition against at least one FAS-II enzyme. The aerial parts of the completely unstudied endemic Turkish plant *Anthemis pestalozzae* (Asteraceae) showed the most interesting profile and were selected for bioactivity-guided fractionation using a variety of chromatographic methods. Four compounds could be isolated and were identified as the cyanogenic glycoside lucumin, the benzoic acid derivative 2,6-dihydroxybenzoic acid, the indole glycoside 3-carboxymethyl-indole-1-N-β-D-glucopyranoside and a sesquiterpene lactone. The first three compounds were identified for the first time in the genus *Anthemis*. The sesquiterpene lactone was identified to possess the structure which was postulated for sivasinolide, however comparison of NMR data revealed significant differences. Our data suggest that the compound originally declared to be sivasinolide is very likely a different compound. In addition, the presence of the flavonoid rutin in the aqueous methanol subextract could be shown by HPLC analysis.

An extensive analysis of the fatty acid composition of several fractions from the aerial parts of *Anthemis pestalozzae* showed the presence of 37 different fatty acids.

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# *LIST OF ABBREVIATIONS*

| | |
|---|---|
| **#** | |
| **δ** | Chemical shift |
| **7-AAD** | 7-aminoactinomycin D |
| **A** | |
| **A** | Absorption |
| **ACP** | Acyl carrier protein |
| **APS** | Ammonium persulphate |
| **C** | |
| **CoA** | Coenzyme A |
| **COSY** | Proton correlation spectroscopy |
| **cpm** | Counts per minute |
| **D** | |
| **Da** | Dalton |
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **DEPT** | Distortionless enhancement by polarisation transfer |
| **DHA** | Dihydroartemisinin |
| **DMSO** | Dimethyl sulfoxide |
| **dw** | dry weight |
| **E** | |
| **EDTA** | Ethylenediaminetetraacetic acid |
| ***e.g.*** | lat. *exempli gratia* (for example) |
| **EGCG** | (-)-Epigallocatechin gallate |
| **ESI** | Electrospray ionisation |
| ***et al.*** | lat. *et aliter* (and others) |
| **EtOAc** | Ethylacetate |
| **EtOH** | Ethanol |
| **F** | |
| **FA** | Formic acid |
| **FAME** | Fatty acid methyl esters |
| **FAS-I** | Type I fatty acid biosynthesis |
| **FAS-II** | Type II fatty acid biosynthesis |
| **FC** | Flow cytometry |
| **FCC** | Flash column chromatography |
| **Fig.** | Figure |
| **G** | |
| **g** | 1. Gram; 2. Gravitational acceleration |
| **GC-MS** | Gas chromatography coupled with mass spectrometry |
| **GFP** | Green fluorescent protein |
| **H** | |
| **HEPES** | 4-(2Hydroxyethyl)piperazine-1-ethanesulfonic acid |
| **His** | Histidine |
| **HMBC** | Heteronuclear multiple bond coherence |
| **HMQC** | Heteronuclear multiple quantum coherence |
| **I** | |
| **$IC_{50}$** | 50% inhibitory concentration |
| ***i.e.*** | lat. *id est* (that is) |
| **IFA** | Immunofluorescence analysis |
| **IPTG** | Isopropyl-β-D-thiogalactopyranoside |
| **K** | |
| **kb** | Kilobase |
| **kDa** | Kilodalton |
| **L** | |
| **LC-MS** | Liquid chromatography coupled with mass spectrometry |
| **M** | |
| **$M_r$** | Molecular weight |
| ***m/z*** | Mass-to-charge ratio |
| **MeOH** | Methanol |
| **mmu** | Milli-mass units |
| **MS** | Mass spectrometry |
| **N** | |
| **NADH** | Nicotinamide adenine dinucleotide, reduced form |
| **NADPH** | Nicotinamide adenine dinucleotide phosphate, reduced form |
| **Ni-NTA** | Nickel-Nitrilotriacetic acid |
| **nm** | Nanometre |
| **NMR** | Nuclear magnetic resonance |
| **NOE** | Nuclear Overhauser effect |
| **NOESY** | Nuclear Overhauser enhancement spectroscopy |
| **O** | |
| **OCC** | Open column chromatography |
| **P** | |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PBS** | Phosphate buffered saline |
| **pH** | lat. *potentia hydrogenii* |
| **pmol** | Picomole |
| **PMSF** | Phenylmethanesulfonylfluoride |
| **ppm** | Parts per million |
| **pTLC** | preparative thin layer chromatography |
| **R** | |
| **rpm** | Revolutions per minute |
| **S** | |
| **SDS** | Sodium dodecyl sulphate |
| **SI** | Selectivity index |
| **SPE** | Solid phase extraction |
| **SQL** | Sesquiterpene lactone |
| **T** | |
| **Tab.** | Table |
| **TEMED** | Tetramethylethylenediamine |
| **TLC** | Thin layer chromatography |
| **TRIS** | Trishydroxymethylaminomethane |
| **U** | |
| **UV** | Ultraviolet |
| **V** | |
| **VLC** | Vacuum liquid chromatography |
| **W** | |
| **w/v** | Weight/volume |
| **WHO** | World Health Organisation |

## *ACKNOWLEDGMENTS*

I would like to thank all the people who contributed, in whatever manner, to the success of this work.

First, I would like to thank my supervisor Dr. Deniz Tasdemir, for giving me the opportunity to work on this very interesting topic and providing excellent working conditions.

I would like to thank my second supervisor Prof. Michael Heinrich, for his help and support and the possibility to always knock on his door.

My special thanks go to Dr. Remo Perozzo of the University of Geneva, for providing me with glycerol stocks of the FAS-II enzymes and his help in the initial phase of the plasmodial FAS-II system.

I would like to thank Dr. Sergio Wittlin, Dr. Matthias Rottmann and Sonja Keller-Märki at the Swiss Tropical and Public Health Institute for introducing me to antiplasmodial assays.

I would like to thank Prof. Simon Croft and Dr. Livia Vivas from the London School of Hygiene and Tropical Medicine for allowing me to use their facilities for *Plasmodium* blood stage assays and cytotoxicity studies.

I would like to thank Dr. Mark O'Neil-Johnson for performing LC-MS experiments, Prof. Nestor M. Carballeira and Dr. Elsie A. Orellano for the fatty acid methyl ester analysis and Dr. Alice Tarun and Assoc. Prof. Stefan H. Kappe for *Plasmodium* liver stage assays.

I would like to thank Prof. Peter J. Tonge from the Stony Brooks University for the provision of hFAS baculovirus stock.

I would like to thank all my friends and colleagues from the School of Pharmacy and other institutions for their various kinds of help and support with special thanks to Dr. Abdul Basit, Dr. Sibylle Ermler, Dr. Stephen Hilton, Shereen Nasser, Dr. Gary Parkinson, Sinikka Rahte, Eloise Thompson and Dr. Mire Zloh.

I would like to thank the School of Pharmacy for the PhD studentship.

Last but not least, I would like to thank my parents and my family for their strong support, encouragement and love.

# Part 1

# Introduction

## 1.1 General Introduction

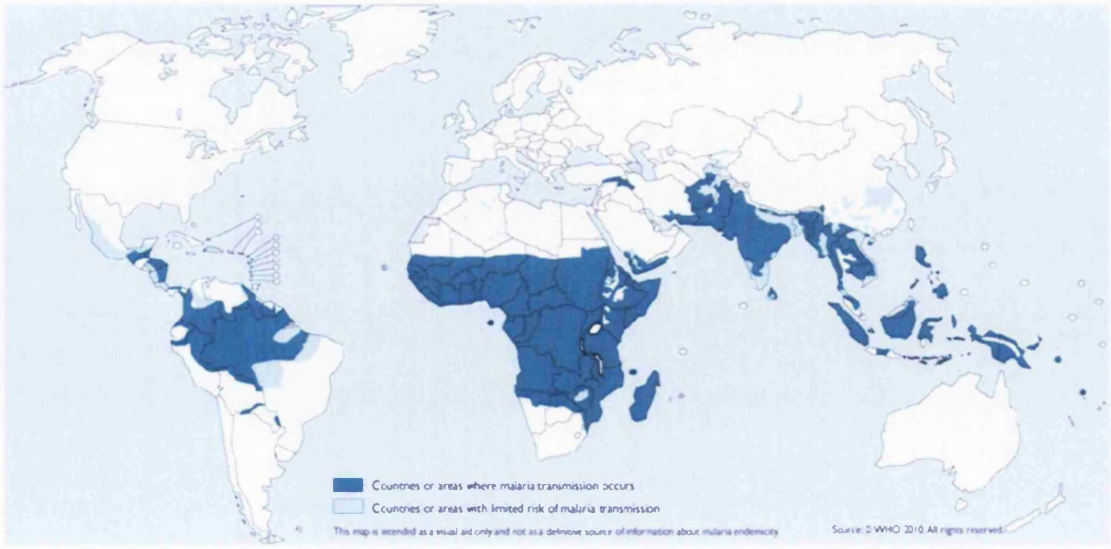
Malaria is the number one parasitic disease worldwide. Natural products have had an enormous impact in malaria chemotherapy. The majority of current antimalarial agents are natural products or derive from a natural product scaffold, which was isolated from plants traditionally used against malaria. The development of resistance against many antimalarial agents has become a great concern for global public health. Hence, new antimalarial drugs for chemotherapy and prophylaxis are urgently needed.

This introduction will give an overview about malaria, the parasite causing the disease (*Plasmodium*), treatment strategies, current antimalarial drugs and developments, and the importance of natural products for the antimalarial drug discovery.

### 1.1.1 Malaria

Malaria is a disease that has been recorded throughout history, and even though more is currently known about the process of infection and the parasites causing the disease than ever before, it remains one of the great health problems, affecting 3 billion people worldwide. In their latest report, the World Health Organisation (WHO) estimated that in 2006 247 million cases of malaria led to nearly 881,000 deaths (WHO, 2008). In 2009, 108 countries were reported to be endemic for malaria with 43 in the African region (Fig. 1.1).

Figure 1.1. Distribution map of malaria in 2009. Dark green: malaria transmission, light green: limited malaria transmission, white: no malaria transmission. (WHO, 2010c)




The infectious disease is caused by pathogenic parasites from the genus *Plasmodium* and is considered to be the most dangerous infectious disease in the world. By far the most deadly species is *Plasmodium falciparum*, which in tropical and subtropical regions remains a serious

problem. 81 of the countries affected by malaria, including 42 in the African region, are endemic for this species (WHO, 2009). More than 80% of all malaria cases due to *P. falciparum* are observed in Africa, leading to childhood death in 20% of cases. However the largest population at risk with 687 million people is located in Southeast Asia where an estimated 90-160 million infections occur annually (Narain, 2008).

Despite the high death rate, malaria should not only be considered as a disease with possible fatality. It also has a major impact on the economic growth rate of the affected countries. Poverty caused by malaria and its consequences for the individual life as well as the inability to cure and prevent malaria due to poverty of the country are creating a vicious circle. It has been estimated that malaria has an impact on the global economy with direct losses of more than 12 billion USD annually in African countries (RBM, 2008). Up to 40% of the African health budget is spent on malaria each year (WHO, 2010a) and a malaria-stricken family loses on average a quarter of its income due to loss of earning and the costs for treatment and prevention (WHO, 2007). Developing countries cannot break free by themselves and often depend on industrialised countries, pharmaceutical companies and foundations to help them.

There have been previous attempts to eradicate malaria and improve treatment and prevention, but so far none has achieved the set targets or ambitions. The first eradication program was started in the 1950s with cloroquine and DDT. It was successful in Europe, North America, the Caribbean and parts of Asia and South-Central America (Carter & Mendis, 2002). However, in other parts of the world, especially sub-Sahara Africa, mainly technical challenges led to failure of malaria eradication, and subsequent resistance development of the parasite against chloroquine caused the resurgence of the disease (Sharma, 1996). Since then, the main focus has shifted to control of malaria for almost 55 years. At the end of 2007, the Gates Foundation refocused on the eradication of malaria which was supported by many other organisations (Roberts & Enserink, 2007). The approach is carried out on several fronts by malaria prevention with insecticide-treated bed nets, indoor residual spraying, intermitted preventive treatment during pregnancy, vector control measures and treatment with artemisinin-based combination therapy (ACT) (RBM, 2008).

Funding for malaria has increased significantly from 0.3 billion USD in 2003 to 1.7 billion USD in 2009 (WHO, 2009). Since the eradication agenda was announced, research in the area of developing novel antimalarial drugs has increased dramatically (Enserink, 2010a). Due to resistance development in the parasite an urgent need for drugs with novel

mechanisms of action has arisen. Ideally, such a candidate should, in addition to curing the patient, block the transmission of malaria by targeting gametocytes of the blood stage parasites. Drugs that cause causal prophylaxis and prevent the outbreak of the disease by targeting liver stage parasites are also desperately needed.

Since malaria is predominantly a problem in developing countries, several key issues forgotten by the Western world have to be kept in mind when looking at treatment strategies.

Many people affected by malaria live in remote, rural areas and do not have access to a health centre. Thus, the diagnosis of malaria is often done presumptuously leading to prescription of antimalarial drugs to uninfected patients (Amexo *et al.*, 2004; Whitty *et al.*, 2008; Gosling *et al.*, 2008). The WHO recommends the diagnosis of malaria by microscopy or with rapid diagnostic tests before treatment, so that only patients with malaria receive antimalarial drugs. However, a recent study in 18 high-burden African countries showed that only 22% of reported malaria cases were confirmed by test (WHO, 2009). Even though antimalarial drugs should only theoretically be available with a prescription, in sub-Sahara Africa it is common practice to purchase medication directly over the counter or even from shops, private practitioners or other outlets (Craft, 2008).

Renewed efforts in better training of medical staff is also of importance, as it is still common practice that patients with negative test results receive prescriptions of antimalarial drugs (Reyburn *et al.*, 2004; Kokwaro, 2009). This is especially significant in countries with declining malaria burden since fever, the main symptom of malaria, might be due to other diseases leading to incorrect treatment endangering the patient and increasing the risk of resistance development (Kokwaro, 2009).

The wide availability and low price for antimalarial drugs are also of great importance and might help to fight substandard and counterfeit antimalarial drugs. It is thought that up to 35% of all antimalarial treatments sold in Africa are substandard (Bate *et al.*, 2008). An alarming survey from Cameroon found 12% of sulphadoxine-pyrimethamine, 38% of chloroquine and 74% of quinine drugs had either no active ingredient present or contained insufficient amounts (Basco, 2004). In Southeast Asia the situation is not better, it was reported that between 38% and 52% of artesunate drugs had no active ingredient (Newton *et al.*, 2006). Currently the price for chloroquine is less than 0.20 USD, compared to 5-6 USD for artemisinin monotherapy and 7-8 USD for ACT (Laxminarayan *et al.*, 2010). Subsiding ACT is crucial, however even if ACT are sold at 1 USD, it is still 5-10 times

more expensive than chloroquine, thus too expensive for many communities and governments in poor countries (Arrow *et al.*, 2004).

### 1.1.2 *Plasmodium*

#### 1.1.2.1 General overview

*Plasmodium* is a single-cell eukaryotic parasite, which infects mammals, birds, reptiles and diptera. It belongs to the phylum of Apicomplexa, a large group of intracellular parasites. The malaria parasite was first discovered in 1880 by Laverna (Cox, 2010). Currently over 175 species are known and new species continue to be described. The four most common species infecting humans are *Plasmodium ovale* (discovered 1918 by John Stephens), *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium falciparum* (described and differentiated between 1885-1890 by Golgi) (Cox, 2010). In addition to these four malarial parasites, the primate species *P. knowlesi* has recently been shown to cause malaria in humans (Cox-Singh *et al.*, 2008). However, it is unclear whether human-mosquito-human infections are possible (Wells *et al.*, 2009).

Severe malaria, also known as cerebral malaria or falciparum malaria, is caused by the most dangerous species *P. falciparum* and is responsible for the majority of malaria-associated deaths. It is the only species, infecting humans, with gorilla origin (Liu *et al.*, 2010). All other human *Plasmodium* species descended from monkey parasites (Rich *et al.*, 2009). *Plasmodium vivax* causes 25-40% of the global malaria burden mainly in South and Southeast Asia and Central and South America (Price *et al.*, 2007). Traditionally, it has been labelled benign and was assumed to only rarely lead to death. However, the mortality reports most likely have to be updated since it was shown that children with vivax malaria have similar mortality figures as the ones infected with falciparum (Poespoprodjo *et al.*, 2009).

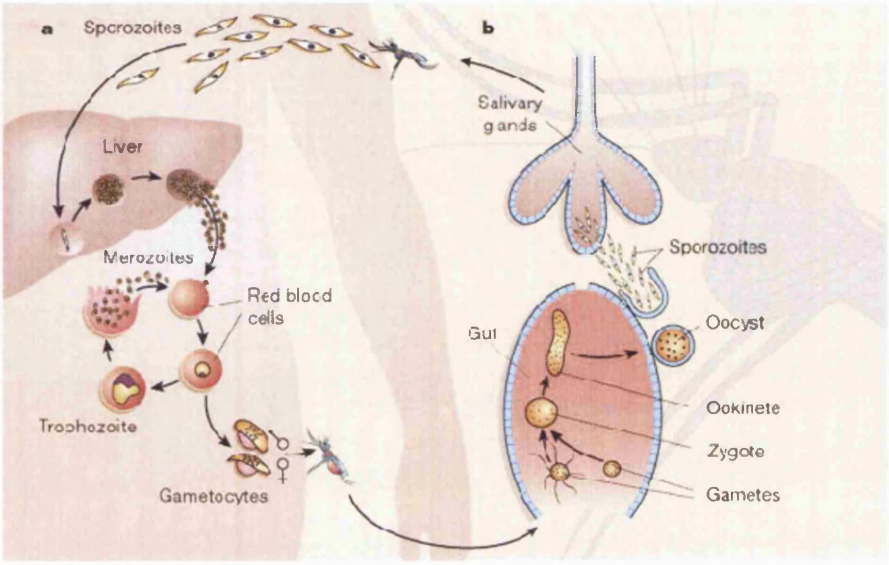
*Plasmodium* parasites are transmitted by female mosquitoes of the genus *Anopheles*. There are over 400 *Anopheles* species worldwide but only about 20 species act as a vector for malaria (WHO, 2010a). *Anopheles gambiae* is one of the best studied species because of its predominant role as a vector for *Plasmodium falciparum*.

#### 1.1.2.2 Life cycle

The life cycle of *Plasmodium* species is complex and includes a host change from human to mosquito (Fig. 1.2). The asexual development of *Plasmodium* occurs in humans. A small numbers of parasites firstly infect the liver and multiply (asymptomatic stage) before infecting red blood cells and starting replication cycles which result in an explosive increase

of parasites within days (symptomatic stage). The sexual stage of the parasite development occurs in the mosquito.

Figure 1.2. Life cycle of *P. falciparum* (Wirth, 2002).




When an infected *Anopheles* mosquito bites, it injects 10-15 sporozoites with its saliva into the avascular skin tissue of the recipient which then invade blood vessels. The sporozoites migrate to the liver by passing through the tissue via plasma membrane disruption and invade hepatocytes (liver stage). This initial asymptomatic stage is latent for *P. vivax* and *P. ovale* and the parasites can lay dormant and survive for weeks, months or even years in the host without causing the typical malaria symptoms. In the hepatocytes, the parasites form a parasitophorous vacuole and differentiate into tens of thousands of merozoites which are released into the bloodstream to invade erythrocytes and starting the erythrocytic infection (blood stage) (Vaughan *et al.*, 2008).

In the erythrocytic cycle (blood stage), *Plasmodium* grows from a ring-shaped form over a larger trophozoite to the schizont form. In this stage the parasite divides several times and produces 8-32 merozoites until the cell bursts. The merozoites travel within the bloodstream to invade new cells. Most merozoites continue this replication cycle, which takes about 48 hours to complete for *P. falciparum* and *P. vivax*, 72 hours for *P. malariae* and 24 hours for *P. knowlesi*. The rupture of erythrocytes corresponds with the symptomatic fever attacks (Miller *et al.*, 2002). Several days after the initial infection of erythrocytes, some merozoites differentiate into gametocytes. For *P. vivax* this process is concurrent or even prior to the development of asexual parasites (Wells *et al.*, 2009). Gametocytes are taken up by a female mosquito at the next bite (Cowman & Crabb, 2006).

In *Anopheles* the sexual development of the gametocytes into gametes takes place in the midgut. The gametes fertilize each other, forming motile zygotes called ookinetes. The ookinetes escape the midgut and embed themselves onto the exterior of the gut membrane where they divide many times to produce up to 1000 small sporozoites. These sporozoites migrate to the salivary glands of the mosquito where they are ready to be injected into the blood stream of the next host.

### 1.1.3 Drug targets

Most of the antimalarial drugs that are currently available target the asexual blood stage parasites. However, the mode of action and target of many drugs are still not known. Resistance against antimalarial drugs usually results from point mutations that affect drug accumulation/efflux in erythrocytes or lead to reduced drug affinity to the target (Hayton & Su, 2004). Drug targets include the parasite replication machinery, translation processes, invasion and egression from erythrocytes as well as the apicoplast and the pathways within (Ralph *et al.*, 2001; Waller & McFadden, 2005; Cowman & Crabb, 2006; Yeoh *et al.*, 2007). Since the apicoplast is the most recently described organelle with many potential drug targets, it will be described in more detail.

#### 1.1.3.1 Apicoplast

The apicoplast is a unique organelle and was first described in 1960, but it was not until the mid 1990s that it was first identified as a novel organelle of symbiotic cyanobacteria origin, analogue to plant plastids (McFadden *et al.*, 1996; Wilson *et al.*, 1996).

The evolution of the apicoplast is complex and presumed to have occurred by multiple endosymbiosis events (Fig. 1.3). In the primary endosymbiosis, a photosynthetic cyanobacterium was incorporated into an eukaryotic phagotroph (alga). The incorporation of this so-called primary plastid (or chloroplast) enables the recipient to photosynthesis and autotrophic survival. Primary plastids can be found in land plants, red algae, green algae and freshwater algae. In a secondary endosymbiosis, a primary endosymbiont (containing the primary plastid) was engulfed by a second eukaryote, *i.e. Plasmodium.* Whether this primary endosymbiont was a red or green alga is still controversial and open to debate (Wilson *et al.*, 1996; Kohler *et al.*, 1997; Funes *et al.*, 2002; Waller *et al.*, 2003; Lau *et al.*, 2009). However, a recent publication suggests that both theories are correct and that a green algal endosymbiont was replaced by a red alga (Moustafa *et al.*, 2009). Another very recent study provided additional evidence that the apicoplast in apicomplexan derived from

a common red alga, but does not reject the earlier hypothesis of Moustafa *et al.* (2009) (Janouskovec *et al.*, 2010).

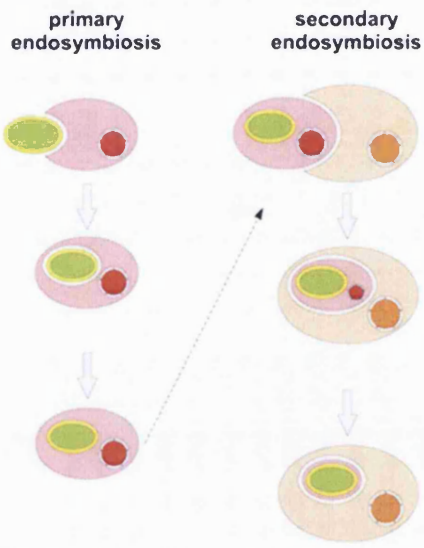


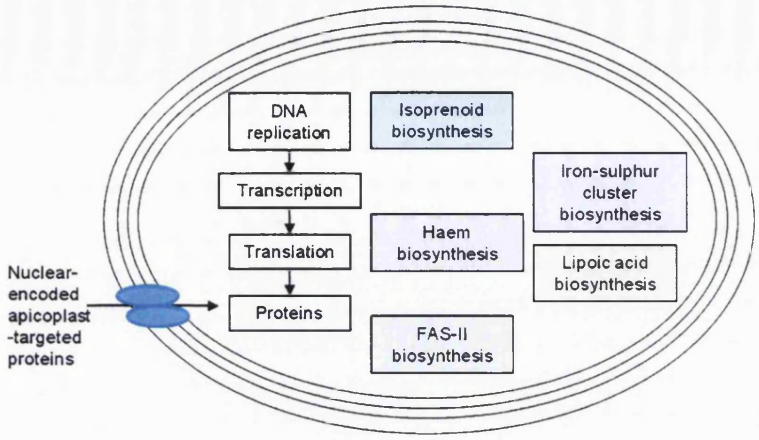

Figure 1.3. The origin of the apicoplast. Modified from (Bodyl *et al.*, 2010)

During both endosymbiosis processes, genes were transferred from the engulfed organism to the host nucleus (Martin *et al.*, 1998; McFadden, 1999). These events resulted in a greatly reduced and truncated 35-kb apicoplast genome that has lost the ability of photosynthesis, which is redundant for a parasitic living organism. The apicoplast is semi-autonomous by encoding 30 proteins and has its own replication, transcription and translation mechanisms. However, the great majority of proteins (predicted 10% of the parasite genome) are nuclear-encoded and targeted posttranslationally to the apicoplast (Pradel & Schlitzer, 2010). Thus, complex transport and import mechanisms for the nuclear-encoded proteins over the four membranes of the apicoplast to their point of origin had to be established (Waller *et al.*, 1998; Waller *et al.*, 2000; Kalanon & McFadden, 2010).

The apicoplast has been shown to be indispensable for the blood stage parasites (Goodman *et al.*, 2007) as well as the liver stage parasites (Yu *et al.*, 2008; Vaughan *et al.*, 2009) as it is the site of several fundamental anabolic pathways. Due to its evolutionary origin from cyanobacteria, the apicoplast genome harbours several bacteria-like housekeeping genes and bacteria-like biosynthetic pathways such as the type II fatty acid biosynthesis (FAS-II), isoprenoid biosynthesis via the methylerythriol phosphate pathway (synonyme: 1-deoxy-D-xylulose-5-phosphate pathway), haem biosynthesis and iron-sulphur cluster biosynthesis (Fig. 1.4) (Ralph *et al.*, 2004).

Due to the evolutionary connection with chloroplasts in plants, it was shown that phytotoxins and herbicides have been effective against *P. falciparum* (Bajsa *et al.*, 2007).

Figure 1.4. Biosynthetic pathways in the apicoplast.




One of the best studied pathway and the first pathway identified in the apicoplast is type II fatty acid biosynthesis (FAS-II). Crucial structural and organisational differences between the type I human fatty acid synthase and the plasmodial type II fatty acid biosynthesis enzymes make this pathway an attractive target for antimalarial research and it will be described in more detail in the following section.

### 1.1.3.2 Fatty acid biosynthesis

The fatty acid biosynthesis pathway is vital for the parasite and all living organisms, as fatty acids are the central constituents of most lipids and are therefore essential for membrane formation. Apart from that, fatty acids are an important form of energy and have other key roles, such as the one in signal transduction.

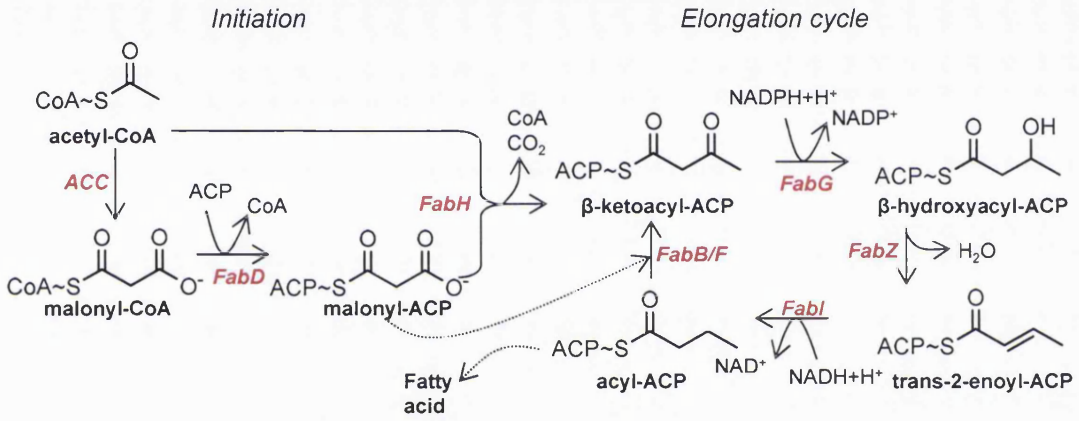
Fatty acid biosynthesis is a highly conserved process with major structural and organisational differences between the plasmodial type II and the human type I. Humans and other higher eukaryotes employ a large single multifunctional enzyme. This cytosolic enzyme has discrete subunits, each one catalysing a different enzymatic reaction (Maier *et al.*, 2008). Bacteria, plants, algae and some apicomplexan parasites such as *P. falciparum* use the FAS-II system, which consists of several small, monofunctional enzymes, each catalysing one single reaction (White *et al.*, 2005).

The FAS-II system from *Plasmodium falciparum* is shown in Figure 1.5 and can be divided into an initiation phase and an elongation cycle. In the first step in the initiation, acetyl-Coenzyme A (acetyl-CoA) is carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACC) which is then transferred to an acyl-carrier protein (ACP) through FabD (malonyl-CoA:ACP transacylase). By condensation of malonyl-ACP with acetyl-CoA the fatty acid elongation cycle is initiated. This reaction is catalysed by FabH (β-ketoacyl-ACP synthase III). The resulting β-ketoacyl-ACP is reduced in a NADPH-dependant reaction to β-

hydroxyacyl-ACP by FabG (β-ketoacyl-ACP reductase), before FabZ (β-hydroxyacyl-ACP dehydratase) catalyses a dehydration to form *trans*-2-enoyl-ACP. In the final elongation step FabI (enoyl-ACP reductase) reduces *trans*-2-enoyl-ACP to acyl-ACP in a NADH-dependent reaction. Subsequent cycles are started by either β-ketoacyl-ACP synthase I (FabB) or II (FabF) through the condensation of malonyl-ACP with acyl-ACP which results in a two carbon elongation per cycle (Surolia *et al.*, 2004).

A recent report showed that *P. falciparum* blood stage parasites produce $C_{16}$ and $C_{18}$ fatty acids (Yu *et al.*, 2008), whereas an earlier study by Surolia and Surolia (2001) reported $C_{10}$ – $C_{14}$ fatty acids.

Figure 1.5. Type II fatty acid biosynthesis in *P. falciparum*.




The best studied FAS-II enzyme is FabI as it is the rate-limiting enzyme and catalyses the final enzymatic reaction in the fatty acid elongation process (Heath & Rock, 1995). Sequence alignment of plasmodial FabI with that of bacteria (*E. coli* and *M. tuberculosis*) and the plant *Brassica napus* showed great overall similarity with the plant enzyme (48%). The FabI equivalent in *E. coli* (*Ec*FabI) and *M. tuberculosis* (*Mt*FabI) showed 16% and 30% similarity to the plasmodial FabI (*Pf*FabI), respectively (Perozzo *et al.*, 2002). This not only demonstrates the evolutionary link of the plasmodial apicoplast with plant plastids, but also a high conservation of fatty acid biosynthesis.

Structural comparison of the active sites from FabI enzymes demonstrated that *P. falciparum* and *E. coli* shared the greatest similarity and have both adopted a very similar conformation (Perozzo *et al.*, 2002). It is therefore unsurprising that many inhibitors provide cross-activity towards FAS-II enzymes from different organisms. Triclosan, a broad-spectrum biocide, is a known FabI inhibitor against a variety of organisms including *E. coli*, *S. aureus* and *M. tuberculosis* (Heath *et al.*, 1998; Parikh *et al.*, 2000; Heath *et al.*, 2000).

As described above, it has been reasoned that due to the structural and organisational differences between the plasmodial and human fatty acid biosynthesis enzymes, this pathway is a good target for antimalarial drug discovery. However, within the type I fatty acid synthase complex, catalytic domains corresponding to the type II FabH, FabG, FabZ and FabI have been identified and showed some homology to the FAS-II enzymes (Liu *et al.*, 2002). Some FAS-II inhibitors such as cerulenin or triclosan have demonstrated inhibition towards the human FAS-I complex, however these drugs are less potent against the human fatty acid synthase, *e.g.* triclosan has an $IC_{50}$ value of 50 μM for human FAS-I compared to 0.05 μM for plasmodial FabI (Lawrence *et al.*, 1999; Liu *et al.*, 2002).

### Importance of FAS-II for *Plasmodium* survival in different parasite stages

Before the discovery of FAS-II, it was believed that *Plasmodium* is unable to perform *de novo* fatty acid biosynthesis and that fatty acids were acquired through scavenging (Holz, 1977; Vial *et al.*, 1990). The first indication of the presence of a fatty acid biosynthesis pathway came with the *P. falciparum* genome sequencing project (Gardner *et al.*, 1999). Waller *et al.* (1998) used preliminary sequencing data which showed the occurrence of two genes encoding ACP and the FAS-II enzyme FabH. They were able to prove that these two nuclear-encoded FAS-II related proteins were targeted to the apicoplast in *P. falciparum.* The completed genome sequencing showed the presence of further FAS-II enzymes and confirmed that no cytosolic FAS genes or type I FAS complexes were found in the entire plasmodial genome, providing further evidence that the apicoplast is the site of fatty acid biosynthesis (Gardner *et al.*, 2002).

Shortly after the discovery of the FAS-II pathway, experiments were conducted to examine its role in parasite survival. Triclosan, which was known to inhibit bacterial FabI enzymes, was shown to inhibit the incorporation of radiolabelled acetate into fatty acids in *P. falciparum* (Surolia & Surolia, 2001). In addition, triclosan was shown to inhibit the plasmodial FabI enzyme, *in vitro* growth of *P. falciparum* blood stage parasites and *in vivo* growth in *P. berghei.* Thus, it was reasoned that FAS-II is essential for the survival of blood stage parasites and FAS-II enzymes a good target for malaria chemotherapy. This paradigm led to continuous efforts by a wide research community to identify FAS-II inhibitors with antiplasmodial and antimalarial properties. However a lack of correlation between FAS-II enzyme inhibition and antiplasmodial activity against blood stage parasites was repeatedly observed (Kuo *et al.*, 2003; Tasdemir *et al.*, 2006). Recently, two publications refuted the importance of FAS-II in blood stage parasites and showed that the pathway is exclusively crucial for liver stage parasites (Yu *et al.*, 2008; Vaughan *et al.*, 2009). As these two

publications have changed the importance of the FAS-II pathway in the human stages, they will be described in more detail.

Yu *et al.* (2008) mutated the FabI enzyme in *P. falciparum* to be resistant against triclosan and compared the inhibitory potential of the biocide to wild-type parasites and found unchanged susceptibility of the blood stage parasites. To verify the importance of FabI for blood stage parasites, they created FabI knock-out mutants for *P. falciparum* which showed equivalent growth to the wild-type. These findings led the authors to the conclusion that FabI cannot be the target of triclosan and that the enzyme is not essential, *i.e.* FAS-II is dispensable for the development of blood stage parasites. The authors also showed that the results were transferrable to the blood stage development in the rodent *P. berghei* parasites. A nearly simultaneous report published by Vaughan *et al.* (2009) also confirmed that *P. falciparum* blood stage parasites were unaffected by FabI deletion.
These reports have therefore proven that FAS-II is not essential for the blood stage development of *P. falciparum* and *P. berghei* parasites.

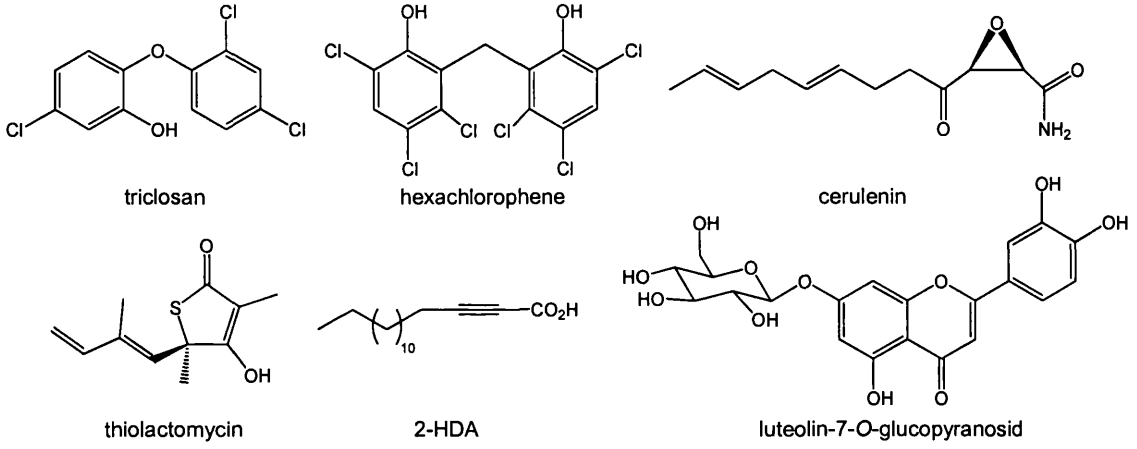
A recent analysis of the *P. yoelii* liver stage transcriptome and proteome by Tarun *et al.* (2008) suggested a possibly vital role of FAS-II for liver stage infections. In a subsequent study, the research group created FabB/F and FabZ knock-out *P. yoelii* parasites and examined the parasite development *in vivo* in all stages of the life cycle. The knock-out mutants showed normal expansion in mosquito and blood stage parasites. However, the parasites were unable to complete liver stage development. A more detailed analysis showed that the parasites arrested in the late liver stage, thus the authors concluded that the FAS-II pathway is vital for liver stage development (Vaughan *et al.*, 2009).
Similarly, the role of FabI in different stages of the *P. berghei* life cycle was investigated *in vivo* by Yu *et al.* (2008) and it was shown that FabI knock-out parasites had a delayed and greatly reduced ability to progress to blood stage infections. Additional *in vitro* tests showed that the knock-out mutants were strongly impaired in the production of merozoites in the late liver stage development.

Thus, the FAS-II pathway was identified as the first target for liver stage parasites and as a good target for the development of prophylactic drugs. Although this finding cannot be confirmed with *P. falciparum in vivo*, it is highly likely that the rodent *in vivo* data are transferable due to the high conservation of FAS-II among *Plasmodium* species (Carlton *et al.*, 2002).

## Compounds targeting FAS-II enzymes in *Plasmodium*

Since the discovery of type II fatty acid biosynthesis in *Plasmodium*, several synthetic compounds and natural products have been tested against plasmodial FAS-II enzymes (Fig. 1.6). Triclosan was the first synthetic compound identified as an inhibitor of FabI (Surolia & Surolia, 2001). Thiolactomycin and cerulenin (both derive from fungi) were the first natural products shown to inhibit the synthases FabF/FabB and FabH, followed by luteolin-7-*O*-glucopyranoside (isolated from *Phlomis brunneogaleata*, Lamiaceae), the first natural product inhibiting FabI (Kirmizibekmez *et al.*, 2004).

Figure 1.6. Chemical structure of selected FAS-II inhibitors.




Analogues from triclosan and thiolactomycin were synthesised and tested for their inhibitory potential against FabI and FabH (Freundlich *et al.*, 2005; Jones *et al.*, 2005; Chhibber *et al.*, 2006). Many other synthetic compounds have been evaluated for their potential against FAS-II enzymes (Sharma *et al.*, 2003; Kumar *et al.*, 2007). In addition, plant and marine extracts, isolated natural products and known natural products (flavonoids, catechins, alkaloids, sesquiterpene lactones) were tested against plasmodial FAS-II enzymes (Tasdemir *et al.*, 2005a; Tasdemir *et al.*, 2006; Sharma *et al.*, 2007; Karioti *et al.*, 2008; Scala *et al.*, 2010; Regalado *et al.*, 2010). The antiplasmodial activity of all identified FAS-II inhibitors was also tested *in vitro* against *P. falciparum* blood stage parasites.

Only recently, antiplasmodial tests against the liver stage parasites have been performed with FAS-II enzyme inhibitors and to date, only three synthetic compounds have been identified as liver stage inhibitors with inhibitory activity towards FAS-II enzymes. The first compound identified was hexachlorophene, which inhibits FabG and shows a similar potential against liver stage parasites *in vitro* (Wickramasinghe *et al.*, 2006; Tarun *et al.*, 2008). Recently, the well know FabI inhibitor triclosan was shown to inhibit the liver stage development *in vitro* and *in vivo* (Singh *et al.*, 2009), and very recently, our group

demonstrated that the synthetic fatty acid 2-hexadecynoic acid (2-HDA) inhibited liver stage development *in vitro* and showed inhibitory potential against the FAS-II enzymes FabI, FabZ and FabG (Tasdemir *et al.*, 2010).

### 1.1.4 Antiplasmodial and antimalarial assays

#### 1.1.4.1 Techniques for *in vitro* assessment against blood stage parasites

The cultivation and maintenance of asexual blood stage parasites of *P. falciparum in vitro* has been established since the late 1970s and shortly afterwards protocols for the quantitative assessment of antimalarial activities were implemented (Trager & Jenson, 1978; Desjardins *et al.*, 1979). The *in vitro* study of *P. falciparum* blood stage parasites is relatively cheap, easy and quick.

The *in vitro* growth inhibition of blood stage parasites can be assessed by different techniques. The incorporation of [$^3$H]hypoxanthine based on the semiautomated microdilution technique developed by Desjardins *et al.* (1979) was used in this study, as it is a highly sensitive method and was the established technique used at the London School of Hygiene and Tropical Medicine, where the assays were conducted. In addition to this radioactivity assay, other techniques for the *in vitro* assessment of antiplasmodial inhibition against blood stage parasites are available and include the lactate dehydrogenase assay, a fluorescence-based high-throughput technique or microscopic techniques using Giemsa staining (Makler *et al.*, 1993; Smilkstein *et al.*, 2004; Le *et al.*, 2008). The main advantage of these methods is that they are inexpensive and do not use radioactivity, which necessitates the implementation of high safety protocols. The drawback of these techniques is that small infection number might not be detected and possible drug interferes with fluorescence excitation and emission.

#### 1.1.4.2 Techniques for *in vitro* assessment against liver stage parasites

In contrast to the blood stage experiments, the examination of liver stage parasites *in vitro* is exceedingly complicated, expensive and requires much more effort and time. Living sporozoites are required for the *in vitro* infection of hepatocytes, which need to be routinely cycled between *Anopheles* mosquitoes and mice. Thus, the establishment of *in vitro* experiments with liver stage parasites is accompanied by the need for mosquito breeding, animal housing and maintenance. The *in vitro* investigation of hepatocytes infected with *P. falciparum* was successfully established, but primary human cells are required for this assay which do not grow continuously in culture and need to be isolated from the liver (Mazier *et al.*, 1985). Advances have been made in the development of human hepatic cell lines, but infection rates are low (Karnasuta *et al.*, 1995; Sattabongkot *et al.*, 2006). Additionally, the *in*

*vitro* models require *P. falciparum* infected *Anopheles* mosquitoes which necessitate the use of high safety protocols. Due to these difficulties, most *in vitro* experiments with liver stage parasites have been performed using the rodent malaria models *P. berghei* and *P. yoelii*. These parasites have the advantage to be infectious not only in mouse hepatoma cell lines (Long *et al.*, 1989; Mota & Rodriguez, 2000), but also in human lung (Hollingdale *et al.*, 1981), HeLa (Calvo-Calle *et al.*, 1994) and hepatoma cell lines (Hollingdale *et al.*, 1983b).

The assessment of the *in vitro* growth inhibition of liver stage parasites is complicated due to the low infection level and complex techniques required for monitoring the parasites development. Several methods have been developed and include quantitative real-time PCR (Bruna-Romero *et al.*, 2001), RNA or DNA hybridisation (Schofield *et al.*, 1987; Li *et al.*, 1991), an infrared fluorescence scanning system (Gego *et al.*, 2006) and indirect or direct immunofluorescent microscopy (Hollingdale *et al.*, 1983a; Carraz *et al.*, 2006). The development of transgenic rodent parasites expressing fluorescent reporter proteins enabled quantitative analysis by flow cytometry (Natarajan *et al.*, 2001; Tarun *et al.*, 2006).

In this study, antiplasmodial activity against *P. yoelii* liver parasites was assessed quantitatively by flow cytometry (FC) and qualitatively by immunofluorescence analysis (IFA) by Dr. A. Tarun and Assoc. Prof. S. Kappe from the Seattle Biomedical Research Institute, USA.

### 1.1.4.3 Techniques for *in vivo* assessment

For the *in vivo* assessment of antimalarial activities, the rodent malaria parasite models *P. berghei* and *P. yoelii* have proven indispensable for the research of liver and blood stage infection. Host and tissue specificity of *P. falciparum* require humans or other higher primates for *in vivo* experiments (Sacci *et al.*, 2006). For obvious ethical reasons humans cannot be used as an *in vivo* model, while other primates are unsuitable due to the complexity of the experiment, availability of animals, high costs and ethical reasons.

While the *in vivo* blood stage models are relatively easy to study (Knight & Peters, 1980), the investigation of liver stage development remained difficult until the introduction of fluorescent parasite strains (Tarun *et al.*, 2006). Recent advances have been made in developing mouse models with human erythrocytes (Angulo-Barturen *et al.*, 2008) or human hepatocytes (Morosan *et al.*, 2006) which are susceptible to *P. falciparum* parasites. However, humanised mouse models still require *P. falciparum* infected *Anopheles* mosquitoes, which will require high safety protocols.

#### 1.1.4.4 Blood stage parasites *versus* liver stage parasites

Due to the technical difficulties of liver stage cultivation, liver stage assays *in vitro* and *in vivo* and high costs for experiments, it is not surprising that most research has so far concentrated on blood stage infection and worldwide only a few groups have established facilities to work on malarial liver stages. As a consequence, only few prophylactic drugs have been developed (*e.g.* primaquine, atovaquone, see 1.1.6) and only one drug has been approved by the FDA for prophylactic treatment targeting liver stage parasites. Hence, research into this area is timely and necessary.

Despite all the technical challenges, targeting liver stage parasites has several advantages. The parasite load in the liver is much smaller compared to the number of parasites in the blood. Only a few (10-15) sporozoites are responsible for the initial infection of hepatocytes. The parasites remain several days in the liver and undergo one replication cycle which results in tens of thousands of parasites that are released into the blood stream. These merozoites numbers are still relatively low compared to the millions of parasites which are present only a few days after the invasion of erythrocytes. Thus, a drug targeting liver stage parasites has to kill a comparatively low number of parasites and has several days before the replication cycle is completed and more parasites are released. In the blood stage the replication cycle is much shorter and completed within 48 h for *P. falciparum*. Newly released parasites infect more erythrocytes, which results in a high drug pressure and the risk of resistance development is exponentially higher in blood stage parasites.
Liver stage parasites are the ideal target for the prevention of malaria, as drugs targeting this clinically silent stage cause causal prophylaxis, *i.e.* preventing the blood stage and clinical symptoms, and thereby stop further spreading of the disease. Targeting the blood stage parasites in antimalarial drug discovery will result in the identification of drugs that can be used for the treatment. Ideally such a drug should, in addition to the asexual stage, also target gametocytes, hence prevent further transmission of malaria.

### 1.1.5 Prevention of malaria

Prevention of malaria includes several different strategies. Prophylactic drugs are especially common for travellers and will be described in 1.1.6. In affected regions, other control measures like the use of bed nets and DDT spraying are commonly in place.

#### 1.1.5.1 Vector control measures

Insecticide-treated bed nets prevent the mosquitoes' access to the human and are highly effective. However, studies showed that only 31% of African households possess an

insecticide-treated bed net and in 2008 only 24% of children under five were sleeping under one, which is below the WHO target of 80% (WHO, 2009). It is also important that in conjunction with the distribution, an awareness on correct use has to be implemented as studies showed that people use the bed nets for other purposes, *e.g.* fishing, as they are inexpensive or free (Minakawa *et al.*, 2008).

Indoor residual spraying of the insecticide DDT is used to control malaria vectors and has been implemented in 19 African and 25 other endemic countries (WHO, 2009). The use of DDT is controversial, in the 1990s it had been replaced with pyrethroids but was reintroduced after the occurrence of resistant *Anopheles* strains (N'guessan *et al.*, 2007). Its effectiveness against the vector stands opposite to potential health effects in residents (van Dyk *et al.*, 2010).

These vector control measures in combination with ACT treatments have led to an at least 50% reduction of malaria inflicted death in some highly endemic countries such as Zambia and Rwanda and to a reduction in malaria cases of over 50% from 2000 to 2008 in 9 African and 29 other endemic countries (WHO, 2009). Successful eradication of malaria in parts of the world was primarily achieved by vector control, thus its impact should not be underestimated.

For successful continuation, new insecticides or strategies are needed as resistance against current chemicals rises in the mosquito (Kokwaro, 2009). Different strategies targeting the mosquito larvae by *Bacillus thuringiensis israelensis* (Fillinger & Lindsay, 2006) or the adult mosquito by entomopathogenic fungi (Scholte *et al.*, 2005) or insect-pathogenic viruses (Ren *et al.*, 2008) are tested. Additionally, genetically engineered mosquitoes and sterile insect techniques are being exploited (Takken & Knols, 2009).

### 1.1.5.2 Malaria vaccine

The research in vaccines against malaria has shown promising developments (Speake & Duffy, 2009; Vanderberg, 2009). In the following, a few recent advances will be described very briefly.

Recently it was shown that sporozoites could immunise people to malaria and clinical trials underlying this principle are on the way (Roestenberg *et al.*, 2009; Hoffman *et al.*, 2010). Advances have been made in the development in partially effective vaccines, offering 50% protection against malaria in the first 8 months. Currently, one such vaccine (RTS,S) is in Phase III clinical trials (Guinovart *et al.*, 2009). A disadvantage of this vaccine is that it does not prevent malaria transmission as it only protects the person from symptoms but does not clear all parasites from the blood. Similar reduction of infection rates can be achieved

by simple control measures with bed nets and residual spraying, thus this approach might have an uncertain future (Vogel, 2010).

Transmission-blocking vaccines are another approach in the vaccine development. They do not prevent the outbreak of the disease, but will produce antibodies in the human which are taken up by the mosquito, preventing the insect from further spreading the disease (Sutherland, 2009; Vogel, 2010). A vaccine of this kind would have to be extremely safe as the persons do not gain protection for themselves. The advantage of this approach is that the antibody only has to target very few parasites in the mosquito compared to several thousands of parasites in the liver or billions in the blood. However, the development of TBV is difficult and most research is directed to proteins within the parasite. Expressing and purifying correctly folded proteins is often complicated but *in vitro* and *in vivo* tests have shown some promising candidates which block 93-96% of parasite development (Vogel, 2010).

An even more promising approach is the targeting of proteins within the mosquitoes as this would possess potential against several *Plasmodium* strains. *In vivo* studies have shown successful candidates which blocked the development of 98% in *P. vivax* and 100% in *P. falciparum* in mosquitoes in Cameroon (Dinglasan *et al.*, 2003; Vogel, 2010).

A very recent study showed that clindamycin and azithromycin caused vaccine-like immunity *in vivo* by causing a cellular defect in the parasites apicoplast during their journey into the liver of the infected host, which disabled the parasites to develop into the blood stage parasites (Friesen *et al.*, 2010).

However, even though there is much research dedicated to the development of vaccines, it is unlikely that one will be available within five years or more realistically, in a decade or two (Laxminarayan *et al.*, 2010; Saleh *et al.*, 2010).

### 1.1.6 Malaria prophylactic drugs

Prophylactic drugs can be divided into two main categories: suppressive prophylactic drugs that eradicate and suppress blood stage parasites and causal prophylactic drugs that eradicate liver stage parasites. True prophylaxis can only be achieved with the latter type, as these drugs are active before the parasites enter the bloodstream, thus preventing the clinical manifestation of the disease and further transmission. Suppressive prophylaxis is a less desirable approach, as these drugs only target the blood stage parasites, thus do not stop further transmission of the disease.

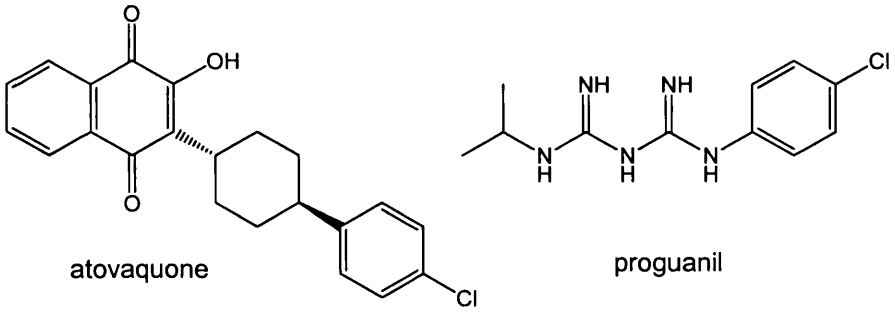
Some drugs, such as the antibiotics tetracycline or rifampicin, have been shown to inhibit the development of liver stage parasites, thus might be good for causal prophylaxis (Tarun

*et al.*, 2008). However, currently these drugs are not included in the treatment guidelines of the WHO for prophylaxis.

### 1.1.6.1 Suppressive prophylactic drugs

Atovaquone in combination with proguanil (Malarone) is recommended by the WHO for the treatment and prophylaxis for travellers (WHO, 2010b). Atovaquone (Fig. 1.7) is a hydroxynaphthoquinone antiparasitic drug active against all *Plasmodium* species. It blocks the parasites cytochrome electron transport system (McKeage & Scott, 2003) and acts as a transmission-blocking drug by inhibiting pre-erythrocytic development in the liver and oocyst development in the mosquito. It is only used in combination with proguanil, with which it acts synergistically.

Figure 1.7. Chemical structure of atovaquone and proguanil.




Proguanil (Fig. 1.7) is a biguanide compound that is metabolised to the active metabolite cycloguanil. Its antimalarial activity was first described in 1945 (Dhanawat *et al.*, 2009). The parent compound has weak intrinsic antimalarial activity through an unknown mechanism, whereas cycloguanil inhibits the plasmodial dihydrofolate reductase (DHFR). Like atovaquone, it is active against the blood stage parasites. Additionally, both drugs have activity against the hepatic form of *P. falciparum* (Berman *et al.*, 2001). Thus, the atovaquone-proguanil combination provides suppressive and causal prophylaxis and blocks further transmission (Nakato *et al.*, 2007).

The use of this combinations is limited to travellers due to fast resistance development against atovaquone after treatment (Fivelman *et al.*, 2002). Thus, widespread use would lead to rapid emergence of resistance to atovaquone (Gebru *et al.*, 2006). Additionally, the use further is limited due to the necessity of daily dosing and high costs and a considerably reduced biotransformation rate of proguanil to cycloguanil was shown for approximately 3% of Caucasian and African population and 20% of Oriental population (Helsby *et al.*, 1990; Kaneko *et al.*, 1999; Polhemus *et al.*, 2008).

All other drugs available for suppressive prophylaxis are also extensively used in the chemotherapy of malaria (see 1.1.7). An additional drawback for these suppressive prophylactic drugs is, that the drug intake needs to be continued for at least four weeks after leaving endemic areas, compared to seven days for Malarone.

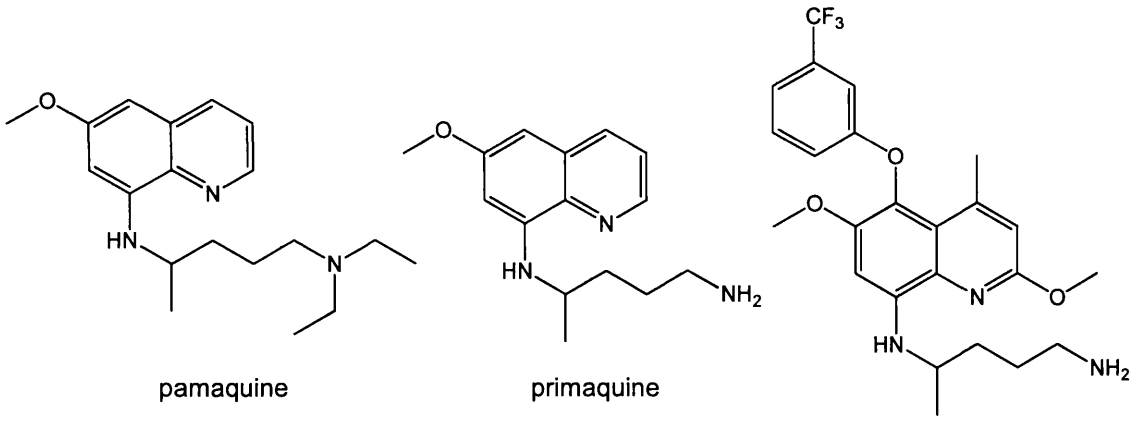
Currently chloroquine, mefloquine and doxycycline are used for suppressive prophylaxis (Arguin & Steele, 2010). The use of chloroquine, alone or in combination with proguanil (see below), is limited to *P. falciparum* chloroquine-sensitive environments because of widespread resistance against this antimalarial drug. The same is true for mefloquine, which is additionally restricted by its harmful neuropsychiatric side effects (Senn *et al.*, 2007). Doxycycline has become an alternative to mefloquine and has recently been shown to inhibit the liver stage development of *P. berghei* parasites *in vitro* (Stanway *et al.*, 2009). The same study also showed liver stage activity for the antimicrobial agents azithromycin and clindamycin. Currently, doxycycline remains the only antibiotic used for prophylaxis, as it was shown to be more effective against falciparum malaria than azithromycin (Taylor *et al.*, 1999) and clindamycin is unsuitable for prophylaxis due to its short half-life (2-4 h) (Pradel & Schlitzer, 2010). The main disadvantage of doxycycline is, that it cannot be used for prophylaxis in children or during pregnancy due to safety reasons and it can potentially cause photo-sensitisation in patients (Arguin & Steele, 2010).

### 1.1.6.2 Causal prophylactic drugs

Only few of the available antimalarial drugs target the liver stage parasites and of all causal prophylactic drugs, primaquine (Fig. 1.8) is currently the only FDA-approved drug on the market which is solely used for malaria prophylaxis. However, it is mainly recommended for the prevention of *P. vivax* malaria and antirelapse therapy after *P. vivax* and *P. ovale* infections (Arguin & Steele, 2010).

Primaquine is a synthetic 8-aminoquinoline derived from quinine and has its origins in the work of Ehrlich who cured malaria patients with methylene blue, a synthetic dye in 1891 (Guttmann & Ehrlich, 1891). Primaquine (introduced in 1951) is a second generation drug from pamaquine which was introduced in the 1920s (Muehlens, 1926; Solomon & Lee, 2009). A third generation molecule (tafenoquine) with a greatly reduced treatment course of 2-3 days compared to the 14 days treatment course of primaquine is currently in clinical development (Brueckner *et al.*, 1998).

Figure 1.8. Chemical structure of pamaquine, primaquine and tafenoquine.




Primaquine is effective against the hepatic forms of the malaria parasite, however, the location, target or mode of action are still unknown (Greenwood *et al.*, 2008). It was also shown to be active against mature gametocytes of *P. falciparum* but lacks activity against asexual stages (Arnold *et al.*, 1955; Pukrittayakamee *et al.*, 2004).

The main disadvantage of this drug and its derivatives is, that they cannot be administered to anyone with glucose-6-phosphate dehydrogenase deficiency (G6PD-deficiency) (Youngster *et al.*, 2010). G6PD is the most common enzyme deficiency in humans and affects approximately 400 million people worldwide and, most importantly, up to 28% of the patients in endemic areas (Beutler & Duparc, 2007; Cappellini & Fiorelli, 2008). Thus, a mass administration of primaquine is not viable. Even though testing for G6PD-deficiency is theoretically possible, in practice it is usually unavailable and primaquine is not given. In addition, the toxicity of primaquine is a limiting factor and a very recent study showed alarming results that haemolysis after primaquine treatment is not restricted to G6PD-deficient individuals (Shekalaghe *et al.*, 2010).

Pyrimethamine is another available drug inhibiting exoerythrocytic parasites and used in combination with dapsone as causal prophylactic drug (Maloprim) (Wells *et al.*, 2009). Dapsone is a sulphone with the same mechanism of action as sulphadoxine (see 1.1.7) (Seydel *et al.*, 1980). Pyrimethamine has also been shown to inhibit the sporozoites development in the mosquito (Teklehaimanot *et al.*, 1985). However, pyrimethamine has been extensively used as companion drug of sulphadoxine for malaria chemotherapy (see 1.1.7) and drug resistance has been reported. In addition, the use of this combination is limited by dapsone, which also has been shown to cause haemolysis in G6PD-deficient patients (Youngster *et al.*, 2010).

### 1.1.7 Chemotherapeutic antimalarial drugs against blood stage parasites

#### 1.1.7.1 Quinine

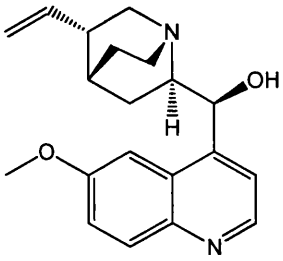



Figure 1.9. Chemical structure of quinine.

Quinine (Fig. 1.9) is an alkaloid derived from the bark of the Cinchona tree *Cinchona calisaya* (Rubiaceae) which has been used for the treatment of fever since the 17th century (Meshnick & Dobson, 2001). The compound was isolated in 1820 by Pelletier and its structure was elucidated in 1944 (Pelletier & Caventou, 1820; Woodward & Doering, 1944).

In the beginning of the 1880s, quinine was first shown to remove the malaria parasites from the blood by Laverna (Cox, 2010). It acts on the mature trophozoite stage but has no effect on the maturation of *P. falciparum* gametocytes. It is ineffective against the liver stages of malaria parasites. Quinine is not only a drug of its own right, but also has been a template structure for many antimalarial drugs (see below).

Quinine interferes with the parasite's haem detoxification in the food vacuole. The parasite takes up erythrocytic haemoglobin into its acidic food vacuole and uses it as a nutrient source. The degradation of haemoglobin produces as a by-product toxic free haem (ferriprotoporphyrin IX). To remove the potentially lytic haem molecules, the parasite polymerises the haem into nontoxic crystals of hemozoin (also called malaria pigment) (Foley & Tilley, 1998). Quinine, and other quinoline drugs, inhibit the crystallization of the toxic ferriprotoporphyrin IX to the nontoxic hemozoin, however the exact molecular mechanisms is still not fully understood (Alumasa *et al.*, 2010).

Even though resistance has been observed, quinine still remains useful for malaria chemotherapy. However, it needs a 7-day treatment course which is frequently not followed due to its well documented severe side effects, three times dosing a day and (especially relevant in children) unpleasant taste (Achan *et al.*, 2009). Quinine combinations with the antibiotics clindamycin or doxycycline are recommended by the WHO for the treatment in travellers and during pregnancy (WHO, 2010b).

#### 1.1.7.2 Chloroquine

Chloroquine (Fig. 1.10), a synthetic compound derived from quinine, was developed in 1934 by Andersag (CDC, 2010). It belongs to the group of 4-aminoquinolines. Chloroquine was ignored for a decade after its discovery as it was thought to be too toxic for clinical use until it was rediscovered during World War II (Solomon & Lee, 2009). Since

then, for many years it became the first line antimalarial drug as it is a cheap, safe and efficient drug. However, its use today is limited due to widespread resistance in Africa.

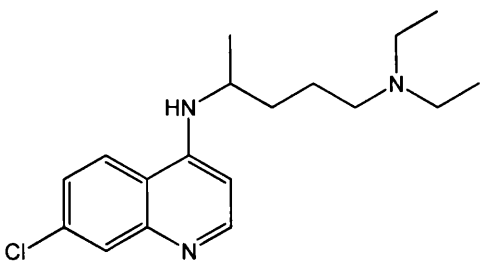



Figure 1.10. Chemical structure of chloroquine.

Chloroquine has rapid schizonticidal activity and acts against immature gametocytes. The mode of action of chloroquine is similar to quinine and results from interference with heme polymerisation in the food vacuole in blood stage parasites (Krugliak & Ginsburg, 1991; Slater & Cerami, 1992; Dorn *et al.*, 1995; Bray *et al.*, 1998).

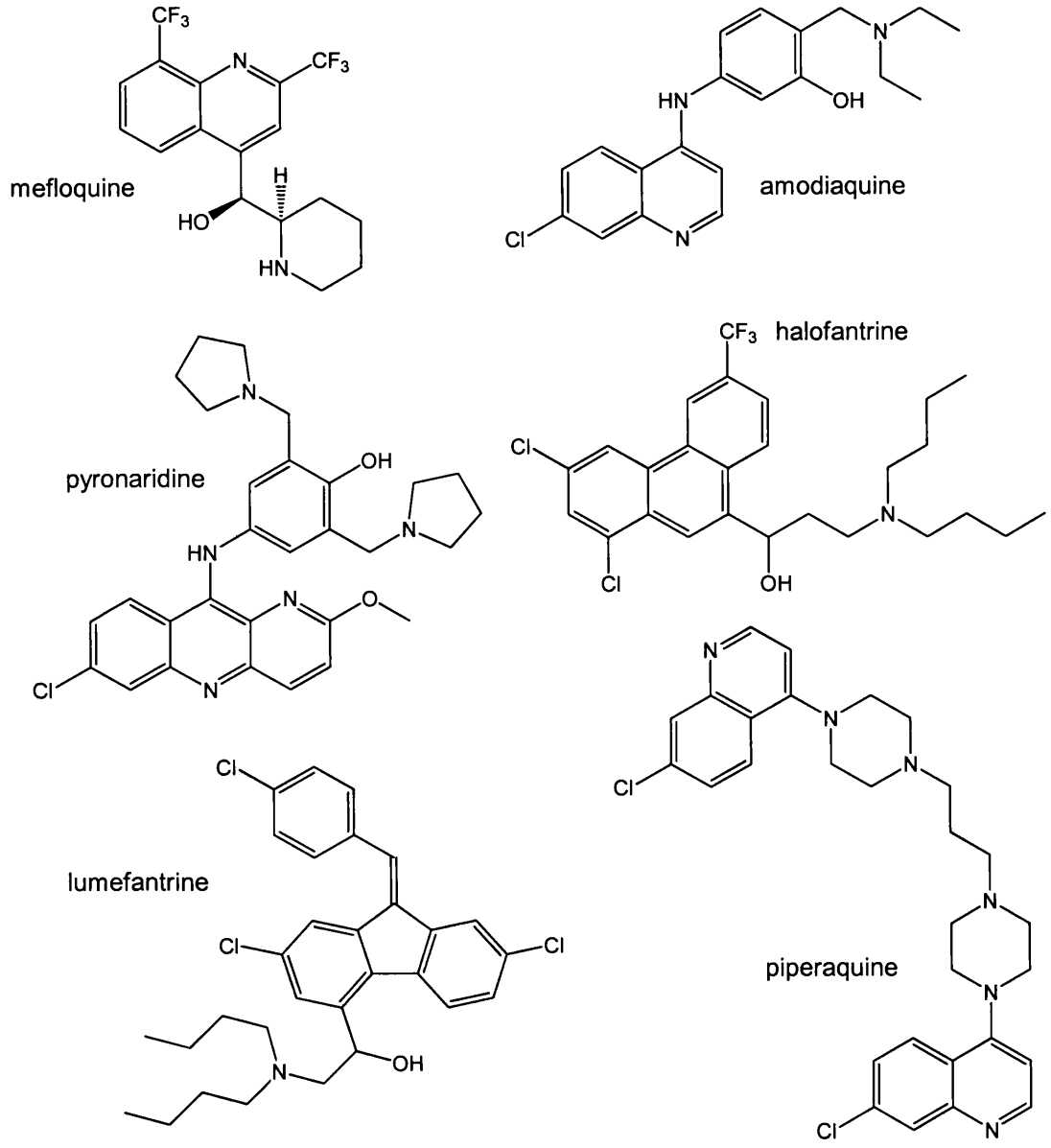
### 1.1.7.3 Drugs derived from chloroquine

Modifications of the molecular structure of chloroquine led to the discovery of lumefantrine, piperaquine and pyronaridine in China, and amodiaquine, mefloquine and halofantrine in the United States of America (Fig. 1.11) (Wells *et al.*, 2009).

Due to their origin, the mode of action has been shown or proposed to be similar to chloroquine by interfering with heme polymerisation (Ringwald *et al.*, 1996; Famin & Ginsburg, 2002; Sisowath *et al.*, 2009).

All drugs with the exception of lumefantrine were used for monotherapy. Today however, all of these chloroquine-like drugs (except halofantrine) are either already available in combination with artemisinin-derivatives (artemether-lumefantrine, piperaquine-DHA, artesunate-amodiaquine and artesunate-mefloquine) or are in development (artesunate-pyronaridine).

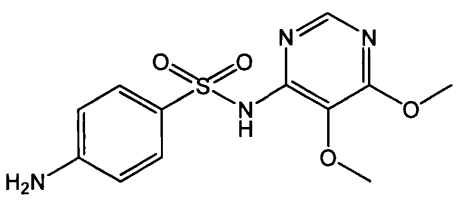
Figure 1.11. Chemical structure of drugs derived from chloroquine.



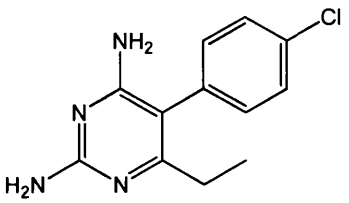

### 1.1.7.4 Sulphadoxine and pyrimethamine (SP)

Sulphadoxine, a slowly eliminated sulphonamide, and pyrimethamine, a 2,4-diamino-pyrimidine and slow-acting blood schizontocide, belong to the group of antifolate drugs (Fig. 1.12). Sulphadoxine and pyrimethamine work synergistically (Chulay *et al.*, 1984) and target enzymes in the parasite cytoplasm involved in folate synthesis, dihydropteroate synthase (Brown, 1971) and dihydrofolate reductase (Ferone, 1970) respectively. Thus these antimalarial drugs indirectly block nucleic acid synthesis.

Figure 1.12. Chemical structure of sulphadoxine and pyrimethamine.




sulphadoxine




pyrimethamine

After the failing of chloroquine due to resistance, sulphadoxine-pyrimethamine (SP, Fansidar) was implemented as the standard antimalarial drug but subsequent resistance quickly developed due to inadequate dosing, a long half-life and the fact that SP stimulated gametocytogenesis, thus increasing transmission of SP-resistant parasites (Terlouw *et al.*, 2003; Schlagenhauf & Petersen, 2009).

### 1.1.7.5 Artemisinin and its derivatives

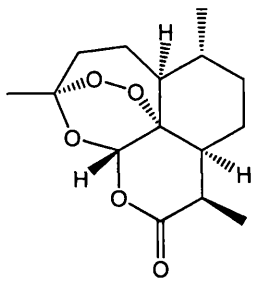



Fig. 1.13. Chemical structure of artemisinin.

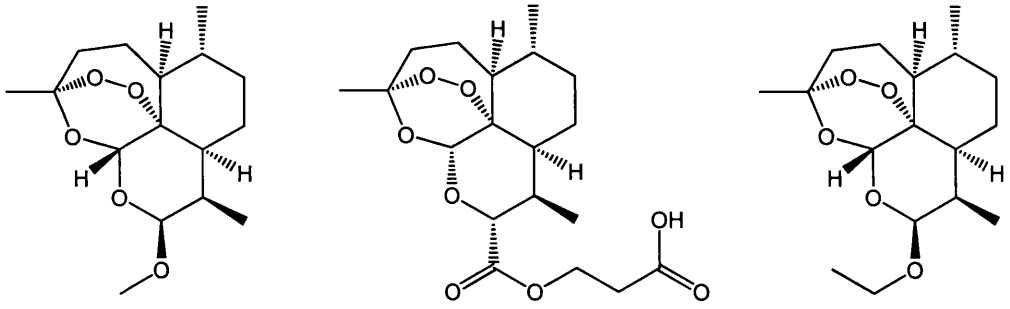
Artemisinin (also known as qing hao su) (Fig. 1.13) is a sesquiterpene lactone extracted in the early 1970s from the leaves of the annual plant *Artemisia annua* (sweet wormwood) (Asteraceae) by Chinese scientists, but was only reported to the rest of the world in 1979 (Anonymous, 1979). By this time the group had characterised the physiochemical properties of artemisinin, with studies performed *in vitro*, *in vivo* and in humans (White, 2008). Reports about the efficacy against uncomplicated and severe malaria followed soon (Jiang *et al.*, 1982; Li *et al.*, 1982). A full chemical synthesis was reported four years after it was introduced to the world but remains too expensive to be commercialised (Schmidt & Hofheinz, 1983). Genetically modified *Saccharomyces cerevisiae* yeast and *E. coli* bacteria are used for large-scale microbial production of artemisinin precursors, which are subsequently transformed into artemisinin by either chemical or biotransformational processes (Zeng *et al.*, 2008). Although these recent advances in bioengineering have been made, the main commercial source of artemisinin still is *Artemisia annua.*

*Artemisia annua* has been used in China for the treatment of febrile illnesses for over two thousand years (Meshnick *et al.*, 1996). In addition to the pure compound artemisinin, infusions of *A. annua* are used for the treatment of malaria in endemic communities. This approach is discussed controversially (Jansen, 2006; Willcox *et al.*, 2007). The main reasons for this controversy are high recrudescence rates in patients and differences in the amount

of extracted artemisinin due to different preparation methods and species varieties, which result in a lower dosage compared to commercially available artemisinin preparations (Mueller *et al.*, 2000; Mueller *et al.*, 2004; Atemnkeng *et al.*, 2009). However, it was shown that with traditional Chinese preparation methods, *A. annua* tea is a good option for patients in remote areas, where it can be used as immediate first-aid until additional medication is available (Willcox *et al.*, 2007).

Artemisinin is a potent and rapidly acting blood schizontocide with an unusual broad activity against asexual parasites, killing all stages from young rings to schizonts (ter Kuile *et al.*, 1993). In *P. falciparum* malaria, artemisinin also kills the gametocytes, including stage four gametocytes, which are otherwise only sensitive to primaquine (Chen *et al.*, 1994b).
The derivatives artemether, the methyl ether of dihydroartemisinin, and artesunate, the sodium salt of the hemisuccinate ester of artemisinin, were produced in 1987 (Fig. 1.14) (Cui & Su, 2009). Initially, these drugs were only given in China as monotherapy, as the WHO and US army decided not to use the developed compounds from the Chinese but to develop their own, less active derivative artemotil, the ethyl ether of artemisinin (Fig. 1.14). Only when resistance to available antimalarial drugs worsened, researchers began to return to the Chinese derivatives (White, 2008).

Figure 1.14. Chemical structure of artemether, artesunate and artemotil.




Artemisinin derivatives are very fast acting drugs and can reduce the parasite load by a factor of approx. 10,000 per asexual cycle, compared to 100 to 1,000-fold reduction per cycle observed for most other antimalarial drugs (White, 1994; White *et al.*, 1999; WHO, 2010b). With a three day treatment, this results in the removal of about 100 million parasites and leaves up to 100,000 parasites for the partner drug (White, 1997). The fast parasite clearance is the biggest advantage of artemisinin derivatives, since they attack ring stage parasites before cytoadherence (Chotivanich *et al.*, 2000). Thus, the clearance rate is used as a marker for artemisinin resistance (White, 1997).

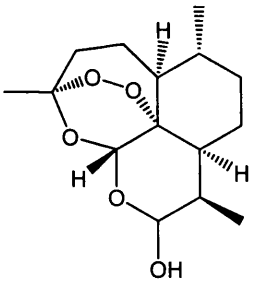


Figure 1.15. Chemical structure of DHA.

*In vivo* artemisinin and its derivatives are rapidly converted into the active principle dihydroartemisinin (DHA) (Fig. 1.15) (Lee & Hufford, 1990). DHA itself was introduced in 1992 (Cui & Su, 2009) and was shown to exhibit neurotoxicity in animal models (Brewer *et al.*, 1994; Nontprasert *et al.*, 2002). However, no such side effect has been observed in humans as the concentrations are not high enough (Kissinger *et al.*, 2000; van Vugt *et al.*, 2000; Hien *et al.*, 2003; Medhi *et al.*, 2009).

The mode of action of artemisinin and its derivatives is still unknown; there are several suggested targets and modes of action but thus far none could be conclusively shown to be the correct one. The endoperoxide pharmacophore is essential for the antimalarial activity but different models have been suggested for the bioactivation of artemisinins (reductive scission model, open peroxide model, iron or heme-dependent bioactivation) (O'Neill *et al.*, 2010).

Several targets for artemisinin have been proposed, heme polymerisation being one of them (Kannan *et al.*, 2002; Robert *et al.*, 2005; Loup *et al.*, 2007). However, these studies have been challenged by other findings showing that artemisinin treatment does not interfere with hemozoin formation *in vivo* (Meshnick, 1996; Haynes *et al.*, 2003). Another possible target is the essential calcium adenosine triphosphatase (PfATPase 6), which is the only sarco/endoplasmic reticulum calcium ATPase (SERCA) found in *P. falciparum* (Eckstein-Ludwig *et al.*, 2003; Jung *et al.*, 2005; Jambou *et al.*, 2005; Uhlemann *et al.*, 2005; Valderramos *et al.*, 2010). The inhibition of the respiratory chain of the mitochondria has also been suggested as a potential target (Krungkrai *et al.*, 1999; Li *et al.*, 2005).

Today, artemisinin has largely given way to DHA and its semisynthetic derivatives artemether and artesunate which are currently the most effective antimalarial drugs for the treatment of multiple-drug-resistant *P. falciparum* strains (White, 2008). In order to overcome the extreme short half-life and high recrudescence rate of these drugs, they have to be combined with a slower acting agent and should only be given as combination therapy to protect them from resistance development (WHO, 2010b).

### 1.1.7.6 Artemisinin-based combination therapy (ACT)

Since 2005, the World Health Organisation strongly recommends artemisinin-based combination therapy (ACT) for the treatment of malaria (WHO, 2009). The advantage of this treatment comes from the combination of a fast acting antimalarial drug, *i.e.*

artemisinin derivative, with a slower acting partner drug. Most parasites are killed by the fast-acting drug, reducing the risk of resistance development to the partner drug. The slower-acting drug kills the remaining parasites, including potential resistant ones. This combination therapy greatly reduces the risk of resistance development against the drugs involved (White, 2004).

Implementation of ACT as first line treatment was successful in national control programs of most malaria endemic countries (WHO, 2009). However, monotherapy with artemisinin derivatives is still practiced in 37 countries, mostly located in Africa (WHO, 2009). In addition, only 50% of the ACT doses were received by health facilities in the public sector in 2008. Notably, a survey in 13 African countries from 2007-2008 showed that in 11 of these countries less than 15% of children under five received ACT when presenting fever, which is considerably below the WHO target of 80% (WHO, 2009). Thus, the practice of ACT as first line treatment needs urgent improvement.

Currently, five artemisinin-based combination therapies are recommended by the WHO as the first line treatment options: artemether-lumefantrine (Coartem), artesunate-mefloquine, artesunate-amodiaquine (Coarsucam or ASAQ), artesunate-SP and DHA-piperaquine (Eurartesim or Artekin). The first fixed-dose ACT artemether-lumefantrine was introduced in 2001 and is currently the only one approved for use in Europe and as recently as 2009 in the USA (Thompson, 2009; Anonymous, 2009).

