Trifluoromethyl-substituted Quinoline and Tetrazole Derivatives: Design, Synthesis, Antimalarial activity and Cytotoxicity

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ABSTRACT

Malaria is a complex parasitic disease caused by the *Plasmodium falciparum.* It has been found to be responsible for the death of many people particularly in under-developed and developing countries. For many years chloroquine and quinine have been the mainstay of therapy for this disease. The research on new therapies against malaria have been hampered by factors such as the development of resistance against these and some of the new drugs or combinations thereof, the lack of adequate knowledge on the exact causes and mechanisms of resistance to the drugs and their mode of action, together with the fact that the disease occurs predominantly in poor countries where there is no adequate funding and monitoring facilities. Residual insecticides where they have been tried are not appropriate because of technical constraints and the vaccine development is still in infancy stage. Of the more than 200 000 compounds developed by Antimalarial Drug Development program of the Walter Reed Army Institute of Research (WRAIR) since its inception in the early 1960s, only 3% have been found to be active in the primary screening tests. Very few of these have reached the Phase III clinical trials.

The successes gained in the use of mefloquine and halofantrine in the treatment of resistant malaria has aroused considerable interest in the contribution made by the trifluoromethyl group as a substituent on antimalarial activity of many molecules. The objective of the current studies was to design, synthesise and evaluate the antimalarial activity of a group of compounds

containing the quinoline, triazine and tetrazole as basic structures but with either one or two trifluoromethyl groups as substituents in addition to other groups. These new compounds were evaluated for activity against the chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum.* The assessments made it possible to construct possible structure-activity relationship profiles.

The new compounds included a series of 2- and 8-trifluoromethyl- and 2,8-*bis*(trifluoromethyl)quinolines and those of trifluoromethyl substituted triazine and tetrazine derivatives with other substituents to form compounds containing the 4-(pyrimidine-5-yl)methanone and 2-(1-ethyl-5-nitro-1*H*-imidazol-4-yl)ethan-1-one moieties, the N,N-*bis*(trifluoromethyl)quinolin-4-yl)diamino alkyl derivatives and 1,2,4-triazine-[5,6b]indole and the 5*H*-1,2,4-triazolo[1',5',2,3]-1,2,4-triazino[5,6b]indole derivatives. All the compounds were characterised by elemental analysis, ^{1}H and ^{13}C NMR, mass and infrared spectrometric determinations. Comparative activities of the compounds were assessed using the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* and cytotoxicity was evaluated using the human promyelocytic leukaemia (HL-60) and Chinese Hamster Ovarian (CHO) cell lines against normal human cells.

In each series of the new compounds, a trifluoromethyl group has been found to enhance antimalarial activity. Except for the tetrazoles, the presence of the two trifluoromethyl groups appears to be essential for activity against the chloroquine-resistant strains of *P. falciparum*. The 2,8-*bis*(trifluoromethyl)-

quinolin-4-yl]-2-(1-ethyl-5-nitro-1*H*-imidazol-4-yl)ethan-1-one also exhibit inhibition of the leukemia cell growth. The N,N-*bis*(trifluoromethylquinolin-4-yl)diaminoalkane series have a high selectivity index. The ferriprotoporphyrin IX-drug complexation and DNA-drug intercalation and binding studies do not provide a convincing support for the actual mode of action of these new compounds.

CHAPTER 1

INTRODUCTION, AIMS AND STUDY DESIGN

1.1 INTRODUCTION AND BACKGROUND INFORMATION

The most recent innovative improvements and developments in health technologies including successes in the discovery and production of new drugs have brought little benefits to the treatment and eradication of malaria in developing and undeveloped countries of the world, where the disease is still a major threat to the health of the population. Approximately 3,2 billion people or more than 50% of the world population is at risk of the infection and between 350 and 500 million clinical cases which result in over 1 million deaths are reported each year. About 80% of these reported cases are African children below the age of five years (The Lancet, 2007). Malaria is by far Africa's most important tropical parasitic disease that kills more people than any other communicable disease, except perhaps tuberculosis and HIV-Aids (Magardie, 2000). Tuberculosis gained prominence recently following the outbreak of the extremely drug resistant (XDR) strain in South Africa (Basu *et al.*, 2007) where since 2005, of the 481 patients who reported for treatment, 216 of them have died by October 2007 (Flanagan, 2007). Serious concerns have been raised regarding the remarkably few drugs available for the treatment of malaria, particularly in rural Africa (White, 1992) where drug resistance is a major problem. Until the 1940's there was only quinine, but the Second World War spawned two new drugs followed by additional two during the war in Vietnam. But now the arsenal for treatment has all but run out, overtaken by the rapid spread of drug-resistant malaria parasites. The pharmaceutical industry seeing little profit in a market confined to poor countries, has also abandoned the disease (Brown, 1992).

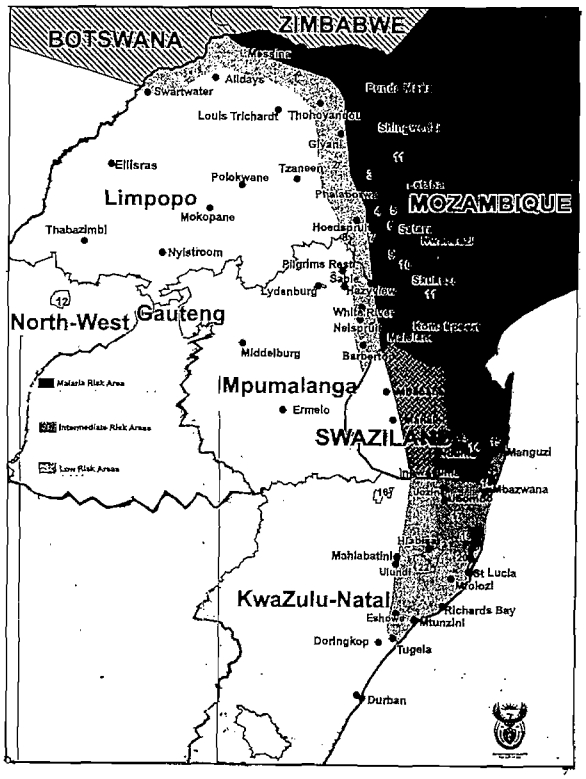


FIGURE 1.1: The malaria risk areas in the Southern Africa region.

Although the geographical area affected by malaria has shrunk over the past 50 years, the Southern Africa region continues to experience resurgence in the malaria transmission, especially in the last few years. Unstable malaria is encountered in Southern Africa below the latitude 20° South in an area encompassing Botswana, Mozambique, Namibia, Zimbabwe, South Africa and Swaziland, with the southernmost limits of transmission extending to northern Limpopo, Mpumalanga, Northwest Province and a portion of KwaZulu-Natal

(Smith *et al.*, 1977) (Figure 1.1). The heavy rainfalls experienced in these regions at the end of 2007 and the beginning of 2008 have also resulted in the re-emergence of this deadly disease.

The National Malaria Research Programme, run by the Medical Research Council (MRC) attributes the sudden increase in malaria manifestations in recent years to factors such as population migration, drug and insecticide resistance and climatic changes leading to heavy rainfalls in Southern Africa and elsewhere. Among the high risk groups are pregnant women, non-immune travellers, displaced people and labourers entering the epidemic areas (Magardie, 2000). In recent years, the risk of malaria is further exacerbated by the fact that many countries use the majority of the health budgets to fight the HIV/Aids pandemic, leaving little money to fight clinical deaths from malaria. According to the World Health Organisation (WHO), the direct and indirect costs of malaria in sub-Saharan countries exceed US$2 billion. In 1987 the estimated annual direct and indirect cost of malaria in Africa was US$ 800 million and this figure was expected to rise. However, despite the extent and severity of the conditions as well as the proven value of research for practical health gain, global expenditure in malaria research is very low, when compared with expenditure on conditions such as cancer, HIV/AIDS or asthma (Anderson *et al.*, 1996). The levels of risks associated with the spread of malaria in the world as shown in Figure 1.2 indicate that over 40% of the population is exposed to malaria with 10% not protected at all by the available programmes. An ambitious new global malaria action plan aimed at reducing the number of deaths from the disease to near zero by 2015 has been launched with world leaders committing nearly $3 billion (about R24 billion) to ensure its success (Thom, 2008). This plan, developed by the Roll Back Malaria Partnership has several short-, medium- and long-term targets, including increasing access to treated bed nests and faster diagnosis, reduction of the number of deaths to zero through continued universal coverage, to complete elimination of malaria in key countries and finally eradication of the disease by finding a vaccine.

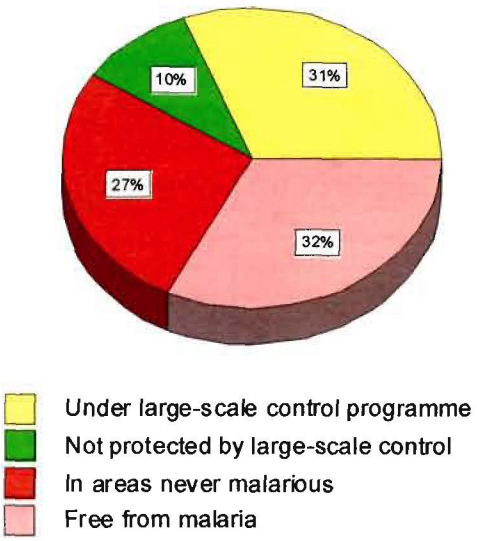


FIGURE 1.2: Population exposed to malaria risk (in percentage of world population) (WHO, 1991)

1.2 CAUSES OF THE DISEASE

Malaria is best thought of as a collective name for different diseases, since the epidemiology of malaria transmission and the severity of the disease vary greatly from region to region, village to village and even from person to person within a village (Anderson *et al.*, 1996). Some of these differences are due to the particular species of the parasite, the degree of compliance of a drug regimen, local patterns of the drug resistance and individual immunity. The disease is caused by four species of the protozoan parasites of the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, with *P. falciparum* being responsible for the most severe manifestation of the disease. Its etiology involves the invasion of the host red blood cells by the parasite (Behere and Goff, 1984). The parasite then matures and reproduces sexually in the mosquito *Anopheles*, and is transmitted through that vector to humans. It has a complex life-cycle, comprising a sexual phase (sporogony) in the mosquito (vector) and an asexual phase (schizogony) in man (Aikawa, 1977; Reynolds, 1993). Infection in man is caused by the injection of the sporozoites from a bite of the infected female ano-

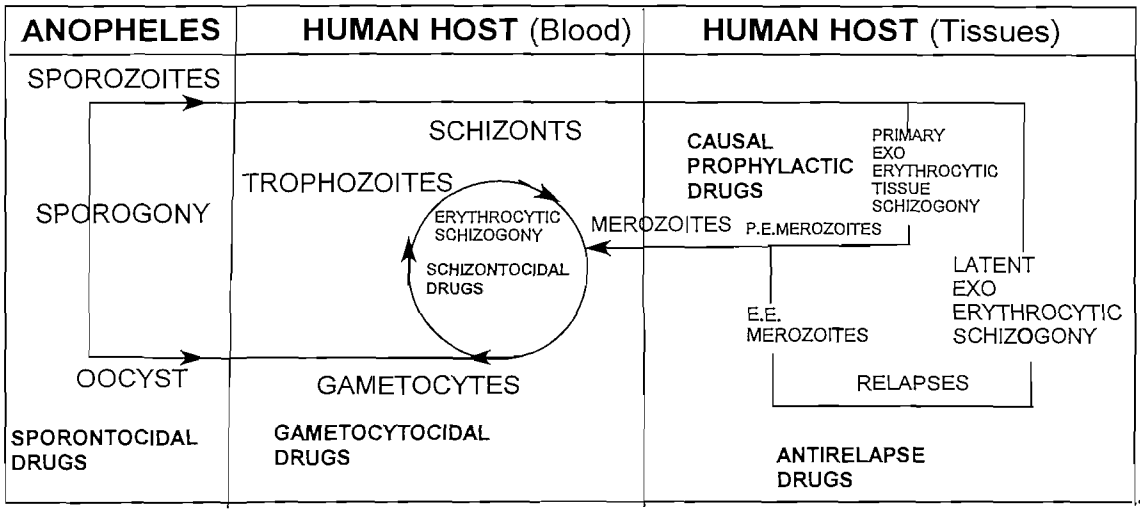
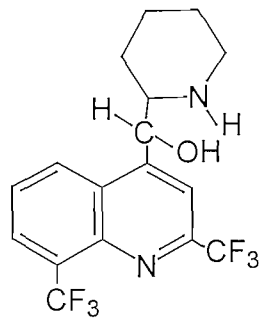


FIGURE 1.3: Simplified diagram of the plasmodial life cycle and the different drugs that can be used (Bruce-Chwatt *et al.*, 1981).

pheles mosquito. Some of these sporozoites rapidly enter the liver parenchymal cells, where they undergo *exoerythrocytic* or pre-*erythrocytic schizogony*, forming tissue *schizonts*, which mature and release thousands of *merozoites* into the blood when cell wall ruptures. When these merozoites invade the erythrocyte, they undergo complex sequence of transformations, beginning with adherence to the host cells and ending when, having reached the cell's interior, they transform themselves into *trophozoites* (Bannister *et al.*, 1977). The produced blood schizonts then rupture to release merozoites into circulation, which will then affect other erythrocytres. The stage is termed the erythocytic cycle and is responsible for the characteristic periodicity of the fever in malaria. After several erythrocytic cycles, and depending on the type of malaria, some erythrocytic forms develop into sexual gametocytes. It is the injection of the infected blood containing gametocytes that eventually gives rise to the sexual cycle in the mosquito (see Figure 1.3). The clinical symptoms of the disease include fever, headache and muscular pains and if not treated promptly can lead to severe complications.

