Synthesis and *in vitro* antimalarial activity of a series of bistriazine compounds

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1.1 Abstract

Malaria persists to proliferate as an economic and social burden in the developing countries despite of a 17% decrease in the estimated number mortalities as reported by the World Health Organization in 2011. In the past decade the annually estimated number of malaria cases has never gone under 216 million, resulting in the mortality rate of more than 8,3 million people, 655 000 in 2011. This worldwide disease is endemic in 109 countries, is dominant in sub-Saharan Africa with 91% of reported cases and 80% of its mortality child and infant related.

Malaria is a preventable and curable disease, however *Plasmodia* mono- and multi-drug resistance towards classic antimalarial drugs such as chloroquine, quinine and sulphadoxine/pyrimethamine etc. has rendered their efficacy useless in certain regions of the world. Five species of *Plasmodia* infects humans with *P. falciparum* being the most virulent, prevalent, carries the highest resistant strains towards antimalarial drugs and is responsible for the majority of malaria associated deaths. *Plasmodium falciparum* resistance towards the last bullet in the gun, artemisinin, has recently been reported in the South-East Asian region. This devastating reality calls for immediate research towards developing novel antimalarials to overcome current predicaments.

In the search for novel drugs with antimalarial activity against the increasingly difficult to treat disease, a medicinal chemistry strategy involving the formation of bis-compounds was applied. This formation is the combination of two identical pharmacophores into a single chemical entity to yield a bis-compound. The bis-compound strategy has the potential advantage of bringing forth two pharmacophore moieties to the drugs' action site, circumventing resistance and restoring the effectiveness of the previous unusable monopharmacophoric drug.

Of the various classes of antimalarials available, the antifolates have been used successfully for more than 5 decades in the struggle against malaria. They are potent antimalarials as they inhibit the production of dihydropteroate (DHP) and tetrahydrofolate (THF), two biologically important folate cofactors in the synthesis of parasitic DNA and RNA. Protozoa are incapable of obtaining these folates by means of absorption from their human hosts, and are dependent on the synthesis of the necessary vitamins *de novo* by means of specific precursors. This metabolic character difference between mammalian and protozoan species results in a perfect drug target for antimalarial therapy. Cycloguanil, the active metabolite of the prodrug proguanil; a dihydrofolate reductase (DHFR) inhibitor, is a commonly used, historically important drug in the

treatment of malaria. Its capabilities have been hindered by *P. falciparum* resistance; nevertheless the folate pathway remains an attractive well recognized target site for drug development. Structural changes to cycloguanils' pharmacophore can overcome the problem of resistance.

The applications of 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride) is nearly endless. It has been studied immensely in a diverse range of applications presenting antibacterial, antitumor and antiparasitic activity. It shares similar chemical properties and biological activity to cycloguanil. By using an appropriate solvent and a hydrochloride acceptor, the chlorine atoms of the cyanuric chloride moiety are easily substituted. Temperature control allows the stepwise formation of mono-, di- or trisubstituted derivatives of triazine with various nucleophiles. The derivatives, including hybrid triazines and triazine related drugs PS15 and WR99210 have revealed promising antimalarial activity against chloroquine sensitive and resistant strains.

The aim of this study was to synthesize a series of disubstituted bistriazines, characterize their physical properties and evaluate their antimalarial activity *in vitro* in comparison to that of chloroquine.

We successfully synthesized nine disubstituted bistriazines by aromatic nucleophilic substitution at C-2, 4 and 6 with ethylenediamine as linker between the two triazine rings. The structures of the prepared bistriazines were confirmed by nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS), infrared spectroscopy (IR) and melting points are reported.

The target bistriazines were screened *in vitro* alongside chloroquine against both chloroquine-sensitive (CQS) 3D7 and chloroquine-resistant (CQR) K1 strains of *Plasmodium falciparum.* Of the nine bistriazines synthesized, 5 showed activity against both CQS and CQR strains. They were less potent than CQ against the 3D7 strain of *P. falciparum.* Against, the K1 strain, however, compounds **16** and **17** had potency comparable to that of CQ, while bistriazine **13** was found to be 2-fold more potent. The bistriazines **15** and **19** featuring aniline and morpholine, and aniline substituents on the triazine rings, respectively, were the most active of all synthesized compounds against the K1 strain of *P. falciparum*. Compounds **15** (EC_{50} = 0.37 µM) and **19** (EC_{50} = 0.26 µM) displayed 4- and 6-fold higher potency than CQ (EC_{50} = 1.67 µM), respectively. The target compounds were screened for cytotoxicity against human fibroblasts (BJ), embryonic kidney (HEK293), liver hepatocytes (Hep G2) and lymphoblast-like (Raji) cell lines

alongside staurosporine as reference drug. All the synthesized active bistriazines were found to be selective towards the parasitic cells and non-toxic to the mammalian ones.

OPSOMMING

Malaria is 'n toenemende ekonomiese en sosiale las ten spyte van 'n 17% afname in die geskatte aantal sterftes, soos gerapporteer deur die Wêreld Gesondheid Organisasie in 2011. In die afgelope dekade het die beraamde aantal malariagevalle nooit een keer onder 216 miljoen jaarliks gedaal nie, wat 'n sterftesyfer van meer as 8,3 miljoen mensetot gevolg gehad het, waarvan 655 000 in 2011 plaasgevind het. Hierdie wêreldwye siekte is endemies in 109 lande, is dominant in sub-Sahara-Afrika met 91% van die gevalle wat aangemeld word en 80% van sy sterftes is kinder- en baba-verwant.

Malaria is 'n voorkombare en geneesbare siekte maar mono- en multi-weerstandigheid van *Plasmodia* teen sekere klassieke malariamiddels, soos chlorokien, kinien en sulfadoksien / pirimetamien ensovoorts het hul doeltreffendheid laat afneem en veroorsaak dat hulle nutteloos in sekere gebiede van die wêreld is. Vyf spesies *Plasmodia* infekteer mense. *P. falciparum* is die mees virulente, is die wydste verspreid, het die mees weerstandige stamme teen malariamiddels en is verantwoordelik vir die meerderheid van malariaverwante sterftes. *Plasmodium falciparum*-weerstandigheid teen artemisinien, die laaste koeël in die geweer, is onlangs in die Suid-Oos-Asiatiese streek gerapporteer. Hierdie vernietigende werklikheid dien as 'n wekroep vir 'n onmiddellike ondersoek na die ontwikkeling van nuwe antimalariamiddels om die huidige probleme te oorkom.

In die soektog na nuwe medisyne met aktiwiteit teen die siekte, wat toenemend moeilik word om te behandel, is 'n medisinale chemiestrategie, wat die vorming van bis-verbindings behels, gevolg. Volgens hierdie strategie word twee identiese farmakofore tot 'n enkele chemiese entiteit gekombineer om 'n bis-verbinding te lewer. Die bis-verbindingstrategie het die potensiële voordeel dat twee farmakofore van die geneesmideel by die area van werking te lewer, weerstand te beperk en die doeltreffende gebruik van vorige onbruikbare monofarmakoforiese middels te bewerkstellig.

Van die verskillende klasse antimalariageneesmiddels wat beskikbaar is, is die antifolate met sukses, vir meer as 5 dekades in die stryd teen malaria gebruik. Hulle is kragtige antimalariamiddels wat die produksie van dihidropteroaat (DHP) en tetrahidrofolaat (THF), twee biologiese belangrike folaatkofaktore in die sintese van parasitiese DNA en RNA, inhibeer. Protosoë is nie in staat om hierdie folate vanaf hul menslike gasheer te absorbeer nie, en is dus afhanklik van die *de novo* sintese van die nodige vitamines deur middel van spesifieke kofaktor-voorlopers. Hierdie metaboliese eienskapsverskil tussen

die soog- en oerdierspesies verskaf 'n perfekte geneesmiddelteiken vir malariaterapie. Sikloguaniel, die aktiewe metaboliet van die progeneesmiddel proguaniel; 'n dihidrofolaatreduktase (DHFR) inhibeerder, is 'n algemeen gebruikte, histories belangrike middel in die behandeling van malaria. Alhoewel dié middel se doeltreffendheid deur *P. falciparum*-weerstandigheid beperk is, bly die folaat-weg steeds 'n aantreklike en bekende teikenarea vir geneesmiddelontwikkeling. Strukturele modifikasies aan sikloguaniel se farmakofoor kan die probleem van weerstandigheid oorkom.

Die gebruike van 2,4,6-trichloor-1,3,5-triasien (siaanuursuurchloried) is byna eindeloos. Dit is intensief in 'n wye verskeidenheid van toepassings bestudeer, wat antibakteriële, antitumor en antiparasitiese aktiwiteit insluit. Dit toon ooreenkomste met die chemiese eienskappe en biologiese aktiwiteit van sikloguaniel. Deur gebruik te maak van 'n geskikte oplosmiddel sowel as 'n waterstofchloriedakseptor, word die chlooratome van die siaanuursuurchloried maklik vervang. Temperatuurbeheer kan die stapsgewyse vorming van mono-, di- of trigesubstituëerde afgeleides van triasien, met verskeie nukleofiele, vergemaklik. Die afgeleides, insluitende hibried-triasiene en triasien-verwante middels, bv. PS15 en WR99210, het al belowende malariaaktiwiteit in chlorokien-sensitiewe en weerstandige rasse geopenbaar.

Die doel van hierdie studie was om 'n reeks digesubstituëerde bis-triasiene te sintetiseer, hul fisiese eienskappe te karakteriseer en hul malariaaktiwiteit *in vitro* te vergelyk met dié van chlorokien.

Ons het nege digesubstituëerde bistriasiene, suksesvol deur aromatiese nukleofiele substitusie by C-2, 4 en 6 met etileendiamien as verbindingsgroep tussen die twee triasien ringe, gesintetiseer. Die strukture van die bereide bistriasiene is deur kernmagnetiese resonans spektroskopie (KMR), massa spektrometrie (MS), Infrarooi spektrometrie (IR) bevestig, en smeltpunte is ook gerapporteer.

Die teiken-bistriasiene is *in vitro,* gesamentlik met chlorokien, teen beide chlorokien-sensitiewe (CQS) 3D7 en chlorokien-weerstandige (CQR) K1 stamme van *Plasmodium falciparum* getoets. Van die nege bistriasiene wat gesintetiseer is, het 5 aktiwiteit teen beide CQS- en CQR–stamme getoon. Teen die K1-stam, het verbindings **16** en **17** egter vergelykbare waardes met dié van chlorokien (CQ) vertoon, terwyl bistriasien **13** 2-keer sterker was. Die bistriasiene, **15** en **19,** met onderskeidelik anilien- en morfolien-, en anilien-substituente op die triasienringe, was die aktiefste van alle gesintetiseerde verbindings teen die K1 stam van *P. falciparum*. Verbindings **15** (EC_{50} = 0,37 µM) en **19**

(EC_{50} = 0,26 µM) vertoon onderskeidelik 'n 4 - en 6-voudige toename in potensie in vergelyking met CQ (EC_{50}= 1,67 µM). Die teikenverbindings is vir sitotoksisiteit teen menslike fibroblast (BJ), embrioniese nier (HEK293), lewerhepatosiet (Hep G2) en limfoblastagtige (Raji) sellyne getoets met staurosporien as verwysing. Dit is bevind dat al die gesintetiseerde aktiewe bistriasiene selektief teenoor die parasitiese selle is en dus nie giftig vir die soogdierselle is nie.

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CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 Introduction

Malaria is one of the three deadly killers on our planet, the others being AIDS and Tuberculosis. This febrile disease endemic to 109 countries (41% of the world); kills an estimated 655 000 people each year, with children under the age of five years being most susceptible to the disease accounting for 80% of the mortality (Murray *et al.*, 2012). This is due to malaria *Plasmodia* rapidly progressing in resistance against classic anti-malarial drugs as well as the continuing of resistance from the vectors (*Anophelene mosquito*) towards insecticides. Malaria is a protozoan parasitic disease caused by the genus *Plasmodium.* There are five species that infect humans namely *P. falciparum, P. malariae, P. ovale, P. vivax* and the newly discovered zoonotic species *P. knowlesi (*Ayouba *et al.,* 2012). These two factors are the key predicaments concerning malaria and unfortunately *P. falciparum,* which is the most virulent of the five species, also has the highest resistant strains resulting in the mortality of thousands (Lehane *et al.*, 2012). The problem with resistant strains however, is not limited to malaria endemic countries but also to travelers that might contract the disease during visits to these countries. Roughly 25 000 travelers are infected each year by the disease causing a 100 avoidable mortalities (Ashley *et al.,* 2006).

Among the classic antimalarials used chloroquine was considered a first line treatment against both uncomplicated and severe malaria alongside the combination therapy sulphadoxine/pyrimethamine. These drugs have been used successfully for more than four decades, however wide spread multidrug resistance has rendered them useless in certain parts of the world. More efficient and potent drugs such as mefloquine have been introduced in the market, however *Plasmodia* resistance swiftly thwarts their efficacy and render them basically useless. Resistance towards the last bullet in the gun, artemisinin, has recently been reported at the South-East Asian region (Kumar *et al.*, 2012). Though years of research have gone into the development of an effective malaria vaccine, it remains non-existent (Schuldt & Amalfitano, 2012). This devastating reality calls for immediate research towards the development of a new efficacious anti-plasmodial.

In the search for novel drugs with anti-plasmodial activity that outwits resistance; new approaches in medicinal chemistry must be taken. The formation of bis-compounds is the combination of two identical pharmacophores into a single chemical entity. The bis-

compound strategy has the potential advantage of bringing forth two pharmacophore moieties to the drugs' action site, circumventing resistance and restoring the effectiveness of the previous ineffective drug.

Cycloguanil, the active metabolite of the prodrug proguanil contains a triazine moiety and was used for the prophylactic treatment of malaria but due to emerging resistance towards the drug, it can no longer be used as monotherapy (Kumar et al., 2009). Malarone® contains proguanil and atovaquone, and is considered a first line treatment of chloroquine-resistant malaria. Cycloguanil is active against protozoa since it is a selective inhibitor of protozoan dihydrofolate reductase enzyme (DHFR) (Katzung *et al.*, 2007). This enzyme is vital for DNA synthesis in both eu- and prokaryotes; however protozoa are incapable of obtaining these folates by means of absorption from their human hosts and are therefore forced to synthesize the necessary vitamins *de novo* by means of specific precursors (Wang *et al.*, 2007). This metabolic character difference between mammalian and protozoan species results in a perfect drug target for antimalarial therapy, although DHFR inhibitors must be highly selective in order to be non-toxic to the human host (Warhurst, 1998).

Cyanuric chloride, containing a triazine moiety, is a six-membered heterocyclic ring containing 3 chlorine and 3 nitrogen atoms. Cyanuric chloride doesn't only share similar chemical properties and appearance to cycloguanil but has also proven itself with antiplasmodial activity (Melato *et al.*, 2008). Various triazine containing drugs have been researched to ascertain whether they had improved anti-plasmodial activity over already existing malaria drugs such as 4-aminoquinoline-based β-carbolines, 9-anilinoacridinetriazines, hybrid 4-aminoquinoline triazines, pyrazole based triazines and trisubstituted triazines (Kumar *et al*., 2011). Although point mutations in the enzyme amino acid sequence have led to resistance to the two well defined antifolates, proguanil (cycloguanil) and pyrimethamine, structural changes to these drugs can overcome this problem. Additionally, studies have shown that by synthesizing bis compounds more efficacious antiplasmodial drugs can be produced (Raynes *et al.*, 1996).

1.2 Aim and objectives of the study

Malaria's resistance towards classical drugs is rapidly progressing, research and development must be devoted towards this field of concern. Cycloguanil has been used extensively in the fight against malaria, despite resistance to this drug; the folate pathway remains an attractive, well recognized target site for drug development. The aim of this study was to synthesize a series of disubstituted bistriazine compounds and to evaluate their *in vitro* antimalarial activity and cytotoxicity alongside chloroquine as reference drug.

In order to achieve the aim of this study, the following objectives were set:

- To synthesize a series of novel disubstituted bistriazine compounds
- To confirm the structures of the target synthesized compounds by means of nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy and mass spectrometry (MS) analysis.
- To evaluate the *in vitro* activity of the target bistriazine compounds against chloroquine-sensitive (CQS) 3D7 and chloroquine-resistant (CQR) K1 strains of *Plasmodium falciparum* alongside chloroquine as reference drug.
- To screen their cytotoxicity against human fibroblasts (BJ), embryonic kidney (HEK293), liver hepatocytes (HepG2) and lymphoblast-like (Raji) cell lines alongside staurosporine as reference drug.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In 2011, a 17% decrease in the estimated number of malaria related mortalities has been reported by the World Health Organization (WHO), 33% less than the initially aimed by the roll back malaria partnership (RBM) in 2010. Even though these are favorable statistics, malaria continues to be a foremost threat to the worlds' health. In the past decade the estimated number of malaria cases never once decreased beneath 216 million annually, resulting in the death of more than 8,3 million people, 655 000 in 2010 (WHO, 2010). Among these dreadful statistics, 80% includes the result of child mortality. This worldwide disease is endemic in 109 countries (Fig. 1), dominant in sub-Saharan Africa (91%), but also prevalent in the South East Asian (6%) and the Eastern Mediterranean Region (3%). However, the morbidity is not only limited to these countries, since malaria also infects more than 25 000 travelers, causing an estimated 100 avoidable mortalities each year (Ashley *et al.*, 2006).

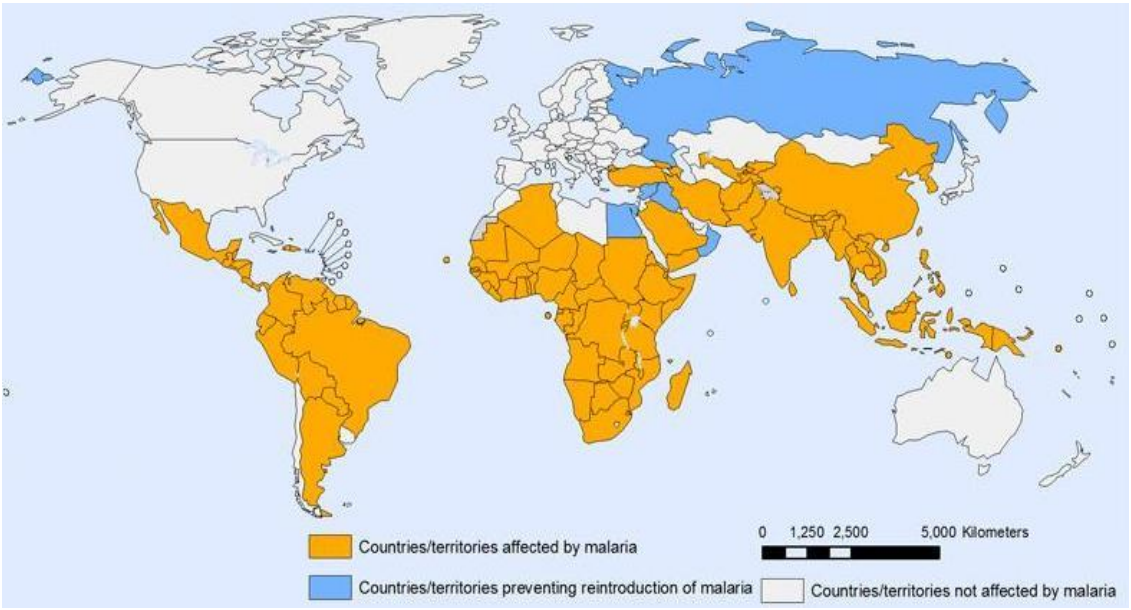


Figure 1 Malaria-free countries/territories and countries/territories preventing reintroduction of the disease, end 2010 (WHO, 2010)

Malaria is transmitted by an infected female mosquito (*Anopheles gambiae), which* obtains the respective *Plasmodia* bodies from a malaria infected individual during the course of a blood meal. After a mere 6 days (in the case of *Plasmodium falciparum*) the vector can transmit this devastating disease.

Five species of the *Plasmodium* genus; *P. falciparum* (a worldwide species, predominately found in Africa)*; P. knowlesi* (a zoonotic species prevalent only in Southeast Asia)*; P. malariae* (found worldwide)*; P. ovale* (mostly prevalent in Western Africa and the western Pacific islands)*;* and *P. vivax* (prevalent in Asia, Latin America, and scattered in Africa) and the zoonotic *P. knowlesi* gives rise to this protozoan parasitic disease. The *Plasmodia* life cycle is sensitive to climate changes therefore brings forth seasonal malaria as well as the geographical distribution of this disease. Of the five species that infect humans, *P. falciparum* is the most virulent, prevalent, caries the highest resistant strains towards antimalarial drugs and is responsible for the majority of malaria associated deaths. *Plasmodium vivax and P. ovale,* on the other hand, can develop dormant liver stages resulting in the recurrence of the disease between symptomless intervals for up to 2 and 4 years respectively.

Oddly enough, malaria is a preventable disease. Methods of prevention include the use of insecticides and bed-nets in vector control and chemoprohylaxis. Chemoprophylactic treatment might be convenient, but there are no regimens with a 100% protection (Baird, 2005). Current research also includes the attempt to design an effective vaccine against malaria. This entails the RTS,S vaccine; to date the most advanced vaccine and which has undergone phase III trials in 7 sub-Saharan African countries enrolling up to 16 thousand infants and children (Casares *et al.*, 2010).

Malaria is a worldwide socio-economic and health burden. *Plasmodium falciparums'* resistance towards classic antimalarial drugs such as chloroquine, pyrimethamine and cycloguanil is contributing to this devastating matter. Chloroquine was the drug of choice in the treatment against *P. falciparum* since the 1940's, however resistance has been reported as early as the 1950's and the call for the development of novel antiplasmodials has been going on for decades (Kain, 1995; Katzung *et al.*, 2007).

2.2 The life cycle of *Plasmodium falciparum*

The lifecycle of *Plasmodium spp.* is complex and consists of different intra- and extracellular environments. The parasite defends itself against the hosts' immune response during this multi-stage event with its 26-30 genome mega bases and more than

5000 proteins. The cycle can be divided into 3 stages, including; a definitive sexual stage in the mosquito, and two intermediate asexual stages in the human host; the initial liver stage followed by the blood stage. Clinical manifestations present themselves during the latter asexual stage (Doolan & Hoffman, 2001).

These stages are:

- The Sporogony in the mosquito;
- Hepatic pre-erythrocytic schizogony (liver stage); and
- Erythrocytic schizogony in the human (blood stage) (Druilhe & Barnwell, 2007)

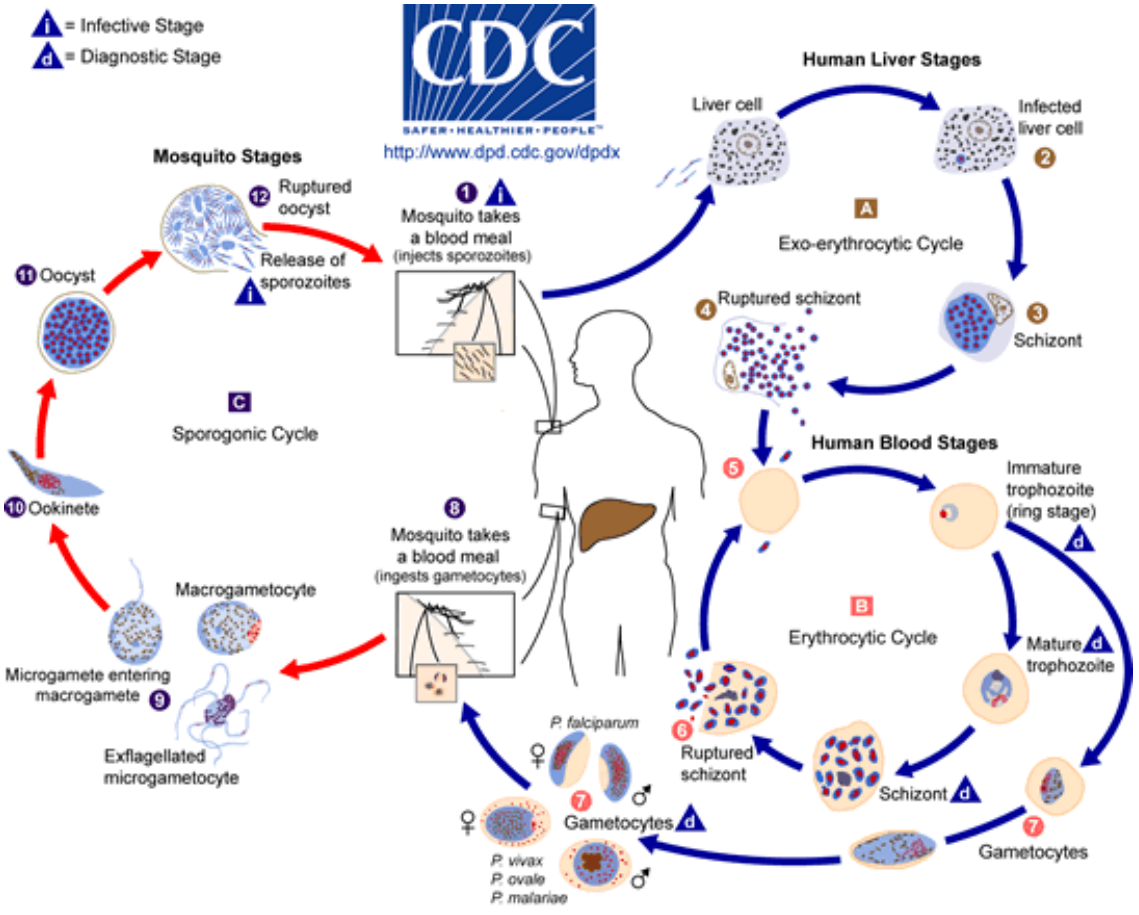


Figure 2 Life cycle of the malaria parasite (dpd.cdc.gov)

2.2.1 The Sporogony in the mosquito

The definite vector of human *Plasmodium* spp. is the *Anopheline* mosquito. During the important sexual stage, gametocytes undergo several morphological changes to finally produce thousands of infectious daughter cells (sporozoites).

Sporogony is the sexual stage that occurs within the mosquito vector. This process starts when the *Anopheline* mosquito engorges on malaria infected human and can be well described in three phases. The first phase is known as "early sporogony" which includes gametocytogenesis (Vaughan, 2007). During the blood meal, macro- (female) and micro- (male) gametocytes are ingested into the mosquito's posterior midgut lumen along with other components of the infected human blood (Baker, 2010; Baton *&* Ranford-Cartwright, 2005). Gametocytogenesis further involves 5 stages and extends over a period of an elongated 8 days (*P. falciparum)* of gametocyte development from sexually committed merozoites entering the red blood cell to mature sexually distinguished gametocytes (Baton & Ranford-Cartwright, 2005). The production and release of micro- and macrogametocytes also differs. Microgametocytes involve a process of exflagellation and assembling of axonemes producing up to eight motile haploid microgametocytes. Intact macrogametocytes then escape the erythrocyte as a relatively large non-motile gametocyte (Baton & Ranford-Cartwright, 2005). Trophozoites and stage 1 gametocytes are difficult to distinguish; however after stage 1, morphological difference can be detected with clear sexual deviations exploited in stage 4 and 5 mature gametes (Baker, 2010). Microgametocytes contain a relatively large nucleus with no nucleolus in contrast to the compact nucleus of macrogametocytes, which are filled with ribosomes, an endoplasmic reticulum, mitochondria and other cytoplasmic organelles whereas the microgametocytes most of these organelles are non-existent (Baton & Ranford-Cartwright, 2005).

The extracellular spherical diploid zygotes are formed during the fertilization of the macrogametes with microgametes. During this fertilization axonemes cease movement and dissemble whilst the microgamete's nucleus merges with the macrogametes' (Baton *&* Ranford-Cartwright, 2005). The formed zygote is believed to be a tetraploid meiotic product which gradually develops into mobile banana shaped ookinetes (Angrisano *et al.*, 2012). During this formation the parasite is protected against the mosquito's digestive proteases *via* a densely coated pellicle known as the inner membrane complex (IMC) (Baton & Ranford-Cartwright, 2005). The mobile ookinetes penetrate the midgut epithelium cell walls and become immobile at the basal lamina surface forming vegetative oocysts.