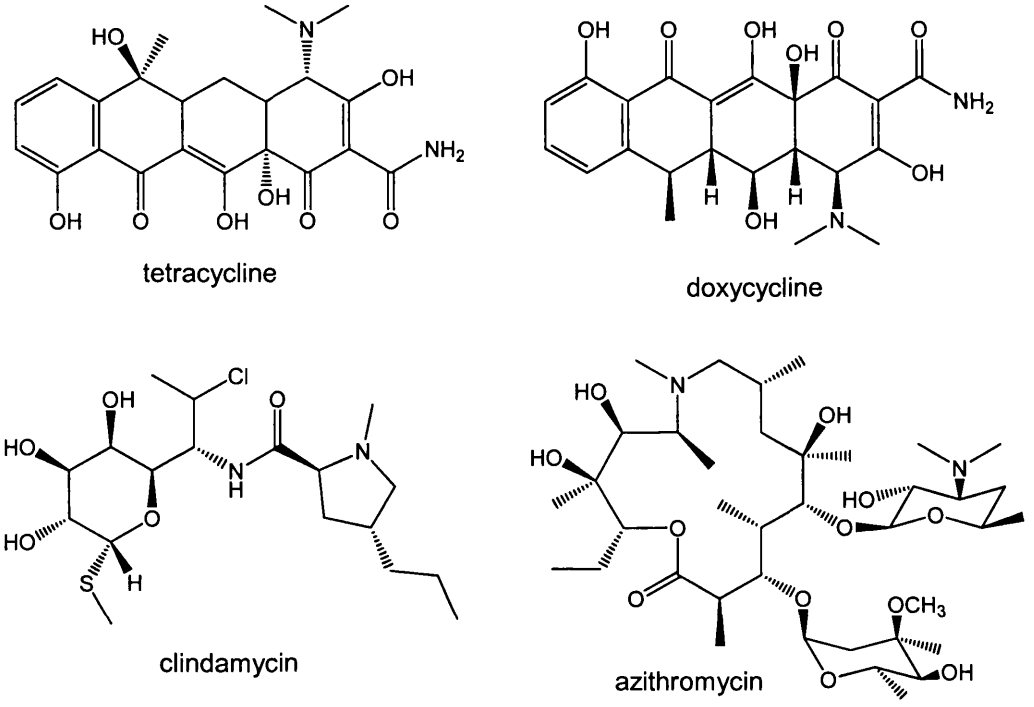
### 1.1.7.7 Antibiotics

Antibiotics have been shown to be effective against *Plasmodium* species by targeting the mitochondrion and apicoplast, two organelles with prokaryotic origin (Pradel & Schlitzer, 2010). The antimalarial effect of antibiotics alone is moderate but in combination with quinine or artesunate, it is greatly enhanced (Ohrt *et al.*, 2002).

Tetracycline, doxycycline, clindamycin and azithromycin (Fig. 1.16) have been shown to kill malaria parasites by targeting genomic functions inside the semi-autonomous apicoplast such as DNA replication, transcription and translation processes (Dahl & Rosenthal, 2007; Pradel & Schlitzer, 2010). In most cases this results in the so called "delayed death" of the parasites which refers to a slow killing during the second replication cycle (Goodman *et al.*, 2007). The delayed death is typical for antibacterial agents that are known to inhibit the prokaryotic translation such as tetracycline or azithromycin (Dahl *et al.*, 2006; Pradel & Schlitzer, 2010). At the point of drug administration, the apicoplast already possesses all the required proteins, and hence the first replication cycle is not interrupted. However, in the second round of replication, translation of essential proteins is disrupted, meaning that

essential functions in the apicoplast are inhibited, ultimately resulting in the parasite's death (Pradel & Schlitzer, 2010). This delayed death effect highlights the need to combine antibiotics with a fast acting partner drug like the artemisinin derivative artesunate (Dahl & Rosenthal, 2007).

Figure 1.16. Chemical structure of antibiotic drugs.



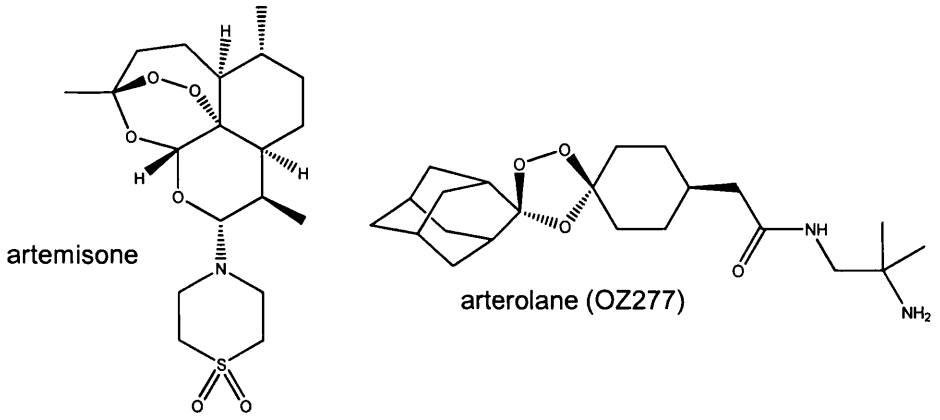

Tetracycline, doxycycline or clindamycin in combination with either quinine or artesunate are recommended by the WHO as second line ACT (WHO, 2010b). Azithromycin, a relatively new macrolide antibiotic derived from erythromycin, has shown high efficacy in combination with quinine and artesunate (Noedl *et al.*, 2006; Miller *et al.*, 2006b). The combination of azithromycin with chloroquine has been shown to be effective against parasites which showed a low response profile to chloroquine alone (Dunne *et al.*, 2005).
The main advantage of azithromycin and clindamycin over tetracyclines are their safe use in children and in pregnant women, the group mainly affected by malaria (WHO, 2010b). Antibiotic combinations are also a good treatment option for severe malaria as it has been shown that the misdiagnosis of malaria and overseen bacterial infections or bacterial co-infections are closely linked with patients deaths (Berkley *et al.*, 1999; Noedl, 2009).
Thus, antibiotics are good chemotherapeutical drugs and, as described previously, have promising potential as prophylactic drugs and vaccines.

### 1.1.8 Pipeline of new antimalarial drugs

The development of new causal prophylactic drugs is restricted to tafenoquine, which is currently in Phase III trials and found to be highly effective for malaria prophylaxis (Nasveld *et al.*, 2010). In addition, a fixed-dose combination for prophylactic use during pregnancy (azithromycin-chloroquine) is currently in Phase III clinical studies (U.S.National Institutes of Health, 2010).

For malaria chemotherapy, one new artemisinin combination, artesunate-pyronaridine (Pyramax), is in advanced stages of clinical testing and drug registration and is expected to be approved in early 2011 (Vivas *et al.*, 2008). Artesunate-pyronaridine is a very promising ACT as there was only minimal monotherapeutical use of pyronaridine (Ramharter *et al.*, 2008). Development of the new combination artesunate-chlorproguanil-dapsone (Dacart) (Phase III) was terminated and the new combination chlorproguanil-dapsone (Lapdap, commercially available in Kenya) and recalled from the market due to significant reductions in haemoglobin levels in G6PD-deficient patients (GlaxoSmithKline, 2009).

Figure 1.17. Chemical structure of artemisone and arterolane.



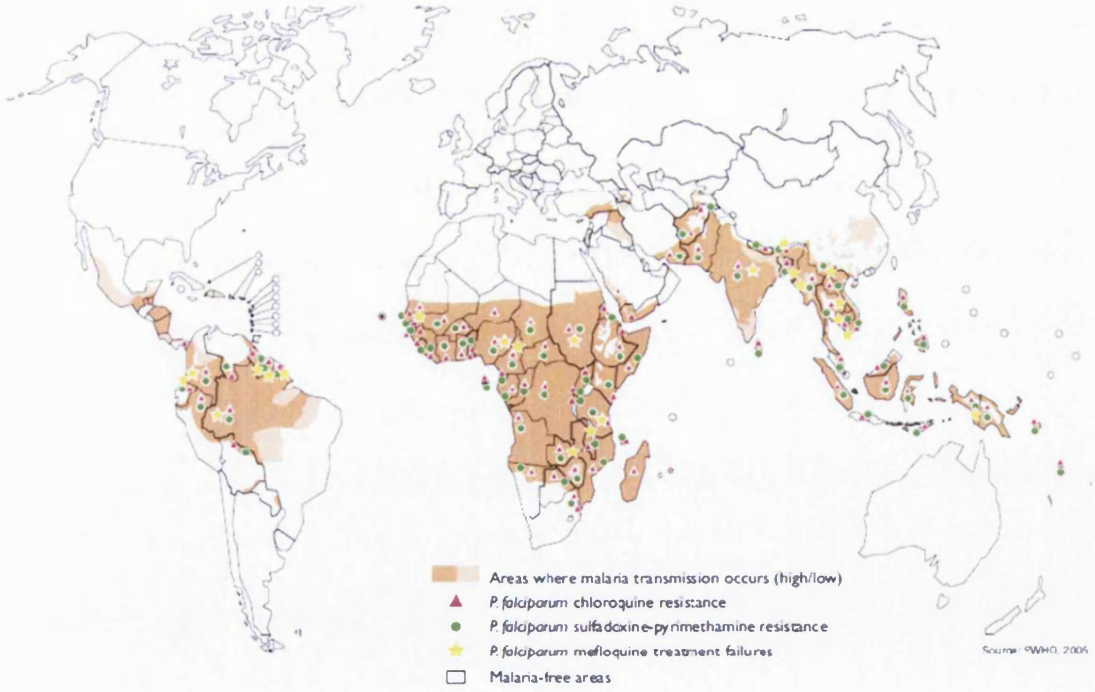

Only a few compounds are in development for new treatment options and all are variations of the artemisinin approach (Fig. 1.17). Artemisone has major structural differences to existing derivatives, arterolane (OZ277) is a synthetic artemisinin derivative containing the endoperoxide bridge and spiroether pharmacophore but otherwise has few similarities with the original compound, and OZ439 (no structure published so far) is a more potent candidate developed from arterolane (Enserink, 2010b). Effectiveness against non-resistant malaria in Phase II studies was disappointing for artemisone and arterolane which is why they had been previously abandoned; the effect of OZ439 still has to be assessed (Enserink, 2010b). However, the rise of artemisinin resistance (see 1.1.9.1) has led to a renewed interest in these compounds in the hope that the structural differences will prevent cross-resistances.

The search for new antimalarial drug leads is intense and numerous new interesting compounds have been discovered (Dhanawat *et al.*, 2009). A very recent GSK screening study with nearly 2 million compounds identified thousands of compounds with novel structures and good antiplasmodial activity (Gamo *et al.*, 2010). However, none of them have yet been tested in Phase I safety studies and the development, if successful, will take at least seven to eight years before any interesting compound will be available to the market as an antimalarial drug (Enserink, 2010b).

### 1.1.9 Resistance against antimalarial drugs

The emergence of resistance against antimalarial drugs is a major problem in fighting malaria (Fig. 1.18) and the traditional monotherapy approach has been abandoned, in order to prevent the development of new resistance. However, in many regions, non-combination drugs are still widely available and in use due to their low price.

Figure 1.18. Malaria transmission areas and reported drug resistance in 2004 (WHO, 2005).




Formerly highly potent drugs such as chloroquine lost their efficacy because of the emergence of resistant strains. Resistance development can be facilitated by inappropriate use, due to discontinuation of the drug (patient feels better or medicine becomes too expensive), underdosing of the drug (sharing drugs with friends or family) and counterfeit drugs (underdosed or no active principle) (Green, 2006). This way the parasites encounter subinhibitory drug concentrations and can accordingly develop resistance.

Resistance against chloroquine in *P. falciparum* was first detected in the late 1950s in Southeast Asia and South America (Wernsdorfer & Payne, 1991) and early 1960s at the Thai-Cambodia border (Moore & Lanier, 1961) which became a hotspot for antimalarial resistance development (Wongsrichanalai & Meshnick, 2008). Thereafter it spread, arriving in East Africa in the late 1970s and subsequently affected the whole continent within a decade (Wellems & Plowe, 2001). Since the 1990s, chloroquine resistance is widespread leading to treatment failure of 70-80% in Africa and Southeast Asia (Vestergaard & Ringwald, 2007). This resistance development is the major cause of increased morbidity and mortality from malaria in recent decades (Trape *et al.*, 1998; Trape, 2001). It has been shown that a reversal of resistance is possible. The discontinued use of chloroquine in Malawi has led to renewed susceptibility of the parasites after seven years (Kublin *et al.*, 2003). However, this strategy requires complete abandonment of the drug for a long time period.

The resistance against chloroquine is linked with mechanisms that can lower the concentration of chloroquine within the parasite's food vacuole (Slater & Cerami, 1992). This results from a single point mutation in the plasmodial transporter *Pfcrt (P. falciparum* chloroquine resistance transporter) (Fidock *et al.*, 2000; Sidhu *et al.*, 2002). More recently, it was shown that another transporter gene named *P. falciparum* multiple-drug resistant gene (*Pfmdr1*) plays a role in modulating the levels of chloroquine resistance (Duraisingh & Cowman, 2005).

Resistance against sulphadoxine-pyrimethamine was first observed at the Thai-Cambodian border in the mid 1960s and results from a single nucleotide polymorphism in dihydrofolate reductase and dihydropteroate synthase (Wongsrichanalai *et al.*, 2002; Gregson & Plowe, 2005). The accumulation of multiple genetic mutations in the parasite led to the failure of this drug (Gatton *et al.*, 2004; Gesase *et al.*, 2009).

Mefloquine resistance was first observed in the early 1980s and is caused by an increased number of the transporter gene Pf*mdr*1 (Wongsrichanalai *et al.*, 2002; Price *et al.*, 2004).

In order to try to prevent the spread of resistant parasite strains, combination drugs with SP and mefloquine were designed (Peters, 1995). However, since resistance against the single drugs already existed, this combination was abandoned in the early 1990s (Schlagenhauf & Petersen, 2009). It is possible that the development of resistance was caused by the long half life of the drugs, which resulted in minimal drug concentrations in patients even weeks or months after treatment. Thus, parasites were able to encounter subinhibitory concentrations (Hastings *et al.*, 2002).

The fact that many antimalarial drugs evolved from similar basic chemical compounds increases the risk of resistance development. It was shown that in areas with mefloquine resistance, reduced efficacy can extend to lumefantrine, halofantrine and quinine (Cowman *et al.*, 1994; Wongsrichanalai *et al.*, 2002; Sidhu *et al.*, 2006).

Resistance against artemisinin *in vitro* has been known for more than two decades (Meshnick, 2002), but only recently first reports about higher recrudescence rates in *P. falciparum* after ACT treatment emerged from observational data (Denis *et al.*, 2006; Vijaykadga *et al.*, 2006). However, already in 2003 susceptibility reduction of the parasites to artesunate was reported to have occurred between 1988 and 1999 in Southwest China where artemisinin and its derivatives have been used excessively for over 20 years (Yang *et al.*, 2003). Initially it was not clear whether the reduced susceptibility was due to the partner drug, artemisinin or unusual host or pharmacokinetic factors (Alker *et al.*, 2007; Wongsrichanalai & Meshnick, 2008).
Very recently, the first clinical case of artemisinin resistance has been reported in Western Cambodia (Dondorp *et al.*, 2009). Reduced sensitivity of *P. falciparum* against ACT was found to be associated with a delayed clearance by artesunate (Dondorp *et al.*, 2009). Most likely this developing resistance is due to widespread monotherapy with artesunate in this region (Yeung *et al.*, 2008; Schlagenhauf & Petersen, 2009; Campbell, 2009) and counterfeit drugs (Newton *et al.*, 2001; Newton *et al.*, 2003; Newton *et al.*, 2006). Another alarming recent study showed a significant decrease of DHA susceptibility at the Thailand-Cambodia border (Noedl *et al.*, 2009).

It is therefore essential that containment steps are implemented to prevent the spread of resistance. In addition, the use of antimalarial drugs, for which resistance has been reported (*e.g.* mefloquine), in ACT has to be monitored carefully, as it can increase the risk of resistance development against the artemisinin derivatives. For example, artesunate-mefloquine combinations were the first ACT implemented (Nosten *et al.*, 1994) and have been successfully used in mefloquine-resistant areas (Carrara *et al.*, 2006). However, it was shown that re-emergence of malaria after artesunate-mefloquine treatment was linked to increased numbers of the plasmodial *Pfmdr1* gene which in turn is responsible for mefloquine resistance (Alker *et al.*, 2007). Recent reports showed declining efficacy and high failure rates of the artesunate-mefloquine combination at the Cambodia-Thailand border and in Southern Cambodia and declining efficacy of this combination from 1995-2007 on the Thai-Myanmar border (Wongsrichanalai & Meshnick, 2008; Carrara *et al.*, 2009; Rogers *et al.*, 2009).

Similarly, relatively high rates of treatment failure with the new DHA-piperaquine combination have been reported (Karunajeewa *et al.*, 2008). Before the establishment of this combination, piperaquine was extensively used in China as a monotherapeutical agent which led to local resistance (von Seidlein & Greenwood, 2003). However, in combination with DHA piperaquine showed good efficacy against malaria (Tran *et al.*, 2004).
Increasing resistance against amodiaquine (Ochong *et al.*, 2003) and the existing widespread resistance of SP should lead to careful monitoring of the effectiveness of the combination of these drugs with artesunate. So far, one study reported problems with artemether-lumefantrine combination in Cambodia, where a high level of mefloquine-resistance exists (Davis *et al.*, 2005).

Currently, ACTs are still effective but the time for successful treatment has increased (Dondorp *et al.*, 2010; Enserink, 2010b). Since the first indication of artemisinin resistance, reduced susceptibility to artemisinin is still confined to the Cambodia-Thailand border, although recently reduced clearance time has been observed on the border of Thailand and Myanmar, even if to a lesser extent (Carrara *et al.*, 2009). Containment steps have been implemented by the WHO in order to try and stop the spreading of these new resistant strains (Enserink, 2008). Failure of ACT would be disastrous as there are no alternative drugs to replace it. Currently, three artemisinin derivatives (artesunate, artemether and DHA) are used in combinations. However, their chemically close relation might lead to cross-resistance. Thus, the development of new drugs with novel structures or mechanisms of action which can replace artemisinin derivatives, or can be used in combination with them, is crucial.

### 1.1.10 Natural products in antimalarial research

Natural products are the major source for new drugs in many areas (*e.g.* inflammatory diseases, infectious diseases, Alzheimer, cancer) and for thousands of years, natural products have been used for the treatment or prevention of diseases. The main source was terrestrial plants, but other natural sources such as terrestrial microorganisms, marine macro- and microorganisms, and terrestrial vertebrates and invertebrates have become more important since the 20$^{th}$ century (Chin *et al.*, 2006).
An investigation of the new approved drugs between January 1981 and June 2006, and an evaluation of their origin, showed 1184 new chemical entities of which 28% were natural products or natural-product derived drugs and 24% were synthetic or natural mimic compounds, based on pharmacophores related to natural products (Newman & Cragg, 2007). The same study showed that in this time frame, nine antimalarial drugs (less than 1%

or the new drugs) were approved and among these only two were considered as totally synthetic drugs (halofantrine and lumefantrine) (Newman & Cragg, 2007).

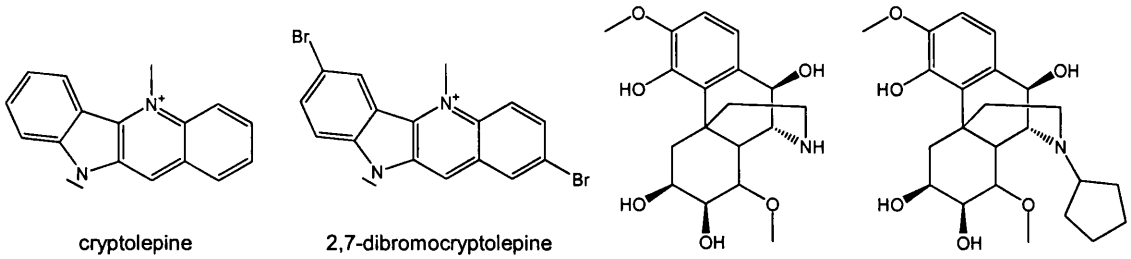
Plants have been proven to be the best sources for antimalarial drug discovery. The chemical diversity offered by plants is enormous and novel compounds with original structures are frequently described. Plants contain a complex mixture of several hundred plant metabolites, which are divided into primary and secondary metabolites. Primary metabolites are molecules needed for normal growth, development and reproduction and include amino acids, nucleotides, sugars and fatty acids. These components are basic building units and are essential for the survival of an organism. They are widely distributed in nature and occur in virtually all organisms. Hence, the primary metabolism of plants, animals, humans and prokaryotes bare significant similarities. Secondary metabolites on the other hand are not *per se* essential for an organism but they play a crucial role for the welfare of the organism and its interaction with the environment. They are derived from primary metabolites, their amounts are generally small and they are often produced for specific purposes such as chemical defence, protection or attractants. Their distribution can be limited to a specific plant part or to a specific family or genus, and can thus be used for chemotaxonomy. Secondary metabolites have been used for centuries as medicines, dyes or perfumes and are mainly responsible for the observed biological activities of plant extracts.

Malaria is an area where natural products have had the highest impact. The ethnobotanical study of plants used traditionally to treat malaria resulted in the isolation and identification of quinine and artemisinin, two blockbuster antimalarial drugs, which were also the template for several synthetic or semi-synthetic derivatives. Many chemical classes have been identified to be accountable for the antimalarial properties of plants, *e.g.* alkaloids, terpenoids (sesquiterpenes, limonoids, quassinoids) and phenolic compounds (chalcones, flavonoids, coumarins) (Kaur *et al.*, 2009).
Reviews summarising the results from antimalarial screenings of natural sources and isolated compounds are published frequently and 40 reviews have been published in the last 5 years about natural products in antimalarial drug discovery (source: PubMed) (Kaur *et al.*, 2009; Gademann & Kobylinska, 2009; Wright, 2010).

The indoloquinolide alkaloid cryptolepine and morphine alkaloid tazopsine (Fig. 1.19) are two examples of antimalarial compounds isolated from plants used traditionally for the treatment of malaria or prophylaxis. Cryptolepine was isolated from *Cryptolepis sanguinolenta* (Periplocaceae) and demonstrated good *in vitro* antiplasmodial activity ($IC_{50}$ of 0.11 μM) and

an *in vivo* effect (80% suppression at 50 mg/kg/d) when administered orally to mice (Wright *et al.*, 1996). However, its toxicity due to intercalation into DNA is a limiting factor, and synthetic derivatives were developed, with 2,7-dibromocryptolepin as best analogue with increased *in vitro* and *in vivo* potential and no toxicity (Bonjean *et al.*, 1998; Wright *et al.*, 2001).

Figure 1.19. Chemical structure of cryptolepine and tazopsine and their derivatives.




Tazopsine was isolated from *Strychnopsis thouarsii* (Menispermaceae), a plant traditionally used during malaria outbreaks as a preventative agent. It showed inhibition of *P. falciparum* blood stage and liver stage parasites ($IC_{50}$ of 4.7 μM and 4.2 μM, respectively) and exhibited some *in vivo* protection in mice (70% suppressive at 100 mg/kg/d) (Carraz *et al.*, 2006). Similarly to cryptolepine, toxicity was a limiting factor for this compound and the development of the semi-synthetic derivative NCP-tazopsine led to reduced toxicity *in vivo* and *in vitro* and stage-specificity towards the hepatic parasites (Carraz *et al.*, 2006).
More than 80 other alkaloids with antiplasmodial or antimalarial potential have been isolated between 1998 and 2008 (Kaur *et al.*, 2009).

The use of natural products for research purposes has to be assessed carefully and a sustainable supply of plant material needs to be ensured to prevent over-collection and species endangerment. An additional challenge with natural products is related to the consistency of the plant material. Chemical composition can be influenced by the collection time, environmental conditions or pathogenic infections and the yield of a drug can vary widely (Kamatou *et al.*, 2008c).

## 1.2 Introduction to this study

After a general introduction into malaria, this section will introduce the plants and natural products selected for this study.

### 1.2.1 Selected Turkish plants

#### 1.2.1.1 The genus *Salvia*

| | |
|---|---|
| Kingdom | Plantae |
| Subkingdom | Tracheobionta |
| Superdivision | Spermatophyta |
| Division | Magnoliophyta |
| Class | Magnoliopsida |
| Subclass | Lamiidae |
| Order | Lamiales |
| Family | Lamiaceae |
| Genus | *Salvia* L. |

The genus *Salvia* is with about 900 species worldwide the largest genus of the mint family Lamiaceae (formerly Labiatae). The genus is found in Central and South America, Eastern and Central Asia, the Mediterranean and South Africa (Walker *et al.*, 2004). Turkey has 87 *Salvia* species of which 44 are endemic (Hedge, 1982).

*Salvia* species are used in many parts of the world to treat various conditions including microbial infections, cancer, malaria, inflammation and amnesia (Kamatou *et al.*, 2008a). Plants from this genus are used in folk medicine for memory enhancement, wound healing, alleviating stomach, liver and rheumatism pains, treatment of common cold and insomnia or the reduction of blood pressure and blood sugar (Goeren *et al.*, 2006; Loizzo *et al.*, 2008). *Salvia* species have been used against perspiration and fever and as antiseptic (Kintzios, 2000). They are an important source of antioxidants in the diet and food industry (Kintzios, 2000). Additionally, *Salvia* species are used in cosmetics, perfumery and the pharmaceutical industry (Azcan *et al.*, 2004).

***Salvia virgata***

*S. virgata* Jacq. (wand sage) is a perennial herb and native in Western and Middle Asia (Iran, Iraq, Syria, Turkey, Kyrgyzstan, Turkmenistan), the Indian subcontinent (Pakistan) and Southeastern Europe (Albania, Bulgaria, Former Yugoslavia, Greece, Italy) (USDA, 2009). It grows in fallow fields, meadows, woodland, scrub and on roadsides.

*S. virgata* has a flowering stems and can become up to 1 m tall. The stems are simple or branched and covered with thickened hairs. Leaves are aromatic when crushed and have an oblong to ovate, slightly lobed form with a slightly heart-shaped base. The upper surface of the leaf is a dull green with sparsely thickened hairs, while the leaf under surfaces is covered with hairs. Flowers are arranged in whorls and have a violet-blue, rarely white colour and are 1-2 cm long (CDFA, 2008).

The traditional use of *S. virgata* decoction in Turkey was for the treatment of leukaemia (Baytop, 1999; Kosar *et al.*, 2008). Additionally, it was used for skin diseases and wound treatment (Akkol *et al.*, 2008). Turkish *S. virgata* has been shown to display antioxidant, antinociceptive and antiinflammatory activity (Kosar *et al.*, 2008; Akkol *et al.*, 2008; Tepe, 2008).

Several phytochemical investigations into this species have been performed and the findings will be briefly summarised. The total phenolic, flavonoids and flavonols contents have been assessed for Turkish and Iranian *S. virgata* (Kan *et al.*, 2007; Nickavar *et al.*, 2007; Kosar *et al.*, 2008; Akkol *et al.*, 2008; Tonsun *et al.*, 2009; Firuzi *et al.*, 2010). Analysis of the seed oil fatty acid composition showed that linoleic (18:2), linolenic (18:3), oleic (18:1) and palmitic acid (16:0) were the predominant fatty acids in this species (Azcan *et al.*, 2004; Bagci *et al.*, 2004; Goeren *et al.*, 2006).

Several compounds were identified in the plant extracts, including rosmarinic acid as main constituent, caffeic acid, gallic acid, *p*-OH benzoic acid, *o*-coumaric acid, luteolin, luteolin-7-*O*-glycoside, oleanolic acid and ursolic acid (Janicsak *et al.*, 2006; Kosar *et al.*, 2008). From Turkish *S. virgata*, flavonoids (5-hydroxy-3',4',7-trimethoxyflavone, salvigine, salvigenin-5-glycoside), diterpenoids (virgatol, horminone, 7-acetylhorminone, cryptanol, ferruginol), triterpenoids (virgatic acid, oleanolic acid, crataegolic acid) and phytosterols (β-amyrin, β-sitosterol) have been isolated (Ulubelen & Ayanoglu, 1975; Ulubelen & Ayanoglu, 1976; Ulubelen, 1989). Isolation of several triterpeneoids (ursolic acid, oleanolic acid, 2α,3α-dihydroxyolean-12-en-28-oic acid, crataegolic acid, 2α-hydroxyursolic acid, 2α,3α,23-trihydroxyolean-12-en-28-oic acid) and the terpenoid blumenol A was demonstrated for Italian *S. virgata* (Delatorre *et al.*, 1990). Interestingly, Delatorre *et al.* (1990) did not find any

virgatic acid, which is a major constituent in *S. virgata* from Turkey (Ulubelen & Ayanoglu, 1976).

Several *Salvia* species, mainly from South Africa, have been shown to posses promising antiplasmodial potential (Kamatou *et al.*, 2005; Kamatou *et al.*, 2008b). So far no study has been conducted examining the antiplasmodial potential of *S. virgata*, which was the reason to include this plant into the screening.

### 1.2.1.2 The genus *Scrophularia*

| | |
|---|---|
| Kingdom | Plantae |
| Subkingdom | Tracheobionta |
| Superdivision | Spermatophyta |
| Division | Magnoliophyta |
| Class | Magnoliopsida |
| Subclass | Lamiidae |
| Order | Scrophulariales |
| Family | Scrophulariaceae |
| Genus | *Scrophularia* L. |

The genus *Scrophularia* comprises about 300 species and belongs to the family Scrophulariaceae (figwort). Plants from this genus inhabit the Northern Hemisphere with most species found in Asia and few in Europe and North America. In Turkey 59 species are found of which 22 are endemic (Lall & Mill, 1978). *Scrophularia* species are perennial herbaceous flowering plants with opposite leaves and open two-lipped flowers forming clusters at the end of square stems.

*Scrophularia* species have been used in traditional medicine particularly as antiinflammatory agent and for dermatosis (Fernandez *et al.*, 1996). In addition, some species were used to treat fever, which is the main symptom of malaria (de Santos *et al.*, 2002). Our group was the first to show antiplasmodial activity and weak FabI inhibition of *S. cryptophila* and *S. lepidota* plant extracts and isolated metabolites (Tasdemir *et al.*, 2005a; Tasdemir *et al.*, 2005b; Tasdemir *et al.*, 2008). In continuation of this work, two unstudied Turkish *Scrophularia* species were selected for the screening.

### *Scrophularia lucida*

*Scrophularia lucida* L. grows on limestone and serpentine cliffs, dry riverbeds, rocky slopes and scree. The plants are distributed in the Arabian Peninsula (Saudi Arabia), Western Asia (Syria, Turkey), Caucasus (Armenia, Azerbaijan, Georgia, Russian Federation) and South Europe (Greece, Italy, France). This species has never been studied for its chemical composition and no biological activities have been reported.

### *Scrophularia pinardii*

*Scrophularia pinardii* Boiss. is endemic to Turkey and grows on limestone rocks and ruins. No studies have investigated the phytochemistry or biological activity of this plant.

### 1.2.1.3 The genus *Anthemis*

| | |
|---|---|
| Kingdom | Plantae |
| Subkingdom | Tracheobionta |
| Superdivision | Spermatophyta |
| Division | Magnoliophyta |
| Class | Magnoliopsida |
| Subclass | Asteridae |
| Order | Asterales |
| Family | Asteraceae |
| Genus | *Anthemis* L. |

The genus *Anthemis* contains around 130 species and belongs to the family Asteraceae (Compositae). Species are distributed predominantly in the Mediterranean, but can also be found in Southwest Asia and South Africa (Heywood & Humphries, 1978). Sixty-two species are located in Europe (Fernandes, 1976) of which 35 can be found in Greece (Fernandes, 1976), 24 in Bulgaria (Andreev *et al.*, 1992), 9 in Serbia (Gajic, 1975), 39 in Iran (Mozaffarian, 1998) and 50 in Turkey, of which 27 are endemic (Grierson & Yavin, 1975). The probably best known and studied species from this genus is *Anthemis nobilis* (synonym: *Chamaemelum nobile*), commonly known as Roman Chamomile. Some *Anthemis* species are referred to as dog-fennel (*A. cotula*) or mayweed (*A. arvensis*), which can lead to misunderstandings as mayweed in botanical terms correctly described the genus *Matricaria* and dog-fennel *Eupatorium capillifolium.*

*Anthemis* species are annual or perennial subshrubs with varying sizes (5-90 cm). Stems are usually branched with mostly cauline leaves. The leaves are obovate to spatulate and alternate. The flowers are usually white, but yellow and pink flowers can also be observed (Grierson & Yavin, 1975). Some *Anthemis* species are considered as weed and pose several problems in agriculture (Pinke & Pal, 2002).

The traditional use of *Anthemis* species goes back to Roman times, where plants from this genus have been used as antispasmodic and sedative agent or for the treatment of digestive and rheumatic disorders (Der Marderosian, 2000). In traditional Anatolian folk medicine *Anthemis* has been used for abdominal pain or as diuretic, antiinflammatory, analgesic, sedative, choleretic or carminative agent (Honda *et al.*, 1996; Baytop, 1999). Other traditional purposes of this genus include the use as dyes, insecticide, food additive, fragrance or as ingredient in cosmetics (Mabberley, 1997).

Over 400 studies have been conducted on *Anthemis* species with regards to their chemical composition and biological activities. In the following a brief overview will be given.

Extracts prepared from *Anthemis* species have been shown to exhibit antibacterial (Konstantinopoulou *et al.*, 2003; Barbour *et al.*, 2004; Akgul & Saglikoglu, 2005), antifungal (Lopez *et al.*, 2008), antioxidant (Povilaityte & Venskutonis, 2000; Djeridane *et al.*, 2007), antiviral (against *Herpes simplex* and *Parainfluenza*) (Orhan *et al.*, 2009) and antileishmanial activities (di Giorgio *et al.*, 2008). Essential oils from *Anthemis* species were shown to exhibit larvicidial activity against *Culex pipiens* (Grace, 2002), antimicrobial (Zani *et al.*, 1991; Albay *et al.*, 2009; Kurtulmus *et al.*, 2010), antifungal (Duarte *et al.*, 2005) and antiinflammatory activities and has shown sedative properties (Rossi *et al.*, 1988)
Biological activities reported from compounds isolated from *Anthemis* species include antibacterial activities of sesquiterpene lactones (Konstantinopoulou *et al.*, 2003; Saroglou *et al.*, 2010) and antiproliferative and cytotoxic activities by sesquiterpene lactones, cyclohexanones and cyclohexenones (Theodori *et al.*, 2006; Rethy *et al.*, 2007; Vuckovic *et al.*, 2010; Hajdu *et al.*, 2010). In addition, antiprotozoal activities of sesquiterpene lactones against *Leishmania* and *Trypanosoma* species was reported (Karioti *et al.*, 2009), but so far only one study from our group examined the antiplasmodial potential of isolated sesquiterpene lactones from *Anthemis auriculata*, including inhibition studies against plasmodial FAS-II enzymes and FabI enzymes of different microorganisms (Karioti *et al.*, 2007; Karioti *et al.*, 2008).

To gain further insights into the antiplasmodial potential of plants from this genus, two unstudied Turkish *Anthemis* species were selected for the screening. It should be noted that *Anthemis* species belong to the same plant family (Asteraceae) as *Artemisia annua*, the plant that provides the antimalarial drug artemisinin.

### Phytochemical investigation

Chemically, *Anthemis* species contain three main classes of bioactive compounds: polyacetylenes, flavonoids and sesquiterpene lactones. Polyacetylenes are predominantly found in the roots, while flavonoids and sesquiterpene lactones are only found in the aerial parts and flowers of the plants.

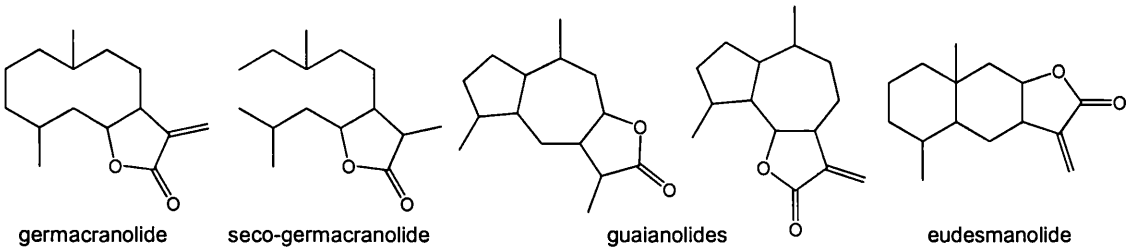
### Sesquiterpene lactones

Sesquiterpene lactones (SQLs) are one of the largest groups of natural compounds with approximately 5000 isolated structures. Their main occurrence is in higher plants of the

family Asteraceae and the majority contains an α-methylene-γ-lactone ring with an α-oriented H7 (Milosavljevic *et al.*, 1999).

The major skeletal types described in *Anthemis* are germacranolides, seco-germacranolides, eudesmanolides and guaianolides (Fig. 1.20). In addition, linear SQLs and some SQLs with unusual skeletons like anthecotulide were described (Staneva *et al.*, 2008).

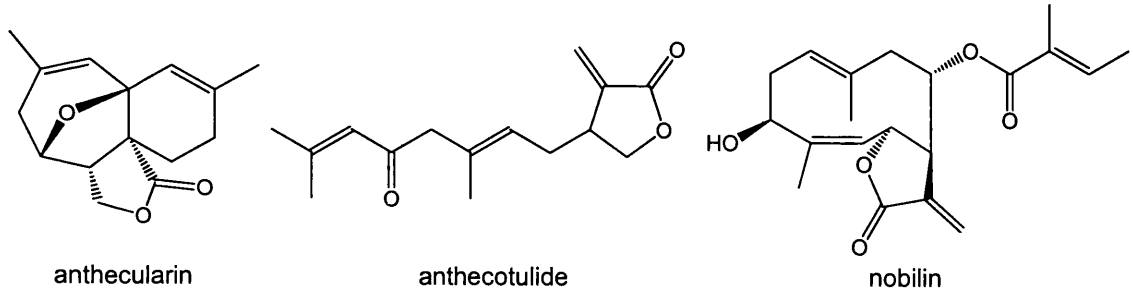
Figure 1.20. Major sesquiterpene lactone skeletons described in the genus *Anthemis*.




Anthecularin (Fig. 1.21), isolated by our group, is one example for a compound with a new ring system and showed antiplasmodial activity and inhibition towards plasmodial FabI and FabG (Karioti *et al.*, 2007). Anthecotulide (Fig, 1.21) is considered to be one of the most potent contact allergens and has been found in *A. auriculata* and *A. cotula* (Hausen *et al.*, 1984; Theodori *et al.*, 2006; Vuckovic *et al.*, 2006a). The first SQL isolated from an *Anthemis* species (*A. nobilis*) was nobilin (Fig. 1.21) (Benesova *et al.*, 1964). Later its hydroxyperoxide 1β-hydroperoxyisonobilin and 4α-hydroperoxyromanolid were discover in flowers of German *A. nobilis* (Rucker *et al.*, 1989).

So far, over 150 SQLs have been found in numerous *Anthemis* species. An extensive list of all SQLs can be found in the appendix, it is based on a publication from Staneva *et al.* (2008) who reviewed 111 SQLs. The list contains all SQLs and other sesquiterpenes isolated from the genus *Anthemis* from English and German reports published between 1964 and November 2010.

Figure 1.21. Chemical structure of selected sesquiterpene lactones from the genus *Anthemis*.

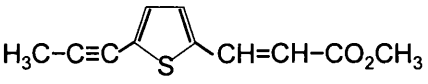


## Polyacetylenes

Polyacetylenes (Fig. 1.22) are organic polymers with repetitive $C_2H_2$ units and often contain only a single acetylenic bond, so the term "polyacetylenes" can be misleading. Most of these compounds have been isolated from the roots of *Anthemis* species in the 1960s and 70s by the research group of Bohlmann (Bohlmann *et al.*, 1962; Bohlmann & Zdero, 1970).

Fig. 1.22. Selected polyacetylenes from the genus *Anthemis*.

$H_3C-[C\equiv C]_3-[CH=CH]_3-H$



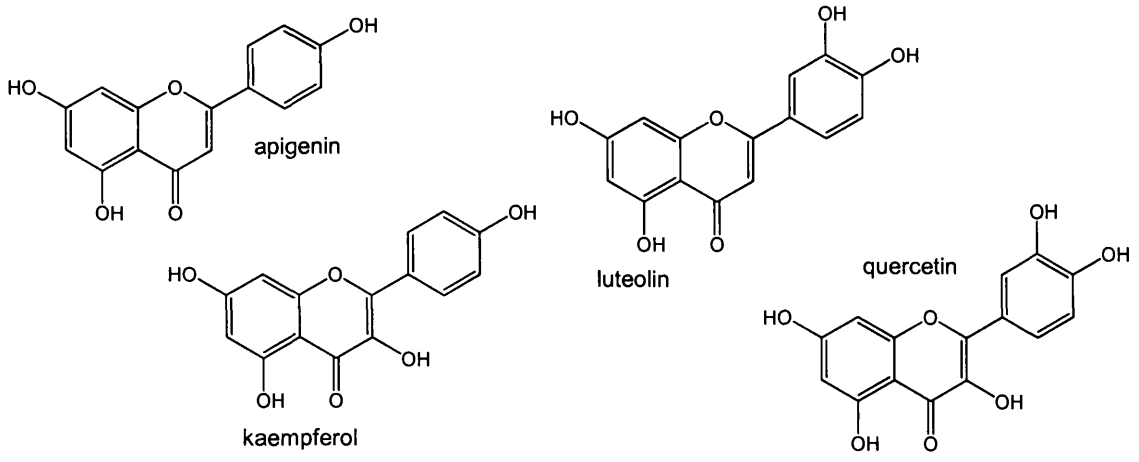
$H_3C-C\equiv C$ S $CH=CH-CO_2CH_3$

$H_3C-[C\equiv C]_3-CH=CH-CONHCH_2CH(CH_3)_2$

## Flavonoids

Flavonoids belong to the big group of phenolic compounds with more than 4000 types identified in vascular plants. More than 70 flavonoids, including apigenin, kaempferol, luteolin and quercetin (Fig. 1.23), have been isolated from *Anthemis* species. A comprehensive list of flavonoids reported from the genus *Anthemis* can be found in the appendix.

Figure 1.23. Chemical structure of selected flavonoids from the genus *Anthemis*.

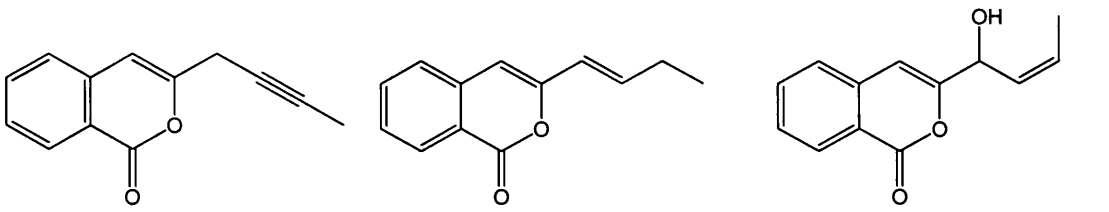



## Coumarins

Only few coumarins and coumarin derivatives have been isolated from *Anthemis* species (Fig. 1.24). In addition to the common coumarins herniarin (Saleh & Rizk, 1974), scopoletin (Abou-Zied & Rizk, 1973; Saleh & Rizk, 1974; Bruno *et al.*, 1991; Pavlovic *et al.*, 2006; Hanganu *et al.*, 2008), scopolin (Pavlovic *et al.*, 2006) and umbelliferone (Pavlovic *et*

*al.*, 2006; Hanganu *et al.*, 2008), the isocoumarins capillarin, (E)-artemidin and 3-(1-hydroxy-buten-(2c)-yl)-isocumarin (Bohlmann & Zdero, 1970), which are only found in the family Asteraceae, have been described.

Fig. 1.24. Chemical structure of isocoumarins from the genus *Anthemis*.




capillarin (E)-artemidin 3-(1-hydroxy-buten-(2c)-yl)-isocumarin

## Other phenolic compounds

The phenolic content of five *Anthemis* species was analysed. Gallic acid was found to be the dominant phenolic metabolite of *A. arvensis* collected from Algeria (Stocker *et al.*, 2004). Further studies on this species showed a total phenolic content of 115.2 mg gallic acid equivalent/g dw with 4% flavonoids (quercetin), 86% hydroxy-cinnamic derivatives (caffeic acid) and 10% hydroxy-benzoic derivatives (gallic acid). Additionally chlorogenic acid, ferulic acid and the flavonoid glycoside rutin were detected (Djeridane *et al.*, 2007).

*Anthemis palestina* from Jordan was shown to possess a total phenolic content of 23.2 mg gallic acid equivalent/g dw (Tawaha *et al.*, 2007), *A. nobilis* from Lithuania 49.6 mg gallic acid equivalent/g dw and 59.6 mg chlorogenic acid equivalent/g dw (Povilaityte & Venskutonis, 2000).

*Anthemis tinctoria* and *A. triumfettii* leaves from Turkey have a total phenolic content of 9.5 and 9.6 mg phenol equivalent/g dw and 146 and 122.9 mg tannic acid equivalent/g dw condensed tannins (proantocyanidins), respectively (Ayaz *et al.*, 2003).

Additionally, the benzoic acid derivatives methyl vanillate (4-hydroxy-3-methoxy benzoic acid methyl ester), isovanillic acid (3-hydroxy-4-methoxy benzoic acid) and *p*-anisic acid (4-methoxybenzoic acid), caffeic acid, chlorogenic acid, protocatechic acid, 3,4-di-*O*-caffeoylquinic acid and 3,5-di-*O*-caffeoylquinic acid were isolated from *Anthemis* species (Power & Browning, 1914; Konstantinopoulou *et al.*, 2003; Theodori *et al.*, 2006; Pavlovic *et al.*, 2006; Skaltsa *et al.*, 2006; Saroglou *et al.*, 2007b).

## Sterols

The sterols found in the genus *Anthemis* are also common in many other plant families. The first sterols identified in the early 20$^{th}$ century in *A. nobilis* were taraxasterol and β-sitosterol-glucoside (Power & Browning, 1914). In addition, campesterol, phytol, β-

sitosterol, stigmasterol and taraxasterol acetate have been isolated from different *Anthemis* species (Baruah *et al.*, 1985; El-Alfy *et al.*, 1989; Zaghloul *et al.*, 1989; Staneva *et al.*, 2004).

### Carotenoids

An analysis of the carotenoid composition from the flowers of *A. tinctoria* showed the presence of neoxanthin, lutein, violaxanthin, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, α-carotene and β-carotene (Hanganu *et al.*, 2008).

### Fatty acids

Early studies with flowers of *A. nobilis* from Belgium showed the presence of cerotic acid (26:0) and suggested the occurrence of oleic (18:1), linoleic (18:2), palmitic (16:0) and stearic (18:0) acids (Power & Browning, 1914). Linoleic acid was later shown to be a constituent in the aerial parts of *A. aciphylla* and the same study detected linoleic acid methyl ester in *A. cretica* subsp. *tenuiloba* (Bohlmann & Zdero, 1975).

More recent reports have shown the presence of myristic (14:0), palmitic (16:0), stearic (18:0), arachidic (20:0), oleic (18:1), linoleic (18:2), and α-linolenic acid (18:3) in flowers of *A. tinctoria* (Hanganu *et al.*, 2008; Orhan *et al.*, 2009). In addition, behenic (22:0), lignoseric (24:0) and cerotic acids (26:0) have been found in the aerial parts of *A. tinctoria* and palmitic, stearic, lignoseric and cerotic acid were shown in *A. austriaca* (Orhan *et al.*, 2009).

### Miscellaneous compounds

Other compounds found in *Anthemis* species include five hydroperoxides (Rucker *et al.*, 1989), cyclohexenones antheminones A and B, cyclohexanone antheminone C, triglyceride 2-*trans*,*trans*-sorbo-1,3-dimyristin (Collu *et al.*, 2008), cyclohexanehexol (Power & Browning, 1914), cyanogenic diglycosides epilucumin and two derivatives (Nahrstedt *et al.*, 1983) and a cyclitol glucoside (Papaioannou *et al.*, 2007).

### *Anthemis pestalozzae*

*Anthemis pestalozzae* Boiss. (synonym: *Cota pestalozzae*) is a completely unstudied annual plant and endemic to Turkey. Its size ranges from 5 to 15 cm. The stem is branched and radiating from the base. Leaves are 2-pinnatisect and ovate-oblong in the outline. The ray flowers are coloured pale violet-pink and the disc corolla is violet-pink. This species grows on limestone and outcrops (Grierson & Yavin, 1975).

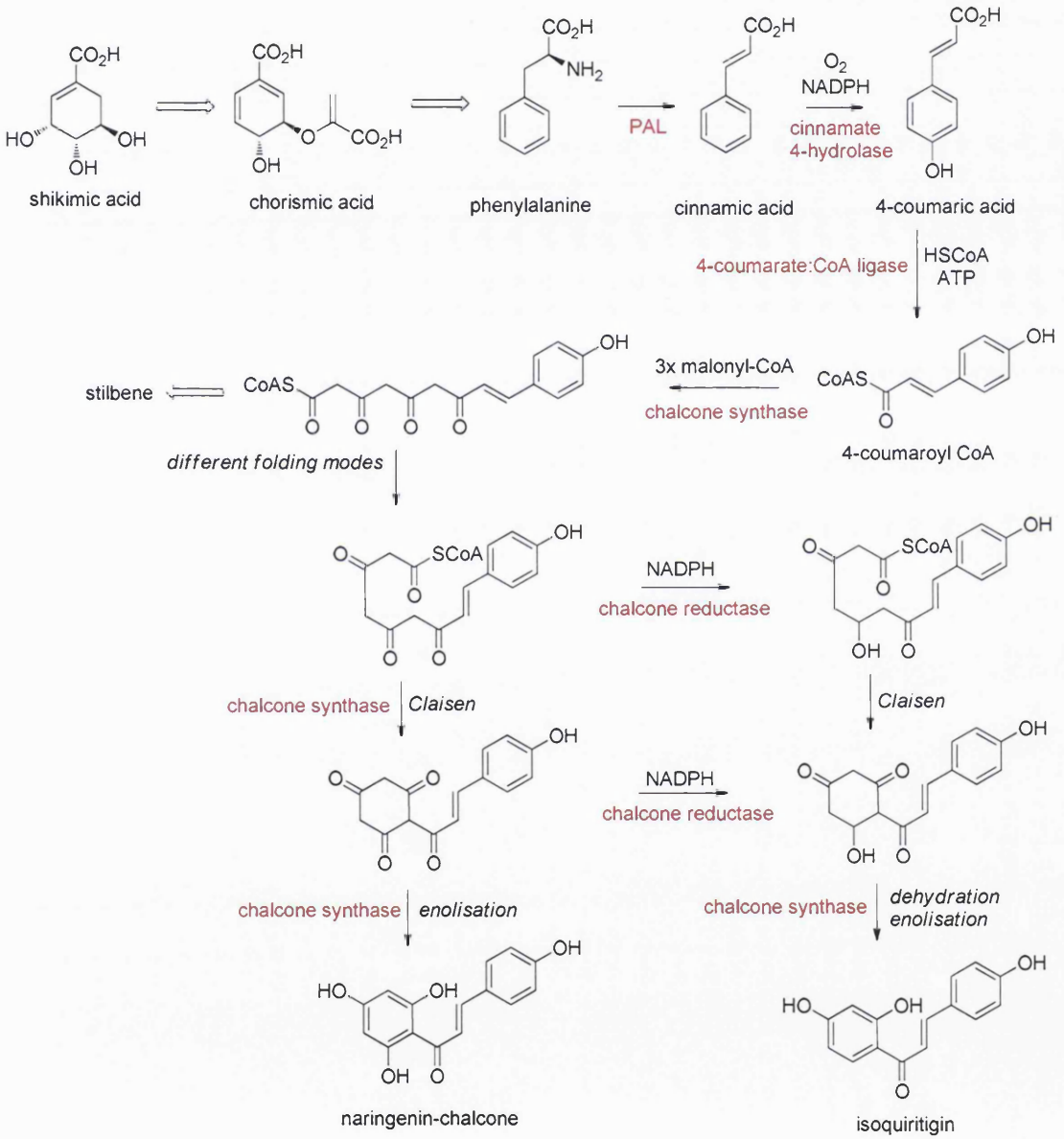
### *Anthemis cretica* subsp. *anatolica*

*Anthemis cretica* L. subsp. *anatolica* (Boiss.) Grierson is a short perennial herb which grows in steppe, on roadsides and limestone hillsides. The stem can be strongly woody at its base and reaches a size of 10-30 cm. Leaves are pinnatisect and generally less than 2 cm in size. The ray flowers are coloured white and the disk flowers are yellow. It can be found in Southeastern Europe and Western Asia (Grierson & Yavin, 1975). So far, only one study in the 1970s has been conducted on this species, showing the present of thioenol ether in the roots (Bohlmann & Zdero, 1975).

### 1.2.2 Natural chalcones

Chalcones are a chemical group that acts as precursors for the well-known family of flavonoids, which have been shown to exhibit *in vitro* activities against FAS-II enzymes and *P. falciparum* blood stage parasites (Tasdemir *et al.*, 2006). Chalcones are biosynthesised via the shikimate pathway as depicted in Figure 1.25.

Figure 1.25. Chalcone biosynthesis pathway (adopted from Dewick (2009)).



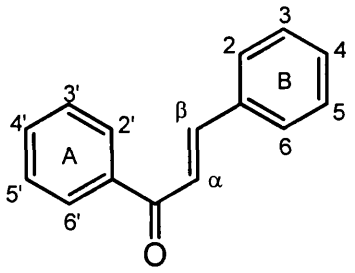

Chalcones have been shown to display wide range of pharmacological activities, including antiprotozoal (Nielsen *et al.*, 1998b; Zhai *et al.*, 1999; Torres-Santos *et al.*, 1999; Kayser & Kiderlen, 2001; Chen *et al.*, 2001; Liu *et al.*, 2003), antibacterial (Nielsen *et al.*, 2004; Nielsen *et al.*, 2005), antiviral (Cheenpracha *et al.*, 2006), antiinflammatory (Hsieh *et al.*, 1998; Barfod *et al.*, 2002; Babu *et al.*, 2002), anticancer (Ducki *et al.*, 1998) and antioxidative activity

(Wang *et al.*, 2009). Cytotoxicity against various human cancer cell lines have also been described (Bhat *et al.*, 2005).

One of the first chalcones shown to possess antiplasmodial potential was phloridzin, a glycosidic dihydrochalcone isolated from *Micromelum tephrocarpum* (Rutaceae) (Phillipson & Wright, 1991). The interest in chalcones as antimalarial agents increased when licochalcone A was isolated from Chinese liquorice roots (*Glycyrrhiza glabra*, Fabaceae), and it was shown to possess good antiplasmodial potential (Chen *et al.*, 1994a). An additional study, published shortly afterwards, looked into synthetic chalcones and their derivatives as antimalarial agents by using structure based modelling studies and *in vitro* testing emphasized the antiplasmodial potential of chalcones (Li *et al.*, 1995).
Since then, large libraries of synthetic chalcones have been tested for antimalarial activity (Chen *et al.*, 1997; Dominguez *et al.*, 2001; Wu *et al.*, 2002; Dominguez *et al.*, 2005a; Dominguez *et al.*, 2005b; Mishra *et al.*, 2008; Dominguez *et al.*, 2009; Casano *et al.*, 2010) and numerous synthetic chalcones and their derivatives were assessed for their structure-activity relationship (Chen *et al.*, 1994a; Li *et al.*, 1995; Nielsen *et al.*, 1998b; Liu *et al.*, 2001; Valla *et al.*, 2006; Wu *et al.*, 2006; Lim *et al.*, 2007). Some chalcones were shown to inhibit malarial cystein proteases, but it is unclear if the antimalarial activity is primarily due to inhibition of these enzymes (Li *et al.*, 1995). More recent publications indicate that inhibition of the heme formation might be involved in the antimalarial activity of chalcones (Dominguez *et al.*, 2005b; Bhattacharjee *et al.*, 2007).

So far no study concentrated on the antiplasmodial activities of natural occurring chalcones and only few natural chalcones have been studied in this respect (Chen *et al.*, 1994a; Yenesew *et al.*, 2004; Frolich *et al.*, 2005; Narender *et al.*, 2005; Joseph *et al.*, 2007; Montenegro *et al.*, 2007). Thus, a screening of 22 commercially available, naturally occurring chalcones with respect to their antiplasmodial potential against erythrocytic parasites was performed in this study. Recently liver stage inhibition was demonstrated for licochalcone A (Mahmoudi *et al.*, 2008), however the FAS-II pathway as possible target in liver stage parasites has not been examined. Thus, FAS-II enzyme inhibition studies were conducted to assess the potential of natural chalcones against this liver stage target and their possible prophylactic potential. Table 1.1 gives an overview about the natural chalcones used in this study.

Table 1.1. Natural chalcones investigated in this study.




| Compound | Ring A | Ring B |
|---|---|---|
| Licochalcone A | 4'-OH | 4-OH, 2-$OCH_3$, 5-$C_4H_8$ |
| Chalcone | H | H |
| 2'-Hydroxychalcone | 2'-OH | H |
| 2-Hydroxychalcone | H | 2-OH |
| 4'-Hydroxychalcone | 4'-OH | H |
| 4-Hydroxychalcone | H | 4-OH |
| 4,2',4'-Trihydroxychalcone (Isoliquiritigenin) | 2',4'-OH | 4-OH |
| 3,4,2',4'-Tetrahydroxychalcone (Butein) | 2',4'-OH | 3,4-OH |
| 4,2',4',6'-Tetrahydroxydihydro-chalcone (Phloretin) | 2',4',6'-OH | 4-OH |
| 3,4,2',4',6', -Pentahydroxy-chalcone (Eriodictyolchalcone) | 2',4',6'-OH | 3,4-OH |
| 4'-Methoxychalcone | 4'-$OCH_3$ | H |
| 4-Methoxychalcone | H | 4-$OCH_3$ |
| 3,4-Dimethoxychalcone | H | 3,4-$OCH_3$ |
| 4,4'-Dimethoxychalcone | 4'-$OCH_3$ | 4-$OCH_3$ |
| 2,3-Dimethoxy-2'-hydroxy-chalcone | 2'-OH | 2,3-$OCH_3$ |
| 4,2'-Dihydroxy-4',6'-dimethoxy-chalcone | 2'-OH, 4',6'-$OCH_3$ | 4-OH |
| 2'-Hydroxy-4,4',6'-trimethoxy-chalcone | 2'-OH, 4',6'-$OCH_3$ | 4-$OCH_3$ |
| 2',6'-Dihydroxy-4,4'-dimethoxy-chalcone | 2',6'-OH, 4'-$OCH_3$ | 4-$OCH_3$ |
| 2',6'-Dihydroxy-4,4'-dimethoxy-dihydrochalcone | 2',6'-OH, 4'-$OCH_3$ | 4-$OCH_3$ |
| 4,2',4'-Trihydroxy-3-methoxy-chalcone (Homobutein) | 2',4'-OH | 4-OH, 3-$OCH_3$ |
| 4,4',6'-Trihydroxydihydro-chalcone-2'-*O*-glucoside (Phloridzin) | 4',6'-OH, 2'-glucoside | 4-OH |
| 3,4,2',3',4'-Pentahydroxy-4'-glucosylchalcone (Marein) | 2',3',-OH, 4'-glucoside | 3,4-OH |

Several of the natural chalcones selected for this study have been shown to display biological activities. Table 1.2 summarizes some of the reported activities. Seven of the natural chalcones (licochalcone A, phloridzin, 4'-hydroxychalcone, 4'-methoxychalcone, 4,4'-dimethoxychalcone, chalcone, homobutein) have been tested *in vitro* for their

antiplasmodial potential against blood stage parasites previously. In addition, antimalarial *in vivo* activity has been reported for licochalcone A (Chen *et al.*, 1994a; Ziegler *et al.*, 2004).

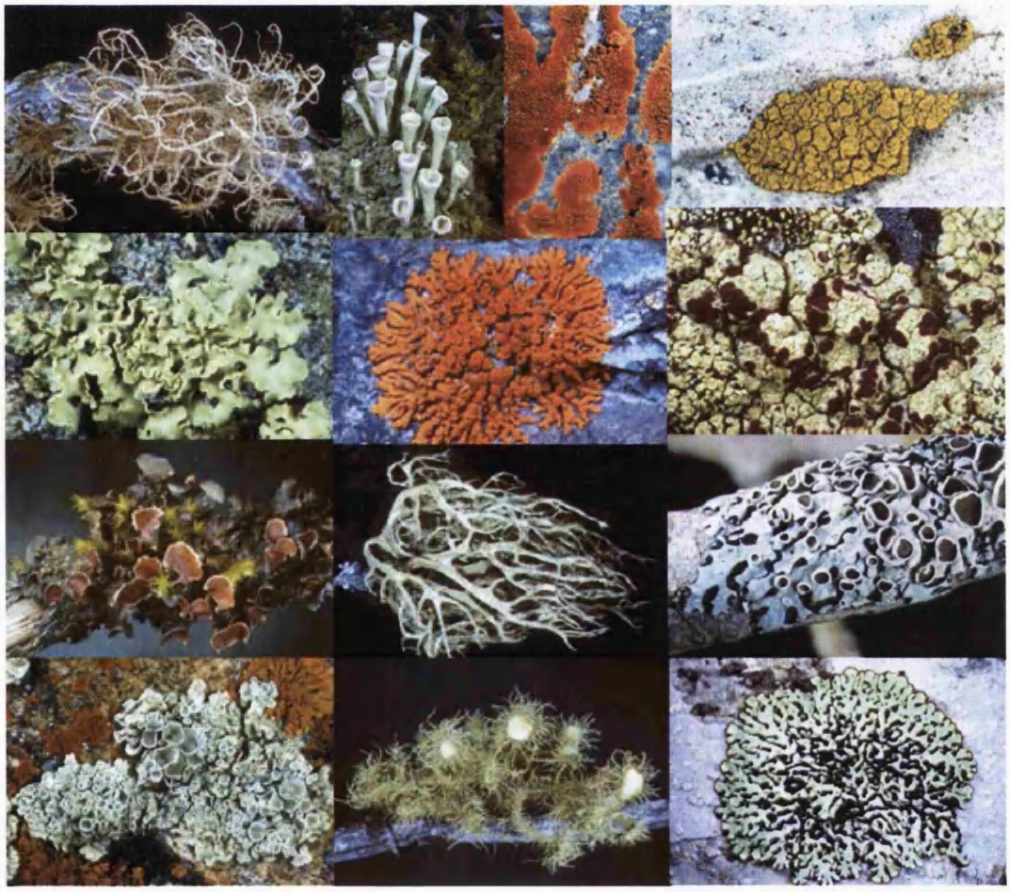
Table 1.2. Reported biological activities of natural chalcones selected for this study.

| Chalcone | Biological activity | References |
|---|---|---|
| Butein | antioxidative | (Wang *et al.*, 2009) |
| | antileishmanial | (Nielsen *et al.*, 1998a) |
| Phloridzin | antiplasmodial | (Cabantchik *et al.*, 1983; Kutner *et al.*, 1987; Loyevsky & Cabantchik, 1994; Montenegro *et al.*, 2007) |
| | antileishmanial | (Kayser & Kiderlen, 2001) |
| Phloretin | antileishmanial | (Kayser & Kiderlen, 2001) |
| Licochalcone A | antiplasmodial | (Chen *et al.*, 1994a; Nielsen *et al.*, 1998b; Mahmoudi *et al.*, 2008; Bhattacharya *et al.*, 2009) |
| | antibacterial | (Okada *et al.*, 1989; Tsukiyama *et al.*, 2002; Friis-Moller *et al.*, 2002; Fukai *et al.*, 2002; Nielsen *et al.*, 2004; Nielsen *et al.*, 2005) |
| | antileishmanial | (Chen *et al.*, 1993; Zhai *et al.*, 1995; Nielsen *et al.*, 1998a; Nielsen *et al.*, 1998b; Chen *et al.*, 2001) |
| | antitrypanosomal | (Troeberg *et al.*, 2000) |
| | immunomodulatory | (Barfod *et al.*, 2002) |
| 4'-Hydroxychalcone | antiplasmodial | (Liu *et al.*, 2001) |
| | antileishmanial | (Nielsen *et al.*, 1998a; Liu *et al.*, 2003) |
| 4-Hydroxychalcone | antiinflammatory | (Hsieh *et al.*, 1998) |
| 4,4'-Dimethoxychalcone | antiplasmodial | (Liu *et al.*, 2001) |
| | antileishmanial | (Nielsen *et al.*, 1998a; Liu *et al.*, 2003) |
| | cytotoxic | (Bhat *et al.*, 2005) |
| 4'-Methoxychalcone | antiplasmodial | (Liu *et al.*, 2003) |
| | antileishmanial | (Liu *et al.*, 2003) |
| | cytotoxic | (Bhat *et al.*, 2005) |
| 4-Methoxychalcone | cytotoxic | (Bhat *et al.*, 2005) |
| | antileishmanial | (Kayser & Kiderlen, 2001) |
| Homobutein | antiplasmodial | (Yenesew *et al.*, 2004) |
| | antileishmanial | (Nielsen *et al.*, 1998a) |
| Chalcone | antiplasmodial | (Wu *et al.*, 2006) |
| | cytotoxic | (Bhat *et al.*, 2005) |
| | antileishmanial | (Nielsen *et al.*, 1998a) |
| Isoliquiritigenin | antileishmanial | (Kayser & Kiderlen, 2001) |
| 2'-Hydroxychalcone | antileishmanial | (Kayser & Kiderlen, 2001) |

### 1.2.3 Lichen secondary metabolites

Lichens (Fig. 1.26) are symbiotic associations between an exhabitant fungus (mycobiont) and one or more inhabitant photosynthetic partners (photobionts). In most cases the fungi are Ascomycota, and only a small numbers of lichens (approximately 2%) have Basidiomycota or anamorphic fungi as mycobionts. The lichen name refers to the fungal partner and about 21% of all fungi are able to life in this complex ecosystem (Honegger, 1991). The number of possible photobionts is much smaller, with 25 algae genera and 15 cyanobacteria genera (Kirk *et al.*, 2008). In most cases the photobionts in a lichen species is not known (Honegger, 1991), although cyanobacteria are typically found in lichen inhabiting low-resources environments.

Figure 1.26. Different lichen species.



About 18,500 lichen species are known to date with new species discovered frequently (Molnar & Farkas, 2010). Lichens can be found worldwide from tropical to arctic and from aquatic to xeric regions. They live in the most extreme environmental conditions (Nash, 2008), and even have been shown to survive in space (Sancho *et al.*, 2007). Lichens colonise numerous different substrates such as rocks, soil, metal, leaves, bark, plastic or glass (Cocchietto *et al.*, 2002).

Secondary lichen metabolites are chemically diverse aliphatic and aromatic compounds with a relatively low molecular weight (Turk *et al.*, 2003). They are produced by the mycobiont, but the photobionts might have an influence on the secondary metabolism (Huneck, 1999; Brunauer *et al.*, 2007). So far approximately 1050 secondary lichen metabolites have been identified and their amounts range from 0.1% to 10%, or even can account for up to 30% of the dry weight (Stocker-Worgotter, 2008). The distribution of the secondary metabolites is usually specific and can be used for chemotaxonomy and systematic analysis (Molnar & Farkas, 2010).

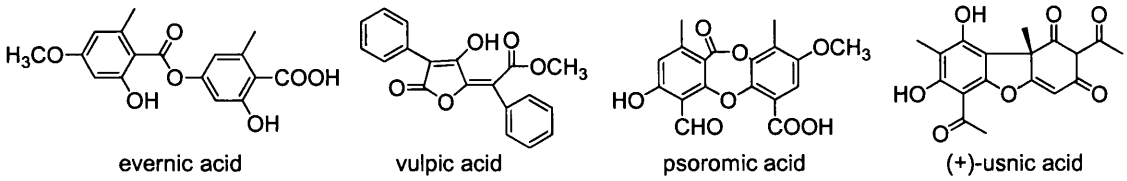
The biosynthesis of lichen secondary metabolites occurs via the acetyl-polymalonyl, the mevalonic acid and shikimic acid pathway (Molnar & Farkas, 2010). Most lichen secondary metabolites are derived from acetyl-CoA via the acetyl-polymalonyl pathway. Compounds can be synthesised via this pathway directly (*e.g.* usnic acid, anthraquinones, xanthones, chromones) or via orsellinic acid as a pre-cursor (*e.g.* depsides, depsidones, dibenzofuranes). Alternatively, a small number of lichen secondary metabolites is derived from the pentose phosphate cycle and amino acid biosynthesis and synthesised via the shikimic acid pathway (*e.g.* vulpinic acid, pulvinic acid derivatives). Most compounds synthesised via these pathways are unique to lichens. Secondary metabolites derived from acetyl-CoA via the mevalonic acid pathways are also commonly found in other organisms and include terpenes, carotenoids and steroids (Nash *et al.*, 2002).

Lichens are extremely slow-growing and long-living organisms and over the centuries have been used for a variety of traditional medicinal purposes, *e.g.* for Egyptian wound dressing (Vartia, 1973), as antibiotics (Vartia, 1950), for coughing (including that associated with tuberculosis) (Vartia, 1973) or as antifebrile agent (Okuyama *et al.*, 1995). Other applications include the use for embalming by the ancient Egyptians, as food, as dye or to monitor pollution levels in industrial areas and cities (Cocchietto *et al.*, 2002; Molnar & Farkas, 2010).

Lichens and their secondary metabolites have been the subject of many studies and a broad range of biological activities have been reported (Molnar & Farkas, 2010). By far the most extensive studied compound of all secondary metabolites identified in lichens is (+)-usnic acid and its enantiomer (-)-usnic acid.

For this study, the four commercially available secondary lichen metabolites evernic acid, vulpic acid (synonym: vulpinic acid), psoromic acid and (+)-usnic acid (Fig. 1.27) were selected.

Figure 1.27. Secondary lichen metabolites




Usnic acid was first isolated in 1844 by Knop (Knop, cited in Ingolsdottir (2002)) and it is widely distributed in several lichen genera including *Usnea* and *Evernia* (Ingolfsdottir, 2002). Evernic acid was described in *Evernia prinastri* in 1883 (Schwarz, 1883) and later also found other genera, including *Usnea* (Nishitoba *et al.*, 1987). Vulpinic acid was first identified in 1831 by Bebert and later isolated from *Letharia vulpina*, liverwort and non-lichenised fungi (Duncan *et al.*, 2003; Toyota *et al.*, 2004). Psoromic acid was first described in *Psoroma crassum* in 1882 by Spica and later described in several other genera (Huneck & Sargent, 1976; Krebs *et al.*, 2004).

Several studies were conducted to assess the *in vitro* microbial activity of these lichen compounds and only selected publications will be referenced in order to give an overview. (+)-Usnic acid was found to demonstrate activity against a variety of gram-positive bacteria, pathogenic anaerobic gram-negative bacilli, anaerobic gram-positive bacteria and *Mycobacterium* species, but showed no effect against gram-negative bacteria such as *E. coli* (Lauterwein *et al.*, 1995; Francolini *et al.*, 2004; Honda *et al.*, 2010). Vulpinic acid, psoromic acid and evernic acid also have been shown to inhibit the growth of gram-positive bacteria (Shibata *et al.*, 1948; Krog, 1954; Lauterwein *et al.*, 1995). Antimycobacterial activity of a mixture containing usnic acid and evernic acid was found (Klosa, 1948), and vulpic acid was also shown to display antimycobacterial properties (Naito *et al.*, 1953), whereas psoromic acid was inactive against *M. tuberculosis* (Shibata *et al.*, 1948).

In addition to these antimicrobial activities, several other biological activities have been described for the secondary lichen metabolites (+)-usnic acid, evernic acid, vulpic acid and psoromic acid and will be listed briefly.

- Analgesic and antipyretic properties for (+)-usnic acid *in vivo* (Okuyama *et al.*, 1995)
- Antiplasmodial activity of (+)-usnic acid *in vitro* (Verotta *et al.*, 2007)
- Antileishmanial activity of (+)-usnic acid *in vitro* and *in vivo* (Fournet *et al.*, 1997)
- Cytotoxicity against murine hepatocytes *in vitro* of (+)-usnic acid (Han *et al.*, 2004)

- Cytotoxicity against human cancer cell lines *in vitro* of (+)-usnic acid, vulpic acid and psoromic acid (Nakazawa *et al.*, 1962; Mayer *et al.*, 2005; Burlando *et al.*, 2009; Backorova *et al.*, 2010)
- Antifungal activity for (+)-usnic acid and evernic acid (Halama & van Haluwin, 2004; Rankovic *et al.*, 2008)
- Antiviral activities for (+)-usnic acid against Junin, Tacaribe and Epstein-Barr viruses and for evernic acid against the Epstein-Barr virus (Yamamoto *et al.*, 1995; Fazio *et al.*, 2007)
- Antiinflammatory activity of (+)-usnic acid in rats (Vijayakumar *et al.*, 2000)
- Wound closure effect of (+)-usnic acid and vulpic acid (Burlando *et al.*, 2009)
- Gastroprotective effect of (+)-usnic acid *in vivo* (Odabasoglu *et al.*, 2006)
- Photoprotection of the photobiont against UV radiation by usnic acid and vulpinic acid (Galloway, 1993)
- Phytotoxic activity of evernic acid, psoromic acid and vulpinic acid (Huneck & Schreibe, 1972; Whiton & Lawrey, 1982; Nishitoba *et al.*, 1987)
- Antifeedant activity of usnic acid and vulpinic acid against *Spodoptera littoralis* insect larvae (Emmerich *et al.*, 1993)

The lichen compounds evernic acid, vulpic acid, psoromic acid and (+)-usnic acid have been selected because lichens from the genera *Usnea* are mentioned in the ancient Chinese text "Shen Nong's Herbal Classic" as a crude drug and have been used for centuries to treat fever and for pain relief on many continents. In the mid 1990s usnic acid isolated from *Usnea diffracta* was identified as the compound responsible for the antipyretic effect in mice *in vivo* (Okuyama *et al.*, 1995), which is particularly interesting, as fever is a dominant symptom of malaria. The earliest malaria cases go back 3500 years (Nerlich *et al.*, 2008) whereas the parasite has only been identified in 1880, thus natural products with known antipyretic properties might have been used as antimalarial drugs.

In addition, recently the *in vitro* antiplasmodial activity of usnic acid against erythrocytic parasites was demonstrated (Verotta *et al.*, 2007). However, so far the antiplasmodial potential of the other three lichen metabolites has not been assessed and no detailed study has been undertaken on malaria prophylactic activity of lichen secondary metabolites.

### 1.3 Aim of the current study

The aim of this thesis was the identification of potentially new natural products and evaluation of existing natural products for their potential as antiplasmodial and malaria prophylactic agents. We employed antiplasmodial assays against blood stage and liver stage parasites, cytotoxicity studies to determine selectivity and inhibition assays against the FAS-II elongation enzymes FabG, FabZ and FabI as potential targets in liver stage parasites.
In addition, to conclusively show the selectivity of our extracts and compounds, we attempted to introduce the human fatty acid synthase (hFAS) into our screening panel. It has been reasoned that the structural and organisational differences between the plasmodial and human FAS enzymes render the fatty acid biosynthesis pathway as a good target, but thus far no study has assessed potential plasmodial FAS-II enzyme inhibitors against the human FAS-I enzyme in the same study.

As described in the introduction (see 1.1.10), natural products are excellent sources for novel drugs to treat malaria as they provide a great range of structural diversity. Artemisinin and quinine are the two major examples for compounds isolated from plants which are invaluable antimalarial drugs, confirming the potential of natural products in malaria chemotherapy. Semi-synthetic and synthetic derivatives of these compounds, such as chloroquine, artesunate or amodiaquine, have been developed to improve their therapeutic potential. The malaria prophylactic potential of natural products merits detailed investigation for drug discovery as so far only two natural products (licochalcone A and tazopsine) have been identified with activity against liver stage parasites.

Previous studies have shown that members of the genera *Salvia*, *Scrophularia* and *Anthemis*, the latter belongs to the same family as the artemisinin-producing plant *Artemisia annua*, display significant antiplasmodial potential (Tasdemir *et al.*, 2005b; Karioti *et al.*, 2008; Kamatou *et al.*, 2008b).
Thus, in order to identify new potential lead compounds, the five Turkish plants *Anthemis anatolica* subsp. *cretica*, *Anthemis pestalozzae* (Asteraceae), *Scrophularia lucida*, *Scrophularia pinardii* (Scrophulariaceae) and *Salvia virgata* (Lamiaceae) were chosen for this study.
Assessment of their chemotherapeutical potential was performed by screening the extracts against *P. falciparum* blood stage parasites and against the human cancer cell line KB for the evaluation of unspecific cytotoxicity. Examination of these extracts against crucial plasmodial liver stage enzymes from the FAS-II pathway was included, to assess their malaria prophylactic potential. After the initial evaluation, the most promising plant was to

be selected for in-depth analysis by bioactivity-guided fractionation for the isolation and structure elucidation of the active principles.

The second approach was the screening of two groups of commercially available natural products, of which at least one member had shown antimalarial potential against the blood stage parasites. The first group were a small number (due to commercial unavailability) of selected lichen secondary metabolites. One of them, (+)-usnic acid, was previously shown to possess antiplasmodial activity against the erythrocytic parasite stage (Verotta *et al.*, 2007). In addition, usnic acid was identified as the principle responsible for the antipyretic effect from *Usnea diffracta*, which was used in Asia, Africa and Europe for fever control (Okuyama *et al.*, 1995). So far, only few natural products have been assessed for their potential against liver stage parasites, *i.e.* prophylactic potential. Thus, the selected lichen metabolites were tested for their antiplasmodial activity towards blood and liver stage parasites and their inhibitory potential against FAS-II enzymes as potential target of liver stage activity.

Natural occurring chalcones were the second group of natural products selected for this study. Chalcones are the precursors of flavonoids, which are known for their *in vitro* potential against FAS-II enzymes and *P. falciparum* blood stage parasites (Tasdemir *et al.*, 2006). In addition, licochalcone A is a well known natural chalcone with antiplasmodial activity against blood stage and liver stage parasites (Chen *et al.*, 1993). Numerous synthetic chalcone derivatives have been shown to exhibit antiplasmodial activity against erythrocytic parasites (Liu *et al.*, 2003). However, no study has yet been performed solely on natural chalcones. Thus, the antiplasmodial activity against *P. falciparum* blood stage parasites and inhibitory potential against FAS-II enzymes was examined for 22 commercially available natural chalcones. Selected chalcones with good FAS-II inhibition were to be assessed against liver stage parasites. Structure activity relationships for antiplasmodial activity and enzyme inhibition were evaluated.

# Part 2

# Material & Methods

## 2.1 Materials

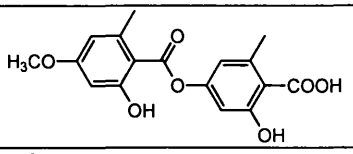
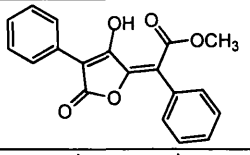
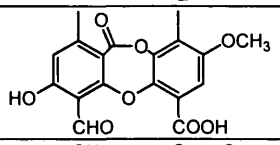
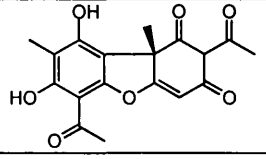
### 2.1.1 Plant material

The plant materials were collected in Turkey between April 2007 and April 2008 and identified by Assoc. Prof. R.S. Göktürk from the Akdeniz University, Turkey. The voucher specimens were deposited at the Akdeniz University, Department of Botany, Antalya, Turkey. *Anthemis cretica* L. subsp. *anatolica* (Boiss.) Grierson (Asteraceae) was collected from C3 Antalya: Geyikbayırı, Feslikan-Karçukuru plateau, step, 2000 m (voucher specimen number 6052); *Anthemis pestalozzae* Boiss. (Asteraceae) from C3 Antalya: Akseki, Cevizli, Çakıllı pass, 1200 m (voucher specimen number 6050); *Salvia virgata* Jacq. (Lamiaceae) from C3 Antalya: Geyikbayırı, Feslikan plateau, step, 1772 m (voucher specimen number 6065); *Scrophularia lucida* L. (Scrophulariaceae) from C3 Antalya, between Antalya-Kemer, Göynük, Göynük valley, 36° 40' 46.1"N, 30° 34' 07.7'E, 10 m (voucher specimen number 6483); *Scrophularia pinardii* Boiss. (Scrophulariaceae) between C3 Antalya-Manavgat, on rocks, 36° 49' 34.3"N, 31° 26' 27.0'E, 15 m (voucher specimen number 6286).

Collected plant material was air-dried and stored in a dry and dark environment at room temperature. Prior to the extraction, the plant material was ground to a fine power.