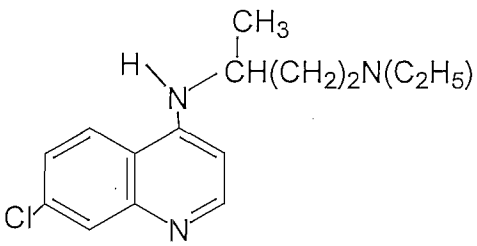
Several principal antimalarial drugs and combinations thereof used in the various stages of the parasite life cycle have been identified and include the following:

1. The 4-methanolquinoline derivatives such as cinchona alkaloids and mefloquine **(1)** that are rapidly acting blood schizontocides. This compound was introduced for routine use in 1985.



(1)

2. The 4-aminoquinolines such as chloroquine **(3)** and amodiaquine **(9)** that are rapidly acting blood schizontocides with some gametocytocidal activity. The basic chemical structure of these compounds forms the basis of this investigation.



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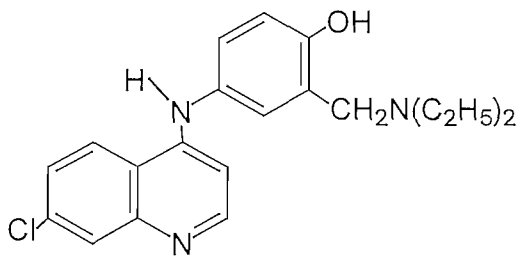


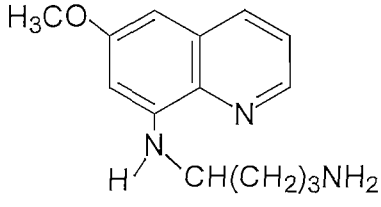
(9)

3. The 8-aminoquinolines such as primaquine **(12)** that are used primarily as tissue schizontocides to prevent relapses of the *ovale* and *vivax* malarias.
4. The biguanides such as proguanil and chlorproguanil, which have dihydrofolate reductase inhibitory activity and act as tissue schizontocides mainly for the prophylaxis of *falciparum* malaria. They act on both the pre-

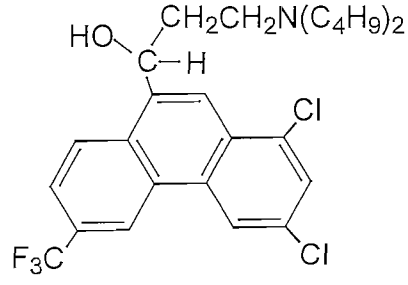
erythrocytic and erythrocytic stages of the parasite in the host, as well as on the phase of development in the mosquito (White, 1988).

5. The diaminopyrimidines such as pyrimethamine that are dihydrofolate reductase inhibitors (Hitchings, 1952; Rollo, 1955; Gutteridge and Triggs, 1971) and which have similar action as the biguanides.

6. The 9-phenanthrenemethanols such as halofantrine **(2)** that compares favourable with mefloquine (Cosgriff *et al.*, 1982; Boudreau *et al.*, 1988). These are blood schizontocides.



(12)



(2)

7. The sesquiterpene lactone (Brossi *et al.*, 1988) artemisinin, known as *Ginghaosu* in China, an extract of the wormwood plant, *Artemesia annua* (Brown, 1992; White, 1992) and its derivatives such as artemether and arteether. These are blood schizontocides, which are very effective, rapid acting, well-tolerated and on administration, the patient feels better almost immediately (Baker and Burgin, 1996). While the mode of action of these compounds is not known with absolute certainty *in vitro* studies have shown that these derivatives are more potent than the parent compound, artemesinin (Averi *et al.*, 1996). For centuries in China, the roots of *Dichroa febrifuga* Lour have been employed against malaria fevers and no parasite resistant to it were isolated (Takaya *et al.*, 1999). Febrifugine and isofebrifugine that exit in equilibrium are isolated as active principles against malaria.

8. The sulphonamides especially sulphadoxine are dihydropteroate and folate synthesis inhibitors. Sulphadoxine has been used in combination

with pyrimethamine (Lewis and Ponnampalam, 1979; Pearlman *et al.*, 1977; Doberstyn *et al.*, 1979; Nguyen-Dinh *et al.*, 1982). The combination shows synergy through sequential blockage of folic acid synthesis (White, 1988).

9. Antibiotics like tetracyclines and doxycyclines are blood schizontocides and also have activity against tissue forms (Meek *et al.*, 1986).
10. Sulphones such as dapsone have similar action as the sulphonamides. They are chemical analogues of *p*-aminobenzoic acid (PABA), an essential precursor for the *de novo* synthesis of folic acid (Milhous *et al.*, 1985).
11. 9-Aminoacridines such as mepacrine were also used in the malaria treatment (Bruce-Chwatt *et al.*, 1981).

1.3 THE CONTROL OF MALARIA

Malaria control is the reduction of malaria to a level at which it does not any more constitute a major health problem. This process usually includes operations that are unlimited in time and which aim at different levels of achievement in the reduction of malaria according to local conditions. On the other hand, malaria eradication is considered to be successful when no autochthonous cases have occurred for three consecutive years. Since the malaria parasite undergoes cyclical development through sporogony in the female anopheles mosquito and tissue schizogony, blood schizogony and gametocytogony in man as indicated in §1.2, control measures need be directed at interfering with any of the phases of the cycle or with the transfer from one host to the other as illustrated in Figure1.3. Measures against the vector proved to be most effective through the use of residual insecticides such as dichlorodiphenyltrichloroethane (DDT), organophosphorus compounds (Malathion) and carbamates (Propoxu) (Wernsdorfer, 1980). The basis for these attempts to eradicate malaria follows from the hypothesis that the complete interruption of malaria transmission over an adequate span of time could prevent new infections and permit spontaneous

disappearance of existing infections (Wernsdorfer and Payne, 1991). In 1996, pressured by environmental groups, South Africa dropped DDT for use in controlling malaria in affected areas for less toxic alternatives. However, four years later the country was facing its first malaria epidemic and country resumed spraying in 2000 and through the Lubombo Spatial Development Initiative (LSDI), the malaria outbreak declined (Johnson, 2007, Thom, 2008). Drastic reduction in the use of these agents because of financial and technical difficulties in some countries including pressures from the environmental groups led to the increase and spread of malaria (Smith *et al.*, 1977). It is still believed that measures to prevent mosquito bites are still the mainstay of prophylaxis. It has been recommended that insecticide-treated nets (ITNs) being cheap and highly effective way of reducing the burden of malaria, must be used (WHO, 2008) and that the eradication campaign if well conceived and thoughtfully implemented with close coordination and co-operation from all stake-holders, particularly with the nations most afflicted by the disease could complement and even strengthen other initiatives, including the building of national health systems (The Lancet, 2007). Bill and Melinda Gates, whose foundation has donated US$ 1 billion have called for the world to launch a new campaign for its eradication.

The usual cited practical problems associated with the control of malaria include factors such as the diagnosis of the disease in populations exposed to the risk. In developing countries, where malaria is prevalent, tertiary health care centres harbour excellent laboratory facilities with experts who are readily available to read and interpret Giemsa-stained thick and thin blood films (Anderson *et al.*, 1996). However, in the majority of less developed countries, the use of microscopic procedures for the diagnosis of malaria at the primary health care level, is hindered by lack of funds, equipment, and trained personnel. Consequently the staff in these areas often lacks the skill and expertise required to make a definite diagnosis of malaria by thin and thick blood smears (Makler and Hinrichs, 1993). On the other hand, in the developed countries, the primary health care clinics are usually well equipped and staffed with trained personnel,

but malaria is a rare disease. It is thus imperative that, in both the developing and developed countries of the world, simple and reasonable sensitive screening tests for the detection of the malaria infections be available in primary health care centres. It is only over the past decade when an increasing interest in controlling malaria through strengthened national and local health care systems, attempts to quantify malaria's importance epidemiologically were made (MIM, 2001). However, it is too early to be excited about these developments as much more precision is needed before a full understanding of malaria's burden is made available. The interface between the parasite biology, and immunology, pathogenesis of infection, clinical manifestation, epidemiologic features, impact of interventions, economic consequences and relationship of malaria with other health problems, particularly HIV/AIDS and nutrition, bear more intense investigation. The battle against malaria requires an intensified research program to develop improved understanding of the infection and disease.

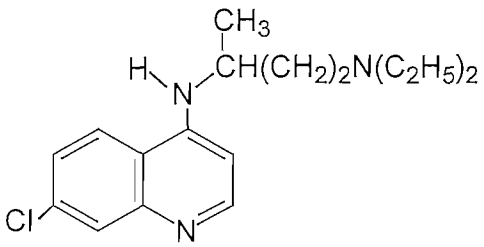
1.4 PROSPECTS OF MALARIA VACCINE DEVELOPMENT

Based on the different stages of the parasite life cycle, several *P. falciparum* vaccine candidate antigens have been identified with successes on the development of the multistage and multi-component recombinant malaria vaccine over the last two decades (Shi *et al.*, 1999). The vaccine is highly immunologenic and the protective efficacy in non-human primates and then human, has been evaluated. During 2001, the Malaria Vaccine Development Programme (MVDP) of the USAID, the Malaria Vaccine Initiative (MVI) at the Program for Appropriate Technology in Health (Path), and the European Malaria Vaccine Initiative (EMVI) announced an alliance with the purpose of accelerating the global effort for producing malaria vaccine for the developing world. The MVI also announced a tripartite collaboration with India's International Centre for Genetic Engineering and Biotechnology (ICGEB) and Bharat Biotech International Limited for the development and evaluation of a *P. vivax* candidate vaccine. The candidate vaccine targeted the functional portion of the parasites' Duffy binding protein,

which allows the *Plasmodium* to bind onto red blood cells and providing the only path for the parasite to enter the cells. Recently it was reported that the RTS,S/ASO2D vaccine candidate appears to cut the severity of the diseases by 58% among the young Mozambican children (Beresford, 2007). Following the confirmation of its safety and effectiveness in small-scale trails, the vaccine could be licensed for use particularly in young children within four years if its effectiveness can be confirmed in large-scale Phase II clinical trials. These trials are expected to start in eight African countries in 2008 (The Lancet, 2007). At this stage, the vaccine appears to reduce clinical malaria episodes by 35% and severe disease by 49% (Beresford, 2008).