Inside each oocysts, DNA replication is initiated, which results in the formation of thousands of mature haploid sporozoites. This can last 7 days or more, and is known as 'mid-sporogony' (Baton *&* Ranford-Cartwright, 2005; Vaughan, 2007). The final phase of sporogony namely, 'late sporogony', is accomplished when the mature sporozoites are released into the heamocoelic cavity and then migrates to the salivary glands of the mosquito (Vaughan, 2007).

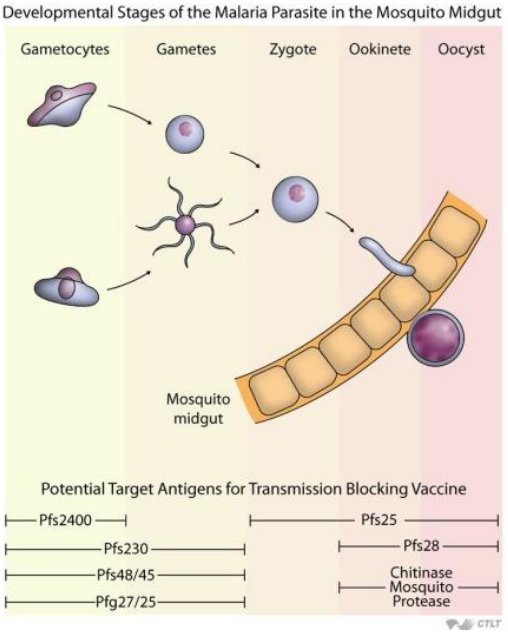


Figure 3 Developmental stages of the malaria parasite in the mosquito midgut (http://ocw.jhsph.edu.)

2.2.2 Hepatic pre-erythrocytic schizogony (liver stage)

The hepatic pre-erythrocytic schizogony stage, better known as the intermediate stage, occurs in the human liver. This is also the first of the two asexual stages and is a clinically silent expansion phase (Schwenk & Richie, 2011).

Ten to hundred sporozoites from sporogony in the mosquitoes' salivary glands are introduced into the humans' cutaneous tissue during a blood meal. Some of these sporozoites become dormant in the cutaneous tissue whereas others alternatively migrate to the spleen or the lymphatic system, where they induce the production of CD4 T immune reactions (Ménard *et al.*, 2008). The biologically important sporozoites find their way into capillary veins, where they are then rapidly transported to the liver *via* the

human circulatory system. They cross the liver's endothelium passing through Kupffer cells, which remove foreign and/or degraded substances from blood, traverse through several hepatocytes (some become necrotic because of cell wounding) until they reach a terminal hepatocyte wherein they will proliferate and evade the immune response of the host (Schwenk & Richie, 2011).

The asexual differentiation of sporozoites to merozoites in the infected terminal hepatocytes, produces a schizont; a hepatocyte bulging with merozoites, that is larger in size than the original uninfected hepatocyte. Hepatocytes being protein and glycol factories, supply sufficient energy for this differentiation thus bulging with thousands of merozoites in the cytoplasm. A mature schizont releases its contents into the sinusoidal lumen of the liver. Here, the merozoites are ready for the invasion of erythrocytes, the setting of the next stage of schizogony (Frevert, 2004).

This is known as the liver stage schizogony of *Plasmodium falciparum. Plasmodium vivax* and *P. ovale* can undergo a formation of developing dormant hypnozoites from merozoites in the early stages of hepatic invasion. This phenomenon of dormancy can cause the recurrent infection of malaria in humans for up to a year (Markus, 2012).

2.2.3 Erythrocytic schizogony

The last stage of the *Plasmodia* life cycle is an asexual multiplication of merozoites in the host red blood cells. This stage is also known as the 'blood-stage' and is exclusively responsible for the pathology and clinical manifestations of malaria (Downie *et al.*, 2008; Mitamura *et al.*, 2000; Sattler *et al.*, 2011; Wang *et al.*, 1996).

As soon as the merozoites are released from the hepatic schizont, they actively invade the erythrocytes and multiply in three phases: (i) ring; (ii) trophozoites and (iii) the erythrocytic schizont phase. In the case of *P. falciparum,* this development takes approximately 48 h. With the rupture of the erythrocytic schizont, an average of 20 merozoites is released per cycle (Arnot *et al.*, 2011; Tilley *et al.*, 2011). The majority of the merozoites will infect new red blood cells, repeating the cycle of inter-erythrocytic schizogony. In turn, the minority of merozoites not infecting erythrocytes will initiate gametocytogenesis and develop into micro- and macrogametocytes. The developed gametocytes can be ingested by a mosquito consequently repeating the life cycle of *Plasmodium* spp (Sattler *et al.*, 2011; Tilley *et al.*, 2011).

2.3 The *Pathology of Plasmodium falciparum malaria*

Although information regarding the specific pathogenesis of benign malaria isn't particularly broad, the severe form of this disease has been studied intensively. The aggregation, sequestration, rosetting and destruction of erythrocytes during the asexual blood stage of the *Plasmodia* life cycle, as discussed below, lead to the pathology of severe malaria (Cook *et al.*, 2009; Walker *et al.*, 2010).

Malaria was thought to release a toxin during schizont rupture. Although no toxin has been identified, this disease does induce elevated levels of cytokines by means of hemozoin (malaria pigment) released during schizont rupture (Clark *et al.*, 1981). Many of the symptoms and signs associated with the disease including fever and malaise can be correlated to this phenomenon. During schizont rupture, pulse releases of tumor necrosis factor (TNF) can be observed, which causes the characteristic cyclic spike fevers of *P. falciparum* malaria (Riley, 1999).

Infected erythrocytes can cytoadhere to microvascular endothelium. This process is well known as sequestration, and is thought to be central to malaria pathology (Newton & Krishna, 1998). Cytoadherence can briefly be described as the tethering of an erythrocyte to microvascular endothelium, followed by rolling and then the firm adherence between these two cells. Sequestrated cells firmly adhere to the microvascular endothelium and does not re-enter circulation (Yipp *et al.*, 2000), which in turn leads to a twofold increase in the viscosity of blood and promotes additional sequestration by increasing infected erythrocyte contact to the microvascular endothelium (Flatt *et al.*, 2005). Vital organs are prone to this process and cell sequestration is asynchronously non-homogeneously distributed in the brain, heart, eyes, liver, kidneys, intestines, adipose tissue and the smallest amount in the bone marrow and skin (Trape *et al.*, 2002).

The formation of rosettes and the adherence of infected to uninfected erythrocytes share some common characteristics to cytoadherence. Reduction in circulation is achieved with rosetting, which starts in the venules (Dondorp *et al.*, 2000). This is considered to accommodate cytoadherence by aggregating anaerobic glycolysis, reducing the pH and thus facilitating sequestration (Dondorp *et al.*, 2004).

Aggregation of infected erythrocytes is mediated *via* platelet CD36 (Chotivanich *et al.*, 2004). The phenomena of sequestration, rosetting and aggregation lead to microcirculatory obstruction that results in anaerobic glycolysis, lactic acidosis and cellular dysfunction (Cook *et al.*, 2009).

Erythrocyte deformability differs in *P. falciparum* and *P. vivax.* The deformed erythrocytes of *P. falciparum* malaria are anatomically rigid, larger in size and spherical. These cells are less filterable by the spleen, and are readily removed. The pathology of malaria is complex, affects nearly all of the organs of the human host and further research on this subject is required, which is beyond the scope of this study.

2.4 Signs, symptoms & complications of *P. falciparum malaria*

Malaria is an acute febrile disease. The onset of the initial symptoms of uncomplicated malaria can be vague, with symptoms resembling that of an influenza virus infection. Uncomplicated malaria presents itself with fever, chills, headache, loss of appetite, lethargy and muscular aches (Walker *et al.*, 2010). The disease can normalize if untreated and form paroxysmal spikes of tertian or quartan fevers, represented with a cold first stage, followed by a hot, then a sweating stage. This phenomenon is rarely seen in *P. falciparum* infections, however it can occur in a 48 h tertian cycle (Jerrard *et al.*, 2002). As the disease progresses, the liver and spleen enlarge and anaemia commences. A dry cough can be present as well as an increase in respiratory rate. Constipation or diarrhoea is normal and must be treated effectively to avoid dehydration. When any of these symptoms and signs is present in a patient located in an endemic malaria area, the infection must be regarded as malarial, and immediate treatment must be set in place accordingly (Ashley *et al.*, 2006).

Untreated uncomplicated malaria can rapidly progress in to the severe form of the disease. This fatal progression can lead to complications such as cerebral malaria, acidosis, acute renal failure, hypoglycaemia, pulmonary oedema, severe anaemia and/or bleeding, resulting in the death of the infected individual.

Of above mentioned complications, cerebral malaria (malarial coma) is the most common cause of malarial deaths (Cook *et al.*, 2009; Walker *et al.*, 2010), and the onset of malarial coma can be sudden. Unconsciousness can manifest after a series of gradual or generalized seizures. Initial signs of this condition include drowsiness, confusion, disorientation, agitation and/or delirium. Extreme agitations, signs of bleeding and sustained hyperventilation are indications of a poor prognosis. The mortality rate amongst pregnant woman infected with cerebral malaria is poor with over 50% of the said population succouring to the disease, although not always fatal, this can induce fever and contractions, resulting in the abortion or premature birth of the foetus. Children born from malaria infectious mothers are at risk of developing congenital malaria, a very

rare and the least known manifestation of malaria (Cook *et al.*, 2009; Menendez & Mayor, 2007).

Individuals surviving cerebral malaria are also at risk of developing post-malaria neurological syndromes (PMNS). Common complications of PMNS include; psychosis, epilepsy in children, encephalopathy, parkinsonian rigidity and cerebellar dysfunctions (Cook *et al.*, 2009).

2.5 Diagnosis

In order to increase the chances of a successful treatment and positive prognosis, an efficient and accurate diagnosis is an absolute and must be performed promptly if the disease is suspected. Malaria must not be diagnosed by means of clinical symptoms for the disease can easily be misinterpreted as its signs and symptoms are common and present in almost all infections. Generally the disease must be diagnosed employing microscopy; thereafter a treatment regime must be implemented to avoid progression of the assumed disease. An exception to this rule is applied to infants and children located in malaria endemic areas, because this disease is responsible for the majority of fever related cases (Cook *et al.*, 2009).

For over a decade light microscopy of blood stained smears has been regarded as the "golden standard" in malarial diagnosis. Thick and thin film blood smears are prepared using Giemsa, Field or Wright's stain methods (Ashley *et al.*, 2006; Moody & Cchiodin, 2000). Giemsa stains are more accurate than Field's and thick smears approximately 30 times more sensitive than thin (Cook *et al.*, 2009). Thick smears are used for the detection of *Plasmodia* while thin smears are for speciation (Moody & Cchiodin, 2000). The main disadvantages concerning these methods are that they require a skilled microscopist, acquire time, laboratory facilities and false positive diagnosis can be made during the duration when most of the infected cells are sequestrated.

In recent years, the introduction of rapid malaria diagnostic tests has been very beneficial for the diagnosis of this disease e.g. they are relatively inexpensive, practical; don't require much skill to operate, with a high rate of success. Specific malaria antibodies (*P. falciparum* histidine-rich protein 2{*Pf*HRP2}, parasite-specific lactate dehydrogenase and aldolase) are identified and coloured. Of these *Pf*HRP2-tests are mainly used, the least expensive and most robust under tropical conditions (Cook *et al.*, 2009).

Although more modern techniques exist for the diagnosis, they are not readily available and expensive. They are based on the fluorescence of DNA and RNA microscopy under ultra-violet light. PCR detection of *Plasmodia* is very sensitive (greater than that of microscopy), and is particularly useful for research concerning resistance, mutations and strain differences, however these methods are used to a lesser extent for the diagnosis of malaria. (Moody & Cchiodin, 2000). Post mortem malaria diagnosis can also be done by morticians, however this is not favourable.

2.6 Malaria prevention and control

Malaria is a curable disease, yet more importantly a preventable disease. An enormous amount of time and money has been dedicated to the treatment, prevention and control of this fatal disease.

Prevention starts by prohibiting an infectious *Anophelene* mosquito from engorging on a human. This, however, can be a difficult process as mosquitoes have a tendency to take their blood meal at night when their victims are either asleep or not aware of them.

Preventative strategies have been implemented over the last decades in malaria endemic countries. These include the use of insecticide treated bed nets (ITN), insect repellents, chemoprophylaxis, indoor residual spraying (IRS), intermittent preventative treatment in pregnancy (IPT) and infancy (IPTi) and mass treatment and experimental vaccines (Cook *et al.*, 2009).

Bed nets have been used for decades and technology has improved their efficacy and ease of use over the past few years. They are currently treated with modern pyrethroid insecticides (that repels and kills mosquitoes) (Lengeler, 2004), are long lasting (LLIN's) and can be washed. Studies have indicated a decrease of 20% in child mortality and can halve malarial cases, especially maternal malaria by making use of these specially treated bed nets and curtains (Crawley & Nahlen, 2004; Greenwood, 2008). Indoor residual spraying works on the same principal as ITN's. Indoor walls are literally sprayed with either pyrethroids or DDT. Mosquitoes have a tendency to linger on walls and trees between blood meals, thus this method is of value because mosquitoes are repelled and killed during this procrastination (Greenwood, 2008).

Intermittent preventative treatment in pregnancy (IPT) and infants (IPTi) can be described as the administration of relevant anti-malarial therapy as a treatment regardless of whether these groups of individuals are infected or not. Problems

concerning IPT's include safety for use over an extended amount of period, cost effectiveness and aggravation of resistance towards antimalarials (Crawley & Nahlen, 2004). Research continues for the development of an effective vaccine against this disease. The actuality that humans have acquired clinical immunity towards this disease, in fact contributes to the research of an antimalarial vaccine (Crawley & Nahlen, 2004).

2.7 Malaria treatment

The main objective in uncomplicated malaria treatment is to cure the infected individual as soon as possible, and with severe malaria to prevent death (WHO, 2010). For effective malaria treatment, a prompt diagnosis is vital. This includes identification of the infecting species of *plasmodia,* level of infection, patient malarial exposure history, clinical status and drug susceptibility of the patient, all according to the region where the infection was acquired (Winstanley & Ward, 2006). Currently no single drug can eradicate the most prevalent species of malaria (*P. falciparum* and *P. vivax*) because of their anatomical differences and widespread multi-drug resistance.

The antimalarial drugs used for malaria treatment can be sub categorised according to their action on the different stages of the *Plasmodia's* life cycle:

- Blood schizonticides: drugs that act on the asexual erythrocytic forms of malaria parasites;
- Tissue schizonticide drugs: also termed casual prophylaxis, are used to prevent erythrocytic infections by eliminating sporozoites or primary tissue forms of *plasmodia;*
- Gametocides: drugs that act on the sexual forms of malaria. Eliminating the transmission of gametocytes to mosquitos;
- Sporontocides: drugs administered to the infected host, to prevent/interrupts oocyst formation and thus parasitical development in the mosquito (Bruce-Chwatt, 1955)

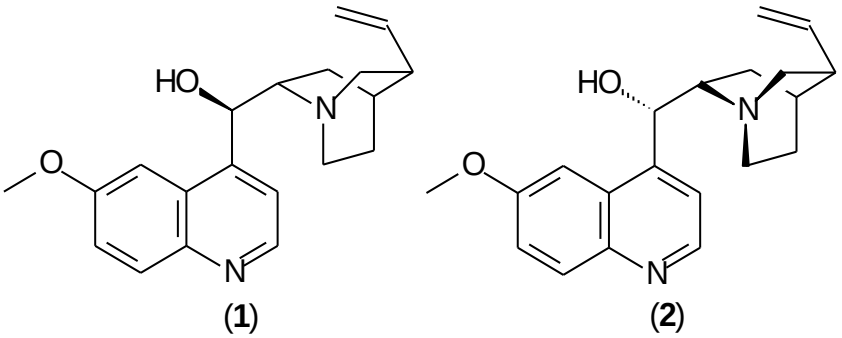
2.8 Antimalarial drugs

The current antimalarial drugs can be divided into sub classes according their mechanism of action and/or pharmacophores as follows:

2.8.1 The hemozoin aryl amino-alcohol inhibitors

2.8.1.1 The quinoline alkaloids

The first naturally occurring organic compound identified as an antimalarial drug, quinine (**1**) dates back more than three centuries (Stepniewska *et al.*, 2001). Quinine has proven itself as a powerful antimalarial drug since its isolation in 1820 from the bark of the *Cinchona* tree. This alkaloid is still used as first line treatment of *P. falciparum* resistant malaria in some countries, however more effective and safer synthetic compounds have been synthesized and its overall use has decreased. Quinine is active against *falciparum* malaria as it is a fast acting, potent blood schizonticide. It has gametocidal properties but not against *P. falciparum* (Katzung *et al.*, 2007). Although quinine resistance has been reported in South East Asia and in Africa, it remains an effective treatment against uncomplicated malaria (Okombo *et al.*, 2011). Quinine is generally well tolerated, despite adverse effects such as cinchonism, gastrointestinal side effects, cardiac dysrhythmias and central nervous system adverse effects (Huston & Levinson, 2006). Its dextrorotary diastereoisomer; quinidine (**2**) is active against severe malaria, yet it is primarily used to treat cardiac arrhythmias (Spikes, 1998).



(**1**) (**2**)

Figure 4 The diastereoisomers quinine (**1**) and quinidine (**2**)

2.8.1.2 The aminoquinolines

Structural modifications to quinines' pharmacophore led to one of the most effective antimalarials to date (Kumar & Srivastava *et al.*, 2010). Chloroquine (**3**) has been the drug of choice for treatment and prophylaxis of malaria since the 1940's (Mohanty *et al.*, 2009). Similar to quinine, it is also an active blood schizonticide antimalarial. The mechanism of antimalarial activity is still considered controversial, but it has been observed that chloroquine accumulates in the digestive vacuole of the parasite, where it is thought to interact with the haemoglobin degradation pathway. Usually heme is

metabolized to hemozoin and is released during schizont rupture. Parasite toxicity is thus elicited by the accumulating breakdown product of haemoglobin i.e. heme. This 4-aminoquinoline sustain against non *falciparum* malaria as well as chloroquine sensitive strains of *falciparum* malaria. Generally, it is very well tolerated; however nausea, pruritus, vomiting, abdominal pain, visual disturbances and headaches are common (Katzung *et al.*, 2007). Despite its efficacy, resistance has been reported as early as in the 1950's and has now become a major widespread problem (Awasthi *et al.*, 2011).

Regardless of the widespread resistance to chloroquine, the 4-aminoquinoline pharmacophore remains an active substrate for antimalarial research (Sahu *et al.*, 2011). Among the libraries of 4-aminoquinolines that have been synthesized, amodiaquine (**4**) illustrates potent antimalarial activity, and is effective in chloroquine resistant treatment. The mechanism of action, resistance and side effects of amodiaquine are reported to be similar to those of chloroquine. Several studies have documented, that it is more potent against malaria than chloroquine (Checchi *et al.*, 2002; Checchi *et al.*, 2002; Meshnick & Aalker, 2005). In an effort to circumvent the emergence of resistance, uncomplicated malaria can be treated with amodiaquine (**4**) and sulphadoxine/pyrimethamine combinations to improve overall efficacy (Obonyo *et al.*, 2007).

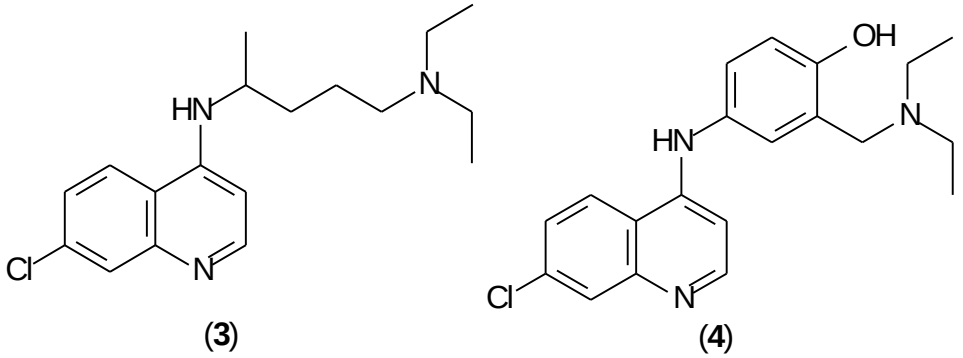
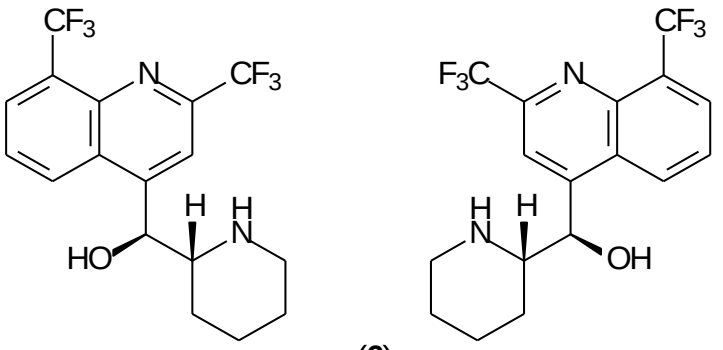


Figure 5 Structure of chloroquine (**3**) and amodiaquine (**4**)

2.8.1.3 The 4-methanolquinolines

The fluorinated 4-methanolquinoline, mefloquine (**6**) was introduced in the 1970's. This synthetic analogue of quinine is mainly used in the treatment and prevention of chloroquine resistant malaria. During the drugs' synthesis a 50:50 racemic mixture is formed (**6**), both isomers are equally active against malaria but differ in pharmacokinetic properties in which the (-) enantiomer exhibits higher blood concentrations. (Vakily *et*

al., 2004). While the mechanism of action is not clearly defined it is thought to form toxic complexes with heme resulting in the degradation of parasitic organelles and membranes (Mungthin *et al.*, 1998). Although resistance to mefloquine has been reported in South East Asia, it does not seem to relate with chloroquine but rather with quinine and halofantrine. This chemotherapeutic drug shares side effects similar to other quinolines and strong character concerning neuropsychiatric adverse effects is dominant. It is a potent inducer of depression, confusion, acute psychosis, nightmares and seizures. Mefloquine is thus contra-indicated in epilepsy and patients with psychiatric disorders.

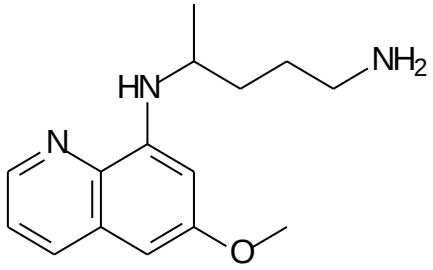


(**6**)

Figure 6 The structure of mefloquine, as a racemate (**6**)

2.8.1.4 The 8-aminoquinolines

The synthetic 8-aminoquinoline primaquine (**7**) is a tissue schizonticide indicated for liver eradication of all species of malaria. Known as the only drug to be effective against the dormant hypnozoites of *P. vivax* and *P. ovale* it is gametocidal and requires a single dose for complete gametocyte clearance from the human host. Although this treatment carries no clinical value it plays a vital role in the control of malaria by preventing sexual gametocyte transmission to mosquitoes (Baird & Surjadjaja, 2011). Studies have illustrated its use prophylactically but a concern regarding toxicity prevents this treatment regime. Primaquine carries some risk of safety for example; it should be withheld from infected individuals until their glucose-6-phosphate dehydrogenase (G6PD) status is known. A single dose of primaquine can cause hemolysis or methomoglobinemiea in G6PD deficient individuals. Treatment with this drug can also cause serious adverse reactions e.g. leucopenia, agranulocytosis, leukocytosis and cardiac arrhythmias and should be avoided during pregnancy (Katzung *et al.*, 2007).

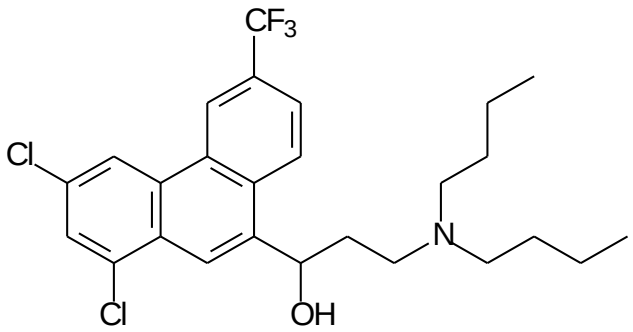


(**7**)

Figure 7 Structure of primaquine (**7**)

2.8.1.5 The 9-phenanthrene methanols

The blood schizonticide halofantrine (**8**) is a 9-phenanthrene methanol derivative which is active against all five species of malaria infecting humans. Halofantrine is used against *P. falciparum* resistant strains and is intrinsically more potent than quinine and mefloquine. Unfortunately, its use has caused rare and sudden death of treated individuals. The QT and PR intervals of the heart's electrocardiogram (ECG) are dose related prolonged, thus resulting in altered cardiac conduction. Although the drugs' absorption is improved when taken with a meal, administration is recommended on an empty stomach to avoid malabsorption (Kain, 1995).



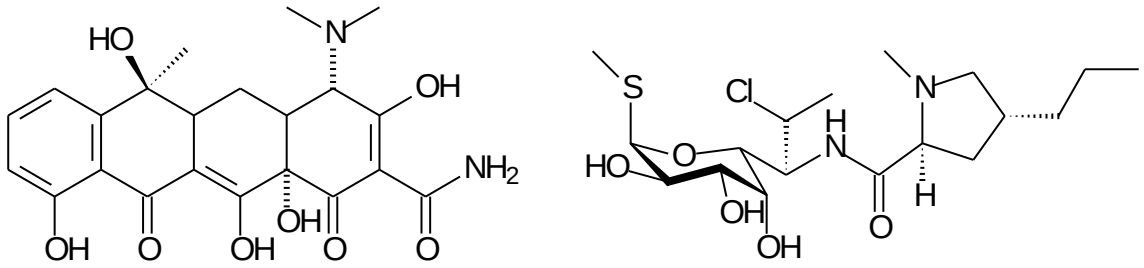
(**8**)

Figure 8 Structure of halofantrine (**8**)

2.8.2 Antibiotics

Numerous antibiotics are used for the treatment of malaria. The mechanism of action of these drugs is unclear and still open for discussion. Tetracycline (**9**) and doxycycline are erythrocytic schizonticides, and are mostly used for the prophylactic treatment of both chloroquine- and mefloquine-resistant *P. falciparum* (Katzung et al., 2007). Although

these antibiotics are generally well tolerated, side effects including photosensitivity, infrequent gastrointestinal symptoms and candidal vaginitis are common. Clindamycin (**10**) is another antibiotic of the lincosamide class effective against malaria. Tetracyclines are known to interact with and form calcium complexes causing tooth enamel dysplasia and irregularities in bone growth, making these drugs contraindicated during pregnancy and adolescence. Clindamycin must be administrated alternatively in these instances. Tetracycline/doxycycline and clindamycin are used in combination with quinidine or quinine against uncomplicated malaria, resulting in a better-tolerated and shorter course of quinine treatment (Katzung *et al.*, 2007).



(**9**) (**10**)

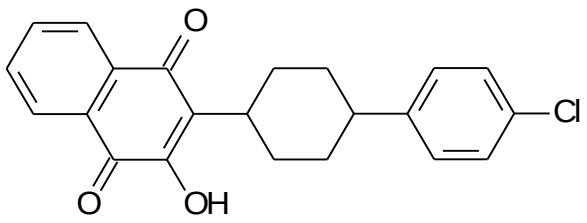
Figure 9 Structures of tetracycline (**9**) and clindamycin (**10**)

2.8.3 The Naphthalenes

Atovaquone (**11**), a hydroxynaphthoquinone is as a blood schizonticide. It was initially developed for its broad-spectrum antiparasitic activity against pneumonia and toxoplasmosis but was later selected for its antimalarial activity. To achieve a synergistic effect against the *Plasmodium* parasite, atovaquone and proguanil are used in combination (Malarone®) (Mustafa & Agrawal, 2008). This naphthalene selectively interrupts mitochondrial electron transport via cytochrome *b*, disrupting mitochondrial trans-membrane potential, resulting in the loss of nucleic acid synthesis in the malaria parasite (McKeage *et al.*, 2003).