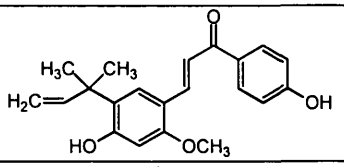
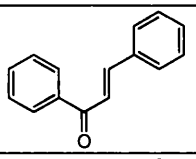
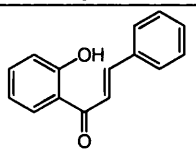
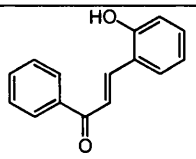
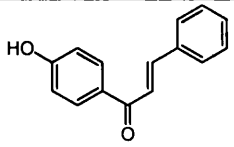
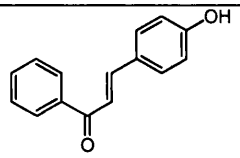
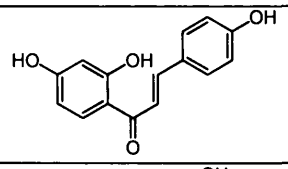
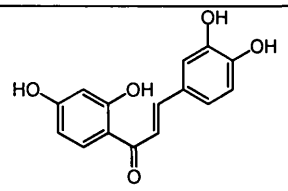
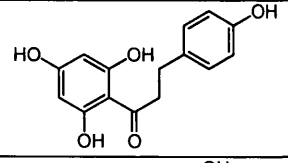
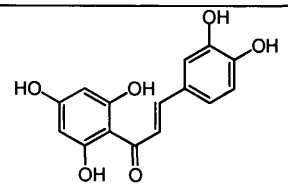
### 2.1.2 Lichen compounds

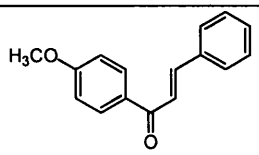
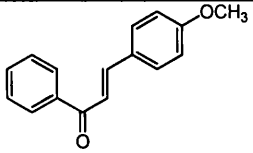
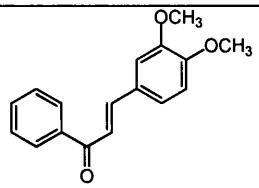
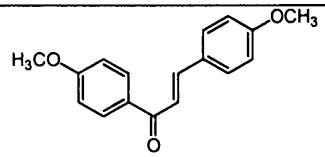
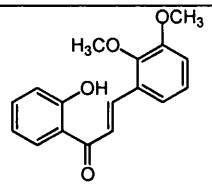
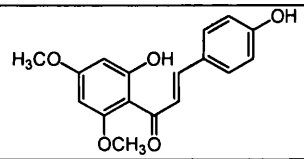
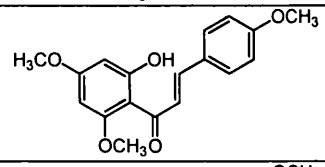
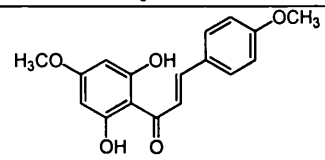
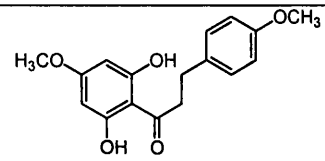
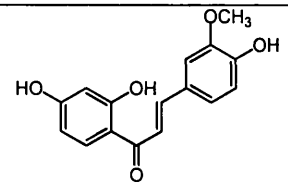
All compounds were commercially available. Evernic acid, vulpic acid and psoromic acid were purchased from Extrasynthese, whereas (+)-usnic acid was purchased from Aldrich.

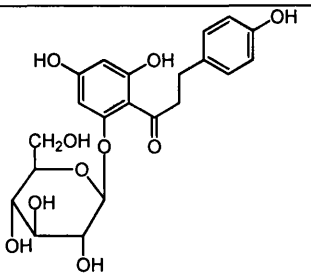
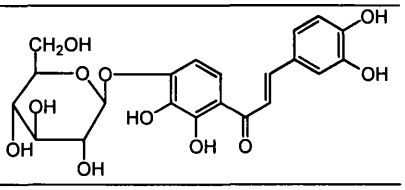
| Compound | Formula | Mw | Structure |
|---|---|---|---|
| Evernic acid | $C_{17}H_{16}O_7$ | 332.32 |  |
| Vulpic acid | $C_{19}H_{14}O_5$ | 322.32 |  |
| Psoromic acid | $C_{18}H_{14}O_8$ | 358.31 |  |
| (+)-Usnic acid | $C_{18}H_{16}O_7$ | 344.32 |  |

### 2.1.3 Chalcones

All compounds were commercially available and were purchased from Extrasynthese with the exceptions of chalcone (Aldrich), 2'-hydroxychalcone (Fluka) and licochalcone A (Calbiochem).

| Compound | Formula | Mw | Structure |
|---|---|---|---|
| Licochalcone A | $C_{21}H_{22}O_4$ | 338.40 |  |
| Chalcone | $C_{15}H_{12}O$ | 208.26 |  |
| 2'-Hydroxychalcone | $C_{15}H_{12}O_2$ | 224.25 |  |
| 2-Hydroxychalcone | $C_{15}H_{12}O_2$ | 224.27 |  |
| 4'-Hydroxychalcone | $C_{15}H_{12}O_2$ | 224.27 |  |
| 4-Hydroxychalcone | $C_{15}H_{12}O_2$ | 224.27 |  |
| 4,2',4'-Trihydroxychalcone (Isoliquiritigenin) | $C_{15}H_{12}O_4$ | 256.26 |  |
| 3,4,2',4'-Tetrahydroxychalcone (Butein) | $C_{15}H_{12}O_5$ | 272.27 |  |
| 4,2',4',6'-Tetrahydroxydihydro-chalcone (Phloretin) | $C_{15}H_{14}O_5$ | 274.28 |  |
| 3,4,2',4',6'-Pentahydroxy-chalcone (Eriodictyolchalcone) | $C_{15}H_{12}O_6$ | 288.27 |  |

| Compound | Formula | Mw | Structure |
|---|---|---|---|
| 4'-Methoxychalcone | $C_{16}H_{14}O_2$ | 238.29 |  |
| 4-Methoxychalcone | $C_{16}H_{14}O_2$ | 238.29 |  |
| 3,4-Dimethoxychalcone | $C_{17}H_{16}O_3$ | 268.32 |  |
| 4,4'-Dimethoxychalcone | $C_{17}H_{16}O_3$ | 268.32 |  |
| 2,3-Dimethoxy-2'-hydroxy-chalcone | $C_{17}H_{16}O_4$ | 284.32 |  |
| 4,2'-Dihydroxy-4',6'-dimethoxy-chalcone | $C_{17}H_{16}O_5$ | 300.32 |  |
| 2'-Hydroxy-4,4',6'-trimethoxy-chalcone | $C_{18}H_{18}O_5$ | 314.34 |  |
| 2',6'-Dihydroxy-4,4'-dimethoxy-chalcone | $C_{17}H_{16}O_5$ | 300.32 |  |
| 2',6'-Dihydroxy-4,4'-dimethoxy-dihydrochalcone | $C_{17}H_{18}O_5$ | 302.33 |  |
| 4,2',4'-Trihydroxy-3-methoxy-chalcone (Homobutein) | $C_{16}H_{14}O_5$ | 286.29 |  |

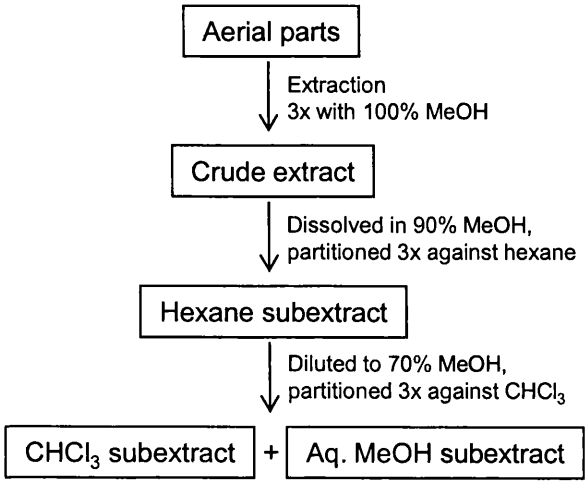
| Compound | Formula | Mw | Structure |
|---|---|---|---|
| Phloretin-2'-*O*-glucoside (Phloridzin) | $C_{21}H_{24}O_{10}$ | 436.41 |  |
| 3,4,2',3',4'-Pentahydroxy-4'-glucosylchalcone (Marein) | $C_{21}H_{22}O_{11}$ | 450.40 |  |

## 2.2 Extraction and liquid-liquid partition

A variety of techniques exist for the extraction of plant material. Traditional techniques include maceration, percolation and decoction. The advantage of these methods is their low costs for reagents and equipment. The main drawbacks are the long extraction time and unsatisfactory efficiency. Newer techniques including supercritical fluid extraction, ultrasound assisted extraction and microwave assisted extraction have the advantage of much shorter extraction times and a reduction in solvent consumption. However, most of these modern techniques require expensive equipment or extract only a specific type of compounds. In addition to the method used for the extraction of plant material, the yield and composition of the extract are greatly influenced by the solvent(s) chosen. For an exhaustive extraction strong solvents (*e.g.* alcohols) in which nonpolar and polar principles will dissolve are recommended. In this study the plant material was extracted by maceration with methanol (MeOH). Even though maceration is a time consuming method it is effective to exhaustively extract plant material with minimal risk of sample degradation.

After solid-liquid extraction the resulting crude extract was partitioned by a modified Kupchan partitioning (Kupchan *et al.*, 1973). In a Kupchan partitioning the percentage of the aqueous phase is increased sequentially. Solvents with different polarities were chosen in order to ensure a coarse separation of the organic principles (Fig. 2.1).

Figure 2.1. Extraction and Kupchan partition scheme.




Protocol

Air-dried and powdered plant material for small scale (10 g) and large scale (422 g) extraction was performed under continuous stirring with MeOH (Fisher, HPLC grade) at room temperature. Solvent amounts were chosen to ensure that all plant material was covered and stirring possible (Tab. 2.1). After at least 18 h the supernatant was decanted, collected, filtered and evaporated to dryness under vacuum at 30°C with a rotary

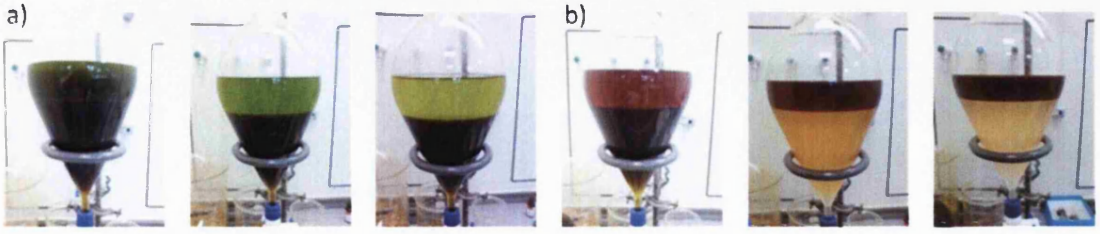
evaporator (Laborota 4003, Heidolph). The extraction was repeated until the plant material was exhaustively extracted as shown in Table 2.1 and resulting crude MeOH extracts combined, evaporated to dryness and stored at -20°C until further workup.

Table 2.1. Methanol extraction of plant material of *Anthemis*, *Scrophularia* and *Salvia* species.

| Material | Solvent amount for extraction | Extraction time |
|---|---|---|
| **Small scale extractions** | | |
| *A. cretica anatolica*, aerial | 150 ml, 150 ml, 100 ml | each time 18 h |
| *A. cretica anatolica*, roots | 150 ml, 150 ml | each time 18 h |
| *A. pestalozzae*, aerial | 180 ml, 180 ml | each time 18 h |
| *A. pestalozzae*, roots | 150 ml, 150 ml | each time 18 h |
| *S. pinardii*, aerial | 100 ml, 100 ml, 100 ml | each time 18 h |
| *S. pinardii*, roots | 100 ml, 100 ml, 100 ml | each time 18 h |
| *S. lucida*, aerial | 100 ml, 100 ml, 100 ml | each time 18 h |
| *S. lucida*, roots | 100 ml, 100 ml, 100 ml | each time 18 h |
| *Salvia virgata*, aerial | 150 ml, 150 ml | each time 18 h |
| **Large scale extraction** | | |
| *A. pestalozzae*, aerial | 5 l, 4.2 l, 4.2 l | 3 d, 18 h, 18 h |

For the modified Kupchan partitioning, the crude MeOH extract was dissolved in 90% MeOH and partitioned three times against hexane (Fisher, HPLC grade). The hexane subextracts were combined and evaporated to dryness. The aqueous methanolic partition (aq. MeOH) was diluted to 70% MeOH and partitioned three times against $CHCl_3$ (Fisher, HPLC grade). The increase of water ensured immiscible solvent mixtures which resulted in two distinct solvent layers (Fig. 2.2). Both subextracts were collected separately and evaporated to dryness. The aq. MeOH subextract was dissolved in minimal amounts of water and freeze dried. The solvent amounts were 100 ml for the small scale extractions and 700 ml for the large scale extraction. All dried subextracts were stored at -20°C.

Figure 2.2. Modified Kupchan partitioning. a) hexane and 90% MeOH , partitioned three-times, b) $CHCl_3$ and 70% MeOH, partitioned three times.



## 2.3 Bioactivity-guided fractionation and isolation

For the bioactivity-guided fractionation, all subextracts and fractions obtained by the initial fractionation step of the subextracts were tested against the plasmodial FAS-II enzymes FabI, FabG and FabZ, *P. falciparum* blood stage parasites and for cytotoxicity as described in 2.5 to monitor biological activity. Activity studies in addition to the chemical profile of the fractions determined which fractions were selected for further investigation.

For the fractionation, a variety of chromatographic methods and materials were used, which will be briefly described in the following sections. The techniques were chosen in accordance to the polarity, complexity and nature of the fractions and preliminary studies by TLC (see 2.3.1.1) and $^{1}$H NMR (see 2.4.1.1). The column size and dimensions and the amount of chromatography material depended on the amount of the fraction to be separated. All information about the chromatographic methods and specifications used for the fractionation are summarised in Figures 2.3 – 2.5.

Figure 2.3. Fractionation scheme for hexane subextract and $CHCl_3$ subextract (FCC fractions 4+5 and 6ab+7). Red fractions were submitted to LC-MS, blue fractions were analysed by GC-MS, pure compounds are shown bold in a box. H: column height

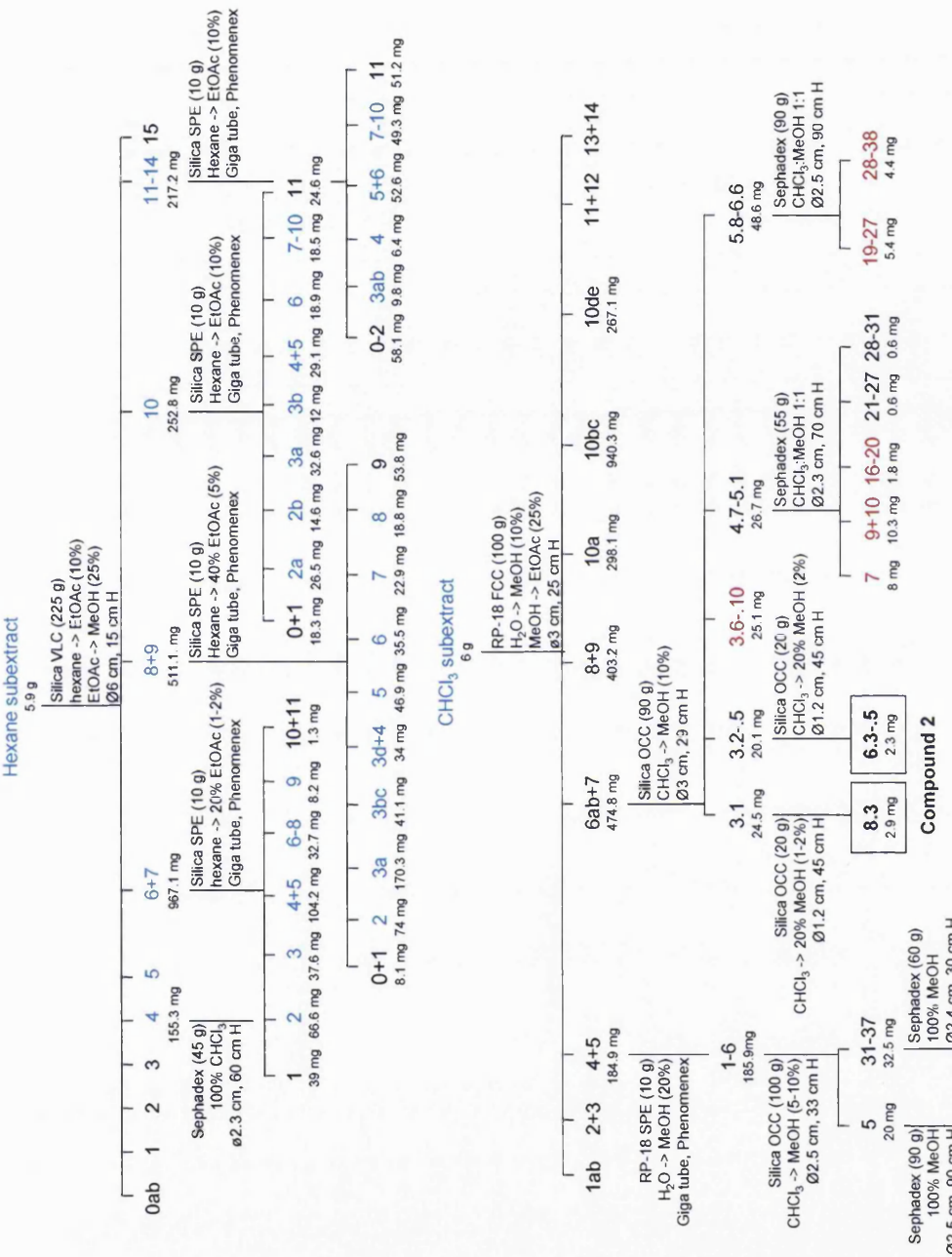


Figure 2.4. Fractionation scheme for $CHCl_3$ subextract (FCC fractions 8+9, 10a, 10bc and 10de). Red fractions were submitted to LC-MS, blue fractions were analysed by GC-MS, pure compounds are shown bold in a box. H: column height

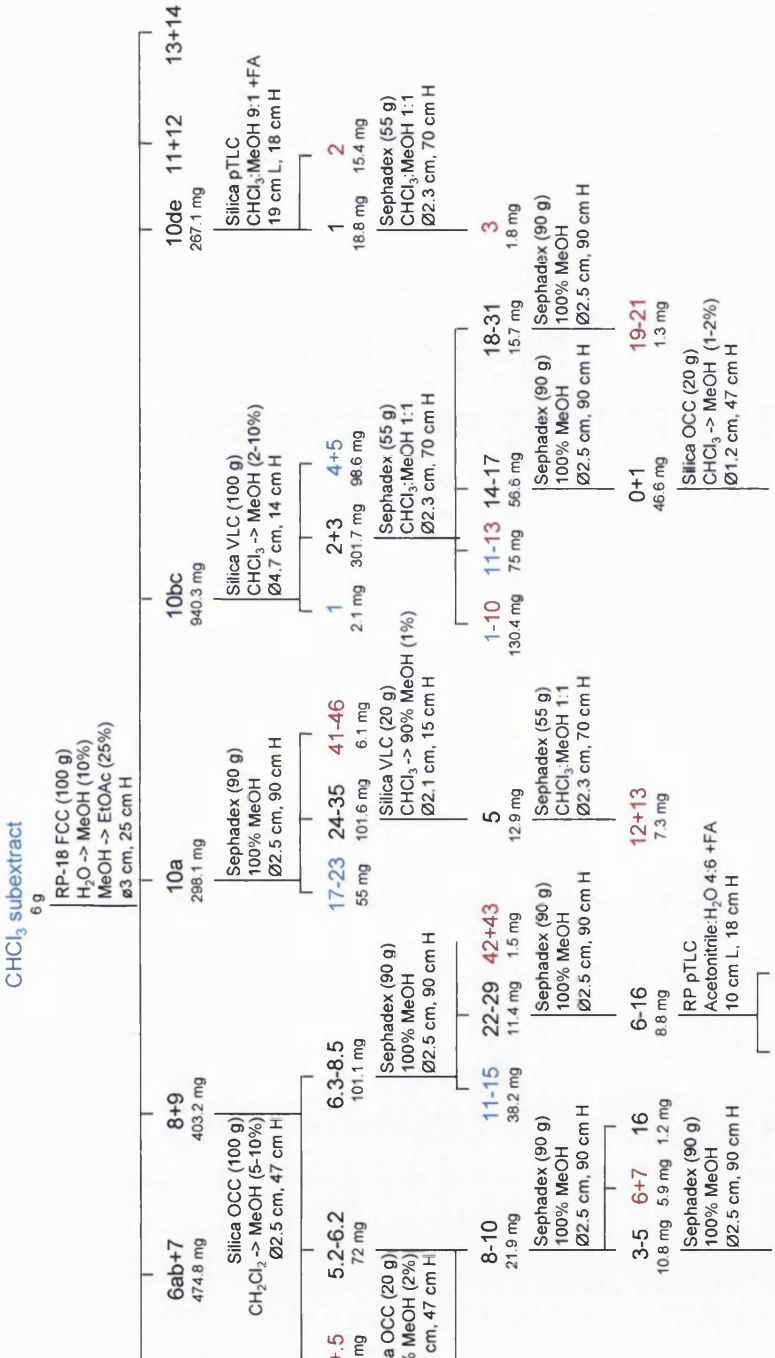
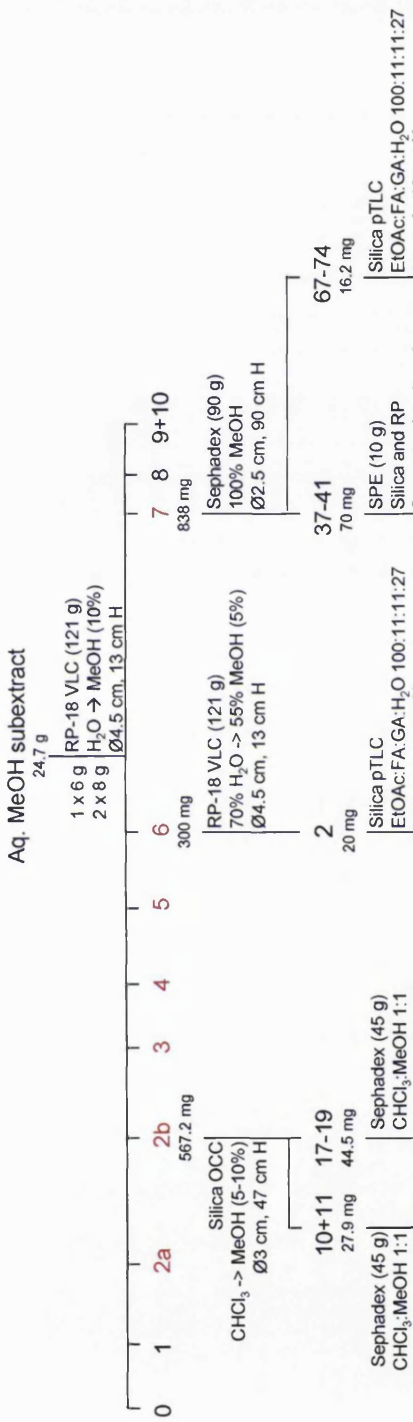


Figure 2.5. Fractionation scheme for aq. MeOH subextract. Red fractions were submitted to LC-MS, pure compounds are shown bold in a box. H: column height



### 2.3.1 Chromatographic methods

A total of eight different preparative and analytical liquid chromatography techniques were employed for the fractionation and analysis of the subextracts: analytical and preparative thin layer chromatography (TLC), vacuum liquid chromatography (VLC), flash column chromatography (FCC), solid phase extraction (SPE), gravity-based open column chromatography (OCC), size-exclusion chromatography (Sephadex LH-20), analytical high pressure liquid chromatography (HPLC) and liquid chromatography coupled with mass spectrometry (LC-MS). In addition gas chromatography coupled with mass spectrometry (GC-MS) was applied for fractions dominated by fatty acids.

As stationary phase for liquid chromatography techniques silica gel, reversed-phased silica gel (RP-18) or, in case of size-exclusion chromatography, Sephadex LH-20 were used as adsorbents. The mobile phase (generally a mixture of solvents) flows through the stationary phase and carries the component(s). The basic principle for the separation of components are different migration properties, which are determined by the choice of the solvent system and adsorbent material (Gibbons & Gray, 1998).

Silica gel is a three-dimensional polymer of silicon oxide tetrahydral units. It has a hydrophilic surface due to the presence of silanol groups (SiOH), which can interact with compounds by forming hydrogen bonds, dipole-dipole interactions or via van der Waals dispersion forces. The stronger the ability of a compound to form these bonds, the stronger it is retained by the silica gel. Thus, polar compounds bind to silica gel with a strong affinity and require polar solvents to recover them. The surface of reversed-phase silica material is modified with alkyl chains (in this study with octadecane, 18 carbon atoms) resulting in higher affinity to non-polar compounds. Hence, non-polar compounds are retained longer by this material. As the mobile phase moves through the absorbent, an equilibrium is established for each compound in the mixture between the molecules that are adsorbed on the stationary phase and the molecules which are in solution. The components of a mixture differ in their solubility in the mobile phase and the strength of their adsorption to the stationary phase, thus the migration of the compounds differs (Gibbons & Gray, 1998).

For the size-exclusion chromatography, Sephadex LH-20 was used as adsorbent. Sephadex it a hydrophilic beaded dextran that is cross-linked by glycerin-ether bonds. Sephadex LH-20 has been hydroxypropylated to yield a chromatographic media with both hydrophilic and lipophilic character. Due to its dual character it swells in water and a number of organic solvents. The wet particle size and therefore the exclusion limit for the gel varies depending on the solvent used. Small molecules retain much longer in the three-dimensional polymer network whereas big compounds travel fast through the adsorbent.

The molecular exclusion limit for Sephadex LH-20 is $M_r$ 4000 – 5000 Da. In addition to the separation by size, a partitioning effect occurs when solvent mixtures ($CHCl_3$/MeOH) are used.

### 2.3.1.1 Thin layer chromatography (TLC)

TLC is a method which can be used for chemical profiling, to separate and analyse mixtures, to isolate compounds and to test the purity of a compound. The stationary phase in TLC is a thin layer of adsorbent material immobilised on a plate. Due to capillary forces the mobile phase is drawn up to the top of the plate. Compounds of acidic nature can interact strongly with the polar silanol groups on a silica TLC plate, which results in tailing of the compound. This effect can be reduced by the addition of formic acid (FA) (Gibbons & Gray, 1998).

After the development of a TLC plate, it was inspected under UV light at 254 nm. Silica plates contain a fluorescent compound and compounds absorbing UV will appear as a dark spot or band on a bright background (quenching effect). Additionally, the plates were inspected at 365 nm to detect fluorescent compounds. For the staining of TLC plates a variety of agents are available. These can be used to detect a specific compound class (*e.g.* Dragendorff reagent for alkaloids, ninhydrin for amino acids, *Naturstoffreagenz* for flavonoids and other phenolic compounds) or as general detection agent, *e.g.* vanillin/sulphuric acid which is a universal spray reagent and stains numerous compound classes. Thus, in addition to the information about the polarity of components in a fraction, TLC can give indications about the constituents in a mixture by the application of a detection agent.

Protocol for analytical TLC

For the analytical TLC a small amount of sample (few mg of extract, fraction or compound) was dissolved in an appropriate solvent. The samples were applied in similar concentrations as a small dot or slim band onto aluminium TLC plates coated with unmodified silica gel 60 $F_{254}$ (Merck, 105554) or onto glass plates coated with modified silica gel 60 RP-18 $F_{254s}$ (Merck, 115423) and placed into a glass chamber which was saturated with an appropriate solvent mixture. The composition of the solvent system depended on the nature and polarity of the samples. When the solvent front reached the top of the plate (1 cm left) the plate was removed, dried and viewed under UV at short (254 nm) and long wavelength (365 nm). Spots that appeared under these wavelengths were marked carefully left ($UV_{254}$) or right ($UV_{365}$). The TLC plate was sprayed with 4% (w/v) vanillin (Sigma-Aldrich, V1104) in sulphuric acid (Sigma-Aldrich, 320501) and heated

for 1 min at 170°C. Alternatively, the *Naturstoffreagenz* was used as staining agent for samples containing flavonoids. For this, the TLC plate was first sprayed with 1% (w/v) methanolic diphenylborinic acid 2-aminoethyl ester (Acros Organics, 155420050) followed by 5% (w/v) ethanolic polyethylene glycol 4000 grade (Fisher, P/3680/53) and the fluorescence was observed at 365 nm.

### 2.3.1.2 Column chromatography with silica material

Column chromatography with silica material was used for the fractionation of the subextracts and subsequent fractions. As first fractionation step for all subextracts, VLC or FCC were used. These methods are a quick and simple way for a coarse separation of subextracts or complex mixtures by applying vacuum or compressed air. For subsequent fractionation OCC or SPE was used. SPE was applied to give a quick and fine separation of complex mixtures (Hostettmann *et al.*, 1997). OCC is a more time intense method, as the solvent mixture flowed through the column by gravity with a greatly reduced flow rate. The advantage of the slow speed is generally a better separation of compounds, however if the flow rate is too low, a broadening of compounds can occur.

To determine the solvent system for column chromatography with silica material, TLC was performed and a system selected where the spots of the fraction showed a good separation with a retention factor of 0.30 or smaller (Gibbons & Gray, 1998). For VLC and FCC, silica gel 60 (40-63 µm) (Merck, 109385) or reversed-phase silica gel LiChroprep RP-18 (40-63 µm) (Merck, 113900) were used as adsorbent, while for OCC silica gel was used exclusively. For SPE 10 g Giga tubes pre-packed with either normal phase silica (Strata SI-1 silica, 55 µm, 70 Å) (Phenomenex, 8B-S012-MFF) or reversed-phase silica (Strata C18-E, 55 µm, 70 Å) (Phenomenex, 8B-S001-MFF) were used.

Protocol VLC and FCC

The silica material was packed wet under vacuum (VLC) or compressed air (FCC). For VLC, vacuum was applied beneath the column (Fig. 2.6 a), while for FCC compressed air was applied on top of the column (Fig. 2.6 b).

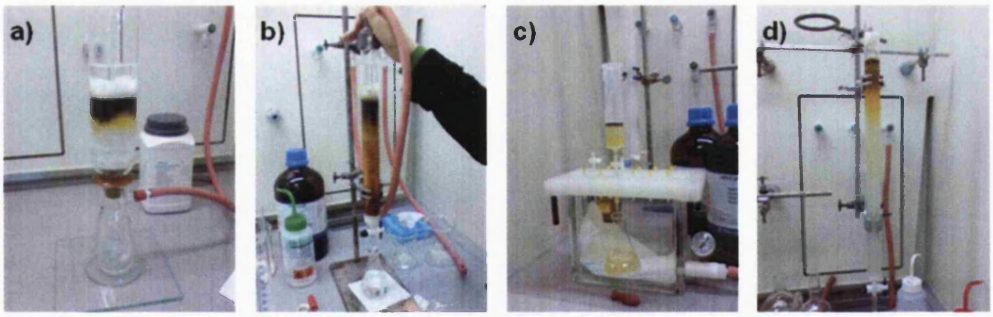
For the hexane subextract silica gel was packed in hexane (Fisher, HPLC grade) into a column fitted with a frit (porosity 4) at the base. For the $CHCl_3$ and aq. MeOH subextracts reversed-phase silica gel was packed in MeOH (Fisher, HPLC grade). It was ensured that the column was tightly and evenly packed without cracks or pockets of air to warrant good separation (Pelletier *et al.*, 1986). The column was washed with solvent used for packing to remove silica fines. For reversed-phase columns the solvent system was conditioned in 25% steps from 100% MeOH to 100% water ($H_2O$). The starting sample was dissolved in

a minimal amount of appropriate solvent (ideally starting solvent) and carefully applied on top of the column. A step gradient with 10% increments from hexane to ethylacetate (EtOAc) (Fisher, HPLC grade) or $H_2O$ to MeOH was employed for the subextracts and for each gradient step 100-200 ml were collected. In subsequent fractionations, smaller increments (1-10%) were employed and smaller amounts (50-100 ml) collected for each gradient step. To ensure good separation and no compound bleeding the column was allowed to go to dryness prior to the next gradient step. Collected fractions were examined with TLC and $^1H$ NMR. Similar fractions were combined, dried under nitrogen and evaporated to dryness under vacuum at 30°C with a rotary evaporator. Additionally, fractions containing water were freeze dried to ensure complete dryness. All fractions were stored at -20°C.

Protocol SPE

In addition to VLC, SPE (Fig. 2.6 c) was used for subsequent fractionation steps. The columns were run using a vacuum manifold (Phenomenex) with a maximum of 10 bar. Collected fractions (50-200 ml) were treated as described above.

Figure 2.6. Different column chromatography methods using silica material. a) VLC, b) FCC, c) SPE, d) OCC




Protocol OCC

For OCC (Fig. 2.6 d), silica gel was wet packed with $CHCl_3$ or dichloromethane ($CH_2Cl_2$) (Fisher, HPLC grade) into a column. Cotton wool was used to create a stopper and retain the silica material. As described above, it was ensured that the column was tightly and evenly packed without cracks or pockets of air. The column was washed and equilibrated with the starting solvent. The sample was dissolved in a minimal amount of appropriate solvent (ideally starting solvent) and applied onto the column. A step gradient towards MeOH was applied in 1-10% increments. The solvent flowed through the column by gravity with a flow rate of 1-2 ml/min. Fractions were collected in small amounts (10-50

ml), if possible also according to bands. Collected fractions were treated as described above.

### 2.3.1.3 Size-exclusion chromatography

In addition to the fractionation with silica material, size-exclusion chromatography was applied. This method was used to separate fractions according to their molecular size and shape and to purify compounds. One advantage of Sephadex material is its inertness which results in no reactivity between the adsorbent and compounds and leads to the complete recovery of the applied material. Disadvantages are the possibility of contamination of Sephadex material with microorganisms when water is used for swelling and the slow flow rate.

Protocol

Sephadex LH-20 (GE Healthcare, 17-0090) was soaked in MeOH, $CHCl_3$ or a $CHCl_3$:MeOH (1:1) mixture and allowed to swell for at least 6 h before packing the column. Cotton wool was used to create a stopper. The Sephadex LH-20 material was poured in one continuous motion into the column to avoid air bubbles and allowed to settle. For the isocratic elution of the compounds the soaking solvents were used, applying a flow rate of maximal 1 ml/min. Fractions were collected in small amounts (5-20 ml), if possible also according to bands. Collected fractions were treated as described above.

### 2.3.1.4 Preparative thin layer chromatography (pTLC)

Preparative thin layer chromatography (pTLC) was a method used in order to isolate compounds by running the fraction on TLC and scraping separated bands off the TLC plate (Gibbons & Gray, 1998). This methods was solely used when fractionation by the above described column chromatography methods did not result in the isolation of compounds. The main disadvantage of pTLC is the loss for material and possible contamination of isolated compounds with silica.

Protocol

Aluminium TLC plates coated with unmodified silica gel 60 $F_{254}$ (Merck, 105554) or glass plates coated with modified silica gel 60 RP-18 $F_{254s}$ (Merck, 115423) were used for pTLC. The dimensions were 20 x 20 cm for silica plates and 10 x 20 cm for reversed-phase silica plates. Samples were applied as a thin band 1 cm from the bottom and leaving 0.5 cm to each sides of the TLC plate. The plates were allowed to dry before run with an appropriate solvent system. Not more than 20 µg of a fraction were applied as a long band to one TLC

plate. Plates were removed from the glass tank once the solvent front reached the top. Bands were scraped off based on UV detection at 254 nm and fluorescence at 365 nm. In addition a thin stripe of the plate was cut off and stained with 4% vanillin/sulphuric acid to ensure detection of all compounds. The compounds were eluted with appropriate solvents and filtered to remove the chromatography material. Collected fractions were treated as described above.

### 2.3.1.5 High performance liquid chromatography (HPLC)

Analytical high performance or high pressure liquid chromatography (HPLC) was used to examine the purity of isolated compounds, for chemical profiling and to identify known compounds in complex fractions. In addition, HPLC is widely used for quantitative and qualitative analysis of extracts or for the separation of compounds.

HPLC is a column chromatography technique, where sample and solvents are pumped with high pressure through a tightly packed column with much smaller particle size (3-10 μm) (Stead, 1998). The increased density in the column results in a good separation on short columns. Behind the column a diode array detector monitored the output of the column at a wavelength of 254 nm and provided a spectrum with absorbance over time. Compounds elute from the column with different retention times, depending on the strength of the interaction with the column material, solvent gradient and flow rate. It is possible to get an indication about the compound composition in a mixture, by measuring retention times for known compounds and comparing them to retention times of compounds in mixtures. The purity of a compound can also be assessed by this method, as each impurity will show an additional peak in the spectrum, which can subsequently be quantified.

Protocol

The samples were run on a 1200 series quaternary liquid chromatography system (Agilent) consisting of a degasser (G1322A), quaternary pump (G1311A), autosampler (G1329A), fraction collector/autosampler thermostat (G1330B), thermostatted column compartment (G1316A), diode array detector (G1315D), refractive index detector (G1362A) and analytical fraction collector (G1364C). Experiments were run and analysed using ChemStation (Agilent).

Samples were prepared at a concentration of 1 mg/ml in an appropriate solvent and filtered. 10 μl of sample were injected into a reversed-phase silica column (Synergi 4 μm Polar-RP, 80 Å, 150 x 3.0 mm) (Phenomenex, 00F-4336-Y0) and eluted with a flow rate of 1 ml/min. Eluent systems used were $H_2O$ and MeOH or $H_2O$ and acetonitrile (Fisher,

HPLC grade). All solvents were degassed by 15 min sonification prior to their use. The gradient and time were optimised for each sample, but generally the gradient program started with 100% $H_2O$ and reached 100% of the second solvent (MeOH or acetonitrile) in 30 min. Compounds were detected with UV at 254 nm.

### 2.3.1.6 Liquid chromatography-mass spectrometry (LC-MS)

Liquid chromatography coupled with mass spectrometry (LC-MS) is an analytical method which combines liquid chromatography (HPLC) and mass spectrometry (MS) (see 2.4.2). This results in high sensitivity and specificity. It is a powerful tool in the specific detection and identification of compounds in a complex mixture by first separating the sample in an HPLC and then directly analysing the compounds by MS.

LC-MS analyses are being performed in collaboration with Dr. Mark O'Neil-Johnson from Sequoia Science, USA.

### 2.3.1.7 Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography coupled with mass spectrometry (GC-MS) is another analytical methods used to detect and analyse compounds in a mixture, which combines gas chromatography (GC) and mass spectrometry (MS). The difference to LC-MS is, that in order for a compound to be analysed by GC-MS, it must be able to be vaporised without decomposition. In this study, GC-MS was used to analyse the fatty acid composition of mixtures. For this, the fatty acids extracted in a mixture were transformed to methyl esters, which are volatile derivatives of the fatty acids. In GC-MS the lengths, bonds, rings and branches of the fatty acid methyl esters can be determined by the comparison with compound libraries.

GC-MS analyses was performed in collaboration with Prof. Nestor Carballeira by Dr. Elsie A. Orellano from the University of Puerto Rico, Puerto Rico. First, fatty acids were converted to fatty acid methyl esters (FAME) with methanolic HCl for 2 h, followed by column chromatography on silica gel with hexane/ether (9:1). The FAME composition was analysed qualitatively and quantitatively by GC-MS by comparing the obtained characteristic mass spectra with authentic samples in the literature (NIST/EPA/NIH Mass Spectral Library) and comparing their equivalent chain length values with known commercial standards (Sigma). The double bonds and methyl branching in these compounds were determined by pyrrolidide derivatisation following the preparation procedure. GC-MS analyses were recorded at 70 eV using either a Hewlett Packard 5972A

MS ChemStation or an Agilent 5975C MS ChemStation coupled to an Agilent 7890A GC where both instruments were equipped with a 30 m x 0.25 mm special performance capillary column (HP-5MS) of polymethyl siloxane crosslinked with 5% phenyl methylpolysiloxane. The GC-temperature program was: 130°C for 1 min, increased at a rate of 3°C/min to 270°C, and maintained for 30 min at 270°C.

## 2.4 Structure elucidation

### 2.4.1 Nuclear magnetic resonance spectroscopy (NMR)

One of the most important methods for structure analysis and chemical profiling is the nuclear magnetic resonance (NMR) spectroscopy. The nuclear spin of atoms is the basic for this technique. All nuclei carry a charge and if this charge spins on the nuclear axis it generates a magnetic dipole along it. Atoms with odd nucleon numbers (*e.g.* $^{1}H$, $^{13}C$) have a spin, thus the magnetic properties of the nuclei can be exploited. The nuclei of $^{1}H$ for example, have two different possible states, a higher energy level or a lower energy level. Nuclei in a lower energy state can be brought to a higher state with electromagnetic radiation. This energy intake can be detected and seen in a spectrum, where the amount of energy relates to the chemical binding properties of the atom (Silverstein & Webster, 1997).

#### 2.4.1.1 Proton NMR ($^{1}H$ NMR)

$^{1}H$ NMR spectroscopy and can be applied to crude extracts, subextracts and fractions to ascertain the presence of various chemical classes within these complex mixtures. Additionally, it is an extremely powerful tool for the structure elucidation and identification of pure compounds and gives an indication about the purity of isolated compounds (Heinrich *et al.*, 2003).

The chemical shift δ of a proton is expressed in parts part million (ppm) and corresponds to the frequency required to bring a proton into resonance, hence it provides information about the environment of a proton in the molecule relative to the solvent used. Protons appearing upfield (right side of the spectrum) are in an environment with high electron density, *i.e.* the nuclei are shielded, and to bring the protons into resonance a higher field strength is required which results in low δ values (Kemp, 1978). Different types of functional groups show typical chemical shift values (*e.g.* aldehyde protons δ 9.5-10.5 ppm, aromatic protons δ 6-8 ppm, methyl protons δ 0.7-1.5 ppm), which can be used to draw conclusion about the type of molecule.

For structure elucidation, integration of the area under the peaks gives a relative number associated with each signal, resulting in the total number of protons in the molecule. The multiplicity of a peak provides information about adjacent protons from spin-spin coupling. The split pattern is determined by the rule $2nI+ 1$, with $n$ being the number of neighbouring equally coupled protons and $I$ the nuclear spin number. The coupling constant $J$ is the difference in frequencies between the component peaks in a multiplet and is measured in hertz (Hz). The $J$ value provides information about the position of protons with respect to each other, *i.e. cis* or *trans* of neighbouring protons in the case of double

bonds or ring junctions or *ortho*, *meta* or *para* position in aromatic ring systems (Silverstein & Webster, 1997).

### 2.4.1.2 Carbon NMR ($^{13}$C NMR)

Carbon NMR ($^{13}$C NMR) was used in addition to $^{1}$H NMR for the elucidation of structures. It is far less sensitive compared to $^{1}$H NMR, which is due to the small natural abundance of $^{13}$C with only 1.1%. For NMR, only nuclei with odd numbers as $^{1}$H or $^{13}$C are of importance as the $^{12}$C or $^{2}$H nucleus have a spin number of zero and are magnetically inactive. $^{13}$C spectrometry became widely popular after its sensitivity was improved by the introduction of pulsed Fourier transform instrumentation in the early 1970s (Silverstein & Webster, 1997). Fourier transform permits the simultaneous excitement of all $^{13}$C nuclei followed by simultaneous collection of all signals. The broadband decoupling of protons by irradiation removes the carbon-proton coupling and results in single sharp peaks for each chemically non-equivalent $^{13}$C atom. Therefore no integration of the peaks is required as the peaks number correlates to the carbon number present in the molecule. However infrequently carbon signals can overlap which results usually in a higher peak. Due to the wide range in which the carbon signals can come into resonance, it is possible to identify carbon-containing functional groups by characteristic shift values. In addition, the appearance of carbon functionalities may be slightly altered if they are attached to electronegative atoms (as oxygen) or in a highly strained ring system, shifting the carbon atoms downfield. This method is an easy way to identify the number of carbon atoms and their nature in a molecule, thus giving an indication of the compound class (Silverstein & Webster, 1997).

Distortionless enhancement by polarisation transfer (DEPT) is another $^{13}$C NMR technique and the most effective method for detecting the types of carbons present in a compound as it can distinguish between methyl ($CH_3$), methylene ($CH_2$), methane (CH) and quaternary (C) carbons. DEPT135° shows positive peaks for $CH_3$ and CH and negative peaks for $CH_2$, whereas with DEPT90° only CH signals are detected. Quaternary atoms show no signal. The numbers 135° and 90° describe the angle of the final $^{1}$H decoupler pulse.

### 2.4.1.3 Two-dimensional NMR experiments

Two-dimensional NMR experiments are the backbone for the structure elucidation of a compound and were applied in addition to one-dimensional $^{1}$H and $^{13}$C NMR experiments. In two-dimensional NMR experiments experimental data are shown in addition to the x (chemical shift) and y (intensity) axes, on the z axis, displaying the intensity of signals from

homonuclear experiments (COSY and NOESY), showing proton-proton connections, and heteronuclear experiments (HMQC and HMBC), showing proton-carbon connections.

Heteronuclear multiple quantum coherence (HMQC) is a proton-detected $^{1}H$-$^{13}C$ correlation analysis, in which only direct proton carbon coupling is observed. Thus, this technique can be used to assign the protons to a specific carbon atom. In this method the $^{1}H$ spectrum of a compound is correlated with the $^{13}C$ spectrum. All proton signals must be accounted for in HMQC and facilitate the structure elucidation of the compound (Silverstein & Webster, 1997).

Proton correlation spectroscopy (COSY) is a crucial technique to elucidate the structure of a compound. All peaks that are mutually spin-spin coupled are shown by cross-peaks that are symmetrical placed along a diagonal, *i.e.* the cross peaks correlate coupled protons. This provides a proton-proton correlation and coupling can occur through two (geminal coupling, $^{2}J$) or three bonds (vicinal coupling, $^{3}J$). COSY analysis is used to determine spin systems within a compound which can be used to built initial fragments of the structure (Silverstein & Webster, 1997).

The heteronuclear multiple bond coherence (HMBC) is a long-range proton-detected $^{1}H$-$^{13}C$ correlation analysis, in which two ($^{2}J$) and three-bond ($^{3}J$) correlations between $^{1}H$ and $^{13}C$ atoms are observed. It is an extremely powerful tool for unambiguous assignment of a compound and to identify the backbone structure, as indirectly carbon-carbon correlations and correlations of quaternary carbons with nearby protons can be obtained. It is used to generate the gross structure of a compound (Silverstein & Webster, 1997).

The nuclear Overhauser enhancement spectroscopy (NOESY) is similar to COSY in terms of the provision of information about proton-proton correlations. It differs from COSY, as the detection of proton-proton interactions is through space rather than through coupling along bonds. This is due to the nuclear Overhauser effect (NOE) which is the transfer of nuclear spin polarisation from one spin population to another via cross-relaxation. NOE can only be detected for atoms that are in close proximity of each other, in the region of 2-5 Å. This technique is therefore essential for the determination of the relative stereochemistry of a compound (Silverstein & Webster, 1997).

Protocol

The analysis of extracts, subextracts and fractions was performed on an AVANCE 400 MHz spectrometer (Bruker) by $^{1}H$ NMR experiments with 16-128 scans. 50 mg of sample (or the whole sample, if smaller amounts were isolated), were dissolved in 600 µl chloroform-d ($CDCl_3$) (Cambridge Isotopes Laboratories, DLM-7-100) or in 600 µl

methanol-$d_4$ (MeOD) (Cambridge Isotopes Laboratories, DLM-24-10) and transferred into a NMR tube with 5 mm diameter and 17.8 cm length (Aldrich, Z276278). The Bruker software Topspin 1.3 was used for spectra acquisition and processing. The spectra calibration was carried out on the residual solvent peak (MeOD: δ 3.31, δ 4.80 (residual water peak), $CDCl_3$: δ 7.27).

For isolated compounds and structure elucidation studies a variety of one-dimensional ($^1$H NMR, $^{13}$C NMR, DEPT) and two-dimensional (HMQC, HMBC, COSY, NOESY) experiments were performed on an AVANCE 500 MHz spectrometer (Bruker). Analysis of pure compounds by $^1$H NMR was performed with 128 scans, for $^{13}$C NMR with 10,000 – 16,000 scans with a resonance frequency of 125 MHz and for DEPT with 5,000 – 8,000 scans. HMQC was measured with 32 scans, HMBC with 64 scans, COSY with 16 scans and NOESY with 16 scans and 400 ms mixing. The Bruker software Topspin 1.3 was used for spectra acquisition and processing. The spectra calibration was carried out on the residual solvent peak (MeOD: $^1$H= δ 3.31, δ 4.80 (residual water peak), $^{13}$C= δ 40; $CDCl_3$: $^1$H= δ 7.27, $^{13}$C= δ 77; pyridine-$d_5$: $^1$H= δ 8.74, δ 7.58, δ 7.22, $C^{13}$= δ 150.35, δ 135.91, δ 123.87; benzene-$d_6$: $^1$H= δ 7.16, $C^{13}$= δ 128.39; deuterium oxide ($D_2O$): $^1$H= δ 4.80).

### 2.4.2 Mass spectrometry (MS)

Mass spectrometry (MS) is an analytical method used for measuring the molecular mass ($M_r$) of a compound. In MS experiments the molecules are ionised, accelerated, deflected and detected. The deflection of the ions by a magnetic field is according to their masses, *i.e.* the lighter they are, the more they are deflected. In addition, the deflection depends on the number of charges on the ions, *i.e.* the more they are charged, the more they are deflected. The result is a spectrum with the relative abundance of the separated ions shown according to their mass to charge ratios ($m/z$) (Gross, 2004). For the analysis in this study, electrospray ionisation (ESI) was performed. With this technique, samples generate singly charged ions, usually protonated ions $[M+H]^+$ or sodium adduct ions $[M+Na]^+$ in positive ionisation mode and deprotonated ions $[M-H]^-$ in negative ionisation mode. The selection of the ionisation mode depends on the nature of the compound. For example, if the compound has functional groups that readily accept a proton such as amines, the positive ion detection is used; if the compound has functional groups that readily lose a proton such as carboxylic acids and alcohols, the negative ion detection is used (Gross, 2004)

Mass spectrometry measurements were performed by the analytical service of the School of Pharmacy. Positive and negative mode electrospray ionisation (ESI) (low resolution) and accurate mass (high resolution) experiments were applied to isolated compounds.

### 2.4.3 Optical activity

The optical activity is the ability of a chiral molecule in solution to rotate the plane of linear polarised light, which can be clockwise (+) or anti-clockwise (-). This optical rotation can be measured and quantified as the number of degrees an analysing lens needs to rotate in order to reverse the rotation of the light. Enantiomers rotate the light by the same degree, but in opposite directions. In an equal mixture of enantiomers the rotations cancel each other out, therefore the rotation measured will be zero.

The rotation measured with a polarimeter is called observed rotation and affected by the path length of the cell (time the light need to travel through a sample) and concentration of the sample (how much sample is present to rotate the light). These effects can be eliminated and the obtained specific rotation [α] used as a characteristic attribute of a compound.

Protocol

The optical rotation [α] was measured on a polarimeter (PerkinElmer) using a quartz cell. The wavelength of the light was set at 589 nm (sodium D line, $[\alpha]_D$) and measured at room temperature ($[\alpha]_D^{22}$). The sample was dissolved in appropriate solvent with a concentration of 1 mg/ml and the same solvent used to blank the instrument. For the calculation of the specific rotation $[\alpha]_D^{22}$ the measured value was divided by the product of the path length in decimetres (= 1) and the concentration in g/ml (= 0.001).

## 2.5 Bioactivity testing

### 2.5.1 Type II fatty acid biosynthesis enzymes from *P. falciparum*

#### 2.5.1.1 Enzyme expression

One of the oldest and best characterised systems for the expression of recombinant proteins are the mammalian gut bacteria *Escherichia coli*. The most common method uses recombinant plasmids in which the genes of interest can be introduced. The expression is under the control of an inducible promoter (*e.g.* lac promotor). To obtain stabile cell lines and to prevent survival of cells without recombinant plasmid, commonly used plasmids show at least one antibiotic resistance.

Many different *E. coli* strains with different modifications are established. In this work the strain BL21-CodonPlus (DE3)-RIL was used. This strain contains a second plasmid with genes for three, in *E. coli* rarely expressed but in eukaryotic cells commonly used, tRNAs (argU (arginine, R); ileY (isoleucin, I); leuW (leucine, L)). The plasmid also contains the gene for chloramphenicolacetyltransferase which mediates chloramphenicol resistance. Additionally, the BL21-CodonPlus strain features a high transformation efficiency (Hte) allele, which helps to increase transformation efficiency of large and ligated DNA and lacks EndA1, an endonuclease I, and OmpT and Lon, two proteases. A DE3 lysogen is included which has the T7 RNA polymerase under the control of the lacUV5 promoter.

Protocol

The fatty acid biosynthesis enzyme FabI was cloned in pET30b (NcoI, BamHI) (Perozzo *et al.*, 2002), FabG in pET30b (NcoI, EcoRI) (Tasdemir *et al.*, 2006), FabZ in pET28b (Nde, EcoRI ) (Kostrewa *et al.*, 2005) and each construct expressed in a separate batch of *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene). Full-length enzymes had been constructed without the putative *N*-terminal signal and translocation sequence, resulting in a molecular weight of 45.3 kDa for FabI (amino acids 78-432), 32.9 kDa for FabG (amino acids 55-304) and 18.9 kDa for FabZ (amino acids 81-230) (Tasdemir *et al.*, 2006). Glycerol stocks of the bacteria were provided as a generous gift by Dr. R. Perozzo from the University of Geneva, Switzerland. For the enzyme expression the bacteria were grown in separate batches over night at 37°C and 160 rpm in 2 l Erlenmeyer flasks containing 1 l Terrific Broth (Fluka, T0918) with 34 µg/ml chloramphenicol (Sigma, C0378) and 100 µg/ml kanamycin sulphate (Sigma, 60615). Enzyme expression was induced after 16-18 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Calbiochem, 420322) followed by further incubation for 5 h at 37°C and 160 rpm. The bacterial cells were harvested by centrifugation at 4°C and 5000 rpm for 20 min (Avanti J-E centrifuge, rotor 16.250, Beckman Coulter). The bacteria pellets were frozen and kept at -20°C.

### 2.5.1.2 Enzyme purification

For the protein purification nickel-NTA affinity chromatography was used as all FAS-II enzymes were cloned with polyhistidine residues (His-tag). A His-tag consists of approximately six histidine residues which have the ability to bind bivalent cations like $Ni^{2+}$. Thus, proteins co-expressed with a His-tag at the N- or C-terminus of the protein can be purified with nickel-NTA affinity chromatography, where bivalent nickel-ions are bound with four of their six binding sites to nitrilotriacetic acid (NTA) which is coupled to an agarose-matrix. His-tagged proteins are immobilised at the nickel-NTA-matrix due to the fact that the histidine residues can bind with their imidazole ring at the remaining two binding sites of $Ni^{2+}$, while untagged proteins move through the column. To eliminate binding of histidine containing background proteins a washing step with low concentrations of imidazole (10-50 mM) is advisable. Imidazole has a greater affinity to $Ni^{2+}$ ions but at low concentrations His-tagged proteins are still tightly bound and can compete with imidazole. At higher concentrations of imidazole (100-250 mM) the His-tagged proteins dissociate from the $Ni^{2+}$ ions, allowing the elution of the enzymes. Alternatively, a reduction of the pH-value (pH 4.5 – 5.3) can be used to elute His-tagged proteins as the nitrogen of the imidazole ring from histidine gains a positive charge and is repelled by the positive nickel-ions.

Protocol

A bacteria pellet equivalent to 250 ml bacteria culture was thawed in 30 ml cold lysis buffer containing 20 mM trishydroxymethylaminomethane (TRIS) (Aldrich, 154563), 500 mM sodium chloride (NaCl) (Acros Organics, 2077900), 20 mM imidazole (Sigma-Aldrich, 56750), 1% glycerol (Sigma, 49767), pH 8.0. A tip of a spatula deoxyribonuclease I from bovine pancreas (Sigma, DN25) and for FabI and FabG 1 mM phenylmethanesulfonyl-fluoride (PMSF) (Sigma, 78830) were added to the suspension. Cells were lysed by disrupting them twice in a French Press (Thermo) at 1500 psi. The lysate was centrifuged at 4°C and 9000 rpm for 40 min (Avanti J-E centrifuge, rotor 25.50, Beckman Coulter) and the supernatant filtered (cellulose nitrate, ø 0.45 µm). An Econo-Pac chromatography column (Bio-Rad, 732-1010) was packed with nickel-NTA agarose (Qiagen, 30210) to a bed volume of 2 ml. A porous 30 µm polyethylene bed support was applied on top of the column without compressing the material to prevent it from running dry. Once a column was packed it could be used for approximately six month. Before each use the column was regenerated with ten times (20 ml) 8 M Urea (Sigma, U5378), 20 mM TRIS, pH 7.4, washed with ten times (20 ml) lysis buffer containing 500 mM imidazole and equilibrated with ten times (20 ml) cold lysis buffer. The filtered enzyme lysate was applied onto the

column and the column washed with ten times (20 ml) cold lysis buffer and five times (10 ml) cold lysis buffer containing 50 mM imidazole. The enzyme was eluted with cold lysis buffer containing 500 mM imidazole in 1 ml steps until all enzyme was removed from the column. For storage the column was washed and kept in 20% ethanol (Fisher, Analytical Grade). Eluted fractions were tested for their protein content by adding 10 µl of each fraction to a test tube containing 790 µl $H_2O$ and 200 µl Bio-Rad protein assay dye reagent concentrate (Bio-Rad, 500-0006), observing the colour change of the dye. Fractions containing enzyme were combined and if necessary concentrated to 2.5 ml with Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane (Millipore, UFC901024) by centrifugation at 4000 g at room temperature (Megafuge 1.0R, Heraeus). The enzyme fraction was desalted with cold HEPES buffer containing 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (Sigma, 54457), 150 mM NaCl, pH 7.4 using a PD-10 column (GE Healthcare, 17-0851-01) according to the manufacturers gravity protocol. Enzyme concentration was determined by measuring the absorption at 280 nm on a Lambda 25 UV/VIS spectrometer (PerkinElmer). A semi-micro cuvette of quartz glass (45 mm H x 12.5 mm D x 12.5 mm W, volume 1400 µl, light path 10 mm) (Hellma, 104-10-40) was blanked against 990 µl HEPES buffer before 10 µl enzyme were added and measured. Following formula was used for the concentration determination: $[c] = \text{mean } A_{enzyme} \times 100 / A^{0.1\%}_{1}$ with $A^{0.1\%}_{1}$ (FabI) = 0.951, $A^{0.1\%}_{1}$ (FabG) = 0.586 and $A^{0.1\%}_{1}$ (FabZ) = 0.606. Enzymes with a concentration below 4 mg/ml were concentrated with Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane (Millipore, UFC801024) as described above. The enzyme was stored in 50% glycerol at -20°C for a maximum of 3 month.

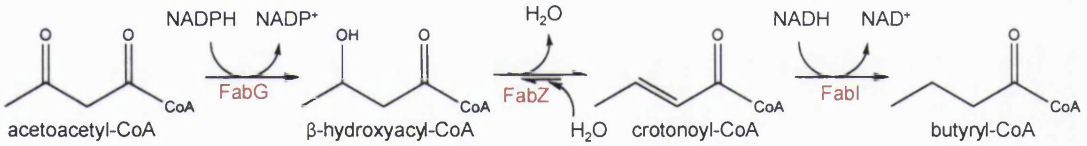
The purification was documented by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Samples were taken from the cell lysate before centrifugation, the supernatant after centrifugation, the flow-through after loading the column (2 µl each), the flow-through after washing the column with 50 mM imidazole (10 µl), from each fraction containing enzyme before combination (10 µl) and the enzyme fractions after desalting (10 µl). To determine the amount of insoluble protein a small amount of the pellet was included as well. All samples were added up to 20 µl with sample buffer containing 166 mM TRIS pH 6.8, 10% glycerol, 2% (w/v) sodium dodecyl sulphate (SDS) (Sigma-Aldrich, L6026), 3.5% 2-mercaptoethanol (Sigma, M7522), 0.17% bromphenol blue-xylene cyanole dye solution (Sigma, B3269) and heated for 5 min at 95°C. As marker a low range SDS-PAGE standard was used (Bio-Rad, 161-0304). The samples were applied into the slots of a 15% polyacrylamide gel. The gel was prepared and run with

200 V (constant) and a maximum of 400 mA for 50 min by using the Mini-PROTEAN Tetra Cell (Bio-Rad, 165-8025). For the separation gel acrylamide 4K solution (30%) (AppliChem, A1672), 1.5 M TRIS pH 8.8, 10% (w/v) SDS, N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma, T9281) and 10% (w/v) ammonium persulphate (APS) (Sigma-Aldrich, 248614) were used, for the stacking gel the TRIS was exchanged for 0.5 M TRIS pH 6.8. The running buffer contained 25 mM TRIS, 192 mM glycine (Sigma, 50046), 0.1% (w/v) SDS. To visualise the proteins in the gel it was stained with 0.1% (w/v) brilliant blue G (Sigma, 27815), 50% methanol, 6% glacial acetic acid (VWR, analytical grade) for 30 sec in a microwave (700 W) and destained for at least 12 h in 5% methanol, 7.5% glacial acetic acid.

### 2.5.1.3 Enzyme inhibition assay

In the enzyme inhibition assays the compound or extract concentration which inhibited 50% of the enzyme ($IC_{50}$ value) was spectrophotometrically determined.

Figure 2.7. Reactions of fatty acid biosynthesis enzymes.




Both FabI and FabG enzymes are reductases. Thus, for FabI the oxidation of the cofactor NAPH to $NAD^+$ during the reduction of the substrate crotonoyl-CoA to butyryl-CoA was measured (Fig. 2.7). Principally the same measurement was performed for FabG, but here the substrate acetoacetyl-CoA was reduced to β-hydroxyacyl-CoA in a NADPH-dependent reaction. The oxidation of the cofactor in these reactions can be observed by changes in the absorption at 340 nm (Fig. 2.8). Full enzyme activity is determined in the absence of compound or extract and compared to the measurements with compound or extract. For FabZ the same principle applies, but as it is a dehydratase and not requires a cofactor, the hydration of crotonoyl-CoA to β-hydroxyacyl-CoA is measured at 263 nm. At this wavelength the reduction measured in the absorption is due to the saturation of crotonoyl-CoA.

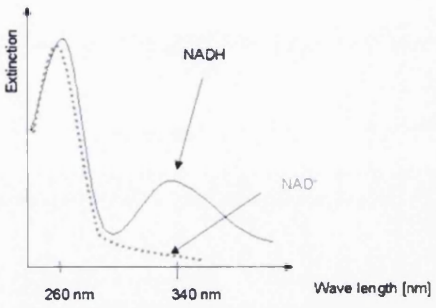



Figure 2.8. Absorption of NADH and $NAD^+$ at different wavelengths.

Two methods, a low-throughput cuvette assay (LTS) and a high-throughput microplate assay (HTS), were used to assess the enzyme inhibition.

Protocol for LTS

The cuvette assay was performed on Lambda 25 UV/VIS spectrometer (PerkinElmer) using a quartz semi-micro cuvette (Hellma, 104-10-40). Cofactor and substrate were dissolved in $H_2O$ and stored at -20°C. During the assay they were kept on ice. A sample of the enzyme was taken out of the -20°C stock and stored at room temperature during the assay, dilution was done with HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4). Samples were dissolved in dimethyl sulfoxide (DMSO) (Sigma, D8418) with a stock concentration of 10 mg/ml. The measurements were performed in a total volume of 1 ml HEPES buffer. The starting concentration of the samples was 10 µg/ml (1 µl of 10 mg/ml stock). Ten-fold dilutions were made with DMSO down to the concentration where full enzyme activity was detected. The highest concentration tested was 50 µg/ml (5 µl of 10 mg/ml stock). For FabI 100 µM (1 µl of 100 mM stock) cofactor β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH) (Sigma, 43420) was added to 1 µg (1 µl of 1 mg/ml stock) enzyme and sample and mixed well. The reaction was started by addition of 50 µM (1 µl of 50 mM stock) substrate 2-butenoyl coenzyme A lithium salt (crotonoyl-CoA) (Sigma, C6146). The mixture was read for 1 min at 340 nm. FabG inhibition was assessed in the same way, using β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH) (Fluka, 93220) as cofactor and acetoacetyl coenzyme A sodium salt hydrate (acetoacetyl-CoA) (Sigma, A1625) as substrate. FabZ was measured at 263 nm for 2 min in the presence of 25 µM (1 µl of 25 mM stock) crotonoyl-CoA and 1 µg (1 µl of 1 mg/ml stock) enzyme. As negative control the enzyme activity in the presence of 0.1% DMSO was determined.

Protocol for HTS

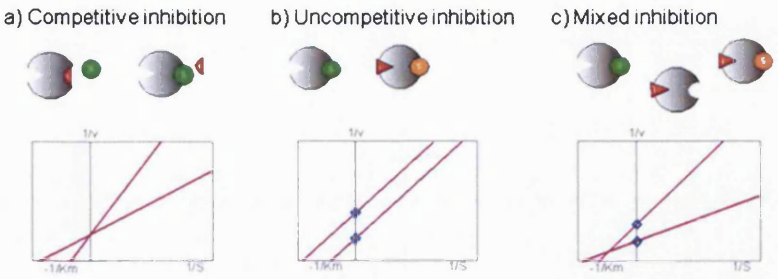
The microplate assay was performed on an EnVision 2101 multilabel reader (PerkinElmer). Enzyme, samples, substrate and cofactor are prepared as described above. For the assay of FabI and FabG polystyrene clear 96-well microplates with a flat bottom (Nunc, 167008) were used, for FabZ UV-transparent microplates (Corning, 3635) had to be used. The measurements were performed in duplicates in a total volume of 200 µl HEPES buffer. As negative control the enzyme activity in the presence of 0.5% DMSO was measured. Starting concentration of the samples was 50 µg/ml and samples were subjected six times to a 10-fold dilution series resulting in test concentrations ranging from 0.0005 µg/ml to 50 µg/ml. For FabI 2 µg enzyme (4 µl of 0.1 mg/ml stock), 400 µM NADH (8 µl of 10 mM

stock) and 300 µM crotonoyl-CoA (12 µl of 5 mM stock) were measured at 340 nm for 37 min. For FabG 0.5 µg enzyme (1 µl of 0.1 mg/ml stock), 400 µM NADPH (8 µl of 10 mM stock) and 300 µM acetoacetyl-CoA (12 µl of 5 mM stock) were measured at 340 nm for 17 min. FabZ was measured at 260 nm for 17 min with 0.5 µg enzyme (1 µl of 0.1 mg/ml stock) and 300 µM crotonoyl-CoA (12 µl of 10 mM stock). Reference compounds for both assays were (-)epigallocatechin gallate (EGCG) (Fluka 50299) for FabG and FabZ and triclosan (Sigma, 72779) for FabI. Fifty percent inhibitory concentrations ($IC_{50}$ values) were estimated from graphically plotted concentration-response curves.

### 2.5.1.4 Enzyme kinetic studies

Kinetic studies were performed in order to elucidate the binding mechanism and site of a compound to an enzyme. Different types of enzyme inhibition are known (Fig. 2.9). In a competitive inhibition the inhibitor and substrate compete for the active site, thus cannot bind the enzyme simultaneously (Fig. 2.9 a). In an uncompetitive inhibition the inhibitor only binds to the enzyme-substrate complex (Fig. 2.9 b). In a mixed inhibition the inhibitor can bind to the free enzyme or enzyme-substrate complex. The binding of the inhibitor results in conformational changes in the enzyme and reduces the affinity of the substrate for the active site, thus affecting the binding of the substrate and *vice versa* (Fig. 2.9 c).

Figure 2.9. Enzyme inhibition types. a) competitive inhibition, b) uncompetitive inhibition, c) mixed inhibition. Illustrated and Lineweaver-Burke plot.




To determine the inhibition type of a compound with respect to the substrate, the effect of different inhibitor concentrations has to be measured against varying substrate concentrations. The results are plotted in a Lineweaver-Burk plot (or double reciprocal plot), a graphical representations of the Michaelis–Menten equation, which visualises the inhibition type (Fig. 2.9).

The dissociation constant of the inhibitor ($K_i$) gives the strength of a bond between an enzyme and its inhibitor and was obtained from a Dixon plot. In a Dixon plot, the 1/v

from the Lineweaver-Burke plot is plotted against the compound concentration for different substrate concentration. The -$K_i$ value is the x-value where the lines intersect.

Protocol

In this study the kinetic of evernic acid on FabZ was determined. The inhibition mechanism with respect to the substrate was determined similar to the enzyme inhibition studies, performing the cuvette assay. Briefly, the enzyme activity of 1 µg FabZ was assessed in the presence of various inhibitor concentrations (0-2 µg/ml) at 263 nm for 2 min. The reaction was initiated by the addition of the substrate crotonoyl-CoA in different concentrations (10-50 µM). To determine the $K_i$ value the measured data are transferred into a Lineweaver-Burke plot. Measured with at least three different compound concentrations, the inhibition type was identified and the $K_i$ value obtained from a Dixon plot. The reported values represent means of two independent experiments.

### 2.5.2 Inhibition assay for *P. falciparum* blood stage parasites

Parasite growth inhibition is measured by incorporation of [$^3$H]hypoxanthine, allowing to determine the compound or extract concentration at which 50% of the growth is inhibited ($IC_{50}$ value). The purine derivative hypoxanthine is a naturally occurring product. *Plasmodium falciparum* depends on hypoxanthine as a source of nitrogen for nucleic acid synthesis and energy metabolism. For the drug assay, the parasites are incubated with different concentrations of compounds or extracts for 48 h, as this is the period *P. falciparum* needs to complete one replication cycle. In the screening medium only a small amount of hypoxanthine is present which is used up after this incubation period. Then [$^3$H]hypoxanthine is added and after a further incubation of 24 h, the parasites are harvested and the incorporated amount of [$^3$H]hypoxanthine counted in a scintillation counter. Compounds or extract with antiplasmodial effects will have killed the parasites in the first 48 h, so no radioactivity will be detected. The gradual increase of the incorporated amount of [$^3$H]hypoxanthine with decreasing compound or extract concentrations is used in order to calculate the $IC_{50}$ values. The incorporated radioactivity of the parasites without compounds or extracts is used as a positive control from which the background radioactivity detected in the negative control (only erythrocytes) is subtracted.

In this study the multiple drug resistant *Plasmodium falciparum* strain K1 was used. The strain was originally found in Thailand and is resistant against the antimalarial drugs chloroquine, sulphadoxine and pyrimethamine.

Protocol

*Plasmodium falciparum* K1 (Malaria Research and Reference Reagent Resource Centre) was cultivated in 75 $cm^2$ flasks in RPMI-1640 (Sigma, R5886) containing 0.03% (w/v) glutamine (Sigma, G8540), 0.2% glucose (w/v) (Sigma, G5767), 0.5% (w/v) albumin (Invitrogen, 11021037) and 150 µM hypoxanthine (Sigma, H9636) with 5% hematocrit in $A^+$ human blood (National Blood Service) and kept at 37°C in 5% $CO_2$. They were subcultured every 48-72 h to 0.5% parasitemia.

*In vitro* parasite growth inhibition in the erythrocytic stage was assessed by the incorporation of [$^3$H]hypoxanthine (Desjardins *et al.*, 1979). All assays were conducted as triplicate measurement in 96-well clear polystyrene flat bottom microplates (BD, 353075) and included control wells with untreated infected erythrocytes (positive control) and uninfected erythrocytes (negative control). Samples were dissolved in DMSO with a stock concentration of 10 mg/ml. Artesunate (Bayer AG, Batch No. DHN060302, 99.2%; dissolved in DMSO) and chloroquine diphosphate (Sigma, 25745; dissolved in water) were used as reference compounds. All samples were diluted to two times the starting concentration with culture medium containing 15 µM hypoxanthine. Samples were subjected twelve times to a to two-fold dilution series, resulting in test concentrations ranging from 0.024 µg/ml to 50 µg/ml. Starting concentrations for artesunate and chloroquine diphosphate were 0.1 µg/ml and 6 µg/ml, respectively. For the assay 50 µl of *P. falciparum* K1 culture (60-80% ring stage) at 0.4% parasitemia were added to each well (except the negative control) containing sample or medium (positive control), reaching a final volume of 100 µl per well and a final hematocrit of 2.5%. Plates were incubated at 37°C in 5% $CO_2$. After 48 h 20 µl (0.2 µCi/well) of [$^3$H]hypoxanthine (Perkin Elmer, NET177005MC) was added to each well and plates were returned to the incubator for an additional 24 h incubation period. The experiment was terminated by placing the plates in a -80°C freezer overnight. Plates were harvested within a week by transferring the well content onto a glassfibre filter (PerkinElmer, 1450-421) using a 96-well cell harvester (Harvester 96 Mach III M, Tomtec). After the addition of MeltiLex solid scintillator (PerkinElmer, 1450-441) to the dry filter mats, the incorporated radioactivity was counted using a 1450 Microbeta Trilux scintillation counter (PerkinElmer). $IC_{50}$ values were estimated by linear interpolation (Huber & Koella, 1993).

### 2.5.3 Cytotoxicity against KB cells

Cytotoxicity assessment was used to determine selective toxicity of compounds or extracts. For this assay KB cells were used, which are derived from a human carcinoma of the nasopharynx. The compound or extract concentration at which the growth of 50% of the

KB cells is inhibited was determined ($IC_{50}$ value). The growth of KB cells was monitored through a chemical reduction of the reagent AlamarBlue. For this, the KB cells were plated out and different concentrations of the compound or extract were added and incubated for 72 h. Toxic compounds or extracts will kill the cells. After this incubation time the reagent AlamarBlue was added and incubated for another 3-6 h. This agent shows cell growth dependant chemical reduction, with living cells continuing their growth and providing a reduced environment (fluorescent, red), whereas the environment of dead cells is oxidised (non-fluorescent, blue). These differences were detected by measuring the fluorescence emission at 585 nm after excitation at 530 nm.

Protocol

KB cells (American Type Culture Collection) were maintained as a monolayer in 25 $cm^2$ flasks in RPMI-1640 (Sigma, R5886) culture medium containing 200 mM L-glutamine (Sigma, G7513) and 10% foetal calf serum (Harlan, Batch no. 09010501). They were subcultured every 7 d to 0.4-1 x $10^4$ cells/ml and kept at 37°C in 5% $CO_2$.

All assays were conducted as triplicate measurement in 96-well clear polystyrene flat bottom microplates (BD, 353075) and included control wells with untreated KB cells (positive control) and medium (negative control). Samples were dissolved in DMSO with a stock concentration of 10 mg/ml. Reference compounds were artesunate, chloroquine diphosphate and podophyllotoxin (Sigma, P4405). For the assay 100 µl cells were seeded to result in 4 x $10^3$ cells per well (except negative control). The plates were incubated at 37°C in 5% $CO_2$ for 24 h. All samples were diluted to two times the starting concentration with culture medium without L-glutamine. Samples were subjected six times to a to ten-fold dilution series, resulting in test concentrations ranging from 0.0005 µg/ml to 50 µg/ml. 100 µl samples were added to the cells and plates incubated for further 72 h at which point 20 µl AlamarBlue (Serotec, BUF012B) were added, followed by a 3-6 h incubation period. The fluorescence emission was measured with a Spectramax Gemini plate reader (Molecular Devices) at 585 nm after excitation at 530 nm. $IC_{50}$ values were calculated by linear interpolation (Huber & Koella, 1993).

### 2.5.4 *P. yoelii* liver stage inhibition assay and cytotoxicity against hepatocytes

The liver stage assays described below were performed in collaboration with Dr. A. Tarun and Assoc. Prof. S. Kappe of the Seattle Biomedical Research Institute, USA.

For the assessment of the *in vitro* liver stage inhibition the human hepatocarcinoma cell line HepG2:CD81, infected with *P. yoelii* parasites, was used. The reasons for using the rodent

malaria model have been explained in detail in the introduction (1.1.4.2). The HepG2:CD81 cell line was chosen because the cells stably express CD81, a protein which is required for high infection rates (Silvie *et al.*, 2003; Bartosch *et al.*, 2003).

For the assessment of the liver stage inhibition, two assays were applied: flow cytometry (FC) and an immunofluorescence analysis (IFA) (Tarun *et al.*, 2008; Tasdemir *et al.*, 2010). In the FC, the infection rates were measured by the detection of GFP-expressing parasites by flow cytometry. The IFA was used to verify the results and determine the effect of the compounds on the morphology and development of liver stage parasites by visualising the parasites with multiple antibody staining.

Protocol for cultivation

Female Swiss Webster and BALB/c mice (Harlan) were kept in a temperature- and humidity-controlled environment under a 12 h light/dark cycle in accordance to the Institutional Animal Care and Use Committee approved protocols. *P. yoelii* wild-type and transgenic sporozoites that express green fluorescent protein (*Py*GFP) parasites were cycled between *Anopheles stephensi* mosquitoes and Swiss Webster mice. Infected mosquitoes were maintained at 24°C and 70% humidity under a 14 h light/10 h dark cycle on sugar and water. *Py*GFP sporozoites were isolated manually from the salivary glands of *Py*GFP infected mosquitoes 14 d post blood meal in RPMI 1640 medium (Sigma) with 5% mouse serum (Antibodies) and 2% penicillin-streptomycin (Sigma) (Tarun *et al.*, 2006).

HepG2:CD81 cells were grown in complete medium containing advanced DMEM/F12, 10% FBS, 2% penicillin/streptomycin, 2% glutamine and 1% amphotericin.

Protocol for flow cytometry

For the flow cytometry (FC) a BD-LSRII flow cytometer (BD Biosciences) was used. One day prior to infection 100,000 cells per well were seeded in 48-well plates (Corning) coated with 20 µg/ml ECL attachment matrix (Upstate Labs) and incubated at 37°C in 5% $CO_2$. Each well was infected with 50,000 *Py*GFP sporozoites (in RPMI media, 100 µl/well) and the plate centrifuged at 1000 rpm for 3 min and incubated at 37°C. Compounds were dissolved at 10 mM in DMSO and tested at concentrations of 10 to 100 µM in complete medium. 2 h postinfectious cells were carefully washed twice with DMEM/F12 medium and cells were exposed to 750 µl complete medium containing varying concentrations of compounds and incubated at 37°C in a $CO_2$ chamber. Medium containing corresponding compound concentrations was changed daily. After 40 h cells were detached with TrypLE (Invitrogen) and added to 300 µl complete medium to prepare a single cell suspension. Cells were centrifuged at 1000 rpm for 1 min and resuspended in 100 µl complete medium

containing 0.1% 7-amino-actinomycin D (7-AAD) (Invitrogen) which is an impermeant nucleic acid dye and acted as viability marker (see below). After transfer to 96-well v-bottom plates, the number of GFP-positive hepatoma cells was determined by flow cytometry for 60 min using a 23 sec acquisition time per well. In most cases the BD-LSRII flow cytometer counted 50,000-100,000 events from each well with 1.0 – 2.0% GFP-positive cells in untreated control wells. Atovaquone was used as a reference compound.

Protocol for immunofluorescence analysis

For the immunofluorescence analysis (IFA) HepG2:CD81 cells from subconfluent cultures were seeded at a density of 75,000 cells/well in 8-well chambered slides (Nunc) and maintained at 37°C in 5% $CO_2$. Each well was infected with 75,000 *P. yoelii* sporozoites. Compounds were dissolved in DMSO and tested at concentrations of 10 to 100 μM in complete media. 3 h postinfectious the cells were treated with varying compound concentrations. After 43 h of incubation at 37°C in a $CO_2$ incubator, the infected cells were fixed with 10% neutral buffered formalin. The cells were incubated with a mouse monoclonal antibody against the *P. berghei* HSP70 protein (Tsuji *et al.*, 1994) (stains parasite cytoplasm) together with a rabbit polyclonal antibody raised against the *P. yoelii* UIS4 protein (Mueller *et al.*, 2005) (stains parasitophorous vacuole membrane). Additionally, the cells were stained with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) (see below). The slides were scanned with a fluorescence microscope and images were captured using a 20x objective. The resulting images were analyzed with the MetaMorph program (Molecular Devices) which allowed automated measurements of liver stage parasites stained with the HSP70 antibody. Approximately 20-25 liver stage parasites were measured for each compound and the average sizes were compared with DMSO treated controls. The normalized reduction in liver stages from infections treated with the compounds compared with DMSO treated controls was calculated and used for $IC_{50}$ estimation using the ICEstimator program (http://bichat.inserm.fr/equipes/ Emi0357/Palu/index.htm). Atovaquone was used as a reference compound.