1.5 AIMS AND OBJECTIVES OF THE PRESENT STUDY

Since the discovery of the non-phototoxic, but highly effective antimalarial quinolinemethanol, mefloquine (**1**) (Ohnmacht *et al.*, 1971) (Figure 1.4), the trifluoromethyl group has aroused considerable and special interest as substituents on organic molecules in the design of quinoline and related compounds used in the treatment of malaria (Strube, 1975) and quinolone antibacterial agents (Sanchez *et al.*, 1992). This is especially so with the realization that its leads to compounds with improved biological activity, particularly against the resistant strains of the plasmodium. Halofantrine (**2**) containing a trifluoromethyl group attached to a phenanthrenemethanol scaffold, compares favourably with **1**, both compounds being effective against the multidrug-resistant *P. falciparum* strain, including that strain that is highly resistant to chloroquine (**3**).



(3)

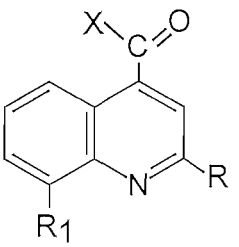
Most of the highly effective quinolone antibacterials contain the trifluoromethyl group or fluorine atom attached to the quinoline ring. Although there is not such a large difference in the size between the trifluoromethyl group (with a van der Waal's radius of 2,44 Å) and methyl group (2,00 Å) groups, but a large difference in electronic effects, the former has been found to contribute greatly to activity, since the fluorocarbon often have different physicochemical properties when compared to the other halocarbons, thus altering the biological properties of the compounds in which they appear (De *et al.*, 1998). Once introduced, the fluorine atom being a sterically demanding atom, with small van der Waal's radius, creates a high carbon-fluorine bond energy which renders the substituent relatively resistant to metabolic transformation (Welch, 1987). It is capable of altering quite drastically, parameters such as basicity or acidity of the neighbouring groups, dipole moments within the molecule, the overall reactivity and stability of neighbouring functional groups, and most importantly, the pKa of the molecule (Hawley *et al*, 1996). These findings regarding the special physicochemical properties of the trifluoromethyl group and their influence on pharmacological activity had a profound impact in our investigations. Thus the focus of these studies was to investigate how one or two trifluoromethyl groups attached to the quinoline and the 1,2,4-triazino[5,6b]indole ring systems influence the activities of the compounds against the chloroquine-senstive and chloroquine-resistant strains of *P. falciparum*.

The number of carbon atoms between the two nitrogen atoms in the diaminoalkane side chain has been observed to be a major determinant of

activity against the chloroquine-resistant *P. falciparum* (De *et al.*, 1998). However, when the length of the alkyl chain exceeds the permissible linear dimension chain, energetically less favourable folding of the chain would occur, leading to decreased activity (Calas *et al.*, 1997). The explanation could be that the alkyl groups could curl up on themselves, leading to expulsion of the quarternary ammonium group out of the anionic site and only the long alkyl chain would be associated, in tightly coiled fashion, with the hydrophobic region on the target (Raynes *et al.*, 1996: 558).

The objective of this investigation was to design, synthesis and conduct preliminary *in vitro* investigations on the structure-activity relationships of a series of quinoline and some selected 1,2,4-triazino[5,6b]indole derivatives the structures of which contain one or two trifluoromethyl groups attached at selected positions on the aromatic ring with heterocyclic groups attached to the carbonyl group at position 4 of the ring or quinoline series in which position 4 of the ring bears diaminoalkyl chains of different lengths or a piperazine ring linking the two quinoline rings in addition to the one or two trifluoromethyl groups as shown by structures **4** and **5**. The third series are derivatives of the 1,2,4-triazino-[5,6b]indoles with one or two trifluoromethyl groups attached at selected positions of the aromatic and/or hetero-aromatic ring systems shown in structures **6** and **7**. The basic structures of the envisaged compounds are shown in Figure 1.4.

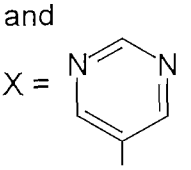
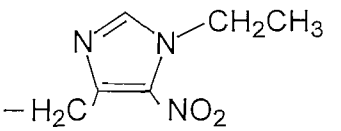
In these studies the antimalarial activity of the compounds will be assessed using the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. Where possible, the antimalarial activity profiles of these compounds will be constructed with reference to their structures particularly the number and position of the trifluoromethyl and other important functional groups. The probable mode of actions of the compounds will be investigated through DNA binding and intercalating and ethidium displacement studies. Cytotoxicity of the compounds will be evaluated against normal and leukemia cells. The studies will be conducted in order to verify on a structure-activity relationship basis the effect of:

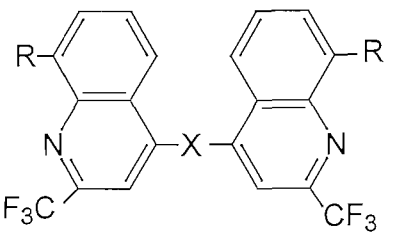


(4)

where R = H or CF_3, R_1 = H or CF_3

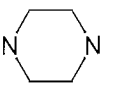
and

X =  or 

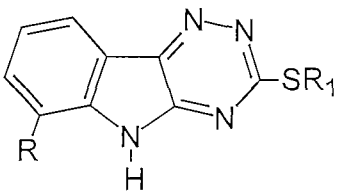


(5)

where R = H or CF_3

and X = $HN(CH_2)_nNH$ or 

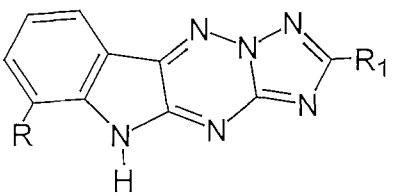
n = 2, 3, 4, or 6



(6)

Where R = H or CF_3

R_1 = CH_3; CH_2CH_3, 



(7)

R = H or CF_3

R_1 = H; CH_3; CF_3 or C_6H_4Cl

FIGURE 1.4: Structures of the target compounds in Tables 4.1a to 4.1d.

1. the presence and positions of the trifluoromethyl groups attached to the quinoline and 1,2,4-triazino[5,6b]indole nuclei including the influence of other functional groups on the indole system,
2. the nature and size of substituents at position 4 of the quinoline ring system as well as the nature and size of the linker when two quinoline ring systems have been linked together, and
3. the role each constituent of the target compounds will play in the possible mechanism of action of these compounds.

It is envisaged that on completion of these studies on each of the series of compounds, the following benefits will accrue for future use:

(a) New chemical entities or lead compounds could be added to the pool of bioactive compounds,

(b) New or improved synthetic pathways could be developed in the area of malaria chemotherapy and related fields.

(c) In the course of the evaluation of the biological activities of these compounds, an understanding of the mechanism of action of these and related compounds will be obtained to assist in future studies in drug design. It was also part of our objective to assess the role of other groups such as the chlorine atom and the nature and size of various functional groups attached at position 4 of the quinoline-type compounds.

(d) To offer advise on an action plan regarding the development of new antimalarial drugs related to these compounds.

1.6 STUDY DESIGN

Each of the studies undertaken consisted of the following stages:

(a) the synthesis stage of the target compounds from available raw materials,

(b) elucidation and confirmation of the structures of the new intermediate and final compounds through the use of elemental analysis, ^{1}H and ^{13}C NMR, mass and infrared spectrophotometric analyses, and

(c) evaluation of biological activity of each series of compounds consisting of *in vitro* assessment of the (i) antimalarial activity using the chloroquine-sensitive (D10) and chloroquine-resistant (K1) strains of P. *falciparum*, (ii) binding and intercalative properties of some of the compounds with DNA or the ability of the compounds to displace ethidium from its complex with haem and (iii) cytotoxicity of some of the compounds against normal and leukemia cells. As these assessments are based on the structures of each investigated compound, all the compounds were coded to prevent investigators' prior knowledge of the structures of the compounds under investigation.

1.7 PRESENTATION OF THE THESIS

Except for the information and data on the quinoline-type compounds substituted with one of two chlorine atoms, the thesis of the study consists of a collection of peer reviewed published papers, each paper, modified slightly for the sake of consistency of reporting in this thesis, constituting a complete chapter of the study. An attempt was made in the synthesis of chlorine-substituted compounds. Although highly active and comparable to chloroquine on their activity against the chloroquine-sensitive strain of the *P. falciparum*, further studies on these compounds were curtailed by short half-lives and other stability problems exhibited by these compounds. The following papers form the basis of this study:

1. 2- AND 8-TRIFLUOROMETHYL- AND 2,8-*BIS*(TRIFLUOROMETHYL) QUINOLINE DERIVATIVES

Joseph L Kgokong and Jaco C. Breytenbach. 2000. Synthesis of novel trifluoromethylquinoline and *bis*(trifluoromethyl)quinoline derivatives. *South African Journal of Chemistry*, **53**(2): 100 – 103.

Joseph L Kgokong, Gilbert M Matsabisa and Jaco C. Breytenbach. 2001. *In vitro* antimalarial activity of novel trifluoromethyl- and *bis*(trifluoromethyl)quinoline derivatives. *Arzneimittel-Forschung/Drug Research,* **51** (1) 163 – 168.

Joseph L. Kgokong and James M Wachira. 2001. Cytotoxicity of novel trifluoromethylquinoline derivatives on human leukaemia cells. *European Journal of Pharmaceutical Sciences*, **12** (4) 369 – 376.

2. N,N-*BIS*(TRIFLUOROMETHYL) AND N,N-*BIS*{*BIS*(TRIFLOUROMETHYL)-QUINOLIN-4-YL}DIAMINO ALKANE DERIVATIVES

Joseph L Kgokong, Gilbert M Matsabisa, Peter P Smith and Jaco C Breytenbach. 2008. N,N-*Bis*(trifluoromethylquinolin-4-yl)diaminoalkanes: Synthesis and antimalarial activity. *Medicinal Chemistry*, **4** (8) 438 – 445.

3. 1,2,4-TRIAZINO[5,6b]INDOLE DERIVATIVES

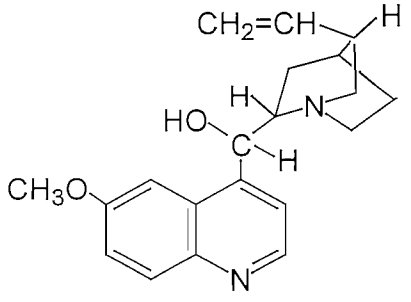
Joseph L Kgokong, Peter P Smith and Gilbert M Matsabisa. 2005. 1,2,4-triazino-[5,6b]indole derivatives: effects of the trifluoromethyl group on *in vitro* antimalarial activity. *Bioorganic & Medicinal Chemistry,* **13**: 2935 – 2942.

CHAPTER 2

LITERATURE REVIEW IN MALARIA RESEARCH

2.1 INTRODUCTION

The first specific and effective impact on the chemotherapy of malaria consisted of the administration of a powder of the bark of Cinchona tree, *Arbor febrifuga,* followed by the use of one or more of the isolated principal alkaloids obtained from the extracts of the bark of this tree (Wernsdorfer and Payne, 1991). These alkaloids (see § 3.2.3 and Table 3.2) were the *laevorotatory* diastereomers, quinine **(8)** and cinchonidine and the *dextrorotatory* quinidine and cinchonine, which were found to rapidly kill mature intraerythrocytic malaria parasites, but had little effect on the gametocytes and did not exhibit any activity at all on the pre-erythrocytic development of the malaria parasites (White, 1988). Subsequently a new generation of synthetic antimalarial drugs in the form of 4-aminoquinolines such as chloroquine and amodiaquine became available. These drugs contain a quinoline ring system as a basic structure and are thus structurally related to the cinchona alkaloids. The primary objective of synthesising new compounds related to these alkaloids through molecular manipulations and other forms of chemical modifications was to improve on the activity of these alkaloids and related compounds and at the same time to offset the development of resistance by the malaria parasites.