Resistance towards atovaquone is a setback and the drug must never be administered as a single agent. Resistance mainly occurs *via* single point mutations on cytochrome *b* and mutations in the catalytic domain of the of the cytochrome *bc*1 complex (Korsinczky *et al.*, 2000; Vaidya & Mather, 2000). Although atovaquone is more expensive than mefloquine and doxycycline, it requires a shorter period of treatment. The following

adverse effects are common with the use of this drug: headaches, nausea, vomiting, fevers, rashes and insomnia (Katzung *et al.*, 2007).



(**11**)

Figure 10 Structure of atovaquone (**11**)

2.8.4 The Artemisinins

The sesquiterpene lactone artemisinin (**12**) was originally isolated from the Chinese herb, *qinghao,* and characterized in 1979. This plant has been used antipyretically for more than two centuries by the Chinese. Artemisinin's low water solubility and short half-life ($t_{1/2}$) causes more frequent oral dosage intake, which result in poor patient compliance. To increase both solubility and half-life structural changes to the pharmacophore have produced more potent and soluble artemisinin derivatives. Of these derivatives, artemether (**14**) and artemotil/arteeter (**16**) are lipid soluble and artesunate (**15**) is water soluble (Eltahir *et al.*, 2010), making these compounds useful for oral, intramuscular and rectal administration (Katzung *et al.*, 2007). All of these artemisinin derivatives are metabolized *in vitro* to the extremely potent rapidly acting blood schizonticide dihydroartemisinin (**13**) (Krungkrai *et al.*, 2010). The mechanism of action resulting in antimalarial activity of the artemisinins is still to be elucidated. However, they are reported to be involved in heme conversions, mitochondrial electron transport systems, sarcoplasmic reticulum inhibition, parasite endocytosis inhibition and hydroxylation of parasite biomolecules (Krungkrai *et al.*, 2010). The lactones appear to be the exceptionally well tolerated in treatment with only minor side effects including nausea, vomiting and diarrhoea. They, however, are teratogenic and should thus not be administered to pregnant women.

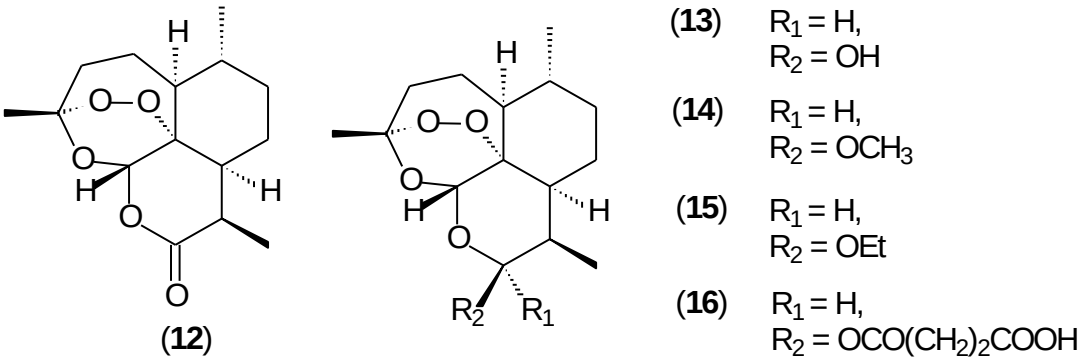


Figure 11 Structures of artemisinin (**12**), dihydroartemisinin (**13**), artemether (**14**), artesunate (**15**) and artemotil (**16**)

2.8.5 Artemisinin Combination Therapy (ACT)

Artemisinins are considered to be the most effective treatment for uncomplicated as well as severe and chloroquine resistant malaria. The half-lives of artemisinins are very short and this makes monotherapy irrelevant because repeated doses must be administered regularly (every 4 hours) (Kauss *et al.*, 2010). Potent combinations of artemisinins with other antimalarial drugs are thus used for therapeutic application and to prevent artemisinin resistance. These include variable combinations of artesunate, artemether and artemotil with pyronaridine (**17**), lumefantrine (**18**) (van Agtmael *et al.*, 1999), piperaquine (**19**), amodiaquine (**4**) (Sowunmi *et al.*, 2009) and sulphadoxine-pyrimethamine.

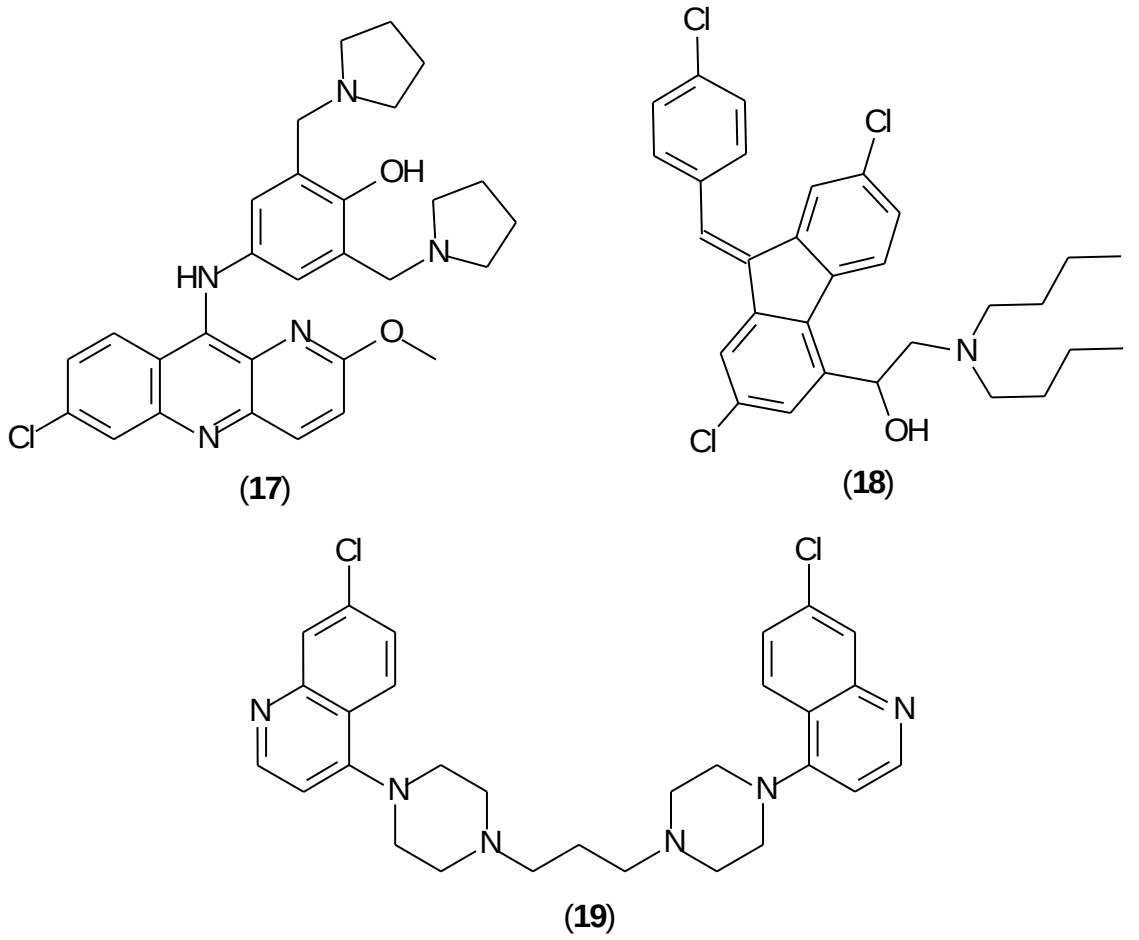


Figure 12 Structures of pyronaridine (**14**), lumefantrine (**15**) and piperaquine (**16**)

2.8.6 The Antifolates

2.8.6.1 The dihydrofolate pathway

The antifolates are active against malaria parasites as they inhibit the production of dihydropteroate and tetrahydrofolate, two important biological folate cofactors in the synthesis of DNA and RNA (Nzila *et al.*, 2005). Humans depend on their folate intake through their diet whereas protozoa lack this characteristic and need to synthesize the folates *de novo* by means of precursors such as *p*-aminobenzoic acid, guanosine triphosphate and glutamate (Wang *et al.*, 2007). This metabolic character difference between mammalian and protozoan species results in a perfect drug target for antimalarial therapy (Biagini *et al.*, 2003). The antifolates that have been used to date only target two enzymes in the folate biosynthesis pathway resulting in two types of antifolates.

They are:

1. The sulphonamides; sulphadoxine and sulphones; dapsone which inhibit the dihydropteroate synthetase enzyme responsible for the catalyses of 6-hydroxymethyl-7,8-dihydropterin pyrophosphate and p-aminobenzoic acid (*p*-AB) to 7,8-dihydropteroate (DHP); and
2. The diaminopyrimidines; pyrimethamine and biguanides; proguanil and chlorproguanil, which inhibit the dihydrofolate reductase enzyme resulting in the reduction of 7,8-dihydrofolate (DHF) to the active cofactor 5,6,7,8-tetrahydrofolate (Wang *et al.*, 2007).

Effective treatment can be achieved against chloroquine resistant strains of *P. falciparum* by combining these two types of antifolates either with each other or with other classes of antimalarial drugs, (Yuthavong, 2002).

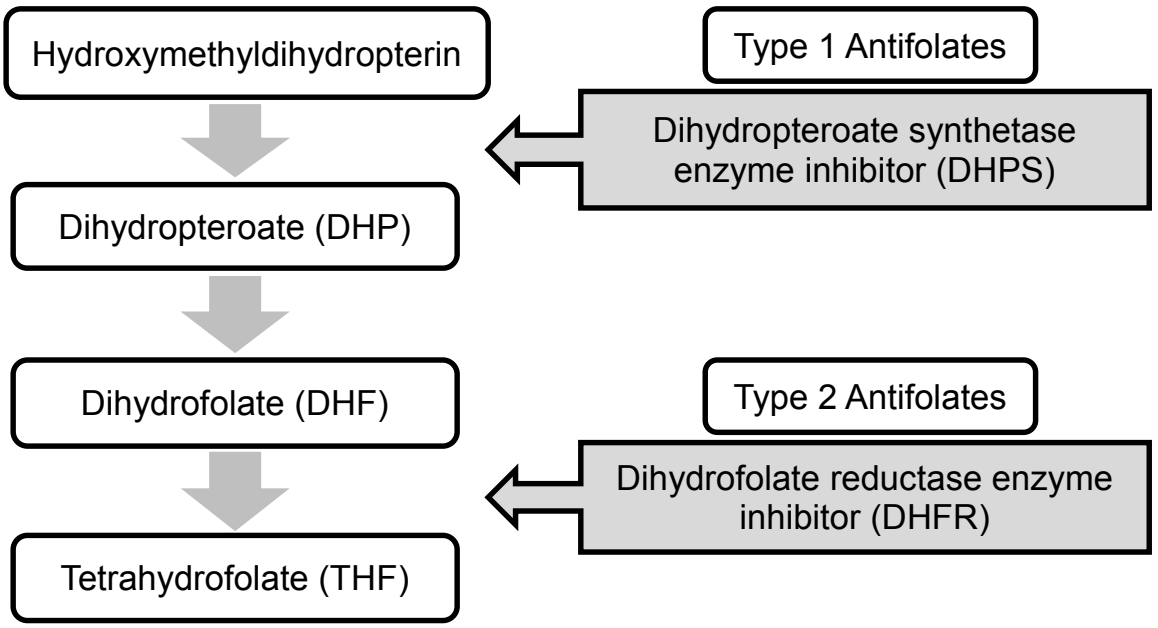
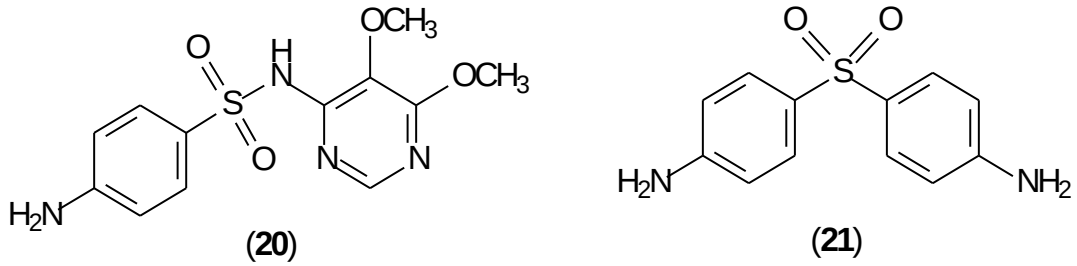


Figure 13 Type 1 & 2 antifolate drug target sites (van Heerden *et al.*, 2012)

2.8.6.2 Type 1 antifolates: dihydropteroate synthetase enzyme inhibitors

Sulphadoxine (**20**) is a long-acting sulphonamide anti-bacterial with an average half-life of about 170 hours. This slow acting drug feature results in the formation of resistance very easily and should never be used as a monotherapy against malaria.

Diaminodiphenylsulfone; dapsone (**21**) is another widely used folate synthesis inhibitor, but with a much shorter half life (30 h) than sulphadoxine, and is generally well tolerated. Type 1 antifolates are active against erythrocytic schizonts but not liver stages and gametocytes. Side effects are common with these dihydropteroate synthetase enzyme inhibitors. They should be withheld from patients with glucose-6-phosphate dehydrogenase deficiencies (G6D) as hemolysis may occur. Other side effects include rare gastrointestinal toxicity, cutaneous toxicity, central nervous system toxicity, renal side effects and haematological disturbances.



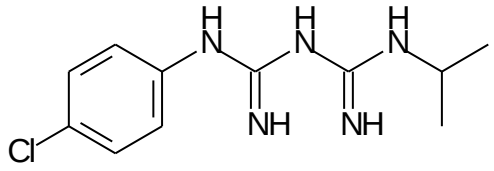
(**20**) (**21**)

Figure 14 Structures of sulphadoxine (**20**) and dapsone (**21**)

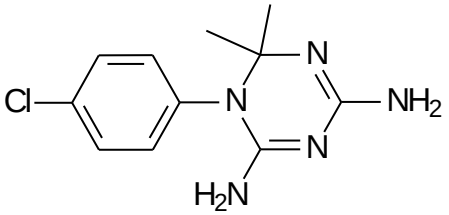
2.8.6.3 Type 2 antifolates: dihydrofolate reductase enzyme inhibitors

The biguanide prodrug (DHFR, dihydrofolate reductase) inhibitors i.e. proguanil (**22**) and chlorproguanil (**24**) are metabolized *in vivo* to their active triazine metabolites; cycloguanil (**23**) and chlorcycloguanil (Watkins & Sixsmith, 1984). These antifolates are considered the safest of all antimalarials (Cook *et al.*, 2009). Proguanil is used in combination with atovaquone (Malarone®) and chlorproguanil in combination with dapsone (lapDap®) for the treatment and chemoprophylaxis of chloroquine-resistant *P. falciparum*. They are active against erythrocytic schizonts and to some extent hepatic forms of malaria with minimal adverse effects such as occasional mouth ulcers and abdominal discomfort emerging.

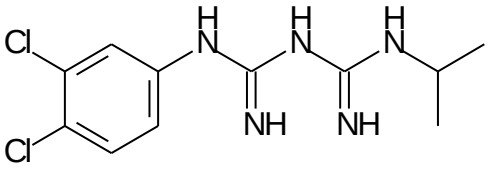
The 2,4-diaminopyrimidine; pyrimethamine (**25**) is related to trimethoprim, a DHFR inhibitor antibiotic. Pyrimethamine is considered a safe drug and adverse effects relate to pre-existing folate deficiencies. In combination with sulphadoxine, it reduces the development of mature asexual trophozoites and also possesses pre-erythrocytic and sporontocide activity (Cook *et al.*, 2009). Type 2 antifolates have extensively been used against uncomplicated chloroquine resistant malaria but emerging resistance remains a problem and a great disadvantage.



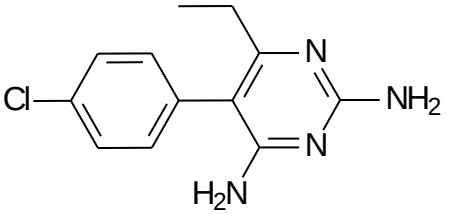
(**22**)



(**23**)



(**24**)

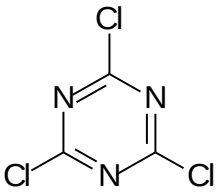


(**25**)

Figure 15 Structures of proguanil (**22**), cycloguanil (**23**), chlorproguanil (**24**) and pyrimethamine (**25**)

2.9 Recent malaria research – Triazine derivatives

The applications of 2,4,6-trichloro-1,3,5-triaizine (cyanuric chloride, CyCl) (**26**) are nearly endless. It has been studied immensely in a diverse range of applications including the pharmaceutical, textile, plastic and rubber industries. Triazine illustrates antibacterial, antitumor and antiparasitic activity (Kumar & Gupta *et al.*, 2010; Srinivas *et al.*, 2005). By employing an appropriate solvent and a hydrochloride acceptor, the chlorine atoms in cyanuric chloride can easily be substituted with various nucleophiles. These substitutions when controlled by temperature can allow the stepwise formation of mono-, di- or trisubstituted analogues of triazine variations (Blotny, 2006).



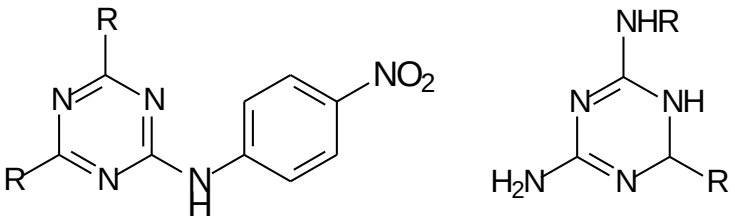
(**26**)

Figure 16 Structure 2,4,6-trichloro-1,3,5-triaizine/cyanuric chloride (**26**)

Cyanuric chloride is structurally related to cycloguanil. This property has been studied indicating that triazines act as DHFR drugs (Sunduru *et al.*, 2010). By combining this pharmacophore to various active antimalarial drugs, resistance was avoided with promising future development of antimalarial chemotherapies. Various triazine substituted analogues have been synthesized and include the following:

2.9.1 2,4,6-Trisubstituted triazine derivatives

A series of trisubstituted triazines were synthesized by Agarwal and colleagues. All of the compounds in this series contained *p*-nitro aniline to replace the chlorobenzene group of cycloguanil along with different nucleophiles (**27**). Among the 19 compounds synthesized, seven showed a MIC (minimum inhibitory concentration) of 21.2 µM and eight in the range of 2.2 – 5.6 µM (Agarwal *et al.*, 2005) against the NF-54 strain of *P. falciparum*. These compounds were also screened for structure activity relationships by means of quantitative analysis (QSAR) which indicated a good correlation between structure and inhibition of DHFR especially the Coulson charge on the N-3 of the triazine ring (Ojha *et al.*, 2011). In another study by Gravestock and colleagues, 2,N^6-disubstituted 1,2-dihydro-1,3,5-triazine-4,6-diamine hydrochloride salts were synthesized (**28**). Among the 28 compounds synthesized nine displayed IC_{50} values better than cycloguanil 5 µM against the cycloguanil-resistant FCR-3 *P. falciparum* strain (Gravestock *et al.*, 2011).



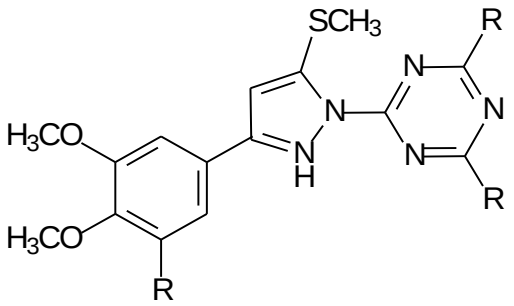
(**27**) (**28**)

R = Various nucleophiles

Figure 17 Structures of trisubstituted triazines (**27**) and 2,N^6-disubstituted 1,2-dihydro-1,3,5-triazine-4,6-diamines (**28**)

2.9.2 Pyrazole based triazines

Kaityar and colleagues synthesized a series of pyrazole based triazines. Among the 22 compounds synthesized, 6 compounds (**29**) showed MIC in the range of 1 – 2 µg/mL and were on an average 32 times more potent than the reference drug cycloguanil against the NF-54 strain of *P. falciparum* (Katiyar *et al.*, 2005).



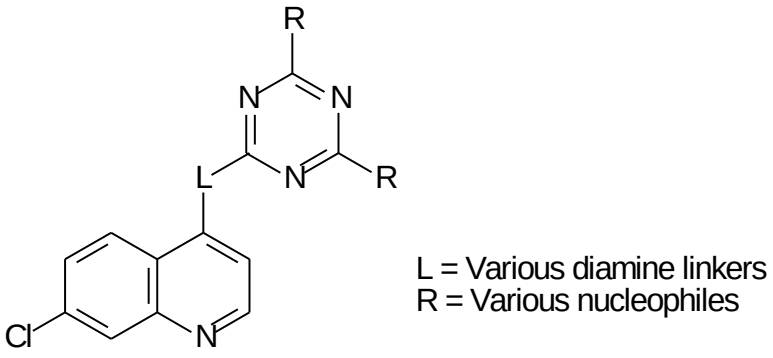
(**29**) R = Various amines

Figure 18 Structures of pyrazole based triazines (**29**)

2.9.3 Hybrid 4-aminoquinoline triazines

4-Aminoquinoline remains an attractive pharmacophore for ongoing investigation for antimalarial activity despite resistance to this scaffold. Various 4-aminoquinoline triazine hybrids containing different linker moieties and nucleophiles, respectively have been synthesized in the past decade. In one study Kumar *et al.* synthesized a series containing 2-(piperazin-1-yl)ethan-1-amine as linker (Kumar *et al.*, 2008). Among the 19 compounds synthesized (**30**), 3 demonstrated $IC_{50's}$ less than 10 ng/mL *in vitro* against the 3D7 chloroquine sensitive strain of *P. falciparum* compared to the reference drug chloroquine 2.6 ng/mL. *In vivo* studies were performed on five of the most active derivatives with two indicating 99.9% suppression of parasitemia on day 4. In another study by the same group, 41 compounds with two different linkers i.e. *p*-phenylenediamine and *m*-phenylenediamine (Kumar *et al.*, 2011) were synthesized. Five of these showed better and 4 had activity comparable to that of chloroquine against the 3D7 strain of *P. falciparum.*

Sunduru and co-workers synthesized twenty 4-aminoquinoline triazine hybrid compounds. Three compounds had improved activity and 5 were comparable to chloroquine against the sensitive 3D7 strain of *P. falciparum* (Sunduru *et al.*, 2009).

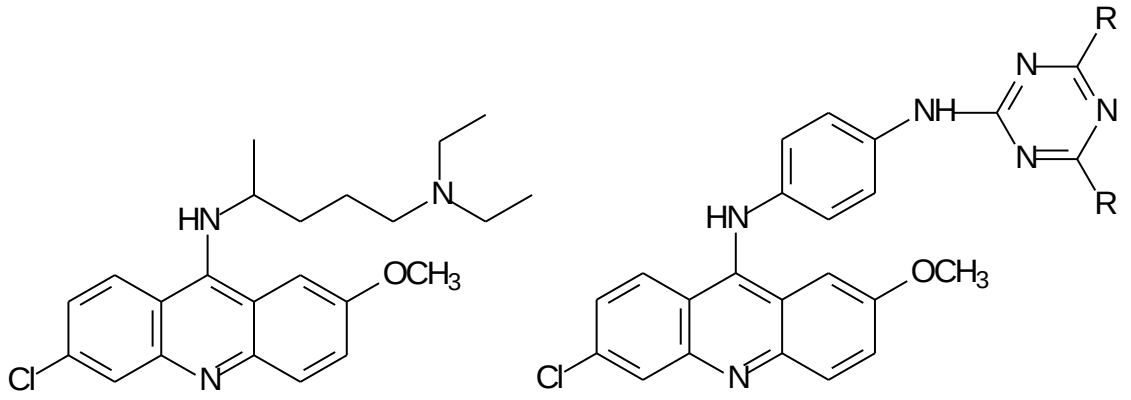


(**30**)

Figure 19 Structures of 4-aminoquinoline-triazine hybrids (**30**)

2.9.4 9-Anilinoacridinetriazines

The first synthetic antimalarial to be used before chloroquine was quinacrine (**31**). Pyronaridine (**14**), another acridine based schizonticide antimalarial has recently been introduced as an active drug against chloroquine resistant *P. falciparum* strains. This demonstrates the pivotal role of the acridine moiety against malaria. Kumar *et al.* synthesized a series of 9-anilinoacridine triazine hybrids using *p*-phenylenediamine as linker and various nucleophiles respectively on the cyanuric chloride scaffold. Among the 22 compounds synthesized (**32**), 4 compounds illustrated enhanced (4.21 – 7.95 nM) and 3 had comparable activity to chloroquine (8.15 nM) against to the 3D7 strain of chloroquine sensitive *P. falciparum* (Kumar *et al.*, 2009).



(**31**) (**32**) R = Various amines

Figure 20 Structures of quinacrine (**31**) and 9-anilinoacridinetriazines (**32**)

2.9.5 1,3,5-triazine-substituted polyamines

In a study by Klenke and colleagues, four series of compounds containing 25 triazine-substituted polyamines were synthesized. The 4 series made use of a common linker between the triazine scaffolds except for series three, which possessed only one triazine moiety. Series 1 and 2 contained 2 triazine moieties and series 4, four moieties. These series were generally more active against the chloroquine-resistant K1 strain, compared to the chloroquine-sensitive NF-54 strain of *P. falciparum*. The addition of simple unbranched analogues and multiple triazine moieties resulted in an increase of activity (Klenke *et al.*, 2003).

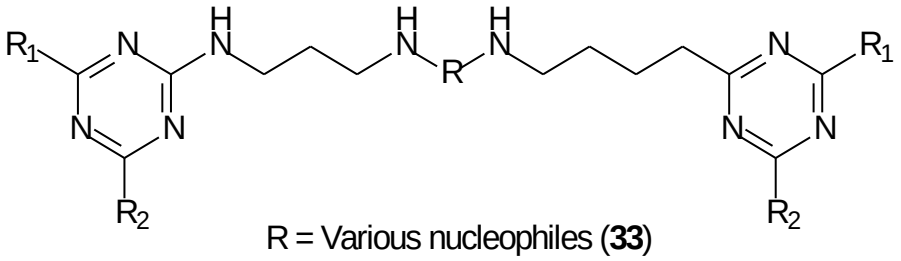
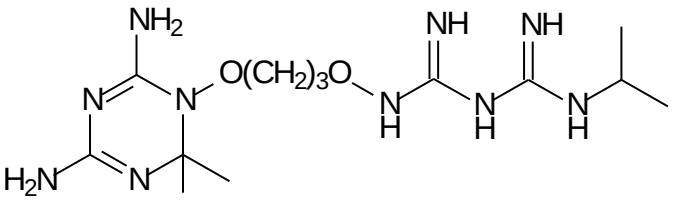


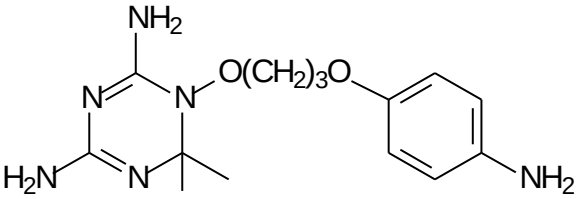
Figure 21 General structure of triazine polyamines (**33**)

2.9.6 Triazine-related drugs

Of the triazine related antimalarial drugs, the prodrug PS-15 (**34**) and its active metabolite WR99210 (**35**) demonstrated promising activity against both sensitive and resistant strains of malaria. These drugs are structurally related to the prodrug proguanil and its active metabolite cycloguanil (Kinyanjui *et al.*, 1999). WR99210 is roughly a thousand times more active than the prodrug PS-15 and 46 times more active than cycloguanil against K1 strains of malaria (Edstein *et al.*, 1997). Unfortunately WR99210 can't be used because of gastrointestinal intolerance however PS-15 was designed to overcome this complication.