Protocol for cytotoxicity against hepatocytes

Cytotoxicity of the compounds was assessed against HepG2:CD81, the same cell line used for the assessment of liver stage inhibition, by using the non-membrane permeant nuclear stain 7-aminoactinomycin D (7-AAD). The proportion of 7-AAD positive cells that was detected in the flow assay was used an indicator for dead cells in the HepG2:CD81 cell culture. Comparison of the 7-AAD positive cell fraction of DMSO-treated cells (less than 5% positive cells) to that of compound-treated cells can be used as an indicator for

cytotoxicity of the compounds in the treated cultures. However, this method gives only a general indication of the cytotoxicity, as floating cells (*i.e.* dead cells) are removed during the daily media exchange during the 40 h incubation period.

In the IFA, cytotoxicity of the compounds were assessed by comparing the 4',6-diamidino-2-phenylindole (DAPI) staining of infected HepG2:CD81 cells treated with DMSO alone or with various concentrations of the compounds. Cytotoxic concentrations of drugs, such as high concentrations of atovaquone, result in cell death and detachment from the culture slides which result in less DAPI-stained cells. Again, this method only gives and indication of cytotoxicity and cannot be used to quantify the cytotoxicity, as cells could have been dislodged during the IFA procedure and the visual assessment is based on subjective observation.

## 2.6 Human FAS-I enzyme

The expression and purification of the complete human FAS-I enzyme has been established via different ways. Expression in *E. coli* (Jayakumar *et al.*, 1996) or the human hepatoma cell line HepG2 (Jayakumar *et al.*, 1995) were unfeasible and therefore the well established Bac-to-Bac baculovirus expression system was chosen (Ciccarone *et al.*, 1997). Baculovirus belongs to the group of double-stranded DNS viruses. The most commonly used virus is the *Autographa californica* multiple nuclear polyhedrosis virus (*Ac*MNPV) who multiplies in cells in the intestinal tract of *Autographa californica* and *Spodoptera frugiperda.* The gene of interest is cloned into a pFastBac vector. The vector used in this study is pFastBac1, where the expression of the gene is controlled by a strong *Ac*MNPV polyhedrin promotor. The pFastBac vector is transformed into the *E. coli* strain DH10Bac, that contains a baculovirus shuttle vector (bacmid) and a helper plasmid, which generates a recombinant bacmid (containing the gene of interest) by transposition of the pFastBac construct. The recombinant bacmid DNA can be isolated and used to infect *Spodoptera frugiperda* cells to generate recombinant baculovirus containing the gene of interest. The baculovirus stock is amplified and can be used to express the protein of interest.

### 2.6.1 *Sf9* cell culture

The cell line chosen for this study were *Spodoptera frugiperda* (*Sf*) cells. The cell line *Sf9* is a clonal isolate of the IPLBSF-21 cell line, which was derived from the pupal ovarian tissue of *Spodoptera frugiperda* (Vaughn *et al.*, 1977). This cell line is traditionally used with baculovirus and grows well in monolayer and suspension culture. The cells are regular in size and have a spherical with some granular appearance.

Protocol for thawing cells into shaking culture

*Spodoptera frugiperda* (*Sf9*) cells were purchased from Invitrogen (11496-015) and stored in liquid nitrogen until thawing. The vial containing frozen cells was placed in a 37°C water bath and thawed rapidly with gentle agitation until the cells were almost thawed. Complete thawing of cells had to be avoided, as this would result in cell death. The vial was removed from the water bath and cells were titrated into 27 ml pre-warmed *Sf*-900 II SFM medium (Invitrogen, 10902-153) with 10 µg/ml gentamycin (Fluka, 46305) in a sterile 125 ml shaking flask and incubated at 28°C with 130 rpm. The cap of the flask was tightened loosely to ensure gas exchange. Cell density and viability was checked every 24 h until a cell density of 3-5 x $10^6$ cell/ml was reached, at which point passaging of cells was started (see below). The viability of revived cells should be 70% or greater and freshly thawed cells should at least been passaged twice before used for any assays.

Protocol for the determination of cell density and viability

To determine cell density and viability 200 µl of cells were added to a mixture of 500 µl 0.4% trypan blue (Sigma, T8154) and 300 µl PBS buffer containing 140 mM NaCl (Sigma, S5886), 2.7 mM KCl (Sigma, P5405), 10 mM $Na_2HPO_4$ (Sigma, S5136), 1.8 mM $KH_2PO_4$ (Sigma, P5655), pH 7.5. The cell suspension was loaded on a Neubauer improved hemacytometer and examined immediately under a microscope at low magnification to check if the cell suspension was spread equally over the hemacytometer. The number of blue stained cells (*i.e.* dead cells) and the number of total cells were counted in several groups of squares and the average was calculated. The cell density and viability was calculated as followed:

cells/ml = viable cells x $10^4$ x 5

% viable cells = [ 1 – (blue cells : total cells) ] x 100

Protocol for the passaging of cells and maintenance of cell culture

Cell density and viability was determined every 48-72 h and cells were subcultured to 0.3-0.5 x $10^6$ cells/ml with pre-warmed *Sf*-900 II SFM medium with 10 µg/ml gentamycin when they reached a density of 2-3 x $10^6$ cells/ml to maintain consistent and optimal cell growth. Every three weeks cells were centrifuged down at 100 g for 5-10 min and resuspend in fresh medium to remove cell debris and metabolic by-products.

Protocol for freezing *Sf9* cells

Cells were frozen with a cell density of 1-2 x $10^7$ cells/ml and at least 90% viability as soon as they established a normal generation time to keep the passage number as low as possible. For this, 2 ml sterile cryovials were set up on ice and cells centrifuged at 100 g for 5-10 min at room temperature. The supernatant was removed and cells were resuspend in freshly prepared, sterile-filtered, cold (4°C) freezing medium containing 50% fresh *Sf*-900 II SFM medium, 50% conditioned medium (medium collected from subculturing procedure) and 7.5% DMSO (Sigma, 472301). 1.5 ml of the cell suspension were transferred into the cold cryovials and frozen in a Nalgen Cryo freezing container (Nalgen, 5100-0001) according to the manufactures protocol. Cells were store in liquid nitrogen.

### 2.6.2 Determination of virus titre of human fatty acid synthase baculovirus stock

The pFastBac1 clone containing the cDNA for the entire human fatty acid synthase (hFAS) with a C-terminal polyhistidin tag was cloned by GlaxoSmithKline (Vazquez *et al.*, 2008). The bacmid and baculovirus stocks containing the hFAS construct were produced with the transposition procedure of the Bac-to-Bac baculovirus expression system (Carlisle-

Moore *et al.*, 2005) and were given as a generous gift by Prof. P. J. Tonge from the Stony Brook University, USA. For the expression of hFAS in *Sf*9 cells, the virus titre (plaque forming units (pfu) per ml) was required, thus as a first step a viral plaque assay was performed to determine the titre.

Protocol viral plaque assay

The viral plaque assay was performed according to the method described in the manual "Bac-to-Bac Baculovirus Expression System" version E (2009) by Invitrogen. Briefly, a 30 ml *Sf*9 cell suspension with 5 x $10^5$ cells/ml was prepared in *Sf*-900 II SFM medium and 2 ml of the cells suspension were aliquoted into each well of 6-well plates. Cells were incubated at room temperature for 1 h to allow them to settle to the bottom of the plate. Attachment of cells and 50% confluence were checked under an inverted microscope. The medium from each well was removed and immediately replaced with 1 ml of appropriate baculovirus stock dilutions, which were prepared with *Sf*-900 II SFM medium in test concentrations of pure virus stock to $10^{-4}$. Pure medium was used as negative control. Cells were incubated with the virus for 1 h at room temperature, before the medium was removed and replaced with warm plaquing medium (40°C) containing 30 ml *Sf*-900 medium (1.3 X) (Invitrogen, 10967) and 10 ml 4% agarose gel (Sigma, A4018). The agarose overlayer was allowed to harden for 1 h at room temperature, before plates were placed in a humid environment and incubated at 28°C. After 4 d 1 ml of a neutral red agarose overlayer containing 1.5 ml of 1 mg/ml neutral red solution (Sigma, N4638), 16.5 ml *Sf*-900 II SFM medium and 6 ml 4% agarose was added to each well and plates were incubated for another 3-6 d at 28°C in a humid environment. Plaques appeared as clear spots and were counted for each dilution. The titre was calculated with the following formula:

titre (pfu/ml) = number of plaques x dilution factor

# Part 3

# Results

## 3.1 Implementation of enzyme related methods

In this chapter the methods implemented or developed at the School of Pharmacy will be presented.

### 3.1.1 Expression and purification of plasmodial FAS-II enzymes

The expression of the enzymes was performed under standard conditions as described in 2.5.1.1. Purification of proteins with a polyhistidine tag (His-tag) with metal chelate affinity chromatography is a well established method (Porath *et al.*, 1975) and was performed as described in 2.5.1.2.

Figure 3.1. Documentation of the purification of FabG by SDS-PAGE. M: low range marker, L: cell lysate before centrifugation, S: supernatant after centrifugation, P: pellet after centrifugation, FT: flow-through after loading column, W: flow-through after washing column, E: fraction containing enzyme before combination, E*: enzyme fractions after desalting.

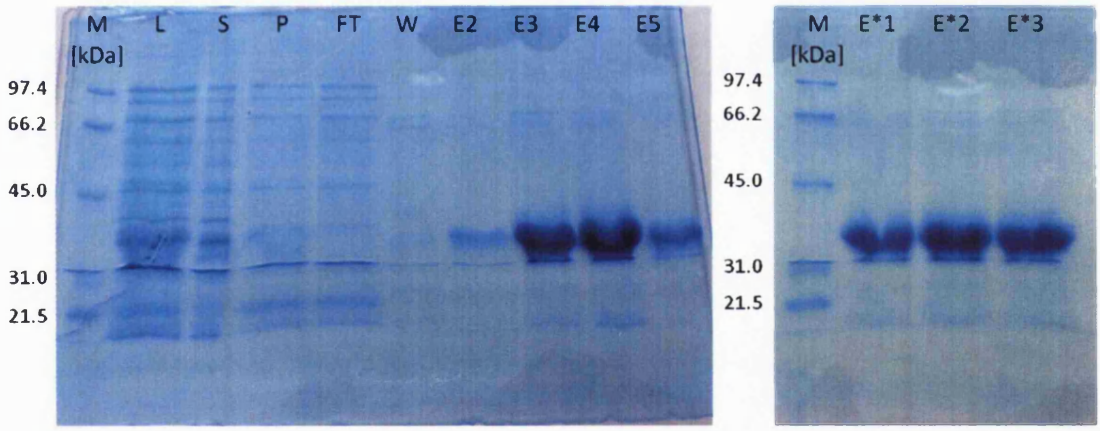


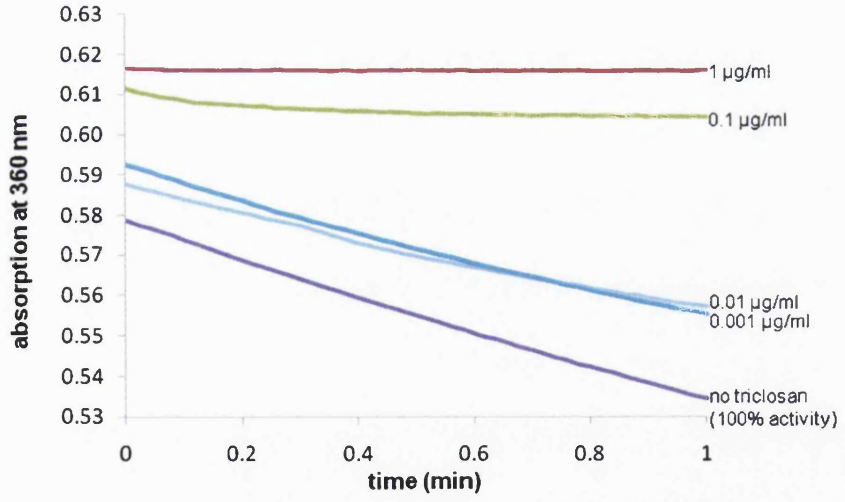

Figure 3.1 shows the purification gels for FabG (32.9 kDa). In order to document the complete purification, samples were taken from all purification steps and ran on SDS-PAGE (2.5.1.2). The first band in each gel contains a low-range marker which was used to assess the size of the protein bands. A sample of the complete cell lysate after disruption of the cells confirmed the presence of numerous proteins, including the protein of interest, in the lysate (L). The supernatant after centrifugation (S) contained all soluble proteins, while the pellet (P) contained insoluble proteins and cell debris. Ideally, and as seen in Figure 3.1, the pellet should not contain the protein of interest. After loading the supernatant onto the nickel-NTA column, the flow-through (FT) was collected and should contain all proteins without His-tag. The column was washed with 50 mM imidazole (W) to remove all proteins without His-tag, before the protein was eluted (E). As a last step, the enzyme

fractions were desalted (E*) and the buffer used for the purification changed to the buffer required for the enzyme assay.

### 3.1.2 Inhibition assay with plasmodial FAS-II enzymes

The inhibition of FAS-II enzymes FabI, FabG and FabZ was measured as described in 2.5.1.3. In the following, the inhibition of FabI by triclosan will be used to demonstrate the assay. The reductase FabI reduces the substrate crotonoyl-CoA to butyryl-CoA in a NADH-dependant reaction. The absorption monitored at 340 nm represents the amount of the cofactor NADH present in the reaction tube. The decrease in the amount of NADH is due to its oxidation to $NAD^+$, which is catalysed by FabI and results in a decreasing slope. High concentrations of triclosan (1 µg/ml) inhibit FabI, thus the reaction and oxidation of NADH is inhibited (Fig. 3.2). Low concentrations of triclosan on the other hand (0.001 µg/ml) do not inhibit FabI, hence NADH oxidation occurs, resulting in a decreasing slope (Fig. 3.2).

Figure 3.2. Absorption of NADH over time for different triclosan concentrations.

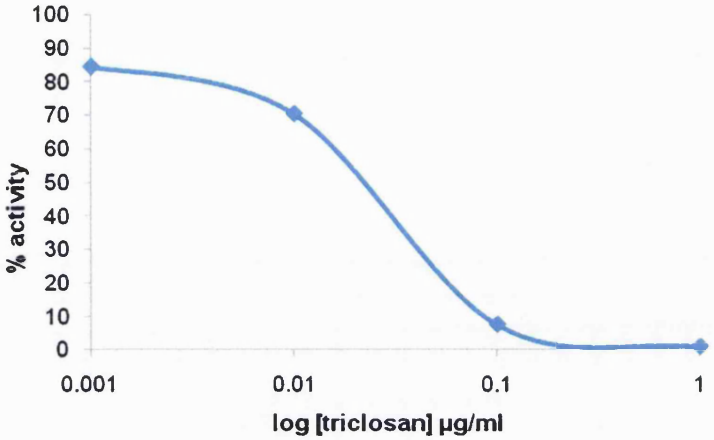



The decrease in the absorption of NADH without the presence of any inhibitor is measured and set as 100% enzyme activity. Thus, the comparison of the slopes from the uninhibited reaction to the slopes from the inhibited reactions can be used to calculate the activity of FabI. The concentration-response curve for FabI inhibition at different triclosan concentrations can be plotted and the $IC_{50}$ value estimated from the graph (Fig. 3.3).

For the low-throughput screening, the conditions required to measure the enzyme inhibitions were known (Tasdemir *et al.*, 2006) and only minor modifications for the FabZ inhibition assay were implemented. The development of the high-throughput screening

required more effort as the extension of the time necessitated adaptation in the enzyme, cofactor and substrate concentrations. Both assays resulted in similar results when the same control drugs was tested.

Figure 3.3. Concentration-response curve for FabI inhibition by triclosan.




### 3.1.3 Expression of hFAS-I

The required baculovirus containing hFAS DNA for the expression of hFAS in the cell line *Sf9* was a generous gift from Prof. P. J. Tonge from the Stony Brook University, USA. A plaque assay was performed in order to determine the titre of the baculovirus (2.6.2). Unfortunately, even undiluted virus was unable to infect *Sf9* cells, thus could not be used for virus amplification or hFAS expression. The design and production of fresh baculovirus would have been a long and complicated process and could not be performed due to time limitations. Hence the expression of the human fatty acid synthase enzyme could not be achieved.

## 3.2 Screening of Turkish plants

This chapter shows the results for the screening of the crude extract and subextracts of *Anthemis cretica* subsp. *anatolica*, *A. pestalozzae*, *Scrophularia lucida*, *S. pinardii* and *Salvia virgata*. The aim of the screening was to investigate the chemical profile and evaluate the biological activity of the plants in order to select the most interesting species for an in-depth phytochemical study.

### 3.2.1 Plant extraction

For the initial screening, 10 g dried plant material of *Anthemis cretica* subsp. *anatolica* (**1** aerial parts, **2** roots), *Anthemis pestalozzae* (**3** aerial parts, **4** roots), *Salvia virgata* (**5** aerial parts), *Scrophularia lucida* (**6** aerial parts, **7** roots) and *Scrophularia pinardii* (**8** aerial parts, **9** roots) were used for extraction as described in 2.2. All crude extracts were subjected to liquid-liquid partitioning using solvents with increasing polarity: hexane (**a**), chloroform (**b**) and aq. methanol (**c**). The obtained extracts and amounts are summarized in Table 3.1.

Table 3.1. Summary of extraction and liquid-liquid partitioning.

| Code | Plant species | Family | Extract | Yield (mg) |
|---|---|---|---|---|
| **1** | *Anthemis cretica* subsp. | Asteraceae | crude | N/A* |
| **1a** | *anatolica* | | hexane | 143.1 |
| **1b** | (aerial parts) | | $CHCl_3$ | 199 |
| **1c** | | | aq. MeOH | 870 |
| **2** | *Anthemis cretica* subsp. | Asteraceae | crude | N/A* |
| **2a** | *anatolica* | | hexane | 63.7 |
| **2b** | (roots) | | $CHCl_3$ | 142.4 |
| **2c** | | | aq. MeOH | 1061.6 |
| **3** | *Anthemis pestalozzae* | Asteraceae | crude | N/A* |
| **3a** | (aerial parts) | | hexane | 163.4 |
| **3b** | | | $CHCl_3$ | 128 |
| **3c** | | | aq. MeOH | 839 |
| **4** | *Anthemis pestalozzae* | Asteraceae | crude | N/A* |
| **4a** | (roots) | | hexane | 49.6 |
| **4b** | | | $CHCl_3$ | 84 |
| **4c** | | | aq. MeOH | 603.5 |
| **5** | *Salvia virgata* | Lamiaceae | crude | N/A* |
| **5a** | (aerial parts) | | hexane | 296.8 |
| **5b** | | | $CHCl_3$ | 433 |
| **5c** | | | aq. MeOH | 345.4 |

* not available, liquid-liquid partitioning was performed immediately after extraction

Table 3.1. continued

| Code | Plant species | Family | Extract | Yield (mg) |
|---|---|---|---|---|
| **6** | *Scrophularia lucida* | Scrophulariaceae | crude | 1950 |
| **6a** | (aerial parts) | | hexane | 164.6 |
| **6b** | | | $CHCl_3$ | 628.3 |
| **6c** | | | aq. MeOH | 1012.5 |
| **7** | *Scrophularia lucida* | Scrophulariaceae | crude | 1580 |
| **7a** | (roots) | | hexane | 35.6 |
| **7b** | | | $CHCl_3$ | 232.5 |
| **7c** | | | aq. MeOH | 971.9 |
| **8** | *Scrophularia pinardii* | Scrophulariaceae | crude | 3040 |
| **8a** | (aerial parts) | | hexane | 169.1 |
| **8b** | | | $CHCl_3$ | 1059.2 |
| **8c** | | | aq. MeOH | 1633.4 |
| **9** | *Scrophularia pinardii* | Scrophulariaceae | crude | 1020 |
| **9a** | (roots) | | hexane | 56.8 |
| **9b** | | | $CHCl_3$ | 146.6 |
| **9c** | | | aq. MeOH | 633.9 |

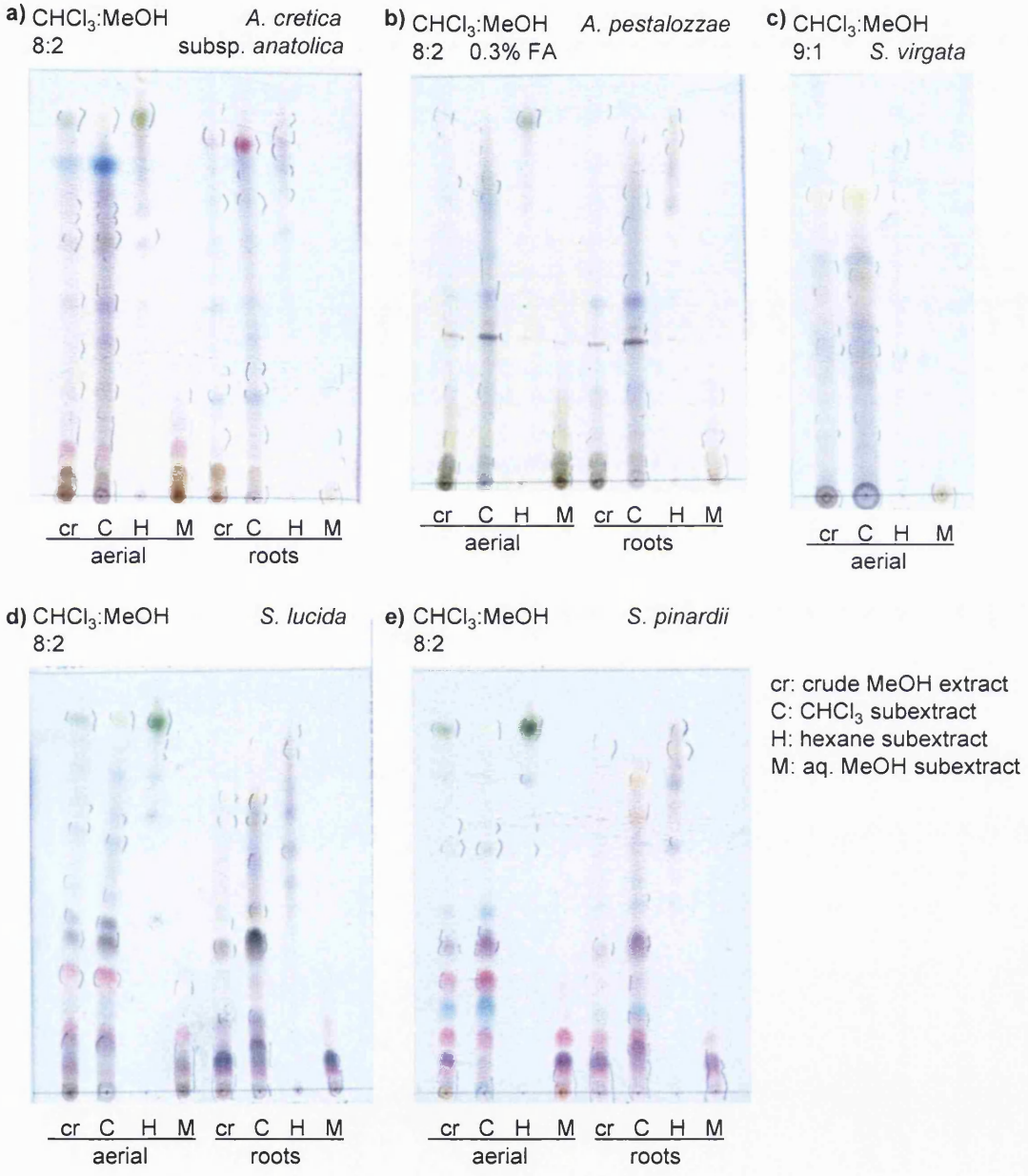
### 3.2.2 Chemical profile

For the chemical profiling the extract and subextracts of the aerial parts and roots from each plant were analysed by TLC and by $^1$H NMR.

#### 3.2.2.1 TLC analyses of extracts and subextracts

The TLC analysis (Fig. 3.4) showed the presence of chlorophyll (green spot) and other nonpolar compounds in the hexane subextracts for all plants. Polar compounds (*e.g.* sugars or glycosides) on the bottom of the TLC plates were observed in the aq. MeOH subextracts. The $CHCl_3$ subextracts contained the widest spectrum of compounds, including some compounds which were also present in the hexane or aq. MeOH subextract. The spray agent used to detect compounds was vanillin/sulphuric acid, which is a universal staining agent and indicated the presence of several secondary metabolites (blue, purple, pink, yellow and orange spots), which were especially rich in the $CHCl_3$ subextracts.

Figure 3.4. TLC analyses of extracts and subextracts. a) *A. cretica* subsp. *anatolica*, b) *A. pestalozzae*, c) *S. virgata*, d) *S. lucida* and e) *S. pinardii*. The first four spots on the TLC plates represent the extracts from the aerial parts, the next four spots the extracts from the roots. Stationary phase: silica gel 60 $F_{254}$, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170°C).



### 3.2.2.2. $^1$H NMR analyses of extracts and subextracts

The $^1$H NMR spectra of the crude extracts of all species are dominated by primary metabolites such as lipids and fatty acids (δ 0-2 ppm) and sugars (δ 3-4 ppm). The spectra for the extracts and subextracts of *Anthemis cretica* subsp. *anatolica* (Fig. 3.5), *A. pestalozzae* (Fig. 3.6), *Salvia virgata* (Fig. 3.7), *Scrophularia lucida* (Fig. 3.8) and *S. pinardii* (Fig. 3.9) showed that the liquid-liquid partitioning resulted in a transfer of most non-polar components such as fatty acids and other lipids into the hexane subextract (blue spectrum), while most sugars can be found in the aq. MeOH fraction (green spectrum). When comparing the subextracts, especially in the downfield, major differences are detectable. Here, characteristic or functional groups such as anomeric protons (δ 4-5 pm), double bonds (δ 5-6 ppm) or aromatic protons (δ 6-8 ppm), which often belong to secondary metabolites such as phenolic compounds or glycosides, can be seen.

The results obtained by TLC and $^1$H NMR analyses showed a rough separation of the crude extracts according to the polarity of the components, which was expected from the liquid-liquid partitioning. All extracts and subextracts were rich in phytochemically interesting secondary metabolites. The extracts from species belonging the to the same genus showed similarities in their $^1$H NMR profiles, suggesting the presence of similar compounds.

Figure 3.5. $^{1}$H NMR spectra (400 MHz) of *Anthemis cretica* subsp. *anatolica*. a) aerial parts, b) roots. black: crude extract in MeOD, blue: hexane subextract in $CDCl_3$, red: $CHCl_3$ subextract in MeOD, green: aq. MeOH subextract in MeOD.

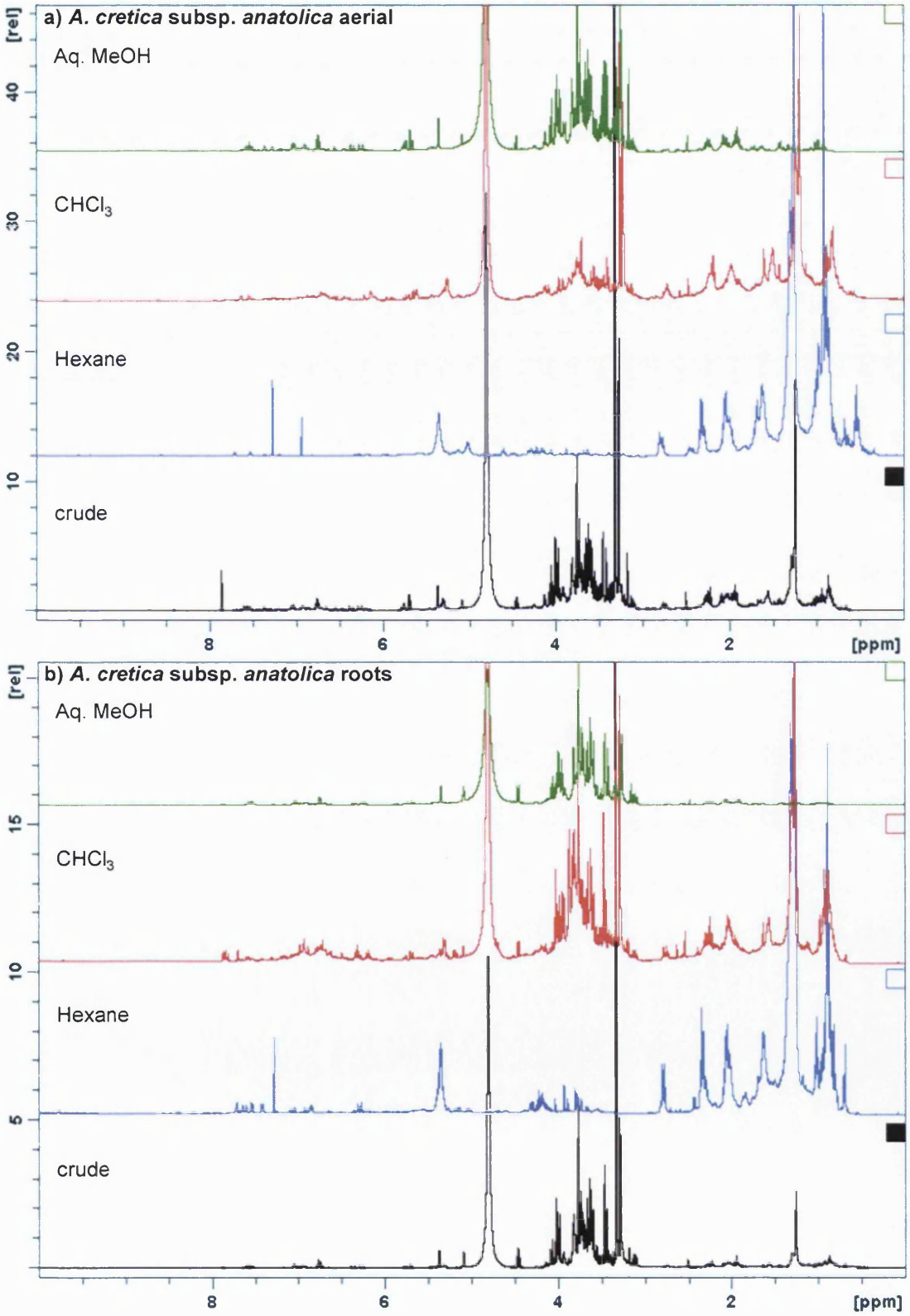


Figure 3.6. $^{1}$H NMR spectra (400 MHz) of *Anthemis pestalozzae*. a) aerial parts, b) roots. black: crude extract in MeOD, blue: hexane subextract in $CDCl_3$, red: $CHCl_3$ subextract in MeOD, green: aq. MeOH subextract in MeOD.

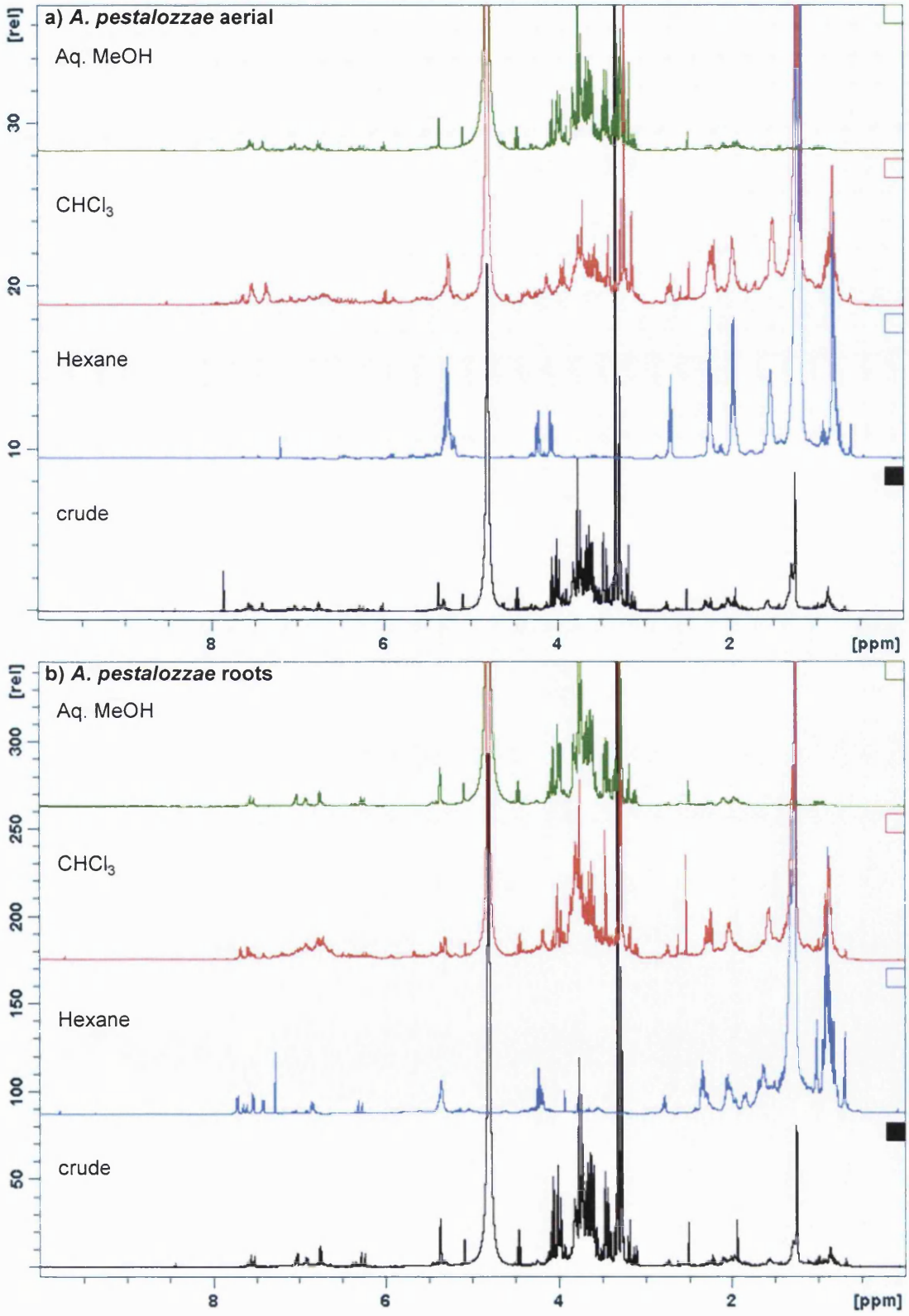


Figure 3.7. $^{1}H$ NMR spectra (400 MHz) of *Salvia virgata* (aerial parts). black: crude extract in MeOD, blue: hexane subextract in $CDCl_3$, red: $CHCl_3$ subextract in MeOD, green: aq. MeOH subextract in MeOD.

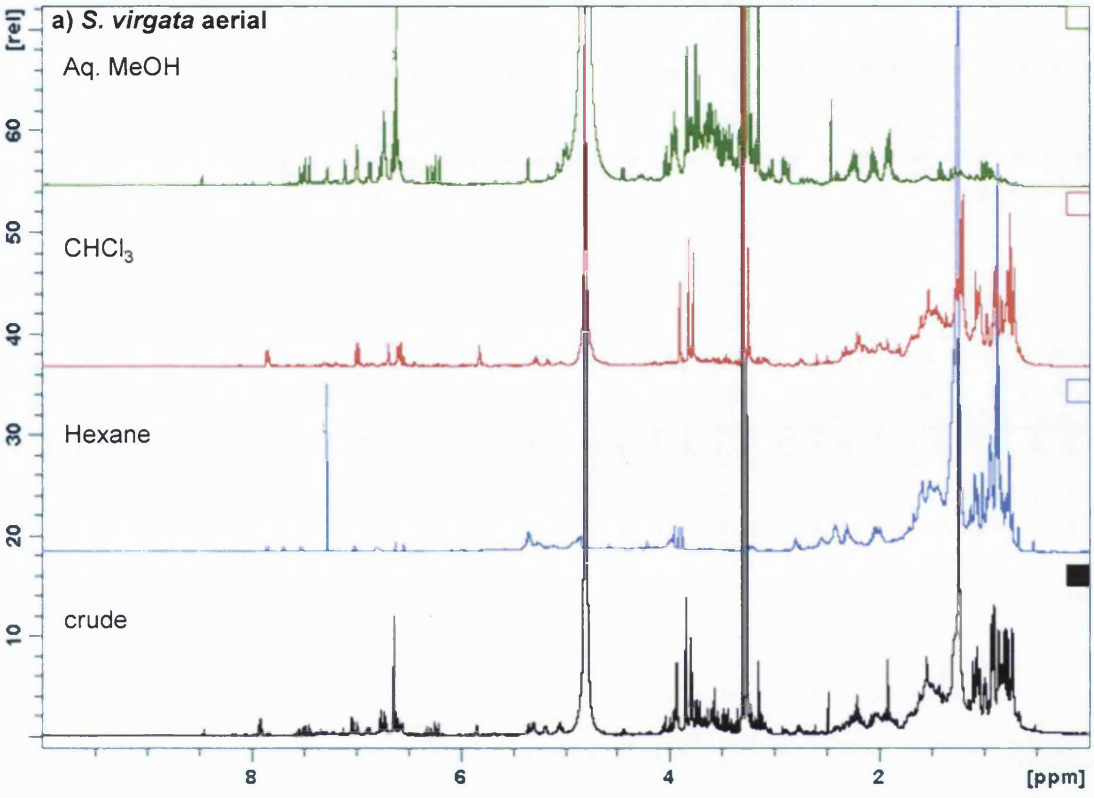


Figure 3.8. $^1$H NMR spectra (400 MHz) of *Scrophularia lucida*. a) aerial parts, b) roots. black: crude extract in MeOD, blue: hexane subextract in $CDCl_3$, red: $CHCl_3$ subextract in MeOD, green: aq. MeOH subextract in MeOD.

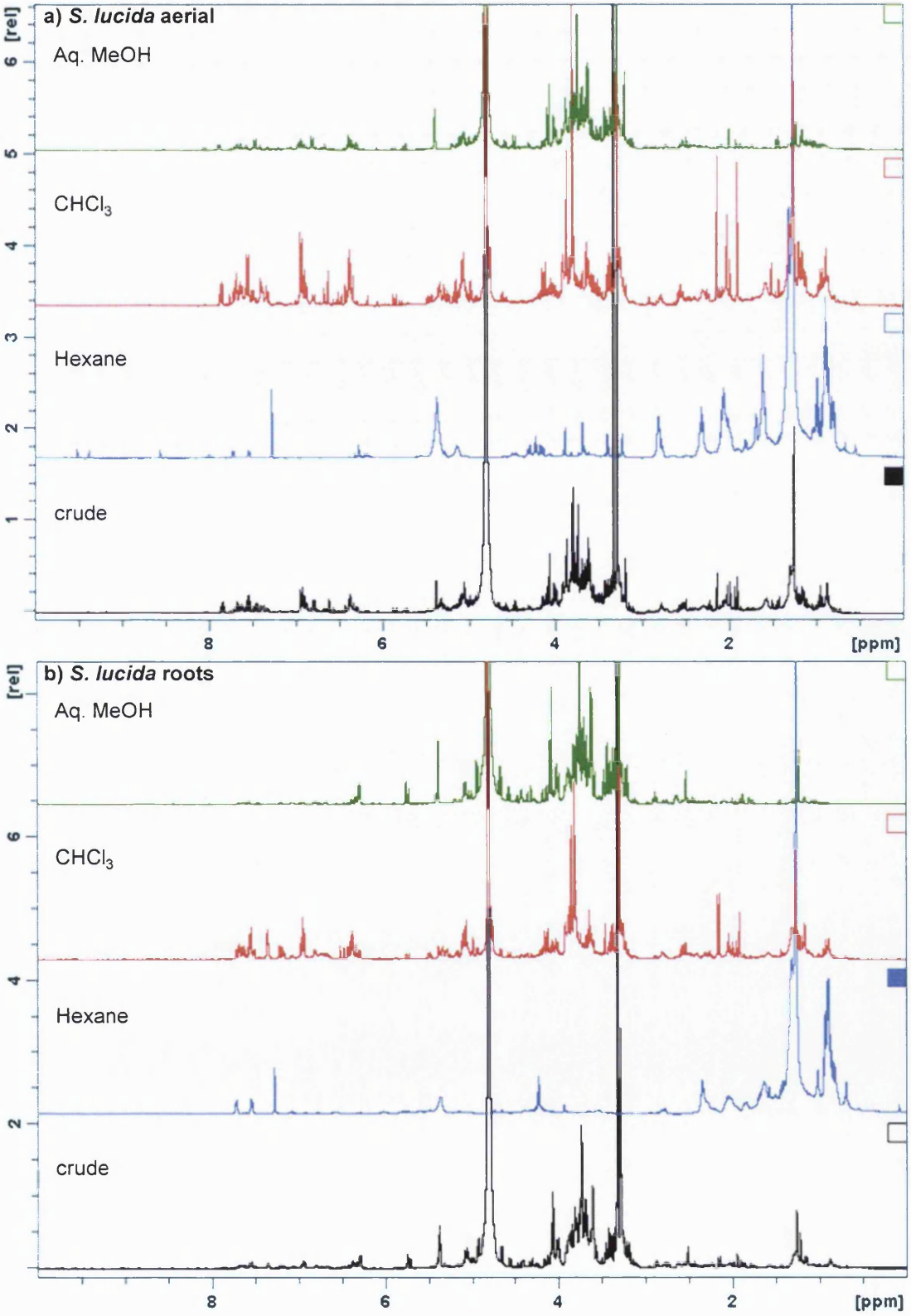
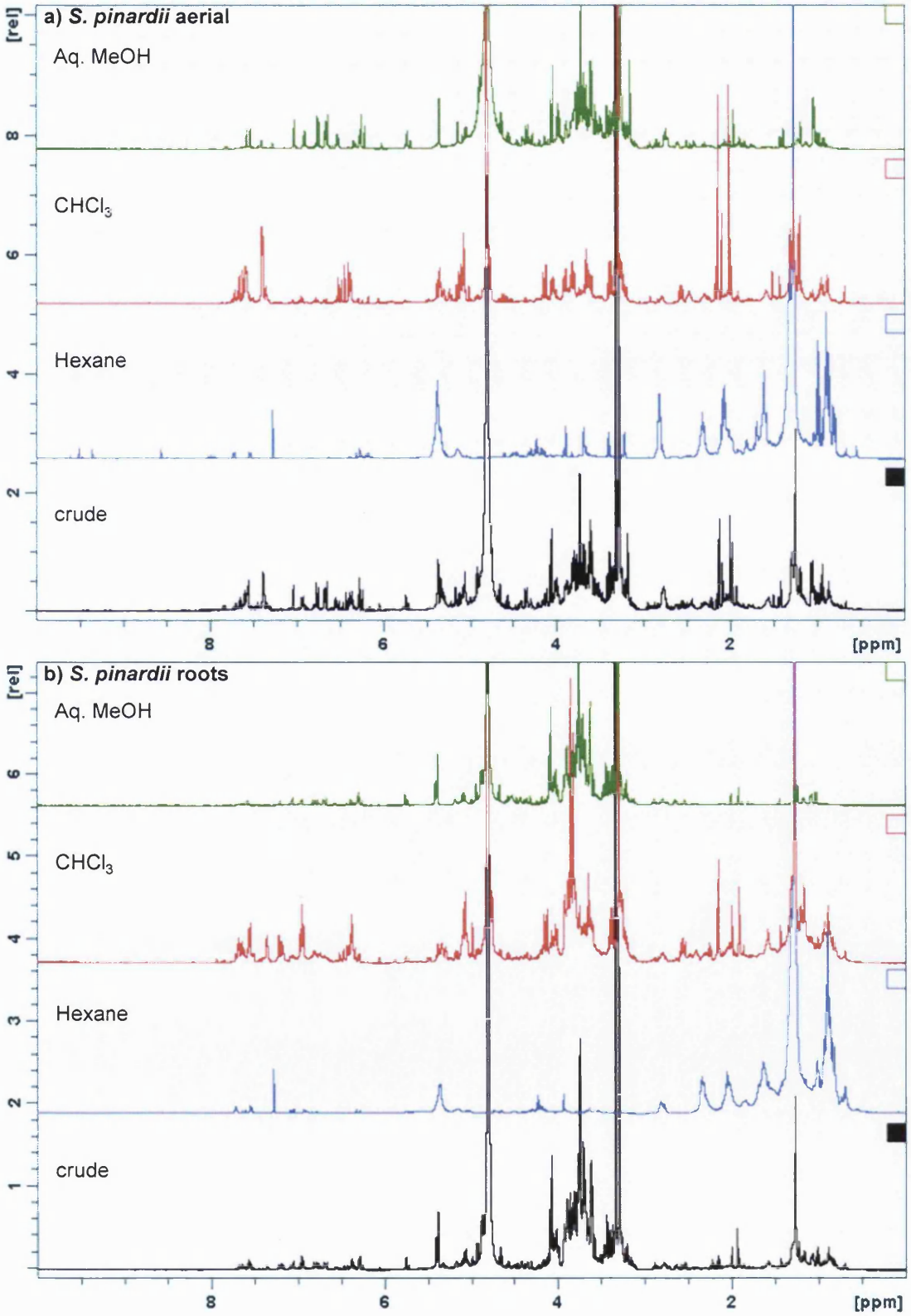


Figure 3.9. $^{1}H$ NMR spectra (400 MHz) of *Scrophularia pinardii*. a) aerial parts, b) roots. black: crude extract in MeOD, blue: hexane subextract in $CDCl_3$ red: $CHCl_3$ subextract in MeOD, green: aq. MeOH subextract in MeOD.



### 3.2.3 Biological screening

The *in vitro* activity of the crude extracts and subextracts were determined against the plasmodial FAS-II enzymes FabI, FabG and FabZ to examine prophylactic potential and against *Plasmodium falciparum* blood stage parasites to investigate chemotherapeutical potential. Cytotoxicity was determined using the human cancer cell line KB to evaluate the selectivity of the extracts. To assess the importance of observed antimalarial activities in the extracts, the recommendation of Gessler *et al.* (1994) was followed. Thus, extracts with an $IC_{50}$ value below 10 µg/ml are considered to show good antiplasmodial activity; $IC_{50}$ values between 10 and 50 µg/ml represent moderate and above 50 µg/ml low activity.

#### 3.2.3.1 Biological activities of crude MeOH extracts

**Antiplasmodial activity and cytotoxicity**

All crude extracts showed good to moderate inhibitory activity against *P. falciparum* blood stage parasites in the range of 4.68 µg/ml to 49.81 µg/ml with no cytotoxicity against KB cells (Tab. 3.2). Both *Anthemis* species had a similar inhibitory potential, which was also observed for the two species from the *Scrophularia* genus. The aerial parts of all species exhibited a better antiplasmodial effect compared to the roots. This was most pronounced for *S. lucida* aerial parts which, with an $IC_{50}$ value of 10.52 µg/ml, were more than four times more potent than the roots (49.81 µg/ml). *S. virgata* (**5**) showed the best antiplasmodial activity with an $IC_{50}$ value of 4.68 µg/ml.

**Plasmodial FAS-II enzyme inhibition**

The highest inhibitory potential against FAS-II enzymes was found for FabZ (Tab. 3.2). For this enzyme all extracts showed good to moderate potency with the exception of the root extracts of *S. lucida* (**7**) and *S. pinardii*, which were inactive against all three enzymes. Similar tendencies as found for the parasite inhibition were observed for the inhibition of the dehydratase FabZ. Again the best inhibitory potential was exhibited by the aerial parts of *S. virgata* (**5**) with an $IC_{50}$ value of 2.5 µg/ml. Both *Anthemis* species had a similar potential (2.8 µg/ml – 4.3 µg/ml) with the aerial parts exhibiting a better effect compared to the roots. The difference in the aerial parts and roots was once more most pronounced for *S. lucida* with an $IC_{50}$ value of 21 µg/ml for the aerial parts (**6**) and no activity for the roots (**7**). The only exception where the roots had a better potential than the aerial parts was found for the inhibition of FabG by *A. pestalozzae*. While none of the root extracts from any of the other species demonstrated activity against FabG, the extracts of the aerial parts of *A. cretica* subsp. *anatolica* (**1**), *S. virgata* (**5**) and *S. lucida* (**6**) showed moderate inhibition towards this enzyme with $IC_{50}$ values of 25 µg/ml, 48 µg/ml and 21 µg/ml,

respectively. Moderate inhibitory potential against FabI was found for *S. virgata* and the roots and aerials of the *Anthemis* species, with the aerial parts of *A. pestalozzae* (**3**) exhibiting the best activity (16 µg/ml). Both *Scrophularia* species showed no effect against FabI.

### 3.2.3.2 Biological activities of subextracts

#### Antiplasmodial activity

The subextracts of all species showed good to moderate antiplasmodial activity for the hexane and $CHCl_3$ fractions (Tab. 3.2). The observed $IC_{50}$ values for the hexane subextracts were up to three times lower compared to the crude extracts and range of 3.53 µg/ml to 7.83 µg/ml for the *Anthemis* and from 14.79 µg/ml to 19.86 µg/ml for the *Scrophularia* species. The only exceptions are the hexane subextracts from aerial parts of *A. pestalozzae* (**3a**) and *S. lucida* (**6a**) where the antiplasmodial activity is slightly reduced compared to the crude extract. As observed for the crude extract, the best potential for the hexane subextracts was found for *S. virgata* (**5a**) with an $IC_{50}$ value of 2.90 µg/ml.

For both *Anthemis* species, the aerial parts of the *Scrophularia* species and *S. virgata* the $CHCl_3$ subextract was the most active fraction with $IC_{50}$ values in the range of 2.34 µg/ml to 5.60 µg/ml for *Anthemis*, 6.81 µg/ml and 17.32 µg/ml for *Scrophularia* and the overall best potential of 2.20 µg/ml for *S. virgata*. For the roots of the *Scrophularia* species moderate antiplasmodial activity (22.06 µg/ml and 19.73 µg/ml) was found. For the hexane and $CHCl_3$ subextracts no trend was observed regarding the activities in the aerial parts and roots of the species.

The aq. MeOH subextracts of both *Scrophularia* species and the roots of *A. pestalozzae* (**4c**) were inactive whereas moderate activity with $IC_{50}$ values of 36.05 µg/ml, 38.77 µg/ml and 47.33 µg/ml were found for the aerial parts of *A. pestalozzae* (**3c**), *A. cretica* subsp. *anatolica* (**1c**) and *S. virgata* (**5c**), respectively. These activities are considerably lower than the antiplasmodial potential observed for the crude extracts.

#### Cytotoxicity against KB cells

Cytotoxicity against KB cells was observed for the hexane subextract of *A. cretica* subsp. *anatolica* (**1a**) but the effect was at least two times less potent than the inhibitory potential against the blood stage parasites (Tab. 3.2). The same applied for the $CHCl_3$ subextracts for aerial parts of *A. cretica* subsp. *anatolica* (**1b**) and *S. virgata* (**5b**), where the antiplasmodial potency was at least three times higher than the observed cytotoxicity.

Table 3.2. Biological activities of the crude extracts and subextracts. $IC_{50}$ values in μg/ml.

| Code | Plant species | Extract | FabI | FabG | FabZ | *P. falciparum* | KB |
|---|---|---|---|---|---|---|---|
| **1** | *Anthemis cretica* | crude | 27 | 25 | **2.8** | 13.43 | >50 |
| **1a** | subsp. *anatolica* | hexane | **8.3** | >10* | >1* | **3.53** | 15.9 |
| **1b** | (aerial parts) | $CHCl_3$ | 12 | **7** | **2** | **3.39** | 11.4 |
| **1c** | | Aq. MeOH | 15 | >50 | **3.9** | 38.77 | >50 |
| **2** | *Anthemis cretica* | crude | 30 | >50 | **3.4** | 18.15 | >50 |
| **2a** | subsp. *anatolica* | hexane | **8.2** | >10* | >1* | **7.77** | 18.6 |
| **2b** | (roots) | $CHCl_3$ | 15 | >50 | **3.4** | **2.34** | >50 |
| **2c** | | Aq. MeOH | 50 | >50 | **4.7** | >50 | >50 |
| **3** | *Anthemis pestalozzae* | crude | 16 | >50 | **4** | 10.74 | >50 |
| **3a** | (aerial parts) | hexane | 17 | >10* | >1* | 13.90 | >50 |
| **3b** | | $CHCl_3$ | **6** | **4.6** | **0.85** | **5.60** | >50 |
| **3c** | | Aq. MeOH | 13 | >50 | **4.3** | 36.05 | >50 |
| **4** | *Anthemis pestalozzae* | crude | 45 | 43 | **4.3** | 24.37 | >50 |
| **4a** | (roots) | hexane | **6.6** | >10* | >1* | **7.83** | >50 |
| **4b** | | $CHCl_3$ | **8.5** | **6** | **2.1** | **3.05** | >50 |
| **4c** | | Aq. MeOH | 50 | >50 | **4.9** | >50 | >50 |
| **5** | *Salvia virgata* | crude | 23 | 48 | **2.5** | **4.68** | >50 |
| **5a** | (aerial parts) | hexane | **9.5** | >50 | **4** | **2.90** | >50 |
| **5b** | | $CHCl_3$ | 39 | 48 | **4.6** | **2.20** | 20.6 |
| **5c** | | Aq. MeOH | 39 | >50 | **5.6** | 47.33 | >50 |
| **6** | *Scrophularia lucida* | crude | >50 | 21 | 21 | 10.52 | >50 |
| **6a** | (aerial parts) | hexane | >50 | 13 | 10 | 12.15 | >50 |
| **6b** | | $CHCl_3$ | >50 | 35 | 12 | **6.81** | >50 |
| **6c** | | Aq. MeOH | >50 | >50 | 28 | >50 | >50 |
| **7** | *Scrophularia lucida* | crude | >50 | >50 | >50 | 49.81 | >50 |
| **7a** | (roots) | hexane | >50 | 10 | **7** | 14.79 | >50 |
| **7b** | | $CHCl_3$ | >50 | >50 | 28 | 22.06 | >50 |
| **7c** | | Aq. MeOH | >50 | >50 | >50 | >50 | >50 |
| **8** | *Scrophularia pinardii* | crude | >50 | >50 | 18 | 24.94 | >50 |
| **8a** | (aerial parts) | hexane | >50 | **8** | **3.8** | 19.86 | >50 |
| **8b** | | $CHCl_3$ | >50 | >50 | 21 | 17.32 | >50 |
| **8c** | | Aq. MeOH | >50 | >50 | >50 | >50 | >50 |
| **9** | *Scrophularia pinardii* | crude | >50 | >50 | >50 | 43.45 | >50 |
| **9a** | (roots) | hexane | >50 | 22 | 42 | 16.10 | >50 |
| **9b** | | $CHCl_3$ | >50 | 31 | 42 | 19.73 | >50 |
| **9c** | | Aq. MeOH | >50 | >50 | >50 | >50 | >50 |
| Control drugs | | | 0.014[1] | 0.47[2] | 0.3[2] | 0.002[3] | 0.002[4] |

[1]triclosan, [2]EGCG, [3]artesunate, [4]podophyllotoxin

*measurement failed at higher concentrations

**Plasmodial FAS-II enzyme inhibition**

For the enzyme inhibition studies (Tab. 3.2) the hexane subextracts of both *Anthemis* species proved to be problematic, thus no $IC_{50}$ values could be determined for FabG and FabZ, as with increasing subextract concentration the measurement failed.

As observed for the crude extracts, the dehydratase FabZ was the best inhibited enzyme. The trend that the aerial parts had a slightly better potential than the roots which was found for the crude extract, was also detected for the subextracts of the *Anthemis* species. The observed $IC_{50}$ values for the *Anthemis* subextracts against FabZ displayed a similar inhibitory potential in the range of 2 µg/ml to 4.9 µg/ml with the $CHCl_3$ subextract of aerial *A. pestalozzae* (**3b**) exhibiting the best activity with an $IC_{50}$ value of 0.85 µg/ml. In the inhibition of the FabG enzyme only the $CHCl_3$ subextracts, except **2b**, exhibited potential with $IC_{50}$ values ranging from 4.6 µg/ml to 7 µg/ml. All *Anthemis* subextracts exhibited good to moderate activity against FabI with similar potential of aerial parts and roots for the hexane and $CHCl_3$ subextracts of *A. cretica* subsp. *anatolica* and the $CHCl_3$ subextracts of *A. pestalozzae*. The hexane subextract of the roots of *A. pestalozzae* (**4a**) showed the overall best inhibitory potential against FabI with an $IC_{50}$ value of 6.6 µg/ml. For both *Anthemis* species the aq. MeOH subextract of the aerial parts showed a similar potential which was more than three times higher compared to the aq. MeOH subextract of the roots.

*S. virgata* showed a similar enzyme inhibition profile with FabZ being the best inhibited enzyme by all three subextracts ($IC_{50}$ value in the range of 4 to 5.6 µg/ml), FabG only being inhibited by the $CHCl_3$ subextract good to moderate inhibition of FabI by all three subextracts.

Both *Scrophularia* species also exhibited the highest activity against FabZ, with $IC_{50}$ values of 3.8 µg/ml – 10 µg/ml for the hexane subextracts and 8 µg/ml – 28 µg/ml for the $CHCl_3$ subextracts. The exception were the roots of *S. pinardii* (**9**) where the hexane and $CHCl_3$ subextracts showed a higher potency towards FabG, with $IC_{50}$ values of 22 µg/ml and 31 µg/ml, respectively. **6c** was the sole aq. MeOH subextracts which showed a moderate potential against FabZ with $IC_{50}$ values of 28 µg/ml. FabG was inhibited by all hexane subextracts from the *Scrophularia* species within a range of 8 µg/ml to 42 µg/ml. The $CHCl_3$ subextracts **6b** and **9b** showed moderate potential against FabG whereas all aq. MeOH subextracts were inactive. Interestingly, no inhibition of FabI was observed for any *Scrophularia* subextract.

Overall the best enzyme inhibition of all subextracts was found towards FabZ. Another similarity between all subextracts was the inactivity of all aq. MeOH subextracts against FabG.

The chemical profiling of the species together with the biological screening were used to identify and select the most interesting species for an in-depth phytochemical investigation. The biological screening demonstrated for all plants good to moderate antiplasmodial activity and good inhibitory potential against at least one FAS-II enzyme. The comparison of the different species showed that *Scrophularia lucida* and *S. pinardii* exhibited overall the lowest potential against blood stage parasites, and even though both plants showed good inhibition against FabG and FabZ, all extracts and subextracts were inactive against FabI. The comparison of both *Anthemis* species and *Salvia virgata* showed that *A. cretica* subsp. *anatolica* and *A. pestalozzae* exhibited a similar antiplasmodial potential, whereas *S. virgata* showed the overall best antiplasmodial activity. *A. pestalozzae* was the only species which provided good inhibitory potential against all three FAS-II enzymes ($CHCl_3$ subextract). Although the $CHCl_3$ subextract of the aerial parts of *A. cretica* subsp. *anatolica* also provided interesting inhibitions against all three enzymes, this subextract as well as the hexane subextract exhibited some cytotoxicity.
Analyses of TLC and $^1$H NMR showed for all extracts a wide variety of compounds. As described in the introduction of the species (1.2.1), a literature review showed that *Salvia virgata* has been previously phytochemically investigated (Delatorre *et al.*, 1990) while *A. pestalozzae* is completely unstudied. Hence, due to its interesting chemical composition, good antiplasmodial activity, the good inhibition of all three FAS-II enzymes and no cytotoxicity *A. pestalozzae* was chosen for in-depth phytochemical and biological investigations.

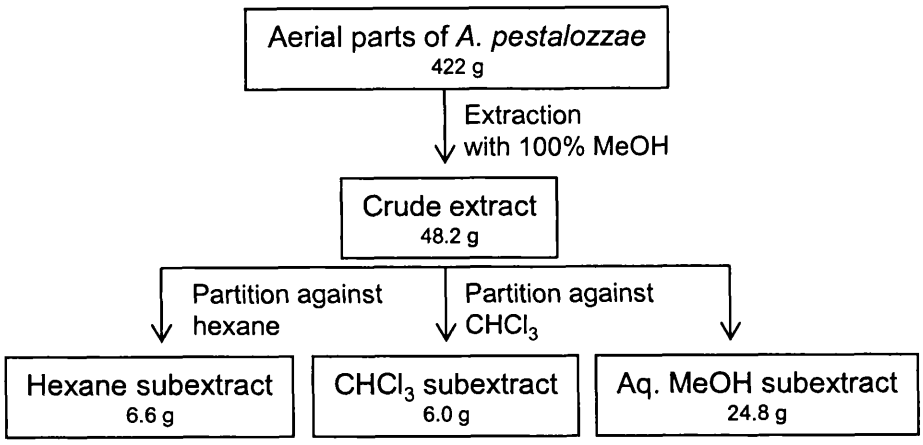
## 3.3 Investigation of *A. pestalozzae* for the phytochemical composition and biological activity

In this chapter the results of the bioactivity-guided fractionation of *A. pestalozzae* and the structure elucidation of isolated compounds will be presented.

### 3.3.1 Large-scale extraction and liquid-liquid partitioning

The extraction and liquid-liquid partition of the air-dried and powdered aerial parts of *Anthemis pestalozzae* (422 g) was performed as described in 2.2 and yielded crude MeOH extract (48.2 g), hexane subextract (6.6 g), $CHCl_3$ subextract (6.0 g) and aq. MeOH subextract (24.8 g) (Fig. 3.10).

Figure 3.10. Extraction scheme for *A. pestalozzae* for phytochemical investigation.




The *A. pestalozzae* extracts were analysed by TLC (Fig. 3.11) and $^1$H NMR (Fig. 3.12) and showed the same chemical profile as described in the screening (3.2.2). All subextracts showed good to moderate antiplasmodial activity and activity against at least one FAS-II enzyme (Tab. 3.3), thus all three subextracts were selected for in-depth investigation. The fractionation of the subextracts, analysis of mixtures, isolation and identification of compounds are presented in the following chapters.

Table 3.3. Biological activities of the crude extract and subextracts of *A. pestalozzae*. $IC_{50}$ values in µg/ml.

| Extract | FabI | FabG | FabZ | *P. falciparum* | KB |
|---|---|---|---|---|---|
| crude | 16 | >50 | 4 | 10.74 | >50 |
| hexane | 17 | >10* | >1* | 13.90 | >50 |
| $CHCl_3$ | **6** | **4.6** | **0.85** | **5.60** | >50 |
| Aq. MeOH | 13 | >50 | **4.3** | 36.05 | >50 |
| Control drugs | 0.014[1] | 0.47[2] | 0.3[2] | 0.002[3] | 0.002[4] |

[1]triclosan, [2]EGCG, [3]artesunate, [4]podophyllotoxin

*measurement failed at higher concentrations

Figure 3.11. TLC analysis of extracts of *A. pestalozzae* (aerial parts). Stationary phase: silica gel 60 $F_{254}$, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170°C). cr: crude extract, C: $CHCl_3$ subextract, H: hexane subextract, M: aq. MeOH subextract.

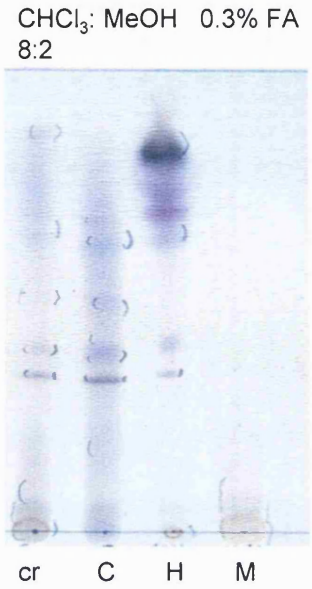
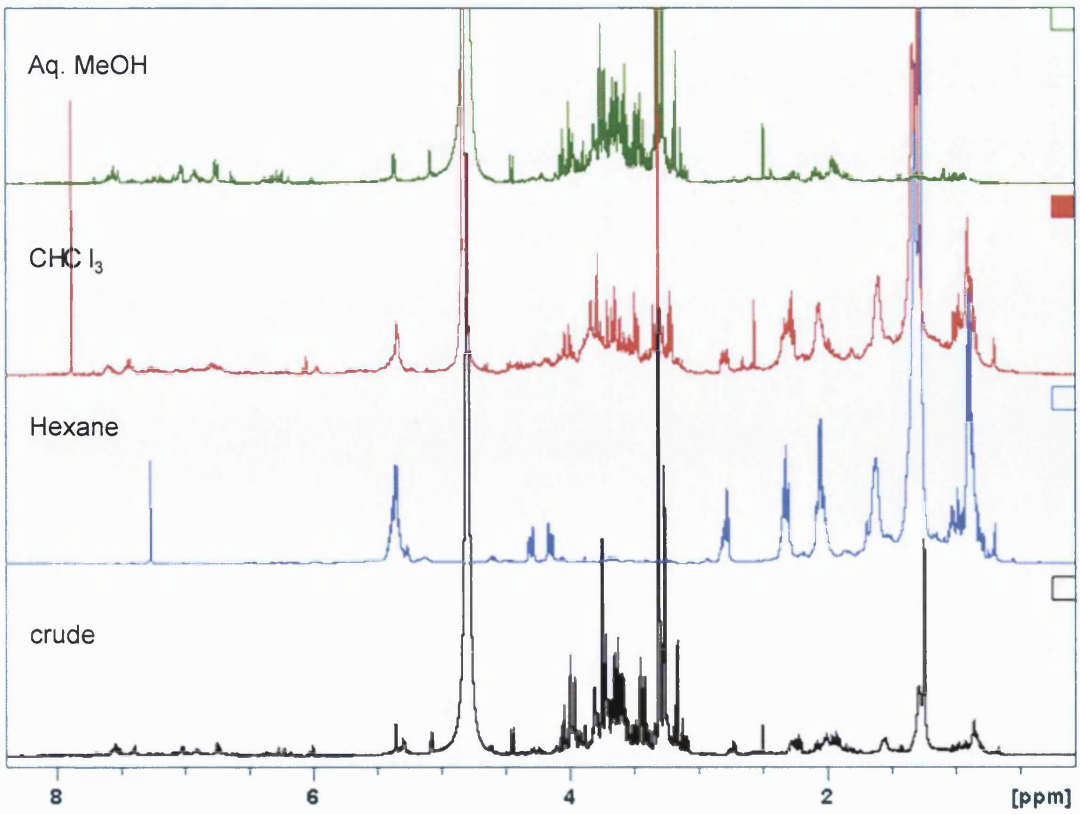



Figure 3.12. $^1H$ NMR spectra (400 MHz) of *A. pestalozzae* (aerial parts). black: crude extract in MeOD, blue: hexane subextract in $CDCl_3$, red: $CHCl_3$ subextract in MeOH, green: aq. MeOH subextract in MeOD.

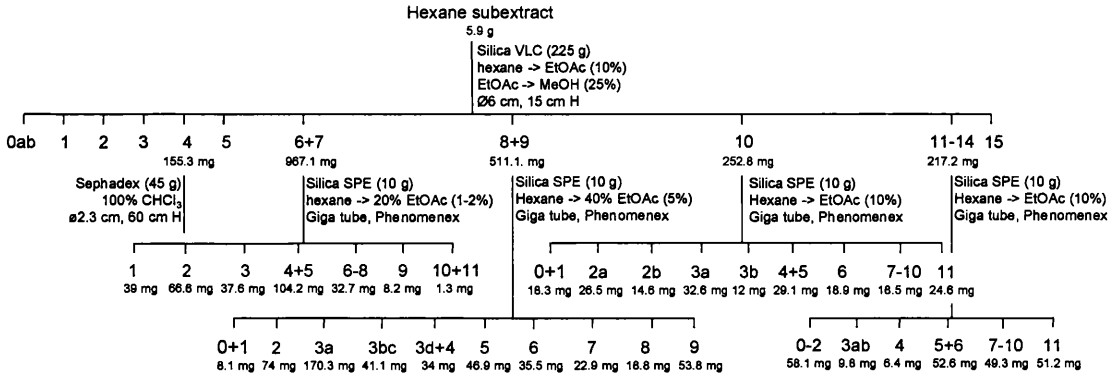


### 3.3.2 Investigation of the hexane subextract

#### 3.3.2.1 Bioactivity-guided fractionation

The hexane subextract was fractionated by VLC with silica gel 60 as stationary phase. A step gradient from 100% hexane to 100% EtOAc in 10% increments was applied, followed by a wash from 100% EtOAc to 100% MeOH in 25% steps and resulted in eleven fractions after combination based on similarities by TLC and $^1$H NMR analyses (Fig. 3.13).

Figure 3.13. Fractionation scheme of the hexane subextract. Blue fractions analysed by GCMS.




The bioactivities of the resulting fractions from the initial VLC fractionation against *P. falciparum* blood stage parasites, plasmodial FAS-II enzymes and cytotoxicity against KB cells for selectivity assessment are shown in Table 3.4.

Table 3.4. Biological activities of the VLC fractions from the hexane subextract. $IC_{50}$ values in µg/ml.

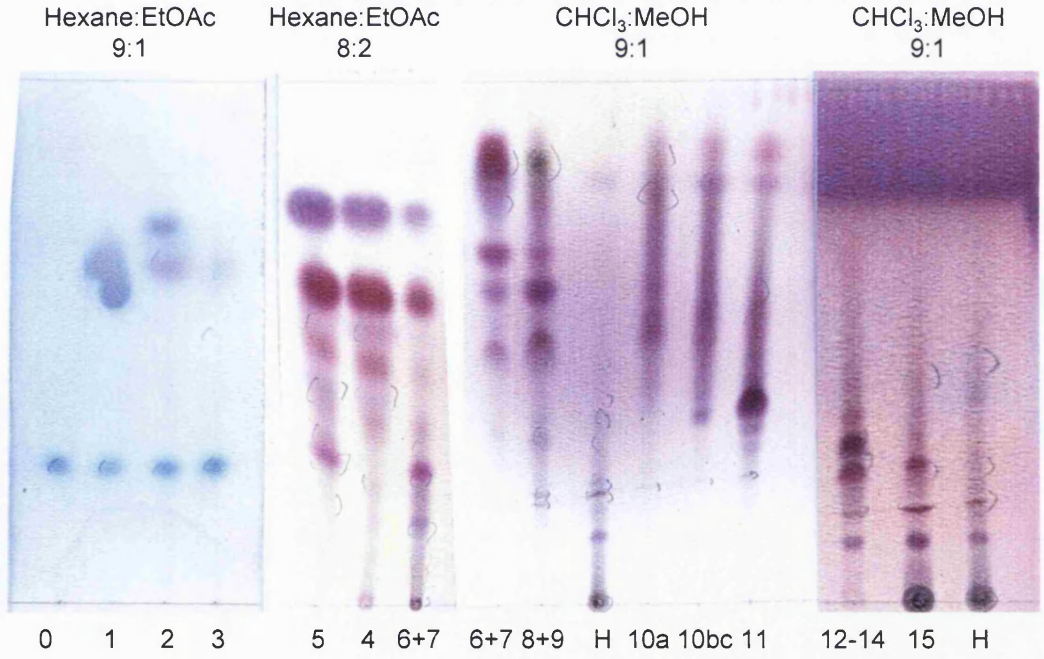
| Fraction | FabI | FabG | FabZ | *P. falciparum* | KB |
|---|---|---|---|---|---|
| hexane | 17 | >10* | >1* | 13.90 | >50 |
| VLC 0ab | >50 | >50 | >50 | **4.42** | >50 |
| VLC 1 | >50 | >50 | >50 | 32.68 | >50 |
| VLC 2 | >50 | >50 | >50 | 10.71 | >50 |
| VLC 3 | >50 | >50 | >50 | **5.10** | >50 |
| VLC 4 | >50 | >50 | >50 | 16.80 | >50 |
| VLC 5 | >50 | >50 | >50 | 26.64 | >50 |
| VLC 6+7 | >50 | 36 | **7.9** | **7.39** | >50 |
| VLC 8+9 | 25 | 8 | **1.9** | **7.03** | 28.81 |
| VLC 10 | >50 | 29 | **2.7** | **2.17** | 28.68 |
| VLC 11-14 | >50 | 17 | **1.5** | **6.64** | >50 |
| VLC 15 | >50 | 11 | **0.28** | 10.01 | >50 |
| Control drugs | 0.024[1] | 0.53[2] | 0.3[2] | 0.001[3] | 0.002[4] |

[1]triclosan, [2]EGCG, [3]artesunate, [4]podophyllotoxin

*measurement failed at higher concentrations

All VLC fractions showed activity against *P. falciparum* blood stage parasites. Good antiplasmodial activity was found for VLC fractions 0ab, 1, 3, 6+7, 8+9, 10 and 11-14 with $IC_{50}$ values in the range of 2.17 μg/ml to 7.39 μg/ml. Moderate antiplasmodial potential was observed for all other fractions. Moderate cytotoxicity was found for fractions 8+9 and 10. The enzyme inhibition studies showed a lack of activity for VLC fractions 0ab – 5. Fractions after 5 exhibited good to moderate inhibition against at least two fatty acid biosynthesis enzymes (FabG and FabZ). Fraction 8+9 was the only fraction inhibiting all three enzymes and showing potential against FabI. Overall, FabZ was the enzyme best inhibited with $IC_{50}$ values between 0.28 μg/ml and 7.9 μg/ml.

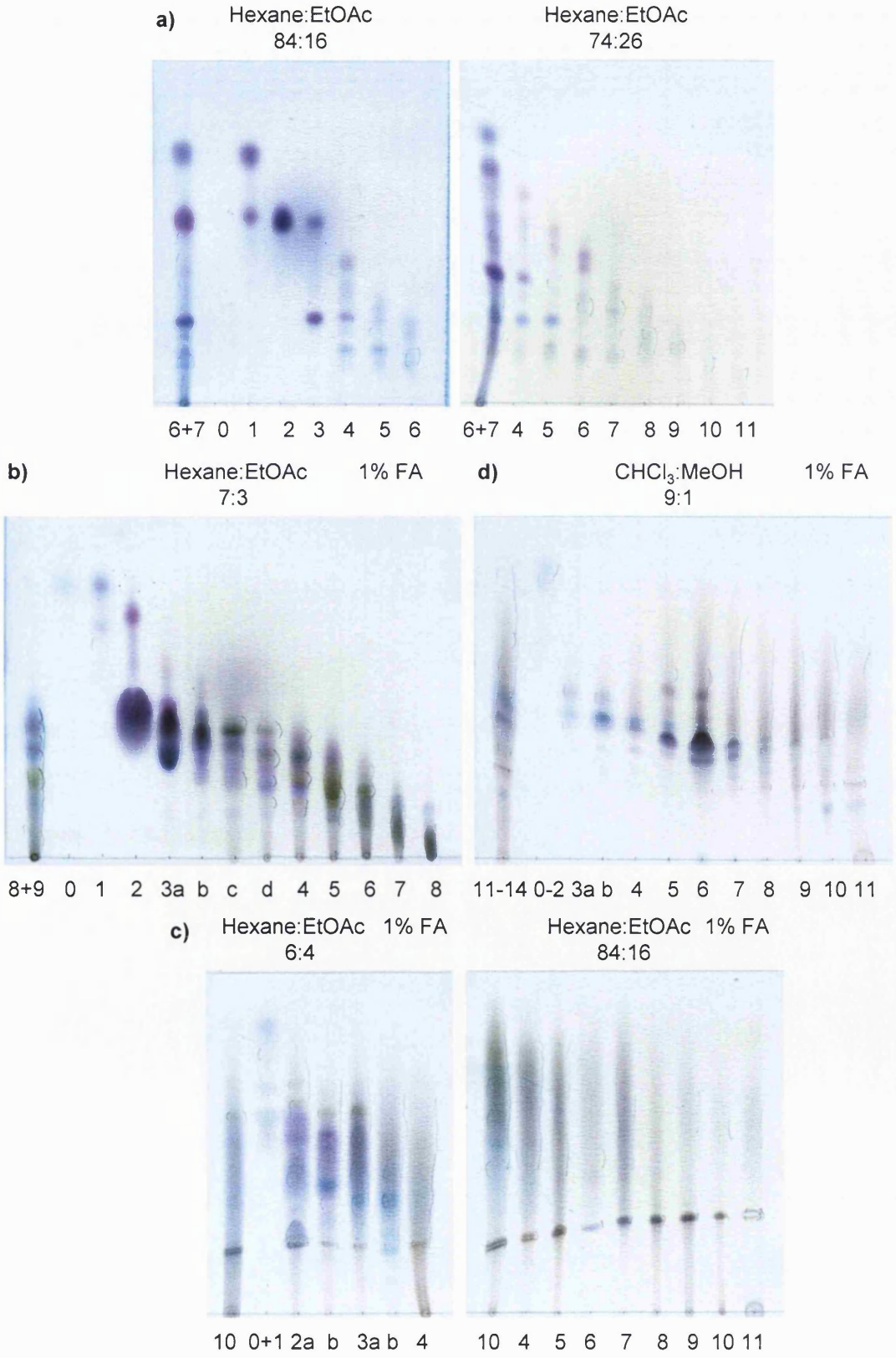
Figure 3.14. TLC analysis of the VLC fractions from the hexane subextract. Stationary phase: silica gel 60 $F_{254}$, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170°C). H: hexane subextract.




TLC analysis (Fig. 3.14) of the VLC fractions indicated significant contamination of fractions 0 – 3 with phthalates, which was confirmed by $^1$H NMR spectroscopy (not shown). Fraction 15, as the last washing fraction, contained mainly debris and no interesting compound were detected in the $^1$H NMR spectrum (not shown). Therefore, these fractions were excluded from further studies. VLC fractions 4, 6+7, 8+9, 10 and 11-14 were selected for fractionation because of their antiplasmodial potential. Their $^1$H NMR spectra were dominated by signals in the high field, which indicated the presence of fatty acids.

Size-exclusion chromatography over Sephadex LH-20 with 100% $CHCl_3$ of fraction 4 did not result in successful separation of compounds (not shown). Fatty acids occur as complex mixtures and often vary only in the number and geometry of double bonds. These variations do not result in detectable size differences, hence fatty acids are very difficult to separate by conventional chromatography methods. Thus, for all other fractions, SPE over silica gel was applied with a step gradient from hexane to EtOAc, successfully removing phthalates and resulting in a rough separation of compounds according to their polarity. TLC analyses of the SPE fractions are shown in Figure 3.15.

Figure 3.15. TLC analyses of SPE fractions from the hexane subextract fractionation. (a) 6+7, (b) 8+9, (c) 10 and (d) 11-14. First spot on the TLC plate is starting material, followed by collected fractions. Stationary phase: silica gel 60 $F_{254}$, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170°C).



All SPE fractions, excluding the phthalate contaminated first fraction and the chemically uninteresting last washing fraction, were tested in bioassays against *P. falciparum* blood stage parasites, plasmodial FAS-II enzymes and for cytotoxicity against KB cells to assess the selectivity. The results are summarised in Table 3.5.