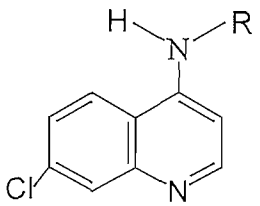
(6)

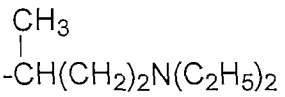
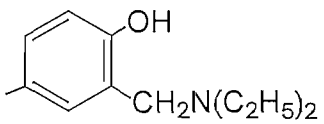
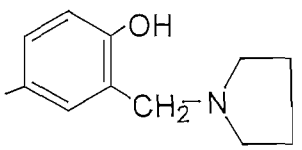
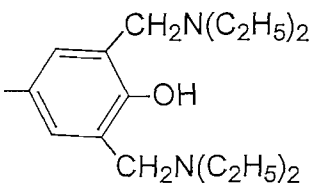
The Antimalarial Drug Development program started in 1963 at the Walter Reed Army Institute of Research (WRAIR) to develop drugs for the prevention and treatment of malaria, but only 3% of the 200 000 compounds tested over a period of 10 years were found to be active in the primary screening tests (Canfield and Rozman, 1974; Schmidt *et al.*, 1978; Canfield and Heiffer, 1979). Some of the compounds investigated belonged to the 4-aminoquinolines (Table 2.1), the 8-aminoquinolines (Table 2.2), the 4-quinolinemethanols (Tables 2.3 and 2.4) and phenanthrenemethanols. The following factors were found to impact negatively on the success of most antimalarial drug development programmes:

1) The malaria parasite has shown a remarkable ability to develop resistance to antimalarial drugs and many of the new drugs show cross-resistance to those developed previously,
2) Novel leads are increasingly difficult to uncover, and efforts to do so often result in more complex organic molecules, with attendant synthetic problems, and
3) The development of new drugs must be accompanied by more sophisticated animal toxicity testing prior to clinical trials to minimize potential toxicity to humans.

However, the development, occurrence and spread of resistance of *P. falciparum* to the drugs currently used, the limited number of alternative drugs, their limitations with respect to adverse events and high costs, all underline the need for research in the field of malaria chemotherapy (Wernsdorfer and Kouznetsov, 1980). The development of resistance to chemotherapeutic agents does not only affect the treatment of malaria, but all facets of chemotherapy including the use of antibiotics and all drugs used in the treatment of tuberculosis, cancer and HIV-Aids. The recent emergence of highly resistant strains of tuberculosis (XDR-strain) in South Africa has created a panic situation in the health sector (Basu *et al.*, 2007). This invariably continues to drive the search for more effective agents that are capable of overcoming or even reversing development of drug resistance

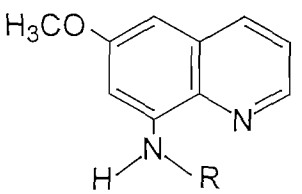
TABLE 2.1 The 4-Aminoquinolines



COMPOUND	SUBSTITUENT (R)
Chloroquine **(3)**	
Amodiaquine **(9)**	
Amopyroquine **(10)**	
Cycloquine **(11)**	

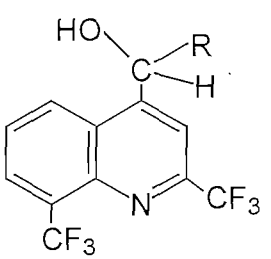
as well as the search for an understanding of the mechanism involve in the development of resistance to drugs. It is hoped that the use of standard medicinal and combinatorial chemical approaches to the synthesis of novel entities, or chemical modification of existing drugs will give way in future to agents that will be used as hits to be turned into useful drugs through structure-activity relationship chemistry approach (Chu *et al.*, 1996).

TABLE 2.2: 8-Aminoquinolines



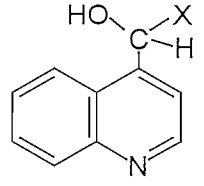
COMPOUND	SUBSTITUENT (R)
Primaquine **(12)**	$-CH(CH_3)(CH_2)_3NH_2$
Quinocide **(13)**	$-(CH_2)_3CH(CH_3)NH_2$
Pamaquine **(14)**	$-CH(CH_3)(CH_2)_3N(C_2H_5)_2$
Pentaquine **(15)**	$-CH(CH_3)(CH_2)_3NHCH(CH_3)_2$

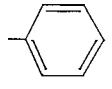
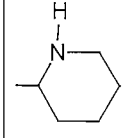
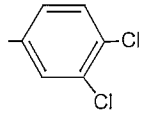
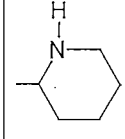
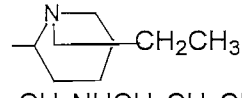
TABLE 2.3: Other quinolinemethanols screened for antimalarial activity



SURVEY No. (WR)	R
177 540	$-(CH_2)_2N(CH_2CH_2CH_2CH_3)_2$
183 544	$-(CH_2)_2NHCH_2CH_2CH_3$
183 545	$-CH_2NHC(CH_3)_3$
183 606	$-(CH_2)_2NHCH_2CH_2CH_3$
184 806	$-CH_2CH_2NHC(CH_3)_3$

TABLE 2.4: 4-Quinolinemethanols



SUBSTITUENTS AND THEIR POSITIONS				
2	6	7	8	X
	-Cl	-H	-Cl	
	-Cl	-H	-Cl	$-CH_2N(CH_2CH_2CH_2CH_3)_2$
$-CF_3$	-Cl	-H	-Cl	
-H	-H	$-CF_3$	-H	
$-CF_3$	-H	-H	$-CF_3$	$-CH_2NHCH_2CH_2CH_3$
$-CF_3$	-H	-H	$-CF_3$	$-CH_2NHC(CH_3)_3$
$-CF_3$	-H	-H	$-CF_3$	$-CH_2N(CH_2CH_2CH_3)_2$
$-CF_3$	-H	-H	$-CF_3$	$-CH_2(CH_3)NHCH_2CH_2CH_3$
$-CF_3$	-H	-H	$-CF_3$	$-CH_2(CH_3)NHC(CH_3)_3$
$-CF_3$	-H	-H	$-CF_3$	$-(CH_2)(CH_3)N(CH_2CH_2CH_2CH_3)_2$

2.2 POSSIBLE MODE OF ANTIMALARIAL DRUG ACTION

One key element in the successful design of new chemical entities required to exhibit a particular pharmacological action is the understanding of and the ability to predict the mechanism of action of such compounds in relation to their

structures and other physicochemical properties. However, in the case of malaria, the emergence of multitudes of sometimes conflicting postulates and theories relating to the explanation of how these drugs exert their activity have created such an unfortunate confusion, making it impossible to simplify both the search for replacement drugs by identifying appropriate vulnerable targets in the parasite, and providing a satisfying intellectual explanation for some of the successful pharmacology of these drugs (Slater, 1993). The successful design of new chemotherapeutic entities coupled with improvements in the pharmacology of existing ones or the creation of a pool of new led compounds depend on a thorough knowledge and understanding of the following essential parameters:

(a) The possible mechanism of action of that particular class of compounds and the ability to relate the structures of the compounds to their pharmacological activity,

(b) An understanding of the mechanism of development of resistance and the ability to predict from available data that such a development will occur,

(c) Other external factors including the chemistry contributing to the pharmacology or the bioavailability of the drugs to the receptor sites.

In spite of the fact that chloroquine has been the mainstay of antimalarial chemotherapy for over 50 years, its mechanism of action remains uncertain, and the following have been proposed to account for its action:

(a) Binding to and intercalating into the DNA double helix (Stollar and Levine, 1963; Whichard *et al*, 1968; Morris *et al*, 1970; Lantz and Van Dyke, 1971; Héléne, 1998),

(b) Alkalisation of the food vacuoles after accumulation of the drugs based on their weak base effects (Homewood *et al*, 1972; Krogstad *et al.*, 1985; Hawley *et al.*, 1996; 1998),

(c) Binding to haem to form a toxic complex (Egan *et al.*, 1996; Sullivan *et al.*, 1996),

(d) Inhibition of haem-dependent protein synthesis (Zarchin *et al*, 1986),

(e) Prevention of iron release from haemoglobin (Slater *et al.*, 1991;

Gabay and Ginsburg, 1993; Rosenthal and Meshnick, 1996),

(f) Inhibition of the food vacuole cysteine protease activity (Vander Jagt *et al.*, 1986; Goldberg *et al.*, 1991; Francis *et al.*, 1994; Asawamahasakda *et al.*, 1994; Rosenthal, 1995; Silva *et al.*, 1996),

(g) Blocking enzymatically or nonenzymatically mediated formation of haemazoin (Egan *et al.*, 1994; Dorn *et al.*, 1995; 1998; Rosenthal and Meshnick, 1996),

(h) Interact specifically with lactate dehydrogenase enzyme of the *P. falciparum* thus depriving it of its ability to regenerate the NAD^+ necessary for use in glycolysis which is a principal source of ATP in the parasite's metabolism (Menting *et al.*, 1997),

(i) Interference with *P. falciparum* phospholipids metabolism (Chevli and Fitch, 1982; Vial *et al*, 1984; Calas *et al.*, 1997) and

(j) Induction of lipid peroxidation by the ferriprotoporphyrin IX-antimalarial drug complex (Sugioka and Suzuki, 1991; de Almeida Ribeiro *et al.*, 1995; Marques, *et al.*, 1996).

Although it is not yet fully and universally accepted, it is believed that the majority of antimalarial drugs of the quinoline-type exert their activity through interaction with ferriprotoporphyrin IX in the acid food vacuoles of the parasites (Egan *et al.*, 1996). However, in some research areas it is still believed that the action of these drugs is through binding to and intercalating with the DNA double helix of the parasite cells, thus preventing the replication of the DNA and RNA during protein synthesis. In the current model (Egan *et al.*, 1998) regarding the pharmacology of chloroquine and related drugs, it is proposed that its action proceeds through the following stages:

(i) The drug owing to its basic properties accumulates in the food vacuoles of the parasites,

(ii) Once in the food vacuole it associates with ferriprotoporphyrin IX,

(iii) The consequent accumulation of the drug and association with ferriprotoporphyrin IX results in the inhibition of β-haematin formation , and

(iv) Destruction of the parasite occurs due to the toxic effects of the ferriprotoporphyrin IX and/or ferriprotoporphyrin IX-drug complex.

2.2.1 THE ROLE OF THE FOOD VACUOLES

All mammalian cells (with the exception of mature erythrocytes) contain membrane bound compartments (vesicles) that have an internal pH of less than 5. The function of these specialised organelles, called food vacuoles, has been illustrated by receptor-mediated endocytosis and by the lysosomal-enzyme targeting procedures (Krogstad and Schlesinger, 1987). The absence of typical lysosomal phosphatases and glycosidases in these vacuoles indicates that these are specialised organelles of the *P. falciparum* and have the following functions:

(a) degrading haemoglobin to provide the iron necessary for the survival of the parasites (Goldberg *et al.*, 1990),

(b) accumulation of the antimalarial drugs during chemotherapy,

(c) providing for the involvement of ferriprotoporphyrin IX in the inhibition of haemazoin formation,

(d) playing a crucial role in the development of chloroquine resistance (Saliba *et al.*, 1998).

2.2.1.1 The Enzymes in the Degradation of Haemoglobin

The cleavage of the intact haemoglobin into small fragments takes place in the food vacuoles through a process that requires the action of endogenous aspartic protease (see Figure 2.1). In the *P. falciparum* food vacuole, at least three proteases have been identified and include the cysteine protease (falcipain) and two aspartic proteases (plasmepsins I and II), each of which probably participates in globin hydrolysis (Rosenthal and Meshnick, 1996).The latter two enzymes are highly site selective when confronted with folded proteins, but not nearly as selective as when

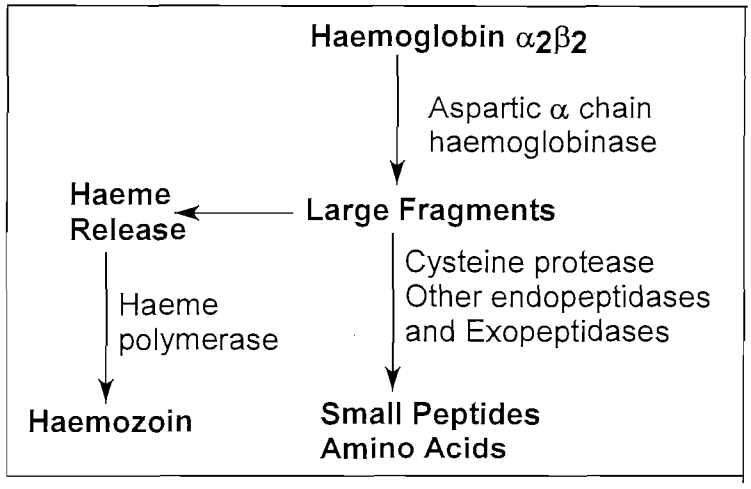


FIGURE 2.1: The proposed pathway of haemoglobin degradation (Slater, 1993).

given unfolded or fragmented proteins. They are thought to cleave the native haemoglobin molecule in order to expose it for completion of the proteolysis (Silva *et al.*, 1996). Plasmepsin II (Plm II) is capable of cleaving native haemoglobin molecule but is more active against denatured or fragmented globin that is produced by the action of plasmepsin I (Plm I).