(**34**)



(**35**)

Figure 22 Structures of triazine antimalarial prodrug PS-15 (**34**) and WR99210 (**35**)

CHAPTER 3

ARTICLE FOR SUBMISSION

Chapter 3 contains the manuscript of an article to be submitted to bioorganic chemistry. The article contains the background, aim, experimental details and results, including the chemical properties and *in vitro* biological results of synthesised compounds of this study. This article is prepared according to the author's guidelines available in the author information pack at the journal homepage:

http://www.journals.elsevier.com/bioorganic-chemistry/

Synthesis and *in vitro* antimalarial activity of a series of bistriazine compounds

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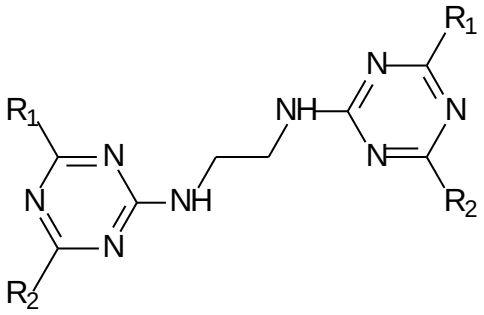
Graphical abstract

A series of bistriazine compounds were synthesised and their *in vitro* antimalarial activity was evaluated against the chloroquine-sensitive (3D7), chloroquine-resistant (K1) strains of *P. falciparum* as well as their cytotoxicity evaluated against BJ, HEK293, HepG2 and Raji cell lines.



11 - 19

R_1, R_2 = Amines

Abstract

A series of amine disubstituted bistriazines were synthesized and their *in vitro* antiplasmodial activity was determined alongside that of chloroquine against various strains of *Plasmodium falciparum.* The cytotoxicity against human cell lines; BJ, HEK293, HepG2 and Raji were also assessed. The bistriazines were synthesized in a two-step reaction by nucleophilic substitution on carbon C-2, 4 and 6 of cyanuric chloride. Five of the nine synthesized compounds were active against both 3D7 and K1 strains of *P. falciparum.* They were found with activity either, comparable or significantly more than chloroquine against the CQR K1 strain, while being less active against the 3D7 strain. They all possessed meaningful selectivity towards the parasitic cells. The bistriazine **19** featuring aniline substituents was the most active (IC_{50} = 0.23 μM) of all, possessing a moderate ~6-fold increased potency against the K1 strains in comparison with chloroquine (IC_{50} = 1.67 μM).

Keywords: Triazine, bistriazine, *Plasmodium falciparum*, malaria, antimalarial activity,

Highlights

- Synthesis of a series of amine substituted bistriazines
- *In vitro* antimalarial activity evaluation against CQS 3D7 and CQR K1 strains
- Five active compounds more potent than CQ against CQR K1 strain
- All compounds non-toxic to human BJ, HEK293, HepG2 and Raji cell lines
- Aniline disubstituted bistriazine 6-fold more potent than CQ against K1

1. Introduction

Malaria is a preventable and curable disease; however its mortality persists at an estimated 1 million people annually. This febrile parasitic disease is a worldwide deadly killer affecting more than a 100 countries (roughly 40% of the world), 255 million people annually [1,2]. Disease treatment is becoming increasingly difficult due to its widespread multidrug resistance to the classic antimalarial drugs such as chloroquine, quinine and sulphadoxine/pyrimethamine [3]. Artemisinin has been declared as the first line treatment regime for chloroquine-resistant malaria by the WHO, but resistance to this drug has recently been reported in the South-East Asian region, calling for an immediate approach to the design of novel and effective antimalarials [4].

For the past four decades the combination drug pyrimethamine/sulphadoxine and chloroquine have been employed as first line treatments against *P. falciparum* infections. However, these drugs have been rendered useless in certain parts of the world as a result of *P. falciparum* resistance. Among the classes of antimalarials available, sulphadoxine and pyrimethamine are designated to the antifolates [5]. They are active against *Plasmodia* parasites as they inhibit the production of dihydropteroate (DHP) and tetrahydrofolate (THF) respectively, two biological important folate cofactors in the synthesis of parasitic DNA and RNA. Humans rely on dietary folate intake whereas protozoa lack this characteristic, and are obliged to synthesize these essential vitamins *de novo* by means of precursors such as p-aminobenzoic acid, guanosine triphosphate and glutamate [6]. This metabolic character difference between mammalian and protozoan species results in a perfect drug target for antimalarial therapy [7].

Malarone® contains proguanil and atovaquone [8], this combination is used for the treatment and chemoprophylaxis of chloroquine-resistant *P. falciparum*. Proguanils' active metabolite, cycloguanil (**A**) is active against protozoa as it is a dihydrofolate reductase (DHFR) inhibitor [9]. This metabolite is a commonly used, well defined historically important drug in the treatment of malaria [10,11]. Although point mutations in the enzyme amino acid sequence have led to resistance to the two well defined antifolates, proguanil (cycloguanil) and pyrimethamine, structural changes to these drugs can overcome this problem [12].

Cyanuric chloride (**1**), containing a triazine moiety, is a six-membered heterocyclic ring with 3 chlorine and 3 nitrogen atoms. The applications of 2,4,6-trichloro-1,3,5-triaizine are nearly endless. It has been studied immensely in a diverse range of applications presenting antibacterial, antitumor and antiparasitic activity [13,14]. It

shares similar chemical properties and biological activity to cycloguanil [15]. By employing an appropriate solvent and a hydrochloride acceptor, the chlorine atoms in cyanuric chloride can easily be substituted with various nucleophiles. These substitutions when controlled by temperature can allow the stepwise formation of mono-, di- or trisubstituted analogues of triazine [16]. Related compounds, including triazine hybrids and triazine related drugs PS15 and WR99210 (**B**) have shown promising antimalarial activity in chloroquine sensitive and resistant strains [17,18]. Studies have shown that by synthesizing bis compounds more efficacious antiplasmodial drugs can be produced [19]

The aim of this study was to synthesize a series of bistriazine compounds containing various amines and to evaluate their *in vitro* antimalarial activity as well as cytotoxicity. We herein report their synthesis and activities in comparison with chloroquine.

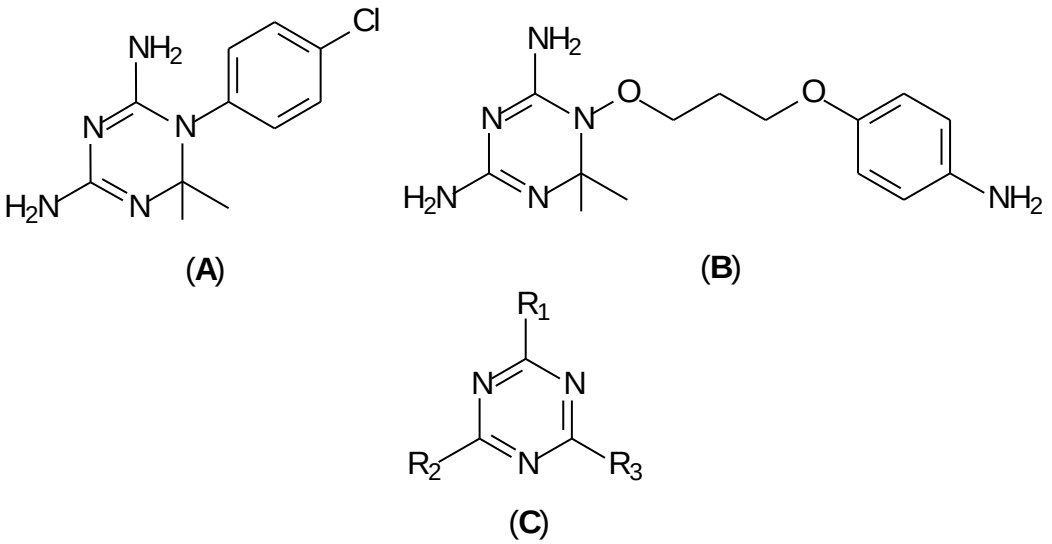


Figure 23 Structures of cycloguanil (**A**), WR99210 (**B**), various trisubstituted triazines (**C**)

2. Materials and methods

2.1. Materials

The reagents 2,4,6-trichloro-1,3,5-triazine, aniline, *p*-anisidine, 2-(diisopropylamino)ethylamine, ethylenediamine (EDA), morpholine, 2-methylpiperidine, potassium carbonate (K_2CO_3), and piperidine and solvents, methanol (MeOH), dichloromethane (DCM), ethyl acetate (EtOAc) and tetrahydrofuran (THF) were purchased from Sigma-Aldrich (Johannesburg, South Africa). All the reagents and chemicals were of analytical grade

2.2. General procedures

Thin-layer chromatography (TLC) was performed using silica gel plates ($60F_{254}$ Merck). Preparative flash column chromatography was carried out on silica gel (230-240 mesh, G60 Merck) and silica gel 60 (70-230 mesh ASTM, Fluka).

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 600 spectrometer (at a frequency of 600.17 and 150.913 MHz, for ^{1}H and ^{13}C respectively) in deuterated chloroform (Chloroform-D_1) and deuterated dimethyl sulfoxide-d_6 (dimethyl sulfoxide-d_6). Chemical shifts are reported in parts per million (δ ppm) using tetramethylsilane (TMS) as internal standard. The splitting pattern abbreviations are as follows: s (singlet), d (doublet), dd (doublet of doublets), dt (doublet of triplets), t (triplet), td (triplet of doublets), tt (triplet of triplet) and m (multiplet). High resolution ESI or APCI (HRESI or HRAPCI) mass spectra (MS) were recorded on a Bruker micrOTOF-Q II 10390 mass spectrometer in ESI and APCI modes and [M+H]+ ions were recorded. Infrared (IR) spectra were recorder on a Bruker alpha-PFTIR an ATR IR instrument. The melting points (mp) were determined on a Büchi melting point B-545 instrument.

3. Experimental procedures

The target amine substituted bistriazine compounds were synthesized in a 2-step process. First, the triazine ring was either bis- or disubstituted by nucleophilic substitution/s of two of the three chlorine atoms of cyanuric chloride using various amines in the presence of potassium carbonate as base. This gave rise to the intermediates **2** - **10**, which are reacted with ethylenediamine in the second step to afford the target bistriazines **11** - **19**.

3.1. Synthesis of disubstituted triazines **2** - **10**

The diamine substituted triazine intermediates were synthesized as depicted in scheme 1 using a reported general method [20] with slight modifications, and described as follows: Cyanuric chloride (**1**) (10.85 mmol) was dissolved in 25 ml of anhydrous THF at room temperature and cooled to -15°C and K_2CO_3 (10.85 mmol, 1.0 equiv.) was added. Amine (secondary/primary) (10.85 mmol, 1.0 equiv.) was dissolved in 5 ml of THF and added drop wise to the cyanuric chloride/K_2CO_3 mixture over a half hour whilst stirring. The reaction mixture was stirred at 0°C for another hour. The reaction progress was monitored *via* thin layer chromatography. After completion, the mixture was evaporated to dryness *in vacuo*. The dried residue was then suspended in 50 ml of distilled water and the monosubstituted intermediate was extracted with ethyl acetate (3 x 50 ml). The organic phase was collected, dried over $MgSO_4$, filtered and evaporated to dryness *in vacuo*. No further purification was conducted. This procedure repeated with the monosubstituted compounds led to the amine disubstituted triazine intermediates **2** - **10** with slight modifications to temperature (room temperature) and duration (6 – 24h). All synthesized intermediates were solids. The NMR, MS, IR data as well as melting point of intermediates **2** - **10** are reported. Intermediates **2** – **10** and compounds **11** – **19** are numbered similar.

3.1.1. *2-chloro-4,6-bis(morpholin-4-yl)-1,3,5-triazine* **2**

Compound **2** was synthesized over a period of 6h and purified by chromatography using DCM as eluent.

Yield: 86%; white powder; mp: 175°C; IR (ATR)/cm^{-1}: 3262, 2927, 2855, 1729; ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.79 – 3.70 (m, 4H, H-9), 3.69 – 3.65 (m, 4H, H-10); ^{13}C NMR (151 MHz, $CDCl_3$): δ 169.59 (C-4,-6), 164.37 (C-2), 66.48 (C-9), 43.81 (C-10); HRMS-APCI *m/z*: 286.1042 (M+1) (M+1, $C_{11}H_{16}ClN_5O_2$, 286.1071 calc.).

3.1.2. 2-chloro-4-(morpholin-4-yl)-6-(piperidin-1-yl)-1,3,5-triazine **3**

Compound **3** was synthesized over a period of 6h and purified by chromatography using DCM as eluent.

Yield: 62%; white powder; mp: 162°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.77 – 3.65 (m, 8H, H-9,-10), 1.62 (q, *J* = 6.2 Hz, 8H, H-11,-12), 1.56 – 1.53 (m, 2H, H-13); ^{13}C NMR (151 MHz, $CDCl_3$): δ 169.50 (C-2), 164.50 (C-4), 163.92 (C-6), 66.60 (C-10), 44.46 (C-12), 43.73 (C-9), 25.67 (C-11), 24.54 (C-13).

3.1.3. 2-chloro-4-(2-methylpiperidin-1-yl)-6-(morpholin-4-yl)-1,3,5-triazine **4**

Compound **4** was synthesized over a period of 6h and purified by chromatography using DCM as eluent.

Yield: 72%; pure white powder; mp: 158°C; IR (ATR)/cm^{-1}: 2923, 2855, 1728, 793; ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.04 - 4.95 (m, 1H, H-15), 4.55 – 4.50 (m, 1H, H-11), 3.76 – 3.72 (m, 8H, H-9,-10), 3.67 (t, *J* = 4.8 Hz, 1H, H-11), 2.87 (d, *J* = 2.9 Hz, 2H, H-13), 1.74 – 1.57 (m, 2H, H-14), 1.54 (dd, *J* = 8.2, 3.0 Hz, 4H, H-12), 1.14 (d, *J* = 7.0 Hz, 3H, H-16); ^{13}C NMR (151 MHz, $CDCl_3$): δ 169.45 (C-2), 164.53 (C-4), 163.89 (C-6), 66.64 (C-10), 45.81(C-15), 43.73 (C-9), 38.46 (C-11), 30.03 (C-14), 25.58 (C-12), 18.80 (C-16); HRMS-APCI *m/z*: 298.1402 (M+1) (M+1, $C_{13}H_{20}ClN_5O$, 298.1435 calc.).

3.1.4. (2-{[4-chloro-6-(morpholin-4-yl)-1,3,5-triazin-2-yl]amino}ethyl)bis(propan-2-yl)amine **5**

Compound **5** was synthesized over a period of 10h and purified by chromatography using the solvent mixture DCM:MeOH 9:1 as eluent.

Yield: 68%; white powder; mp: 124°C; IR (ATR)/cm^{-1}: 3262, 2960, 796; ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.78 (t, *J* = 4.8 Hz, 4H, H-9), 3.67 (dt, *J* = 12.7, 4.7 Hz, 4H, H-10), 3.34 – 3.29 (m, 2H, H-12), 3.02 – 2.96 (m, 2H, H-14), 2.60 (d, *J* = 6.6 Hz, 2H, H-13), 1.00 – 0.96 (m, 12H, H-15); ^{13}C NMR (151 MHz, $CDCl_3$): δ 168.99 (C-2), 165.32 , (C-4), 164.58 (C-6), 66.57 (C-10), 47.85 (C-14), 43.73 (C-13), 43.00 (C-9), 39.89 (C-12), 20.71 (C-15); HR-APCI *m/z*: 343.1989 (M+1) (M+1, $C_{15}H_{27}ClN_6O$, 343.2013 calc.).

3.1.5. 4-chloro-6-(morpholin-4-yl)-N-phenyl-1,3,5-triazin-2-amine **6**

Compound **6** was synthesized over a period of 24h and purified by chromatography using the solvent mixture DCM:MeOH 4:1 as eluent.

Yield: 55%; white powder; mp: 119°C; IR (ATR)/cm^{-1}: 3256, 2978, 1605, 796; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.56 – 7.42 (m, 2H, H-13), 7.36 – 7.29 (m, 2H, H-14), 7.15 – 6.99 (m, 1H, H-15), 3.83 (s, 1H, H-11), 3.72 (t, *J* = 4.1 Hz, 8H, H-9,-10); ^{13}C NMR (151 MHz, $CDCl_3$): δ 164.31 (C-2), 164.00 (C-4), 163.21 (C-6), 137.46 (C-12), 128.91 (C-13), 124.11 (C-14), 120.55 (C-15), 66.52 (C-10), 44.13 (C-9); HRMS-APCI *m/z*: 292.0948 (M+1) (M+1, $C_{13}H_{14}ClN_5O$, 292.0965 calc.).

3.1.6. 4-chloro-N-phenyl-6-(piperidin-1-yl)-1,3,5-triazin-2-amine **7**

Compound **7** was synthesized over a period of 24h and purified by chromatography using the solvent mixture DCM:MeOH 4:1.

Yield: 63%; white powder: mp: 139°C; IR (ATR)/cm^{-1}: 3223, 2936, 2855, 1531, 979, 796; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.58 – 7.50 (m, 2H, H-14), 7.32 (t, *J* = 7.9 Hz, 2H, H-15), 7.07 (t, *J* = 7.4 Hz, 1H, H-16), 3.77 (q, *J* = 5.3 Hz, 1H, H-12), 1.71 – 1.56 (m, 10H, H-9,-10,11); ^{13}C NMR (151 MHz, $CDCl_3$): δ 164.30 (C-2), 163.81 (C-4), 163.33 (C-6), 137.85 (C-13), 128.84 (C-15), 123.69 (C-16), 120.26 (C-14), 44.92 (C-9), 25.69 (C-10), 24.46 (C-11); HRMS-APCI *m/z*: 290.1152 (M+1) (M+1, $C_{14}H_{16}ClN_5$, 290.12 calc.).

3.1.7. 4-chloro-N-(4-methoxyphenyl)-6-(2-methylpiperidin-1-yl)-1,3,5-triazin-2-amine **8**

Compound **8** was synthesized over a period of 10h and purified by chromatography using DCM as eluent.

Yield: 59%; off-white powder; mp: 114°C; IR (ATR)/cm-1: 3208, 2936, 1570, 1508, 1220, 799; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.40 (d, *J* = 8.5 Hz, 2H, H-12), 6.88 – 6.81 (m, 2H, H-11), 5.04 (s, 2H, H-15), 4.93 (s, 1H, H-19), 4.60 (s, 2H, H-14), 4.53 (d, *J* = 13.6 Hz), 2.98 – 2.90 (m, 2H, H-16), 1.66 (m, 2H, H-17), 1.42 (m, 1H, H-18), 1.20 (d, *J* = 7.0 Hz, 3H, H-19); ^{13}C NMR (151 MHz, $CDCl_3$): δ 163.31 (C-2), 163.16 (C-4), 156.10 (C-6), 130.92 (C-13), 130.76 (C-11), 122.23 (C-12), 113.99 (C-10), 38.89, (C-14), 30.03 (C-19), 25.53 (C-15), 18.73 (C-16), 15.62 (C-17), 15.26 (C-20); HRMS-APCI *m/z*: 334.1413 (M+1) (M+1, $C_{16}H_{20}ClN_5O$, 334.1435 calc.).

3.1.8. 6-chloro-2-N,4-N-bis(4-methoxyphenyl)-1,3,5-triazine-2,4-diamin **9**

Compound **9** was synthesized over a period of 6h and purified by chromatography using the solvent mixture DCMMeOH 4:1.

Yield: 67%; off-white powder; mp: >300°C; IR (ATR)/cm^{-1}: 3356, 2834, 1573, 1502, 1217, 788; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.73 (s, 2H, H-12), 7.44 – 7.36 (m, 3H, H-14), 7.27 – 7.21 (m, 2H, H-11), 6.90 (s, 1H, H-9); ^{13}C NMR (151 MHz, $CDCl_3$): δ 164.16 (C-2), 157.64 (C-4,-6), 128.44 (C-13), 123.45 (C-10), 123.10 (C-12), 114.40 (C-11), 55.50 (C-14); HRMS-APCI *m/z*: 358.1043 (M+1) (M+1, $C_{17}H_{16}ClN_5O_2$, 358.1071 calc.).

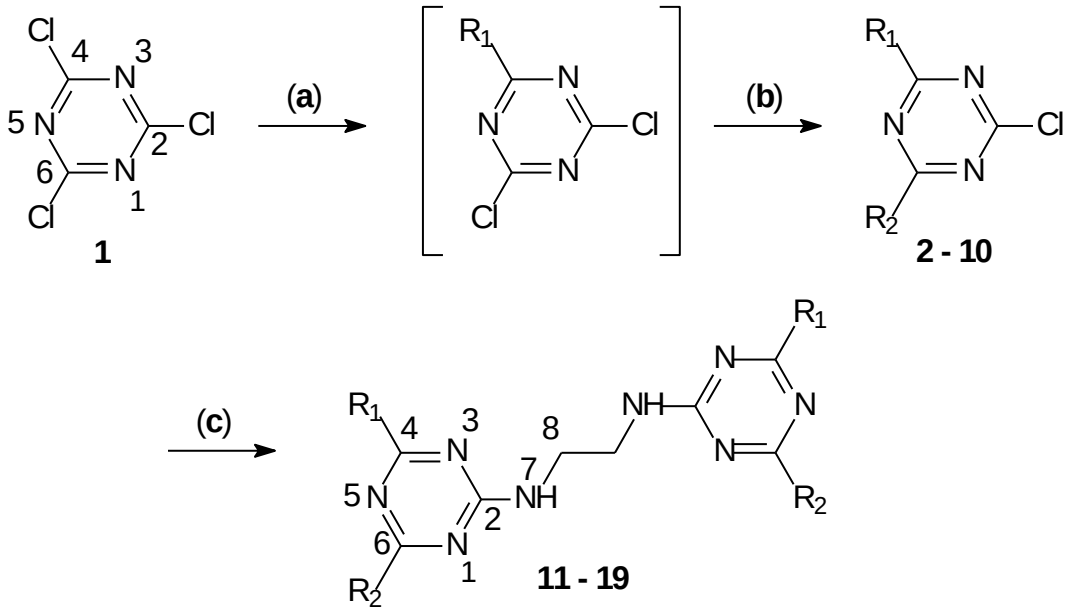
3.1.9. 6-chloro-2-N,4-N-diphenyl-1,3,5-triazine-2,4-diamine **10**

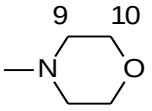
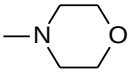
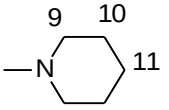
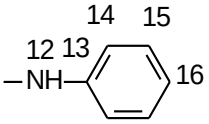
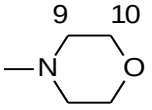
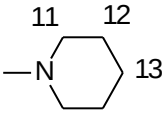
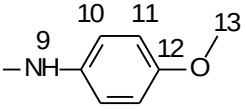
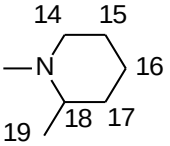
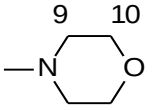
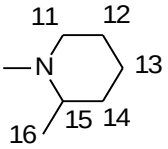
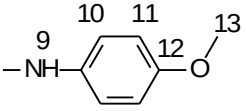
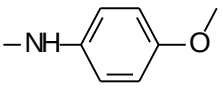
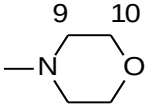
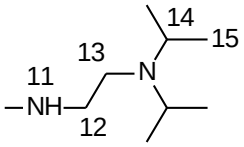
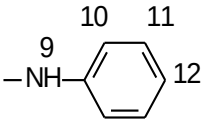
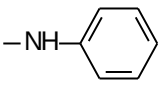
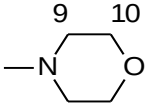
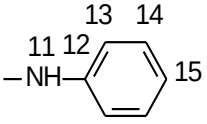
Compound **10** was synthesized over the period of 6h and purified by chromatography using the solvent mixture DCMMeOH 4:1.

Yield: 73%; white powder; mp: 198°C; IR (ATR)/cm^{-1}: 3404, 3260, 1410, 750; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.49 (d, *J* = 8.0 Hz, 1H, H-13), 7.32 (t, *J* = 7.9 Hz, 2H, H-11), 7.09 (t, *J* = 7.4 Hz, 1H, H-12), 2.78 (s, 1H, H-9); ^{13}C NMR (151 MHz, $CDCl_3$): δ 164.42 (C-2), 163.41 (C-4,-6), 137.53 (C-10), 128.90 (C-12), 124.06 (C-11), 120.53 (C-13).

3.2. Synthesis of target bistriazines 11 - 19

The amine substituted bistriazines were synthesized as depicted in scheme 1 and was achieved as follows: Given intermediate **2** - **10** (1.0 equiv.) was dissolved, K_2CO_3 (1.0 equiv.) was added in THF and refluxed. The reaction mixture was heated to 60°C and the EDA (0.5 equiv.) was added to the mixture. The reaction was monitored by TLC and continued for 24h. After 24h, the reaction mixture was evaporated to dryness *in vacuo* and the dried residue suspended in 50 ml of water. The bistriazines were extracted with EtOAc (3 x 50 ml) and the organic phase was collected, dried over $MgSO_4$, filtered and evaporated to dryness *in vacuo.* The desired final compounds **11** - **19** were purified using column chromatography and dried under vacuum. All of the synthesized compounds were solids, except **14**, which was oil. The ^{1}H, ^{13}C NMR, MS and IR data as well as melting points are reported.



Cpd	R_1	R_2	Cpd	R_1	R_2
11			**16**		
12			**17**		
13			**18**		
14			**19**		
15					

Scheme 1 Reagents and conditions: (**a**) aryl/alkyl amines, 0 °C, 1h, THF, K_2CO_3; (**b**) aryl/alkyl amines, 25 °C, 6 – 24h, THF, K_2CO_3; (**c**) intermediates **2** – **10,** ethylenediamine, THF, K_2CO_3, refluxed, 24h.

3.2.1 N-(2-{[bis(morpholin-4-yl)-1,3,5-triazin-2-yl]amino}ethyl)-4,6-bis(morpholin-4-yl)-1,3,5-triazin-2-amine **11**

Compound **11** was purified by chromatography using the solvent mixture MeOH:DCM:NH_4OH (9:1:0.1) as eluent.

Yield: 37%; pure white powder; mp: 275°C; IR (ATR)/cm^{-1}: 3329, 2963; ^1H NMR (600 MHz, $CDCl_3$): δ 3.69 – 3.61 (m, 16H, H-9,-10), 3.48 (d, J = 3.48 Hz, 2H, H-8), 1.84 (s, 1H, H-7); ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.43 (C-4,-6), 165.14 (C-2), 66.79 (C-10), 43.52 (C-9), 41.16 (C-8); HRMS-ESI *m/z*: 559.3208 (M+1) (M+1, $C_{24}H_{38}N_{12}O_4$, 559.3217 calc.).

3.2.2 4-(morpholin-4-yl)-N-(2-{[4-(morpholin-4-yl)-6-(piperidin-1-yl)-1,3,5-triazin-2-yl]amino}ethyl)-6-(piperidin-1-yl)-1,3,5-triazin-2-amine **12**

Compound **12** was purified by chromatography using EtOAc as eluent.

Yield: 22%; white powder; mp: 237°C; IR (ATR)/cm^{-1}:3253, 3149, 2924, 2849; ^1H NMR (600 MHz, $CDCl_3$): δ 3.71 – 3.63 (m, 12H, H-9,-10,-11), 3.51 (t, *J* = 4.1 Hz, 2H, H-8), 2.01 (s, 1H, H-7), 1.61 – 1.58 (m, 2H, H-13), 1.53 – 1.48 (m, 4H, H-12); ^{13}C NMR (151 MHz, $CDCl_3$): δ 166.52 (C-4), 165.32 (C-2), 164.77 (C-6), 66.86 (C-10), 43.99 (C-9), 43.53 (C-11), 41.21 (C-8), 25.75 (C-12), 24.90 (C-13); HRMS-ESI *m/z*: 555.3621 (M+1) (M+1, $C_{26}H_{42}N_{12}O_2$, 555.3632 calc.).