Table 3.5. Biological activities of SPE fractions from the hexane subextract fractionation. $IC_{50}$ values in μg/ml.

| Code | Fraction | FabI | FabG | FabZ | *P. falciparum* | KB |
|---|---|---|---|---|---|---|
| | VLC 6+7 | >50 | 36 | **7.9** | **7.39** | >50 |
| H1a | VLC 6+7: 2 | 50 | >50 | >50 | >50 | >50 |
| H1b | VLC 6+7: 3 | >10* | >10* | >10* | 38.47 | >50 |
| H1c | VLC 6+7: 4+5 | >10* | >10* | >10* | 42.39 | >50 |
| H1d | VLC 6+7: 6-8 | **2.8** | >10* | **5.1** | 17.53 | >50 |
| H1e | VLC 6+7: 9 | **2.7** | >10* | **3.8** | 13.44 | >50 |
| | VLC 8+9 | 25 | **8** | **1.9** | **7.03** | 28.81 |
| H2a | VLC 8+9: 2 | >10* | >50 | >10* | >50 | >50 |
| H2b | VLC 8+9: 3a | >10* | >50 | 23 | >50 | >50 |
| H2c | VLC 8+9: 3bc | >10* | >10* | **8.5** | 10.70 | >50 |
| H2d | VLC 8+9: 3d+4 | >10* | **5.8** | >1* | **8.13** | 16.8 |
| H2e | VLC 8+9: 5 | 16 | >10* | >1* | **8.28** | 27.3 |
| H2f | VLC 8+9: 6 | 18 | >10* | **3.7** | **8.17** | 23.0 |
| H2g | VLC 8+9: 7 | 25 | >10* | 4 | **7.06** | 19.2 |
| H2h | VLC 8+9: 8 | 16 | >10* | **4.5** | **4.91** | 17.4 |
| | VLC 10 | >50 | 29 | **2.7** | **2.17** | 28.68 |
| H3a | VLC 10: 2a | **7.4** | 21 | >50 | 16.82 | 18.8 |
| H3b | VLC 10: 2b | **1.9** | 43 | **7.5** | 10.16 | 18.6 |
| H3c | VLC 10: 3a | **3.4** | 11 | >10* | **9.83** | 46.0 |
| H3d | VLC 10: 3b | **5.1** | >10* | **5.4** | **9.29** | >50 |
| H3e | VLC 10: 4+5 | 10 | >10* | **3.3** | **8.38** | 36.4 |
| H3f | VLC 10: 6 | >10* | >10* | >10* | **8.94** | >50 |
| H3g | VLC 10: 7-10 | **6.3** | >10* | >10* | **7.97** | >50 |
| | VLC 11-14 | >50 | 17 | **1.5** | **6.64** | >50 |
| H4a | VLC 11-14: 3ab | **5.6** | >50 | 30 | 23.08 | >50 |
| H4b | VLC 11-14: 4 | **5** | 38 | 17 | 19.08 | >50 |
| H4c | VLC 11-14: 5+6 | >10* | >10* | >50 | 23.48 | >50 |
| H4d | VLC 11-14: 7-10 | >10* | 47 | >50 | 19.31 | >50 |
| Control drugs | | 0.024[1] | 0.53[2] | 0.3[2] | 0.005[3] | 0.002[4] |

[1]triclosan, [2]EGCG, [3]artesunate, [4]podophyllotoxin

*measurement failed at higher concentrations

All fractions except H1a, H2a and H2b show good to moderate activity against *P. falciparum* blood stage parasites. Comparison of the SPE fractions with their starting fractions showed that multiple fractions retained the antiplasmodial activity. In some cases (*e.g.* H1 and H4), separation of the VLC fractions led to a significant decrease in antiplasmodial potential compared to the starting fraction. Only one SPE fraction (H2h) was found to exhibit a

slightly better antiplasmodial activity with an $IC_{50}$ value of 4.91 µg/ml compared to its starting fraction VLC 8+9 ($IC_{50}$ 7.03 µg/ml).

For the inhibition of FAS-II enzymes, good inhibitory activities could be detected in fractions H1d+e, H3a-d, H3g and H4a+b towards FabI with $IC_{50}$ values in the range of 1.9 µg/ml to 7.4 µg/ml. For FabG and FabZ no increase in the enzyme inhibitory potency was found. On the contrary, while VLC fraction 11-14 showed good activity against FabZ, the further fractionation led to higher $IC_{50}$ values and loss of potency.

### 3.3.2.2 Fatty acid methyl ester analysis

The major components in the hexane subextract and fractions are fatty acids as determined by $^1$H NMR analyses. To identify the fatty acid composition, the hexane subextract, VLC fractions 4-14 and chemically interesting SPE fractions were subjected to gas chromatography coupled with mass spectrometry (GC-MS), which was performed by Prof. N. M. Carballeira from the University of Puerto Rico, Puerto Rico. The fatty acids were methylated as explained in section 2.3.1.7 prior to GS-MS experiments.

Table 3.6. Fatty acid methyl ester abundances of the hexane subextract (H) and VLC fractions in weight percent.

| Fatty acid | H | VLC 4 | VLC 5 | VLC 6+7 | VLC 8+9 | VLC 10a | VLC 10bc | VLC 11 | VLC 12-14 |
|---|---|---|---|---|---|---|---|---|---|
| **saturated** | | | | | | | | | |
| myristic (14:0) | 0.9 | 0.5 | 5.0 | 3.0 | 4.0 | 4.0 | 1.4 | 3.0 | 4.0 |
| pentadecanoic (15:0) | 0.5 | - | - | - | 0.4 | - | - | 0.9 | 1.0 |
| palmitic (16:0) | **57.0** | **26.0** | **23.0** | **37.0** | **52.0** | **56.0** | **72.0** | **65.0** | **58.0** |
| margaric (17:0) | 0.5 | - | - | 0.4 | 0.2 | - | - | 0.7 | 1.0 |
| stearic (18:0) | **10.0** | 3.0 | 7.0 | 5.0 | 2.0 | 4.0 | 1.4 | 4.0 | 5.0 |
| arachidic (20:0) | 0.5 | 0.5 | 2.0 | 2.0 | 0.2 | 1.0 | 0.6 | 0.4 | 1.0 |
| heneicosylic (21:0) | - | - | - | 0.2 | - | - | - | - | - |
| behenic (22:0) | - | - | 0.3 | 0.7 | - | - | - | 0.2 | 0.6 |
| tricosylic (23:0) | - | - | - | 0.1 | - | - | - | - | - |
| lignoceric (24:0) | - | 0.5 | 0.1 | 0.2 | - | - | - | - | 0.4 |
| cerotic (26:0) | - | - | - | 0.1 | - | - | - | - | - |
| **unsaturated** | | | | | | | | | |
| oleic (18:1) | **10.0** | **15.0** | **27.0** | **26.0** | - | - | 7.5 | - | 7.5 |
| linoleic (18:2) | **21.0** | - | **35.0** | **26.0** | **23.0** | **14.0** | **10.0** | **12.0** | **11.0** |
| linolenic (18:3) | - | **55.0** | - | - | **18.0** | **21.0** | 7.5 | **14.0** | 7.5 |
| gadoleic (20:1) | - | - | 0.5 | 0.4 | - | - | - | - | - |
| **hydroxylated** | | | | | | | | | |
| α-OH 16:0 | - | - | - | - | - | - | - | - | 2.0 |

The hexane subextract showed the presence of eight fatty acids, with the highest abundance of palmitic acid (57%), linoleic acid (21%), stearic acid (10%) and oleic acid (10%) (Tab. 3.6). A total of fifteen fatty acids ranging from $C_{14}$ to $C_{26}$ were detected and quantified in the VLC fractions (Tab. 3.6). The major component in VLC fractions 6 – 14 was palmitic acid ranging from 37% (VLC 6+7) to 72% (VLC 10bc). VLC fraction 4 and 5 were the only fractions dominated by unsaturated fatty acids with linolenic acid (55%) and linoleic acid (35%) as main constituents, respectively. All examined SPE fraction showed the presence of the saturated fatty acids myristic acid (0.5 – 4%), palmitic acid (23 – 72%), stearic acid (2 – 10%) and arachidic acid (0.2 – 1%). The VLC fraction 6+7 showed the largest variety of fatty acids and was the only fraction with traces of heneicosylic acid (0.2%), tricosylic acid (0.1%) and cerotic acid (0.1%). Fraction 12-14 was the only fraction in which minor amounts of the hydroxy fatty acid α-OH 16:0 was detected.

In the SPE fractions a greater diversity of fatty acids was observed (Tab. 3.7). A total of 37 fatty acids ranging from $C_{10}$ to $C_{30}$ plus one unknown fatty acid in H2g (2.0%) were detected. All fractions contained palmitic acid (11.2 – 73.2%), stearic acid (3.4 – 37.0%) and behenic acid (0.4 – 12.5%), with palmitic acid being the most abundant fatty acid in most fractions with up to 73.2% in H4b. The exceptions are fraction H1b, H1d and H2c in which linoleic acid (39.7% in H1d and 57.6% in H1b) and linolenic acid (39.8% in H2c) were the main fatty acids, and H3e with stearic acid (23.9%) as most abundant fatty acid. All fractions, with the exception of H4b, contain two to four fatty acids in high abundances (>10%). The very short capric acid (10:0) and undecylic acid (11:0) were detected in small amounts in H1a and H2g, whereas traces of the long melissic acid (30:0) were solely found in H2a and H3d. The latter two, as well as fractions H2e, H2f and H2h, were the only fractions which contained exclusively saturated fatty acids.

Table 3.7. Fatty acid methyl ester abundances of SPE fractions from the hexane subextract fractionation in weight percent.

| Fatty acid | H1a | H1b | H1c | H1d | H1e | H2a | H2b | H2c | H2d | H2e | H2f | H2g | H2h | H3a | H3b | H3c | H3d | H3e | H3f | H3g | H4a | H4b | H4c | H4d |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **saturated** | | | | | | | | | | | | | | | | | | | | | | | | |
| 10:0 | 0.3 | | | | | | | | | | | 0.5 | | | | | | | | | | | | |
| 11:0 | 1.4 | | | | | | | | | | | | | | | | | | | | | | | |
| 12:0 | 0.6 | | 0.3 | | 0.2 | 1.3 | 0.4 | 0.2 | | 0.8 | 1.4 | 4.6 | | 0.6 | | | | 1.0 | | 0.2 | | | | 0.2 |
| 13:0 | 0.5 | | | | | 0.3 | | | | | | | | | | | | | | | | | | |
| 14:0 | 5.1 | 1.4 | 2.9 | 2.9 | 5.4 | 4.1 | 1.3 | 2.1 | 3.7 | 8.8 | 7.6 | 5.7 | | 2.3 | | 2.4 | 1.4 | **14.1** | 2.5 | 5.8 | 0.6 | 1.3 | | 1.2 |
| 15:0 | 0.9 | | 1.3 | 0.6 | | 2.8 | 0.9 | 0.6 | 0.8 | 1.1 | 1.1 | 0.7 | | 1.4 | | 0.6 | 0.3/5.6* | | 0.6 | 0.6 | | 0.6 | 1.5 | 0.7 |
| 16:0 | **40.0** | **14.0** | **65.8** | **26.1** | **38.6** | **52.8** | **38.4** | **20.7** | **31.6** | **47.5** | **55.2** | **50.3** | **42.7** | **57.6** | **46.6** | **40.8** | **34.8** | **11.2** | **34.8** | **38.8** | **28.9** | **43.3** | **73.2** | **40.3** |
| 17:0 | 1.7 | | 1.6 | 0.4 | 0.6 | 2.3 | 0.4 | 0.4 | 0.6 | 1.5 | 1.1 | 0.7 | | 1.7 | | 0.8 | 0.7 | 1.0 | 0.6 | 0.8 | 0.7 | 0.8 | | 0.8/0.5* |
| 18:0 | **19.1** | 3.4 | **12.3** | 4.3 | 9.0 | **10.4** | 3.3 | 3.3 | 9.0 | **19.9** | **13.0** | **11.1** | **37.0** | 9.9 | **13.4** | **16.0** | **10.5** | **23.9** | 8.1 | **12.8** | 7.7 | 7.4 | 7.0 | 7.2 |
| 19:0 | | | 0.3 | | | 0.3 | | | | | | 0.3 | | | | | | | | | | | | |
| 20:0 | 7.0 | 1.1 | 5.2 | 1.0 | 3.1 | 4.4 | 0.7 | 0.8 | 3.1 | 6.5 | 4.6 | 3.1 | 6.2 | 4.0 | 4.0 | | 6.5 | 5.9 | 1.9 | 2.8 | 7.1 | 3.2 | 1.8 | 2.0 |
| 21:0 | | | 0.6 | | | 1.3 | | | 0.3 | | | | | 0.3 | | 0.8 | 0.3 | 1.5 | | | 9.2/0.7* | | | |
| 22:0 | 3.3 | 0.6 | 3.2 | 1.0 | 2.1 | 3.9 | 0.4 | 0.4 | 1.9 | 3.4 | 3.0 | 4.7 | 4.4 | 2.5 | 2.6 | 9.2 | **10.4** | 7.3 | 2.5 | 2.1 | **12.5** | 4.7 | 2.2 | 5.0 |
| 23:0 | | | 1.3 | 0.2 | | | 0.2 | 0.2 | 0.5 | 1.1 | 0.8 | 1.0 | | 1.1 | | 2.0 | 2.4 | 3.4 | 1.2 | 1.1 | 2.1 | 1.3 | | 4.0 |
| 24:0 | 1.6 | 0.6 | 2.9 | 0.4 | 1.7 | 3.1 | 0.2 | 0.2 | 0.9 | 2.7 | 2.4 | 4.6 | 5.3 | 2.0 | | **12.0** | **12.4** | 8.8 | 2.5 | 2.8 | 9.4 | 3.8 | 3.3 | **8.6** |
| 25:0 | | | | | | **10.4** | | | 0.2 | 6.1 | 8.2 | 4.9 | 4.4 | 0.3 | | 1.6 | 3.1 | 5.9 | 0.6 | 0.8 | 1.1 | 0.8 | | 3.2 |
| 26:0 | | | 1.0 | | | 1.3 | | | 0.6 | 0.4 | 1.6 | 4.6 | | 1.1 | | 7.2 | 6.8 | 2.9 | 0.6 | 0.6 | 3.5 | 0.8 | | 1.2 |
| 27:0 | | | | | | | | | | | | | | | | 0.6 | 0.7 | | | | 0.4 | | | |
| 28:0 | | | 0.6 | | | 1.0 | | | | | | 1.0 | | 0.6 | | 4.4 | 3.9 | 1.5 | | 0.3 | 1.1 | | | |
| 30:0 | | | | | | 0.3 | | | | | | | | | | | 0.3 | | | | | | | |
| **unsaturated** | | | | | | | | | | | | | | | | | | | | | | | | |
| 16:1 | 0.6 | | | | | | | 2.1 | 4.4 | | | | | **12.4** | **23.0** | | | | | | | 3.4 | | |
| 17:2 | | | | | | | | | | | | | | | | | | | | | 0.9 | | | |
| 18:1 | 1.2/7.1* | 1.1 | 0.3/0.3* | **11.0/1.4*** | **12.4** | | 0.2 | | 1.1 | | | 0.7 | | 2.0 | 7.1 | 1.6 | | 4.4 | **10.9/1.2*** | **18.0/1.0*** | 6.5 | 6.6 | 5.9 | 0.2/0.3* |
| 18:2 | 7.8 | **57.6** | | **39.7** | **27.0** | | **26.9** | **29.2** | **20.2** | | | | | | 3.4 | | | 7.3 | **14.9** | 8.9 | 7.0/0.6* | **15.3** | 5.1 | |
| 18:3 | | **20.2** | | **11.0** | | | **26.7** | **39.8** | **21.2** | | | | | | | | | | **16.4** | | | 6.6 | | |
| 20:1 | | | | | | | | | | | | | | | | | | | | 0.6 | | | | 0.5 |
| 24:1 | | | | | | | | | | | | | | | | | | | | 0.3 | | | | |
| **hydroxylated or methylated** | | | | | | | | | | | | | | | | | | | | | | | | |
| br-15:0 | 0.2 | | | | | | | | | | | | | | | | | | | | | | | |
| α-OH 16:0 | 0.6 | | | | | | | | | | | | | | | | | | | | | | | 0.7 |
| 2-Me-16:0 | 0.8 | | | | | | | | | | | | | | | | | | | | | | | |
| br-17:0 | 0.3 | | | | | | | | | | | | | | | | | | | | | | | |
| α-OH 22:0 | | | | | | | | | | | | | | | | | | | | | | | | 2.7 |
| α-OH 23:0 | | | | | | | | | | | | | | | | | | | | | | | | 2.0 |
| α-OH 24:0 | | | | | | | | | | | | | | | | | | | 0.6 | 1.0 | | | | **14.4** |
| α-OH 25:0 | | | | | | | | | | | | | | | | | | | | | | | | 2.2 |
| α-OH 26:0 | | | | | | | | | | | | | | | | | | | | | | | | 1.5 |
| α-OH 24:1 | | | | | | | | | | | | | | | | | | | | | | | | 0.7 |

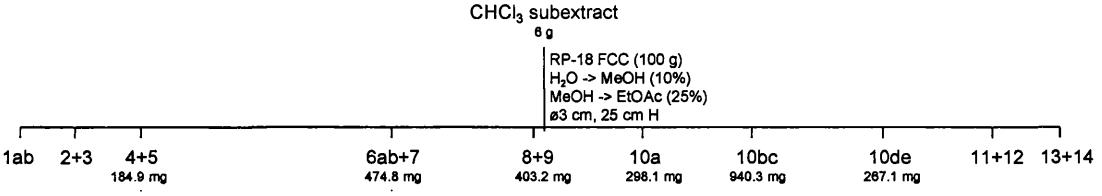
*different isomers

### 3.3.3 Investigation of the chloroform subextract

#### 3.3.3.1 Bioactivity-guided fractionation

The $CHCl_3$ subextract was fractionated by FCC with reversed-phased silica gel (LiChroprep RP-18) as stationary phase. A step gradient from 100% $H_2O$ to 100% MeOH in 10% increments was applied, which was followed by a wash from 100% MeOH to EtOAc in 25% steps. This resulted in ten fractions after combination based on similarities by TLC and $^1H$ NMR analyses (Fig. 3.16).

Figure 3.16. Fractionation scheme of the $CHCl_3$ subextract.




The results from the bioactivity tests against *P. falciparum* blood stage parasites, plasmodial FAS-II enzymes and KB cells for the cytotoxicity assessment are shown in Table 3.8. All FCC fractions exhibited good to moderate inhibition of *P. falciparum* blood stage parasites with fraction 6ab+7 and 10bc showing moderate cytotoxicity. Moderate inhibition of at least one fatty acid biosynthesis enzyme was observed for fractions 1a, 2+3 and 4+5. Fractions 1b, 11+12 and 13+14 displayed good to moderate enzyme inhibition potential. The best activities against FAS-II enzymes were shown from fraction 6ab+7 to fraction 10de which inhibited all three enzymes.

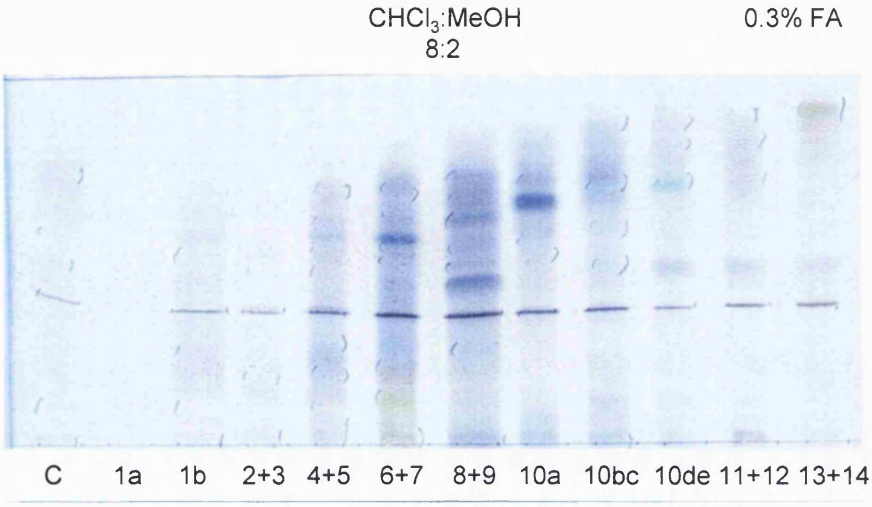
Table 3.8. Biological activities of the FCC fractions from the $CHCl_3$ subextract. $IC_{50}$ values in µg/ml.

| Fraction | FabI | FabG | FabZ | *P. falciparum* | KB |
|---|---|---|---|---|---|
| $CHCl_3$ | **6** | **4.6** | **0.85** | **5.60** | >50 |
| FCC 1a | 50 | >50 | >50 | 28.47 | >50 |
| FCC 1b | 18 | 39 | **8** | **6.97** | >50 |
| FCC 2+3 | >50 | >50 | 45 | 24.01 | >50 |
| FCC 4+5 | 24 | >50 | 14 | 12.75 | >50 |
| FCC 6ab+7 | **3.3** | 13 | **3.9** | **7.37** | 37.4 |
| FCC 8+9 | **5** | 13 | **3.8** | **1.48** | >50 |
| FCC 10a | **2.4** | **5.1** | **1.6** | **2.17** | >50 |
| FCC 10bc | **4** | **3.5** | **0.6** | **1.74** | 21.3 |
| FCC 10de | 10 | **3.8** | **1.2** | **6.04** | >50 |
| FCC 11+12 | 50 | **6** | **2.5** | **6.68** | >50 |
| FCC 13+14 | **7.9** | 8 | >1* | **9.16** | >50 |
| Control drugs | 0.024[1] | 0.53[2] | 0.3[2] | 0.001[3] | 0.002[4] |

[1]triclosan, [2]EGCG [3]artesunate, [4]podophyllotoxine

*measurement failed at higher concentrations

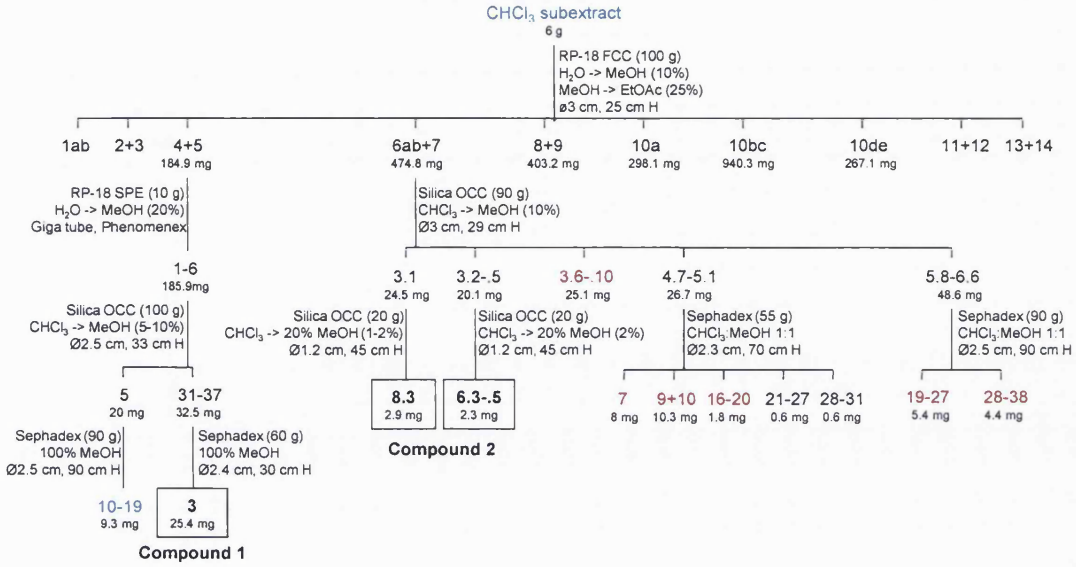
Figure 3.17. TLC analysis of the FCC fractions from the $CHCl_3$ subextract. Stationary phase: silica gel 60 $F_{254}$, staining agent: 4% vanillin/sulphuric acid (heated for 1 min at 170°C). C: $CHCl_3$ subextract




In the first eluted (1 – 3) and last washing fractions (11 – 14) only few compounds were detected on the TLC plate (Fig. 3.17). The $^1$H NMR spectra (not shown) confirmed that these fractions were chemically not interesting as they contained mainly debris and fatty acids (11 – 14) or sugars (1 – 3), hence they were excluded from further separation. Fractions 4+5, 6ab+7, 8+9, 10a, 10bc and 10de showed interesting chemical profiles. In addition, all of these fractions exhibited antiplasmodial activity and were active against at least two FAS-II enzymes. Thus, they were selected for further fractionation (Fig. 3.18 and 3.19).

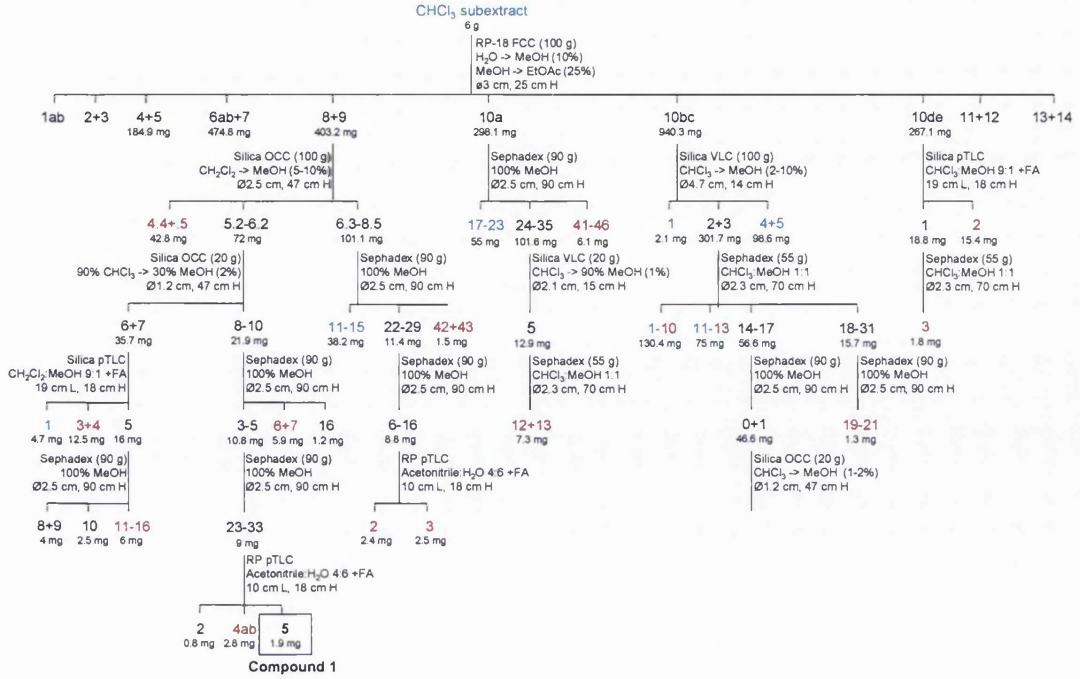
Fraction 4+5 was subjected to SPE on RP-18 silica for a coarse separation. Combined fractions based on TLC and $^1$H NMR analyses were fractionated by OCC over silica gel 60 with a step gradient from 100% $CHCl_3$ to 100% MeOH in 5-10% increments. Resulting fractions 5 (eluted in 25% MeOH) and 31-37 (eluted in 90-100% MeOH) were further purified by size-exclusion chromatography over Sephadex LH-20 with 100% MeOH. The $^1$H NMR spectra of the fractions from 5 indicated the presence of several fatty acids, therefore fraction 10-19, as the main fraction, was analysed by GC-MS in collaboration with Prof. N. M. Carballeira, Puerto Rico. From fraction 31-37 compound **1** (25.4 mg) was isolated (see 3.3.5.1).

Figure 3.18. Fractionation scheme of FCC fractions 4+5 and 6ab+7 from the $CHCl_3$ subextract. Red fractions were submitted to LC-MS, blue fractions were analysed by GC-MS. Pure compounds are shown bold in a box.




The FCC fraction 6ab+7 was fractionated by OCC over silica gel 60 with a step gradient from 100% $CHCl_3$ to 100% MeOH in 10% increments. OCC on silica gel 60 was repeated for the resulting fractions 3.1 and 3.2-.5 (eluted in 30% MeOH) with a step gradient from 100% $CHCl_3$ to 20% MeOH in 1-2% increments and yielded compound **2** (2.9 mg and 2.3 mg, respectively) (see 3.3.5.2). Fractions 4.7-5.1 (eluted in 40-50% MeOH) and 5.8-6.6 (eluted in 50-60% MeOH) from the first OCC were purified by size-exclusion chromatography over Sephadex LH-20 with $CHCl_3$:MeOH (1:1). Further fractionation was unfeasible as the resulting fractions had only small amounts or were inseparable by TLC. In order to identify known compounds, interesting mixtures were submitted to LC-MS analysis, which was performed by Sequoia Sciences, USA. Unfortunately, analyses of the fractions is still on-going, thus no results from the LC-MS analyses can be presented.

Figure 3.19. Fractionation scheme of FCC fractions 8+9, 10a, 10bc and 10de from the $CHCl_3$ subextract. Red fractions were submitted to LC-MS, blue fractions were analysed by GC-MS. Pure compound are shown bold in a box.




The fractionation from FCC fractions 8+9 to 10de yielded no new compounds, but the fractionation of FCC fraction 8+9 yielded 1.9 mg of compound **1**. Several fractions showed interesting chemical profiles but could not be subjected to further fractionation due to small amounts or inseparable mixtures. Depending on their nature, these fractions were further analysed by GS-MS (see 3.3.3.2) or submitted to LC-MS (analyses on-going) in collaboration with Prof. N. M. Carballeira, Puerto Rico and Sequoia Sciences, USA, respectively.

All fractions selected for GC-MS or LC-MS analysis were subjected to bioactivity testing against *P. falciparum* blood stage parasites, plasmodial FAS-II enzymes and cytotoxicity testing against KB cells to determine selectivity (Tab. 3.9).

With the exception of fraction C3f, all fractions showed good to moderate antiplasmodial activity. The best antiplasmodial potential was observed for fraction C3d and C5c with $IC_{50}$ values of 0.3 μg/ml and 0.79 μg/ml, respectively. Five of the twenty-eight fractions (C3a, C4a, C5c-e) were cytotoxic but exclusively for fraction C5d this resulted in a low selectivity index, as the cytotoxic effect was more potent than the antiplasmodial effect. Good inhibition of all three fatty acid biosynthesis enzymes was found for fraction C4b and C5e. Most other fractions had good to moderate potency against at least one enzyme except for fractions C1a, C2e, C2f, C3g-d and C3i where no inhibitory effect was found.

Table 3.9. Biological activities of fractions selected for further analysis by GC-MS or LC-MS. $IC_{50}$ values in μg/ml.

| Code | Fraction | FabI | FabG | FabZ | *P. falciparum* | KB |
|---|---|---|---|---|---|---|
| | FCC 4+5 | 24 | >50 | 14 | 12.75 | >50 |
| C1a | FCC 4+5: p50_10-19 | >50 | >50 | >50 | 31.67 | >50 |
| | FCC 6ab+7 | **3.3** | 13 | **3.9** | **7.37** | 37.40 |
| C2a | FCC 6ab+7: p54_3.6-.10 | 17 | 41 | 20 | **8.92** | >50 |
| C2b | FCC 6ab+7: p68_13+14 | >50 | 27 | >50 | 14.44 | >50 |
| C2c | FCC 6ab+7: p68_19-27 | 12 | 32 | **5** | 11.61 | >50 |
| C2d | FCC 6ab+7: p68_28-38 | **3** | >50 | **4** | **7.92** | >50 |
| C2e | FCC 6ab+7: p72_7 | >50 | >50 | >50 | **6.60** | >50 |
| C2f | FCC 6ab+7: p72_9+10 | >50 | >50 | >50 | 25.22 | >50 |
| C2g | FCC 6ab+7: p72_16-20 | 29 | 50 | 26 | 15.25 | >50 |
| | FCC 8+9 | **5** | 13 | **3.8** | **1.48** | >50 |
| C3a | FCC 8+9: p76_4.4+.5 | 24 | 15 | 32 | **4.12** | 22.45 |
| C3b | FCC 8+9: p84_1 | 36 | >50 | >50 | 32.20 | >50 |
| C3c | FCC 8+9: p84_3+4 | >50 | 30 | >50 | **3.37** | >50 |
| C3d | FCC 8+9: p92_11-16 | >50 | >50 | >50 | **0.30** | >50 |
| C3e | FCC 8+9: p94_6+7 | >50 | >50 | >50 | **6.81** | >50 |
| C3f | FCC 8+9: p100_4ab | >50 | >50 | >50 | >50 | >50 |
| C3g | FCC 8+9: p87_11-15 | >50 | >50 | >50 | 30.57 | >50 |
| C3h | FCC 8+9: p87_42+43 | **8.3** | 17 | 10 | 18.36 | >50 |
| C3i | FCC 8+9: p103_2 | >50 | >50 | >50 | **2.14** | >50 |
| C3j | FCC 8+9: p103_3 | >50 | 43 | >50 | **3.64** | >50 |
| | FCC 10a | **2.4** | **5.1** | **1.6** | **2.17** | >50 |
| C4a | FCC 10a: p107_17-23 | 12 | >50 | **5.3** | **2.63** | 32.11 |
| C4b | FCC 10a: p107_41-46 | **1.2** | **5.8** | **2.5** | **4.40** | >50 |
| C4c | FCC 10a: p113_12+13 | **3.3** | 26 | **0.7** | **8.97** | >50 |
| | FCC 10bc | **4** | **3.5** | **0.6** | **1.74** | 21.30 |
| C5a | FCC 10bc: p123_1 | **9.5** | >10* | >10* | **5.69** | >50 |
| C5b | FCC 10bc: p123_4+5 | **4.6** | >50 | **6.5** | 21.61 | >50 |
| C5c | FCC 10bc: p125_1-10 | **4.3** | >1* | **6.5** | **0.79** | 19.40 |
| C5d | FCC 10bc: p125_11-13 | **1** | >10* | 22 | 33.17 | 18.20 |
| C5e | FCC 10bc: p129_19-21 | **9.3** | **9.5** | **0.25** | **6.15** | 18.84 |
| | FCC 10de | 10 | **3.8** | **1.2** | **6.04** | >50 |
| C6a | FCC 10de: p119_2 | **1.6** | **0.7** | 12 | **4.29** | >50 |
| C6b | FCC 10de: p121_3 | **6.9** | >50 | 32 | **8.78** | >50 |
| Control drugs | | 0.024[1] | 0.53[2] | 0.3[2] | 0.01[3] | 0.002[4] |

[1]triclosan, [2]EGCG, [3]artesunate, [4]podophyllotoxine

*measurement failed at higher concentrations

### 3.3.3.2 Fatty acid methyl ester analysis

Fractions which appeared to be rich in fatty acids by $^1$H NMR analysis (not shown) were subjected to GC-MS analysis (Tab. 3.10). In the $CHCl_3$ subextract six fatty acids were detected, with palmitic acid (16:0, 52%) and linoleic acid (18:2, 28%) as main constituents. In the selected fractions, 16 different fatty acids in the range from $C_{12}$ to $C_{24}$ were found. All fractions showed the highest abundance of palmitic acid (16:0) (34.9 – 100%), which was the sole fatty acid in C5b. The exception is C1a which had almost identical amounts of palmitic and stearic acids. Fraction C1a was also the only fraction containing lignoceric acid (24:0). Fraction C1a, together with C3g, C5a and C5b, contained exclusively saturated fatty acids. The largest diversity in fatty acids was found in C5a, which was the only fraction that contained lauric acid (12:0), tridecylic acid (13:0) and nonadecylic acid (19:0).

Table 3.10. Fatty acid methyl ester abundances of the $CHCl_3$ subextract and fractions in weight percent.

| Fatty acid | $CHCl_3$ | C1a | C3b | C3g | C4a | C5a | C5b | C5c | C5d |
|---|---|---|---|---|---|---|---|---|---|
| **saturated** | | | | | | | | | |
| 12:0 | | | | | | 0.4 | | | |
| 13:0 | | | | | | 0.4 | | | |
| 14:0 | 1.3 | | | 5.3 | 1.5 | 7.2 | | **10.6** | 0.2 |
| 15:0 | | | | | 1.1 | 0.4/6.4* | | 1.1 | |
| 16:0 | **52.0** | **31.9** | **34.9** | **64.2** | **88.6** | **49.2** | **100.0** | **57.8** | **45.6** |
| 17:0 | | 3.5 | | | 0.7 | 4.8 | | | 0.2 |
| 18:0 | 2.6 | **33.9** | **34.7** | **30.5** | 6.3 | **17.6** | | 4.6 | 4.0 |
| 19:0 | | | | | | 1.2 | | | |
| 20:0 | | 4.6 | | | | 3.2 | | | 1.8 |
| 22:0 | | **16.5** | | | | 7.6 | | | |
| 23:0 | | 4.1 | | | | 1.6 | | | |
| 24:0 | | 5.5 | | | | | | | |
| **unsaturated** | | | | | | | | | |
| 16:1 | | | | | | | | | 2.7 |
| 18:1 | 8.0 | | **30.4** | | 1.8 | | | 5.4 | |
| 18:2 | **28.0** | | | | | | | 9.8 | **25.5** |
| 18:3 | 8.0 | | | | | | | **10.7** | **19.9** |

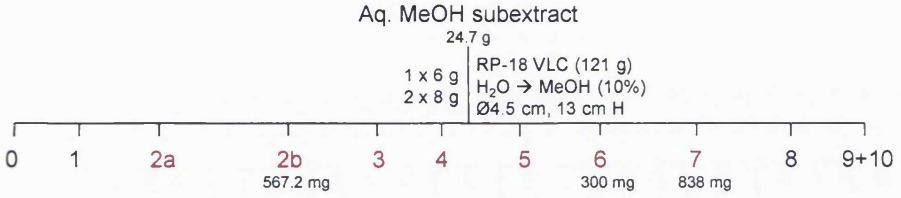
*different isomers

### 3.3.4 Investigation of the aqueous methanolic subextract

#### 3.3.4.1 Bioactivity-guided fractionation

The aq. MeOH subextract was fractionated by VLC over reversed-phase silica gel (LiChroprep RP-18) with a step gradient from 100% $H_2O$ to 100% MeOH in 10% increments, which resulted in eleven fractions after combination according to TLC and $^1H$ NMR analyses (Fig. 3.20).

Figure 3.20. Fractionation scheme of the aq. MeOH subextract.



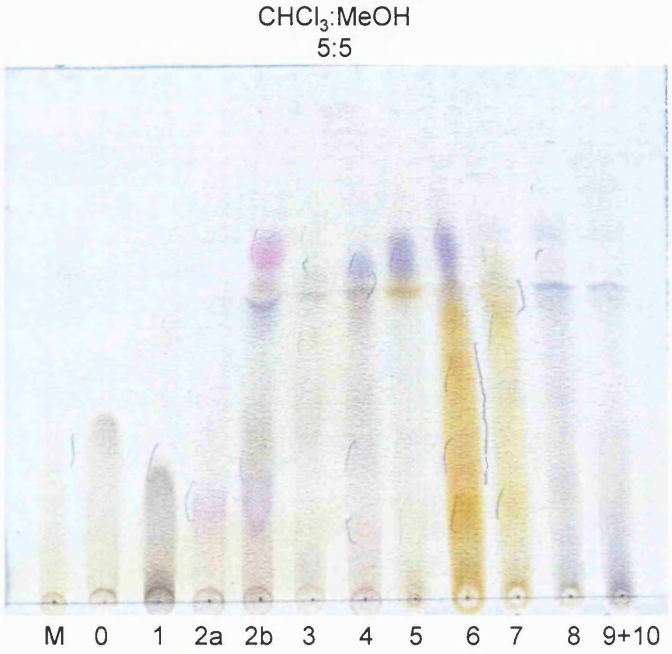

Bioactivity assessments against *P. falciparum* blood stage parasites and plasmodial FAS-II enzymes and cytotoxicity assessment against KB cells for selectivity showed biological activity from VLC fraction 2b onwards (Tab. 3.11). Antiplasmodial activity was found from fraction 6 onwards with moderate $IC_{50}$ values in the range of 17.8 to 37.4 µg/ml. The same fractions also showed good inhibition of FAS-II enzymes FabI and FabZ with $IC_{50}$ values ranging from 4.3 – 0.6 µg/ml. No inhibition of FabG was observed, except for a moderate potential by fraction 9+10 ($IC_{50}$ of 10 µg/ml). Fractions 2b, 3, 4 and 5 exhibited good to moderate activity against the FabZ enzyme, but lacked antiplasmodial potential. No cytotoxicity was observed.

Table 3.11. Biological activities of the VLC fractions from the aq. MeOH subextract. $IC_{50}$ values in µg/ml.

| Fraction | FabI | FabG | FabZ | *P. falciparum* | KB |
|---|---|---|---|---|---|
| Aq. MeOH | 13 | >50 | **4.3** | 36.05 | >50 |
| VLC 0 | >50 | >50 | >50 | >50 | >50 |
| VLC 1 | >50 | >50 | >50 | >50 | >50 |
| VLC 2a | >50 | >50 | >50 | >50 | >50 |
| VLC 2b | >50 | >50 | 13 | >50 | >50 |
| VLC 3 | >50 | >50 | 10 | >50 | >50 |
| VLC 4 | >50 | >50 | **2.7** | >50 | >50 |
| VLC 5 | 26 | >50 | **2.3** | >50 | >50 |
| VLC 6 | **4.3** | >50 | **0.9** | 37.4 | >50 |
| VLC 7 | **1** | >50 | **0.6** | 17.8 | >50 |
| VLC 8 | **7** | >50 | **1.2** | 21.8 | >50 |
| VLC 9+10 | **0.8** | 10 | **1.0** | 23.2 | >50 |
| Reference | 0.024[1] | 0.53[2] | 0.3[2] | 0.004[3] | 0.001[4] |

[1]triclosan, [2]EGCG, [3]artesunate, [4]podophyllotoxine

Figure 3.21. TLC analysis of the VLC fractions from the aq. MeOH fractionation. Stationary phase: silica gel 60 $F_{254}$, staining agent: 4% vanillin/sulphuric acid (heated for 1 min at 170°C).




TLC analysis (Fig. 3.21) of the VLC fractions indicated the presence of flavonoids in fractions 6 and 7 (strong fluorescence, yellow coloured spots). Additional TLC analysis stained with *Naturstoffreagenz* verified the presence of flavonoids (not shown). Flavonoids are known for their *in vitro* potential against FAS-II enzymes and *P. falciparum* blood stage parasites (Tasdemir *et al.*, 2006), and both fractions presented a remarkable inhibitory profile against the plasmodial FabZ enzyme with $IC_{50}$ values of 0.6 µg/ml (fraction 7) and 0.9 µg/ml (fraction 6). In addition, good activity against FabI and moderate antiplasmodial activity was detected, thus fractions 6 and 7 were selected for further analysis and fractionation in order to purify and identify the active components. VLC fraction 2b was also selected for further investigation. Even though this fraction only showed moderate inhibitory potential against FabZ, phytochemical analysis by TLC and $^1$H NMR analyses (not shown) showed an interesting chemical profile. In order to identify known compounds and flavonoids fractions 2a to 7 were subjected to LC-MS analysis by Sequoia Sciences, USA (data not yet available). VLC fraction 8 and 9+10 were excluded from further fractionation, despite their moderate antiplasmodial activity and good inhibition of FabZ and FabI. This was because TLC and $^1$H NMR analyses (not shown) were not interesting.

The fractionation of the VLC fractions is summarised in Figure 3.22. From the fractions 6 and 7 no compounds could be isolated. In order to identify some of the flavonoids present, both fractions were analysed by TLC (Fig. 3.23) and compared to several known flavonoids reported from the genus *Anthemis*.

Figure 3.22. Fractionation scheme of VLC fractions from the aq. MeOH subextract. Red fractions were submitted to LC-MS analysis. Pure compounds are bold in a box

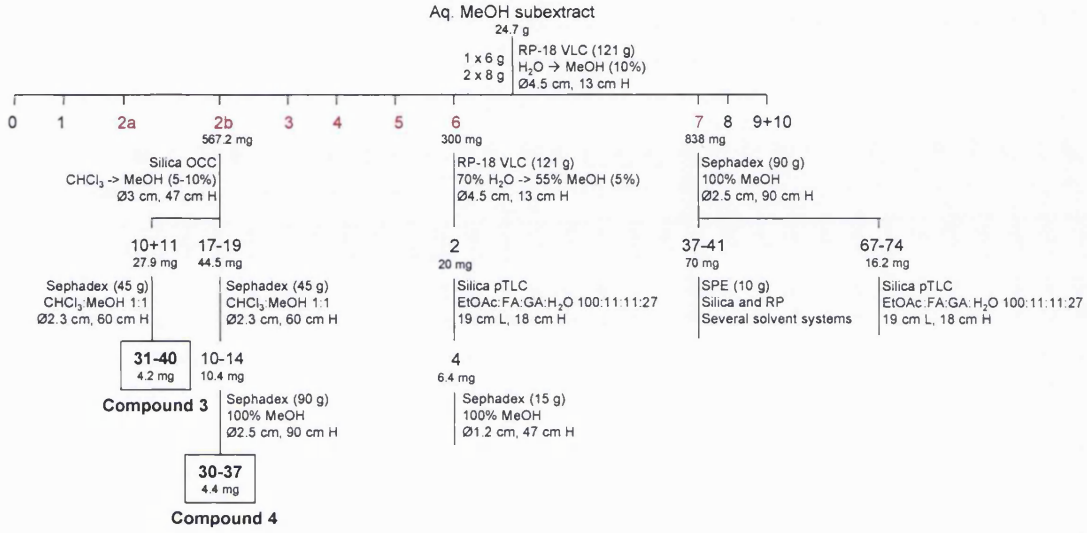
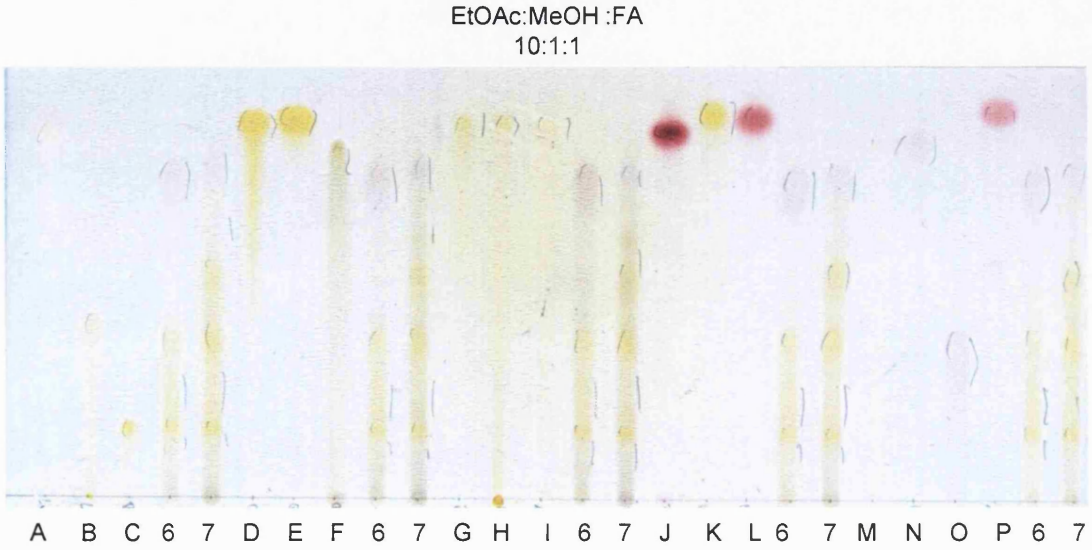
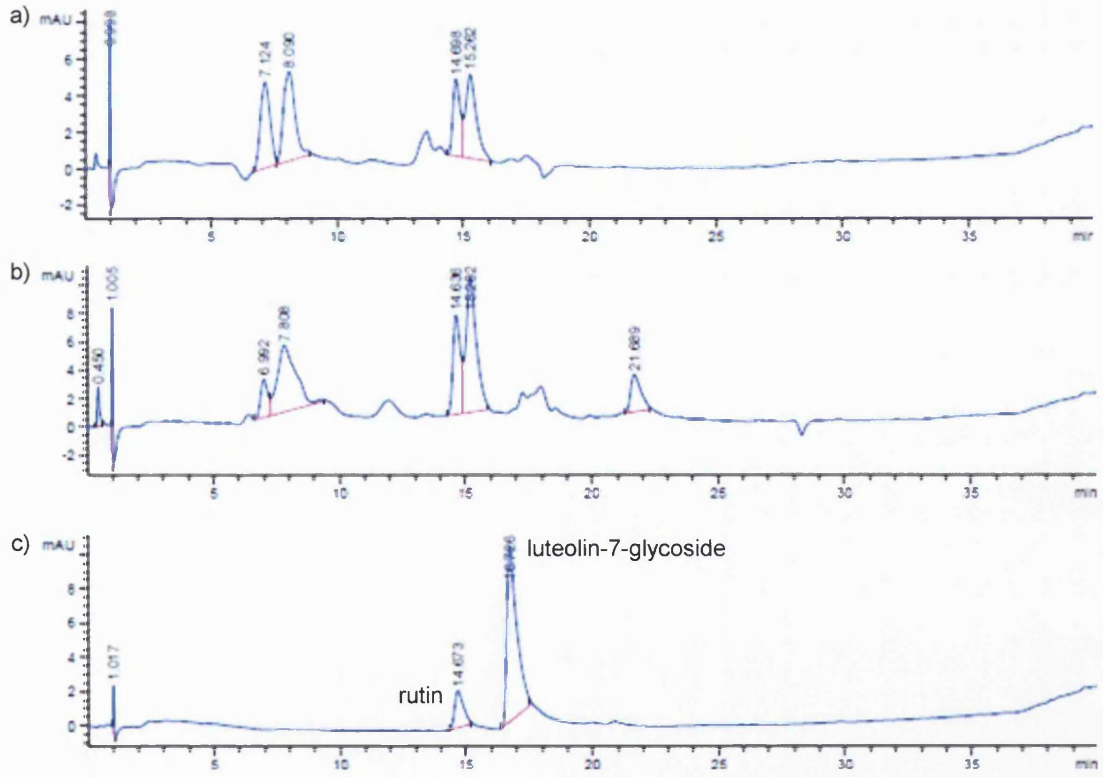



Figure 3.23. TLC comparison of VLC fractions 6 and 7 and several know flavonoids reported from the genus *Anthemis*. A: luteolin, B: luteolin-7-glucoside, C: rutin, D: quercetin, E: taxarixetin, F: quercetagetin, G: rhamnetin, H: isorhamnetin, I: apigenin, J: taxifolin, K: kaempferol, L: eriodictyol, M: cirsimaritin, N: caffeic acid, O: chlorogenic acid, P: naringenin. Stationary phase: silica gel 60 $F_{254}$, staining agent: 4% vanillin/sulphuric acid (heated 1 min at 170°C).



The TLC comparison suggested the presence of rutin (C) and luteolin-7-glycoside (B) in VLC fraction 6 and 7. To confirm the presence of these flavonoids a HPLC analysis with a gradient from 100% $H_2O$ to 30% MeOH in 5 min, 30% MeOH to 70% MeOH in 30 min and 70% MeOH to 100% MeOH in 5 min was applied. VLC fractions 6 and 7 as well as a mixture of rutin and luteolin-7-glycoside were measured. Rutin had a retention time of 14.67 min which correlates well with an observed peak in fraction 6 (14.70 min) and fraction 7 (14.64 min) (Fig. 3.24). Luteolin-7-glycoside with a retention time of 16.73 min could not be detected in fraction 6 or 7 (Fig. 3.24). Additional analysis of these two fractions by LC-MS is ongoing.

Figure 3.24. HPLC analyses of VLC fractions 6 (a) and 7 (b) and flavonoids rutin and luteolin-7-glycoside (c). Monitored with UV-detection at 254 nm.




Fraction 2b was fractionated by OCC over silica gel 60 with a gradient from 100% $CHCl_3$ to 100% MeOH with 5-10% increments. The two resulting fractions 10+11 (eluted in 45-50% $CHCl_3$) and 17-19 (eluted in 30% $CHCl_3$) were further purified by size-exclusion chromatography on Sephadex LH-20 with $CHCl_3$:MeOH (1:1). From fraction 10+11 compound **3** (4.2 mg) was isolated (see 3.3.5.3). Fraction 10-14 from the chromatography of 17-19 was subjected to a second size-exclusion chromatography on Sephadex LH-20 with 100% MeOH, which yielded 4.4 mg of compound **4** (see 3.3.5.4).

### 3.3.5 Isolated compounds

#### 3.3.5.1 Structure elucidation of compound 1: Cyanogenic glycoside

Compound **1** was isolated as pale orange coloured oil from the FCC fractions 4+5 and 8+9 from $CHCl_3$ subextract as described in 3.3.3.1.

TLC analysis of compound **1** on silica gel 60 $F_{254}$ plates showed UV quenching activity at 254 nm and a brownish spot after vanillin/sulphuric acid staining (Fig. 3.25). The positive mode ESI mass spectrometry [(+)-ESI-MS)] spectrum (Fig. 3.26) contained an intense molecular ion peak at $m/z$ 450.57 $[M+Na]^+$, suggesting the molecular formula $C_{19}H_{25}NO_{10}$ and a calculated molecular weight of $m/z$ 427.40. High resolution mass spectrometry (accurate mass) gave a theoretical molecular weight of $m/z$ 450.1376 $[M+Na]^+$ and a measured mass value of $m/z$ 450.1357 resulting in an absolute mass error of Δ -1.9 mmu and relative error of δ 4 ppm.


Figure 3.25. TLC analysis of compound **1**. Left: isolated from FCC fraction 4+5, right: isolated from FCC fraction 8+9. Stationary phase: silica gel 60 $F_{254}$, staining agent: vanillin/sulphuric acid (heated for 1 min at 170°C).

Figure 3.26. (+)-ESI-MS spectrum of compound **1**.

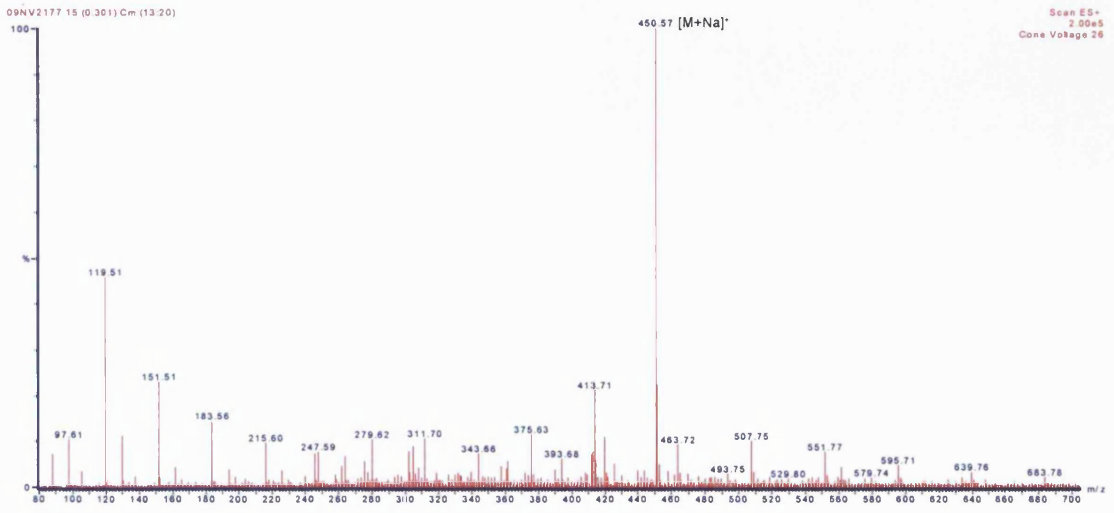
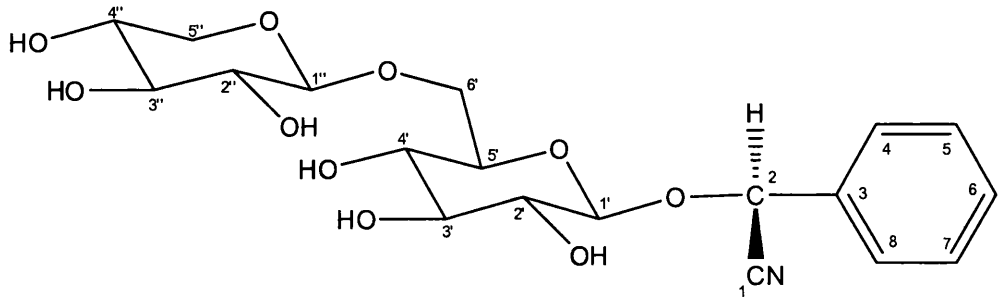


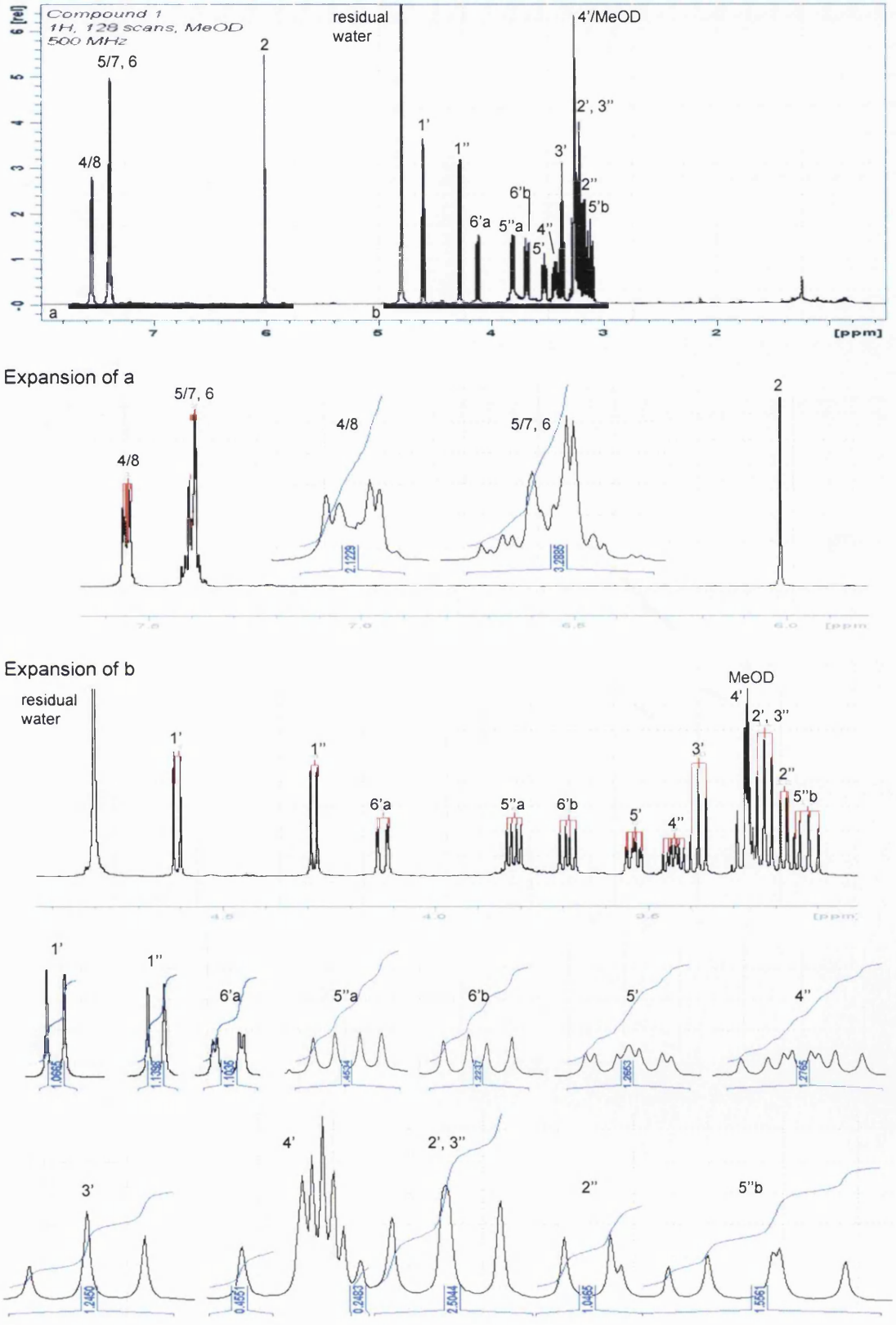
Figure 3.27. Structure of compound **1**.




The $^1$H NMR spectrum of compound **1** in MeOD (Fig. 3.28) showed several signals between δ 3.0 and δ 4.7, which are characteristic for residual sugar protons. The peaks at δ 4.61 (H-1', d, $J$ = 7.8 Hz) and δ 4.28 (H-1", d, $J$ = 7.4 Hz) are characteristic for anomeric protons and suggested the presence of two sugar units. The coupling constants of the anomeric protons (7.8 Hz and 7.4 Hz) indicated the β configuration of both sugar units. Integration of the $^1$H NMR peaks between δ 3.0 and δ 4.2 revealed 11 protons, suggesting the sugar portion of the molecule is composed of a hexose and a pentose. The integration of the peaks in the downfield region of the $^1$H NMR spectrum showed the presence of three protons for δ 7.40 (H-5, H-6, H-7, m) and two protons for δ 7.55 (H-4, H-8, dd, $J$ = 2.0, 7.4), which are characteristic for protons in a monosubstituted phenyl ring.

The uneven number of the molecular weight ($m/z$ 427.40) indicated the presence of a nitrogen atom in the molecule. The molecular formula $C_{19}H_{25}NO_{10}$ suggested eight degrees of unsaturation and since the two sugar units and phenyl ring accounted for six degrees of unsaturation, the presence of a nitrile group is expected. This is in agreement with the observation of an isolated broad singlet signal at δ 6.01 (H-2, s) that indicated the presence of a cyanohydrin proton.

Figure 3.28. $^{1}$H NMR spectrum of compound **1** with expansions of peaks (MeOD, 500 MHz).



The $^{13}C$ NMR spectrum in MeOD (Fig. 3.29) showed 17 signals. Ten signals were observed in the upfield with a chemical shift between δ 67.0 and 77.9 ppm and were identified by DEPT90° and DEPT135° (Fig. 3.29) as methine and methylene groups. The chemical shift values are characteristic for the carbon atoms in sugar units and assigned to the residual sugar protons by HMQC (Fig. 3.30), with the exception of δ 68.8 (d) which was assigned to the cyanohydrin carbon C-2 . The two methine carbon signals observed at δ 102.2 (C-1', d) and δ 106.0 (C-1", d) have typical chemical shift values for the anomeric sugar carbons and their downfield shift is characteristic for *O*-glycosylation. The chemical shift of C-6' (δ 70.3, t) towards the downfield indicated a glycosidic bond between C-6' and C-1". The chemical shift values observed in the $^{1}H$ NMR and $^{13}C$ NMR experiments are characteristic for β-D-glucose and β-D-xylose (Agrawal, 1992).

In the downfield region of the $^{13}C$ NMR spectrum two quaternary carbons and three methine groups were observed. The signal at δ 118.7 (s) is characteristic for a nitrile group (Takeda *et al.*, 1997) and was assigned to the quaternary C-1. The C-3 atom was identified as a quaternary carbon in the phenyl ring with a chemical shift δ 135.3 (s). The remaining three aromatic methine groups at δ 128.8 (C-4, C-8, d), δ 130.0 (C-5, C-7, d) and δ 130.8 (C-6, d) ppm were assigned to the phenyl ring. Carbons C-4 and C-8 as well as C-5 and C-7 have identical chemical shifts, which explains why only 17 peaks, instead of 19, were observed in the $^{13}C$ NMR spectrum.

Figure 3.29. $^{13}C$ NMR (black), DEPT135° (blue) and DEPT90° (red) spectra of compound **1** (MeOD, 125 MHz).

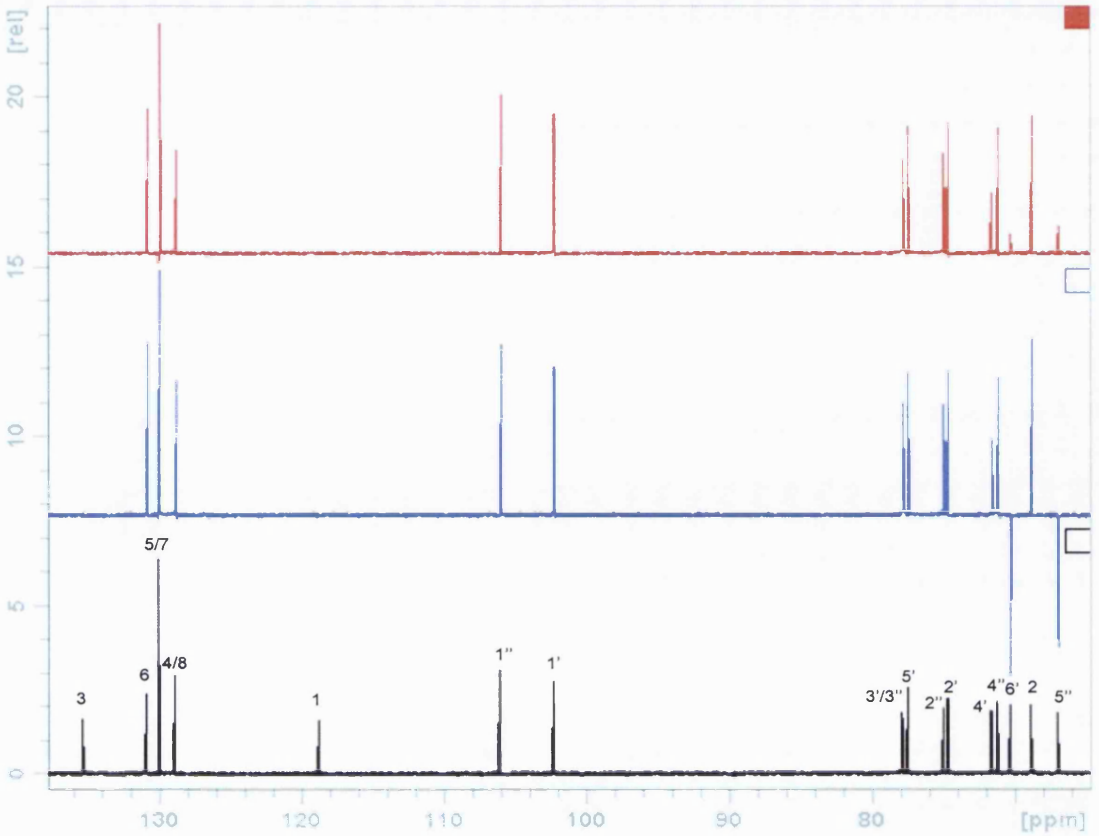
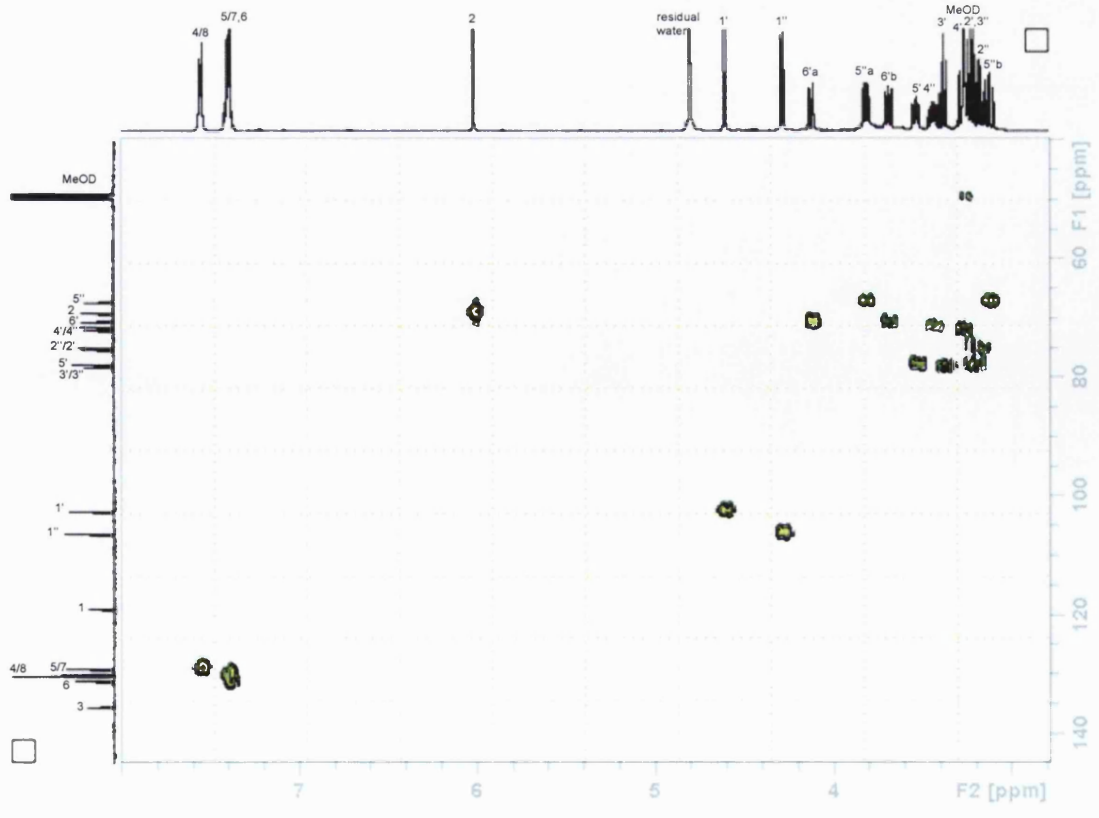



Figure 3.30. HMQC spectrum of compound **1** (MeOD, 500 MHz/125 MHz).



HMBC correlations (Fig. 3.31) were observed between the overlapped H-4/H-8 (δ7.55, dd, $J$ = 2.0, 7.4 Hz) and the aromatic carbons C-5/C-7 and $^3J$ correlations to C-2, C-6 and each other. The coupling constants verified the *ortho* position of H-4/H-8 to H-5 and H-7 (7.4 Hz) and the *meta* position of H-4/H-8 to H-6 (2.0 Hz). For the overlapped H-5/H-7 (δ 7.40, m) $^2J$ correlations to C-4/C-8 and a $^3J$ correlation to C-3 were detected. For the cyanohydrin proton H-2 $^2J$ correlations to C-1 and C-3 and $^3J$ correlations to C-4, C-8 and C-1' were observed. This indicated the attachments of C-2 to C-3 in the phenyl ring and of C-2 to the C-1' carbon in the sugar unit.

Examination of the anomeric proton H-1" of the terminal sugar unit showed a $^3J$ correlation to C-6', verifying the presence of a glycosidic bond between C-6' and C-1".

Figure 3.31. HMBC spectrum of compound **1** (MeOD, 500 MHz/125 MHz).

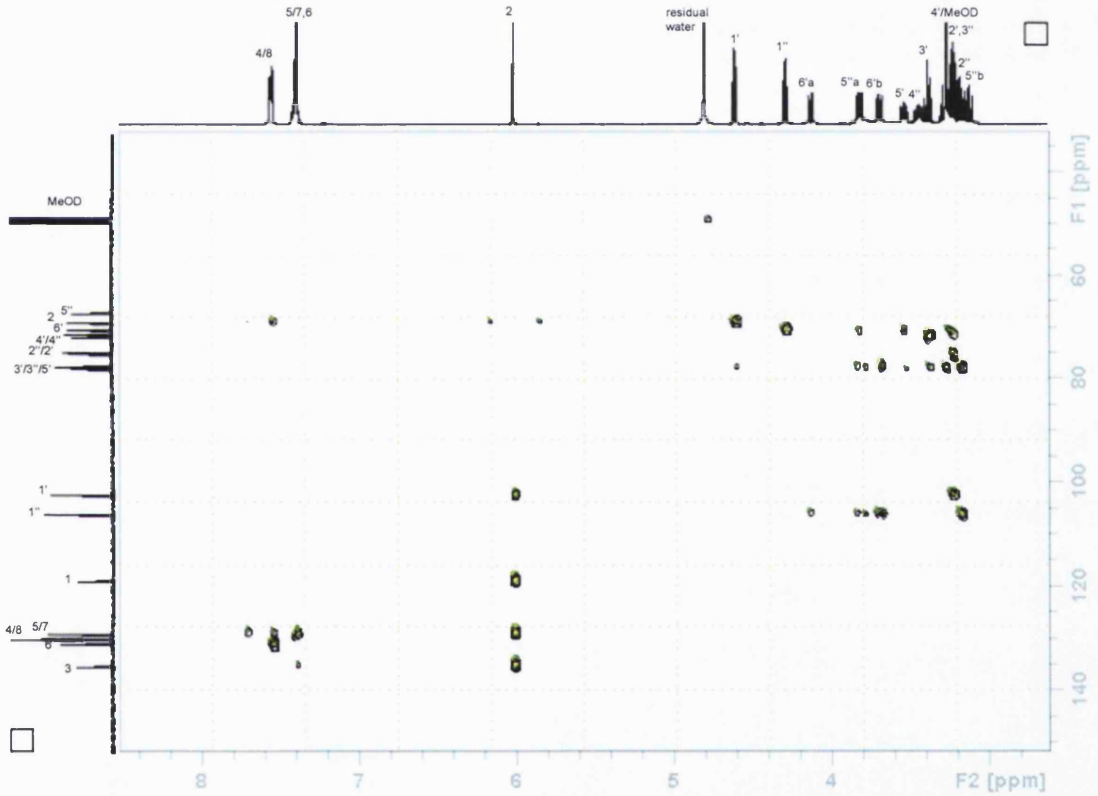


Table 3.12. $^{1}H$ NMR and $^{13}C$ NMR data and $^{1}H$-$^{13}C$ long-range (HMBC) correlations for compound **1** (MeOH, 500 MHz/125 MHz). Chemical shifts δ are reported in ppm, *J* values in Hz.

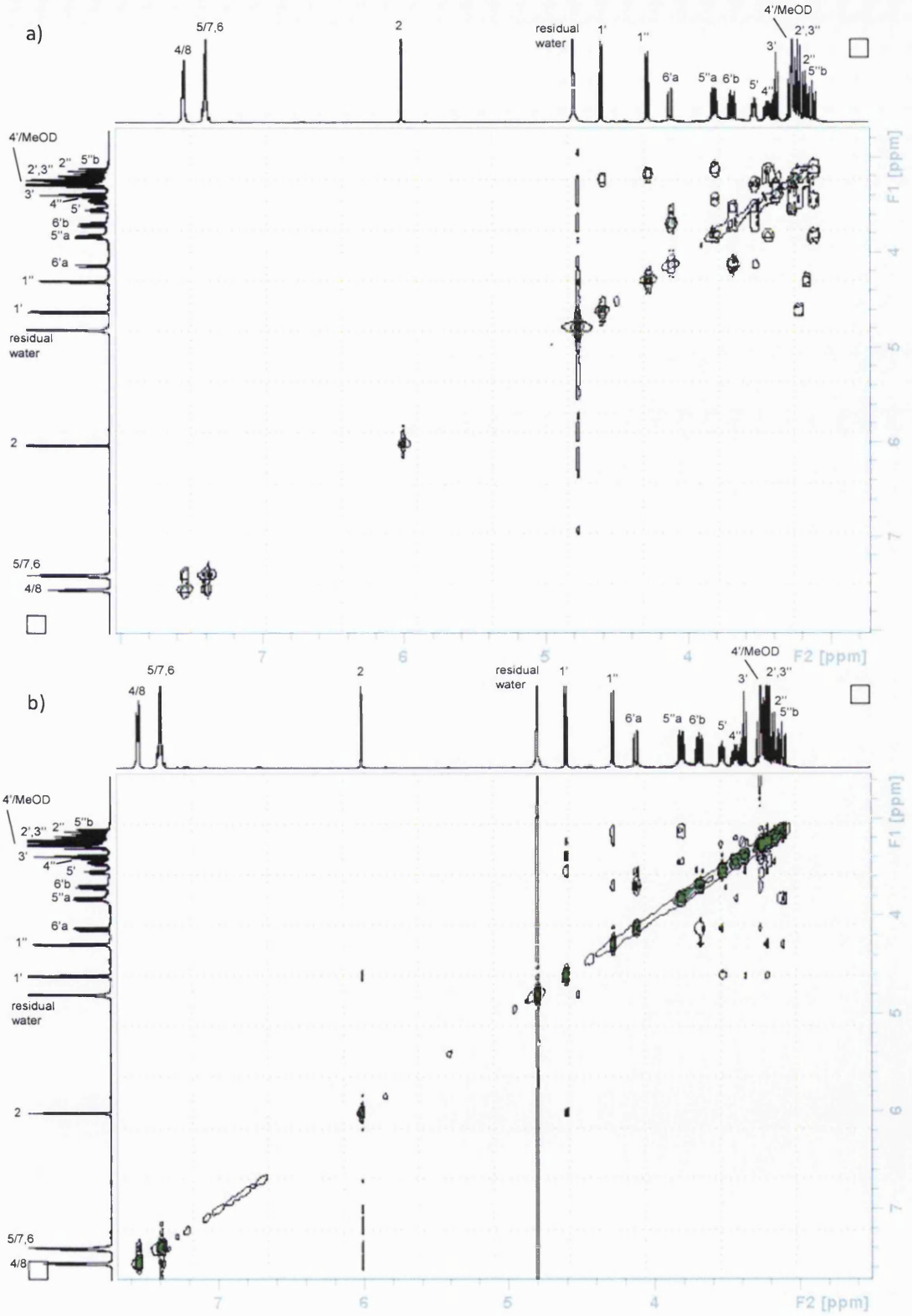
| Position | $^{1}H$ | $^{13}C$ | $^{2}J$ | $^{3}J$ |
|---|---|---|---|---|
| 1 | | 118.7 *s* | | |
| 2 | 6.01 *s* | 68.8 *d* | C-1, C-3 | C-4, C-8, C-1' |
| 3 | | 135.3 *s* | | |
| 4 | 7.55 *dd* (2.0, 7.4) | 128.8 *d* | C-5 | C-2, C-6, C-8 |
| 5 | 7.40 *m* | 130.0 *d* | C-4 | C-3 |
| 6 | 7.40 *m* | 130.8 *d* | | |
| 7 | 7.40 *m* | 130.0 *d* | C-8 | C-3 |
| 8 | 7.55 *dd* (2.0, 7.4) | 128.8 *d* | C-7 | C-2, C-4, C-6 |
| 1' | 4.61 *d* ( 7.8) | 102.2 *d* | | C-2, C-3' or C-5' |
| 2' | 3.22 *t* (8.6) | 74.7 *d* | C-1' | |
| 3' | 3.38 *t* (9.1) | 77.9 *d* | C-4' | C-5' |
| 4' | 3.27* | 71.6 *d* | C-3', C-5' | |
| 5' | 3.53 *m* | 77.5 *d* | C-6' | C-3' |
| 6' | 4.12 *dd* (2.0, 11.8) | 70.3 *t* | | C-1" |
| | 3.69 *dd* (7.0, 11.8) | | C-5' | C-1" |
| 1" | 4.28 *d* (7.4) | 106.0 *d* | | C-6' |
| 2" | 3.18 *d* (7.4) | 75.0 *d* | C-1", C-3" | |
| 3" | 3.22 *t* (8.6) | 77.8 *d* | C-2", C-4" | |
| 4" | 3.44 *m* | 71.2 *d* | | |
| 5" | 3.81 *dd* (5.4, 11.5) | 67.0 *t* | | C-1", C-3" |
| | 3.13 *m* | | C-4" | C-1" |

* obscured by MeOD peak at δ 3.31

The COSY spectrum (Fig. 3.32 a) indicated the presence of three spin systems within compound **1**. Complex $^{1}H$-$^{1}H$ couplings were observed between the protons H4 – H8 in the phenyl ring in the first spin system. The methine and methylene protons in the inner glucoside (H-1' – H-6') and the terminal xylose (H-1" – H-5") were identified as the second and third spin system and showed complex homonuclear cross-peaks. No coupling was observed for H-2.

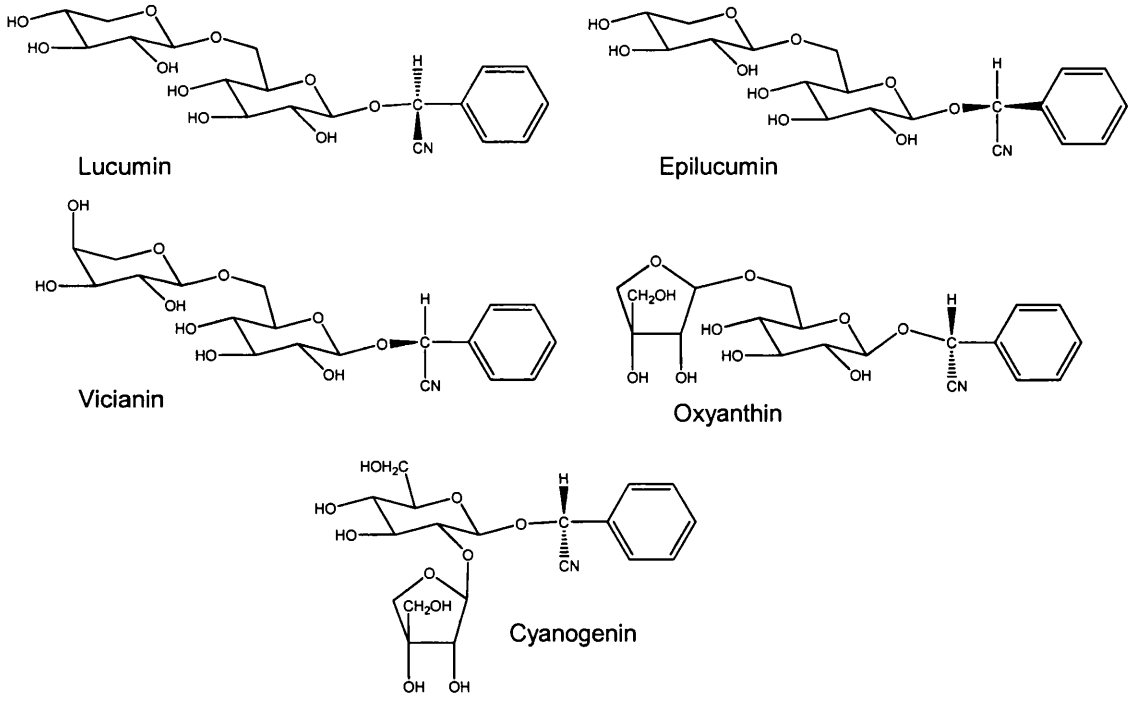
In order to identify the relative stereochemistry of compound **1**, a NOESY experiment was run. As shown in Figure 3.32 b, the cyanohydrin proton (H-2) showed an NOE coupling with the anomeric glucose proton (H-1'), indicating both protons to be on the same side, *i.e.* α.