A vacuolar aspartic haemoglobinase, which has been purified and characterised (Goldberg *et al.*, 1991), recognises haemoglobin, making a single initial cleavage in the α-chain between 33-phenylalanine and 34-leucine,a site which is in the hinge region of haemoglobin and which is involved in maintaining the integrity of the molecule as it bind oxygen (Francis *et al.*, 1994). Cleavage at this site by the malaria haemogobinase appears to unravel the molecule, filleting it open for rapid degradation by other proteases (Goldberg and Slater 1992). It is possible that the inhibition of this enzyme prevents toxic haem release, while the other inhibitors allow toxic haem build-up with consequent membrane damage (Francis *et al.*, 1994). It has been proposed that it is essential that the cleavage of the intact haemoglobin into fragments take place before so that other proteolytic activities can function efficiently (Goldberg *et al*, 1991). Even before globin is

proteolytically degraded and haem is sequestered into haemozoin, the temporal exposure of host cell cytosol to the lysosome-like environment of the food vacuole results in haemoglobin denaturation and consequent iron release (Gabay *et al.*, 1994). Several inhibitors of the degradation processes have been identified. Two of these are Pepstatin A, a specific aspartic protease inhibitor acting against the haemozoin production in trophozoites by about 10% and hypoxanthine incorporation by about 60%, and the E64, a specific cysteine protease inhibitor, which inhibits haemozoin production more than hypoxanthine uptake in both rings and trophozoites at a variety of concentrations (Asawamahasakda *et al.*, 1994). While the proteolysis of globin by the malaria parasites involve both the cysteine and aspartic proteinase activities, the former also plays a role as a toxic agent as it also inhibits the globin hydrolysis (Rosenthal, 1995).

2.2.1.2 The Food Vacuoles as Receptors for Drugs Accumulation

The antimalarial activities of the quinoline-type drugs are a function of both the ability of the drug to interfere with the polymerisation process (Egan *et al.*, 1994; Dorn *et al.*, 1995; Warhurst, 1995; Adams *et al.*, 1996) as well as the capacity to accumulate to pharmacologically relevant concentrations at the site of drug action (Hawley *et al.*, 1998). The direct relationship between the levels of the drug accumulation and drug potency that has been observed, seems to be double exponential and not linear (Hawley *et al.*, 1996). It has also been noticed that the accumulation of the drugs in the acid food vacuoles is influenced by:

(a) the transmembrane proton (pH) gradient that exists between the external environment and the intracellular parasite, and

(b) the physiological properties of the drugs (Martiney *et al.*, 1995; Bray *et al.*, 1996).

2.2.1.2.1 The pH Gradient in the Food Vacuoles.

The transmembrane proton gradient across the parasite's food vacuoles appears to account for virtually all of the specific uptake and action of chloroquine (**3**) and

mefloquine **(1)** (Yayon *et al.*, 1984; Krogstad *et al.*, 1985). This gradient, existing in malaria-infected cells has been found to account for the extensive accumulative uptake of **3**. The acidic pH of the trophozoite vacuole is maintained by a dynamic equilibrium between proton leakage and a vacuolar ATP-dependent proton pump. Raising the pH by pretreatment with other weak bases such as ammonium chloride has been found to reduce subsequent uptake of either **1** or **3** (Krogstad *et al.*, 1985). Qualitative and quantitative evidence for the involvement of the acidic parasite food vacuole in the accumulation of bases due to its acidic nature has been observed (Yayon *et al.*, 1984). It is the existence of this proton gradient across the acidic compartment and/or in the relative permeability of the membranes to the protonated forms of the drug, that account for the spectrum of sensitivity of malaria parasite to chloroquine and other drugs. For tebuquine analogues drug accumulation is found to be significantly correlated with the reciprocal of drug IC_{50} values (r = 0,98) (O'Neill *et al.*, 1997). Although the pH gradient provides the driving force for drug uptake, it is insufficient to account for the full extent of drug accumulation (Hawley *et al.*, 1986).

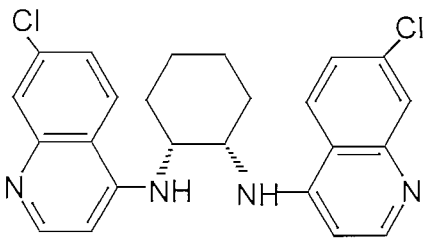
The extent of drug accumulation at the site of haem polymerisation is also a regulator of antimalarial activity, and the principal difference between the quinolines which show good antiparasitic activity *in vitro* and those that do not is a reflection of their ability to accumulate within the parasite rather than their ability to inhibit polymerisation (Hawley *et al.*, 1998). The ability of the drug to accumulate in the food vacuoles profoundly influences the local concentration of the drug in the food vacuole, therefore its ability to inhibit haem polymerisation *in situ*. Kinetic models used to analyse the time-course of chloroquine uptake and the steady-sate levels of drug accumulation in strains of *P. falciparum* that display variable drug resistance have demonstrated that drug resistance is compatible with the existence of a weakened proton pump in the resistant strains, and the drug efflux kinetics cannot distinguish between the possible modes of drug resistance (Ginsburg and Stein,1991). The antimalarials are concentrated by the parasitized red cells, and the pH gradient across the parasite's acid

vesicles appear to account for virtually all of the parasite specific uptake of **1** and **3** (Krogstad *et al.*, 1985). The accumulation of the drug in the parasite vesicle is a unique mechanism by which **3** produces its marked effect (Krogstad and Schlesinger, 1987).

2.2.1.2.2 The Physicochemical Properties of the Drugs

The high levels of accumulation that compounds such as **3** and **9** are able to achieve within the parasite's food vacoule are thought to result at least partially from the fact that they are weak bases (Geary *et al.*, 1986). These compounds are able to exit in both charged (protonated) and uncharged (unprotonated) forms. The unprotonated form or neutral form of the drug is highly membrane permeable, and can diffuse freely and rapidly across biological membranes, whereas the mono- and/or diprotonated forms of the drug are at least an order of magnitude less membrane permeable, and so diffuse across these membranes at a much reduced rate (Hawley *et al.*, 1996). Although it is widely accepted that **3** accumulates by a weak base mechanism and not by binding to haem, once accumulated in the food vacuole it is possible for this compound to interfere with haem processing (Slater, 1993). Since weak bases are protonated rapidly in the acid vesicles because of their high dissociation constants, the total concentration of weak bases in the vesicle increases as additional weak base moves into the vesicle from the cytoplasm, until a steady state is achieved (Krogstad and Schlesinger, 1987). The increased accumulation in the parasite vesicle is a unique mechanism by which **3** produces its marked effects on the pH in that the vesicles at nanomolar extracellular concentrations of **3** that do not affect mammalian cells (Krogstad and Schlesinger, 1987). This protonation of the uncharged weak base entering the acid vesicle raises the intravesicular pH by means of consumption of hydrogen ions. As the drug accumulates in the food vacuole (pH 5), tertiary amino group as well as the heterocyclic nitrogen atom in the quinoline ring become fully protonated (Egan *et al.*, 1998). This also leads to the increase in the pH of the vacuole, inhibiting further haemoglobin catabolism.

The drug uptake characteristics of compounds **3** and **9** show that both drugs exhibit a biphasic accumulation characteristic, comprising a high-affinity saturable component that is parasite-specific and pharmacologically important, and a low-affinity non-saturable component that is not parasite-specific and may be of toxicological relevance only (Hawley *et al.*, 1996). Accordingly, the most ideal drug would be the one that would selectively saturate the high-affinity component at concentrations at which the amount of the drug at the low-affinity site will be negligible. The accumulation of both compounds is significantly reduced in chloroquine-resistant parasites compared to the chloroquine-susceptible parasites, with the reduction in chloroquine accumulation being 3 - 5-fold compared with 2-fold for amodiaquine (Bray *et al.*, 1996). For compound **3** a 10 - 20-fold difference in susceptibility corresponds to only 4 - 5-fold difference in accumulation, while for **9**, a 3 - 6-fold difference in susceptibility corresponds to a 2-fold difference in accumulation. The fact that the *S,S* and *R,R* enantiomers of the bisquinoline, *trans*-N^1,N^2-*bis*(7-chloroquinolin-4-yl)cyclohexane-1,2-diamine **(16)** both inhibit polymerisation with equal potency, but differ in their ability to inhibit chloroquine-resistant parasite growth, may be ascribed to the transport factors across the cell membrane which affect the two enantiomers of the same compound's accumulation in the parasites (Ridley *et al.*, 1997).



(16)

Studies conducted on the uptake of 4-aminoquinoline drugs by malaria parasites have identified a two component system consisting of a saturable portion of high affinity which is stimulated by glucose and a nonsaturable component of low affinity that is not stimulated by glucose (Deribe and Warhurst, 1985). From

these studies a conclusion was made that only a proportion of the drug accumulated at high affinity has antimalarial activity and that the proportion of high affinity uptake is reduced in resistant isolates (Bray *et al.*,1996). It was further proposed that the inclusion of a moiety to the drug molecule that will provide suitable weak basic properties will render such a molecule potentially antimalarial and any newly designed drug would be expected to be active against the parasite until resistance occurs through natural selection (Egan *et al.*, 1996). A combination of high accumulation and favourable heme binding appears to be important for high activity of the 4-aminoquinoline class of drugs (O'Neill *et al.*, 1997). It is therefore essential that in the development of new antimalarial drugs to identify structural and/or physicochemical features of such compounds that greatly enhances the drug's interaction with the low-affinity, low-capacity component, and reducing the compound's interaction with the low-affinity, high-capacity component (Hawley *et al.*, 1996). A combination of high cellular accumulation and favourable binding appears to be important for high activity in the 4-aminoquinoline class of drugs (O'Neill *et al.*, 1997). Once compounds **1**, **3** and **8** are accumulated in the food vacuole, the formation of the complex between each drug and ferriprotoporphyrin IX, followed by a complex-induced lipid peroxidation, plays a key role in the disturbance of the metabolism in the parasite's food vacuole (Sugioka and Suzuki, 1991).

In the investigation of the cellular accumulation ratios of the compounds **3** and **9** in both malaria parasites and human CH1 cancer cells (Hawley *et al.*, 1996) a suggestion emerged that the increased level of compound **9** accumulation in comparison to that of **3**, may be due to its enhanced affinity for an intraparasitic binding site. This enhanced affinity with subsequent increase in drug accumulation is the reason why **9** shows greater inherent activity against *P. falciparum* than **3**. Following from this investigation, a conclusion was based on the derivation of the Henderson-Hasselbach equation with the following assumptions was reached:

1) the proton gradient from extracellular medium to within the acid vacuolar pH of 2,4 units (i.e. extracellular pH of 7,4 and vacuolar pH of 5),
2) Charged (protonated) drugs being membrane impermeable, and
3) There is no intracellular binding of either drug, it is possible to predict compartmental drug distribution using the equation described earlier (Krogstad and Schlesinger, 1986; Hawley *et al.*, 1996):

$$\frac{[Drug]_v}{[Drug]_o} = \frac{1 + 10^{(pKa1 - pHv)} + 10^{(pka1 + pKa2 - 2pHo)}}{1 + 10^{(pKa1 - pHo)} + 10^{(pKa1 + pKa2 - 2pHo)}}$$

Where pH_v represents the pH inside the vacuole (assumed to be pH 5) and pH_o is the external pH (assumed to be pH 7,4).
The ratio $[Drug]_v / [Drug]_o$ is the vacuolar drug accumulation ratio (VAR), and VAR x Fractional cell volume occupied by acid vacuoles = CAR where CAR is the cellular drug accumulation ratio.