3.2.3 4-(2-methylpiperidin-1-yl)-N-(2-{[4-(2-methylpiperidin-1-yl)-6-(morpholin-4-yl)-1,3,5-triazin-2-yl]amino}ethyl)-6-(morpholin-4-yl)-1,3,5-triazin-2-amine **13**

Compound **13** was purified by chromatography using the solvent mixture MeOH:DCM (9.5:0.5) as eluent.

Yield: 56%; off white powder; mp: 157°C; IR (ATR)/cm^{-1}: 3349, 2926, 2849; ^1H NMR (600 MHz, $CDCl_3$): δ 5.04 – 4.95 (m, 1H, H-15), 4.60 – 4.51 (m, 1H, H-11), 3.68 (m, 8H, H-9,-10), 3.54 – 3.48 (m, 2H, H-8), 2.81 (t, *J* = 13.2 Hz, 1H, H-11), 2.14 (s, 1H, H-7), 1.67 – 1.56 (m, 2H, H-13), 1.51 (d, *J* = 8.6 Hz, 2H, H-14), 1.43 – 1.32 (m, 2H, H-12), 1.11 (d, *J* = 7.0 Hz, 3H, H-16). ^{13}C NMR (151 MHz, $CDCl_3$): δ 165.28 (C-2), 164.57 (C-4), 159.28 (C-6), 66.85 (C-10), 45.08 (C-15), 43.59 (C-9), 41.18 (C-8), 37.95 (C-11), 30.18 (C-14), 25.76 (C-12), 19.12 (C-13), 15.08 (C-16); HRMS-ESI *m/z*: 583.3952 (M+1) (M+1, $C_{28}H_{46}N_{12}O_2$, 583.3945 calc.).

3.2.4 2-N-{2-[bis(propan-2-yl)amino]ethyl}-4-N-(2-{[4-({2-[bis(propan-2-yl)amino]ethyl}amino)-6-(morpholin-4-yl)-1,3,5-triazin-2-yl]amino}ethyl)-6-(morpholin-4-yl)-1,3,5-triazine-2,4-diamine **14**

Compound **14** was purified by chromatography using the solvent mixture MeOH:DCM:NH_4OH (9.5:0.5:0.1) as eluent.

Yield: 37%; golden oil; ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.71 – 3.64 (m, 8H, H-9,-10), δ 3.49 (s, 2H, H-8), 3.31 (s, 2H, H-12), 3.31 (d, *J* = 8.6 Hz, 2H, H-14), 2.58 (s, 2H, H-13) 2.13 (s, 1H, H-7), 1.00 (d, *J* = 8.6 Hz, 12H, H-15); ^{13}C NMR (151 MHz, $CDCl_3$): δ 166.19 (C-2,-6), 165.16 (C-4), 66.83 (C-10), 48.54 (C-14), 44.25 (C-13), 41.15 (C-9), 40.26 (C-8,-12), 20.55 (C-15); HRMS (ESI) *m/z*: 673.5096 (M+1) (M+1, $C_{32}H_{60}N_{14}O_2$, 673.5101 calc.).

3.2.5 6-(morpholin-4-yl)-2-N-(2-{[4-(morpholin-4-yl)-6-(phenylamino)-1,3,5-triazin-2-yl]amino}ethyl)-4-N-phenyl-1,3,5-triazine-2,4-diamine **15**

Compound **15** was purified by chromatography using the solvent mixture MeOH:DCM (9.5:0.5) as eluent.

Yield 21%; white powder; mp: 187°C, IR (ATR)/cm^{-1}: 3275, 2919, 2851; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.57 – 7.43 (m, 2H, H-13), 7.31 – 7.18 (m, 2H, H14), 6.98 (m, 1H, H-15), 5.27 (s, 1H, H-11), 3.70 (t, *J* = 4.9 Hz, 8H, H-9,-10), 3.54 (d, *J* = 4.6 Hz, 2H, H-8), 2.15 (s, 1H, H-7); ^{13}C NMR (151 MHz, $CDCl_3$): δ 166.35 (C-4), 164.95 (C-2), 164.13 (C-6), 139.35 (C-12), 128.65 (C-13), 122.38 (C-14), 119.73 (C-15), 66.72 (C-10), 62.91 (C-8), 43.61 (C-9); HRMS-ESI *m/z*: 571.2993 (M+1) (M+1, $C_{28}H_{34}N_{12}O_2$, 571.3005 calc.).

3.2.6 2-N-phenyl-4-N-(2-{[4-(phenylamino)-6-(piperidin-1-yl)-1,3,5-triazin-2-yl]amino}ethyl)-6-(piperidin-1-yl)-1,3,5-triazine-2,4-diamine **16**

Compound **16** was purified by chromatography using the solvent mixture MeOH:DCM (9:1) as eluent.

Yield 28%; white powder; mp; 176°C; IR (ATR)/cm^{-1}: 2931, 2850; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.65 – 7.46 (m, 2H, H-14), 7.24 (dd, *J* = 9.5, 5.3 Hz, 2H, H-15), 6.95 (t, *J* = 7.3 Hz, 1H, H-16), 5.88 (s, 1H, H-12), 3.80 – 3.60 (m, 6H, H-8,-9), 2.11 (s, 1H, H-7), 1.61 (q, *J* = 5.1 Hz, 4H, H-10), 1.54 (s, 2H, H-11); ^{13}C NMR (151 MHz, $CDCl_3$): δ 165.83 (C-2), 164.35 (C-4), 163.76 (C-6), 139.52 (C-13), 128.57 (C-15), 122.12 (C-16), 119.69 (C-14), 44.27 (C-9), 33.18 (C-8), 25.74 (C-10), 24.80 (C-11); HRMS-ESI *m/z*: 567.3410 (M+1) (M+1, $C_{30}H_{38}N_{12}$, 567.3421 calc.).

3.2.7 *2-N-(4-methoxyphenyl)-4-N-[2-({4-[(4-methoxyphenyl)amino]-6-(2-methylpiperidin-1-yl)-1,3,5-triazin-2-yl}amino)ethyl]-6-(2-methylpiperidin-1-yl)-1,3,5-triazine-2,4-diamine* **17**

Compound **17** was purified by gradient chromatography using the solvents DCM and EtOAc as eluents.

Yield 14%; white powder; mp: 157°C; IR (ATR)/cm^{-1}: 3270, 2928; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.40 (d, *J* = 16.7 Hz, 2H, H-12), 6.79 (d, *J* = 8.5 Hz, 2H, H-11), 5.27 (m, 2H, H-15), 4.97 (s, 1H, H-19), 4.56 (s, 1H, H-8), 3.74 (s, 3H, H-14), 2.85 (s, 1H, H-8), 1.77 – 1.46 (m, 6H, H-16,-17,-18), 1.15 (s, 3H, H-20); ^{13}C NMR (151 MHz, $CDCl_3$): δ 165.89 (C-2), 164.35 (C-4), 163.84 (C-6), 155.06 (C-13), 121.63 (C-10), 113.81 (C-11), 99.92 (C-12), 55.42 (C-14), 53.39 (C-19), 45.29 (C-15), 38.12 (C-8), 30.16 (C-18), 25.72 (C-16), 19.07 (C-17), 15.19 (C-20); HRMS-ESI *m/z*: 655.3925 (M+1) (M+1, $C_{34}H_{46}N_{12}O_2$, 655.3945 calc.).

3.2.8 *2-N-[2-({bis[(4-methoxyphenyl)amino]-1,3,5-triazin-2-yl}amino)ethyl]-4-N,6-N-bis(4-methoxyphenyl)-1,3,5-triazine-2,4,6-triamine* **18**

Compound **18** was purified by chromatography using the solvent mixture MeOH:DCM (9:1) as eluent and was recrystalized in DCM.

Yield 27%; mp: 248°C; IR (ATR)/cm^{-1}: 3419, 3340, 2930; ^{1}H NMR (600 MHz, DMSO-*d*6): δ 7.63 (s, 4H, H-12), 6.94 (s, 2H, H-9), 6.80 (d, *J* = 8.7 Hz, 4H, H-11), 3.71-3.67 (m, 6H, H-14), 3.53 – 3.49 (m, 1H, H-7), 3.36 (s, 2H, H-8); ^{13}C NMR (151 MHz, DMSO-*d*6): δ 165.79 (C-4,-6), 163.99 (C-2), 154.28 (C-13), 133.43 (C-10), 121.59 (C-12), 113.46 (C-11), 55.11 (C-14), 40.26 (C-8); HRMS-ESI *m/z*: 703.3217 (M+1) (M+1, $C_{36}H_{38}N_{12}O_4$, 703.321723 calc.).

3.2.9 *2-N-(2-{[bis(phenylamino)-1,3,5-triazin-2-yl]amino}ethyl)-4-N,6-N-diphenyl-1,3,5-triazine-2,4,6-triamine* **19**

Compound **19** was purified by gradient chromatography using the solvents DCM and EtOAc as eluents.

Yield: 25.27%; mp: 226°C; IR (ATR)/cm^{-1}: 3438, 3354, 3254, 2932; ^{1}H NMR (600 MHz, DMSO-d6): δ 9.14 (s, 1H, H-9), 9.02 (s, 1H, H-9), 7.79 (d, *J* = 6.6 Hz, 4H, H-11), 7.23 (t, *J* = 7.8 Hz, 4H, H-12), 7.10 (s, 1H, H-7), 6.92 (q, *J* = 6.7 Hz, 2H, H-13), 3.58 – 3.52 (m, 2H, H-8); ^{13}C NMR (151 MHz, DMSO-d6): δ 165.79 (C-2), 164.12 (C-4,), 163.98 (C-6), 140.30 (C-11), 128.28 (C-12), 121.57 (C-10), 119.89 (C-13), 40.21 (C-8); HRMS-ESI *m/z*: 583.2789 (M+1) (M+1, $C_{32}H_{31}N_{12}$, 583.2795 calc.).

3.3. Methodology for *in vitro* biological evaluation

3.3.1 Determination of antimalarial effective concentration (EC)

The bistriazines **11** - **19** were screened against the chloroquine-sensitive (CQS, 3D7) and chloroquine-resistant (CQR, K1) strains of *P. falciparum*. Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen [21]. Asynchronous parasites were grown in the presence of fresh group O-positive erythrocytes (Lifeblood, Memphis, TN) in Petri dishes at a hematocrit of 4-6% in RPMI-based medium consisting of RPMI 1640 supplemented with 0.5% AlbuMAX II, 25 mM HEPES, 25 mM $NaHCO_3$ (pH 7.3), 100 µg/mL hypoxanthine, and 5 µg/mL gentamicin. Cultures were incubated at 37 °C in a gas mixture of 90% N_2, 5% O_2, and 5% CO_2. For EC_{50} determinations, 20 µL of RPMI 1640 with 5 µg/mL gentamicin were dispensed per well in an assay plate 384-well microtiter plate, clear-bottom, tissue-treated). Next, 40 nL of each compound, previously serial diluted in a separate 384-well white polypropylene plate, were dispensed in the assay plate, and then 20 µL of a synchronized culture suspension (1% rings, 4% hematocrit) was added per well to make a final hematocrit and parasitemia of 2% and 1%, respectively. Assay plates were incubated for 72 h, and the parasitemia was determined by a method previously described [22]. Briefly, 10 µL of 10X Sybr Green I, 0.5% v/v Triton, and 0.5 mg/mL saponin solution in PBS were added per well. Assay plates were shaken for 60 s, incubated in the dark for 90 m, and then read with the Envision spectrofluorometer at Ex/Em 485 nm/535 nm. EC_{50}s were calculated using proprietary software developed in house (RISE) in the Pipeline Pilot environment that pools data from all replicates of the experiment and fits a consensus model.

3.3.2 In vitro cytotoxicity against BJ, HEK293, Hep G2 and Raji cells

BJ, HEK293, Hep G2 and Raji cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured according to recommendations. Cell culture media were purchased from ATCC. Cells were routinely tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza). Exponentially growing cells were plated in Corning 384 well white custom assay plates, and incubated overnight at 37 ºC in a humidified, 5% CO_2 incubator. DMSO inhibitor stock solutions were added the following day to a top final concentration of 25 µM, 0.25% DMSO and then diluted 1/3 for a total of ten testing concentrations. Cytotoxicity was determined following a 72-hour incubation using Promega Cell Titer Glo Reagent according to the manufacturer's recommendation. Luminescence was measured on an

Envision plate reader (Perkin Elmer). EC_{50}s were calculated using proprietary software developed in house (RISE) in the Pipeline Pilot environment that pools data from all replicates of the experiment and fits a consensus model.

3.3.3 Dose-response curve fitting

Dose-response curves were calculated from percent activity values and log10-transformed concentrations, the proprietary robust interpretation of screening experiments (RISE) application written in Pipeline Pilot (Accelrys, v. 8.5) and the R program [23]. Briefly, non-linear regression was performed using the R *drc* package with the four-parameter log-logistic function (LL2.4) [24]. The data for all replicates for each compound was fit three separate times by varying the parameters that were fixed during regression: (1) all parameters free, (2) high response fixed to 100, (3) low response fixed to 0. The best fit from these three nested models was selected using the *anova.drc* function. 95% confidence intervals were produced based upon this fit.

Dose-response curves were assigned a quality score according to the following heuristic. Compounds which failed to fit to any curve, or with curves having efficacy <25% or >150% or hill slope <0.5 or >25 were designated class 'D1'. Compounds passing this first criteria with curves having efficacy <50%, calculated EC_{50} > the highest concentration tested, lower and upper EC_{50} confidence limits > 10-fold EC_{50}, or slope at the highest concentration tested >75% (non-saturating) were designated class 'C1'. Compounds passing previous criteria with curves having lower and upper EC_{50} confidence limits > 5-fold EC_{50} or slope at the highest concentration tested >25% (not completely saturating) were designated class 'B1'. All remaining curves were designated 'A1', which is indicative of ideal, well-behaved sigmoidal response. Curves that were inverted (activity decreased as concentration increased) were prefixed with the letter 'N', such as 'NA1' In tabulating data, EC_{50} values are only called for A1 and B1 class curves with a single value and error being provided for A1 curves and a range provided for B1 curves. C1 and D1 curves were assigned an arbitrary value of either greater than the highest concentration tested or lower than the lowest concentration tested, when appropriate. The target compounds were screened for cytotoxicity against human fibroblasts (BJ), embryonic kidney (HEK293), liver hepatocytes (Hep G2) and lymphoblast-like (Raji) cell lines alongside staurosporine as reference drug.

4. Results and discussion

4.1. Chemistry

The triazine intermediates **2** – **10** were obtained by aromatic nucleophilic substitutions of 2 of the 3 chlorine atoms of cyanuric chloride. The MS, IR and NMR data are in accordance of their structures. The mass spectra of the intermediates showed two molecular ion peaks [M+H]+ and [(M+H)+2]+, which support the identity of these compounds and confirmed the presence of one chlorine atom in their structures. The IR spectra of **2** – **10** showed characteristic absorption in the region 770 - 710 cm^{-1} attributed to the C-Cl group and compounds **6** – **19** in the region 3200 – 2900 cm^{-1} attributed to the N-H groups of the secondary amine substituents and the ethylenediamine linker.

The bistriazine target compounds were synthesized by aromatic substitution (SNAr) involving of cyanuric chloride, various amines and ethylenediamine as linker between the two triazine rings. The yields varied from poor to moderate, and were found in the 14 – 56 % range after purification by silica column chromatography and/or crystallization in dichloromethane. The low yields in these nucleophilic substitution reactions may be attributed to multi-substitutions. The chemical structures of the synthesized compounds were confirmed using ^{1}H NMR, ^{13}C NMR, HRESI and HRMS-APCI spectroscopy. The signal assigned to the linker protons in compounds **11** – **19**, showed double intensity, revealing that their structures are symmetrical. Thus, only half proton and carbon signals are displayed on the spectra.

The ^{13}C NMR spectrum of the cyanuric chloride moiety showed signals at 166.43 (C-4 and 6) and 165.14 (C-2) for **11**, 166.52 (C-2), 165.32 (C-4) and 164.77 (C-6) for **12**, 165.28 (C-2), 164.57 (C-4) and 159.28 (C-6) for **13**, 166.19 (C-2 and 6) and 165.16 (C-4) for **14**, 166.35 (C-2), 164.95 (C-4) and 164.13 (C-6) for **15**, 165.83 (C-2), 164.35 (C-4) and 163.76 (C-6) for **16**, 165.89 (C-2), 164.35 (C-4) and 163.84 (C-6) for **17**, 165.79 (C-4 and 6) and 163.99 (C-2) for **18** and 165.79 (C-4, 6) and 164.12 (C-2) for **19**. These signals confirm the cyanuric chloride moiety for compounds **11** – **19** as well as their di- or bis-substitutions as per symmetrical equivalence. The aromatic carbons of compounds **15**, **16**, **17**, **18** and **19** from the aniline and *p*-anisidine moieties were confirmed between ranges 119.69 – 139.52 and 99.92 – 155.06 respectively. The ethylenediamine linker moiety (C-8) was present in all of the target compounds; **11** (41.16), **12** (41.21), **13** (41.18), **14** (41.15), **15** (43.61), **16** (44.27), **17** (38.12)**, 18** (40.26) and **19** (40.21).

The ^{1}H NMR spectra of the bistriazines displayed peaks in the 1.5-2.6 and 3.4-3.6 ppm regions attributed the resonance of H-7 and H-8, respectively, and confirming the presence of EDA linker in their structures. The secondary amine bearing H-7 is further confirmed by the presence of a stretching band in the 3200-3000 cm^{-1} in the IR spectra, attributable to NH group.

The chemical structures of the disubstituted cyanuric chloride intermediates **2** - **10** were confirmed using MS analysis. The HRAPCI-MS confirmed the exact mass of **2** [286.1042], which corresponds to the formula $C_{11}H_{17}ClN_5O_2$, of **4** [298.1402] for $C_{13}H_{21}ClN_5O$, of **5** [343.1989] for $C_{15}H_{28}ClN_6O$, of **6** [292.0948] for $C_{13}H_{15}ClN_5O$, of **7** [290.1152] for $C_{14}H_{17}ClN_5$, of **8** [334.1413] for $C_{16}H_{21}ClN_5O$, and of **9** [358.1043] for $C_{17}H_{17}ClN_5O_2$.

The chemical structures of the title bistriazines **11** - **19** were further confirmed by MS analysis. The HRESI-MS confirmed the exact mass of **11** [559.3208], which corresponds to the formula $C_{24}H_{39}N_{12}O_4$, of **12** [555.3621] for $C_{26}H_{43}N_{12}O_2$, of **13** [583.3952] for $C_{28}H_{47}N_{12}O_2$, of **14** [673.5088] for $C_{30}H_{61}N_{14}O_2$, of **15** [571.2993] for $C_{29}H_{37}N_{11}O_2$, of **16** [567.3410] for $C_{30}H_{39}N_{12}$, of **17** [655.3925] for $C_{34}H_{47}N_{12}O_2$, of **18** [703.3217] for $C_{36}H_{39}N_{12}O_2$, and of **19** [583.28] for the formula $C_{32}H_{31}N_{12}$.

4.2. In-vitro Antimalarial activity and cytotoxicity

The target bistriazines **11** - **19** were screened *in vitro* alongside chloroquine against the chloroquine sensitive (CQS) 3D7 and chloroquine resistant (CQR) K1 strains of cloned human *Plasmodium falciparum*. (Table 1)

Of the nine bistriazines synthesized, 5 compounds showed activity against 3D7 and K1 strains with EC_{50} values in the 0.23 – 1.39 µM and 0.26 – 1.62 µM range, respectively, while 4 compounds were inactive. Compounds **13** (EC_{50} = 1.39 µM), **15** (EC_{50} = 0.89 µM), **16** (EC_{50} = 1.35 µM), **17** (EC_{50} = 1.32 µM) and **19** (EC_{50} = 0.23 µM) activity were meaningfully less potent than chloroquine (EC_{50} = 0.01 µM) against the CQS 3D7 strain of *P. falciparum* malaria.

Against, the K1 strain, however, compounds **16** (EC_{50} = 1.35 µM) and **17** (EC_{50} = 1.62 µM) had potency comparable to that of CQ (EC_{50} = 1.67 µM), while bistriazine **13** (EC_{50} = 0.84 µM) was found to be 2-fold more potent. The bistriazines **15** (EC_{50} = 0.37 µM) and **19** (EC_{50} = 0.26 µM) featuring aniline and morpholine, and aniline substituents on the triazine rings, respectively, were the most active of all synthesized compounds

against the K1 strain of *P. falciparum*. Compound **19** had similar activity against the CQS and CQR strains (RI = 1.15), while **15** had more than a two fold increase (RI = 0.41) in activity against the CQR K1 as compared to the 3D7 strain. Compounds **13** (RI = 0.6) and **16** (RI = 0.85) also exhibited an increase in activity against the K1 strain. Compounds **15**, **16** and **19**, all featuring aniline substituent proved to be active against both strains. This may suggest that the aniline moiety is necessary for activity of these biscompound types. On contrary the presence of the morpholine substituent may induce loss of activity as compounds **11**, **12** and **14**, all possessing that ring moiety were inactive.

To determine whether the synthesized bistriazine compounds were selective in their action against the *Plasmodia* cells, relative to human host cells, selectivity index (SI) was determined for multiple mammalian cell types by means of cytotoxicity determination. All compounds **11** – **19** were meaningfully less potent against BJ, HEK293, Hep G2 and Raji cell lines compared to the reference drug (staurosporine) and indicate that they are non-toxic to the mammalian cells.

Furthermore the distribution of a drug molecule in the body is influenced by the aqueous solubility and the lipophilicity of the compound. In order for a drug to enter systemic circulation, it must possess balanced lipophilic and hydrophilic properties to permeate the biological membranes as well as to permit an efficient drug delivery [25]. The partition coefficient (logP) is a good measure of this balance with values between 0 and 5 being targeted, and 0-3 being ideal [26,27]. To assess if there was any correlation between logP and the *in vitro* antimalarial activity of the synthesized bistriazines, LogP values of chloroquine and compounds **11** – **19** were calculated using ACD/ChemSketch V5.54. The logP values ranged between -1.16 and 5.66. Compound **19**, while the most active of the synthesized bistriazines possesses a logP value of 3.97, while **15** the second most active compound has a LogP of -1.16. Compounds **12** and **18** lack activity, but have calculated LogP values 4.67 and 3.77, respectively. Thus, based on the calculated logP values, there is no correlation between the synthesized bistriazines **11** – **19** and their *in vitro* antimalarial activity, confirming that the drug's absorption is dependent upon multiple physicochemical parameters [28].

Table 1. EC_{50} values, *in vitro* antimalarial activity and cytotoxicity of synthesized bistriazines

		Activity EC_{50} (uM)[a]			Cytotoxicity EC_{50} (μM)[b]							
Cpd.	**Calc. LogP**	**3D7**	**K1**	**RI[c]**	**BJ**	**HEK 293**	**HEP G2**	**Raji**	**SI_1[d]**	**SI_2[e]**	**SI_3[f]**	**SI_4[g]**
11	1.58	>5.66	>5.66	>1	>9.82	>9.82	>9.82	>9.82	-	-	-	-
12	4.67	>13.66	>13.66	>1	>23.7	>23.7	>23.7	>23.7	-	-	-	-
13	5.66	1.39	0.84	0.6	>21.72	>21.72	>21.72	>21.72	>15.7	>15.7	>15.7	>15.7
14	-0.84	>7.34	>7.34	>1	>12.73	>12.73	>12.73	>12.73	-	-	-	-
15	-1.16	0.89	0.37	0.41	>16.02	>16.02	>16.02	>16.02	>17.9	>17.9	>17.9	>17.9
16	1.93	1.35	1.15	0.85	>23.57	>23.57	>23.57	>23.57	>17.5	>17.5	>17.5	>17.5
17	2.82	1.32	1.62	1.22	>23.18	>23.18	>23.18	>23.18	>17.6	>17.6	>17.6	>17.6
18	3.77	>10.57	>10.57	>1	>18.33	>18.33	>18.33	>18.33	-	-	-	-
19	3.97	0.23	0.26	1.15	>18.1771	>18.1771	>18.1771	>18.1771	>79.1	>79.1	>79.1	>79.1
CQ	4.87	0.01	1.67	126.6								
STP					2.3	2.3	2.3	2.3				

[a]Effective concentration of compound inducing 50% reduction in parasitic cells count; [b]effective concentration of compound selectively inducing 50% reduction in parasitic cells count in the presence of mammalian cells; [c]resistance index (RI) = EC_{50} K1 / EC_{50} 3D7; [d]Selectivity index (SI_1) = EC_{50} BJ/EC_{50} 3D7; [e]selectivity index (SI_2) = EC_{50} Hek 293/EC_{50} 3D7; [f]selectivity index (SI_3) = EC_{50} Hep G2/EC_{50} 3D7; [g]selectivity index (SI_4) = EC_{50} Raji G2/EC_{50} 3D7.

5. Conclusions

A series of diamine substituted bistriazines was synthesized through nucleophilic substitution at carbon atoms C-2, -4 and 6 of cyanuric chloride using various amines. The chemical structures of the synthesized compounds were verified and confirmed by NMR, IR and MS analyses. The target bistriazines were screened for antimalarial activity alongside chloroquine against the CQS 3D7 and CQR K1 strains of *P. falciparum*. Five out of the nine synthesized bistriazine compounds possessed, activity against both the CQS 3D7 and CQR K1 strains of *P. falciparum*, and were selectively toxic to the parasitic cells in the presence of the mammalian ones However, none compound showed potency comparable or higher than CQ against the 3D7 strain. Against the K1 strain, however, all five active bistriazines were either more potent or displayed potency comparable to that of CQ. The bistriazines **13**, **16** and **17** featuring the pairs of substituents; morpholine and 2-methylpiperidine, aniline and piperidine, and 4-methoxyanilne and 2-methylpiperdine, respectively, were found with potency comparable to that of CQ. Compounds **15** and **19** with aniline and morpholine, and aniline substituents, respectively, displayed significantly better activity against the K1 strain. Bistriazine **15** was found with 5-fold higher potency than CQ **(**EC_{50}; 0.37 *vs,* 1.67 μM) while **19** displayed 6 times more activity in comparison to CQ (EC_{50}; 0.23 *vs,* 1.67 μM), and thus lend itself as worthy bistriazine to further investigated in vivo in the search for the so desperately needed potent, cost effective and resistance free alternative to chloroquine for the treatment of *Plasmodium* malaria.

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Conflit of interest

The authors declare that they have no conflit of interest to disclose.

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CHAPTER 4

SUMMARY AND CONCLUSION

This section strives to unify the outcomes of the current study and draws overall conclusions from all data, to point out relevance and specific advances achieved and to propose appropriate prospective studies.

Malaria persists to proliferate as an economic and social burdon despite a 17% decrease in the estimated number of malaria related deaths reported by the World Health Organization in 2011. In the past decade the estimated number of malaria incidence annually, never once has decreased below 216 million, resulting in the death of more than 8,3 million people, 655 000 in 2010 (WHO, 2010). This worldwide disease is endemic to 109 countries [(dominant in sub-Saharan Africa (91%)] and 80% of its mortality is child and infant related.

Five species of *Plasmodium* genus cause this protozoan parasitic disease. Among the five species *P. falciparum* is the most virulent, prevalent, caries the highest resistant strains towards antimalarial drugs and is responsible for the majority of malaria associated deaths.

Malaria is a preventable and curable disease, however *Plasmodia* mono- and multi- drug resistance towards classic antimalarial drugs such as chloroquine, quinine and sulphadoxine/pyrimethamine has rendered their efficacy useless in certain parts of the world, resulting in complex treatment regimens. Resistance towards the last bullet in the gun, artemisinin, has recently been reported at the South-East Asian border. This devastating reality calls for an immediate research plan to develop new antimalarials overcoming previous predicaments.

The antifolates are active against malaria parasites as they inhibit the production of dihydropteroate (DHP) and tetrahydrofolate (THF), two biologically important folate cofactors in the synthesis of parasitic DNA and RNA (Nzila *et al.*, 2005). Humans depend on their folate intake by means of their diet whereas protozoa lack of this metabolic function and are required to synthesize their folates *de novo* (Wang *et al.*, 2007). This metabolic character difference between mammalian and protozoan species results in a perfect drug target for antimalarial therapy (Biagini *et al.*, 2003). Cycloguanil the active metabolite of the prodrug proguanil; a DHFR inhibitor, is a commonly used, well defined historically important drug in the treatment of malaria (Kumar *et al.*, 2011; Vilaivan *et al.*,

2003). Its' capabilities might have been hindered by resistance; nevertheless the folate pathway remains an attractive well understood target site for drug development. Structural modifications to cycloguanil's pharmacophore can overcome the difficulty of resistance (Gravestock *et al*., 2011).