Figure 3.32. COSY and NOESY spectra of compound **1**. a) COSY, b) NOESY (MeOD, 500 MHz).



The comparison of the gross structure of compound **1** with literature data showed the existence of five compounds with the same molecular formula (Fig. 3.33): lucumin, epilucumin, vicianin, oxyanthin and cyanogenin. The differences between the compounds reside in the nature of the terminal sugar group, the position of glycosylation and stereochemistry of C-2. The inner sugar was identified in all cases as glucose, while the terminal sugar unit was xylose (lucumin and epilucumin), arabinose (vicianin) or apiose (oxanthin and cyanogenin). With the exception of cyanogenin, the terminal sugar unit is attached at the C-6' position of glucose. As described above, the glycosylation for compound **1** was identified from C-6' to C-1", which eliminated cyanogenin.

Figure 3.33. Cyanogenic glycosides.




The specific rotation value of compound **1** was determined as $[\alpha]^{D}_{22}$ as -53 (c= 0.1) in $H_2O$ and -45 (c= 0.1) in MeOH. The published specific rotation values are as follows: lucumin (-50.5, c= 0.4 in $H_2O$; Eyjolfsson, 1971), vicianin (-19.6, c= 1.0 in $H_2O$; Nagumo *et al.*, 1985), oxyanthine (-81.1, c= 0.95 in MeO; Rockenbach *et al.*, 1992) and cyanogenin (-73, c 0.7 in EtOH; Dellagreca *et al.*, 2000). No specific rotation has been reported for epilucumin.

Comparison of the published specific rotation values and the spectral data eliminated vicianin and oxyanthin (Nagumo *et al.*, 1985; Rockenbach *et al.*, 1992), leaving lucumin and epilucumin as possible compounds.

To verify the identity of compound **1**, the spectral data for lucumin (Takeda *et al.*, 1997) and epilucumin (Nahrstedt *et al.*, 1983) were compared to compound **1**. $^{13}C$ NMR experiments were repeated with the solvents used in the literature (pyridine-$d_5$ and $D_2O$) as the published $^{1}H$ NMR data showed many overlapping signals, thus was not suitable for comparison. The spectra can be found in the appendix. Table 3.13 shows the comparison of the $^{13}C$ NMR data.

Table 3.13. $^{13}C$ NMR data (125 MHz) for compound **1** compared with lucumin (Takeda *et al.*, 1997) and epilucumin (Nahrstedt *et al.*, 1983). Chemical shifts are reported in ppm.

| | **Compound 1** Pyridin-$d_5$ | **Lucumin**[1] Pyridin-$d_5$ | **Compound 1** $D_2O$ | **Epilucumin**[2] $D_2O$ |
|---|---|---|---|---|
| 1 | 119.0 *s* | 119.4 | 117.9 *s* | 119.1 |
| 2 | 68.6 *d* | 67.8 | 69.2 *d* | 69.8 |
| 3 | 135.1 *s* | 134.5 | 132.4 *s* | 133.5 |
| 4,8 | 128.6 *d* | 129.4 | 127.9 *d* | 130.4[a] |
| 5,7 | 129.5 *d* | 128.2 | 129.3 *d* | 128.9[a] |
| 6 | 130.2 *d* | 130.0 | 130.5 *d* | 131.5 |
| 1' | 103.0 *d* | 102.6 | 100.5 *d* | 101.6 |
| 2' | 75.1*d* | 74.7 | 72.7 *d* | 74.1[b] |
| 3' | 78.8 *d* | 78.3 | 75.7 *d* | 76.8[c] |
| 4' | 72.1 *d* | 71.5 | 69.2 *d* | 70.4[d] |
| 5' | 78.7 *d* | 78.3 | 75.3 *d* | 76.7[c] |
| 6' | 70.6 *t* | 70.0 | 68.5 *t* | 69.7 |
| 1" | 106.9 *d* | 106.2 | 103.7 *d* | 104.8 |
| 2" | 75.5 *d* | 75.2 | 73.0 *d* | 73.8[b] |
| 3" | 78.3 *d* | 78.0 | 75.5 *d* | 76.4[c] |
| 4" | 71.5 *d* | 71.2 | 68.7 *d* | 70.4[d] |
| 5" | 67.5 *t* | 67.1 | 65.2 *t* | 66.3 |

[1]Recorded on JEOL FT-NMR A500 (125 MHz), [2]recorded on Bruker WM 400 (100 MHz). [a]intensity of these resonances indicated the presence of two or more carbons, [b,c,d]resonance assignment interchangeable

The reported chemical shift for C-1 for epilucumin in $D_2O$ is δ 119.1 compared to δ 117.9 measured for compound **1**. For lucumin the chemical shift δ 119.4 was reported in pyridine-$d_5$, which is practically the same value as the observed δ 119.0 for compound **1**. This observation, together with the similarity in the reported specific rotation by Eyjolfsson *et al.* (1971), identified compound **1** as lucumin.

The $^{13}C$ chemical shift values for the pairs of C-4/C-8 and C-5/C-7 were mutually interchanged in lucumin based on $^{1}H$ NMR and HMBC data.

### 3.3.5.2 Structure elucidation of compound 2: Sesquiterpene lactone

Compound **2** was isolated as a yellowish oil from the FCC fraction 6ab+7 of the $CHCl_3$ subextract as described in 3.3.3.1.

TLC analysis on silica gel 60 $F_{254}$ plates showed UV quenching activity at 254 nm for this compound and staining with vanillin/sulphuric acid resulted in a dark purple band (Fig. 3.34).

Figure 3.34. TLC analysis of compound **2**. Stationary phase: silica gel 60 $F_{254}$, staining agent: vanillin/sulphuric acid (heated for 1 min at 170°C).

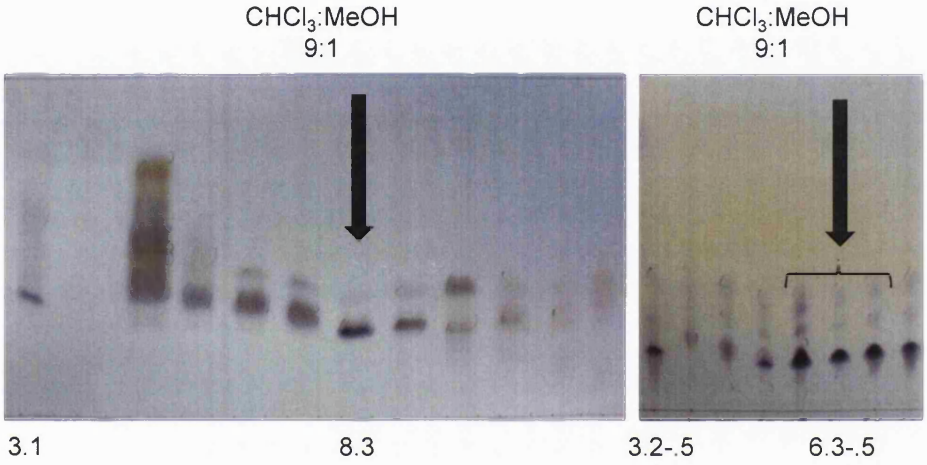
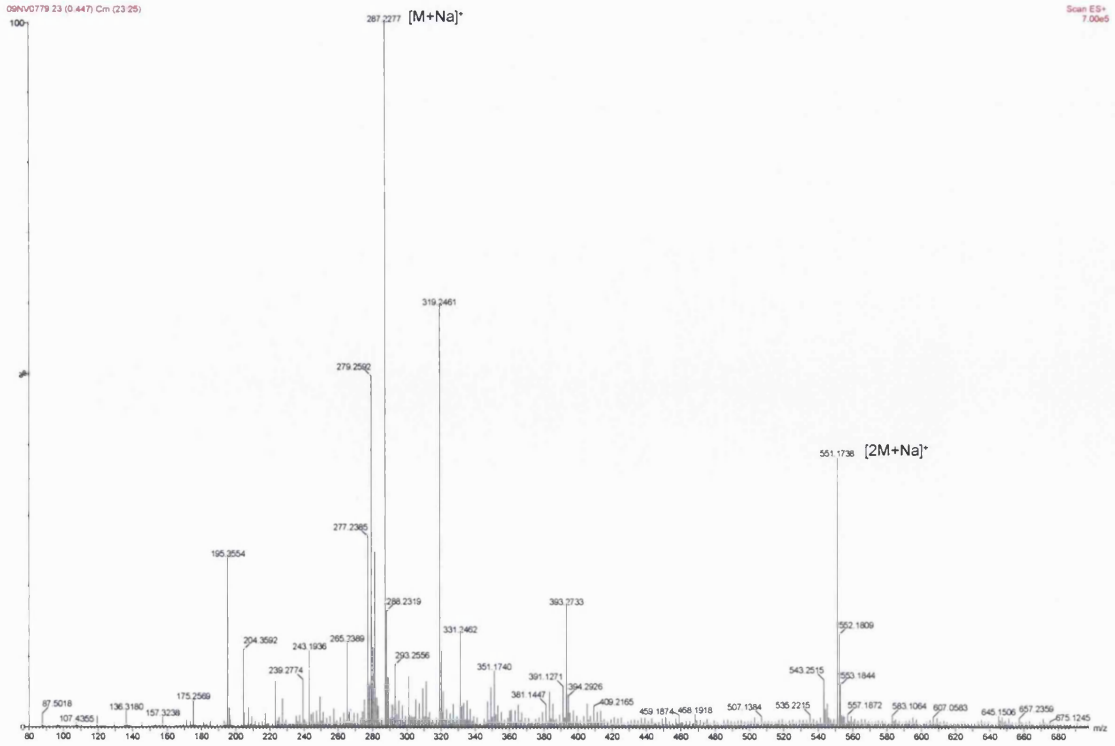



Figure 3.35. (+)-ESI-MS spectrum of compound **2**.



The (+)-ESI-MS spectrum (Fig. 3.35) showed an intense molecular ion peak at $m/z$ 287.23 $[M+Na]^+$, which suggested the molecular formula $C_{15}H_{20}O_4$ and a calculated molecular weight of $m/z$ 264.15. High resolution mass spectrometry (accurate mass) gave a theoretical molecular weight of $m/z$ 287.1259 $[M+Na]^+$ and a measured mass value of $m/z$ 287.1261 resulting in an absolute mass error of Δ +0.2 mmu and relative error of δ 1 ppm. The peak for $[M+H]^+$ was hidden in the baseline and was measured to be $m/z$ 265.1432, which resulted with the theoretical mass value of $m/z$ 265.1440 and in an absolute mass error of Δ -0.8 mmu and relative error of δ 3 ppm.
The molecular formula indicated that compound **2** is a sesquiterpene lactone and suggested the presence of six degrees of unsaturation.

In the downfield region, the $^1$H NMR spectrum in $CDCl_3$ (Fig. 3.36) contained two exocyclic double bond protons at δ 6.17 (H-13a, d, $J$ = 3.1 Hz) and δ 5.99 (H-13b, d, $J$ = 2.9 Hz). A broad singlet at δ 5.36 (H-3, s) suggested the presence of an additional endocyclic double bond. In the midfield, three protons were observed and their chemical shift values at δ 3.70 (H-1, dd, $J$ = 3.9, 9.9 Hz), δ 3.98 (H-6, t, $J$ = 11.1 Hz) and δ 4.17 (H-8, td, $J$ = 4.5, 10.5 Hz) indicated that all three protons were oxygenated. In the upfield region four peaks that integrated for six protons were observed with chemical shift values of δ 1.27 (H-9b, m), δ 1.96 (H-2b, m), δ 2.35 (H-2a, H-5, H-9a, m) and δ 2.57 (H-7, tt, $J$ = 3.1, 10.6 Hz). In addition, two methyl groups at δ 0.90 (H-14, s) and δ 1.85 (H-15, s) were detected. The chemical shift value of H-15 indicated it to be an olefinic methyl group.

The $^{13}$C NMR spectrum in $CDCl_3$ (Fig. 3.37) showed 15 signals. Analysis of the DEPT135° (Fig. 3.37) and the HMQC (Fig. 3.38) spectra showed the presence of one olefinic methyl (C-15, δ 23.5), one olefinic methylene (C-13, δ 119.9), one olefinic methine (C-3, δ 121.6), two olefinic quaternary carbons (C-4, δ 132.9 and C-11, δ 137.0), one carbonyl group (C-12, δ 170.4), two aliphatic methylenes (C-2, δ 32.6 and C-9, δ 45.5), one oxymethine (C-8, δ 67.4), two hydroxymethines (C-1, δ 75.1 and C-6, δ 79.1), one methyl (C-14, δ 12.3), two methines (C-5, δ 50.4 and C-7, δ 56.5), and one quaternary carbon (C-10, δ 40.5). The carbons at δ 170.4 (C-12) and δ 119.9 (C-13) were readily assigned to the lactone ring.

Figure 3.36. $^{1}H$ NMR spectrum of compound **2** with expansions of peaks ($CDCl_3$, 500 MHz). X: residual MeOH

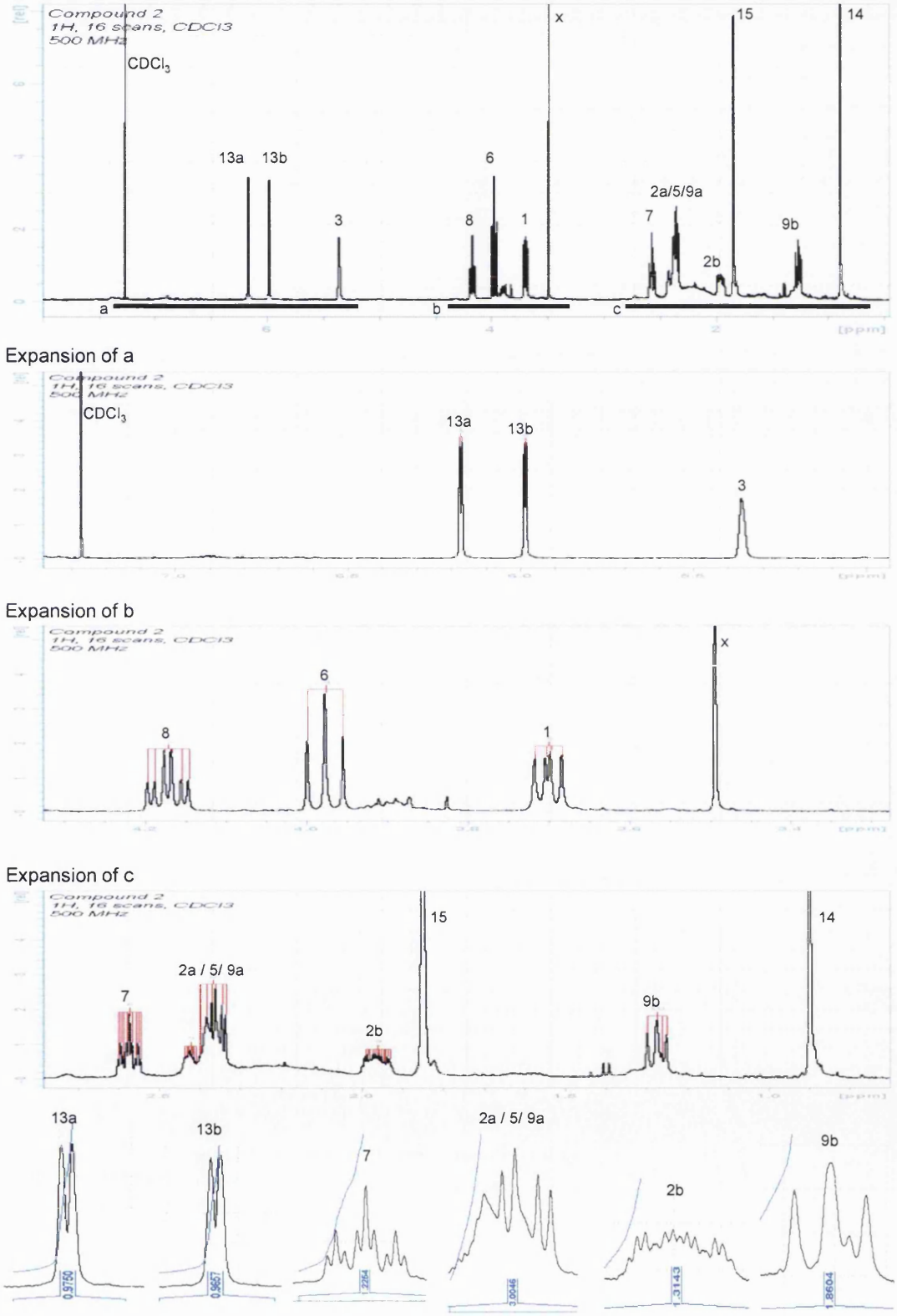


Figure 3.37. $^{13}C$ NMR (black) and DEPT135° (blue) spectra of compound **2** ($CDCl_3$, 125 MHz).

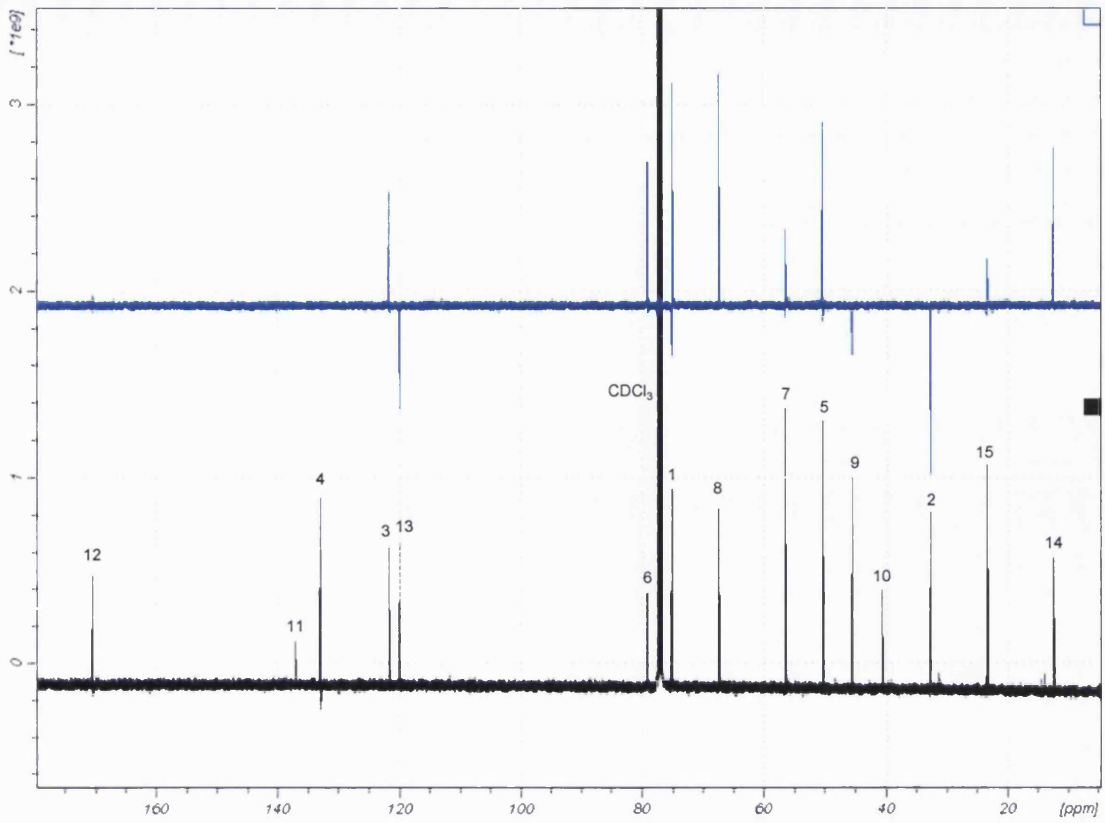
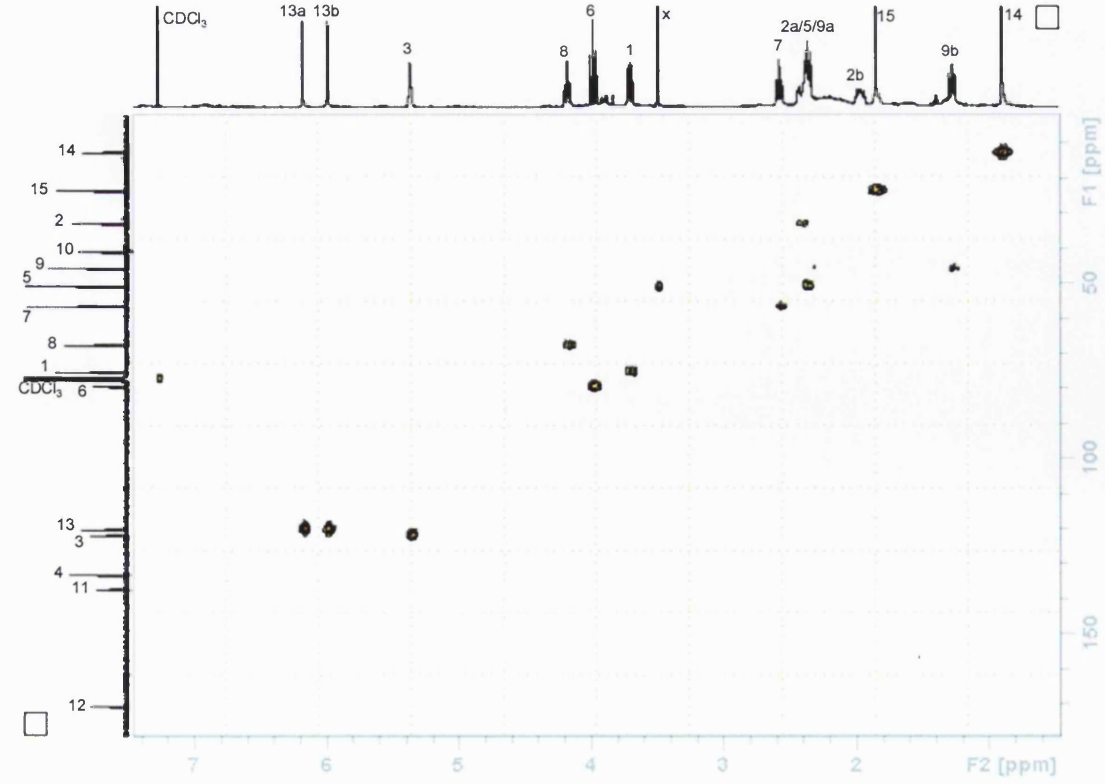



Figure 3.38. HMQC spectrum of compound **2** ($CDCl_3$, 500 MHz/125 MHz).



In the COSY spectrum (Fig. 3.39) three proton spin systems were detected. The first spin system started with H-5 (δ 2.35) which showed a homonuclear cross-peak to the hydroxylated H-6 (δ 3.98), which in turn coupled with H-5 and H-7 (δ 2.57). Proton H-7 showed coupling with H-6 and H-8 (δ 4.17). For H-8 homonuclear cross-peaks were observed with H-7, H-9a (δ 2.35) and H-9b (δ 1.27), completing fragment A (Fig. 3.40). The second spin system started with H-1(δ 3.70) which showed a homonuclear cross-peak to H-2a (δ 2.35) and H-2b (δ 1.96), which in turn showed coupling to H-1 and H-3 (δ 5.36) (fragment B). The methylene protons H-13a (δ 6.17) and H-13b (δ 5.99) represent an isolated third spin system (fragment C).

Figure 3.39. COSY spectrum of compound **2** ($CDCl_3$, 500 MHz).

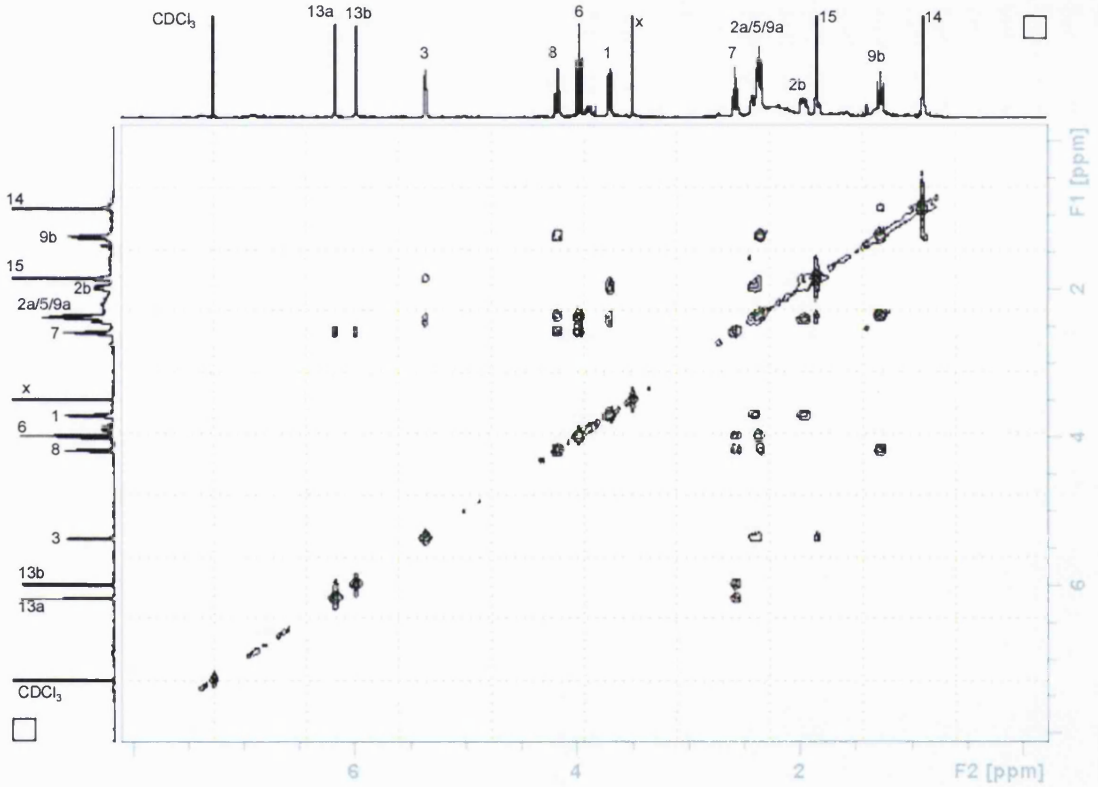
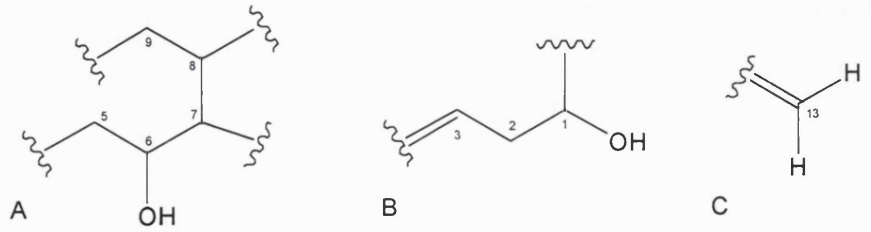



Figure 3.40. Fragments of compound **2** deducted by COSY.



To connect the fragments obtained by the COSY experiment and determine the planar structure of compound **2**, the HMBC spectrum (Fig. 3.42) played a crucial role. The H-13a proton showed a $^{2}J$ correlation to the quaternary carbon C-11 (δ 137.0) and a $^{3}J$ correlation to the carbonyl carbon C-12. Both H-13 protons show $^{3}J$ correlations to the methine carbon C-7, placing the lactone moiety between C-7 and C-8. The methyl group H-14 showed a $^{2}J$ correlation to the quaternary carbon C-10 (δ 40.5) and $^{3}J$ correlations to C-5, C-9 and C-1, thus fragments A and B could be connected. The HMBC correlations between the olefinic methyl group H-15 and the olefinic quaternary carbon C-4 (δ 132.9), the olefinic methine carbon C-3 (δ 121.6) and the methine carbon C-5 (δ 50.4) complete the decalin ring, completing the planar structure of a tricyclic sesquiterpene lactone with one double bond between C-3 and C-4 (Fig. 3.41).

Figure 3.41. Gross structure of compound **2**.

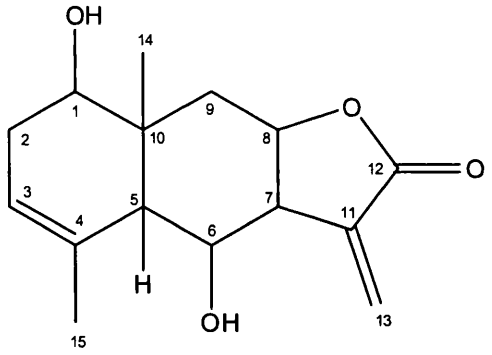


Figure 3.42. HMBC spectrum of compound **2** ($CDCl_3$, 500 MHz/125 MHz).

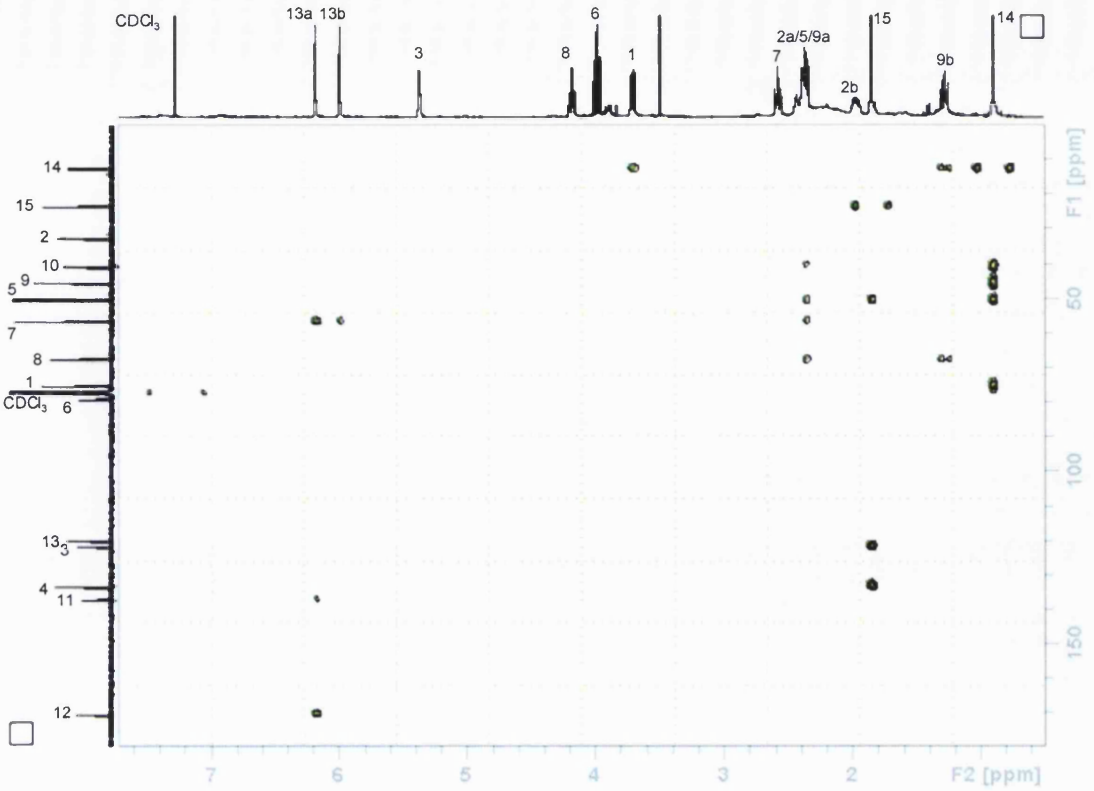



Table 3.14. $^{1}H$ NMR and $^{13}C$ NMR data and $^{1}H$-$^{13}C$ long-range (HMBC) correlations and couplings from COSY for compound **2** ($CDCl_3$, 500 MHz/125 MHz). Chemical shifts δ are reported in ppm, *J* values in Hz.

| | $^{1}H$ | $^{13}C$ | COSY | $^{2}J$ | $^{3}J$ |
|---|---|---|---|---|---|
| 1 | 3.70 *dd* (6.7, 9.9) | 75.1 *d* | H-2a, H-2b | | C-14 |
| 2a | 2.35 *m* | 32.6 *t* | H-1, H-2b, H-3 | | C-10 |
| 2b | 1.96 *m* | | H-1, H-2a | | |
| 3 | 5.36 *s* | 121.6 *d* | H-2a, H-2b | | |
| 4 | | 132.9 *s* | | | |
| 5 | 2.35 *m* | 50.4 *d* | H-6, H-15 | C-10 | C-7 |
| 6 | 3.98 *t* (11.1) | 79.1 *d* | H-5, H-7 | | |
| 7 | 2.57 *tt* (3.1, 10.6) | 56.5 *d* | H-6, H-8 | | |
| 8 | 4.17 *td* (4.5, 10.5) | 67.4 *d* | H-7, H-9a, H-9b | | |
| 9a | 2.35 *m* | 45.5 *t* | H-8, H-9b | C-8, C-10 | C-5, C-7 |
| 9b | 1.27 *m* | | H-8, H-9a, H-14 | C-8 | C-14 |
| 10 | | 40.5 *s* | | | |
| 11 | | 137.0 *s* | | | |
| 12 | | 170.4 *s* | | | |
| 13a | 6.17 *d* (3.1) | 119.9 *t* | H-7 | C-11 | C-7, C-12 |
| 13b | 5.99 *d* (2.9) | | H-7 | | C-7 |
| 14 | 0.9 *s* | 12.3 *q* | H-9b | C-10 | C-1, C-5, C-9 |
| 15 | 1.85 *s* | 23.5 *q* | H-3, H-5 | C-4 | C-3, C-5 |

The relative stereochemistry of compound **2** was determined through NOE correlations observed in the NOESY spectrum (Fig. 3.44). Spatial couplings were observed between H-14 and H-6 and H-8, indicating these protons to be on the same side, *i.e.* β. This left the stereochemistry of H-1, H-5 and H-7 to be determined. No NOE correlation was detected between H-1 and H-7 due to the spatial distance between these protons. Thus, H-5 is a crucial proton in the assignment for the relative stereochemistry, but the overlapping of the proton signal for H-5 with H-2a and H-9a in $CDCl_3$ complicated the task. Thus, to determine the relative stereochemistry of H-1, H-5 and H-7 and confirm the relative stereochemistry in the remaining three chiral centres, NMR experiments were repeated in benzene-$d_6$ ($^1$H NMR spectrum in Fig. 3.45). (The assignment of the protons to the peaks was done by analysis of the COSY spectrum (Fig. 3.46) and comparison of the multiplicity of the proton signals, as the solubility of compound **2** in benzene-$d_6$ was problematic and no heteronuclear spectra could be recorded.)

In benzene-$d_6$ the proton peak for H-5 was clearly separated from H-9a and H-2a (Fig. 3.47), and NOE couplings were observed between H-5 and H-1 and H-7. Thus, H-5 was readily assigned to be positioned α. NOE correlations between H-14 and H-6 and H-8 further confirmed that these protons are on the opposite side of the molecule, resulting in the relative stereochemistry shown in Figure 3.43.

Figure 3.43. Structure of compound **2**.

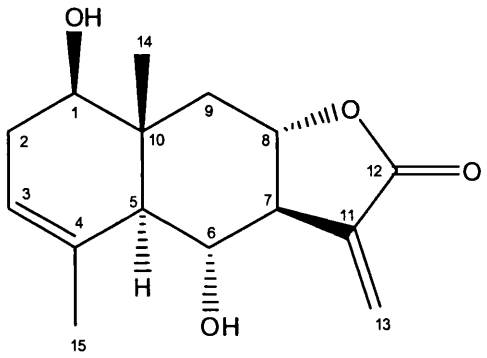


Figure 3.44. NOESY spectrum of compound **2** ($CDCl_3$, 500 MHz).

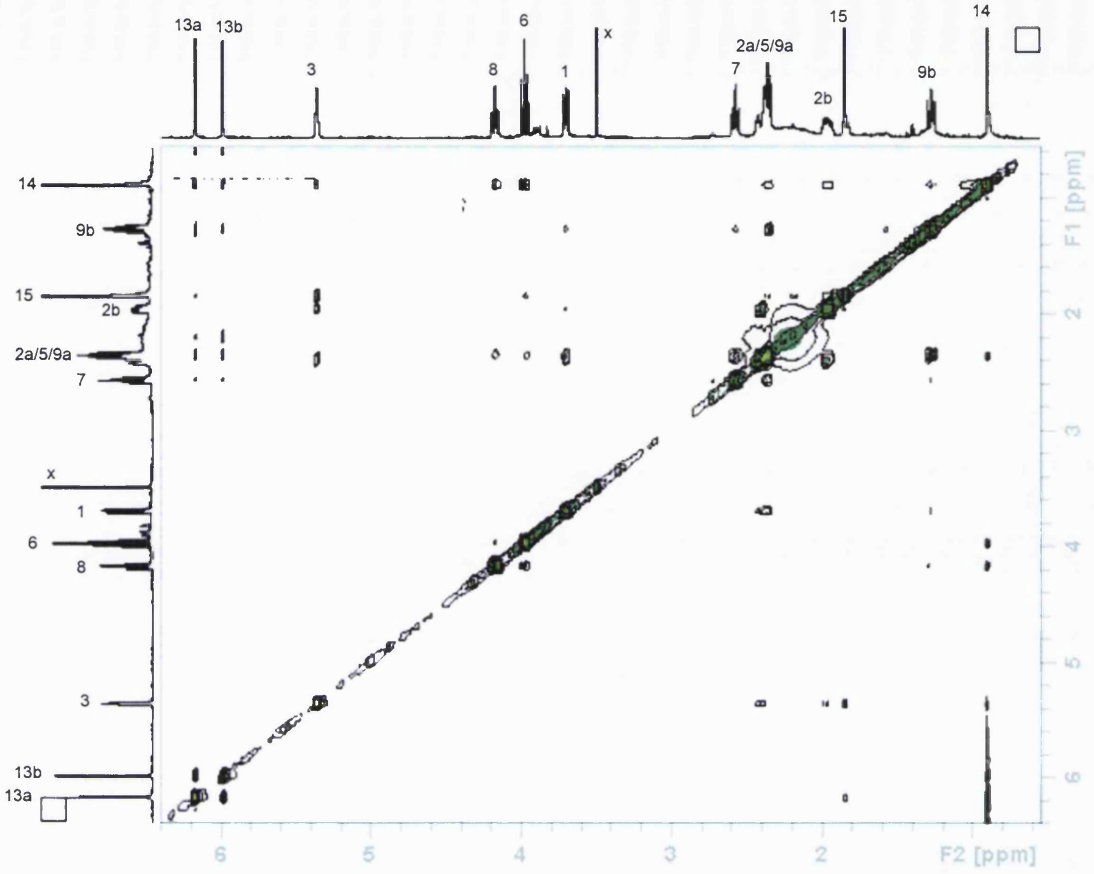
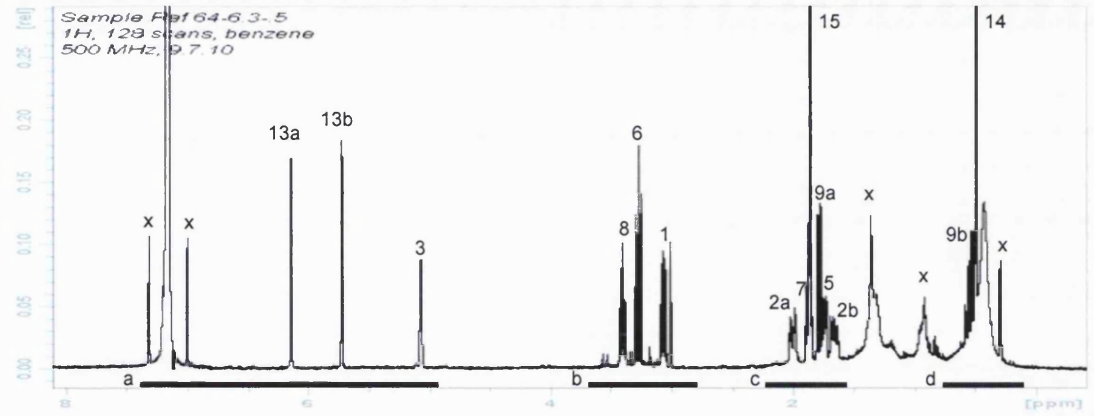
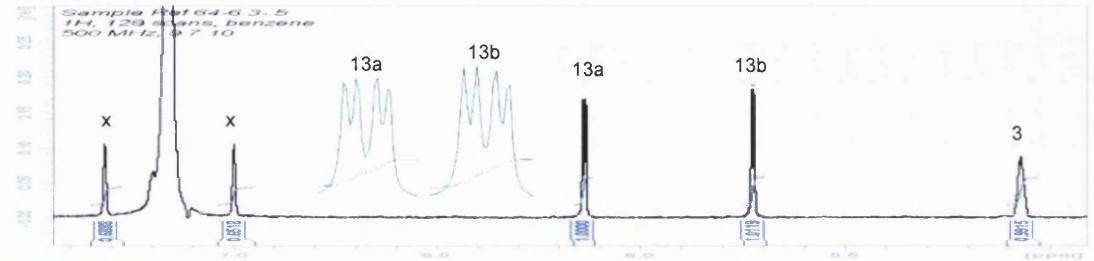


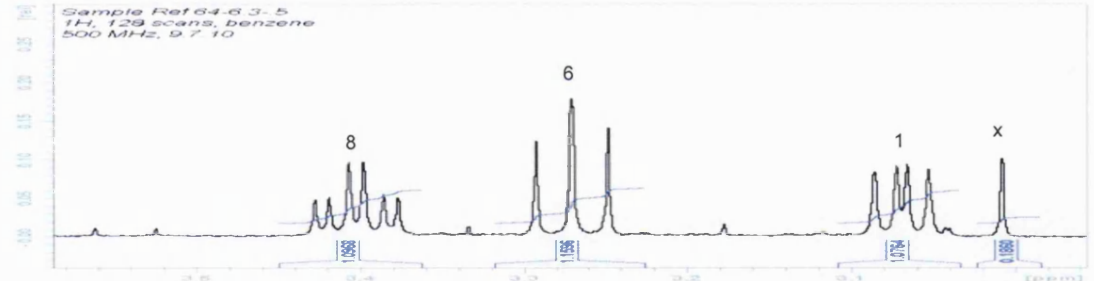
Figure 3.45. $^{1}$H NMR spectrum of compound **2** (benzene-$d_6$, 500 MHz). X: impurity or ghost peaks



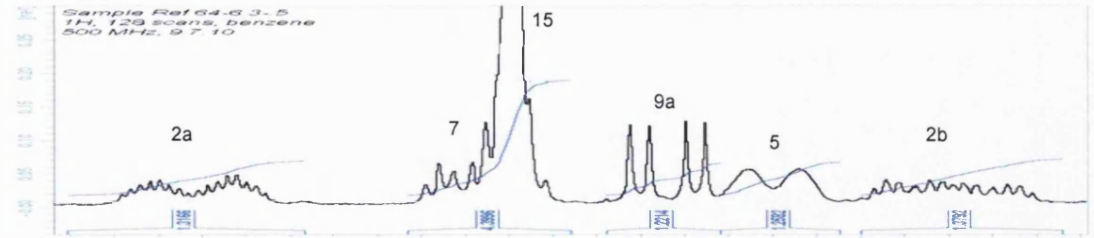

Expansion of a




Expansion of b




Expansion of c




Expansion of d

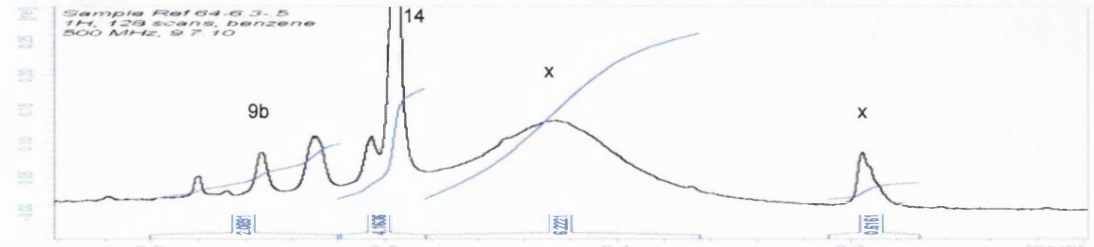


Figure 3.46. COSY spectrum of compound **2** (benzene-$d_6$, 500 MHz)

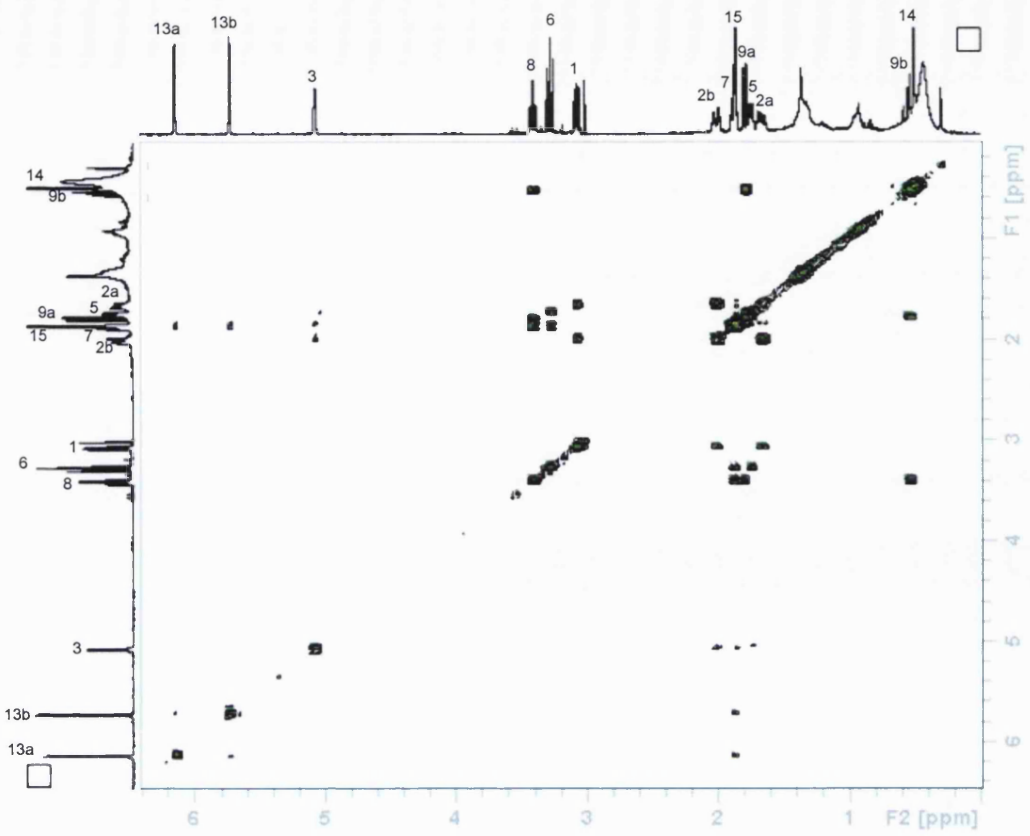
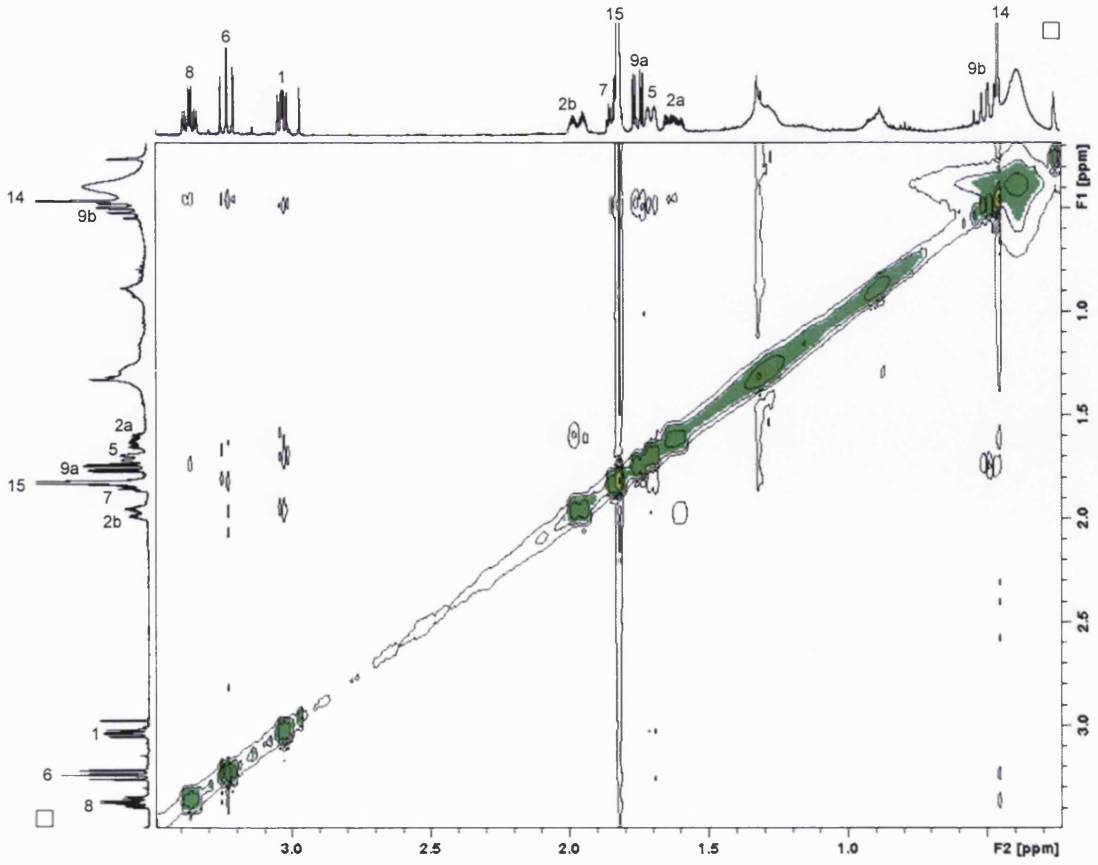



Figure 3.47. NOESY spectrum of compound **2** (benzene-$d_6$, 500 MHz).



A literature search revealed that compound **2** has the same gross structure and relative stereochemistry as sivasinolide, which has been first isolated from *Tanacetum densum* subsp. *sivasicum* (Asteraceae) (Goeren *et al.*, 1992) (Fig. 3.48). In addition, altissin, which only differs in the relative stereochemistry at C-1 and C-14, has been isolated from *Anthemis altissima* (Konstantinopoulou *et al.*, 2003). The spectral data of compound **2** were compared to sivasinolide and altissin and are presented in Table 3.15.

Figure 3.48. Structures of compound **2**, sivasinolide and altissin.

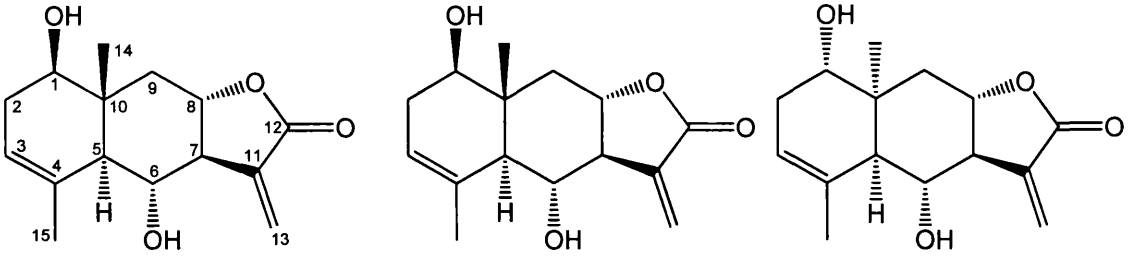



Table 3.15. One-dimensional NMR data for compound **2** ($CDCl_3$, 500 MHz/125 MHz) compared with altissin (Konstantinopoulou *et al.*, 2003) and sivasinolide (Goeren *et al.*, 1992) (measured in $CDCl_3$). Chemical shifts δ are reported in ppm, *J* values in Hz.

| | **Compound 2** | | **Altissin**[1] | | **Sivasinolide**[2] | |
|---|---|---|---|---|---|---|
| | **$^1$H** | **$^{13}$C** | **$^1$H** | **$^{13}$C** | **$^1$H** | **$^{13}$C** |
| 1 | 3.70 *dd* (6.7, 9.9) | 75.1 *d* | 3.78 *dd* (8.9, 6.4) | 66.4 *d* | 3.73 *dd* | 77.6 *d*[a] |
| 2a | 2.35 *m* | 32.6 *t* | 2.44 *m* | 33.3 *t* | | 40.2 *t*[b] |
| 2b | 1.96 *m* | | 1.95 *m* | | | |
| 3 | 5.36 *s* | 121.6 *d* | 5.40 *s* | 121.5 *d* | 5.40 *m* | 123.0 *d* |
| 4 | | 132.9 *s* | | 137.5 *s* | | 140.2 *s* |
| 5 | 2.35 *m* | 50.4 *d* | 1.85 *d* (9.6) | 58.6 *d* | 2.09 *d* | 57.0 *d* |
| 6 | 3.98 *t* (11.1) | 79.1 *d* | 3.88 *t* (9.8) | 75.1 *d* | 4.07 *dd* | 75.7 *d*[a] |
| 7 | 2.57 *tt* (3.1, 10.6) | 56.5 *d* | 2.57 *tt* (11.0, 2.9) | 54.9 *d* | 2.51 *m* | 54.7 *d* |
| 8 | 4.17 *td* (4.5, 10.5) | 67.4 *d* | 4.03 *dt* (3.8, 12.1) | 70.6 *d* | 4.07 *dd* | 69.8 *d*[a] |
| 9a | 2.35 *m* | 45.5 *t* | 2.76 *dd* (12.4, 3.8) | 36.9 *t* | 2.51 *dd* | 39.9 *t*[b] |
| 9b | 1.27 *m* | | 1.37 *t* (12.4) | | 1.45 *dd* | |
| 10 | | 40.5 *s* | | 40.6 *s* | | 41.6 *s* |
| 11 | | 137.0 *s* | | 134.0 *s* | | 135.5 *s* |
| 12 | | 170.4 *s* | | 170.4 *s* | | 169.9 *s* |
| 13a | 6.17 *d* (3.1) | 119.9 *t* | 6.13 *d* (3.2) | 120.0 *t* | 6.18 *d* | 118.3 *t* |
| 13b | 5.99 *d* (2.9) | | 5.91 *d* (3.2) | | 5.95 *d* | |
| 14 | 0.90 *s* | 12.3 *q* | 0.98 *s* | 20.2 *q* | 0.99 *s* | 13.1 *q* |
| 15 | 1.85 *s* | 23.5 *q* | 1.91 *s* | 26.2 *q* | 1.95 *s* | 24.0 *q* |

[1] recorded on Bruker DRX 400 (400 MHz) for $^1$H NMR and Bruker AC 200 (100 MHz) for $^{13}$C NMR, [2]manufacturer not specified (200 MHz for $^1$H NMR, 100 MHz for $^{13}$C NMR)

[a,b] interchangeable

The comparison of the NMR data of compound **2** with sivasinolide and altissin showed overall many similarities, but significant differences were observed in the chemical shift values at positions C-4, C-5, C-6 and C-9. In addition, considerable discrepancies were found in the $^{13}C$ NMR data between compound **2** and sivasinolide at position C-2, and between compound **2** and altissin at positions C-1 and C-14. Major differences in the $^{1}H$ NMR spectrum were observed for H-5 and H-9.

The $^{13}C$ NMR data for the quaternary carbon atoms, *i.e.* C-4 and C-11, in compound **2** merit some discussion. In the published literature, C-4 was assigned to the chemical shift values at δ 137.5 (altissin) and δ 140.2 (sivasinolide), which is similar to C-11 (δ 137.0) in compound **2**; C-11 was assigned to δ 134.0 (altissin) and δ 135.5 (sivasinolide), which is similar to C-4 (δ132.9) in compound **2**. The unmistakable HMBC correlations between C-4 (132.9 ppm) and H-15 and between C-11 (137.0 ppm) and H-13a confirmed the NMR data the assignment of C-4 and C-11 in compound **2** was correct, hence the NMR data of C-4 and C-11 might be incorrect assigned in the literature.

The specific rotation $[\alpha]^{D}_{22}$ for compound **2** was found to be +70 (c= 0.1, $CHCl_3$), which differs greatly from the published specific rotation of altissin with -10.5 (c= 0.15, $CHCl_3$). Unfortunately no specific rotation has been published for sivasinolide so far.

The considerable differences in the NMR data between compound **2** and sivasinolide prompt serious ambiguity in structural elucidation of sivasinolide. Several other authors have isolated sivasinolide and identified it by the comparison of the published literature data from Goeren *et al.* (1992) without a detailed investigation of the NMR data (Konstantinopoulou *et al.*, 2003; Triana *et al.*, 2003; Pillay *et al.*, 2007).

A single crystal X-ray diffraction experiment is currently being performed to conclusively confirm the suggested relative stereochemistry of compound **2**.

### 3.3.5.3 Structure elucidation of compound 3: Benzoic acid derivative

Compound **3** was isolated as a white-yellowish powder from the VLC fraction 2b of the aq. MeOH subextract as described in 3.3.4.1.

TLC analysis on a silica gel 60 $F_{254}$ plate showed UV quenching activity at 254 nm and staining with vanillin-sulphuric acid resulted in a bright pink spot (Fig. 3.49). The (-)-ESI-MS spectrum (Fig. 3.50) contained an intense molecular ion peak at $m/z$ 329.59 $[2M-2H+Na]^-$ and a second molecular ion peak at $m/z$ 153.64 $[M-H]^-$, which suggested the molecular formula $C_7H_6O_4$ with a calculated molecular weight of $m/z$ 154.12. High resolution mass spectrometry (accurate mass) gave a theoretical molecular weight of $m/z$ 153.0188 $[M-H]^-$ which resulted with the measured mass value of $m/z$ 153.0196 in an absolute mass error of Δ +0.8 mmu and a relative error of δ 5 ppm.

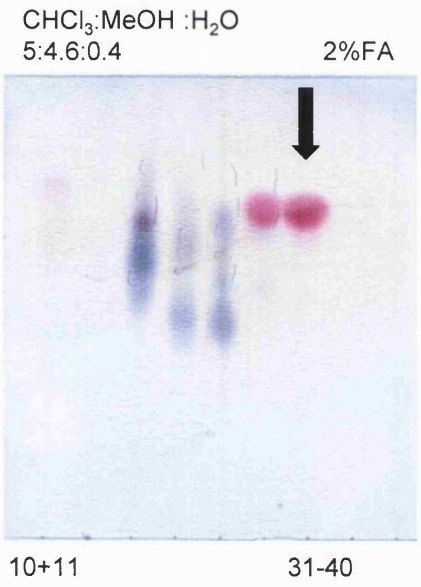



Figure 3.49. TLC analysis of compound **3**. Stationary phase: silica gel 60 $F_{254}$, staining agent: vanillin/sulphuric acid (heated for 1 min at 170°C).

Figure 3.50. (-)-ESI-MS spectrum of compound **3**.

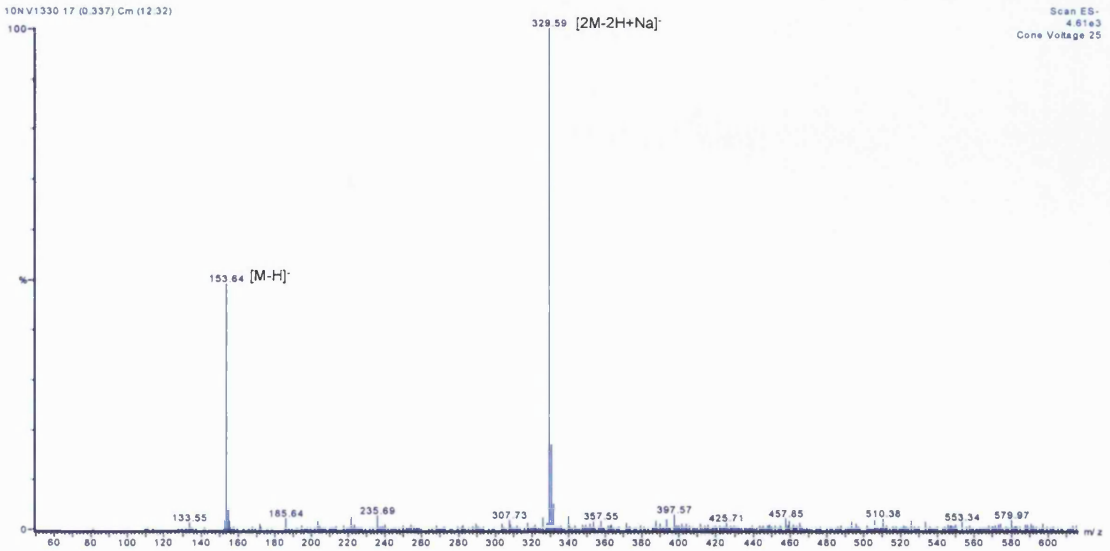
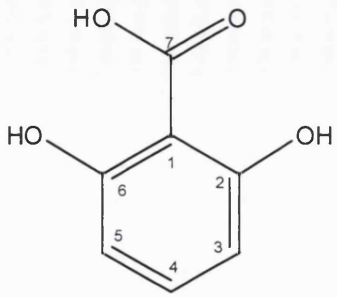


Figure 3.51. Structure of compound **3**.




The $^{1}H$ NMR and $^{13}C$ NMR spectra show that compound **3** is a very simple compound (Fig. 3.51). The $^{1}H$ spectrum in MeOD (Fig. 3.52) showed two peaks at δ 6.17 (H-3, H-5, d, $J$ = 8.2) and δ 7.00 (H-4, t, $J$ = 8.2), which indicated the presence of a phenyl ring. Integration of the proton peaks showed two protons in peak δ 6.17, suggesting chemically equivalent protons. The COSY spectrum (Fig. 3.53) confirmed the presence of only one spin system within compound **3** with homonuclear cross-peaks for H-4 and H-3/H-5.

Figure 3.52. $^{1}H$ NMR spectrum of compound **3** with expansions of peaks (MeOD, 400 MHz).

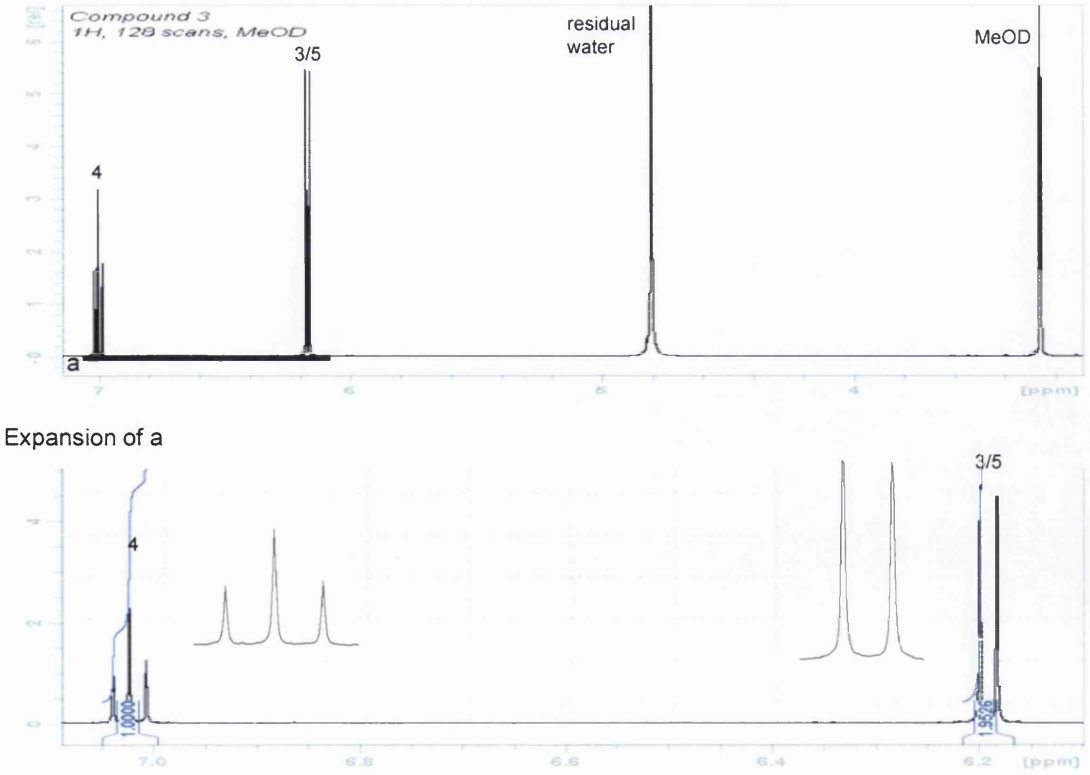
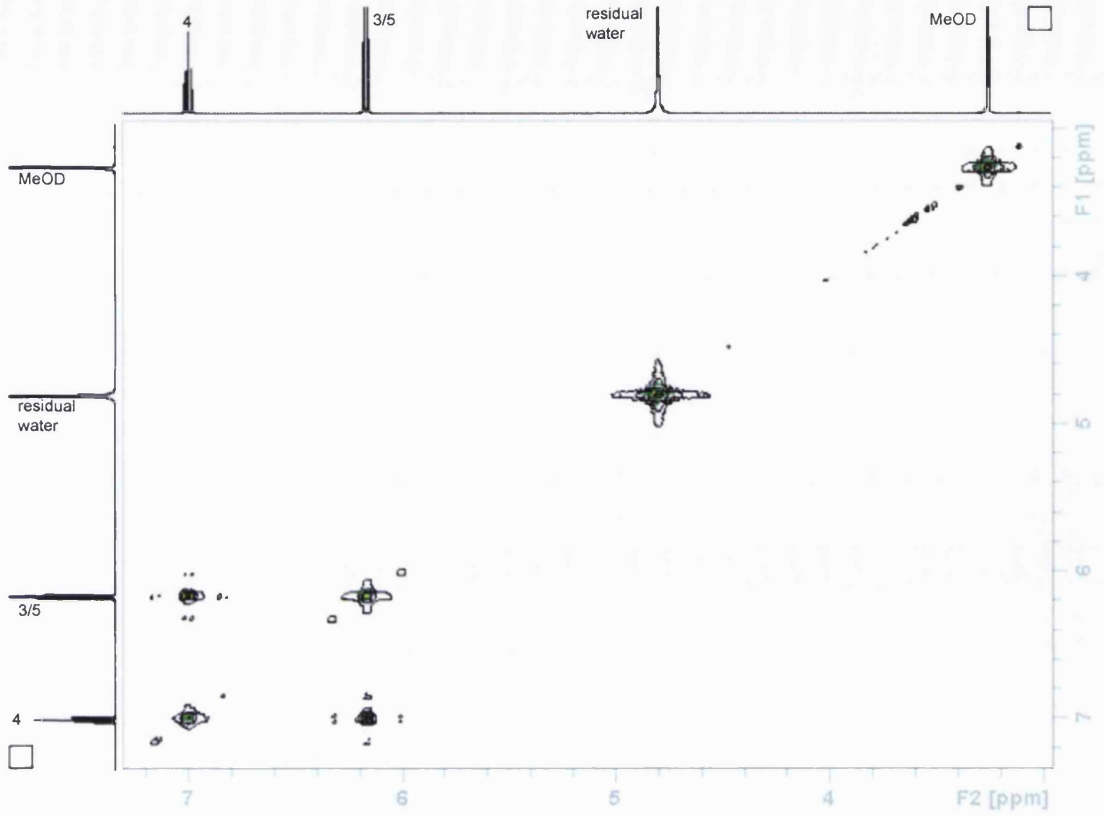
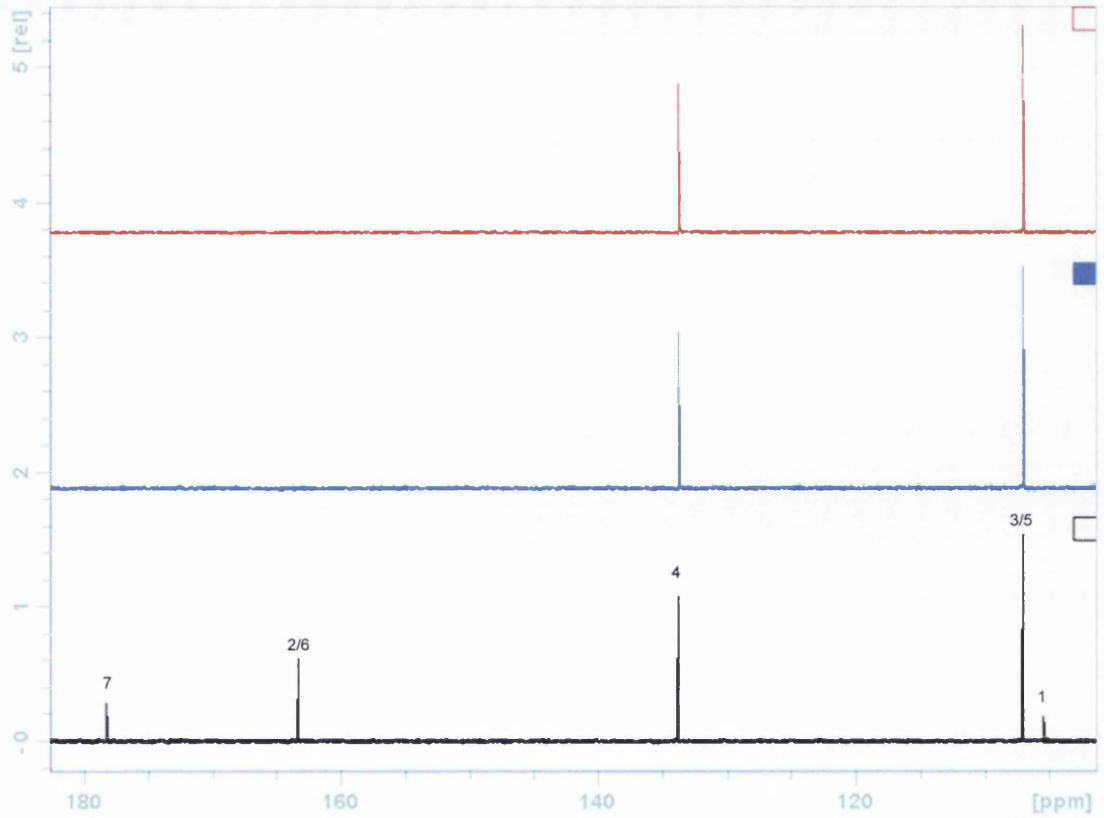


Figure 3.53. COSY spectrum of compound **3** (MeOD, 500 MHz).




The $^{13}C$ spectrum in MeOD (Fig. 3.54) showed 5 signals. Inspection of the DEPT spectra revealed the presence of in total three aromatic methine carbons, two at δ 107.0 (C-3/C-5) and one at δ 133.7 (C-4) which were assigned to the protons H-3/H-5 and H-4 in the phenyl ring by analysis of the HMQC and COSY spectra (Fig. 3.55 a and 3.53). In addition, the $^{13}C$ NMR spectrum showed three quaternary carbons, of which δ 178.2 was readily assigned to a carboxyl group (C-7). The chemical shift of C-2 and C-6 (δ 163.3) indicated the substitution with hydroxyl groups on these carbons. The quaternary C-1 was assigned to δ 105.3. The observation of four peaks in the $^{13}C$ NMR spectrum for the phenyl ring instead of six, suggested compound **3** to be a symmetric compound with four chemically equivalent carbons (C-2/C-6 and C-3/C-5).

Figure 3.54. $^{13}C$ NMR (black), DEPT135° (blue) and DEPT90° (red) spectra of compound **3** (MeOD, 125 MHz).




The HMBC spectrum (Fig. 3.55 b) revealed $^{3}J$ correlations between H-4 and C-2/C-6 and a weak long-range correlation between H-4 and C-1. For H-5/H-3 $^{2}J$ correlations to C-2/C-6, $^{3}J$ correlations to each other and C-1 and a weak correlation to C-7 were observed. This led to the proposal that compound **3** is 2,6-dihydroxybenzoic acid.

To confirm the proposed structure of 2,6-dihydroxybenzoic acid, the coupling constants and multiplicity of the protons were examined. The proton signal at δ 6.17 (H-3, H-5) split into a doublet with a coupling constant of 8.2 Hz. Proton H-4 (δ 7.00) coupled with H-3 and H-5 which resulted in a triplet and a coupling constant of 8.2 Hz. The $J_{3,4}$ and $J_{4,5}$ values indicated the *ortho* coupling of H-4 with both H-3 and H-5. The expected doublet of doublets for protons H-3 and H-5 and additional coupling constant for the *meta* substitution between these two protons were too small to be detected.

Comparison of the 1D NMR data of compound **3** (Tab. 3.16) with those published in the literature (Soares-Santos *et al.*, 2003) further confirmed that compound **3** is 2,6-dihydroxybenzoic acid.

Figure 3.55. HMQC (a) and HMBC (b) spectra of compound **3** (MeOD, 500 MHz/125 MHz).

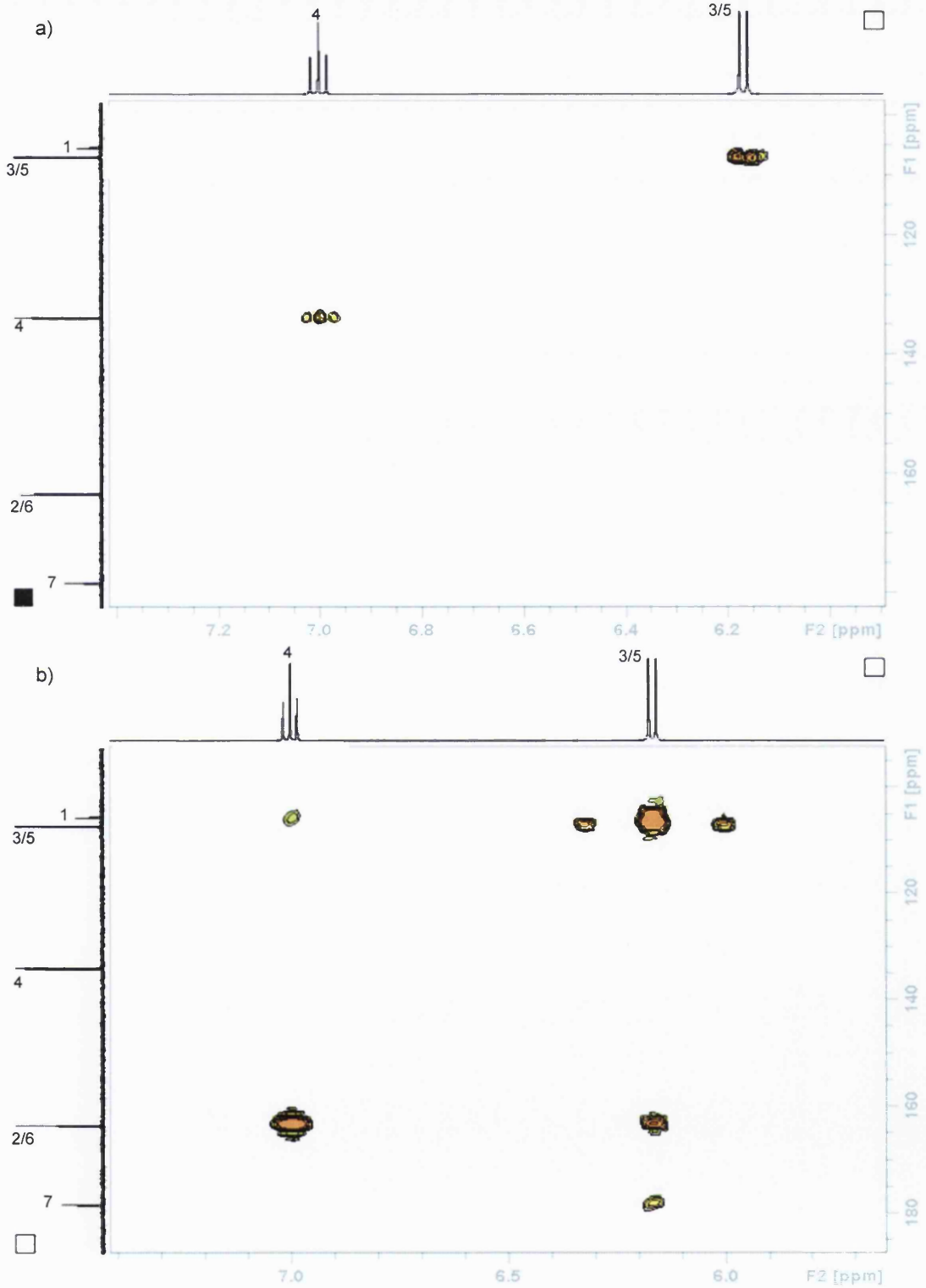


Table 3.16. $^{1}H$ NMR, $^{13}C$ NMR data and $^{1}H$-$^{13}C$ long-range (HMBC) correlations for compound **3** (MeOD, 500 MHz/125 MHz). [1] refers to the published data for 2,6-dihydroxybenzoic acid in DMSO-$d_6$ taken from Soares-Santos *et al.* (2003). Chemical shifts δ are reported in ppm, *J* values in Hz.

| Position | $^{1}H$ | $^{13}C$ | $^{2}J$ | $^{3}J$ | $^{1}H$ [1] | $^{13}C$ [1] |
|---|---|---|---|---|---|---|
| 1 | | 105.3 *s* | | | | 102.6 |
| 2 | | 163.3 *s* | | | | 160.7 |
| 3 | 6.17 *d* (8.2) | 107.0 *d* | C-2 | C-1, C-5, C-7 | 6.32 *d* | 106.7 |
| 4 | 7.00 *t* (8.2) | 133.7 *d* | C-3, C-5 | C-2, C-6 | 7.20 *t* | 134.3 |
| 5 | 6.17 *d* (8.2) | 107.0 *d* | C-6 | C-1, C-3, C-7 | 6.32 *d* | 106.7 |
| 6 | | 163.3 *s* | | | | 160.7 |
| 7 | | 178.2 *s* | | | | 172.6 |

### 3.3.5.4 Structure elucidation of compound 4: N-glucosylated indole alkaloid

Compound **4** was isolated as a white oil from the VLC fraction 2b of the aq. MeOH subextract as described in 3.3.4.1.

Development of this compound on a silica gel 60 $F_{254}$ TLC plate showed UV quenching activity and a bright purple band after vanillin/sulphuric acid staining (Fig. 3.56). The (+)-ESI-MS spectrum (Fig. 3.57) showed a molecular ion peak at $m/z$ 360.5 $[M+Na]^+$. The uneven molecular weight suggested the presence of a nitrogen atom in the molecule and the molecular formula $C_{16}H_{19}NO_7$ with a calculated molecular weight of $m/z$ 337.32.