In addition, the curves of drug uptake versus external concentration of a saturable component of high affinity and against nonsaturable component of low affinity can be simulated by superimposing a rectangular hyperbola onto a straight line, described by the equation (Bray *et al*, 1996):

$$[TD] = [ED].C_{ap}/([ED] + K_d) + m \,.\, [ED]$$

where [TD] is the total concentration of the drug taken up, [ED] is the concentration of the drug in the external medium and is proportional to the concentration of the drug available to bind the high affinity component, C_{ap} is the capacity of the high affinity component, K_d is the apparent dissociation constant of the high affinity component and m is the slope of the line describing the low affinity component. CAR is equivalent to total intracellular drug concentration divided by the extracellular drug concentration and is given by:

$$CAR = [TD]/[ED]$$

The plot of CAR at IC_{50} against the reciprocal of [ED] at IC_{50} for the isolates will give a linear relationship if the amount of high affinity uptake at IC_{50} is the same. The slope of the line corresponds to the amount of high affinity uptake at IC_{50} and the intercept corresponds to the low affinity CAR *m*.

2.2.1.3 THE HAEM RELEASE AND HAEMOZOIN FORMATION

The catabolism of haemoglobin also leads to the deposition of malaria pigment called haemozoin within the food vacuoles (Fitch and Kanjanangulpan, 1987; Brémard *et al.,* 1993; Wood and Eaton, 1993). The haemozoin accumulates exponentially over time in livers and spleen of *P. berghei* NK65-infested ICR mice (Sullivan *et al.*, 1996), and this accumulation can affect the regulation of many immune-mediated processes. It has been noticed that the quantity of haemozoin in chloroquine-susceptible parasites is higher than in chloroquine-resistant parasites (Goldberg and Slater, 1992), meaning that haemoglobin degradation in chloroquine-resistant *P. falciparum* remains tightly coupled to haemozoin production, despite exposure to chloroquine (Orjih and Fitch, 1993). Research findings have shown that the *P. falciparum* FCR-3 trophozoites contain approximately 339 ± 69 ng haemozoin/10^6 parasites, while rings contain 23 ± 7 ng haemozoin/10^6 parasites (Sullivan *et al.,* 1996). The formation of haemozoin or malaria pigment is thought to constitute a detoxification pathway for the highly toxic ferriprotoporphyryn IX (Brémard *et al.*, 1993; Adams *et al.*, 1996).

2.2.1.3.1 The Sources of Iron for the Parasites

The erythrocytic malaria parasites reside in an environment rich in haemoglobin that is a ready source of nutrients for the parasites (Rosenthal and Meshnick, 1996). These parasites, like any other organism, need a balanced intracellular composition of amino acids to optimally synthesise the proteins (Zarchin *et al.*, 1986). The achievement of this balance depends on several interdependent factors which control the intracellular levels of each amino acid. These include

factors such as the rate of amino acid production and consumption, the established concentration gradient and the relative permeability of each individual acid. However, as amino acid synthesis and uptake are apparently insufficient to satisfy metabolic needs, the parasites utilises haemoglobin through hydrolysis of its globin portion. Thus haemoglobin constitutes the principal source of iron required for the synthesis of iron-containing proteins such as ribonucleotide reductase, superoxide dismutase and cytochrome, and for the *de novo* haem biosynthesis (Dominguez *et al.*, 1997). Despite the involvement of only a small percentage of denatured haemoglobin, the abundance of haemoglobin relative to the other iron source in the host cell, makes this the major source of intracellular supply of iron to the growing parasite (Gabay *et al.*, 1994) as it does not depend on exogenous iron. The fact that internally generated iron reduces the sensitivity of the parasite to compound **3** indicates that the vacuolar production of iron from digested haemoglobin should be considered as a possible target in the design of quinoline-containing drugs. The parasites show haem dependency for protein synthesis, and the addition of **3** *in vitro* inhibits this haem dependent protein synthesis (Surolia and Padmanaban, 1991). Parasite lysate from cultures treated with therapeutic concentrations of chloroquine *in sutu* manifest enhanced phosphorylation of the parasite eukarytic initiation factor 2α (eIF-2α) under conditions of cell-free protein synthesis. The process is inhibted by the addition of haemin to the lysate leading to a decrease in general protein synthesis.

2.2.1.3.2 The Structure and Role of Ferriprotoporphyrin IX

Structurally trapped within the haemazoin is the parasite's endogenous antimalarial agent called ferriprotoporphyrin IX in the form of haematin (Banyal and Fitch, 1982; Dorn *et al.*, 1995; Egan *et al.*, 1996). This agent contains a five coordinate iron (III) complex in a high spin state, with four of the iron (III) bonds in each haematin subunit linked to the planar porphyrin ring, and the fifth is believed to be linked to a propionic acid side chain of the adjacent haematin unit as shown in Figure 2.2 (Dorn *et al.*, 1998; Slater *et al.*, 1991). The carboxylate component originates from the glycosylation of haemoglobin (Goldie *et al.*, 1990). Raman

resonance microspectrometry carried out on a single haemozoin particle or a bulk material has shown frequencies analogous to those exhibited by haemin with a high spin pentacoordinate (square pyramidal) iron (III) (Warhurst, 1995).

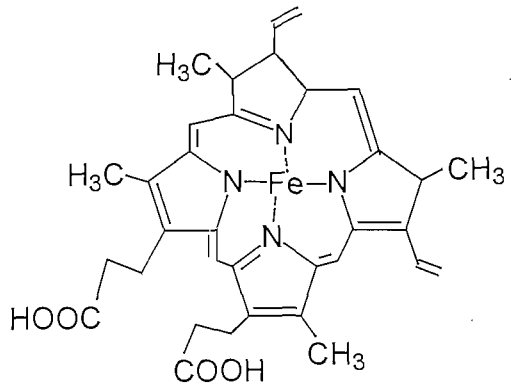


FIGURE 2.2: The structure of ferriprotoporphyrin IX (FP)

(a) The Ferriprotoporphyrin IX-Antimalarial Drug Complex Formation

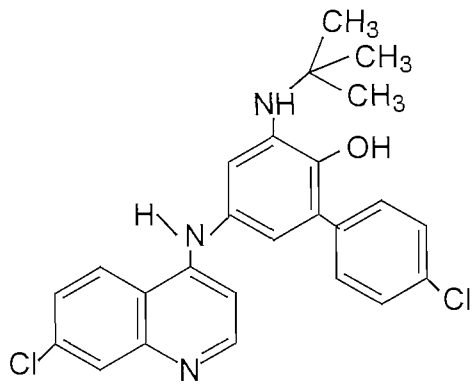
In an aggregated form, ferriprotoporphyrin IX (FP) apparently serves as a receptor for the concentration of antimalarial drugs (Chou *et al.*, 1980; Behere and Goff, 1984). It is for this reason that the parasite's food vacuole is believed to be the locus of activity for antimalarial drugs (Adams *et al.*, 1996). Malaria parasites avoid the accumulation of FP within the parasite's vacuole by converting it to the insoluble inert haem polymer called haemozoin (Olliaro and Goldberg, 1995; Raynes *et al.*, 1996). Free FP is a toxic agent, and parasites that are lacking in haem oxygenase are unable to detoxify the free FP by metabolism but the malaria parasites have evolved an autocatalytic detoxification process in which FP is oxidised to haematin (Hawley *et al.*, 1998).

It is currently, although not universally, accepted that the mode of action of the quinoline-type antimalarials can be accounted for by their ability to disrupt the formation of haemozoin by the parasites (Egan *et al.*, 1994; Dorn, *et al.*, 1995; Warhurst, 1995; Adams *et al.*, 1996), possibly, but not necessarily, by inhibiting the participation of schizontocidal haem polymerase enzyme. The drugs are thought to coordinate to the FP monomers, blocking the formation of haemozoin,

and allowing a significant concentration of haem-antimalarial complex to remain in solution where it exercises a plasmodiotoxic effect by catalysing the formation of active oxygen species (Adams *et al.*, 1996). The release of this "active" oxygen is also connected with the mode of action of artemesinin related drugs (Brossi *et al.*, 1988). Compound **3** acts by diverting FP complexes with soluble parasitic products into a toxic FP-chloroquine complex which then impairs the ability of the parasite and the host red blood cells to maintain cationic gradients, leading to the death of the parasites as a result of these ionic changes or of outright lysis (Verdier *et al.*, 1985). The mode of interaction of these compounds appears to occur by the π-π complexation through interaction of the π-electron cloud of the aromatic ring of the drug with that of the haem molecule (Egan *et al.*, 1996; Marques *et al.*, 1996). It has been proposed that the activities of these compounds are a function of both the ability of the compound to interfere with the polymerisation process and its capacity to accumulate to pharmacological relevant concentrations at the site of drug action (Hawley *et al.*, 1998). The polymerisation of haematin to haemozoin (malaria pigment) is a crucially important chemical reaction in the malaria parasite (Egan *et al.*, 1997). The principal difference between the quinolines which show good antimalarial activity *in vitro* and those that do not is a reflection of their ability to accumulate within the parasite rather than their ability to inhibit the formation of haemozoin (Hawley *et al.*, 1998). This increased concentration of the FP and FP-chloroquine complex causes hemolysis by a colloidal osmotic mechanism (Chou and Fitch, 1981; Orjih *et al.*, 1981; Fitch *et al.*, 1982).

The interaction of the drug with FP appears to be coplanar π–π interaction between the aromatic ring systems of the drug and of the macrocyclic porphyrin (Egan *et al.*, 1998; Marques *et al.*, 1996) and this appears to occur strongly at pH 5,6 within the food vacuole. The essential feature or principal binding interaction for **9** in water appears to be planar π–π stacking, whereas for tebuquine **(17)**, a combination of hydrogen bonding to the side-chain carboxylate of haem and π–π

stacking over the porphyrin ring system constitute the most favourable mode of interaction with large *p*-chlorophenyl function positioned away from the bonding sites (O'Neill *et al.*, 1997). The quinine-uroporphyrin I complex as well as the chloroquine complex share the common feature of being π–π type complexes,



(17)

and the induced-shift pattern of the quinoline carbons being different for each drug, leading to different complex structures (Constantinidis and Satterlee, 1988). On the other hand, quinine on its own interacts at the porphyrin periphery because its quinoline carbons exhibit both upfield and downfield induced shifts. Compound **3** is thought to lie at the porphyrin centre because all of its quinoline carbons exhibit upfield shifts. The most likely reason for this difference could be the steric hindrance. In compound **8**, the quinuclidine part of the molecule is much more rigid than the aliphatic part (carbons 1 - 7) of **3**. The quinine association is cooperative, while the chloroquine association is nonco-operative, with the source of co-operativity being postulated to be due to the 9-OH group coordination to the haem iron in addition to the π–π association. From the results of the proton NMR characterisation (Constantinidis and Satterlee, 1988) it was postulated that conformations of quinine in which the quinuclidine 2-, 6-, and 8-position proton resonances existed would be perturbed due to 9-OH coordination to the haem iron ion. At a pH value of 5,6 the iron (III) protoporphyrin moiety could exist in two forms - the unstable monomeric complex Fe(PP)OH of the synthetic iron (III) porphyrin is considered to proceed successively via oxy stable

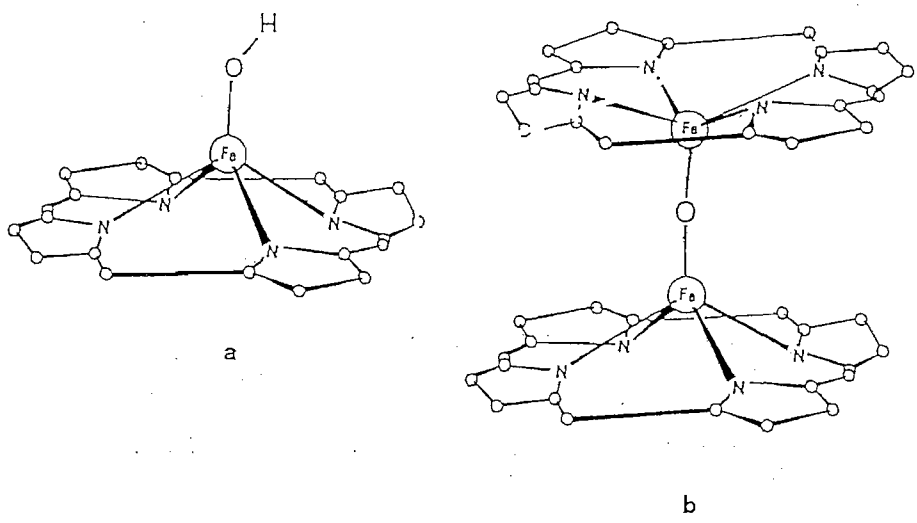


FIGURE 2.3: Molecular structure of FP: (a) Monomeric hydroxyl complex and (b) dimeric μ-oxo complex (Brémard *et al.*, 1993).

bimetallic complex $[Fe(PP)]_2O$ and the autoxidation of the oxyhaemoglobin to the Fe(PP)OH occurring when the haemoprotein releases haem from its binding pocket (Brémard *et al.*, 1993) (Figure 2.3). The autoxidation mechanism of the ferrous, peroxo, and oxoferryl forms and finally to μ-oxo bridged species is initiated and maintained. There is also a suggestion that quinolines act by incorporation of drug-haem complex into the growing polymer of haemozoin and this haem-quinoline complex interaction with haemozoin is non-covalent (Sullivan *et al.*, 1996).