The applications of 2,4,6-trichloro-1,3,5-triaizine (cyanuric chloride) is nearly endless. It has been studied immensely in a diverse range of applications presenting antibacterial, antitumor and antiparasitic activity (Srinivas *et al*., 2005; Kumar *et al*., 2010) and it shares similar chemical properties and biological activity to cycloguanil (Melato *et al.*, 2008). By using an appropriate solvent and a hydrochloride acceptor, the chlorine atoms of the cyanuric chloride moiety are easily substituted. When they are controlled by temperature it allows stepwise formation of mono-, di- or trisubstituted derivatives of triazine with various nucleophiles (Blotny, 2006). The derivatives, including hybrid triazines and triazine related drugs PS15 and WR99210 have shown promising antimalarial activity in chloroquine sensitive and resistant strains (Kinyanjui *et al*., 1999; Edstein *et al*., 1997).

The aim of this study was to synthesize a series of bistriazine compounds containing various amines and to evaluate their *in vitro* antimalarial activity. Studies have shown that by synthesizing bis compounds more efficacious antiplasmodial drugs can be produced (Raynes *et al.*, 1996).

In order to achieve this goal, the following objectives were set:

- Synthesis and physical characterization of disubstituted bistriazine derivatives by means of NMR, MS and melting point analysis.
- Evaluate the *in vitro* anti-malarial efficacy of the bistriazine derivatives against the chloroquine sensitive 3D7 and chloroquine resistant K1 strains of *Plasmodium falciparum* in comparison to the reference drug chloroquine.
- Asses the selectivity index of the synthesized compounds.

The disubstituted bistriazine derivatives were successfully synthesized by means of synthetic organic methods and their structures were verified by NMR and MS spectroscopy.

The target bistriazines **11** - **19** were screened *in vitro* alongside chloroquine against the chloroquine sensitive (CQS) 3D7 and chloroquine resistant (CQR) K1 strains of human cloned *Plasmodium falciparum*.

Among the nine bistriazines synthesized, 5 compounds showed activity against 3D7 and K1 strains with an EC_{50} value range of 0.23 – 1.39 μM and 0.26 – 1.62 μM respectively and 4 compounds were inactive. Compounds **13**, **15**, **16**, **17** and **19** activity were meaningfully less potent than chloroquine (EC_{50} = 0.01 μM) against the CQS 3D7 strain of *P. falciparum* malaria. Against, the K1 strain, however, compounds **16** (EC_{50} = 1.15 μM) and **17** (EC_{50} = 1.62 μM) had potency comparable to that of CQ (EC_{50} = 1.67 μM), while bistriazine **13** (EC_{50} = 0.84 μM) was found to be 2-fold more potent. The bistriazines **15** (EC_{50} = 0.37 μM) and **19** (EC_{50} = 0.26 μM) featuring aniline and morpholine, and aniline substituents on the triazine rings, respectively, were the most active of all synthesized compounds against the K1 strain of *P. falciparum*. Of the nine target compounds; **15** and **19** were the most active, however **19** revealed a decrease (RI = 1.15) and **15** more than a two fold increase (RI = 0.41) in activity against the CQR K1 strain of *P. falciparum*. Compounds **13** (RI = 0.6) and **16** (RI = 0.85) also exhibited an increase in activity towards the CQR K1 strain. Bistriazine compounds **18** and **19** were disubstituted with aromatic nucleophilic substitutions; aniline and *p*-anisidine respectively. It can be concluded that **19** is active, however **18** lacks activity. In literature, triazine is reported to be active against *Plasmodia* since it is a DHFR inhibitor (Melato *et al.*, 2008). If the bistriazines of this study act as DHFR inhibitors, the inactivity of compound 18 may suggest that either the methoxy group of *p*-anisidine in its structure did not favor the DHFR pathway, that it is not susceptible or that the solubility of **18** prevented absorption into the *Plasmodia* cell. All of the active compounds displayed selectivity indexes towards the *Plasmodia*, relative to human host cells.

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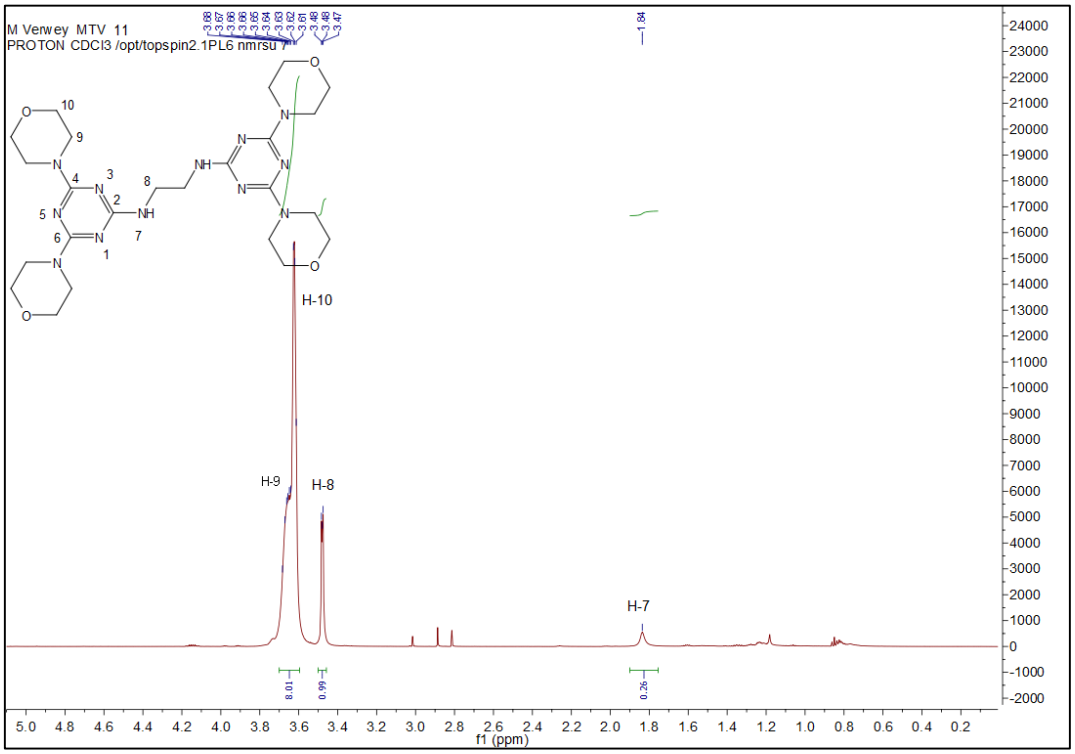
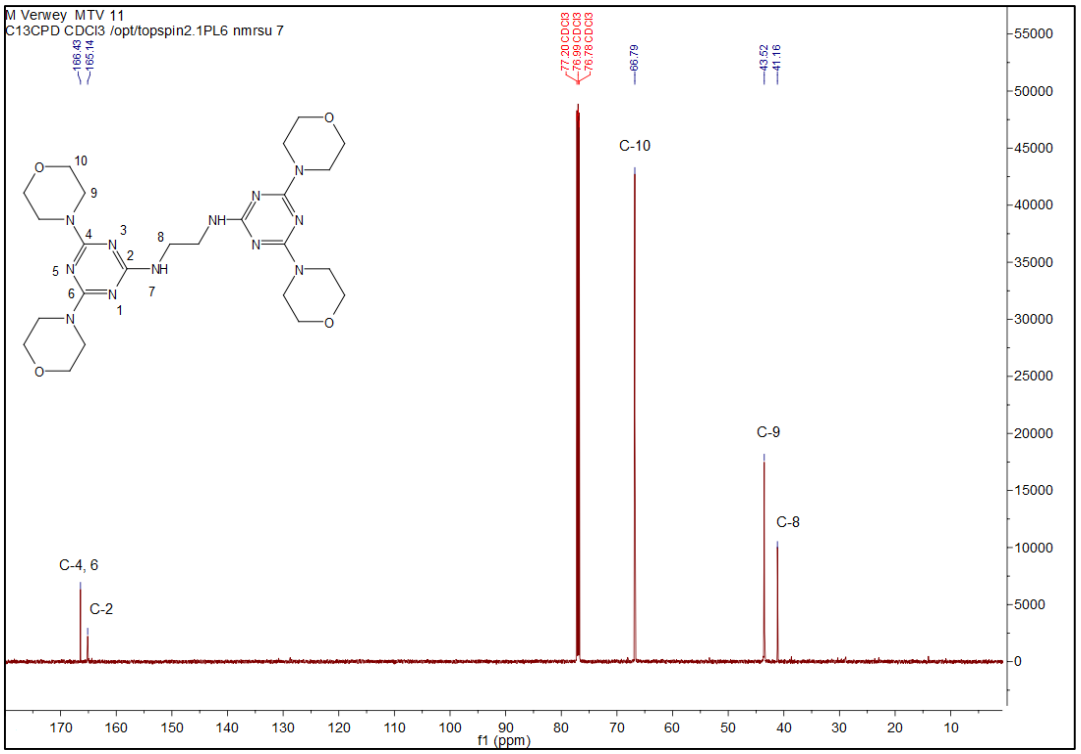
APPENDIX A: SPECTRA