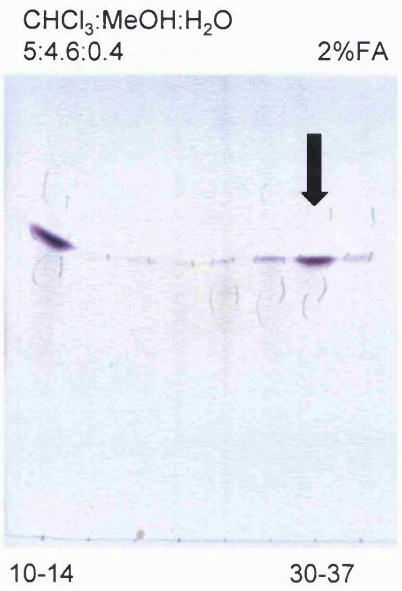



Figure 3.56. TLC analysis of compound **4**. Stationary phase: silica gel 60 $F_{254}$, staining agent: vanillin/sulphuric acid (heated for 1 min at 170°C).

Figure 3.57. (+)-ESI-MS spectrum of compound **4**.

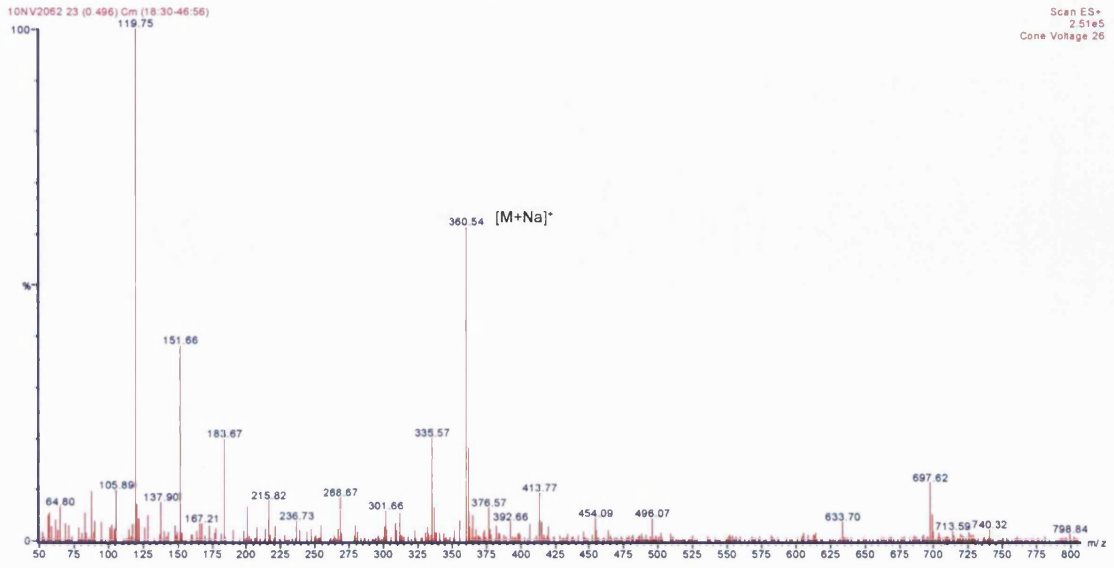
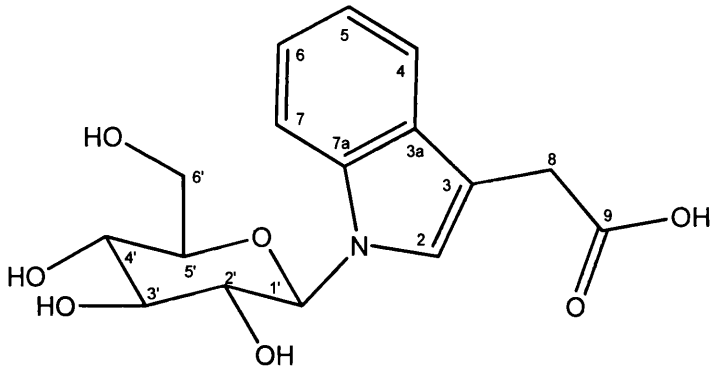


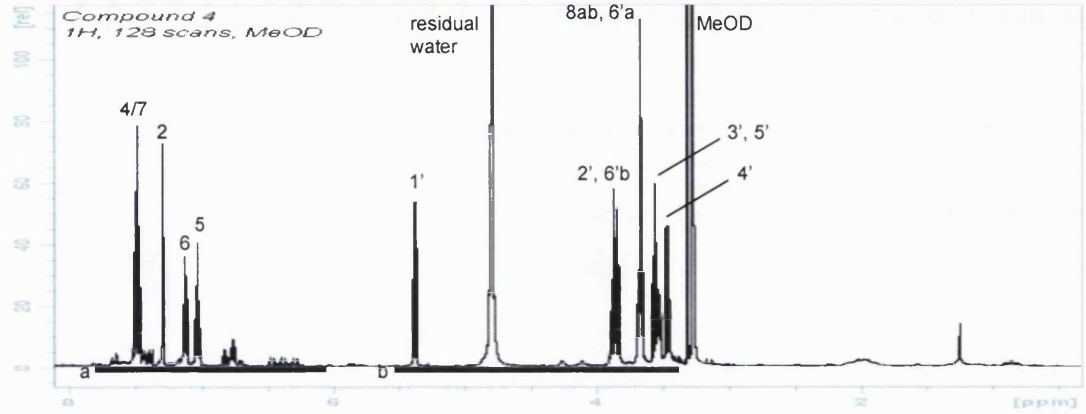
Figure 3.58. Structure of compound **4**.



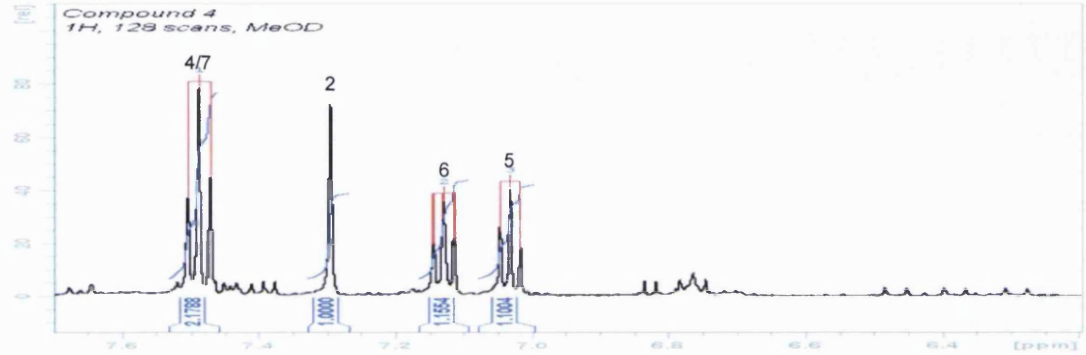

The $^{13}C$ NMR spectrum of compound **4** in MeOD (Fig. 3.60) showed 15 signals, plus a carboxyl signal retrieved from the HMBC spectrum. In the downfield region of the $^{13}C$ NMR spectrum, five methine groups in the range of δ 111.5 and δ 125.4 and three quaternary carbons between δ 111.2 and δ 138.5 suggested the aromatic nature of the compound. Accordingly, the $^{1}H$ NMR spectrum of compound **4** in MeOD (Fig. 3.59) contained four aromatic signals in the downfield region between δ 7.0 and δ 7.6, which integrated for five protons. The singlet at δ 7.30 (H-2, s) was characteristic for a proton next to a nitrogen atom in a C-3 substituted indole ring (Silverstein & Webster, 1997). This data, together with the four aromatic protons observed at δ 7.03 (H-5, dd, $J$ = 0.8, 7.5 Hz), 7.13 (H-6, dd, $J$ = 1.0, 7.7 Hz) and 7.49 (H-4, H-7, t, $J$ = 8.5 Hz) suggested the presence of a monosubstituted indole ring. The coupling constant of 8.5 Hz for H-4/H-7 signal indicated the *ortho* position of H-4 to H-5 and of H-7 to H-6. The H-5 and H-6 protons appeared as a doublet of doublets with $J$ values ≥ 7.5 Hz, hence had to be *ortho* positioned to one another. Additional weak couplings observed between H-7 and H-5 ($J_{5,7}$ = 0.8 Hz), as well as H-4 and H-6 ($J_{6,4}$ = 1.0 Hz) confirmed their *meta* substitution to each other in the benzene ring.

Further investigation of the $^{13}C$ NMR and DEPT spectra (Fig. 3.60) identified one methylene and five methine carbons with characteristic chemical shift values for β-D-glucose (Agrawal, 1992). In the $^{1}H$ NMR spectrum a signal observed at δ 5.38 (H-1', d, $J$ = 8.7 Hz) was readily assigned to the anomeric sugar proton. The coupling constant of H-1' (8.7 Hz) indicated its β conformation. The unusual chemical shift of C-1' (δ 86.9) was indicative of the attachment of the glucose unit on the nitrogen in the indole ring. In the upfield region of the $^{1}H$ NMR spectrum, four signals were observed between δ 3.4 and δ 4.0, which integrated for eight protons. Examination of the peaks by HMQC (Fig. 3.61) revealed in addition to six residual sugar protons, a highly deshielded methylene group at δ 3.67 (H-8, m), which corresponded to a weak signal in the $^{13}C$ NMR spectrum at δ 32.7 (t).

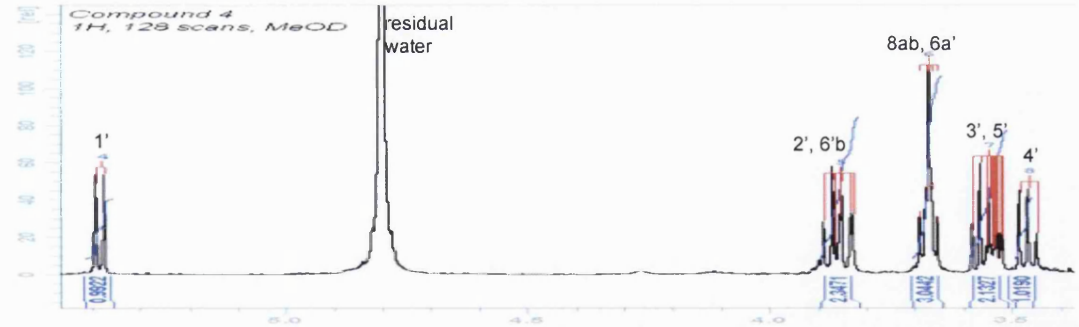
Figure 3.59. $^{1}$H NMR spectrum of compound **4** with expansions of peaks (MeOD, 500 MHz).




Expansion of a




Expansion of b



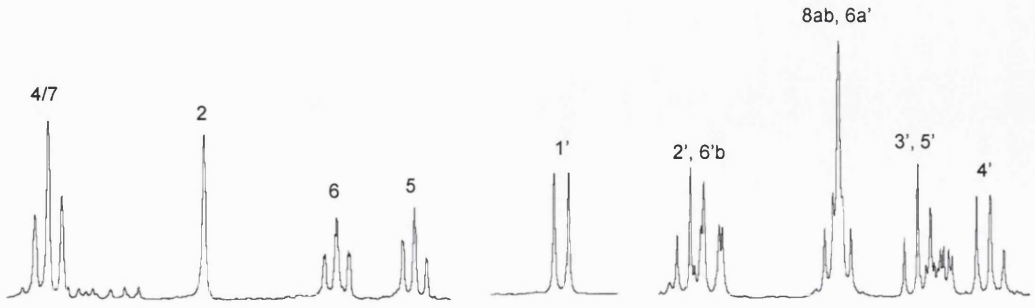



Figure 3.60. $^{13}C$ NMR (black), DEPT135° (blue) and DEPT90° (red) spectra of compound **4** (MeOD, 125 MHz).

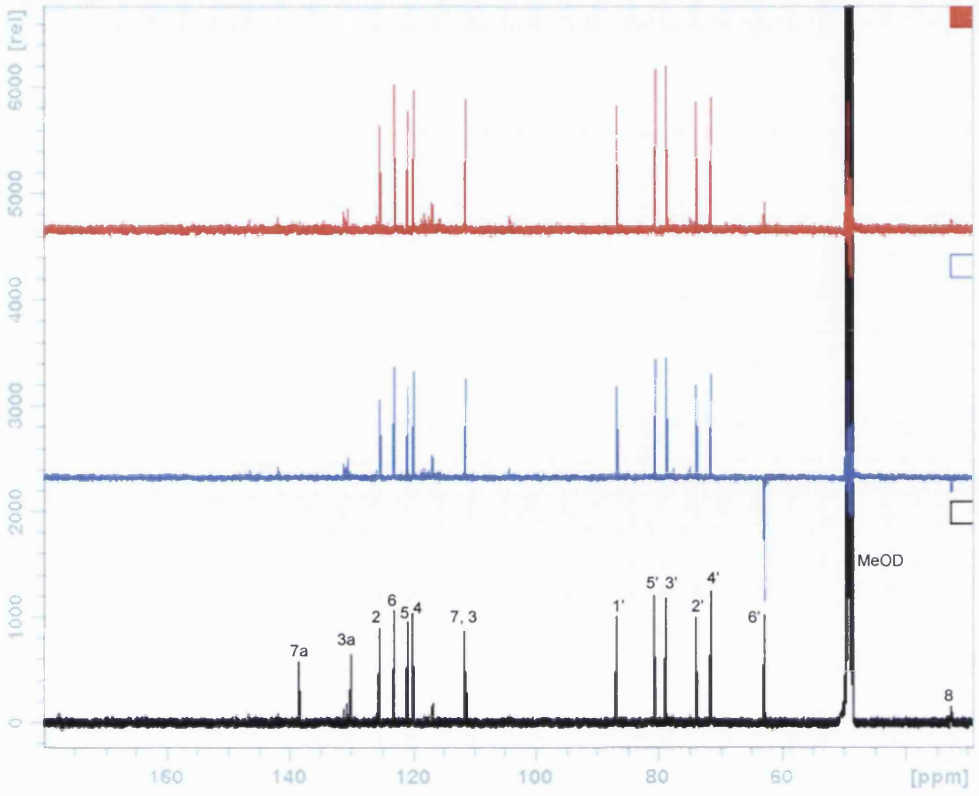
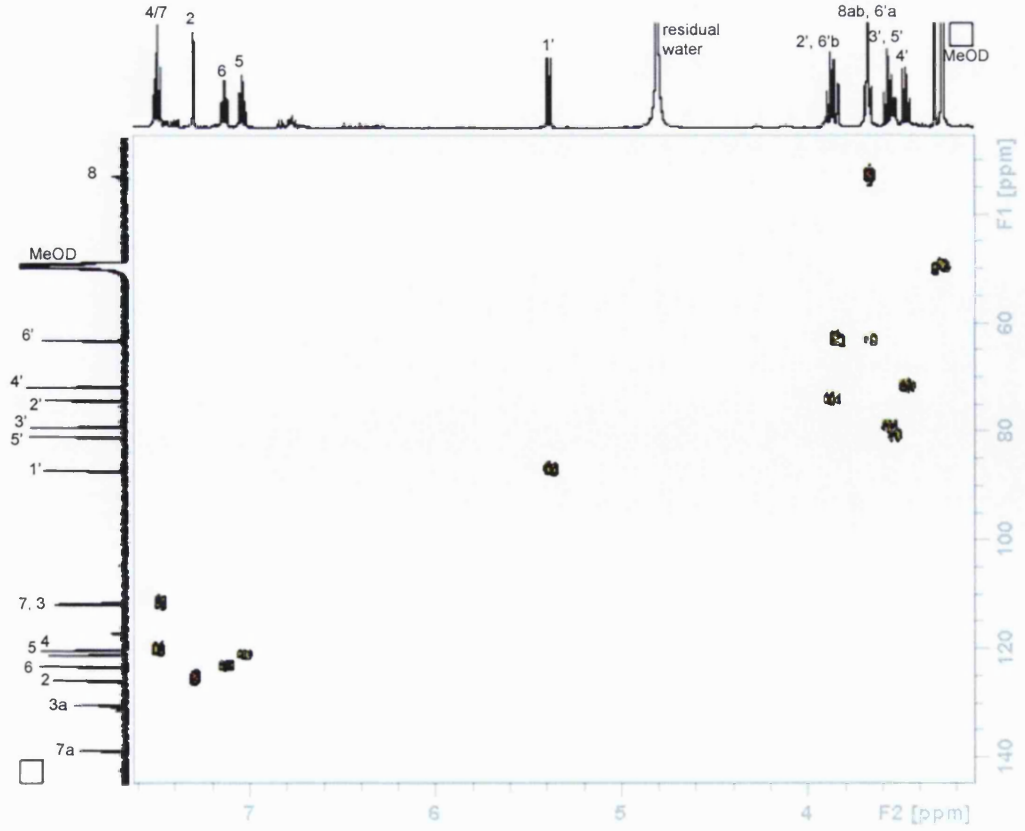
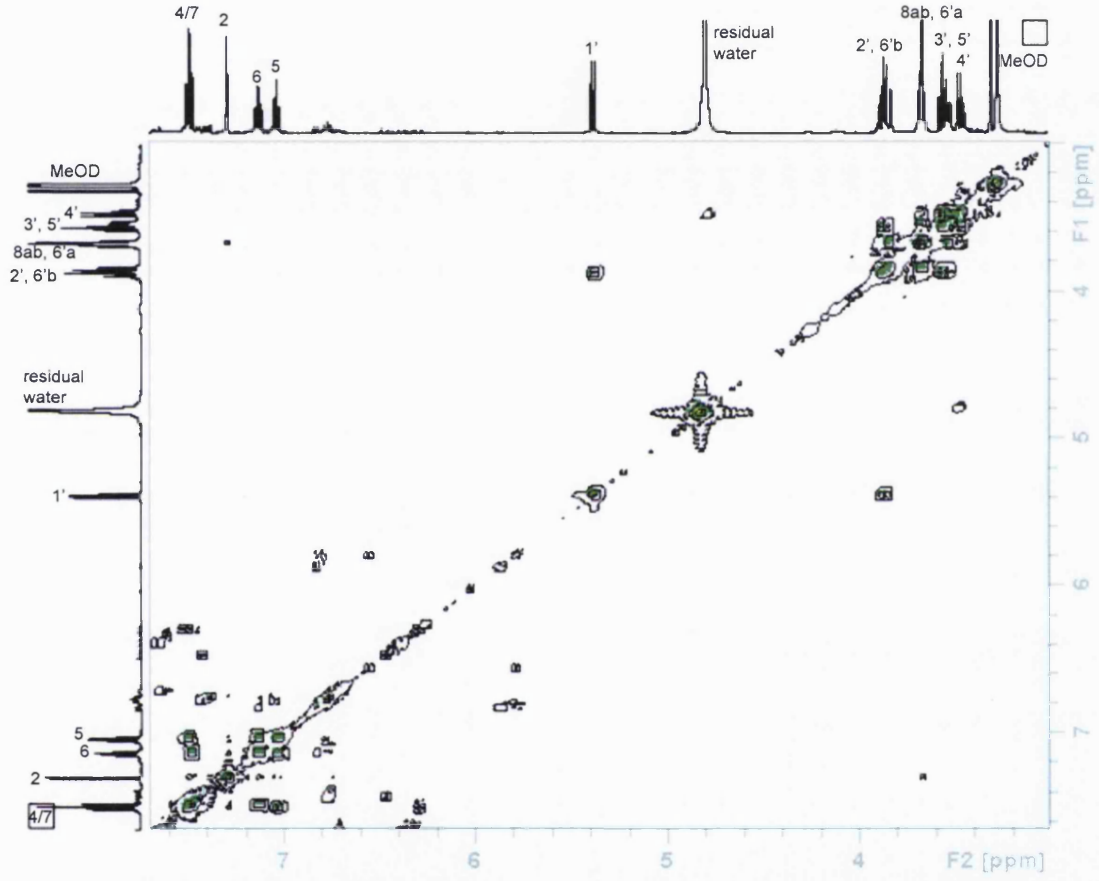



Figure 3.61. HMQC spectrum of compound **4** (MeOD, 500 MHz/125 MHz).



Analysis of the COSY spectrum (Fig. 3.62 and Tab. 3.17) showed the presence of two full proton spin systems, plus two isolated proton systems within compound **4**. The first spin system is composed of four aromatic protons in the benzene ring (H-4 – H-7), the second spin system of the protons in the glucose (H-1' – H-6'). The methylene protons H-8a and H-8b represent an isolated third spin system. The final methine proton H-2, indicated a substitution on the C-3 position of the pyrrole ring.

Figure 3.62. COSY spectrum of compound **4** (MeOD, 500 MHz).




The inspection of the HMBC spectrum (Fig. 3.63 and Tab. 3.17) revealed a $^{2}J$ correlation between H-8 and C-3 (δ 111.2, s) and $^{3}J$ correlations between H-8/C-2 (δ 125.4, d) and H-8/C3a (δ 130.1, s). Additional heteronuclear coupling was observed between H-8 and the carboxyl carbon C-9 at δ 177.7. These observations confirmed the attachment of a carboxymethyl group to the C-3 position in the indole system.

The anomeric sugar proton H-1' showed long-range correlations to C-2 and C-7a, verifying the sugar attachment on the nitrogen of the indole system. All these observations resulted in the structure of 3-carboxymethyl-indole-1-N-β-D-glucopyranoside for compound **4**.

Figure 3.63. HMBC spectrum of compound **4** (MeOD, 500 MHz/125 MHz).

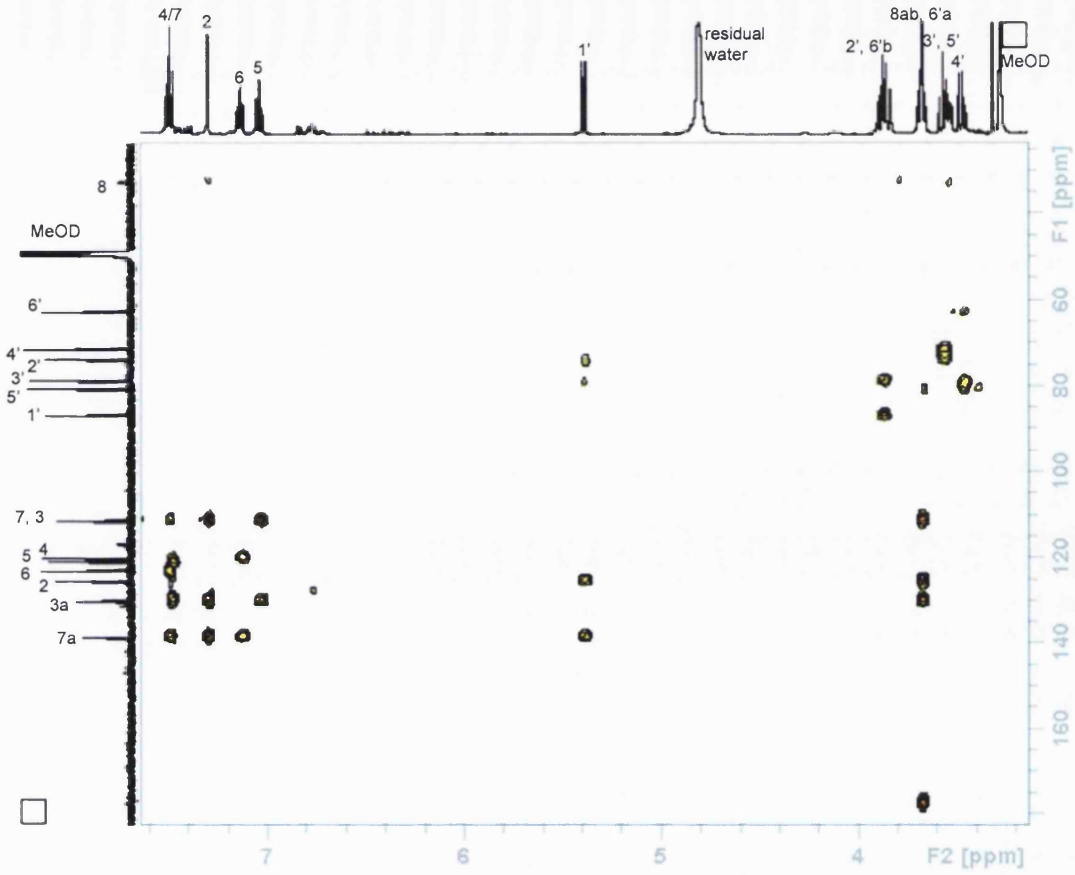



Table 3.17. $^{1}$H NMR and $^{13}$C NMR data and $^{1}$H-$^{13}$C long-range (HMBC) correlations for compound **4** (MeOD, 500 MHz/125 MHz). Chemical shifts δ are reported in ppm, *J* values in Hz.

| | $^{1}$H | $^{13}$C | $^{2}J$ | $^{3}J$ | COSY |
|---|---|---|---|---|---|
| 2 | 7.30 *s* | 125.4 *d* | C-3 | C-3a, C-7a, C-8 | H-8ab |
| 3 | | 111.2 *s* | | | |
| 3a | | 130.1 *s* | | | |
| 4 | 7.49 *t* (8.5) | 120.0 *d* | C-3a, C-5 | C-3 | H-5 |
| 5 | 7.03 *dd* (0.8, 7.5) | 120.9 *d* | | C-3a, C-7 | H-4, H-6 |
| 6 | 7.13 *dd* (1.0, 7.7) | 123.1 *d* | | C-4, C-7a | H-5, H-7 |
| 7 | 7.49 *t* (8.5) | 111.5 *d* | C-6, C-7a | | H-6 |
| 7a | | 138.5 *s* | | | |
| 8ab | 3.67 *m* | 32.7 *t* | C-3, C-9 | C-2, C-3a | H-2 |
| 9 | | 177.7* *s* | | | |
| 1' | 5.38 *d* (8.7) | 86.9 *d* | C-2' | C-7a, C-2, C-3' | H-2' |
| 2' | 3.86 *m* | 74.0 *d* | C-1', C-3' | | H-1', H-3' |
| 3' | 3.54 *m* | 78.9 *d* | C-2', C-4' | | H-2', H-4' |
| 4' | 3.47 *m* | 71.6 *d* | C-3', C-5' | C-6' | H-3', H-5', H-6'a |
| 5' | 3.54 *m* | 80.7 *d* | C-6' | | H-4', H-6'a, H-6'b |
| 6'a | 3.67 *m* | 62.9 *t* | C-5' | | H-4', H-5', H-6'b |
| 6'b | 3.86 *m* | | | | H-5', H-6'a |

*no signal in $^{13}$C NMR (value from HMBC)

A literature search revealed that 3-carboxymethyl-indole-1-N-β-D-glucopyranoside is a known natural product and has been isolated previously from the fruits of *Ribes rubrum* (red currant, Grossulariaceae) by Schwarz and Hofmann (2007) and the fruiting bodies of the basidiomycete *Cortinarius brunneus* (Teichert *et al.*, 2008). In order to verify that compound **4** is identical to the published 3-carboxymethyl-indole-1-N-β-D-glucopyranoside, the $^{13}C$ NMR data were compared (Tab. 3.18).

Table 3.18. $^{13}C$ NMR data for compound **4** (125 MHz) compared with those from (Schwarz & Hofmann, 2007). Chemical shifts are reported in ppm.

| | **Compound 4 MeOD** | **Schwarz *et al.*[1] MeOD** |
|---|---|---|
| 2 | 125.4 *d* | 124.5 |
| 3 | 111.2 *s* | 110.0 |
| 3a | 130.1 *s* | 128.0 |
| 4 | 120.0 *d* | 119.1 |
| 5 | 120.9 *d* | 122.5 |
| 6 | 123.1 *d* | 120.6 |
| 7 | 111.5 *d* | 110.2 |
| 7a | 138.5 *s* | 136.4 |
| 8 | 32.7 *t* | 30.9 |
| 9 | 177.7* *s* | 177.6 |
| 1' | 86.9 *d* | 84.4 |
| 2' | 74.0 *d* | 71.6 |
| 3' | 78.9 *d* | 77.1 |
| 4' | 71.6 *d* | 69.3 |
| 5' | 80.7 *d* | 77.1 |
| 6' | 62.9 *t* | 60.6 |

*no signal in $^{13}C$ NMR (value from HMBC),[1]recorded on Bruker DPX-400 (100 MHz)

Comparison of the spectral data of compound **4** with those published in the literature (Tab. 3.18), showed minor differences in the observed and published values. However, these differences are consistent, which is often the case when NMR equipments with different field strengths are used (125 MHz, for compound **4**, 100 MHz for reference compound).

Thus, the chemical structure of the compound **4** was confirmed to be 3-carboxymethyl-indole-1-N-β-D-glucopyranoside.

### 3.3.5.5 Bioactivities of isolated compounds

The bioactivities of chemically interesting (flavonoid-rich) fractions and inseparable fatty acid mixtures were given in chapter 3.3.2, 3.3.3 and 3.3.4. In this chapter the bioactivities of isolated and purified compounds will be reported.

Bioactivity studies of the isolated pure compounds were performed against *P. falciparum* blood stage parasites, plasmodial FAS-II enzymes and KB cells for the assessment of cytotoxicity The results are presented in Table 3.19.

Compound **1** (lucumin) showed moderate antiplasmodial activity with an $IC_{50}$ value of 15.31 µg/ml (35.82 µM). No cytotoxicity or inhibitory effect against plasmodial FAS-II enzymes was observed. Compound **2** exhibited good activity against the blood stage parasites with an $IC_{50}$ value of 5.13 µg/ml (19.42 µM). However, observed cytotoxicity ($IC_{50}$ of 4.80 µg/ml or 18.17 µM) resulted in a low selectivity index (SI 0.9). In addition, moderate inhibition of FabG with an $IC_{50}$ value of 39 µg/ml (147.7 µM) was found for compound **2**. Compound **3** (2,6-dihydroxybenzoic acid) and compound **4** (3-carboxymethyl-indole-1-N-β-D-glucopyranoside) showed no antiplasmodial activity or cytotoxicity. Compound **3** showed moderate inhibition of FabI with an $IC_{50}$ value of 35 µg/ml (227.1 µM), while compound **4** was inactive against all three enzymes. Even though only moderate FAS-II enzyme inhibition was observed for compound **2** and **3**, all compounds are currently being tested against liver stage parasites to assess their prophylactic potential.

Table 3.19. Biological activities of the isolated compounds. $IC_{50}$ values in µg/ml.

| Fraction | FabI | FabG | FabZ | *P. falciparum* | KB |
|---|---|---|---|---|---|
| Compound **1** | >50 | >50 | >50 | 15.31 | >50 |
| Compound **2** | >50 | 39 | >50 | **5.13** | **4.80** |
| Compound **3** | 35 | >50 | >50 | >50 | >50 |
| Compound **4** | >50 | >50 | >50 | >50 | >50 |
| Control drug | 0.024[1] | 0.5[2] | 0.3[2] | 0.002[3] | 0.002[4] |

[1]triclosan, [2]EGCG, [3]artesunate, [4]podophyllotoxine

## 3.4 Biological activities and structure-activity relationships of natural chalcones

In this chapter the antiplasmodial screening of 22 commercially available, selected natural chalcones and the investigation of structure-activity relations will be presented. In addition, FAS-II enzyme inhibition studies were conducted to assess their possible potential against liver stage parasites and cytotoxicity was assessed against KB cells to determine selectivity.

### 3.4.1 Biological activities of chalcones

Selected commercially available natural chalcones were tested for their inhibitory potential against plasmodial FAS-II enzymes FabI, FabG and FabZ, *P. falciparum* blood stage parasites and for cytotoxicity against KB cells. Chalcones with good FAS-II inhibition were to be tested against liver stage parasites, however this was not possible due to the end of funding of the collaboration partners.

All 22 chalcones showed potential to kill blood stage parasites (Tab. 3.20). However, half of the selected compounds also displayed strong cytotoxicity resulting in low selectivity indices (SI <1.0). The best antiplasmodial potential was exhibited by licochalcone A and 2',6'-dihydroxy-4,4'-dimethoxydihydrochalcone with $IC_{50}$ values of 1.0 μM and 3.7 μM, respectively. Both compounds displayed relatively low toxicity (18.1 μM (SI 18.1) and 61 μM (SI 16.5), respectively). Nine other chalcones, namely isoliquiritigenin (SI 1.5), phloretin (SI >7), phloridzin (SI >3.6), eriodictyolchalcone (SI >2.7), marein (SI >5.7), homobutein (SI 5.1), 2',4-dihydroxy-4',6'-dimethoxychalcone (SI 6.5), 2',6'-dihydroxy-4,4'-dimethoxy-chalcone (SI >27) and 2'-hydroxy-4,4',6'-trimethoxychalcone (SI 4.3), showed moderate antimalarial activities in the range of 10 μM to 40 μM with low or no cytotoxicity.

In addition to the antiplasmodial potential, eleven chalcones, namely licochalcone A, 2-hydroxychalcone, 4-hydroxychalcone, isoliquiritigenin, butein, phloretin, homobutein, eriodictyolchalcone, marein, 3,4-dimethoxychalcone and 4,2'-dihydroxy-4',6'-dimethoxy-chalcone, inhibited one or more FAS-II enzyme (Tab. 3.20). Five of these chalcones (licochalcone A, phloretin, eriodictyolchalcone, marein and homobutein) showed interesting enzyme potential, *i.e.* the inhibitory potential against the enzymes was lower than the cytotoxicity of the compound. Licochalcone A, eriodictyolchalcone and marein inhibited FabZ with $IC_{50}$ values of 6.5 μM, 4.5 μM and 13.3 μM, respectively. Homobutein and phloretin showed in addition to FabZ inhibition (6.6 μM and 46.8 μM), inhibition against FabG with moderate $IC_{50}$ values of 29.7 μM and 76.6 μM, respectively. Butein was the only chalcones that was active against all three enzymes and the sole chalcone with activity against FabI ($IC_{50}$ 12.9 μM), however its cytotoxicity will be a limiting factor.

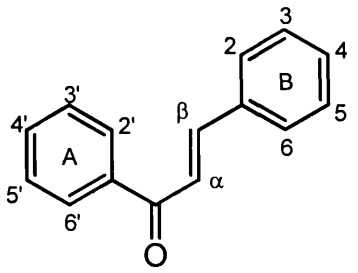



Table 3.20. Biological activities of natural chalcones. $IC_{50}$ values in μM.

| | FabI | FabG | FabZ | *P. falciparum* | KB |
|---|---|---|---|---|---|
| Licochalcone A | >100 | 65.0 | **6.5** | **1.0** | 18.1 |
| Chalcone | >100 | >100 | >100 | 46.9 | 12.1 |
| 2-Hydroxychalcone | >100 | >100 | 35.7 | 30.5 | **8.0** |
| 2'-Hydroxychalcone | >100 | >100 | >100 | 40.8 | 20.8 |
| 4-Hydroxychalcone | >100 | >100 | 75.8 | 29.3 | 10.8 |
| 4'-Hydroxychalcone | >100 | >100 | >100 | 12.3 | **8.5** |
| 4,2',4'-Trihydroxychalcone (Isoliquiritigenin) | >100 | 66.3 | 58.5 | 17.9 | 26.7 |
| 3,4,2',4'-Tetrahydroxychalcone (Butein) | 12.9 | 13.2 | **6.2** | **9.6** | **5.8** |
| 4,2',4',6'-Tetrahydroxydihydro-chalcone (Phloretin) | >100 | 76.6 | 43.8 | 14.2 | >100 |
| 3,4,2',4',6'-Pentahydroxychalcone (Eriodictyolchalcone) | >17.3* | >100 | **4.5** | 37.2 | >100 |
| 4-Methoxychalcone | >100 | >100 | >100 | 49.3 | 12.4 |
| 4'-Methoxychalcone | >100 | >100 | >100 | 22.2 | **6.7** |
| 3,4-Dimethoxychalcone | >100 | >100 | 96.9 | 23.4 | **7.2** |
| 4,4'-Dimethoxychalcone | >100 | >100 | >100 | 26.7 | 25.9 |
| 2,3-Dimethoxy-2'-hydroxychalcone | >100 | >100 | >100 | 18.4 | **6.1** |
| 4,2'-Dihydroxy-4',6'-dimethoxy-chalcone | >1.7* | >100 | 79.9 | 11.6 | 74.9 |
| 2',6'-Dihydroxy-4,4'-dimethoxy-chalcone | >16.6* | >100 | >16.6* | 31.6 | >100 |
| 2',6'-Dihydroxy-4,4'-dimethoxy-dihydrochalcone | >100 | >100 | >100 | **3.7** | 61.0 |
| 4,2',4'-Trihydroxy-3-methoxy-chalcone (Homobutein) | >100 | 29.7 | **6.6** | 12.4 | 63.4 |
| 2'-Hydroxy-4,4',6'-trimethoxy-chalcone | >100 | >100 | >100 | 10.8 | 46.4 |
| 4,2',4',6'-Tetrahydroxydihydro-chalcone-2'-*O*-glucoside (Phloridzin) | >100 | >100 | >100 | 27.4 | >100 |
| 3,4,2',3',4'-Pentahydroxy-4'-glucosylchalcone (Marein) | >100 | >100 | 13.3 | 17.4 | >100 |
| Control drugs | 0.028[1] | 0.63[2] | 0.15[2] | 0.01[3] | 0.003[4] |

[1]triclosan, [2]EGCG, [3]artesunate, [4]podophyllotoxin

*measurement failed at higher concentrations

### 3.4.2 Antiplasmodial structure-activity relationship

The parent compound chalcone showed low antiplasmodial potential with an $IC_{50}$ value of 46.9 μM. The only substitution resulting in a lower $IC_{50}$ value (49.3 μM) was the single methoxylation on the B ring for 4-methoxychalcone. However, the addition of a second methoxy group on either ring A or B, as in the case of 3,4-dimethoxychalcone and 4,4'-dimethoxychalcone, resulted in an increased antiplasmodial potential with $IC_{50}$ values of 23.4 μM and 26.7 μM, respectively. Single methoxylation on the A ring at the position C-4' in 4'-methoxychalcone led to a similar improvement ($IC_{50}$ 22.2 μM).

Hydroxylation of the parent compound chalcone resulted in an increase in the antiplasmodial potential. Single hydroxylation in the B ring led to minor improvement ($IC_{50}$ of 30.5 μM for 2-hydroxychalcone and 29.3 μM for 4-hydroxychalcone) while in the A ring position C-4' is favoured, resulting in an $IC_{50}$ value of 12.3 μM for 4'-hydroxychalcone, compared to 40.8 μM for 2'-hydroxychalcone. 4,2',4'-trihydroxychalcone has an $IC_{50}$ value of 17.9 μM. The addition of a fourth hydroxyl group on the position C-3 (as in the case of butein) results in an additional increase in antiplasmodial activity (9.6 μM). Substitution with a fifth hydroxyl group at position C-6' (as in the case of eriodictyolchalcone) significantly decreased the antiplasmodial potential ($IC_{50}$ 37.2 μM).

The addition of hydroxyl and methoxyl groups on both rings showed improved antiplasmodial potential compared to the parent compound chalcone. The multiple methoxylation of 2'-hydroxychalcone resulted in an approximately four-fold increase in antiplasmodial activity from an $IC_{50}$ value of 40.8 μM for 2'-hydroxychalcone to 10.8 μM for 2'-hydroxy-4,4',6'-trimethoxychalcone. The replacement of the methoxyl group with a hydroxyl group at the position C-4, as in case of 4,2'-dihydroxy-4',6'-dimethoxychalcone, did not influence the activity ($IC_{50}$ 11.6 μM) whereas at the position C-6' (2',6'-dihydroxy-4,4'-dimethoxychalcone) it resulted in considerable decrease of potential ($IC_{50}$ 31.6 μM). The reduction of this chalcone to 2',6'-dihydroxy-4,4'-dimethoxydihydrochalcone led to a significantly better $IC_{50}$ value with 3.7 μM, which is next to licochalcone A ($IC_{50}$ 1.0 μM) the best natural chalcone in this study.

The dihydrochalcone phloretin and its glycoside phloridzin exhibited antiplasmodial activity with $IC_{50}$ values of 14.2 μM and 27.4 μM, respectively. A second chalconeglycoside (marein) with a different hydroxylation pattern and sugar position also showed increased potential compared to the parent compound chalcone with an $IC_{50}$ value of 17.4 μM.

### 3.4.3 Plasmodial FAS-II enzyme inhibition structure-activity relationship

The parent compound chalcone lacked inhibitory activity against all three FAS-II enzymes. The substitution of chalcone with one or more methoxyl groups did not result in a

significant increase of inhibitory activity and only 3,4-dimethoxychalcone showed low potential against FabZ ($IC_{50}$ of 96.9 μM).

Hydroxylation in ring B, as in case of 2-hydroxychalcone and 4-hydroxychalcone, led to inhibition against FabZ with $IC_{50}$ values of 35.7 μM and 75.8 μM, respectively, while hydroxylation in ring A on position C-2' or C-4' did not influence the activity. Multiple hydroxylation on both rings resulted in increased enzyme inhibition potential. This was most pronounced for chalcones with a hydroxyl group at position C-3 such as eriodictyolchalcone (3,4,2',4',6'-pentahydroxychalcone) and butein (3,4,2',4'-tetrahydroxy-chalcone) which exhibited overall the best potential against FabZ with $IC_{50}$ values of 4.5 μM and 6.2 μM, respectively. Comparison of these compounds with the chalcones phloretin (4,2',4',6'-tetrahydroxydihydrochalcone) and isoliquiritigenin (4,2',4'-trihydroxy-chalcone) showed roughly a ten-fold decrease in the enzyme inhibitory potential against FabZ with $IC_{50}$ values of 43.8 μM and 58.5 μM, respectively. Butein showed in addition to good FabZ inhibition the best inhibitory potential against FabG ($IC_{50}$ of 13.2 μM) and was the only compound exhibiting activity against FabI ($IC_{50}$ of 12.9 μM).

The substitution of the chalcone structure with hydroxyl and methoxyl groups did not show a clear pattern of structure-activity relations in the enzyme inhibitory potential. Compound 4,2'-dihydroxy-4',6'-dimethoxychalcone showed FabZ inhibition with an $IC_{50}$ value of 79.9 μM, while homobutein (4,2',4'-trihydroxy-3-methoxychalcone) exhibited good activity against FabZ ($IC_{50}$ of 6.6 μM) and moderate activity against FabG ($IC_{50}$ of 29.7 μM).

Glycosylation resulted in case of phloridzin (4,2',4',6'-tetrahydroxydihydrochalcone-2'-*O*-glucoside) in the loss of FabZ activity (>100 μM) compared to phloretin (4,2',4',6'-tertahydroxydihydrochalcone, $IC_{50}$ of 43.8 μM). For marein (3,4,2',3',4'-pentahydroxy-4'-glucosylchalcone) inhibition against FabZ with an $IC_{50}$ value of 13.3 μM was measured, indicating that the sugar position might play a role in enzyme inhibition.

### 3.4.4 Cytotoxicity structure-activity relationship

The parent compound chalcone exhibited cytotoxicity against KB cells with an $IC_{50}$ value of 12.1 μM. No clear trend was found for the cytotoxicity-structure relations of chalcones. However, it was observed that α,β-saturated chalcones (dihydrochalcones) and chalconeglycosides had significantly reduced cytotoxicity compared to the parent compound chalcone ($IC_{50}$ values >100 μM).

## 3.5 Biological activity studies with lichen secondary metabolites

Commercially available lichen metabolites evernic acid, vulpic acid, psoromic acid and (+)-usnic acid were tested for their inhibitory potential against plasmodial FAS-II enzymes FabI, FabG and FabZ, *P. yoelii* liver stage parasites, *P. falciparum* blood stage parasites and for their cytotoxicity against KB and HepG2:CD81 cells. The liver stage experiments and cytotoxicity studies against HepG2:CD81 cells were performed by Assoc. Prof. S. Kappe and Dr. A. Tarun from the Seattle Biomedical Research Institute, USA.

### 3.5.1 Inhibition of plasmodial FAS-II enzymes as potential liver stage targets and kinetic studies

The assessment of the compounds against crucial plasmodial FAS-II enzymes demonstrated good inhibitory activity against FabZ by evernic acid and vulpic acid with $IC_{50}$ values of 10.7 μM and 20.5 μM, respectively (Tab. 3.21). In addition, evernic acid showed moderate inhibition against FabI ($IC_{50}$ 36.1 μM). Psoromic acid was the only compound exhibiting an inhibitory effect against all three plasmodial enzymes, with an $IC_{50}$ value of 35.2 μM against FabZ, 71.4 μM against FabI and 183.0 μM against FabG. (+)-Usnic acid did not show any activity even at the highest test concentrations of 200 μM.

Table 3.21. Biological activities of lichen secondary metabolites against plasmodial FAS-II enzymes. $IC_{50}$ values in μM.

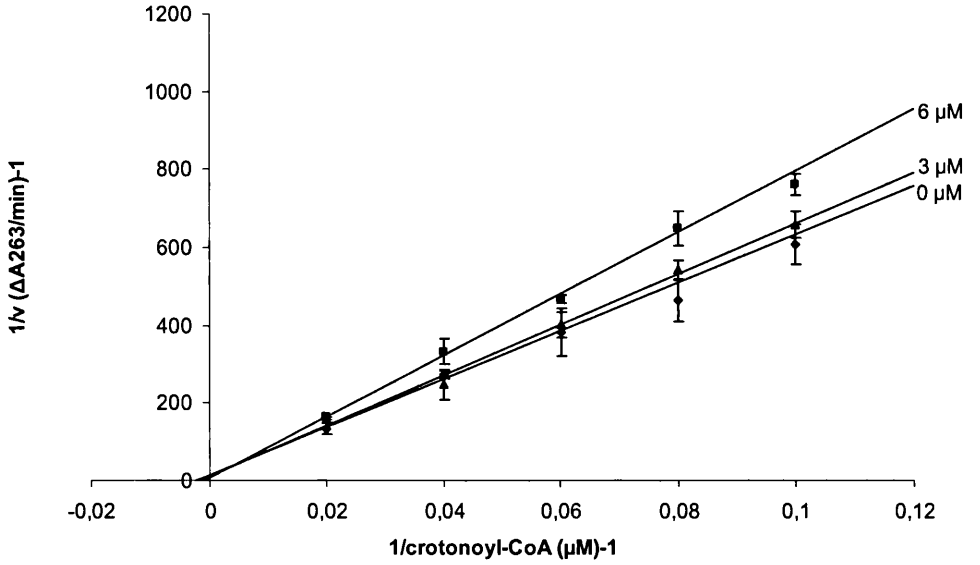
| | **FabI** | **FabG** | **FabZ** |
|---|---|---|---|
| Evernic acid | 36.1 | >200 | 10.7 |
| Vulpic acid | >139.5 | >200 | 20.5 |
| Psoromic acid | 71.4 | 183.0 | 35.2 |
| (+)-Usnic acid | >200 | >200 | >200 |
| Control drugs | 0.05[1] | 1.1[2] | 0.7[2] |

[1]triclosan, [2]EGCG

Evernic acid was identified as the compound with the best potential against the FAS-II enzymes with the best activity against FabZ. Thus, in order to elucidate the binding site and mechanism of inhibition of evernic acid on the FabZ enzyme, kinetic studies were performed.

Evernic acid was identified as competitive inhibitor with respect to the substrate crotonoyl-CoA (Fig. 3.64), indicating that the substrate and the inhibitor have the same binding site in the free enzyme and bind in a mutually exclusive way. Data analysis by Dixon plot provided a $K_i$ value of 19.9 μM.

Figure 3.64. Lineweaver-Burk plot for FabZ and evernic acid. The graphs result from substrate-velocity curves of FabZ activity with different substrate concentrations (10 - 50 µM) in the absence and presence of evernic acid. The lines intercepted on the 1/v axis indicate competitive inhibition for the substrate.




### 3.5.2 Inhibition of liver stage parasites and cytotoxicity against hepatocytes

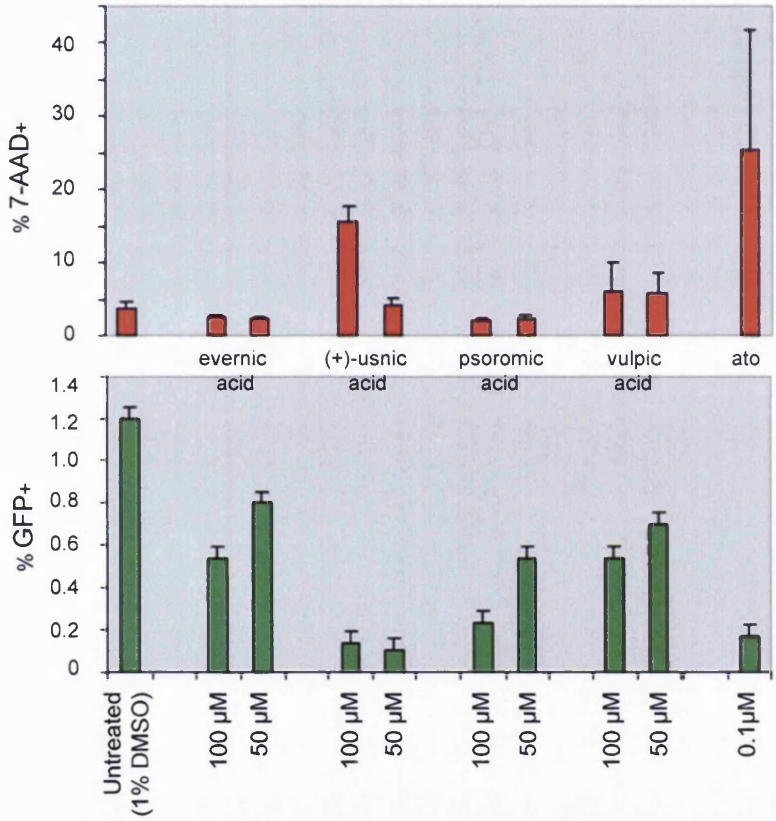
For the assessment of the *in vitro* infections of HepG2:CD81 cells by *P. yoelii* parasites two assays, flow cytometry (FC) and immunofluorescence analysis (IFA), were used.

In the FC, the cells were infected with transgenic *P. yoelii* sporozoites expressing green fluorescent protein (GFP) prior to exposing them to varying compound concentrations. After 40 h the trypsinised cell suspension was stained with the impermeant nucleic acid dye 7-aminoactinomycin (7-AAD) in order to assess the cell viability by staining membrane-compromised, *i.e.* dead cells. However, it should be noted that the cytotoxicity assessment was not quantitative, as dead cells were removed during the daily media exchange during the 40 h incubation period. Cells were analysed by flow cytometry and the number of GFP-positive and 7-ADD-positive cells counted and compared to untreated cells and cells treated with atovaquone, an antimalarial drug in use and known for liver stage inhibition, which served as positive control (Fig. 3.65).

For the untreated control (1% DMSO), 1.3% of the cells were GFP-positive, *i.e.* infected, and less than 5% of the cells were 7-ADD-positive, *i.e.* dead (Fig. 3.65). Cells treated with evernic acid showed 60% inhibition (0.52% GFP-positive cells) at the highest tested concentration of 100 µM and 28% inhibition at 50 µM (0.95% GFP-positive cells). Vulpic acid demonstrated 56% inhibition at 100 µM (0.53% GFP-positive cells) and 42% inhibition at 50 µM (0.70% GFP-positive cells). Psoromic acid inhibited 81% of the liver

stage parasites at 100 μM (0.26% GFP-positive cells) and showed 59% inhibition at 50 μM (0.54% GFP-positive cells). (+)-Usnic acid showed 89% and 93% inhibition of liver stage parasites at 100 μM and 50 μM, respectively (0.14% and 0.09% GFP-positive cells). Atovaquone showed 0.17% GFP-positive cells at a concentration of 0.1 μM, which represented 86.1% inhibition of liver stage parasites.

Figure 3.65. Results FC. Percentage of 7-AAD-positive cells (*i.e.* dead cells) (top panel) and GFP-positive cells (*i.e.* cells infected with parasites) (bottom panel) at different compound concentrations. Atovaquone (ato) was used as reference drug.




For evernic acid and psoromic acid no cytotoxicity against HepG2:CD81 cells was detected at 100 μM (<5% 7-AAD-positive cells), whereas vulpic acid had a minimal increase in the percentage of dead cells at 100 μM and 50 μM (<10% 7-AAD-positive cells) (Fig. 3.65). (+)-Usnic acid showed some cytotoxicity at a concentration of 100 μM with 15% of 7-AAD-positive cells, but no cytotoxicity was found at 50 μM (<5% 7-AAD-positive cells). Atovaquone exhibited the highest cytotoxicity with 25% 7-AAD-positive cells.

In the immunofluorescence analysis (IFA), the effect of the compounds on the morphology and development of liver stage parasites was assessed to verify the results from the FC. Figure 3.66 shows representative images of 43 h postinfectious liver stage HepG2:CD81 cells treated with different compound concentrations. Untreated cells in the

presence of 0.5% DMSO were used as control and atovaquone as reference drug. Cells were stained with antibodies against plasmodial HSP70 (green) and UIS4 (red) to detect infected cells. HSP70 is highly expressed in liver stage parasites and its staining was used to quantify the effect of the treatments (shown in green, Fig. 3.66 a)-d), upper panels). This was achieved by comparison of the average size of the parasites detected in the treated samples to their size in untreated cells, and size reductions by the treatment was used for the $IC_{50}$ estimations. UIS4 is a protein which is expressed exclusively in infective sporozoites and developing liver stages and allowed to confirm the presence of liver stage parasites in the infected cells. The nuclear counterstain DAPI (blue) was used to visualise the hepatocytes and also allowed to visually assess cytotoxicity of the compounds. An overlay of all three channels is shown in the lower panel for each treatment (Fig. 3.66, lower panels).

Treatment of cells with atovaquone resulted in much smaller parasites compared to untreated cells, indicating the inhibition of liver stage development with an estimated $IC_{50}$ value of 0.7 nM. For all compounds, except vulpic acid, a decrease in the size of parasites was observed (Fig. 3.66). The concentration dependant size reductions resulted in estimated $IC_{50}$ values of 19.5 μM for evernic acid, 10.9 μM for psoromic acid and 4.9 μM for (+) usnic acid (Tab. 3.22).

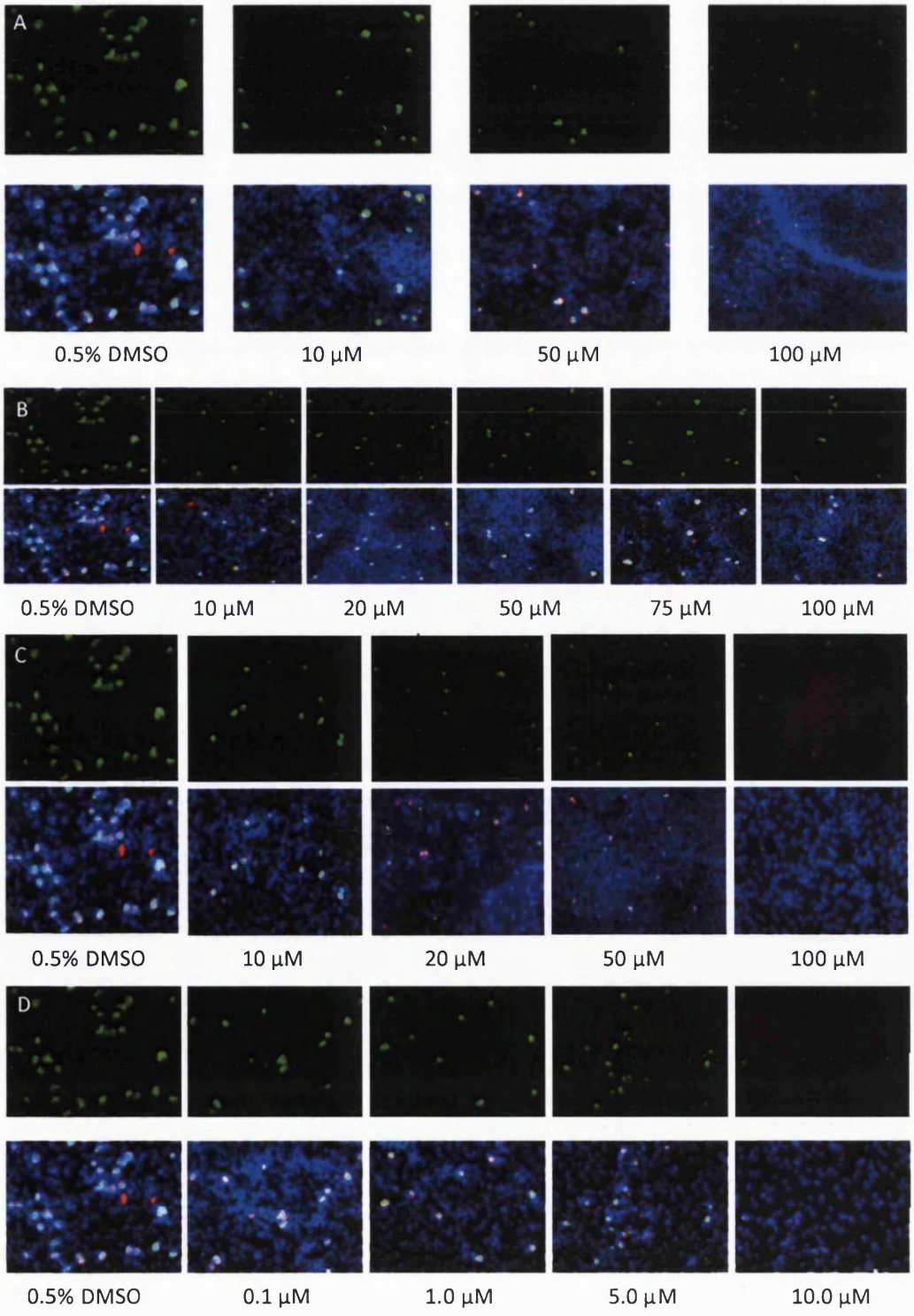
Nuclear staining (Fig. 3.66) indicated that psoromic acid showed some toxicity against the hepatocytes at concentrations higher than 20 μM. For (+)-usnic acid cytotoxicity was found at concentrations higher than 10 μM. Evernic acid and vulpic acid showed no toxic effects at the highest tested concentrations (>100 μM).

Table 3.22. Biological activities of lichen secondary metabolites. Tested against plasmodial FAS-II enzymes, *P. yoelii* liver stage (LS) parasites, *P. falciparum* blood stage (BS) parasites and KB cells. $IC_{50}$ values in μM.

| | **FabI** | **FabG** | **FabZ** | ***P. falciparum* BS** | ***P. yoelii* LS (IFA)** | **KB** |
|---|---|---|---|---|---|---|
| Evernic acid | 36.1 | >200 | 10.7 | 142.1 | 19.5 | 190.4 |
| Vulpic acid | >139.5 | >200 | 20.5 | 48.5 | >100 | 58.4 |
| Psoromic acid | 71.4 | 183.0 | 35.2 | 29.2 | 10.9 | 31.6 |
| (+)-Usnic acid | >200 | >200 | >200 | 47.3 | 4.9 | 127.9 |
| Control drugs | 0.05[1] | 1.1[2] | 0.7[2] | 0.005[3] | 0.0007[4] | 0.012[5] |

[1]triclosan, [2]EGCG, [3]artesunate, [4]atovaquone, [5]podophyllotoxin

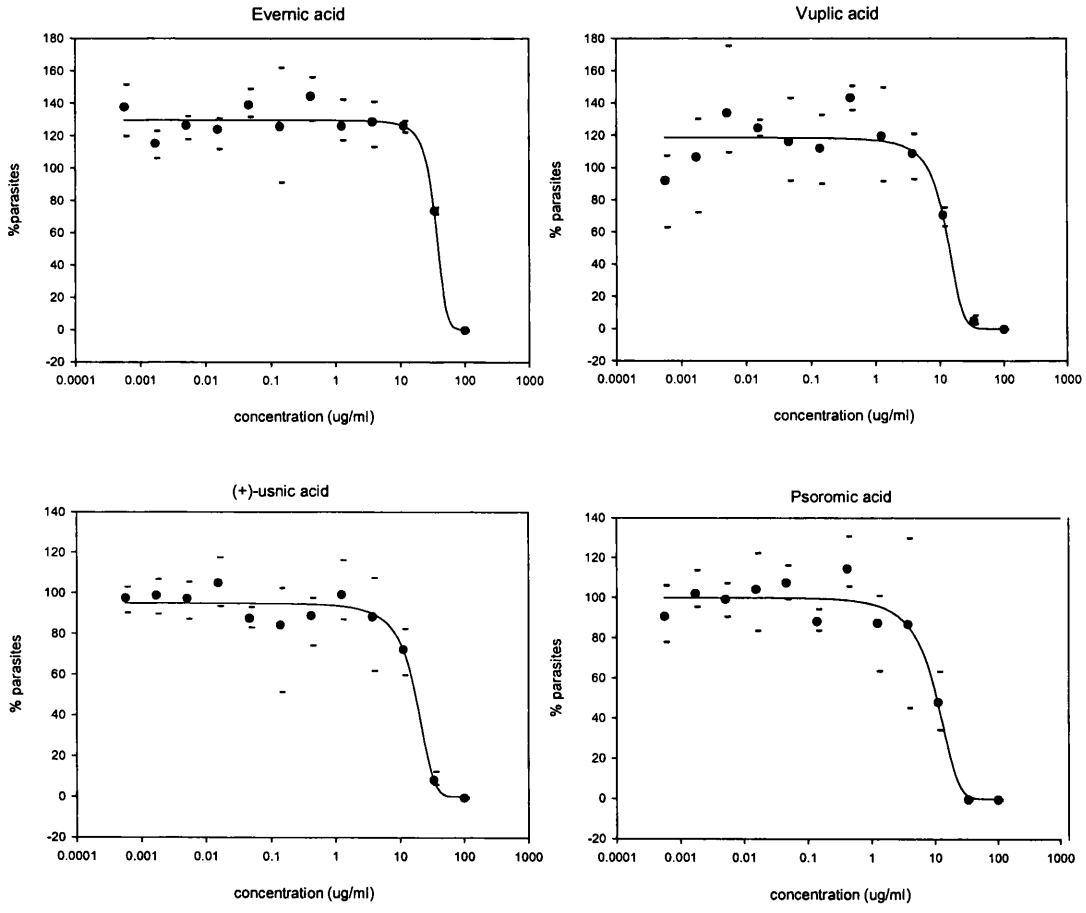
Figure 3.66. Immunofluorescence microscopy images (20x objective magnification) of HepG2:CD81 cells infected with *P. yoelii*. A) evernic acid, B) vulpic acid, C) psoromic acid and D) (+)-usnic acid. Images are taken at 43 h postinfectious. Top panels show liver stage parasites detected by antibody against plasmodial HSP70 (FITC, green). Lower panels show hepatocytes stained with DAPI (blue) and liver stage parasites doubly stained with antibodies against HSP70 (FITC, green) and UIS4 protein (Texas red, red). Each panel represents a different compound concentrations.



### 3.5.3 Inhibition of blood stage parasites and cytotoxicity against KB cells

The assessment of the inhibitory potential of the lichen metabolites against blood stage *P. falciparum* parasites showed moderate to low antiplasmodial activity for all compounds. Concentration-response curves for the growth inhibition were determined as the percentage of parasites after treatment with the different compounds at various concentrations (Fig. 3.67). Psoromic acid exhibited the best potential with an $IC_{50}$ value of 29.2 µM, followed by (+)-usnic acid with 47.3 µM, vulpic acid with 48.5 µM and evernic acid with 142.1 µM (Tab. 3.22).

Figure 3.67. Blood stage parasite numbers at different compound concentrations. Graphs show the mean of triplicates, bars indicate the highest and lowest parasite number detected.




In addition to the qualitative assessment of cytotoxicity against HepG2:CD81 cells, the lichen compounds were tested against the human cancer cell line KB. Psoromic acid showed the highest cytotoxic effect with an $IC_{50}$ value of 31.6 µM (SI 1.1), followed by vulpic acid with 58.4 µM (SI 1.2), (+)-usnic acid with 127.9 µM (SI 2.7) and evernic acid with 190.4 µM (SI 1.3) (Tab. 3.22).

# Part 4

# Discussion

## 4.1 General discussion of the methods

For the enzyme expression, purification and assays, all techniques had to be implemented or developed at the School of Pharmacy. All other methods used in this study are established and evaluated techniques. In the following some of the problems and limitations experienced will be discussed.

### 4.1.1 Expression and purification of recombinant FAS-II and FAS-I enzymes

The expression and purification of the plasmodial FAS-II enzymes FabI, FabG and FabZ (see 2.5.1.1 and 2.5.1.2) was successfully implemented. Implementation of the expression of human FAS-I on the other hand failed, as the baculovirus stock containing hFAS-I was no longer infectious. The inactivity of the baculoviruses is most likely caused by the age of the stock (produced in 2003). The production of new baculovirus required hFAS-I DNA and would have been too time-consuming. Thus, the addition of hFAS-I into the screening panel could not be achieved.

### 4.1.2 Plasmodial FAS-II enzyme inhibition studies

The enzyme inhibition studies were performed either by a cuvette assay (LTS) or a high-throughput assay (HTS) (see 2.5.1.3). The cuvette assay is a well established method and was modified to ensure steady-state conditions (Tasdemir *et al.*, 2006). The high-throughput assay is based on the same principles and was developed and implemented in the course of this study. For FabI, previously described screening methods using a 96-well plate format were modified to ensure steady-state conditions (Freundlich *et al.*, 2005; Kumar *et al.*, 2007). No high-throughput assays have been previously described for plasmodial FabG or FabZ and were developed as part of this study.

The advantage of the cuvette assay are comparably low costs for reagents. The enzyme inhibition of a sample was assessed at a starting concentration of 10 μg/ml. If no inhibition or an effect resulting in less than 50% inhibition was observed, the concentration of the sample was increased to 50 μg/ml. Thus, for inactive samples, only two measurements were performed. For active samples, *i.e.* more than 50% enzyme inhibition at 10 μg/ml, lower sample concentrations were measured until full enzyme activity was recovered. In the high-throughput assay, all samples were measured with concentrations ranging from 0.0005 μg/ml to 50 μg/ml. The use of 96-well plates necessitated an increase in the reaction time from 1 or 2 min in the LTS to 17 or 34 min in the HTS. This required the use of higher cofactor and substrate concentrations to ensure steady-state conditions during the whole assay, thus resulting in higher costs for reagents. The advantage of the HTS is the quick assessment of several compounds at various concentrations simultaneously.

For some extracts, fractions and compounds the inhibitory potential could not be assessed as with increased sample concentration the measurements became unreliable (shown as >1 or >10). The assessment of enzyme inhibition is monitored by the decrease in absorption of the concentration of the cofactor NADH (FabI) or NADPH (FabG) or the decrease in absorption by the saturation of the substrate (FabZ). Thus, a fully active enzyme showed a time-dependant reduction in absorption which could be quantified by the negative slope. For active compounds the negative slope became shallower, *i.e.* the enzymatic reaction was slower. For some chalcones it was observed that the slope became steeper, *i.e.* the enzymatic reaction was quicker. This could be explained if the compounds act as an activator on the enzyme and increase the reaction speed. A second observation, for some extracts, fractions and chalcones, was a positive slope. This could be explained by an increase of NADH, which is not possible as no $NAD^+$ was present in the reaction mixture. Another explanation could be that some chemical reaction between compounds in the fraction or extract occurred. However, it was not within the scope of this work to investigate this further.

The absorbance-based technique is currently the only available method to assess plasmodial FAS-II enzyme inhibition. As in most absorbance-based systems, the self-absorbance properties of the extracts, fractions or compounds were the limitations. Obstruction by self-absorbance generally occurred above 50 µg/ml, hence this concentration was chosen as the highest test concentration. A second reason was that DMSO, in which the samples were dissolved, showed unspecific enzyme inhibition at concentrations above 1%, which would be equivalent to a test concentration of 100 µg/ml. Higher stock concentrations, which would result in a lower DMSO concentration, could not be prepared due to solubility problems.

### 4.1.3 Antiplasmodial and cytotoxicity assays

The *in vitro* growth inhibition of blood stage parasites was assessed by the incorporation of [$^3$H]hypoxanthine based on the semiautomated microdilution technique developed by Desjardins *et al.* (1979). In addition to previously described modification (Vivas *et al.*, 2005), the incubation time of sample and parasites was increased from 24 h to 48 h. Despite extending the overall assay duration, this longer incubation time was chosen to ensure sample exposure throughout all parasite stages in the blood stage.

The highest sample concentration measured were 50 µg/ml, which is the concentration above which activity is no longer relevant on a therapeutical level (Gessler *et al.*, 1994). In addition, similar to the enzyme assay, DMSO showed unspecific parasite inhibition at

concentrations above 1%, which would be the equivalent to a sample concentration of 100 µg/ml.

A chloroquine-resistant *P. falciparum* strain was used for the assessment of antiplasmodial activity. Due to time and expenses limitations, the addition of a chloroquine-sensitive strain, as suggested by Wright (2010), was not feasible. Resistant strains and resistance development are the major problems in malarial treatment, hence the K1 strain which has multi-drug resistant against chloroquine, sulphadoxine and pyrimethamine was chosen for this study.

In addition to the plasmodial growth inhibition studies, the cytotoxicity was assessed against the human cancer cell line KB to determine unspecific effects, resulting from cytotoxicity.

The assessment of *in vitro* growth inhibition of liver stage parasites was assessed by flow cytometry and immunofluorescence analysis and performed by our collaboration partners Dr. A. Tarun and Assoc. Prof. S. Kappe.

In this study the rodent parasite *P. yoelii* and the human hepatoma cell line HepG2:CD81 were used. Protocols with other cell lines of mouse or human origin are available (Hollingdale *et al.*, 1981; Mota & Rodriguez, 2000). The advantage of a human hepatoma cell line is, that the same cell type is used as the parasites infect in humans. In addition, cytotoxicity against liver cells can be assessed concurrently and the rodent model is an accepted model for this type of study. The assessment of *in vitro P. falciparum* liver stage inhibition, which would be even closer to the real life situation, necessitates the use of primary human cells isolated from the liver, as infection rates in human hepatic cells lines are extremely low with 0.07% compared to 1-2% in the rodent system (Sattabongkot *et al.*, 2006). In addition, high safety procedures are required due to the use of *P. falciparum* infected *Anopheles* mosquitoes.

### 4.1.4 Units to report biological activity

Many phytochemical investigations or reviews report the observed activities of isolated compounds in µg/ml (Innok *et al.*, 2009; Oliveira *et al.*, 2009; Scala *et al.*, 2010). In this study two units were used to report the observed biological activities: microgram per millilitre (µg/ml) and micromolar (µM).

For all plant extracts and fractions the activity was reported in µg/ml. Extracts are complex mixtures and in order to report an activity in µM, the molecular weight ($M_r$) of the active compound is required. As the compounds and their percentage within the complex mixtures of extracts and fractions are unknown, their reported activity can only be applied

to the whole sample and therefore reported in μg/ml. For the purpose of comparing the activity of isolated compounds with the activity of the fraction or extract from which they are derived, it is sensible to report the compound activity in μg/ml. However, for the comparison of different compounds with each other, it is prudent to compare the activity in μM, as the compounds differ in their molecular weight. The importance of this is demonstrated with the example of chalcone and marein. The antiplasmodial activity for chalcone ($IC_{50}$ 9.8 μg/ml) and marein ($IC_{50}$ 7.9 μg/ml) is very similar when reported in μg/ml. The molecular weight of marein ($M_r$ 450.40) is more than two-fold higher than the molecular weight of chalcone ($M_r$ 208.26), *i.e.* a smaller number of marine molecules are present in the sample compared to chalcone. Thus, to be able to compare the actual effect of the same numbers of molecules, the unit μM has to be used. For marein and chalcone this results in $IC_{50}$ values of 17.4 μM and 46.9 μM, respectively. Hence marein is more than twice as potent as chalcone and its potency would be underestimated when reported in μg/ml.

The use of μM is especially important when the potencies of new compounds are compared to known drugs or for the assessment of synthetic or semi-synthetic derivatives developed from a compound. Furthermore, from a mechanistical point of view, in order to understand the mode of action of a compound, it is important to know the number of molecules interacting with targets (*e.g.* enzymes, receptors), rather than the mg needed to achieve an effect.

## 4.2 Discussion of the screening of Turkish plants

The screening of the Turkish plants *Anthemis cretica* subsp. *anatolica*, *A. pestalozzae*, *Scrophularia lucida*, *S. pinardii* and *Salvia virgata* demonstrated antiplasmodial activity for all plant extracts and subextracts. The crude methanol extract is a complex mixture of several hundred plant metabolites. The liquid-liquid partition provided a very basic separation of nonpolar principles (hexane) from middle polar ($CHCl_3$) and polar (aq. MeOH) constituents. However, each subextract is a multi-component mixture with active, partially active and inactive compounds. Hence, synergistic and/or antagonistic effects may play an important role in the observed activities. Chemically interesting secondary metabolites generally represent a minor portion of extracts and subextracts. Thus, the result that all plant extracts exhibit antiplasmodial potential against *P. falciparum* blood stage parasites is encouraging and further investigation and bioactivity-guided fractionation might yield pure compounds with clear bioactivity profiles. In this study, the bioactivity-guided fractionation of *A. pestalozzae* was performed and the results will be discussed in 4.3.

The observed activities against plasmodial FAS-II enzymes also indicate that the plants contain compounds that potentially can inhibit the liver stage of *Plasmodium*, which is interesting for the development of prophylactic drugs.

This study reports for the first time the antiplasmodial activity of the selected Turkish plants. Previous studies against *P. falciparum* with plants from the same genera have been reported and will be summarised briefly and compared to the results obtained in this study.

For several South African *Salvia* species good to moderate antiplasmodial activity was shown (Pillay *et al.*, 2008). The reported $IC_{50}$ value for the crude extract of *S. virgata* from this study is 4.68 µg/ml, which is only marginally higher compared to *S. radula* ($IC_{50}$ 3.91 µg/ml), that has been previously reported as the most active solvent extract (MeOH-$CHCl_3$) (Kamatou *et al.*, 2008b). Overall, the highest antiplasmodial potential for *Salvia* species have been detected in the essential oils, with *S. runcinata* exhibited the best potential ($IC_{50}$ 1.23 µg/ml) (Kamatou *et al.*, 2005).

So far the antiplasmodial potential of only two *Scrophularia* species has been reported. The aerial parts of *S. lepidota* showed good activity for the hexane and $CHCl_3$ crude extract with $IC_{50}$ values of 3.9 µg/ml and 4.1 µg/ml, respectively, while the $H_2O$ crude extract showed moderate potential ($IC_{50}$ 18.6 µg/ml) (Tasdemir *et al.*, 2005a). The EtOH crude extract from the roots of this species demonstrated moderate activity ($IC_{50}$ 17.5 µg/ml) and a low inhibitory potential against plasmodial FabI ($IC_{50}$ 80 µg/ml) (Tasdemir *et al.*, 2005b). For the aerial parts of *S. cryptophila* only the $CHCl_3$ crude extract exhibited good activity with an $IC_{50}$ value of 1.8 µg/ml, whereas the hexane crude extract was moderately active ($IC_{50}$ 17.4

μg/ml) and the $H_2O$ crude extract inactive (>20 μg/ml) (Tasdemir *et al.*, 2005a). The trend that either the hexane or $CHCl_3$ extracts seem to exhibit the best antiplasmodial potential was also observed in this study and was especially dominant for the roots subextracts. Interestingly, the lack of FabI inhibition reported in this study, was also observed by Tasdemir *et al.* (2008) for isolated compounds from *S. cryptophila.*

Even though *Anthemis* is a widely studied genus, so far only one investigation about the antiplasmodial potential of isolated compounds from *A. auriculata*, including FAS-II enzyme inhibition studies, has been reported (Karioti *et al.*, 2007; Karioti *et al.*, 2009). Thus, this is the first study, in which the antiplasmodial activity of *Anthemis* extracts is reported.

## 4.3 Discussion of phytochemical and biological investigation of *A. pestalozzae*

This is the first study investigating the chemical composition of *Anthemis pestalozzae*. Four compounds were isolated and identified as the cyanogenic glycoside lucumin (**1**), a sesquiterpene lactone (**2**), the benzoic acid derivative 2,6-dihydroxybenzoic acid (**3**) and the N-glucosylated indole alkaloid 3-carboxymethyl-indole-1-N-β-D-glucopyranoside (**4**). Compounds **1**, **3** and **4** were identified for the first time in *Anthemis*. Compound **2** has the identical planar structure and relative stereochemistry as sivasinolide which has been previously reported in *A. altissima*. However our data suggest that the original published data identifying sivasinolide are ambiguous, hence all compounds identified as sivasinolide could be a different compound (see below). In addition, the presence of the well known flavonoid rutin could be shown in the aq. MeOH subextract by HPLC analysis and an extensive analysis of the fatty acid composition of several fractions of the hexane and $CHCl_3$ subextract was performed by GC-MS.

Lucumin (**1**) has been isolated previously from the seeds of *Lucuma mammosa* (Sapotaceae) (Bachstez *et al.*, 1949; Eyjolfsson, 1971), the seeds of *Calocarpum sapota* (Sapotaceae) (Takeda *et al.*, 1997) and the leaves of *Clerodendrum grayi* (Lamiaceae) (Miller *et al.*, 2006a). Lucumin belongs to a small group of cyanogenic diglycosides found in plants, which are generally restricted to generative parts of the plant (fruits and seeds). Epilucumin and the more complex diglycosides *Anthemis* glycoside A and B, isolated from the seeds of *Anthemis cairica*, have been the only cyanogenic glycosides found in *Anthemis* species so far (Nahrstedt *et al.*, 1983). This is the first study showing the presence of lucumin in the genus *Anthemis* and the second study identifying this compound in a vegetative tissue.

The sesquiterpene lactone sivasinolide was previously isolated from the aerial parts of *Tanacetum argenteum* and *T. densum* (Lamiaceae) (Goeren *et al.*, 1992; Goeren & Tahtasakal, 1997), aerial parts of *Gonospermum elegans* and *G. canariense* (Asteraceae) (Triana *et al.*, 2000; Triana *et al.*, 2003), aerial parts of *Oncosiphon piluliferum* (Asteraceae) (Pillay *et al.*, 2007) and aerial parts of *Anthemis altissima* (Asteraceae) (Konstantinopoulou *et al.*, 2003). Identification of isolated compounds as sivasinolide in all published reports was based on the comparison of $^{1}H$ and $^{13}C$ NMR data with those published by Goeren *et al.* (1992). Detailed examination of the NMR spectra for compound **2** resulted in the gross structure and relative stereochemistry which has been postulated for sivasinolide. However, comparison of the NMR data showed clear discrepancies between compound **2** and sivasinolide. Thus, compound **2** and the compound published as sivasinolide appear to be different compounds. Crystallisation experiments are currently ongoing to conclusively determine

the relative stereochemistry of compound **2** and resolve whether it has the structure proposed for sivasinolide, which would imply that Goeren *et al.* (1992) have isolated a different compound.

2,6-dihydroxybenzoic acid (**3**) has previously been isolated from the roots of *Rumex japonicus* (Polygonaceae) (Jiang *et al.*, 2007), the roots of *Polygonum cuspidatum* (Polygonaceae) (Xiao *et al.*, 2002), the leaves of *Alangium platanifolium* var. *trilobum* (Alangiaceae) (Otsuka *et al.*, 1989) and the fruits of *Aniba riparia* (Lauraceae) (Barbosa-Filho *et al.*, 1987). This is the first time 2,6-dihydroxybenzoic acid was shown in the genus *Anthemis* and in the family Asteraceae.

3-carboxymethyl-indole-1-N-β-D-glucopyranoside (**4**) is a metabolite of the plant hormone indole-3-acetic acid, which is an important molecule in the regulation of plant growth and development. So far it was isolated from the fruits of *Ribes rubrum* (red currant, Grossulariaceae) (Schwarz & Hofmann, 2007) and the fruiting bodies of the basidiomycete *Cortinarius brunneus* (Teichert *et al.*, 2008). Kai *et al.* (2007) showed that this compound is present in the roots of *Oryza sativa* (Poaceae), *Arabidopsis thaliana* (Brassicaceae), *Lotus japonicus* (Fabaceae) and *Zea mays* (Poaceae). This is the first report of this compound in the genus *Anthemis* and in the family Asteraceae, and the first study identifying it in the aerial parts of a plant.