A complex formation also occurs between FP and compound **9**, and the stability of this complex is considered to be due to the combined effect of different non-covalent interactions such as hydrophobic and electrostatic interactions. These interactions involve charged nitrogenous groups on amodiaquine and FP carboxylates as well as possible hydrogen bonding between the aromatic hydroxyl and amino groups and the corresponding hydrogen-bonding groups on

FP (Blauer *et al.*, 1993). There is an indication that the haem-drug complex is apparently stabilised by intermolecular H-bonding of the protonated side chain to one of the carboxylate functions in haem with the quinoline nitrogen away from the central iron atom (O'Neill *et al.*, 1997). The orientation of the amino side chain function in tebuquine is governed by intramolecular H-bonding between one of the protons on the nitrogen with the lone pair of electrons on oxygen. This internal hydrogen bonding in compound **17** allows the side chain to adopt a conformation where the second proton on the charged ammonium atom forms a favourable hydrogen bonding interaction with one of the carboxylates of haem. The coordination of chloroquine to FP is essential for the drug to acquire a lipid peroxidative ability (de Almeida Ribeiro *et al.*, 1995).

(b) The Inhibition of Haemazoin Formation

The conversion of haematin to β-haematin (haemozoin or malaria pigment) is thus a crucially important chemical reaction in the malaria parasite (Adams *et al.*, 1996). It is essential to the survival of the parasite as it detoxifies the haem released during host haemoglobin degradation. However, this conversion is normally blocked by intraerythrocytic antimalarial drugs of the quinoline type (Egan *et al.*, 1994). Both the oxidation state of the FP and pH of the content of the food vacuoles are also important factors in determining the ready formation of haemazoin/β-haematin in the food vacuoles of the parasites (Dorn *et al.*, 1998). Under physiological conditions, the polymerisation of haem is not a spontaneous process, but requires the presence of some parasitic or biological material for the initiation and/or continuous formation of haemazoin (Pandey and Tekwani, 1996). Haematin exists predominantly in its μ-oxo dimer form and this form cannot be incorporated into a growing haemozoin chain as the two central iron atoms are bound together by the bridging oxygen atoms (Dorn *et al.*, 998). If the μ-oxo dimer equilibrium-dependent haematin monomer concentration is a key factor in the initiation of haematin polymerisation, then there is a possibility that lipids catalyse the initiation process by shifting the equilibrium to the monomeric form . The bisquinolines inhibit haematin polymerisation by binding to haematin μ-oxo

dimer, shifting the equilibrium between haematin monomer and haematin-μ-oxo dimer, thereby decreasing haematin monomer incorporation into haemozoin (Vennerstrom *et al.*, 1998). A similar hypothesis seems to suggest that the binding of **3** to haematin produces a forward shift in the equilibrium between the haematin monomer and haematin μ-oxo dimer, thereby decreasing the concentration of haematin monomer available for incorporation into haemazoin, a molecular mechanism, to account for chloroquine mediated inhibition of haematin polymerisation (Vippagunta *et* al., 1999). However, while earlier findings suggested that polymerisation of haem to form haemozoin/β-haematin is a spontaneous process (Egan *et al.*,1994), it subsequently emerged that this process requires the presence of some parasitic or biological material for the formation of haemozoin (Pandey and Tekwani, 1996). As the process of conversion of FP to an insoluble form of haematin apparently determines the concentration of FP available to bind to **3**, it may also determine whether the parasite is susceptible or resistant to **3** (Fitch and Kanjananggulpan, 1987). Evidence sourced from kinetic experiments indicates the conversion of haematin to β-haematin is an apparent zero-order process which is not autocatalytic (Adams *et al.*, 1996).

The strong correlation observed between the ability of quinoline blood schizonticides to inhibit haematin polymerisation, and to inhibit malarial parasite growth in culture, supports the concept that these compounds exert their antimalarial activity through the inhibition of haematin polymerisation (Dorn *et al.*, 1998). However, other processes may include the inhibition of both glutathione- and glutathione/hydrogen peroxidase-mediated iron release from haematin, inhibition of haematin-dependent lipid peroxidation or that of polyamine transport. It is generally thought that these types of inhibitions require that the drugs concentrate in the parasite food vacuole to the levels greater than micromolar levels normally expected to inhibit haematin polymerisation *in vitro* (Ridley *et al.*, 1997). Majority of quinoline blood schizonticides are known to inhibit haematin polymerisation (Dorn *et al.*, 1998). The bisquinolines are also believed to inhibit

haematin polymerisation by binding to haematin μ-oxo dimer, shifting the equilibrium between haematin monomer and haematin μ-oxo dimer (see Fig. 2.3), thereby decreasing the concentration of haematin monomer available for incorporation into haemozoin (Vennerstrom *et al.*, 1998). This may have other consequences that may or may not contribute to the antimalarial properties of the drugs. It is also feasible that the binding of the quinoline antimalarials stabilises the μ-oxo dimer relative to the monomer, shifting the dimerisation equilibrium to the right, and reducing the amount of haematin monomer available for incorporation into the growing haemazoin (Dorn *et al.*, 1998). This is part of the molecular mechanism to account for chloroquine-mediated inhibition of haematin polymerisation (Vippagunta *et al.*, 1999). Molecular modelling data suggest that enthalpy-driven chloroquine-haematin μ-oxo dimer binding is derived largely from energetically favourable π–π molecular recognition interaction between quinoline heterocycle of chloroquine and the metalloporphyrin ring of haematin μ-oxo dimer (Vippagunta *et al.*, 1999), with the hydrophobicity of the aromatic ring of the drug playing a predominant role (Egan *et al.*, 1997). The bonding of 4-aminoquinolines (**3** and **9**) to ferriprotoporphyrin IX is predominantly entropically driven, while bonding of the amino alcohols (**1**, **8**, and **8.5**) is enthalpically driven (Egan *et al.*, 1997). For most drugs the drug-haemain μ-oxo dimer binding is enthalpy-driven, a phenomenon attributed largely to the π–π interactions between the drugs and haematin, while for compounds **1** and **2** the μ-oxo binding is also entropy driven (Dorn *et al.*, 1998). Enthalpy-entropy compensations are a consequence of weak associations where the bonding enthalpy is much lower than typical covalent strengths (Searle *et al.*, 1995).

2.2.2 THE BINDING TO AND INTERCALATION OF DRUGS WITH DNA

The pre-1980 conventions on the mode of action of the quinoline-type drugs were based on the formation of molecular complexes between the drug and the native, double-stranded DNA and thus block the enzymatic syntheses of DNA and RNA (Hahn *et al.*, 1966). Then it was believed that compound **3** formed a

complex with DNA and all non-primary diamines and in the process stabilising the DNA helix by ionic interaction with the phosphoric acid groups (Allison *et al.*, 1965). This compound and its analogues were according to these theories acting by intercalating with and stabilising the DNA double helix (Hahn *et al*, 1966). The stabilisation of the DNA double helix is achieved through the formation of the drug-DNA complex such that the molecule of the drug spans the minor grooves between the complementary strands of the DNA double helix (Hélène, 1998), thus blocking the enzymatic synthesis of the DNA and RNA as well as the biosynthesis of these nucleic acids in susceptible cells (Allison et al., 1965). The process was thought to occur through the formation of a complex between the drug and DNA through intercalation of the aromatic ring system and the interaction of the positively charged side-chain with negatively charged DNA phosphate groups (Panter *et al.*, 1973). As a final stage in its mode of action, **3** was thought to intercalate with nucleic acids of the parasites and in so doing, prevent the normal replication of the plasmodial nucleic acids (O'Brien and Hahn, 1965; Peters, 1973). This view is also supported by the fact that the antitumour agents currently in use are thought to exert their cytotoxic activity by interfering with DNA metabolism (Baguley, 1982). The antimetabolites and certain other related compounds inhibit the DNA replication either directly or indirectly, while the alkylating agents react chemically with the DNA. The DNA double helix can bind different types of ligands and these ligands are classified as intercalators because they insert their aromatic rings between the two adjacent base pairs and rove binders which bind with DNA within either groove of the double helix (Héléne, 1998). The DNA intercalating agents are thought to inhibit cell growth by either poisoning the topoisomerases or inhibiting transcription (Watcelin *et al.*, 2003). Thus it will be helpful to design molecules that can recognise specific sequence on the DNA double helix that will provide new tools to control gene expression and a rational basis for fresh approach to the development of new drugs.

2.3 THE DEVELOPMENT OF RESISTANCE TO ANTIMALARIAL DRUGS

When the dose of a drug that is required to control an infection reaches or exceeds that which is fully tolerated by the host, one may justifiably say that the pathogens concerned are "drug resistant". The basic response of the organism to a drug may vary considerably from one geographical area to another (Peters, 1982). Resistance of human plasmodia to modern synthetic antimalarials was observed soon after these drugs had become widely used. Resistance of *P. falciparum* to the 4-aminoquinolines was reported from Asia and South America in the early 1960's, but extensive and regular use of residual insecticides kept the chloroquine-resistant strains to a few small isolated foci (Wernsdorder and Kouznetsov, 1980). A drastic reduction of these operations because of financial and technical difficulties resulted in the spread of malaria. In addition, the emergence of multidrug-resistant strains of *Plasmodia* has created a near-desperate situation, where the need for new inexpensive antimalarials to circumvent the parasite's resistance mechanism has become vital (Raynes *et al.*, 1999) as chloroquine resistance spreads from East Asia through Africa to Argentina (see Figure 2.4). By 1982 in Thailand, patients with *P. falciparum* malaria showed decreased sensitivity *in vitro* to both mefloquine and quinine, and *in vivo*, it showed an RII pattern of drug resistance in mefloquine (Boudreau *et al.*, 1982). Some of the factors that are considered more likely to play an important role in the emergence and propagation of drug resistance are (1) mass drug administration,(2) inadequate treatment, (3) intensity of transmission, (4) population mobility and(5) relative immunity (Wernsdorfer and Payne, 1991).

Failure of a malaria infection to respond to treatment results either from intrinsic resistance of the parasite to the drug treatment, or host factors (pharmacokinetics, compliance) which results in insufficient concentration of the drug in the blood (White, 1992). The design and subsequent synthesis of the new antimalarials are hindered by the fact that the mechanism of resistance is not fully understood (Raynes *et al.*, 1999).