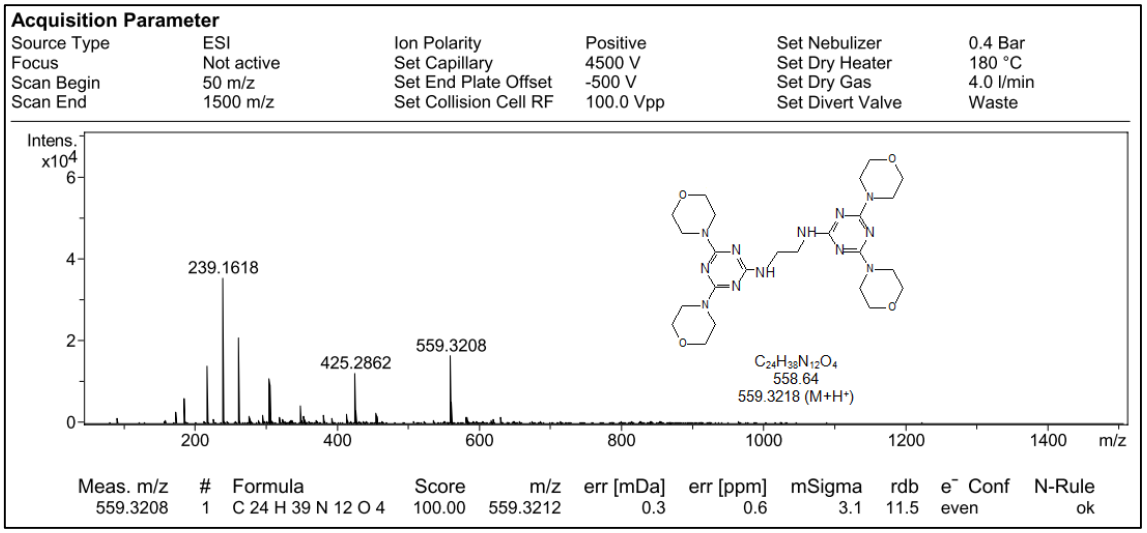
^{1}H NMR of compound 11



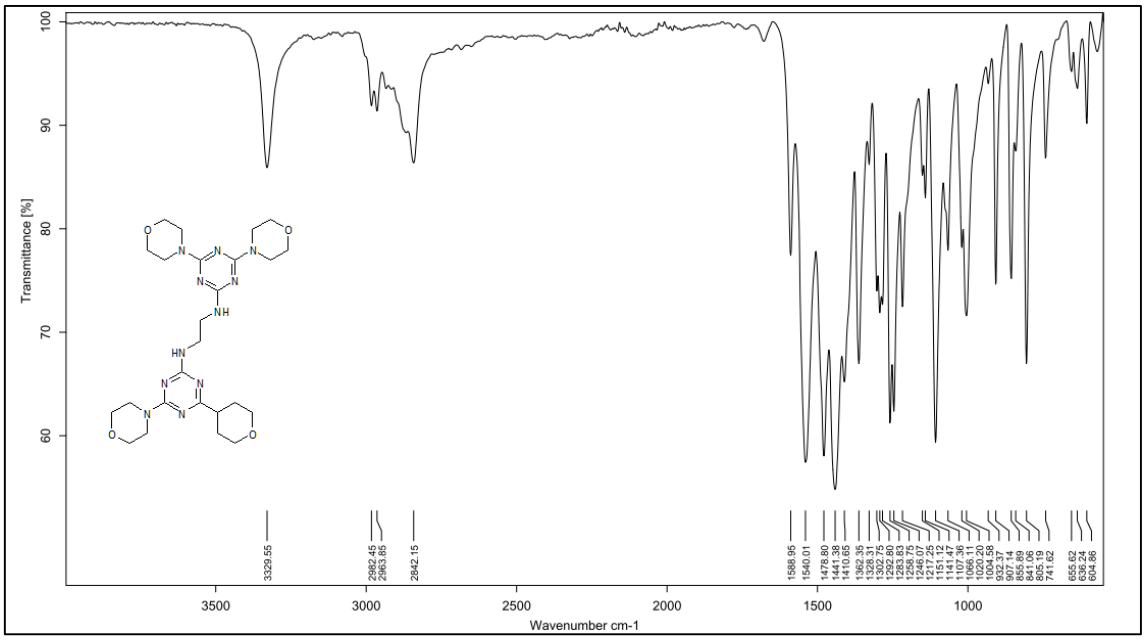
^{13}C NMR of compound 11



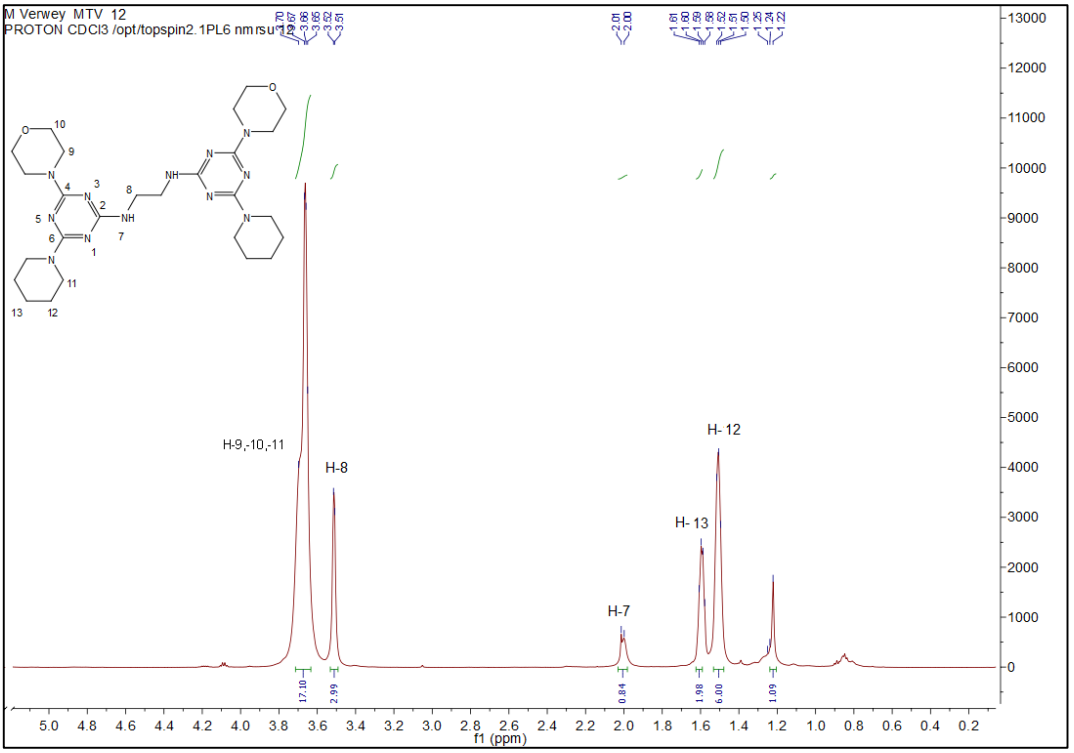
MS ES+ of compound 11



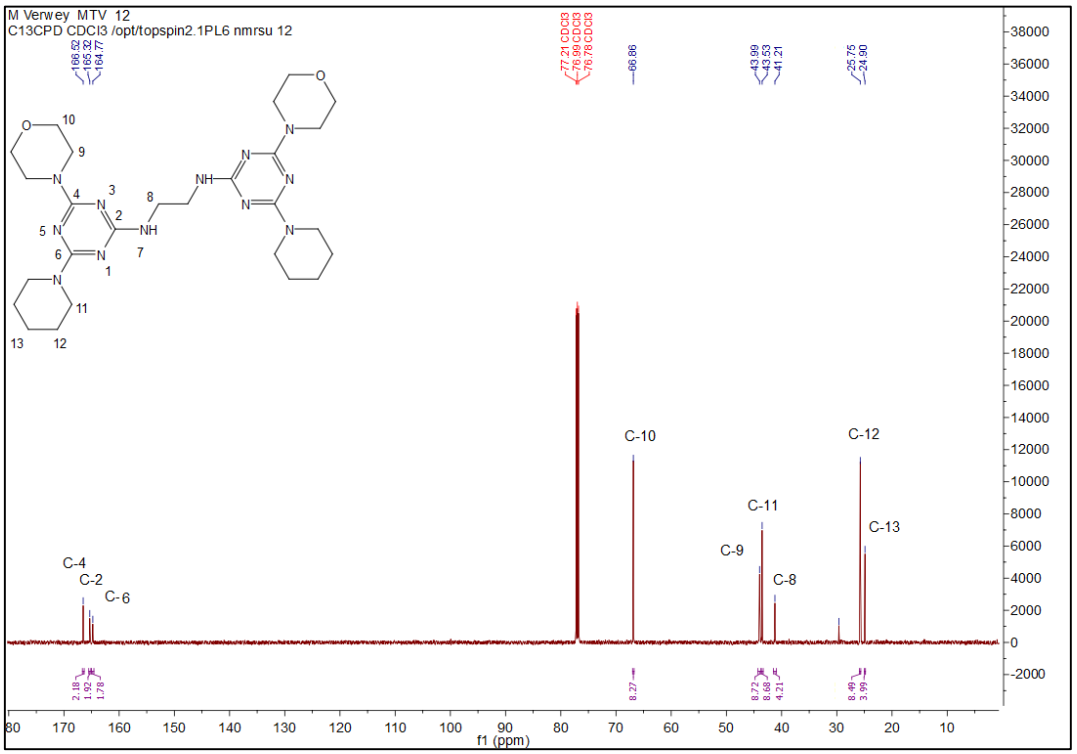
IR of compound 11



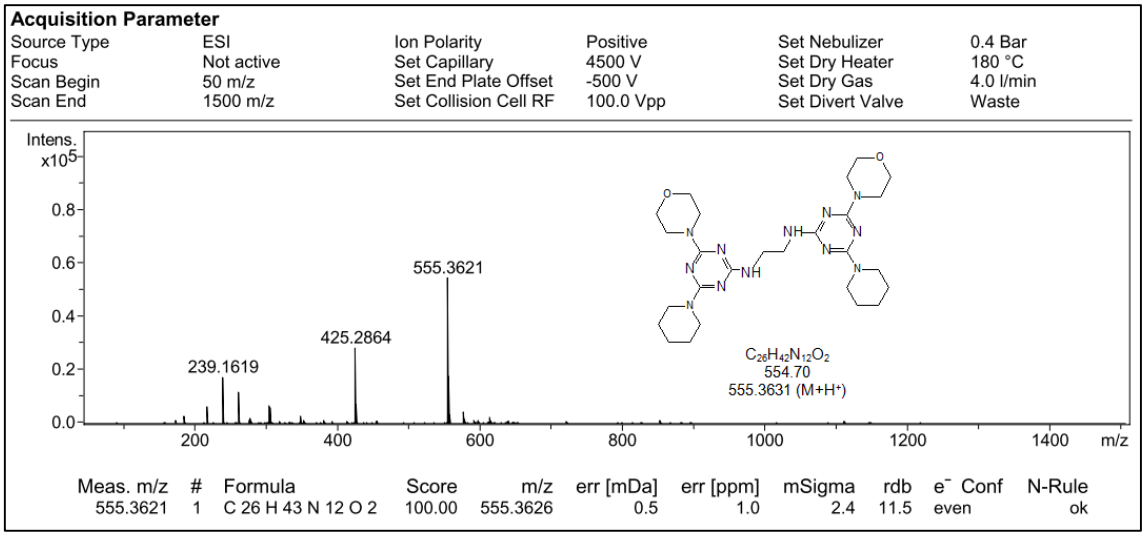
^{1}H NMR of compound 12



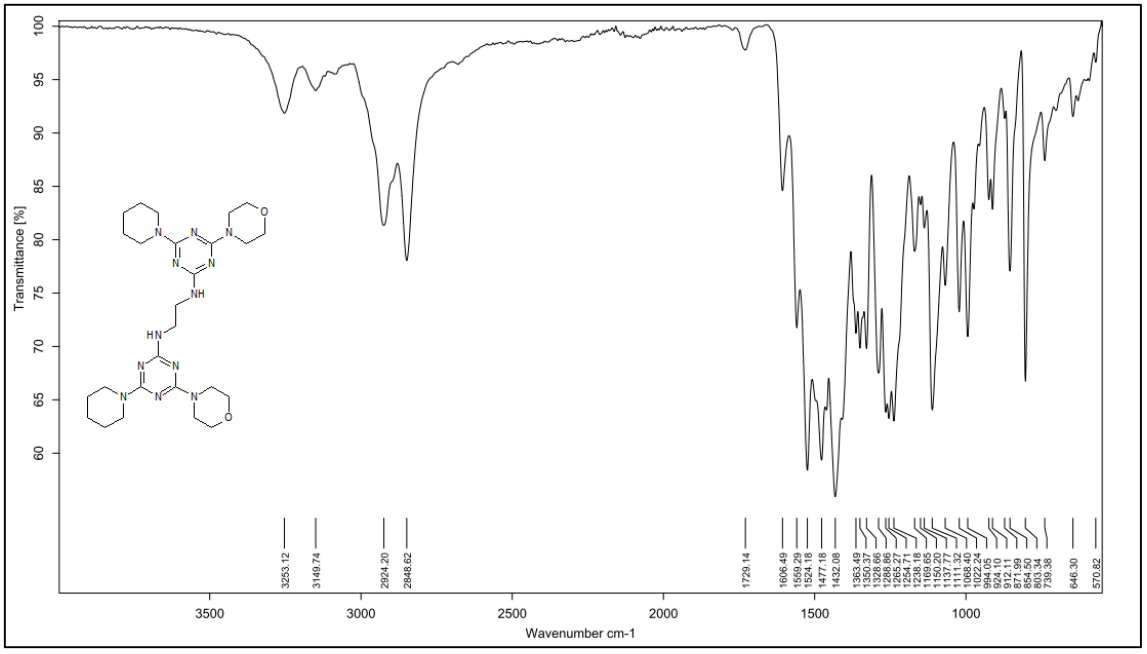
^{13}C NMR of compound 12



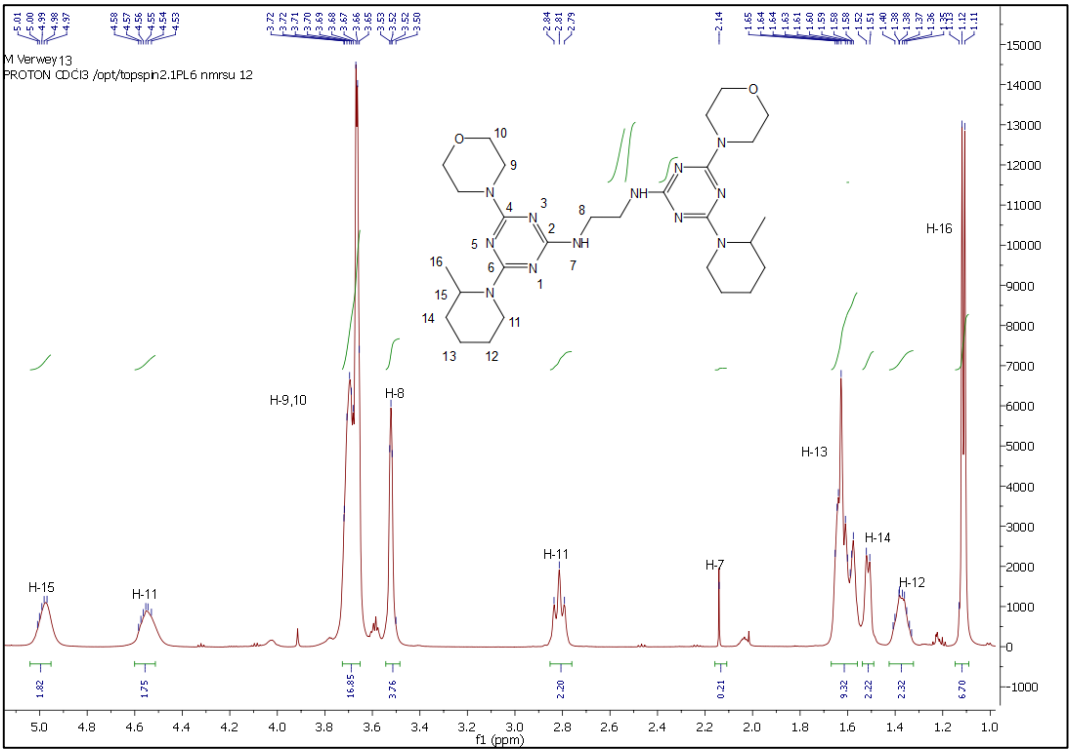
MS ES+ of compound 12



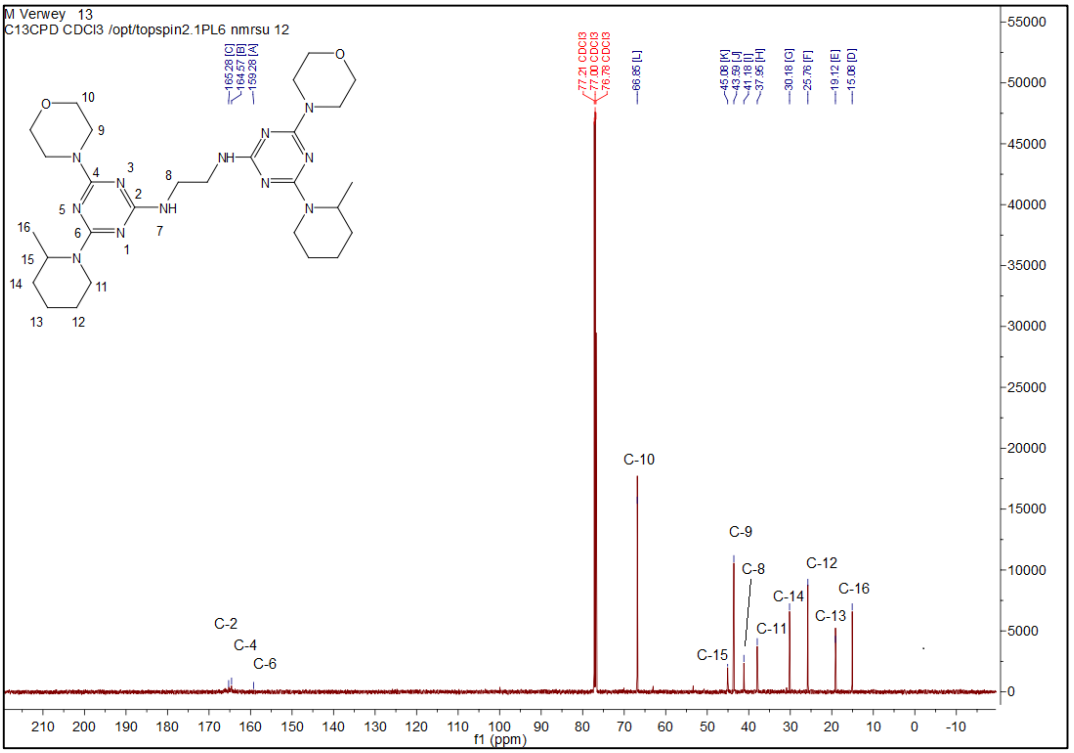
IR of compound 12



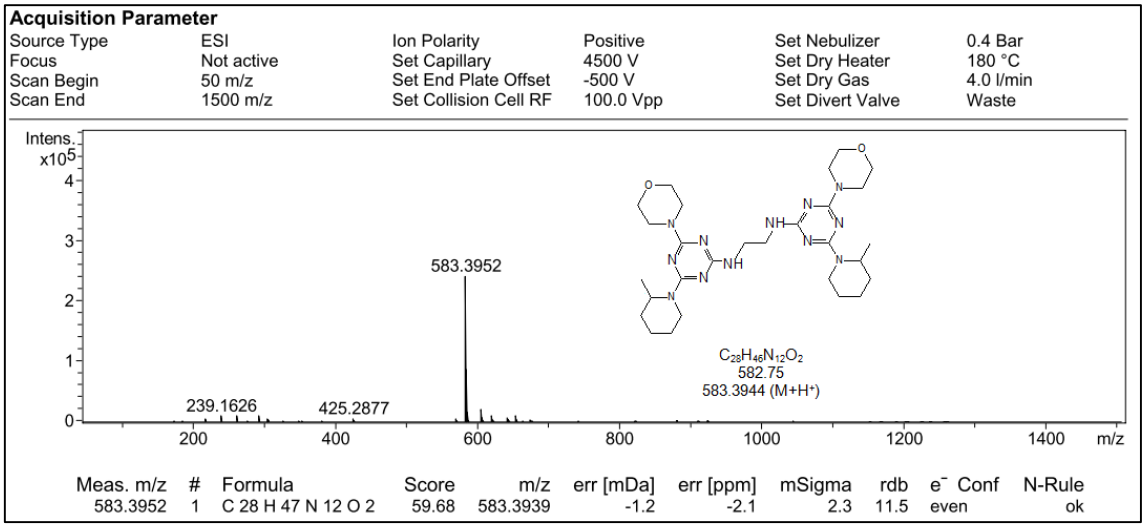
^{1}H NMR of compound 13



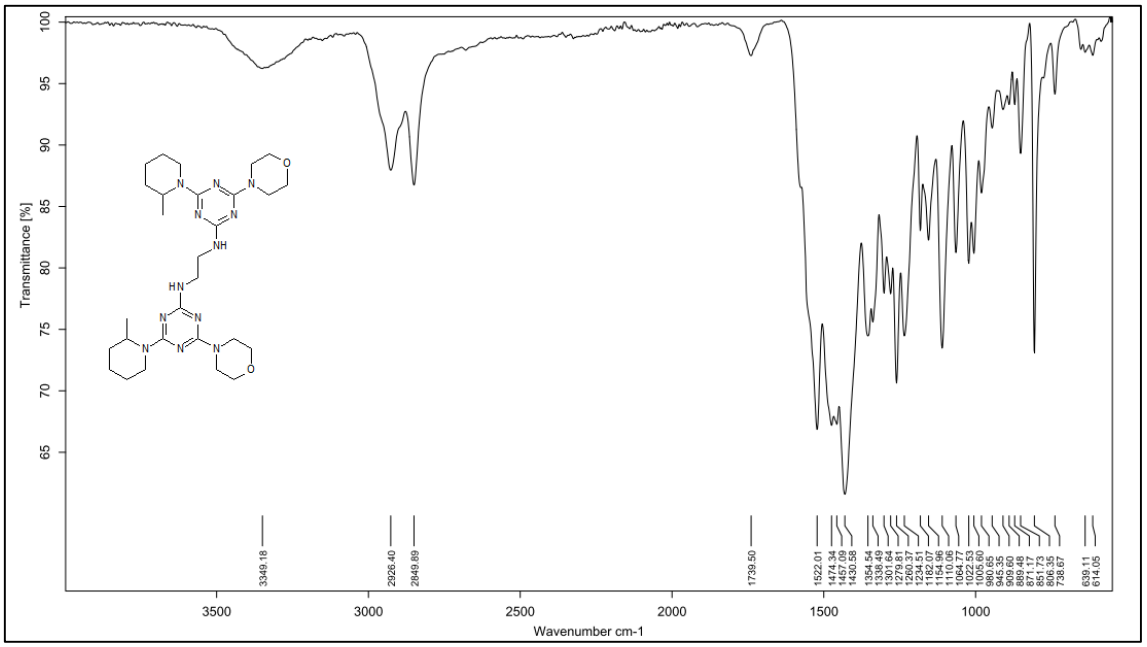
^{13}C NMR of compound 13



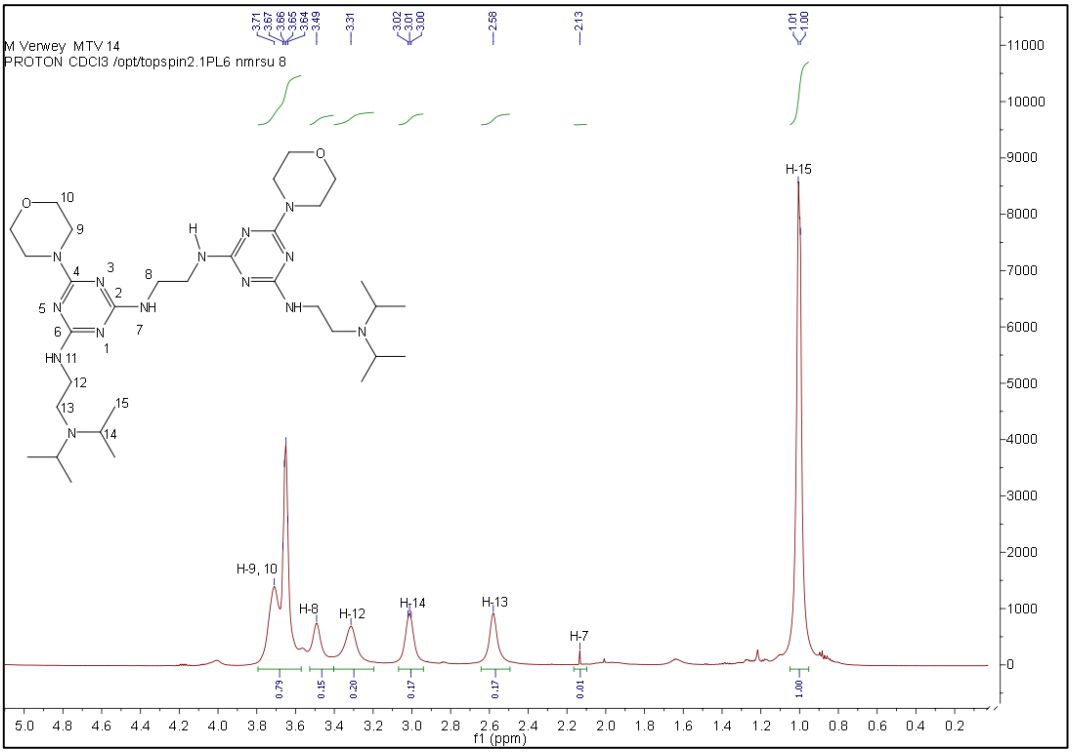
MS ES+ of compound 13



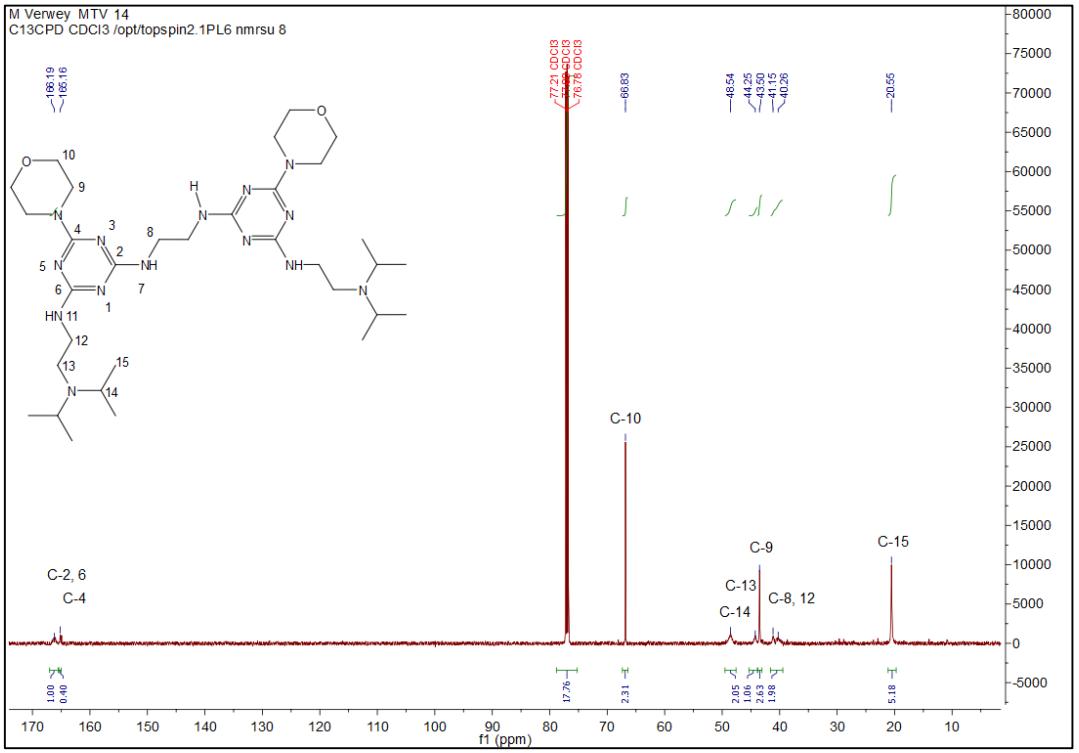
IR of compound 13



^{1}H NMR of compound 14



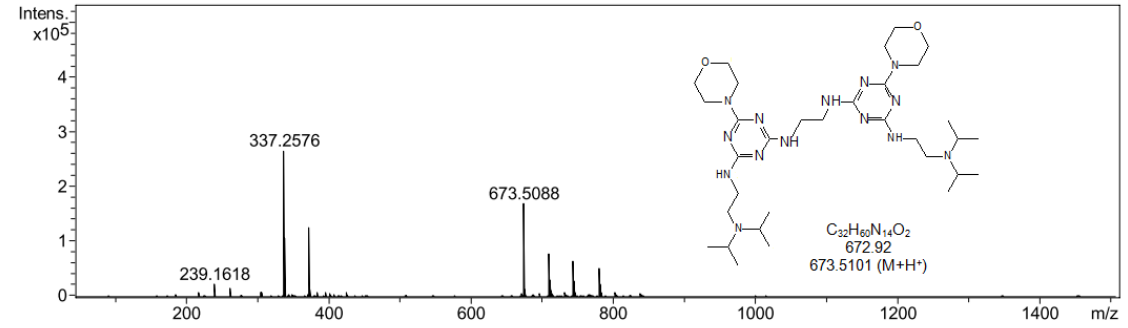
^{13}C NMR of compound 14



MS ES+ of compound 14

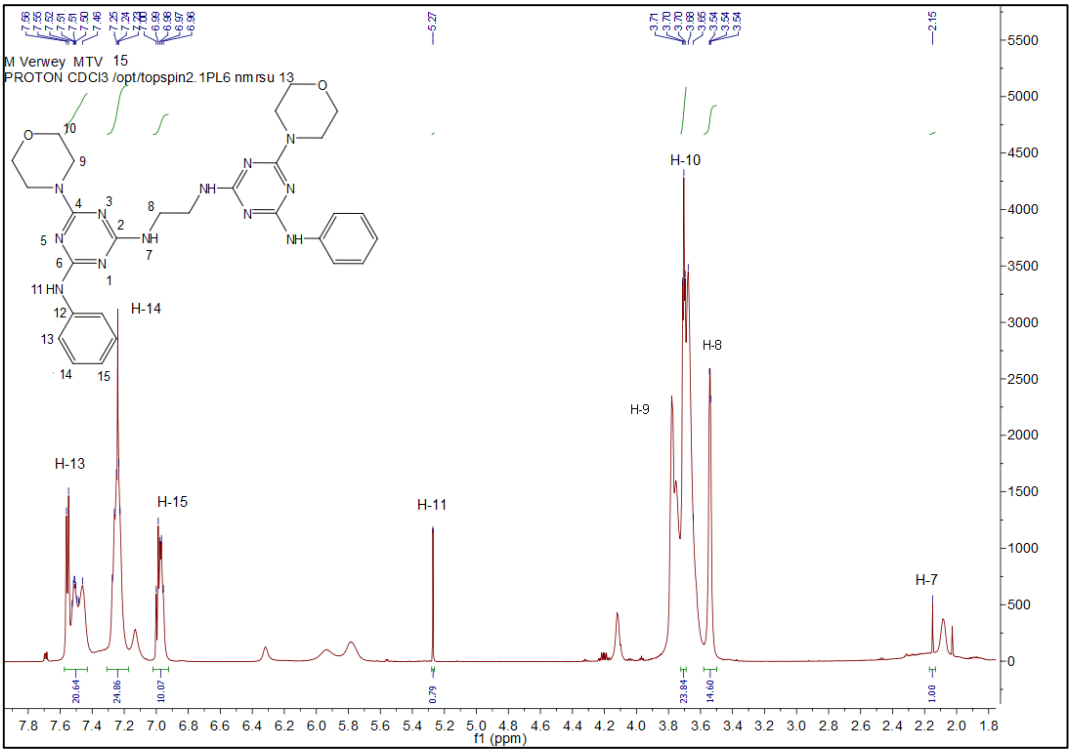
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

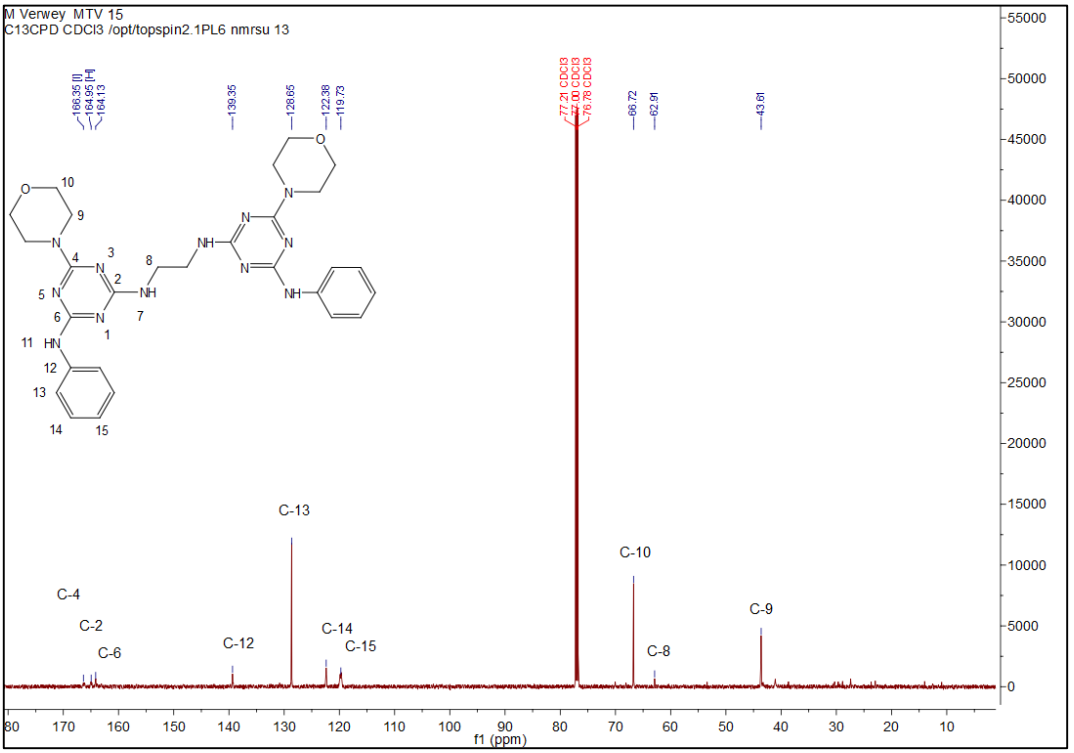


Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
673.5088	1	C 30 H 59 N 17 O	100.00	673.5083	-0.5	-0.8	3.1	10.0	odd	ok