GC-MS analysis of the hexane subextract and subsequent fractions showed the presence of 37 different fatty acids, whereas in the $CHCl_3$ subextract and fractions 16 fatty acids were detected. Extensive analysis of the fatty acid composition from extracts of *Anthemis* has so far only been performed in one recent study from Orhan *et al.* (2009), where the hexane extracts of *Anthemis tinctoria* var. *tinctoria* and *A. austriaca* were examined. The major constituent identified in the flowers of *A. tinctoria* was oleic acid (18:1) with 5.8%, while in the aerial parts cerotic acid (26:0) with 8.3% is the most dominant fatty acid. For *A. austriaca* (unspecified which plant parts) only four fatty acids were found: palmitic acid (3.0%), cerotic acid (2.2%), lignoceric acid (1.3%) and stearic acid (0.9%).
In the hexane and $CHCl_3$ subextract of *A. pestalozzae* presented in this study, palmitic acid was found to be the main constituent with 57% and 52%, respectively. In most fractions this fatty acid was predominant, with four exceptions in which linoleic acid (18:2), linolenic acid (18:3) and stearic acid (18:0) showed the highest abundance.

The comparison of the biological activity with the fatty acid composition of the examined fractions suggests complex interactions within the mixtures. It is not possible to relate the observed antiplasmodial activity to the presence or absence of just one fatty acid.
Fatty acids have previously been linked with antiplasmodial activity (Kumaratilake *et al.*, 1992; Krugliak *et al.*, 1995; Tasdemir *et al.*, 2007). Linoleic acid and other polyunsaturated fatty acids have been shown to inhibit the growth of *P. falciparum* blood stage parasites *in vitro* while oleic acid and behenic acid only had little or no effect (Kumaratilake *et al.*, 1992). Palmitic acid was shown to inhibit the blood stage parasites of *P. falciparum in vitro* with an $IC_{50}$ value of 3.8 µg/ml (Tasdemir *et al.*, 2010). Another study examined the influence of the unsaturation level in $C_{18}$ fatty acids oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). All three were shown to inhibit *P. falciparum* blood stage parasites *in vitro* with $IC_{50}$ values of 23 µg/ml, 76 µg/ml and 92 µg/ml, respectively (Krugliak *et al.*, 1995). A recent work examined which structural characteristics provide the best antiplasmodial potential by examining isomeric $C_{16}$ acetylenic fatty acids and the presence and position of the triple bond in the acyl chain was found to be a vital feature (Tasdemir *et al.*, 2010).

The results of the bioactivity-guided fractionation of *A. pestalozzae* presented here, show that fractionation, isolation and identification of active principles from plant extracts represents great challenges. Good biological activity in an extract can be lost in the process of fractionation due to the loss of synergistic interactions or decomposition of active compounds (Houghton *et al.*, 2007). Thus, it is not unusual that biological activity observed in extracts cannot be assigned to specific compounds, as shown for the plant of interest in this thesis (see below). However, although the fractionation of the subextract did not lead to the identification of specific compounds responsible for the initial antiplasmodial activity and enzyme inhibition, some fractions showed a noteworthy increase in biological activity. Significant increase (up to six-times) in antiplasmodial activity was observed for fractions of the $CHCl_3$ and hexane subextracts. Overall, C3d followed by C5c from the $CHCl_3$ subextract showed the best activity against *P. falciparum* with good $IC_{50}$ value of 0.30 µg/ml and 0.79 µg/ml, respectively. GC-MS analysis of C5c showed the presence of seven fatty acids in this fraction: myristic acid (14:0), pentadecanoic acid (15:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). This suggests that the specific composition of fatty acids and their abundance in this fraction might be responsible for the observed antiplasmodial activity. However, it cannot be excluded that additional compounds are involved, as the fatty acid composition in the $CHCl_3$ subextract ($IC_{50}$ 5.60 µg/ml) is very similar to C5c. It is speculative whether the concentration of active principles or the removal of antagonistic components in the C5c

fraction play a role and cannot be answered at this point. Analysis of these fractions by LC-MS, as well as the LC-MS analysis of fraction C3d, which are currently carried out and thus could not be included into this thesis, might be able to answer this question.
In addition to the increased antiplasmodial activity observed for some fractions, the increase in the FAS-II inhibitory potential up to 16-times in fractions from the aq. MeOH subextract are also noteworthy. This is likely due to the removal of primary metabolites, such as sugars. Identification of known compounds in these fractions by LC-MS analysis is also still in progress.

As mentioned above, the bioactivity-guided fractionation of *A. pestalozzae* led to the identification of four compounds. From these compounds only lucumin (**1**) and the sesquiterpene lactone (**2**) showed antiplasmodial activity with $IC_{50}$ values of 15.31 µg/ml (35.82 µM) and 5.13 µg/ml (19.41 µM), respectively. Cytotoxicity of compound **2** ($IC_{50}$ of 4.8 µg/ml or 18.17 µM) however resulted in a low selectivity index (SI 0.9). None of the isolated compounds showed significant inhibition of plasmodial FAS-II enzymes.
Antibacterial activity and antiplasmodial activity against the chloroquine-sensitive strain D10 with an $IC_{50}$ value of 2.6 µg/ml (9.8 µM) have previously been reported for sivasinolide (Konstantinopoulou *et al.*, 2003; Pillay *et al.*, 2007). However, as it was shown that sivasinolide and compound **2** isolated in this study must be different compounds, the antiplasmodial activity cannot be compared. Altissin has been shown to have antibacterial activity, but has not been tested so far against *Plasmodium* (Konstantinopoulou *et al.*, 2003).
For lucumin (**1**) and 3-carboxymethyl-indole-1-N-β-D-glucopyranoside (**4**) no biological activities have been reported so far. Several biological studies have been performed with 2,6-dihydroxy benzoic acid (**3**) including antiviral, antibacterial and antifungal experiments, but no antiplasmodial activities have been reported (Turner *et al.*, 2004; Kalinowska *et al.*, 2008).

## 4.4 Discussion of biological activities and structure-activity relationships of natural chalcones

In this study the antiplasmodial activity of selected natural chalcones and their structure-activity relationships have been assessed for the first time.

Recently, licochalcone A and synthetic chalcone derivatives have been shown to act synergistically with artemisinin *in vitro*, opening new options for novel combinational chemotherapy against malaria (Bhattacharya *et al.*, 2009; Mishra *et al.*, 2009). This study identified 2',6',-dihydroxy-4,4'-dimethoxydihydrochalcone as an interesting candidate for future studies, as it showed next to licochalcone A the best antiplasmodial activity ($IC_{50}$ value 3.7 μM) with a similar selectivity index.

Of the seven know natural chalcones to demonstrate antiplasmodial potential, the reported $IC_{50}$ values of chalcone (47.4 μM), 4,4'-dimethoxychalcone (21.7 μM) and homobutein (15.0 μM) are in agreement with the results presented in this study (Liu *et al.*, 2001; Yenesew *et al.*, 2004; Wu *et al.*, 2006). However, the observed antiplasmodial effect for licochalcone A, phloridzin, 4'-hydroxychalcone and 4'-methoxychalcone differ from previous findings (Cabantchik *et al.*, 1983; Nielsen *et al.*, 1998b; Liu *et al.*, 2001; Liu *et al.*, 2003). One possible explanation might be the use of different *P. falciparum* strains. Licochalcone A for example, was tested against the chloroquine-susceptible strain 3D7 and reported to display an $IC_{50}$ value of 5.6 μM as compared to 1.0 μM reported here (Nielsen *et al.*, 1998b). Phloridzin was assessed against a chloroquine-resistant strain (FCR-3TC) and an $IC_{50}$ value of 16 μM reported (Cabantchik *et al.*, 1983), but a more recent study using a different chloroquine-resistant strain (W2) reported a much higher $IC_{50}$ value of 44 μM (Montenegro *et al.*, 2007). The $IC_{50}$ value obtained in this study was with 27.4 μM in between the previous reported values, which suggests variations in sensitivity between different *P. falciparum* strains. A second possible influence on the results could be the length of incubation times. Liu *et al.* (2001) incubated the parasites for 24 h before adding the radioactive hypoxanthine, while in this study the first incubation period is 48 h long to ensure exposure of the compound to all parasite stages. Thus, stage specific inhibition could explain why the observed $IC_{50}$ values in this study for 4'-hydroxychalcone (12.3 μM) and 4'-methoxychalcone (22.2 μM) are lower than the reported values of 29.6 μM for 4'-hydroxychalcone and 55.5 μM for 4'-methoxychalcone (Liu *et al.*, 2001; Liu *et al.*, 2003).

Of all tested chalcones, butein was the sole compound which has been previously assessed against plasmodial FabI and a similar result ($IC_{50}$ value of 12.5 μM as compared to 12.9 μM here) was reported (Sharma *et al.*, 2007).

Several studies have been performed with synthetic chalcone compounds to assess structure-activity relations (Li *et al.*, 1995; Liu *et al.*, 2001; Liu *et al.*, 2003; Lim *et al.*, 2007). The advantage of such studies for drug development is that specific modifications can be made revealing the importance of different positions in the rings. Due to the limited number of naturally occurring chalcones that are commercially available, such specific modifications were not obtainable, making it difficult to draw clear structure-activity relations. However, it was possible to conclude some general trends from the obtained results.

The first observation was substitution of chalcone with a single hydroxyl group in ring B led to inhibition of the FAS-II enzyme FabZ, while single hydroxylation in ring A did not result in any enzyme inhibition. Multiple hydroxylation in both rings resulted in increased enzyme inhibition activity, especially for chalcones with a hydroxyl group at position C-3 (3,4,2',4',6'-pentahydroxychalcone and 3,4,2',4'-tetrahydroxychalcone). The position of the sugar unit might also play a role in observed enzyme inhibition activity, as phloridzin (2'-glycoside) showed no enzyme inhibition while marein (4'-glycoside) inhibited FabZ with an $IC_{50}$ value of 13.3 μM.

The only observations regarding cytotoxicity structure-activity relationships were that α,β-saturated chalcones and chalcone-glycosides showed reduced cytotoxicity.

Any substitution on chalcone with one or more hydroxyl and or methoxyl groups led to an increase of antiplasmodial activity. The exception is the single methoxylation on the B ring (4-methoxychalcone), but if this reduced antiplasmodial activity is due to the position C-4 or generally true for single methoxylation on the B ring cannot be answered at this stage. Another observation was, that the hydroxylation of C-6' on the A ring seems to be accompanied with a loss of antiplasmodial activity. Two examples are 2'-hydroxy-4,4',6'-trimethoxychalcone (10.8 μM) and butein (9.6 μM) which showed $IC_{50}$ values of 31.6 μM (2',6'-dihydroxy-4,4'-dimethoxychalcone) and 37.2 μM (eriodictyolchalcone) after hydroxylation at the C-6' position, respectively. As a third trend for antiplasmodial structure-activity relationships, it was observed that α,β-saturated chalcones showed improved antiplasmodial activity. This was most pronounced for 2',6'-dihydroxy-4,4'-dimethoxydihydrochalcone with an $IC_{50}$ value of 3.7 μM, which is roughly a ten-fold increase in potency compared to the observed activity for 2',6'-dihydroxy-4,4'-dimethoxychalcone (31.6 μM). Phloretin, another dihydrochalcone in this study, showed an $IC_{50}$ value of 14.2 μM, which is marginally lower compared to isoquiritigin (17.9 μM). However, with the additional hydroxyl group on the position C-6' in phloretin, a decrease in activity would have been expected.

As mentioned above, several structure-activity studies have been performed with synthetic chalcones. Some studies looked into the importance of the α,β-double bond with contradicting results. Li *et al.* (1995) evaluated over 200 synthetic chalcones and concluded that the α,β-unsaturated ketone bridge is essential for antimalarial activity because of an at least 10-fold decrease in the antimalarial effect in α,β-saturated compounds. Another report claimed that modifications of the double bond have no significant effect on the antiprotozoal activity and showed that licochalcone A and its dihydro derivative display similar antiplasmodial potential (Nielsen *et al.*, 1998b). Both findings differ from the observations in this study, which implies that α,β-saturated compounds are favoured. It is possible that synthetic chalcones with chloro, fluoro or other substitutions show a different preference when compared with natural chalcones.

This is the first study investigating the malaria prophylactic potential of a panel of natural chalcones through inhibition of FAS-II enzymes as potential drug targets. It has shown that licochalcone A, eriodictyolchalcone, homobutein and marein demonstrate good activity against the FAS-II enzyme FabZ with low cytotoxicity. The assessment of selected natural chalcones against liver stage parasites is ongoing.

Licochalcone A was recently described to be highly active *in vitro* against the liver stages of *P. yoelii* with an $IC_{50}$ value of 0.9 nM and an $IC_{50}$ value of 6.3 nM against mouse hepatocytes in cytotoxicity studies (Mahmoudi *et al.*, 2008). The comparison of the activity against liver stage parasites with the FAS-II enzyme inhibition obtained in this study, suggests the antiplasmodial effect against liver stage parasites does not solely result from inhibition of the fatty acid biosynthesis, as licochalcone A inhibits FabZ with an $IC_{50}$ value of 6.5 μM, which is more than 7000-times less potent than the reported activity against liver stage parasites.

## 4.5 Discussion of biological activities of lichen secondary metabolites

The lichen secondary metabolites evernic acid, vulpic acid, psoromic acid and (+)-usnic acid were evaluated for their potential in malaria chemotherapy (*P. falciparum* blood stage) and prophylaxis (*P. yoelii* liver stage). Additionally, selected FAS-II enzymes were tested as potential targets in malaria liver stage parasites.

The activity against the blood stage *P. falciparum* parasites for evernic acid, vulpic acid and psoromic acid is reported for the first time. The previously described antiplasmodial activity of (+)-usnic acid against blood stage parasites of the K1 strain ($IC_{50}$ 15.3 μM) is slightly better than the observed value in this study ($IC_{50}$ 47.3 μM) (Verotta *et al.*, 2007).
All lichen compounds showed activity against liver stage parasites in at least one of the assays (see 3.5.2). The results acquired by the different assays showed incongruity. This can be explained by the aim and nature of both assays. In FC the infection rate was assessed, *i.e.* how many cells are infected after exposure to the compounds. In the IFA the effect of the compounds on the morphology and development of liver stage parasites were determined. As the effect of a compound on the liver stage development, by comparing the reduction in liver parasite sizes, is a more sensitive method, the $IC_{50}$ values found in the IFA are used as the basis for the discussion of liver stage inhibition.
Evernic acid, psoromic acid and (+)-usnic acid showed good inhibition of parasite liver stage development. However, for the latter two cytotoxicity against hepatocytes was observed, resulting in narrow therapeutic indices for these compounds.

Of the four tested lichen metabolites, evernic acid was the only one without any relevant cytotoxicity against human cancer cells and human hepatocytes. This observation is in agreement with recent findings from Burlando *et al.* (2009) who found a low toxic profile for evernic acid against two human tumour cell lines. In the same study they showed that (+)-usnic acid and vulpic acid possessed strong and moderate cytotoxicity, respectively. Cytotoxicity of (+)-usnic acid against several murine and human cancer cell lines has been reported before (Bazin *et al.*, 2008). The evaluation of data obtained in this study for the human carcinoma cell line KB resulted in a slightly different cytotoxicity profile of the compounds. Here, psoromic acid presented the highest cytotoxicity, followed by vulpic acid and (+)-usnic acid.
However, the observed cytotoxicity trends of the compounds in hepatocytes showed a different picture, with highest cytotoxicity for (+)-usnic acid, followed by psoromic acid. These observations are in agreement with previously published data reporting hepatotoxicity for (+)-usnic acid in rats and rat primary hepatocytes (Pramyothin *et al.*,

2004) and the finding that psoromic acid induced apoptotic activities in primary rat hepatocytes in sub-cytotoxic concentrations (Correche *et al.*, 2004).

Evernic acid was identified as a highly interesting compound. It showed inhibitory activity against the development of liver stage parasites with an $IC_{50}$ value of 19.5 μM, which correlates well with the inhibition of the FAS-II enzyme FabZ with a similar potency (10.7 μM). Investigation of the inhibition kinetic, revealed that evernic acid is a competitive inhibitor of FabZ with respect to the substrate crotonoyl-CoA. This indicates that evernic acid and the substrate have the same binding site in the free enzyme and bind in a mutually exclusive way. Modelling experiments to obtain further insights into the binding of this compounds are currently being performed in collaboration with Dr. M. Zloh from the School of Pharmacy.
The similar activity of evernic acid against liver stage parasites and the FAS-II enzyme FabZ, indicate that the activity against the liver stage parasites results from the inhibition of this FAS-II enzyme. In addition, the absence of cytotoxicity against hepatocytes and low cytotoxicity against KB cells make this compound very interesting for further studies as potential prophylactic drug lead.

The low potency of evernic acid against blood stage parasites is noteworthy ($IC_{50}$ 142.1 μM). One could argue that this is a disadvantage and a compound that is active against both stages would be preferred, as it subsequently can kill arisen blood stage parasites if not all liver stage parasites were successfully removed by the drug. On the other hand, the risk of resistance development against a drug inhibiting both stages is much higher. The replication cycle of *P. falciparum* in the blood takes 48 h and results in millions of parasites within a short period of time. In the liver only few dozens of parasite initiate the infection and undergo one multiplicative expansion which takes several days to complete (about 1-2 weeks) (Singh *et al.*, 2009). Thus, a drug solely targeting liver stage parasites would have to deal with a much smaller parasite load and has more time to kill the parasites before the cells burst and parasite numbers increase.

So far, only very few natural products have been identified as inhibitors of liver stage parasites. Carraz *et al.* (2006) isolated the morphinan alkaloid tazopsine from *Strychnopsis thouarsii* (Menispermaceae) stem bark. They reported the inhibition of liver stage development in primary hepatocytes infected with *P. yoelii* and *P. falciparum in vitro* with $IC_{50}$ values of 3.1 μM and 4.2 μM, respectively. Blood stages of *P. falciparum* were inhibited with a similar potency resulting in $IC_{50}$ values of 4.7 μM against the chloroquine-sensitive strain

3D7 and 5.7 μM against the chloroquine-resistant strain FCR3. Cytotoxicity was reported with $IC_{50}$ values of 43.7 μM for mouse hepatocytes (*P. yoelii*) and 28.4 μM for human hepatocytes (*P. falciparum*). *In vivo* experiments demonstrated 70% protection of mice against *P. yoelii* infection treated with 100 mg/kg tazopsine daily for 4 d (>100 mg/kg toxic). With few relatively easy chemical modifications Carraz *et al.* (2006) developed a semi-synthetic derivative of tazopsine, NCP-tazopsine, which showed reduced toxicity and was exclusively effective against parasites in the liver stage. And even though they found a 10-fold decrease in the antiparasitic effect against *P. falciparum* infected hepatocytes, they showed that the derivative prevented *P. yoelii* infected mice to develop blood stage infection.

Mahmoudi *et al.* (2008) developed a prediction model for liver stage activity and they tested selected heterogeneous drug molecules. Among them was licochalcone A, a natural chalcone, which is discussed in the previous section of this thesis (4.4).

In addition to these natural products, extracts from *Triphyophyllum peltatum* (Dioncophyllaceae), *Ancistrocladus abbreviates* and *A. tectorius* (Ancistrocladaceae) were shown to inhibit *P. berghei* liver stage parasites *in vitro* (Francois *et al.*, 1997).

As described in the introduction (1.1.3.2), to date only three synthetic compounds have been identified as *in vitro* liver stage inhibitors with inhibitory activity towards FAS-II enzymes. The first compound identified was hexachlorophene, which inhibits FabG with an $IC_{50}$ value of 2.1 μM (Wickramasinghe *et al.*, 2006) and showed a similar potential against blood stage parasites ($IC_{50}$ value 6.2 μM) (Wickramasinghe *et al.*, 2006) and liver stage parasites ($IC_{50}$ value 4.8 μM) (Tarun *et al.*, 2008). Recently, triclosan, which inhibits plasmodial FabI with an $IC_{50}$ value of 0.05 μM, was shown to inhibit the liver stage development (Singh *et al.*, 2009). Singh *et al.* (2009) assessed the inhibition over three days when triclosan was added 24 h before the infection ($IC_{50}$ 6.8 μM), and over two days when triclosan was added after the infection of cells ($IC_{50}$ 39.4 μM). The potential against blood stage parasites was shown *in vitro* ($IC_{50}$ 0.7 μM) and *in vivo* (complete clearance with a single injection of 38 mg/kg) (Surolia & Surolia, 2001). Very recently, our group demonstrated that the synthetic acetylenic fatty acid 2-hexadecynoic acid (2-HDA) inhibited liver stage development with an $IC_{50}$ value of 60.7 μM (FC) and 19.4 μM (IFA) (Tasdemir *et al.*, 2010). The same study determined the inhibitory potential of 2-HDA against FAS-II enzymes with $IC_{50}$ values of 1.5 μM against FabI, 2.3 μM against FabZ and 13.9 μM against FabG. Antiplasmodial activity against blood stage parasites was shown with an $IC_{50}$ value of 41.3 μM.

With evernic acid, this study was able to identify a fourth compound and the first natural product with liver stage activity with the FAS-II system as potential target. In addition, it is the first compound with a much lower antiplasmodial potential against blood stage parasites compared to the other FAS-II liver stage inhibitors.

# Part 5

# Summary and conclusion

## 5.1 Summary and conclusion

In this study the antimalarial and malarial prophylactic potential of selected Turkish plants and pure natural products was investigated.

The crude extracts of all plants (*A. pestalozzae*, *A. cretica* subsp. *anatolica*, *S. lucida*, *S. pinardii*, *S. virgata*) showed an effect against *P. falciparum* blood stage parasites, which was most pronounced in the hexane or $CHCl_3$ subextracts. In an in-depth phytochemical investigation of *Anthemis pestalozzae*, four compounds could be isolated: lucumin, 2,6-dihydroxybenzoic acid, 3-carboxymethyl-indole-1-N-β-D-glucopyranoside and a sesquiterpene lactone. The first three compounds were identified for the first time in the genus *Anthemis*. The sesquiterpene lactone was identified to possess the identical gross structure and relative stereochemistry which was proposed for sivasinolide, however the comparison of NMR data for the compound isolated in this study with the NMR data for sivasinolide showed that the two compounds are likely to be different compounds. The sesquiterpene lactone showed the best antiplasmodial potential against blood stage parasites, followed by lucumin. However, cytotoxicity for the former compound resulted in a low selectivity index. No significant plasmodial FAS-II enzyme inhibition was observed. An extensive analysis of the fatty acid composition of *A. pestalozzae* identified 37 fatty acids in subsequent fractions from the hexane and chloroform subextracts. Although no correlation could be shown between the fatty acid composition of mixtures and observed antiplasmodial activities, a good effect against *P. falciparum* parasites was demonstrated for several fractions, highlighting the potential of fatty acids in antimalarial drug development.

The lichen metabolites evernic acid, vulpic acid, psoromic acid and (+)-usnic acid showed moderate inhibition against the blood stage parasites *P. falciparum* for all compounds. Good inhibition against the liver stage development of *P. yoelii* was demonstrated for evernic acid, psoromic acid and (+)-usnic acid. Cytotoxicity against hepatocytes was found for psoromic and (+)-usnic acids resulting in low selectivity indices. Evernic acid was identified as the first natural product with liver stage inhibition and the FAS-II enzyme FabZ, for which it is a competitive inhibitor, as possible target.

In a third approach, 22 natural chalcones were assessed for their antiplasmodial potential and all compounds demonstrated inhibition against *P. falciparum* blood stage parasites; for 11 compounds significant cytotoxicity was shown. In addition to the well known antimalarial chalcone licochalcone A, a second natural chalcone (2',6'-dihydroy-4,4'-dimethoxydihydrochalcone) was identified with a good inhibition profile. Four chalcones

(butein, homobutein, eriodictyolchalcone and licochalcone A) were identified as remarkable inhibitors of the FAS-II enzyme FabZ. Structure-activity relationship studies were performed, and despite the limited number of available compounds, the following conclusions could be drawn: I) It was observed that any modification to chalcone, with the exception of single methoxylation on the B ring, resulted in an increase of antiplasmodial potential. II) The hydroxylation of position C-6' in ring A seemed to be less favourable and resulted in lower antiplasmodial activity. III) The results suggest that α,β-saturated chalcones show increased antiplasmodial activity and reduced cytotoxicity. IV) Hydroxylation in ring B or multiple hydroxylation in both rings resulted in increased enzyme inhibition activity. V) Glycosidation resulted in lower cytotoxicity and the position of the sugar unit might have an influence on the enzyme inhibition activity. However, investigation of a larger number of chalcone derivatives is required to confirm and expand these observed trends.

Natural product research can be performed by investigating biologically active plants and isolate and identify their active principles, or by the investigation of a known biologically active group of natural products from a selected organism, family or species or according to their chemical class. In this study both approaches, the screening and investigation of Turkish plants and the investigation of selected natural products from lichens and the chemical group of chalcones, were applied in order to maximise the possibility to identify new compounds with antimalarial potential.

Both approaches however, have their advantages and disadvantages. The phytochemical investigation of a plant is time intense and it might not be possible to identify a compound responsible for the activity. On the other hand, without this type of study of plants (or microorganisms, marine organisms, fungi...), drug discovery would stagnate soon as more than 50% of all new drugs approved within the last 25 years were natural products, derived from natural products or mimic compounds based on phamacophores related to natural products (Newman & Cragg, 2007). It is impossible to imagine how our lives would be, if all drugs with a background in natural products were gone. By studying known natural products from the same origin or chemical class, it might be possible to identify more potent compounds and draw conclusions from the structure to the activity. This might help in the development of synthetic and semi-synthetic derivatives with increased biological potential, bioavailability or reduced cytotoxicity.

This study demonstrates promising results for both approaches, as all plants, lichen compounds and chalcones exhibited an antiplasmodial effect. Although the *in vivo* potential

of interesting compounds has not yet been examined, the sesquiterpene lactone sivasinolide from *A. pestalozzae*, evernic acid as potential prophylactic compound with a suggested target and 2',6'-dihydroxy-4,4'-dimethoxydihydrochalcone were identified as new good antimalarial compounds. In addition, these compounds have different structures compared to the available antimalarial drugs, which due to the resistance development, is an important feature in the search for potential drugs in the fight against malaria.

## 5.2 Future work

All plant extracts from the initial screening showed antiplasmodial and enzyme inhibition potential and a bioactivity-guided fractionation to identify and isolate the active principles could be the starting point for future studies.

Future work regarding the investigation of *Anthemis pestalozzae* should include the testing of compounds identified by LC-MS from active fractions to determine their contribution to the observed activity of these fractions. Despite the lack of significant activity of the isolated compounds against plasmodial FAS-II enzymes, these compounds could be tested against liver stage parasites to assess their prophylactic potential as the fatty acid biosynthesis is only one potential target in liver stage parasites.

With the results from the chalcone screening, an interesting task for future studies would be to test other chalcones with different hydroxylation and methoxylation pattern and their dihydro derivatives to verify the observed trends and investigate them in more detail.
In addition, the findings from the enzyme inhibition studies could provide a basis for studies assessing the potential of eriodictyolchalcone, homobutein, marein and other natural chalcones against *Plasmodium* liver stages to examine their prophylactic potential.

To validate evernic acid as the first natural product active against *Plasmodium* liver stages with a potential target, the next step would be to investigate the effect of evernic acid *in vitro* against *P. falciparum* infected hepatocytes and *in vivo* in *P. yoelii* infected mice. Modelling studies could provide further insights into the binding mode of this compound to FabZ and physiochemical properties. Future work should also include the synthesis of derivatives of evernic acid, psoromic acid and (+)-usnic acid by a medicinal chemistry approach in order to reduce toxicity and increase antiplasmodial activity against liver stage parasites.

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Table A1. Table of sesquiterpene lactones from the genus *Anthemis*. [a] relative stereochemistry, A: aerial parts, F: flowers, L: leaves, R: roots

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| (1R,3S,6S,7R,8R)[a]-1-hydroperoxy-3-acetoxy-8-hydroxygermacra-4Z,10(14),11(13)-trien-6,12-olide | *A. aetnensis* | Sicily | A | (Bruno *et al.*, 1997) |
| (1R,3S,6S,7R,8R)[a]-1-hydroxy-3-acetoxy-8-hydroxygermacra-4Z,10(14),11(13)-trien-6,12-olide | *A. aetnensis* | Sicily | A | (Bruno *et al.*, 1997) |
| (1R,5R,6R,7S,8R,9S,10S)[a]-8,9,10-trihydroxyguaia-3,11(13)-dien-6,12-olide | *A. plutonia* | Cyprus | A | (Bruno *et al.*, 1998) |
| (1S,2R,5R,6R,7S,8R,9R,10S)[a]-,8,9,10-Tetrahydroxyguia-3,11(13)-dien-6,12-olide | *A. alpestris* | Spain | A | (Bruno *et al.*, 2002) |
| (1S,2R,5R,6R,7S,8R,9R,10S)[a]-10-hydroxy-2,8,9-triacetoxyguaia-3,11(13)-dien-6,12-olide | *A. aetnensis*<br>*A. cretica* subsp. *cretica* | Sicily<br>Serbia | A<br>A | (Bruno *et al.*, 1997)<br>(Vajs *et al.*, 1999) |
| (1S,2R,5R,6R,7S,8R,9R,10S)[a]-2,10-dihydroxy-8-angeloxy-9-acetoxyguia-3,11(13)-dien-6,12-olide | *A. alpestris* | Spain | A | (Bruno *et al.*, 2002) |
| (1S,2R,5R,6R,7S,8R,9R,10S)[a]-2,9-diacetoxy-8,10-dihydroxyguaia-3,11(13)-dien-6,12-olide | *A. alpestris*<br>*A. aetnensis*<br>*A. cretica* subsp. *cretica* | Spain<br>Sicily<br>Serbia | A<br>A<br>A | (Bruno *et al.*, 2002)<br>(Bruno *et al.*, 1997)<br>(Vajs *et al.*, 1999) |
| (1S,2R,5R,6R,7S,8R,9R,10S)[a]-2-acetoxy-8,10-dihydroxy-9-(2-methylbutyryloxy)-guaia-3,11(13)-dien-6,12-olide | *A. alpestris*<br>*A. aetnensis*<br>*A. cretica* subsp. *cretica* | Spain<br>Sicily<br>Serbia | A<br>A<br>A | (Bruno *et al.*, 2002)<br>(Bruno *et al.*, 1997)<br>(Vajs *et al.*, 1999) |
| (1S,2R,5R,6R,7S,8R,9R,10S)[a]-2-acetoxy-8,10-dihydroxy-9-(2-methylpropanoyloxy)-guaia-3,11(13)-dien-6,12-olide | *A. alpestris*<br>*A. aetnensis*<br>*A. cretica* subsp. *cretica* | Spain<br>Sicily<br>Serbia | A<br>A<br>A | (Bruno *et al.*, 2002)<br>(Bruno *et al.*, 1997)<br>(Vajs *et al.*, 1999) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| (1S,2R,5R,6R,7S,8R,9R,10S)[a]-2-acetoxy-8,10-dihydroxy-9-isovaleryloxyguaia-3,11(13)-dien-6,12-olide | *A. alpestris*<br>*A. aetnensis*<br>*A. cretica* subsp. *cretica* | Spain<br>Sicily<br>Serbia | A<br>A<br>A | (Bruno *et al.*, 2002)<br>(Bruno *et al.*, 1997)<br>(Vajs *et al.*, 1999) |
| (1S,5R,6R,7S,8R,9R,10S)[a]-2,9-diacetoxy-10-hydroxy-8-(2-methylbutyryloxy)-guaia-3,11(13)-dien-6,12-olide | *A. aetnensis* | Sicily | A | (Bruno *et al.*, 1997) |
| (1S,5R,6R,7S,8R,9R,10S)[a]-2,9-diacetoxy-10-hydroxy-8-(isovaleryloxy)-guaia-3,11(13)-dien-6,12-olide | *A. aetnensis* | Sicily | A | (Bruno *et al.*, 1997) |
| (1S,5R,6R,7S,8R,9R,10S)[a]-9-acetoxy-2,8,10-trihydroxyguaia-3,11(13)-dien-6,12-olide | *A. aetnensis* | Sicily | A | (Bruno *et al.*, 1997) |
| (4S,5R,6S,7R,8S)-8-(2-methylbutanoyloxy)-4,5-epoxygermacra-1(10),11(13)-dien-6,12-olide | *A. punctata* subsp. *cupaniana* | Sicily | A | (Bruno *et al.*, 1991) |
| (4S,5R,6S,7R,8S)-8-angeloxy-4,5-epoxygermacra-1(10),11(13)-dien-6,12-olide | *A. punctata* subsp. *cupaniana*<br>*A. macedonica* | Sicily<br>Bulgaria | A<br>A | (Bruno *et al.*, 1991)<br>(Staneva *et al.*, 2006) |
| (4S,5R,6S,7R,8S)-8-isobutyryloxy-4,5-epoxygermacra-1(10),11(13)-dien-6,12-olide | *A. punctata* subsp. *cupaniana* | Sicily | A | (Bruno *et al.*, 1991) |
| (4S,5R,6S,7R,8S)-8-isovaleryloxy-4,5-epoxygermacra-1(10),11(13)-dien-6,12-olide | *A. punctata* subsp. *cupaniana* | Sicily | A | (Bruno *et al.*, 1991) |
| (4S,5R,6S,7R,8S)-8-tigloxy-4,5-epoxygermacra-1(10),11(13)-dien-6,12-olide | *A. punctata* subsp. *cupaniana* | Sicily | A | (Bruno *et al.*, 1991) |
| (4S,5R,6S,7S,9R,11R)-9-acetoxy-4,5-epoxygermacra-1(10)-en-6,12-olide | *A. punctata* subsp. *cupaniana* | Sicily | A | (Bruno *et al.*, 1991) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| (5R + 5S) 5-hydroxy-5,6-dihydro-6,13-dehydro-antheindurolide A | *A. arvensis* | Serbia | A | (Vuckovic *et al.*, 2006b) |
| (5R + 5S)-5-hydroperoxy-5,6-dihydro-6,13-dehydro-antheindurolide A | *A. arvensis* | Serbia | A | (Vuckovic *et al.*, 2006b) |
| (5R,6R+5S,6S) antheindurolide A-5,6-oxide | *A. arvensis* | Serbia | A | (Vuckovic *et al.*, 2006b) |
| (6R or 6S) 6-hydroxy-5,6-dihydro-4,5-dehydro-antheindurolide A | *A. arvensis* | Serbia | A | (Vuckovic *et al.*, 2006b) |
| (6R+6S) (4E) 6-hydroperoxy-5,6-dihydro-4,5-dehydro-antheindurolide A | *A. arvensis* | Serbia | A | (Vuckovic *et al.*, 2006b) |
| (E)-1α,10β-epoxy-3β-acetoxy-6α-hydroxygermacra-4,11(13)-dien-12,8α-olide | *A. carpatica* | Serbia | A | (Vajs *et al.*, 2000) |
| 1,10-epoxynobilin | *A. nobilis* | not given | F | (Holub & Samek, 1977) |
| 1,10-epoxyparthenolide | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989) |
| 10α-hydroxy-11β,13-dihydroxerantholide | *A. austriaca* | Bulgaria | A | (Staneva *et al.*, 2004) |
| 10α-hydroxy-9α-acetoxyguaia-3,11(13)-dien-6,12-olide | *A. carpatica* | Bulgaria | A | (Staneva *et al.*, 2002) |
| 1-*epi*-tatridin B | *A. wiedemanniana*<br>*A. altissima*<br>*A. melanolepis* | Turkey<br>Greece<br>Greece | A<br>A<br>A | (Celik *et al.*, 2005)<br>(Konstantinopoulou *et al.*, 2003)<br>(Saroglou *et al.*, 2010) |
| 1α,10β-epoxy-6α-hydroxy-1,10H-inunolide | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| 1α,10β-epoxy-8β-hydroxygermacra-4(15),11(13)-dien-12,6α-olide (melanolepin C) | *A. melanolepis* | Greece | A | (Skaltsa *et al.*, 2006) |
| 1α,4β,8α-trihydroxyeudesm-11(13)en-12,6α-olide (melanolepin B) | *A. melanolepis* | Greece | A | (Skaltsa *et al.*, 2006) |
| 1α-hydroxydeacetylirinol 4α,5β-epoxide | *A. melanolepis* | Greece | A | (Skaltsa *et al.*, 2006) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| 1α-hydroxyxerantholide | *A. austriaca* | Bulgaria | A | (Staneva *et al.*, 2004) |
| 1β,4α,6α-trihydroxy-eudesm-11-en-8α-12-olide | *A. melanolepis* | Greece | A | (Saroglou *et al.*, 2007b) |
| 2α-hydroperoxy-8-*O*-isobutyryl-9α-acetoxycumambrin B | *A. carpatica* | Serbia | A | (Bulatovic *et al.*, 1997; Vajs *et al.*, 2000) |
| 2β-hydroxyepiligustrin | *A. carpatica* | Serbia | A | (Vajs *et al.*, 2000) |
| 3-dehydronobilin | *A. nobilis* | not given | F | (Holub & Samek, 1977) |
| 3-epinobilin | *A. nobilis* | not given | F | (Holub & Samek, 1977) |
| 3α-hydroxy-8α-isobutyryloxyreynosin | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1988) |
| 3α-hydroxy-8α-methacryloyloxyreynosin | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1988) |
| 3β-acetoxycostunolide | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989) |
| 3β-hydroxycostunolide | *A. melampodina*<br>*A. ruthenica* | Egypt<br>Hungary | A<br>A | (El-Alfy *et al.*, 1989; Sarg *et al.*, 1990)<br>(Hajdu *et al.*, 2010) |
| 4-hydroxyanthecotuloide | *A. auriculata* | Greece | A | (Theodori *et al.*, 2006) |
| 4-*O*-acetylanthecotuloide = 4-acetoxyanthecotulide | *A. auriculata* | Greece | A | (Theodori *et al.*, 2006) |
| 5,8-dehydro-4-oxonerolidol | *A. austriaca* | Turkey | A | (Bohlmann *et al.*, 1974) |
| 5-hydroperoxy-6,13-dehydro-5,6-dihydroanthecotuloide | *A. cotula* | Bulgaria<br>Serbia | A<br>A | (Staneva *et al.*, 2005)<br>(Vuckovic *et al.*, 2006a) |
| 5-oxo-6,13-dehydro-5,6-dihydroanthecotuloide | *A. cotula* | USA<br>Bulgaria<br>Serbia | A<br>A<br>A | (Baruah *et al.*, 1985)<br>(Staneva *et al.*, 2005)<br>(Vuckovic *et al.*, 2006a) |
| 5-oxo-6,7 Z-dehydro-5,6-dihydroanthecotuloide | *A. cotula* | USA<br>Bulgaria | A<br>A | (Baruah *et al.*, 1985)<br>(Staneva *et al.*, 2005) |
| 6-deacetyl-β-cyclopyrethrosin | *A. melanolepis* | Greece | A | (Saroglou *et al.*, 2010) |
| 6,7 E-dehydro-5,6-dihydroanthecotuloide | *A. cotula* | Bulgaria | A | (Staneva *et al.*, 2005) |
| 6,7 Z-dehydro-5,6-dihydroanthecotuloide | *A. cotula* | USA<br>Bulgaria | A<br>A | (Baruah *et al.*, 1985)<br>(Staneva *et al.*, 2005) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| 6-hydroxy-4,5-dehydro-5,6-dihydroanthecotuloide | *A. cotula* | Serbia | A | (Vuckovic *et al.*, 2006a) |
| 6α-hydroxyxerantholide | *A. austriaca* | Bulgaria | A | (Staneva *et al.*, 2004) |
| 8,9-di-*O*-acetylanthemolide B | *A. cretica* subsp. *cretica*<br>*A. carpatica* | Serbia<br>Serbia | A<br>A | (Vajs *et al.*, 1999)<br>(Bulatovic *et al.*, 1997) |
| 8-acetoxy-4-oxonerolidol | *A. austriaca* | Turkey | A | (Bohlmann *et al.*, 1974) |
| 8-deoxy-9-*O*-acetylanthemolide B | *A. carpatica* | Serbia | A | (Vajs *et al.*, 2000) |
| 8-deoxycumambrin B | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989; Zaghloul *et al.*, 1989) |
| 8-*O*-angeloyl-9-*O*-acetylanthemolide B | *A. cretica* subsp. *cretica* | Serbia | A | (Vajs *et al.*, 1999) |
| 8-*O*-dihydroanthecotuloide | *A. cotula* | USA<br>Bulgaria<br>Serbia | A<br>A<br>A | (Baruah *et al.*, 1985)<br>(Staneva *et al.*, 2005)<br>(Vuckovic *et al.*, 2006a) |
| 8-*O*-isobutyryl-9-*O*-acetylanthemolide B | *A. cretica* subsp. *cretica*<br>*A. carpatica* | Serbia<br>Serbia | A<br>A | (Vajs *et al.*, 1999)<br>(Bulatovic *et al.*, 1997; Vajs *et al.*, 2000) |
| 8-*O*-isobutyryl-9α-acetoxycumambrin B | *A. carpatica* | Bulgaria<br>Serbia | A<br>A | (Staneva *et al.*, 2002)<br>(Vajs *et al.*, 2000) |
| 8-*O*-isobutyrylfuscatin | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1988) |
| 8-*O*-methacryloylfuscatin | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1988) |
| 8-*O*-tigloyl-9-*O*-acetylanthemolide B | *A. carpatica* | Serbia | A | (Vajs *et al.*, 2000) |
| 8-*O*-tigloyl-9α-acetoxycumambrin B | *A. carpatica* | Bulgaria | A | (Staneva *et al.*, 2002) |
| 8α-acetoxy-4α-hydroxyguaia-1(10),2,11(13)-trien-12,6α-olide | *A. segetalis* | Montenegro | A | (Vuckovic *et al.*, 2010) |
| 8α-acetoxy-4β-hydroxyguaia-1(10),2,11(13)-trien-12,6α-olide | *A. segetalis* | Montenegro | A | (Vuckovic *et al.*, 2010) |
| 8α-acetylanthemolide B (8-*O*-acetylanthemolide B) | *A. cretica* subsp. *cretica*<br>*A. carpatica* | Serbia<br>Serbia | A<br>A | (Vajs *et al.*, 1999)<br>(Bulatovic *et al.*, 1997) |
| 8α-angeloyloxydouglanin | *A. carpatica* | Bulgaria | A | (Staneva *et al.*, 2002) |
| 8α-hydroxydouglanin | *A. carpatica* | Bulgaria | A | (Staneva *et al.*, 2002) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| 8α-isobutyryloxyanthemolide A | *A. carpatica* | Serbia | A | (Vajs *et al.*, 2000) |
| 8α-isobutyryloxyanthemolide C | *A. carpatica* | Serbia | A | (Vajs *et al.*, 2000) |
| 8α-isobutyryloxyarmefolin | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1986) |
| 8α-isobutyryloxyarmexifolin | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1986) |
| 8α-isobutyryloxydouglanin | *A. carpatica* | Bulgaria | A | (Staneva *et al.*, 2002) |
| 8α-isobutyryloyloxybalchanin | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1986) |
| 8α-methacryloyloxyarmefolin | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1986) |
| 8α-methacryloyloxyarmexifolin | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1986) |
| 8α-methacryloyloxybalchanin | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1986) |
| 8α-propionyloxyanthemolide C | *A. carpatica* | Serbia | A | (Vajs *et al.*, 2000) |
| 8α-tigloyloxyanthemolide C | *A. carpatica* | Serbia | A | (Vajs *et al.*, 2000) |
| 8α-tigloyloxydouglanin | *A. carpatica* | Bulgaria | A | (Staneva *et al.*, 2002) |
| 8α-xydroxyxeranthemolide | *A. austriaca* | Bulgaria | A | (Staneva *et al.*, 2004) |
| 9-*O*-acetylanthemolide B | *A. carpatica* | Serbia | A | (Bulatovic *et al.*, 1997) |
| 9-*O*-deacetylanthemolide D | *A. carpatica* | Serbia | A | (Vajs *et al.*, 2000) |
| 9α-acetoxycumambrin A | *A. carpatica* | Bulgaria<br>Serbia | A<br>A | (Staneva *et al.*, 2002)<br>(Bulatovic *et al.*, 1997; Vajs *et al.*, 2000) |
| 9α-acetoxycumambrin B | *A. plutonia*<br>*A. carpatica*<br><br>*A. cretica* subsp. *cretica* | Cyprus<br>Serbia<br>Bulgaria<br>Serbia | A<br>A<br>A<br>A | (Bruno *et al.*, 1998)<br>(Bulatovic *et al.*, 1997)<br>(Staneva *et al.*, 2002)<br>(Vajs *et al.*, 1999) |
| 9α-acetoxyparthenolide | *A. stribrnyi* subsp. *tracica*<br>*A. macedonica*<br>*A. cretica* subsp. *tenuiloba*<br>*A. punctata* subsp. *cupaniana* | Bulgaria<br>Bulgaria<br>Turkey<br>Sicily | A<br>A<br>A<br>A | (Staneva *et al.*, 2006)<br>(Staneva *et al.*, 2006)<br>(Bohlmann & Zdero, 1975)<br>(Bruno *et al.*, 1991) |
| 9α-hydroxyparthenolide | *A. punctata* subsp. *cupaniana* | Sicily | A | (Bruno *et al.*, 1991) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| 9α-hydroxycumambrin A | *A. plutonia* | Cyprus | A | (Bruno *et al.*, 1998) |
| | *A. carpatica* | Serbia | A | (Bulatovic *et al.*, 1997) |
| | | Bulgaria | A | (Staneva *et al.*, 2002) |
| | *A. cretica* subsp. *cretica* | Serbia | A | (Vajs *et al.*, 1999) |
| acetylisospiciformin | *A. stribrnyi* subsp. *tracica* | Bulgaria | A | (Staneva *et al.*, 2006) |
| altissin | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| anthecotuloide | *A. auriculata* | Greece | A | (Theodori *et al.*, 2006) |
| | *A. cotula* | Not given | A | (Bohlmann *et al.*, 1969) |
| | | USA | A | (Baruah *et al.*, 1985) |
| | | Bulgaria | A | (Staneva *et al.*, 2005) |
| | | Serbia | A | (Vuckovic *et al.*, 2006a) |
| anthecotuloide-5,6-oxide | *A. cotula* | Serbia | A | (Vuckovic *et al.*, 2006a) |
| anthecularin | *A. auriculata* | Greece | A | (Theodori *et al.*, 2006) |
| antheindurolide A | *A. arvensis* | Serbia | A | (Vuckovic *et al.*, 2006b) |
| | *A. cotula* | Bulgaria | A | (Staneva *et al.*, 2005) |
| | *A. indurata* | Egypt | A | (Abou El-Ela *et al.*, 1990) |
| | *A. pseudocotula* | Egypt | A | (Abou El-Ela *et al.*, 1990) |
| antheindurolide B (corrected by Vuckovic) | *A. arvensis* | Serbia | A | (Vuckovic *et al.*, 2006b) |
| | *A. cotula* | Bulgaria | A | (Staneva *et al.*, 2005) |
| | *A. indurata* | Egypt | A | (Abou El-Ela *et al.*, 1990) |
| | *A. pseudocotula* | Egypt | A | (Abou El-Ela *et al.*, 1990) |
| anthemin A | *A. melanolepis* | Greece | A | (Saroglou *et al.*, 2007b) |
| anthemin B | *A. melanolepis* | Greece | A | (Saroglou *et al.*, 2007b) |
| anthemin C | *A. melanolepis* | Greece | A | (Saroglou *et al.*, 2008) |
| anthemolide A | *A. carpatica* | Serbia | A | (Bulatovic *et al.*, 1997; Vajs *et al.*, 2000) |
| anthemolide B | *A. carpatica* | Serbia | A | (Vajs *et al.*, 2000) |
| | *A. cretica* subsp. *cretica* | Serbia | A | (Vajs *et al.*, 1999) |
| anthemolide C | *A. carpatica* | Serbia | A | (Bulatovic *et al.*, 1997; Vajs *et al.*, 2000) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| anthemolide D | *A. cretica* subsp. *cretica* | Serbia | A | (Vajs *et al.*, 1999) |
| | *A. carpatica* | Serbia | A | (Bulatovic *et al.*, 1997) |
| anthemolide E | *A. carpatica* | Serbia | A | (Bulatovic *et al.*, 1997) |
| anthemolide F | *A. carpatica* | Serbia | A | (Vajs *et al.*, 2000) |
| anthepseudolide | *A. pseudocotula* | Egypt | A | (Abou El-Ela *et al.*, 1990) |
| anthepseudolide-6-*O*-acetate | *A. pseudocotula* | Egypt | A | (Abou El-Ela *et al.*, 1990) |
| armefolin | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1988) |
| armexifolin | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1986) |
| *cis-cis*-3α-acetoxy-8β-hydroxycustonolid | *A. cretica* subsp. *montana* | Turkey | A | (Bohlmann & Zdero, 1975) |
| chrysanin | *A. ruthenica* | Hungary | A | (Hajdu *et al.*, 2010) |
| costunolide | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989; Sarg *et al.*, 1990) |
| creticacoumarine | *A. cretica* subsp. *cretica* | Yugoslavia | R | (Hofer & Greger, 1985) |
| | *A. cretica* subsp. *montana* | Turkey | R | (Bohlmann & Zdero, 1975) |
| cumambrin A | *A. carpatica* | Bulgaria | A | (Staneva *et al.*, 2002) |
| cumambrin B | *A. carpatica* | Serbia | A | (Vajs *et al.*, 2000) |
| deacetylludalbin | *A. melanolepis* | Greece | A | (Skaltsa *et al.*, 2006) |
| dehydrolanuginolide | *A. punctata* subsp. *cupaniana* | Sicily | A | (Bruno *et al.*, 1991) |
| | *A. macedonica* | Bulgaria | A | (Staneva *et al.*, 2006), |
| dentatin A | *A. melanolepis* | Greece | A | (Saroglou *et al.*, 2007a) |
| desacetoxymatricarin | *A. pseudocotula* | Egypt | A | (Abou El-Ela *et al.*, 1990) |
| desacetyllaurenobiolide | *A. melanolepis* | Greece | A | (Saroglou *et al.*, 2007a) |
| desacetyl-β-cyclopyrethrosin | *A. wiedemanniana* | Turkey | A | (Celik *et al.*, 2005) |
| | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| douglanin | *A. carpatica* | Bulgaria | A | (Staneva *et al.*, 2002) |
| | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989) |
| elagalactone A | *A. melanolepis* | Greece | A | (Saroglou *et al.*, 2010) |
| epi-(4E) 6-hydroperoxy-5,6-dihydro-4,5-dehydro-antheindurolide A | *A. auriculata* | Bulgaria | F | (Todorova *et al.*, 2008) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| epi-(5R + 5S) 5-hydroxy-5,6-dihydro-6,13-dehydro-antheindurolide A | *A. auriculata* | Bulgaria | F | (Todorova *et al.*, 2008) |
| epi-5-hydroperoxy-5,6-dihydro-6,13-dehydro-antheindurolide A | *A. auriculata* | Bulgaria | F | (Todorova *et al.*, 2008) |
| epi-6-hydroxy-5,6-dihydro-4,5-dehydro-antheindurolide A | *A. auriculata* | Bulgaria | F | (Todorova *et al.*, 2008) |
| epi-antheindurolide A | *A. auriculata* | Bulgaria | F | (Todorova *et al.*, 2008) |
| epi-antheindurolide A-5,6-oxide | *A. auriculata* | Bulgaria | F | (Todorova *et al.*, 2008) |
| epi-antheindurolide B | *A. auriculata* | Bulgaria | F | (Todorova *et al.*, 2008) |
| estafiatin-8-*O*-angelate | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989) |
| estafiatin-8-*O*-isovalerate | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989) |
| hydroxyisonobilin | *A. nobilis* | not given | L | Samek *et al.* (1976) |
| hydruntinolide A | *A. aetnensis* | Sicily | A | (Bruno *et al.*, 1997) |
| | *A. hydruntina* | Italy | A | (di Benedetto *et al.*, 1991) |
| hydruntinolide B | *A. cretica* subsp. *cretica* | Serbia | A | (Vajs *et al.*, 1999) |
| | *A. aetnensis* | Sicily | A | (Bruno *et al.*, 1997) |
| | *A. hydruntina* | Italy | A | (di Benedetto *et al.*, 1991) |
| hydruntinolide C | *A. hydruntina* | Italy | A | (di Benedetto *et al.*, 1991) |
| isospiciformin | *A. stribrnyi* subsp. *tracica* | Bulgaria | A | (Staneva *et al.*, 2006) |
| ludalbin | *A. macedonica* | Bulgaria | A | (Staneva *et al.*, 2006) |
| | *A. carpatica* | Bulgaria | A | (Staneva *et al.*, 2002) |
| michelenolide | *A. macedonica* | Bulgaria | A | (Staneva *et al.*, 2006) |
| nobilin | *A. nobilis* | not given | F | (Benesova *et al.*, 1964; Holub & Samek, 1977) |
| parthenolide | *A. cretica* subsp. *tenuiloba* | Turkey | A | (Bohlmann & Zdero, 1975) |
| | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989; Zaghloul *et al.*, 1989; Sarg *et al.*, 1990) |
| | *A. macedonica* | Bulgaria | A | (Staneva *et al.*, 2006) |
| | *A. stribrnyi* subsp. *tracica* | Bulgaria | A | (Staneva *et al.*, 2006) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| reynosin | *A. macedonica* | Bulgaria | A | (Staneva *et al.*, 2006) |
| santamarine | *A. macedonica* | Bulgaria | A | (Staneva *et al.*, 2006) |
| | *C. fuscatum* (*A. fuscatum*) | Spain | A | (De Pascual Teresa *et al.*, 1986) |
| sivasinolide | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| sivasinolide-6-*O*-angelate | *A. ruthenica* | Hungary | A | (Hajdu *et al.*, 2010) |
| spiciformin | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| stizolin | *A. punctata* subsp. *cupaniana* | Sicily | A | (Bruno *et al.*, 1991) |
| | *A. macedonica* | Bulgaria | A | (Staneva *et al.*, 2006), |
| tanacin | *A. ruthenica* | Hungary | A | (Hajdu *et al.*, 2010) |
| tatridin A | *A. wiedemanniana* | Turkey | A | (Celik *et al.*, 2005) |
| | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| | *A. melanolepis* | Greece | A | (Saroglou *et al.*, 2010) |
| xeranthemolide | *A. austriaca* | Bulgaria | A | (Staneva *et al.*, 2004) |
| xerantholide | *A. austriaca* | Poland | A | (Holub *et al.*, 1982) |
| | | Turkey | A | (Bohlmann *et al.*, 1974) |
| | | Bulgaria | A | (Staneva *et al.*, 2004) |
| β-amyrin | *A. pseudocotula* | Egypt | A | (Balbaa *et al.*, 1975) |
| β-cyclopyrethrosine | *A. melanolepis* | Greece | A | (Skaltsa *et al.*, 2006) |
| β-hydroxy-1-desoxotamirin | *A. melanolepis* | Greece | A | (Skaltsa *et al.*, 2006) |

Table A2. Table of other sesquiterpenes from the genus *Anthemis*. A: aerial parts, R: roots

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| **sesquiterpene acids** | | | | |
| methyl 8α-(2methylbutanoyloxy)-3-oxo-4,11(13)-guaiadien-12-oate | *A. austriaca* | Bulgaria | A | (Staneva *et al.*, 2004) |
| methyl 8α-3,8-dioxo-4,11(13)-guaiadien-12-oate | *A. austriaca* | Bulgaria | A | (Staneva *et al.*, 2004) |
| methyl 8α-isobutyryloxy-3-oxo-4,11(13)-guaiadien-12-oate | *A. austriaca* | Bulgaria | A | (Staneva *et al.*, 2004) |
| methyl pechueolate | *A. austriaca* | Bulgaria | A | (Staneva *et al.*, 2004) |
| **sesquiterpene-coumarin ethers** | | | | |
| 7-acetoxypectanone | *A. aetnensis*<br>*A. cretica* subsp. *cretica* | Sicily<br>Yugoslavia | A<br>R | (Bruno *et al.*, 1997)<br>(Hofer & Greger, 1985) |
| dehydronerolisovalerat | *A. cretica* subsp. *montana*<br>*A. cretica* subsp. *tenuiloba*<br>*A. cretica* subsp. *anatolica* | Turkey<br>Turkey<br>Turkey | R<br>R<br>R | (Bohlmann & Zdero, 1975)<br>(Bohlmann & Zdero, 1975)<br>(Bohlmann & Zdero, 1975) |
| didehydropectanone | *A. cretica* subsp. *cretica* | Yugoslavia | R | (Hofer & Greger, 1985) |
| drimanthone | *A. cretica* subsp. *cretica* | Yugoslavia | R | (Hofer & Greger, 1985) |
| epoxyfarnochrol | *A. cretica* subsp. *cretica* | Yugoslavia | R | (Hofer & Greger, 1985) |
| isodrimachone | *A. cretica* subsp. *cretica* | Yugoslavia | R | (Hofer & Greger, 1985) |
| pectanone | *A. cretica* subsp. *cretica* | Yugoslavia | R | (Hofer & Greger, 1985) |

Table A3. Table of flavonoids from the genus *Anthemis*. A: aerial parts, F: flower heads, L: leaves, R: roots, D: disks, Ra: ray, P: petal

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| 5,7,3'-trihydroxy-3,6,4'-trimethoxyflavonol | *A. melanolepis* | Greece | A | (Skaltsa *et al.*, 2006) |
| 5-methoxy-3,7-dihydroxy-dihydroflavonol | *A. melanolepis* | Greece | A | (El-Alfy *et al.*, 1989) |
| 5-methoxy-3,7-dihydroxy-dihydroflavonol-3-*O*-glucoside | *A. melanolepis* | Greece | A | (El-Alfy *et al.*, 1989) |
| 6-hydroxy-kaempferol-3,6,4'-trimethly ether | *A. tinctoria* | not given<br>Slovakia | L<br>F | (Wollenweber & Mayer, 1991)<br>(Masterova *et al.*, 1993) |
| 6-hydroxyluteolin 6,3'-dimethyl ether | *A. cretica* subsp. *saxatilis*<br>*A. dumetorum* | UK<br>UK | L<br>L | (Williams *et al.*, 2001)<br>(Williams *et al.*, 2001) |
| 6-hydroxyluteolin 6,7,4'-trimethyl ether | *A. cretica* subsp. *saxatilis*<br>*A. monantha* | UK<br>UK | L<br>L | (Williams *et al.*, 2001)<br>(Williams *et al.*, 2001) |
| 6-hydroxyluteolin 6-methyl ether | *A. cretica* subsp. *saxatilis* | UK | L | (Williams *et al.*, 2001) |
| 6-hydroxyquercetin 3,6,4'-trimethyl ether | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| 7,4'-di-*O*-methylapigenin | *A. melanolepis* | Greece | A | (Saroglou *et al.*, 2010) |
| apigenin | *A. biebersteiniana*<br>*A. carpatica*<br>*A. cotula*<br><br>*A. cretica*<br>*A. maritima*<br>*A. pseudocotiola*<br>*A. dumetorum*<br>*A. altissima*<br>*A. auriculata*<br>*A. nobilis*<br><br>*A. melanolepis* | not given<br>not given<br>Serbia<br>not given<br>not given<br>not given<br>Egypt<br>UK<br>Greece<br>Greece<br>Egypt,<br>Belgium<br>Greece | L<br>L<br>A<br>L<br>L<br>L<br>F<br>L, Ra<br>A<br>A<br>F<br><br>A | (Greger, 1969)<br>(Greger, 1969)<br>(Vuckovic *et al.*, 2006a)<br>(Greger, 1969)<br>(Greger, 1969)<br>(Greger, 1969)<br>(Saleh & Rizk, 1974)<br>(Williams *et al.*, 2001)<br>(Konstantinopoulou *et al.*, 2003)<br>(Theodori *et al.*, 2006)<br>(Abou-Zied & Rizk, 1973)<br>(Power & Browning, 1914)<br>(Skaltsa *et al.*, 2006) |
| apigenin 7-diglucuronide | *A. chia* | Greece | Ra | (Williams *et al.*, 2001) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| apigenin 7-glucoside | *A. pseudocotiola* | Egypt | F | (Saleh & Rizk, 1974) |
| | *A. nobilis* | Egypt | F | (Abou-Zied & Rizk, 1973) |
| | | not given | | (Pietta *et al.*, 1991) |
| | | Belgium | F | (Power & Browning, 1914) |
| | *A. cretica* subsp. *saxatilis* | UK | Ra | (Williams *et al.*, 2001) |
| | *A. chia* | Greece | Ra | (Williams *et al.*, 2001) |
| apigenin 7-glucoside-6"-(3"'-hydroxy-3"'-methyl-glutarate (chamaemeloside) | *A. nobilis* | not given | F | (Tschan *et al.*, 1996) |
| apigenin 7-glucuronide | *A. nigrescens* | UK | D, Ra | (Harborne *et al.*, 1970) |
| | *A. cretica* subsp. *saxatilis* | UK | Ra | (Williams *et al.*, 2001) |
| | *A. dumetorum* | UK | D, Ra | (Williams *et al.*, 2001) |
| | *A. chia* | Greece | Ra | (Williams *et al.*, 2001) |
| | *A. triumfetti* | Montenegro | A | (Pavlovic *et al.*, 2006) |
| apigenin 7-methyl ether | *A. dumetorum* | UK | Ra | (Williams *et al.*, 2001) |
| apigenin-4'-methyl ether | *A. tinctoria* | not given | L | (Wollenweber & Mayer, 1991) |
| | | Slovakia | F | (Masterova *et al.*, 1993) |
| apigenin-7-apioglucoside (apiin) | *A. nobilis* | not given | F | (Wagner & Kirmayer, 1957; Pietta *et al.*, 1991) |
| | | Egypt | F | (Abou-Zied & Rizk, 1973) |
| axillarin | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989; Zaghloul *et al.*, 1989) |
| centauridin | *A. ruthenica* | Hungary | A | (Hajdu *et al.*, 2010) |
| centaureidin | *A. ruthenica* | Hungary | A | (Hajdu *et al.*, 2010) |
| chrysoeriol | *A. dumetorum* | UK | L | (Williams *et al.*, 2001) |
| chrysoeriol 7-glucoside | *A. monantha* | UK | Ra | (Williams *et al.*, 2001) |
| chrysoeriol 7-glucuronide | *A. monantha* | UK | Ra | (Williams *et al.*, 2001) |
| cirsimaritin | *A. maritima* | Italy | L | (Collu *et al.*, 2008) |
| dihydrokaempferol | *A. melanolepis* | Greece | A | (Skaltsa *et al.*, 2006) |
| eupatilin | *A. maritima* | Italy | L | (Collu *et al.*, 2008) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| eriodictyol | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| | *A. auriculata* | Greece | A | (Theodori *et al.*, 2006) |
| | *A. melanolepis* | Greece | A | (Saroglou *et al.*, 2010) |
| hispidulin | *A. cotula* | Serbia | A | (Vuckovic *et al.*, 2006a) |
| iso-cvercetrine | *A. tinctoria* | not given | F | (Hanganu *et al.*, 2008) |
| isorhamnetin | *A. cotula* | not given | L | (Greger, 1969) |
| | *A. biebersteiniana* | not given | L | (Greger, 1969) |
| | *A. carpatica* | not given | L | (Greger, 1969) |
| | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| kaempferol | *A. cotula* | not given | L | (Greger, 1969) |
| | *A. carpatica* | not given | L | (Greger, 1969) |
| | *A. tinctoria* | not given | L | (Greger, 1969) |
| | *A. woronowii* | not given | L | (Greger, 1969) |
| | *A. altissima* | not given | L | (Greger, 1969) |
| | *A. nobilis* | Egypt | F | (Abou-Zied & Rizk, 1973) |
| kaempferol 4'-methyl ether | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| kaempferol-3,4'-dimethyl ether | *A. tinctoria* | not given | L | (Wollenweber & Mayer, 1991) |
| | | Slovakia | F | (Masterova *et al.*, 1993) |
| lupeol | *A. melampodina* | Egypt | A | (Sarg *et al.*, 1990); (El-Alfy *et al.*, 1989) |
| lupeol acetate | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989) |
| lupeyl acetate taraxasterol | *A. melampodina* | Egypt | A | (Sarg *et al.*, 1990) |
| luteolin | *A. biebersteiniana* | not given | L | (Greger, 1969) |
| | *A. carpatica* | not given | L | (Greger, 1969) |
| | *A. cotula* | not given | L | (Greger, 1969) |
| | *A. cretica* | not given | L | (Greger, 1969) |
| | *A. maritima* | not given | L | (Greger, 1969) |
| | *A. auriculata* | Greece | A | (Theodori *et al.*, 2006) |
| | *A. nobilis* | Egypt | F | (Abou-Zied & Rizk, 1973) |
| | *A. melanolepis* | Greece | A | (Saroglou *et al.*, 2007b) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| luteolin 7-glucoside | *A. nobilis* | not given | | (Pietta *et al.*, 1991) |
| | *A. cretica* subsp. *saxatilis* | UK | D, L | (Williams *et al.*, 2001) |
| | *A. chia* | Greece | Ra | (Williams *et al.*, 2001) |
| | *A. monantha* | UK | Ra | (Williams *et al.*, 2001) |
| | *A. nobilis* | Egypt | F | (Abou-Zied & Rizk, 1973) |
| | *A. tinctoria* | not given | F | (Hanganu *et al.*, 2008) |
| | *A. triumfetti* | Montenegro | A | (Pavlovic *et al.*, 2006) |
| luteolin 7-glucuronide | *A. nigrescens* | UK | D, Ra | (Harborne *et al.*, 1970) |
| | *A. monantha* | UK | Ra | (Williams *et al.*, 2001) |
| luteolin 7-*O*-β-D-glucopyranoside | *A. auriculata* | Greece | A | (Theodori *et al.*, 2006) |
| naringenin | *A. melanolepis* | Greece | A | (Skaltsa *et al.*, 2006) |
| nicotiflorin | *A. tinctoria* subsp. *tinctoria* | Greece | A | (Papaioannou *et al.*, 2007) |
| patuletin (quercetagetin-6-methyl ether) | *A. tinctoria* | Slovakia | L, F | (Harborne *et al.*, 1970; Masterova *et al.*, 1993; Masterova *et al.*, 2005) |
| | *A. carpatica* | Spain/Portugal | F | (Harborne *et al.*, 1976) |
| | *A. nigrescens* | Spain/Portugal | F | (Harborne *et al.*, 1976) |
| | *A. woronowii* | Spain/Portugal | L | (Harborne *et al.*, 1976) |
| | *A. melampodina* | not given | A | (Greger, 1969) |
| | | Egypt | | (Zaghloul *et al.*, 1989) |
| patuletin 7-glucoside (patulitrin) | *A. tinctoria* | Slovakia | P, L, D, Ra, F | (Harborne *et al.*, 1970); (Williams *et al.*, 2001); (Masterova *et al.*, 2005) |
| | *A. carpatica* | UK | D, Ra | (Harborne *et al.*, 1970) |
| | *A. nigrescens* | UK | D, Ra | (Harborne *et al.*, 1970) |
| | *A. chia* | Greece | D | (Williams *et al.*, 2001) |
| | *A. monantha* | UK | D | (Williams *et al.*, 2001) |
| | *A. triumfetti* | Montenegro | A | (Pavlovic *et al.*, 2006) |
| | *A. tinctoria* subsp. *tinctoria* | Greece | A | (Papaioannou *et al.*, 2007) |
| patuletin 3-glucoside | *Anthemis tinctoria* subsp. *subtinctoria* | UK | L | (Williams *et al.*, 2001) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| patuletin 3-rutinoside | *Anthemis tinctoria* subsp. *subtinctoria* | UK | L | (Williams *et al.*, 2001) |
| patuletin 7-*O*-β-D-(6"-caffeoylglucoside) | *A. tinctoria* | Slovakia | F | (Masterova *et al.*, 2005) |
| pectolinaringenin | *A. auriculata* | Greece | A | (Theodori *et al.*, 2006) |
| quercetagetin | *A. tinctoria* | not given | L | (Greger, 1969) |
| | *A. woronowii* | not given | L | (Greger, 1969) |
| | *A. altissima* | not given | L | (Greger, 1969) |
| quercetagetin 3,6,3',4'-tetramethyl ether | *A. monantha* | UK | L | (Williams *et al.*, 2001) |
| | *A. tinctoria* subsp. *subtinctoria* | UK | L | (Williams *et al.*, 2001) |
| quercetagetin 3,6,3'-trimethyl ether | *A. chia* | Greece | L, Ra | (Williams *et al.*, 2001) |
| | *A. dumetorum* | UK | L, D | (Williams *et al.*, 2001) |
| | *A. monantha* | UK | D | (Williams *et al.*, 2001) |
| | *A. tinctoria* subsp. *subtinctoria* | UK | L | (Williams *et al.*, 2001) |
| quercetagetin 3,6,4'-trimethyl ether | *A. tinctoria* | not given | L | (Wollenweber & Mayer, 1991) |
| | | Slovakia | F | (Masterova *et al.*, 1993) |
| quercetagetin 3,6-dimethyl ether | *A. tinctoria* | not given | L | (Wollenweber & Mayer, 1991) |
| | | Slovakia | F | (Masterova *et al.*, 1993) |
| quercetagetin 3'6-dimethyl ether (spinacetin) | *A. tinctoria* | Slovakia | L | (Masterova *et al.*, 1993) |
| quercetagetin 7-glucoside | *A. chia* | Greece | D | (Williams *et al.*, 2001) |
| | *A. monantha* | UK | D | (Williams *et al.*, 2001) |
| quercetin 3,3'-dimethyl ether | *A. tinctoria* | not given | L | (Wollenweber & Mayer, 1991) |
| | | Slovakia | F | (Masterova *et al.*, 1993) |
| quercetin 3-methyl ether | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| quercetin 3-*O*-glucoside (isoquercitrin = isoquercetrin) | *A. triumfetti* | Montenegro | A | (Pavlovic *et al.*, 2006) |
| | *A. tinctoria* subsp. *tinctoria* | Greece | A | (Papaioannou *et al.*, 2007) |
| | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| quercetin | *A. tinctoria* | Slovakia | L | (Masterova *et al.*, 1993) |
| | | not given | L | (Greger, 1969) |
| | *A. biebersteiniana* | not given | L | (Greger, 1969) |
| | *A. carpatica* | not given | L | (Greger, 1969) |
| | *A. cotula* | not given | L | (Greger, 1969) |
| | | UK | L | (Glennie & Harborne, 1971) |
| | *A. woronowii* | not given | L | (Greger, 1969) |
| | *A. altissima* | not given | L | (Greger, 1969) |
| | | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| | *A. triumfetti* | Montenegro | A | (Pavlovic *et al.*, 2006) |
| | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989); (Zaghloul *et al.*, 1989) |
| quercetin 4'-methyl ether (taxarixetin) | *A. tinctoria* | Slovakia | L | (Masterova *et al.*, 1993) |
| quercetin 4'-*O*-glucoside | *A. triumfetti* | Montenegro | A | (Pavlovic *et al.*, 2006) |
| quercetin 5-glucoside | *A. cotula* | UK | L | (Glennie & Harborne, 1971) |
| quercetin 7-*O*-glucoside | *A. triumfetti* | Montenegro | A | (Pavlovic *et al.*, 2006) |
| rhamnetin | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |
| | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989); (Zaghloul *et al.*, 1989) |
| rhamnetin-3-*O*-glucoside | *A. melampodina* | Egypt | A | (El-Alfy *et al.*, 1989) |
| rutin (quercetin 3-*O*-rutinoside) | *A. dumetorum* | UK | L | (Williams *et al.*, 2001) |
| | *A. monantha* | UK | L | (Williams *et al.*, 2001) |
| | *A. triumfetti* | Montenegro | A | (Pavlovic *et al.*, 2006) |
| | *A. tinctoria* subsp. *tinctoria* | Greece | A | (Papaioannou *et al.*, 2007) |
| | *A. tinctoria* | not given | F | (Hanganu *et al.*, 2008) |
| salvigenin | *A. maritima* | Italy | L | (Collu *et al.*, 2008) |
| santin | *A. chia* | Greece | L, D, Ra | (Williams *et al.*, 2001) |
| | *A. dumetorum* | UK | L, D | (Williams *et al.*, 2001) |

| Compound | Species | Origin of material | Plant part | Reference |
|---|---|---|---|---|
| scutellarein-6,4'-dimethyl ether | *A. tinctoria* | not given | L | (Wollenweber & Mayer, 1991) |
| | | Slovakia | F | (Masterova *et al.*, 1993) |
| | *A. dumetorum* | UK | L, D | (Williams *et al.*, 2001) |
| | *A. monantha* | UK | D | (Williams *et al.*, 2001) |
| scutellarein-6-methyl ether (hispidulin) | *A. tinctoria* | not given | L | (Wollenweber & Mayer, 1991) |
| | | Slovakia | F | (Masterova *et al.*, 1993) |
| | *A. cretica* subsp. *saxatilis* | UK | L | (Williams *et al.*, 2001) |
| | *A. triumfetti* | Montenegro | A | (Pavlovic *et al.*, 2006) |
| taxifolin | *A. altissima* | Greece | A | (Konstantinopoulou *et al.*, 2003) |

Figure A1. $^{13}C$ NMR spectrum of compound **1**. Measured in deuterium oxide.

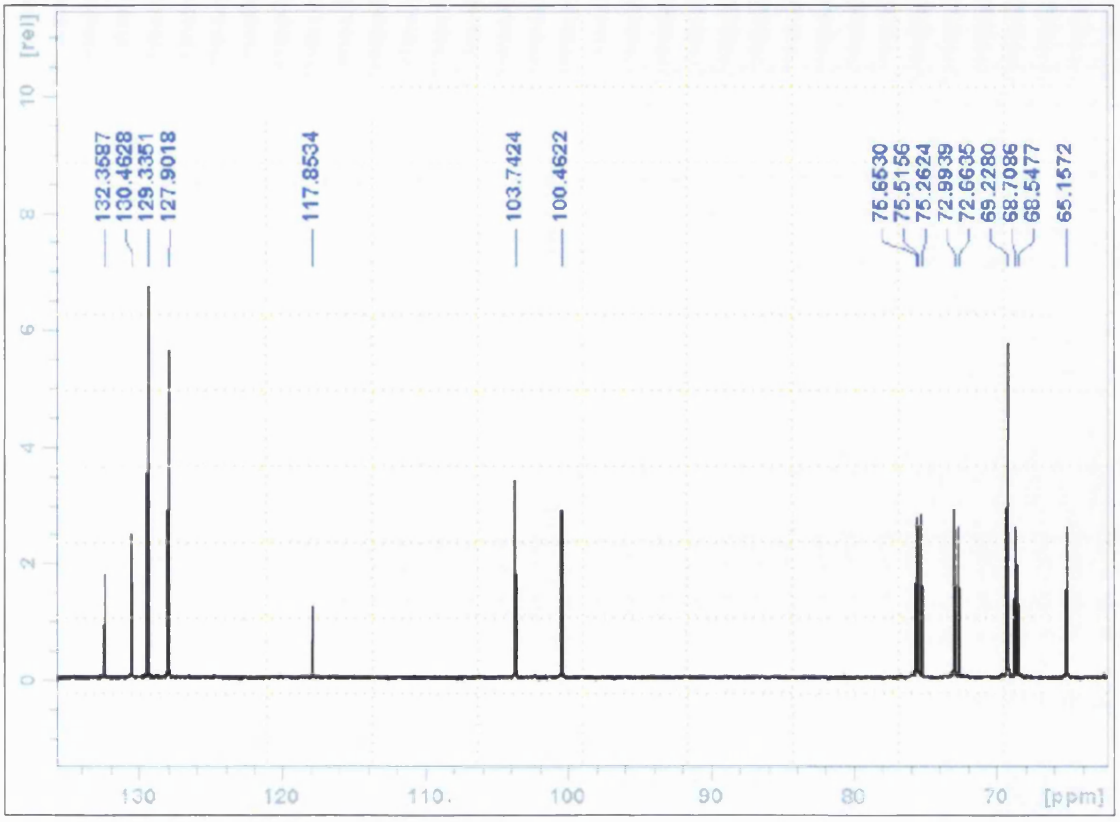
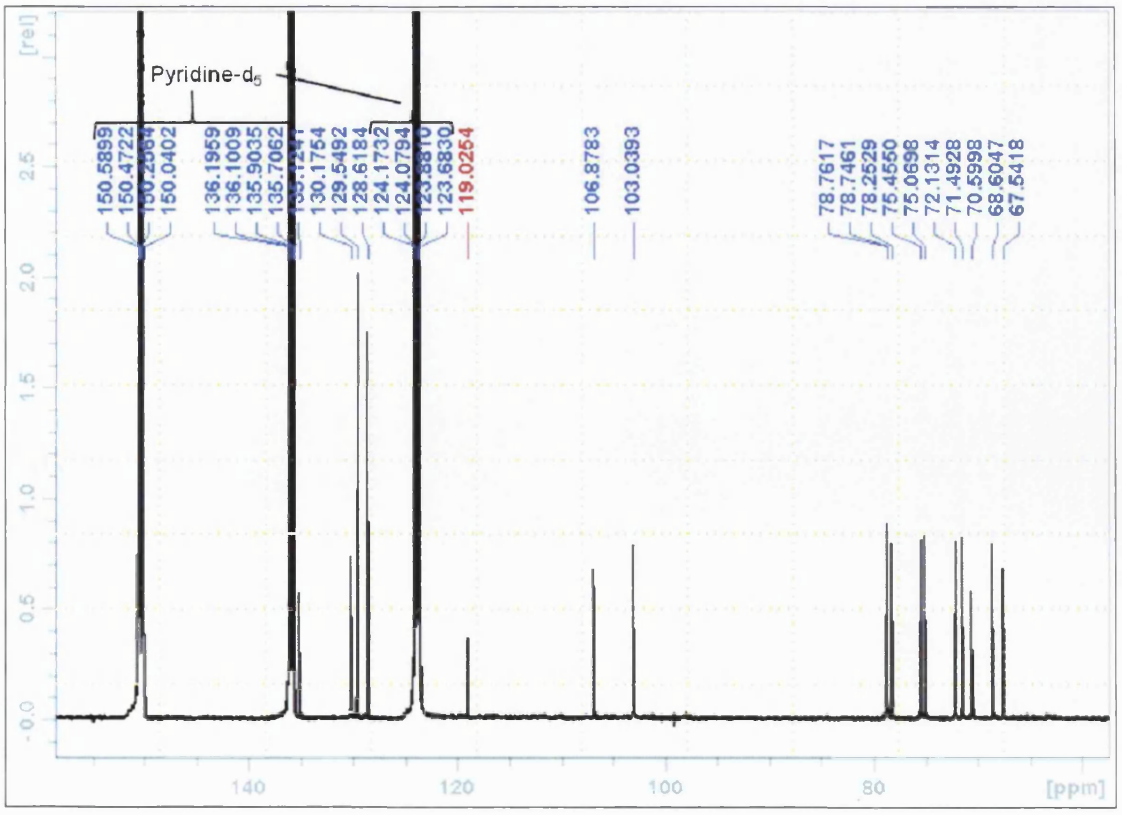



Figure A2. $^{13}C$ NMR spectrum of compound **1**. Measured in pyridine-$d_5$.



## *LIST OF PUBLICATIONS AND PRESENTATIONS*

**Publications**

D. Tasdemir, D. Sanabria, I.L. Lauinger, A. Tarun, R. Herman, R. Perozzo, M. Zloh, S. H. Kappe, R. Brun and N.M. Carballeira

**2-Hexadecynoic acid inhibits plasmodial FAS-II enzymes and arrests erythrocytic and liver stage Plasmodium infections**

Bioorganic & Medicinal Chemistry (2010) 18(21):7475-7485

**Poster presentations**

I.L. Lauinger, L. Vivas, R.S. Göktürk and D. Tasdemir

**Screening of Turkish plants for antimalarial and cytotoxic effects**

International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, 29.8. – 2.10.2010, Berlin (Germany)

I.L. Lauinger and D. Tasdemir

**Targeting fatty acid biosynthesis II enzymes of *Plasmodium falciparum***

Summer School of the European University Consortium for Pharmaceutical Sciences, 26.6. – 3.7.2009, Copenhagen (Denmark)

I.L. Lauinger and D. Tasdemir

**Targeting malaria through inhibition of plasmodial fatty acid biosynthesis enzymes**

Molecular Pharmacy opening at the School of Pharmacy, 14.5.2009, London

I.L. Lauinger and D. Tasdemir

**Targeting fatty acid biosynthesis II enzymes of *Plasmodium falciparum*: Inhibition studies with secondary lichen metabolites**

PhD Research Day, Internal Symposium at the School of Pharmacy, 21.10.2008, London

**Talks**

**Searching for natural products against malaria**

PhD Research Day, Internal Symposium at the School of Pharmacy, 13.4. 2010, London