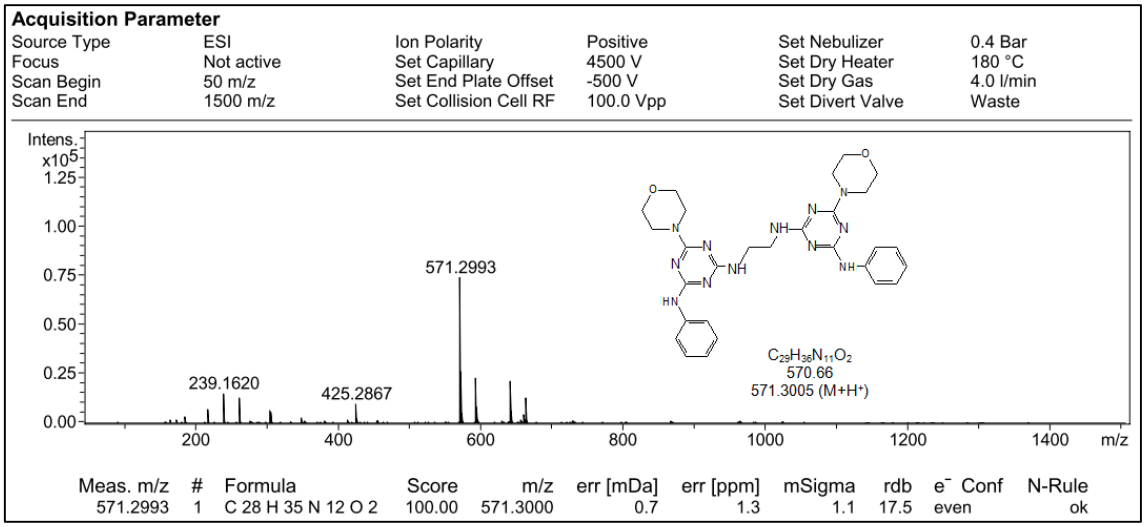
^{1}H NMR of compound 15



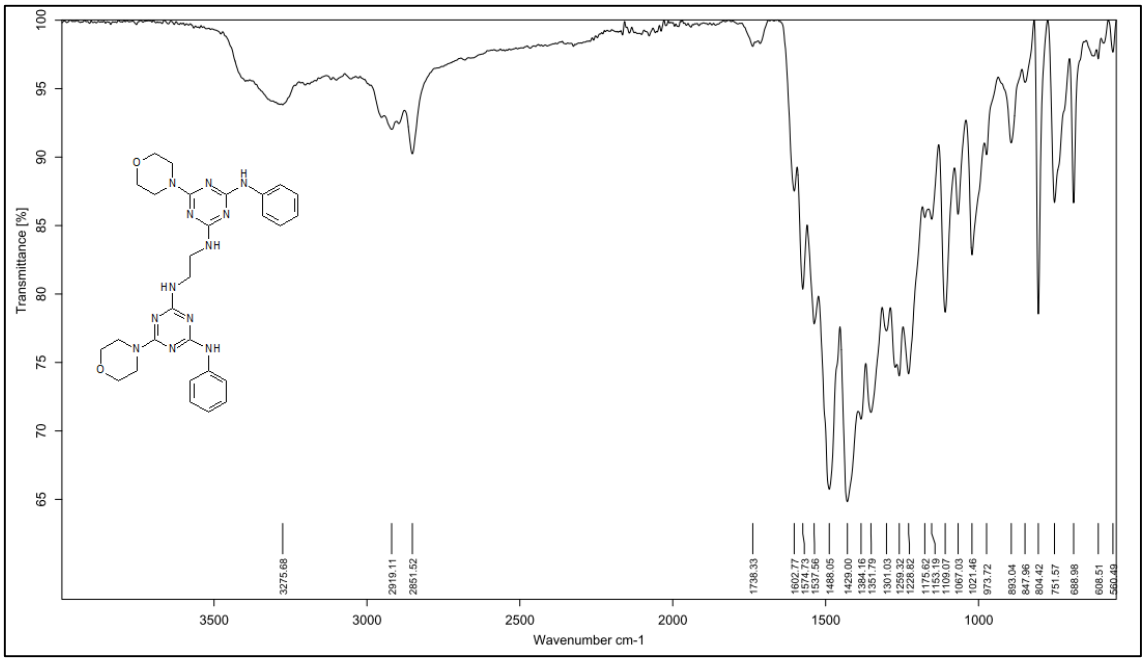
^{13}C NMR of compound 15



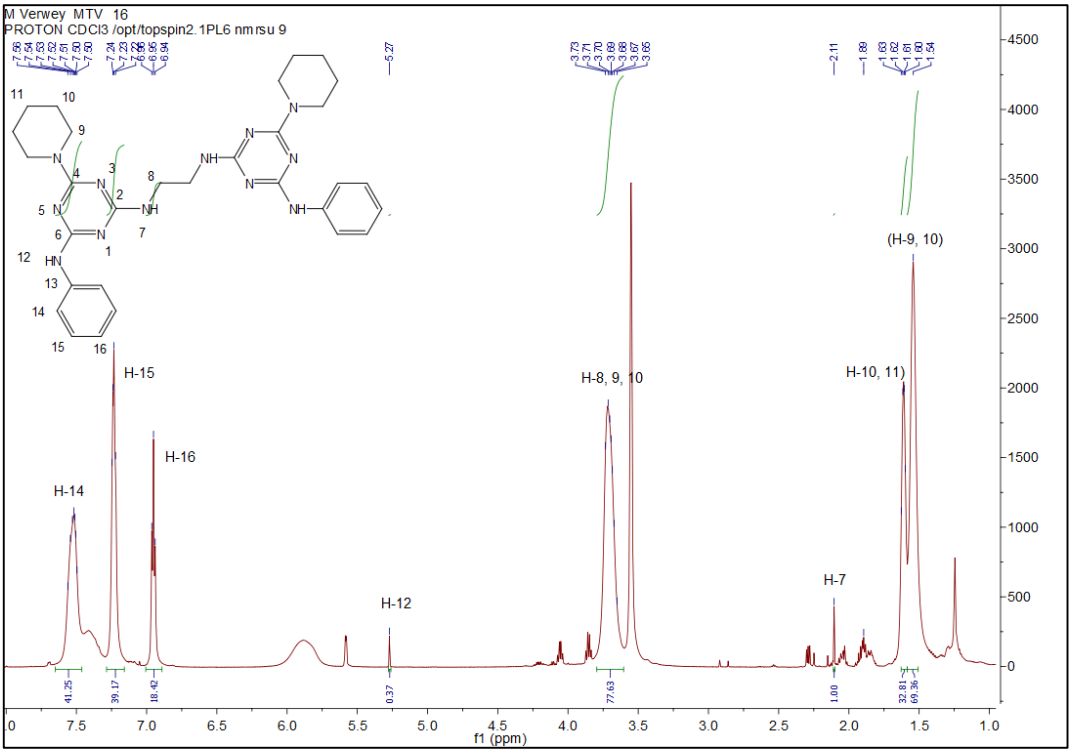
MS ES+ of compound 15



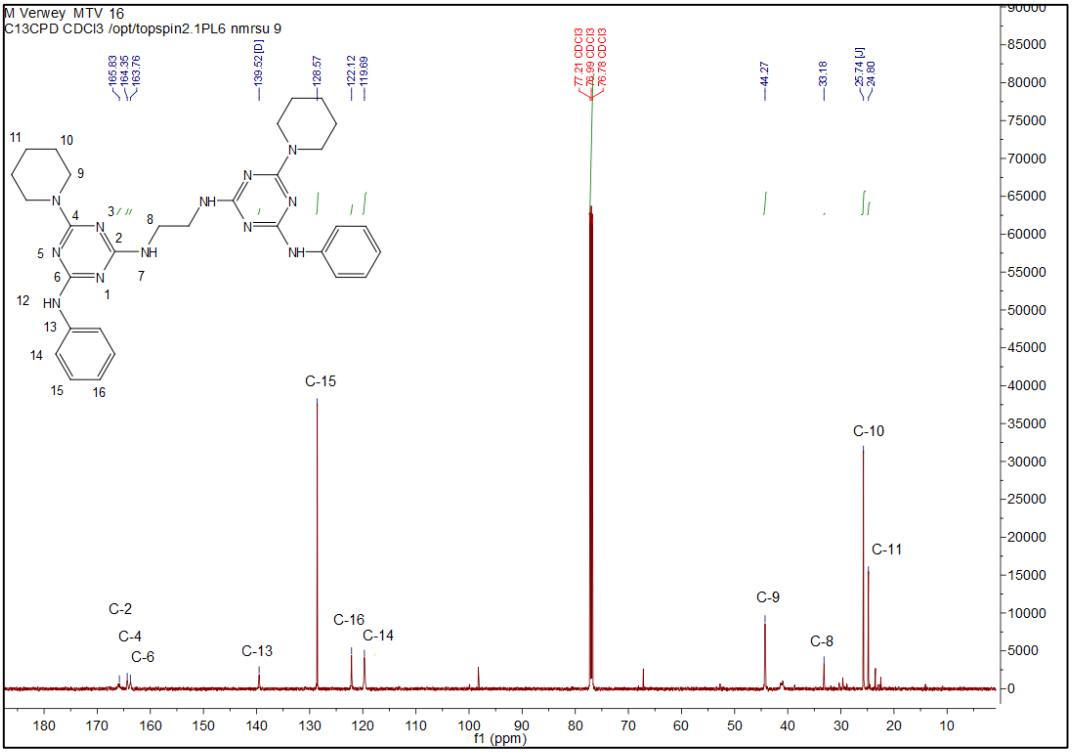
IR of compound 15



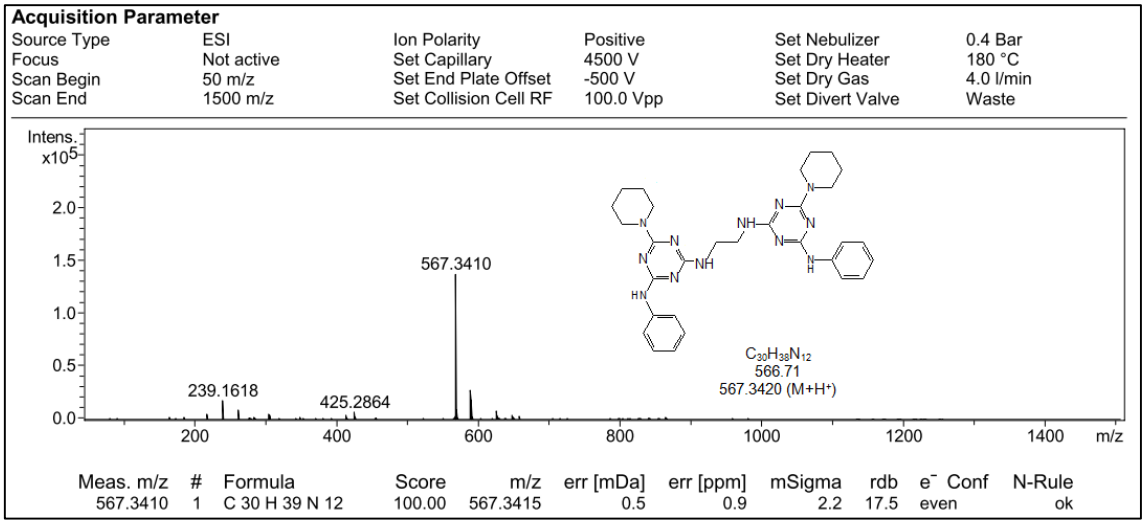
^{1}H NMR of compound 16



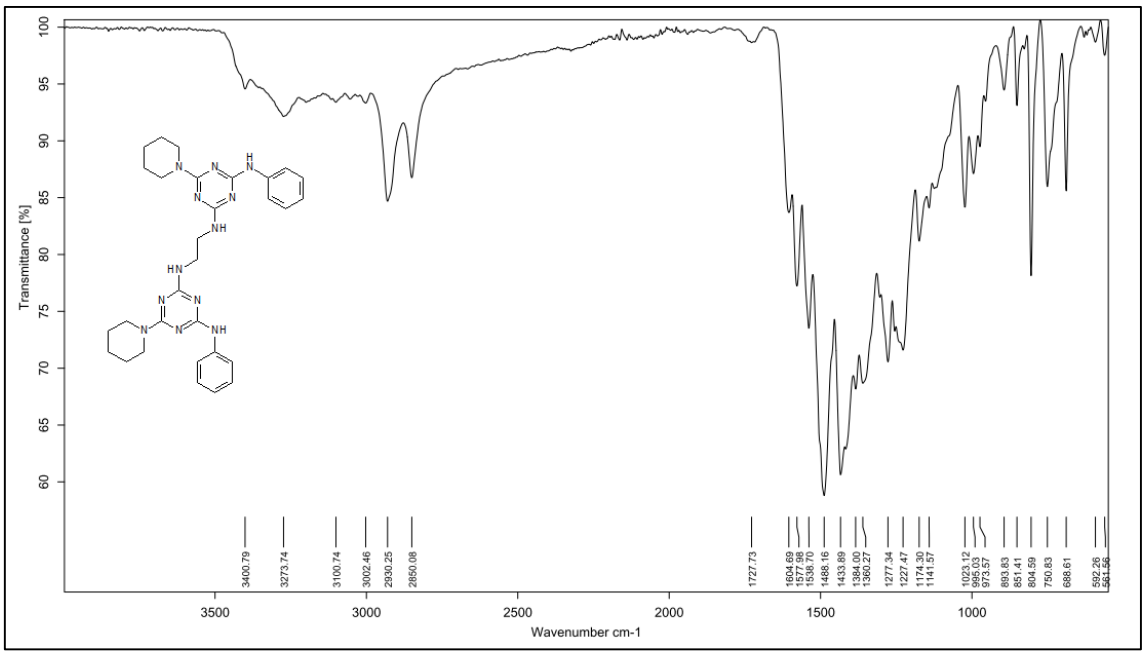
^{13}C NMR of compound 16



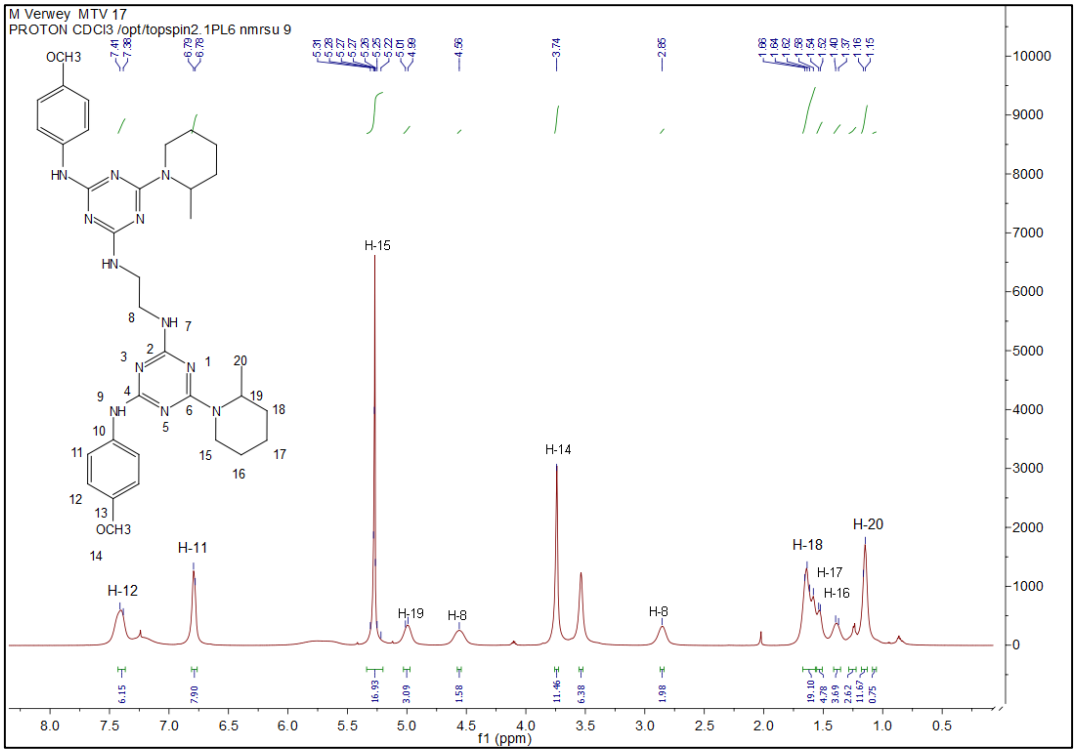
MS ES+ of compound 16



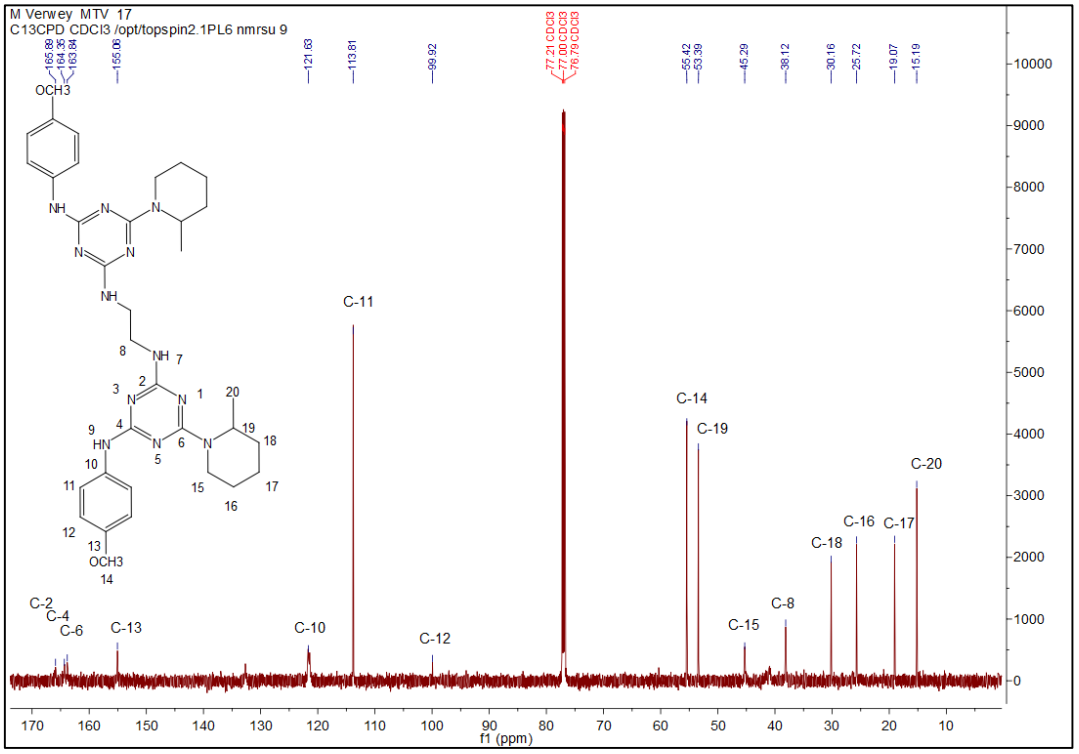
IR of compound 16



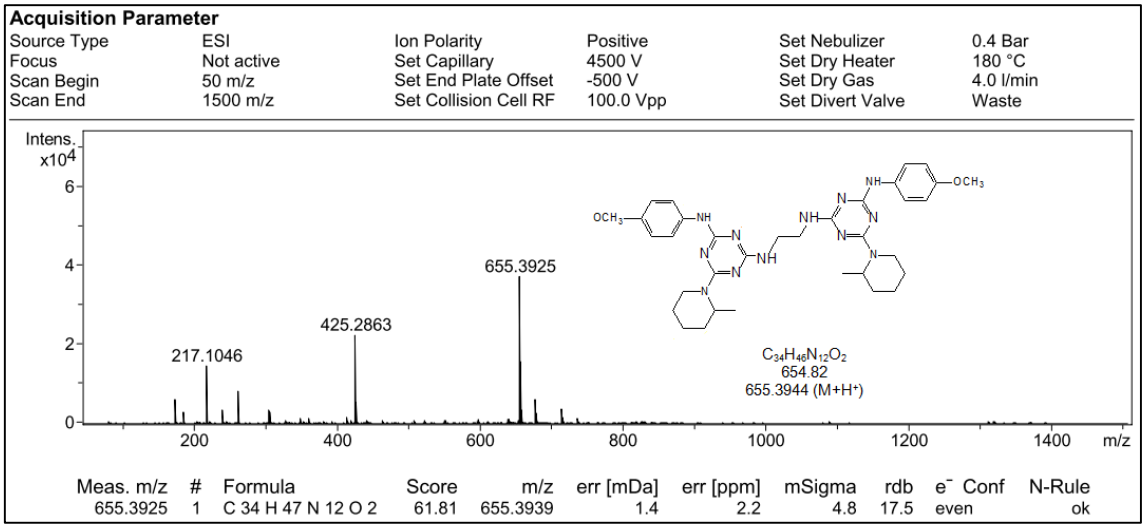
^{1}H NMR of compound 17



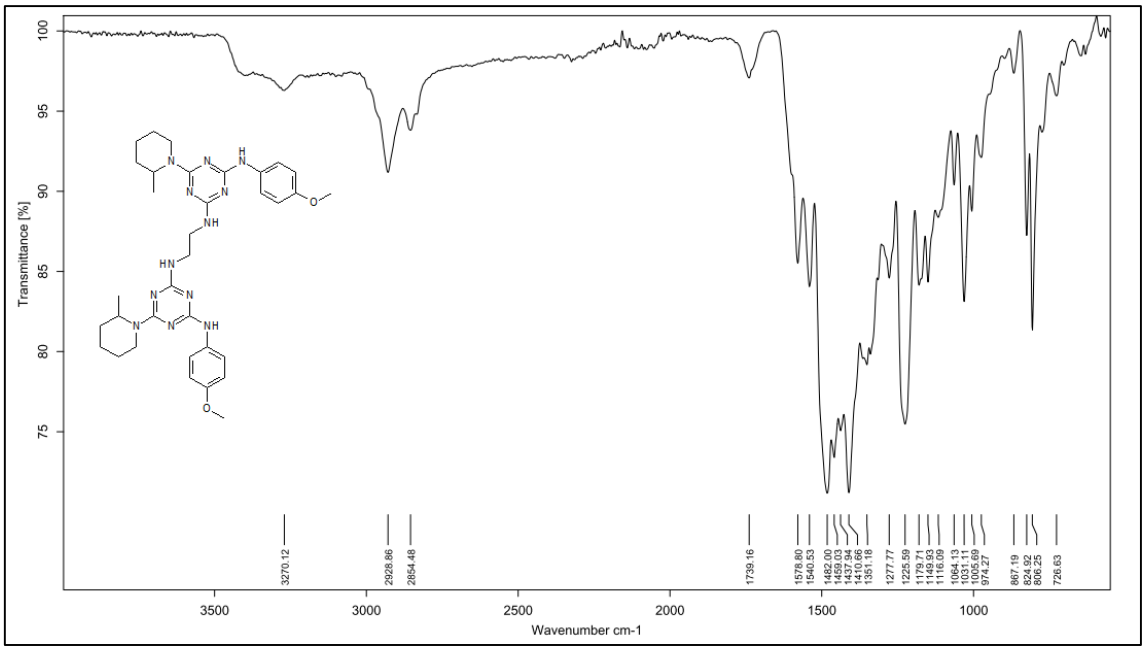
^{13}C NMR of compound 17



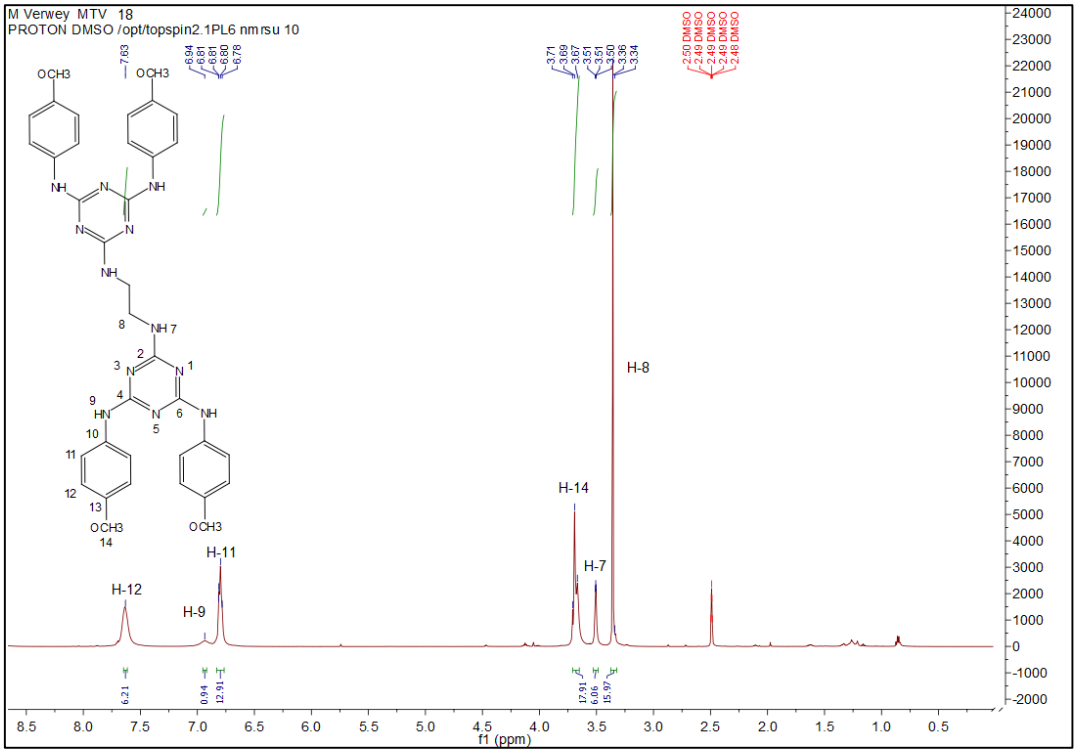
MS ES+ of compound 17



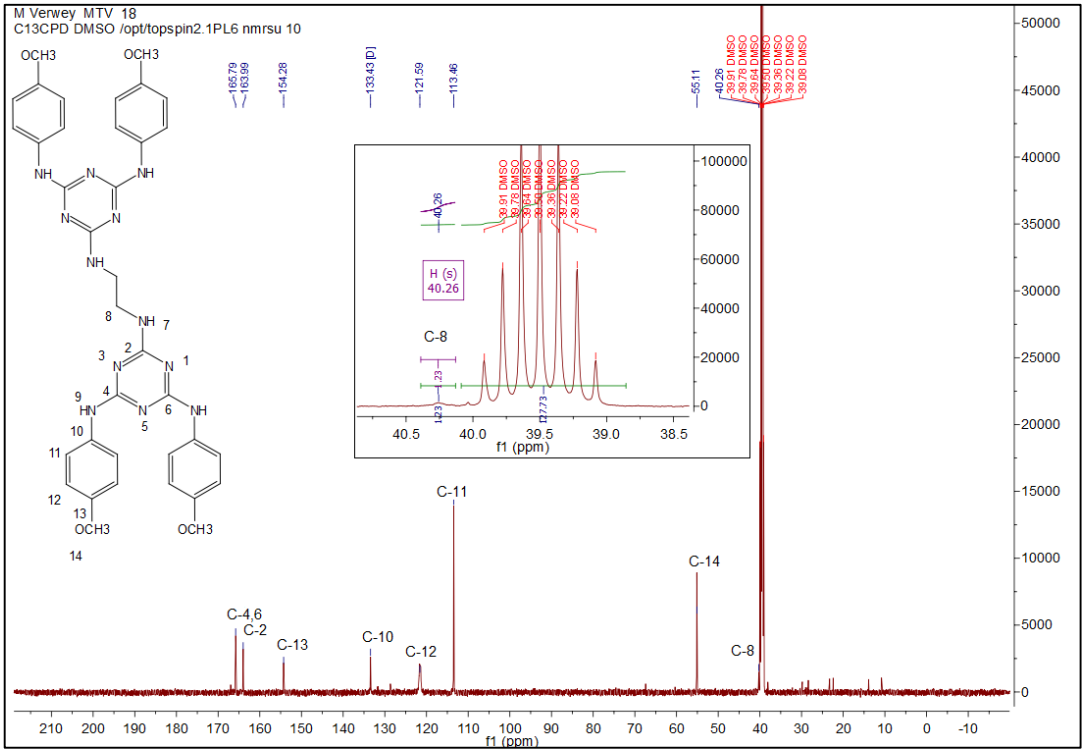
IR of compound 17



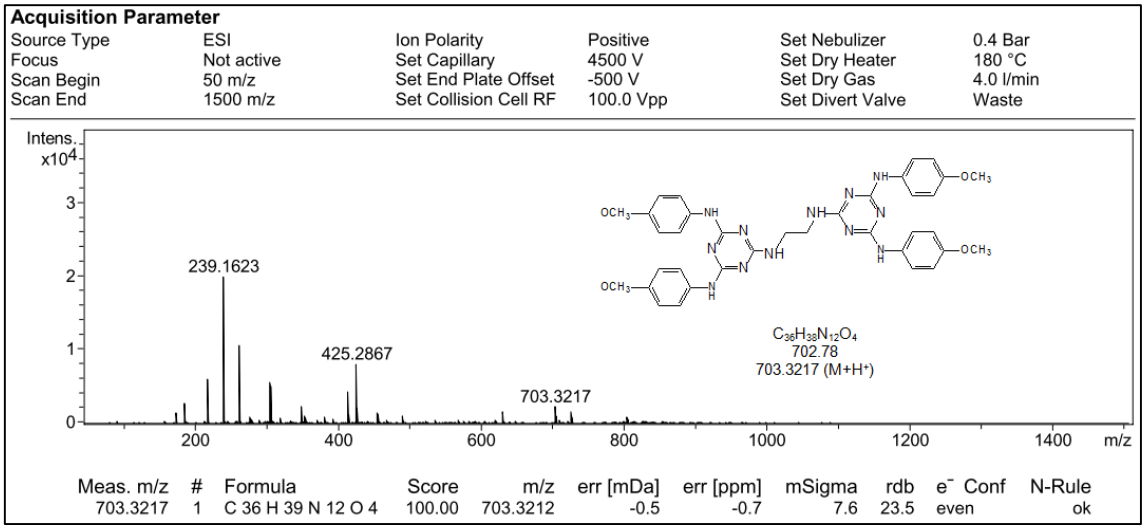
^{1}H NMR of compound 18



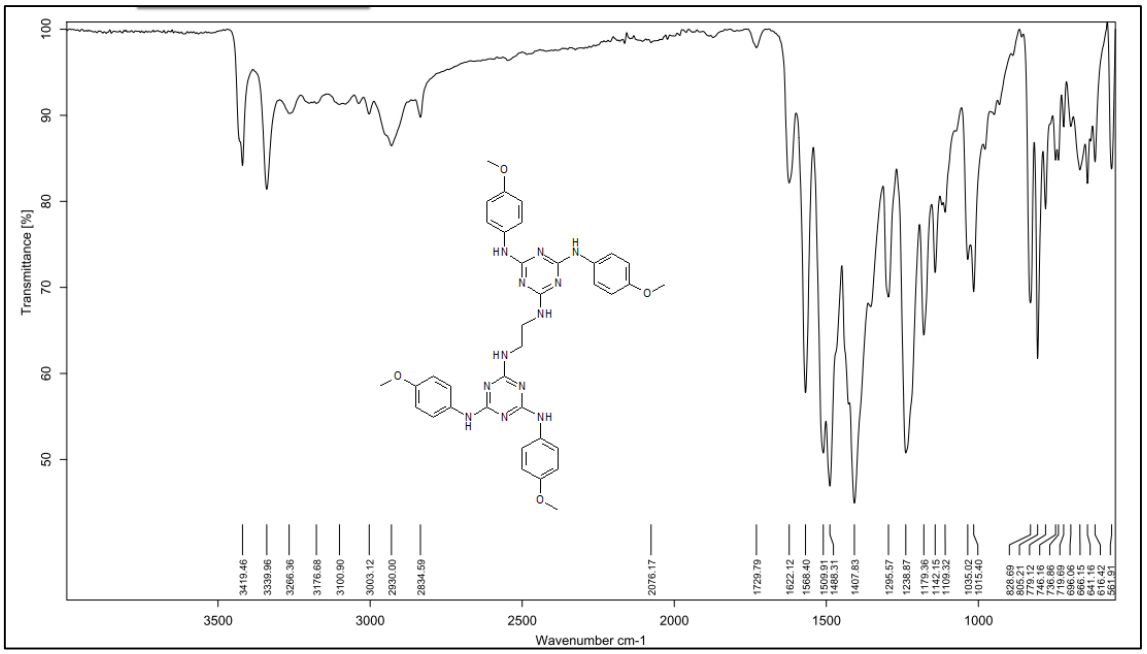
^{13}C NMR of compound 18



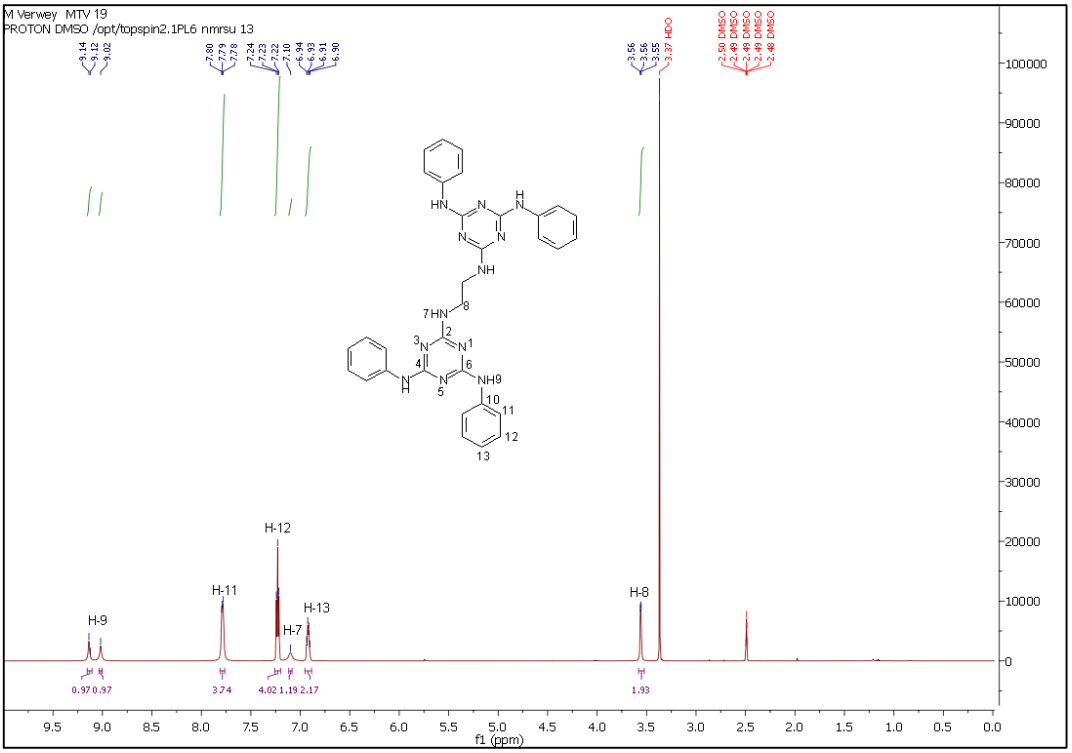
MS ES+ of compound 18



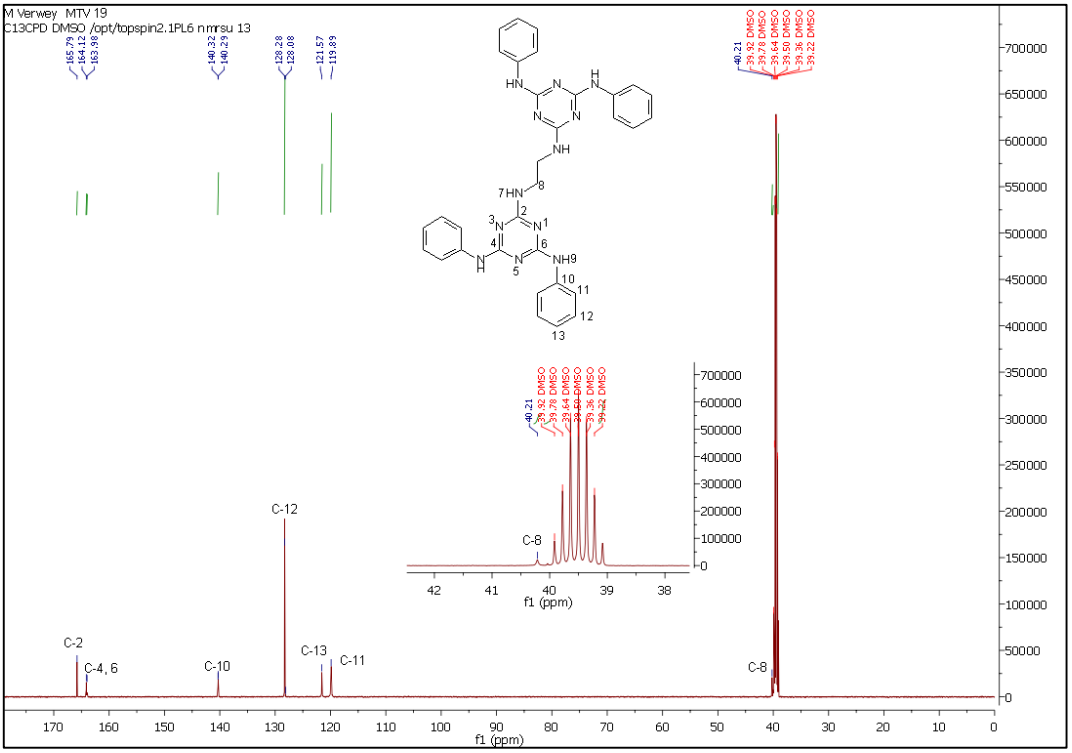
IR of compound 18



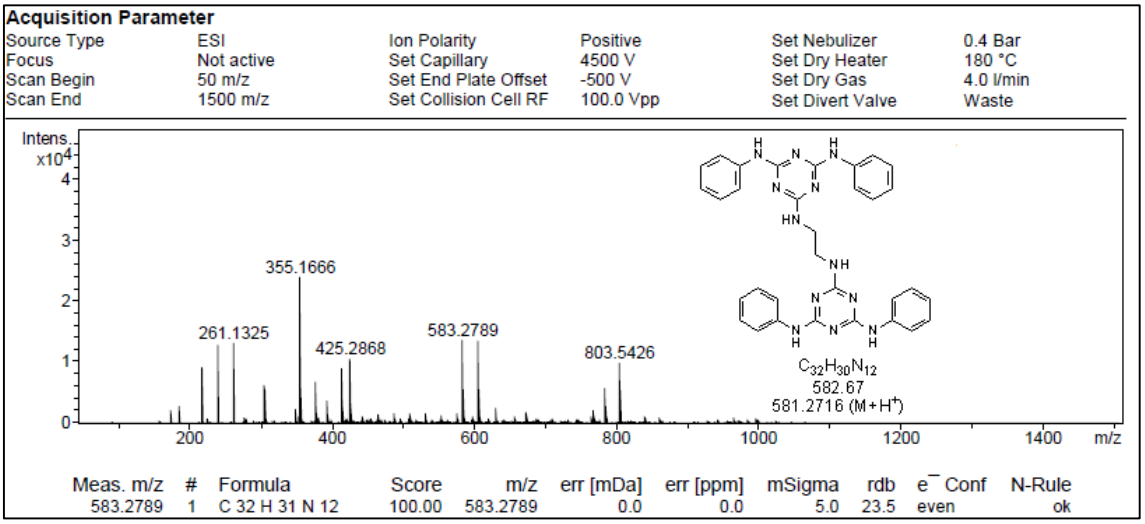
^{1}H NMR of compound 19



^{13}C NMR of compound 19



MS ES+ of compound 19



IR compound 19