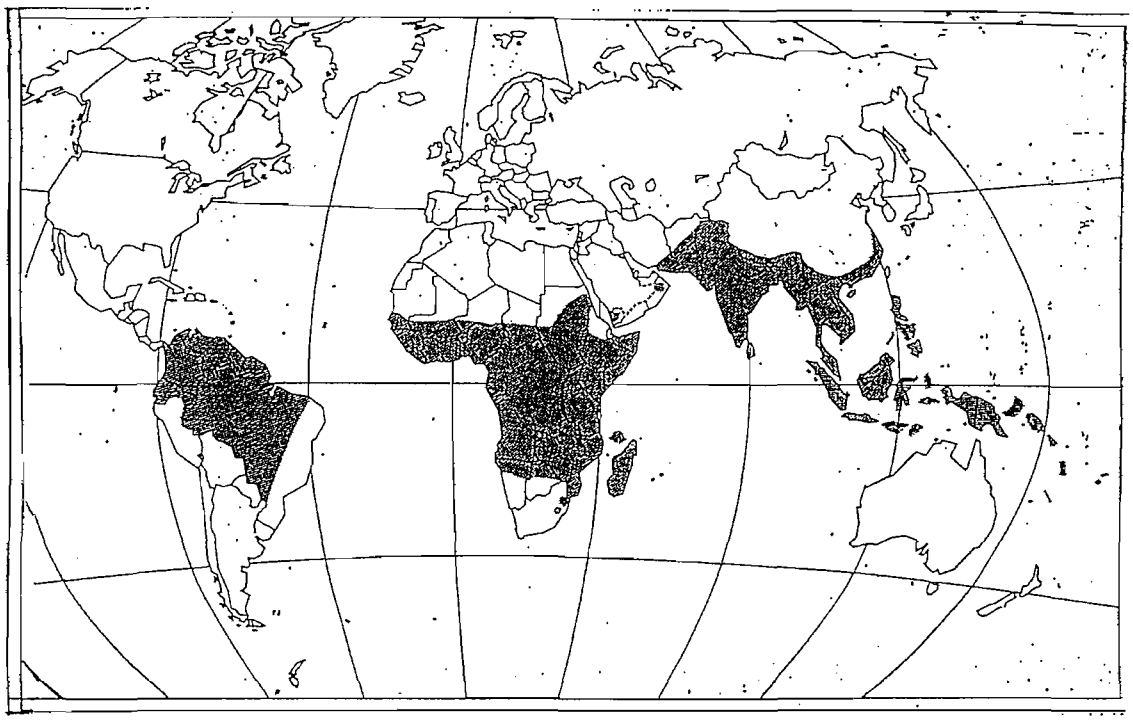


FIGURE 2.4: The extent of the spread of chloroquine resistance by 1989 (Wernsdorfer and Payne, 1991)

2.3.1 Alteration in Drug Accumulation in the Food Vacuoles

Although the molecular basis for resistance has not been adequately established, it appears to involve a decreased accumulation of the drug by the chloroquine-resistant parasites (Geary *et al.*, 1986). Chloroquine resistance is associated with reduced concentration of the drug in the acid food vacuole of the parasite (Krogstad *et al.*, 1987), a process which is not associated with reduced uptake, but with increased efflux from the cell (White, 1992). The *P. falciparum* digestive food vacuole accumulates **3** in an ATP-dependent manner and appears to play a crucial role in chloroquine resistance (Saliba *et al.*, 1998). Resistant parasites have a 40- to 50-fold more rapid release of **3** than susceptible parasites (Krogstad *et al.*, 1987). These strains are distinguished from susceptible ones by their ability to rapidly excrete chloroquine (Gluzman *et al.*, 1991). This phenomenon could apparently be due to an alteration of the plasma membrane,

with the observation of double minute chromosomes or homogeneously staining regions in some of the multidrug-resistant cell lines suggesting that gene amplification could underly this phenomenon (Roninson *et al.*, 1984).

2.3.2 The Energy-dependent reduced Binding of Drugs

The alkaloid resistance in leukemic cells are thought to be due to primarily energy-dependent reduced binding as the energy is required to keep the receptor proteins in an "off" state and removal of the source of energy may alter the structure of the receptor in some manner (Beck *et al.*, 1983). Inhibitors of energy metabolism augment the rate of net accumulation of the drug in both mutant and wild-type cells (Riordan and Ling, 1985).

2.3.3 The Differences in Phospholipid Composition

Previous findings (Wood and Eaton, 1993) indicate that chloroquine-resistant *P. berghei* accumulate significantly less parasite-associated haem, which is not accompanied by substantial differences in proteolytic activities or decrease in host haemoglobin compared with the chloroquine-sensitive infection. Different parasite strains have different phospholipid composition, suggesting that enzymes involved in phospholipid synthesis may be expressed to different levels in diverse lines, or that their regulation may be different, or that the transport of substrates/products of phospholipid metabolism is strain-dependent (Shalmiev and Ginsburg, 1993). There is also the possibility that mutations of genes, such as those controlling membrane phospholipid composition, can modulate the activity of the putative parasite efflux pump, allowing it to work more efficiently in resistant isolate. This occurs with a possible involvement of a P-glycoprotein-type transporter (Bray *et al.*, 1996).

2.3.4 The Role of P-glycoprotein

An alternative proposal is that chloroquine resistant parasites have mutated their mdr gene such that the P-glycoprotein present becomes more efficient at pumping out **3** (Foote *et al.*, 1990). This P-glycoprotein localised in the digestive

vacuole (Cowman *et al.*, 1991) is thought to transport cytotoxic drugs out of the cell, and thereby prevent their accumulation in toxic concentrations (Slater, 1993). The findings which are consistent with a model in which the mdr-P-glycoprotein acts to pump drug out of the cell in an energy dependent manner, is that, when multidrug-resistant cell lines are grown in the presence of metabolic inhibitors that deplete intracellular ATP, the drug-resistant cells accumulate the cytotoxic agents to levels approaching the parent drug-sensitive cell lines, but when the inhibitors of ATP production are removed, there is a rapid efflux of cytotoxic agents out of the cell (Croop *et al.*, 1988). Genetic evidence has implicated mdr1 as the gene responsible for the multidrug-resistant phenotype (Chen *et al.,*1986).

The analysis of P-glycoprotein sequence together with the data on increased drug efflux in multidrug-resistant cells, and the sensitivity of the efflux to inhibitors of energy metabolism, has provided a strong argument that the active drug efflux is the main mechanism of multidrug resistance. The P-glycoprotein, a 170 kDa protein, is an ATP-dependent cell membrane efflux pump that actively transport a wide range of structurally diverse compounds, most of which are hydrophobic, lipid-soluble, organic cations of natural origin (Kamath and Morris, 1998). This protein is 1280 amino acids long and consists of two homologous parts of approximately equal length, and each half includes a hydrophobic region with six predicted transmembrane segments and a hydrophilic region. The hydrophilic regions share homology with peripheral membrane components of bacterial active transport systems and include potential nucleotide-binding sites (Chen *et al.*, 1986). The protein can also facilitate the secretion of drugs to the intestinal lumen from the enterocytes (Yasuhara *et al.*, 1998). However, other findings (Wellems *et al.*,1990) seem to indicate that the genetic locus governing chloroquine efflux and resistance is independent of the known mdr-like genes. It was also concluded that a mutated pfmdr1 gene is one of at least mutated genes requires for chloroquine resistance (Foote *et al.,* 1990). The pfmdr1 gene encodes a 160 000 D protein that is localised at the surface of the digestive

vacuole of the trophozoites, and the stage specificity and subcellular localisation of this P-glycoprotein supports the contention that it is involved in efflux of chloroquine from the digestive vacuole (Cowman *et al.*, 1991). Unlike the MDR phenotype in mammalian tumor cells, there is no direct correlation between the overexpression of the P-glycoprotein and the level of chloroquine resistance. A transporter molecule such as this, present on the surface of the digestive vacuole, would be able to remove chloroquine from its site of action. Efflux proteins are integral plasma membrane proteins that are encoded by at least nine genes distributed among at least three subfamilies, and each protein has the capacity to actively extrude from the cell a large variety of drugs with different pharmacological and physiological properties (Taipalensuu *et al.*, 2004).

2.3.5 Other Matters for Consideration in Malaria Chemotherapy

The chloroquine resistant line of *P. berghei* differs from the chloroquine-sensitive strains in utilising the citric acid cycle due to the lack of the succinate dehydrogenase (SDH) activity (Howells *et al.*, 1970). In recent years the chloroquine resistance locus was mapped to a 400 kilobase region of chromosome 7 in *P. falciparum* (Wellem *et al.,*1990) and it has been suggested that the information from these findings can be used to provide for the eventual identification of genetic change that is responsible for chloroquine resistance in this parasite (Slater, 1993). Using thin layer chromatography, it was found that the ^{3}H-chloroquine accumulated in the resistant *P. falciparum* parasites was indistinguishable from the parent compound, indicating that drug resistance does not involve chemical transformation (Gluzman *et al.,*1987). The resistance mechanism is much less effective for compounds related to **9** than for **3** itself, a factor ascribed to the greater lipophilicity of the former compounds (Bray *et al.*, 1996).

CHAPTER 3

STRUCTURES OF COMPOUNDS IN RELATION TO DRUG DESIGN

3.1 INTRODUCTION

Antimalarial activity has been reported for many derivatives of compounds **3** and **8** together with those of the 4-quinolinemethanols and 4- and 8-aminoquinolines (§ 2.1), but very little information has been collated regarding the structure-activity relationships of these agents. It is well known that compound **3** with a chlorine atom at position 7 and amino alkyl substituent at position 4 is very effective against the chloroquine-sensitive strain of *P. falciparum*, but not against the chloroquine-resistant strains, while compound **3** with trifluoromethyl groups at positions 2 and 8 show the opposite effects. However, the design of more effective antimalarials has of necessity been largely based on empiricism, and any biological information that might suggest a more rational approach has been worth exploring (Rapport *et al.*, 1946). Mono- or disubstitutions at the 6-, 7-, or 8-position of the quinoline nucleus enhances activity relative to the unsubstituted compound, while substitution at the 2-position appears necessary to retard metabolic oxidation of the quinoline skeleton (Novotny *et al.*, 1974).

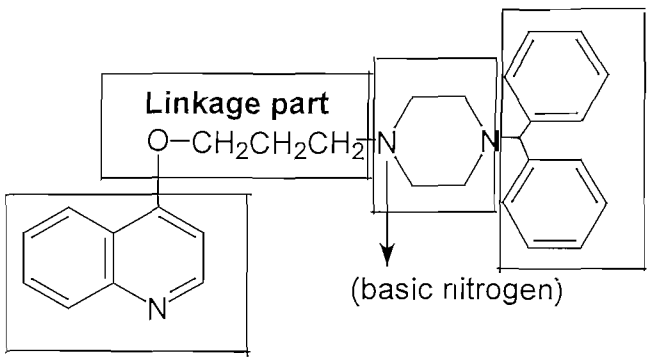


FIGURE 3.1: Important structural features of the quinoline derivatives

3.2 PHYSICOCHEMICAL PROPERTIES AND PHARMACOLOGICAL ACTION

The investigation of the structure-activity relationship of compounds with the structure represented in Figure 3.1 indicated that compounds of this structure enhanced the accumulation of [^{3}H]vincristine in K562/ADM cells and reversed tumor cell MDR (Suzuki *et al.*, 1997). The basic nitrogen atom is thought to be responsible for the accumulation of the compounds in the food vacuoles. It was found that the MDR reversing activity of these compounds was about 3 - 4 times higher than that of verapamil *in vitro* and the structure-activity relationship study was based on (i) a quinoline ring, (ii) a linkage moiety (2-propanol), (iii) piperazine ring, and (iv) a hydrophobic moiety (Figure 3.2). Replacement of the quinoline ring by either a naphthyl or a phenyl ring resulted in the loss of activity. The activity of the compounds is also affected by the distance between the hydrophobic moiety and the basic nitrogen, Nb of the piperazine group, and must normally be 5 Å. The atom coordinating with haemin iron is the side chain quinuclidine or piperidine nitrogen, with the aromatic nitrogen atom of the quinoline ring playing a relatively unimportant role in complexation reaction since the phenanthrene methanols with extra aromatic ring form haemichrome without its help. However, there is a possibility that a hydrogen bond is formed between this group and one of the carboxyl groups of haemin (Warhurst, 1981). There is also a suggestion that the amino groups in the side chains of quinolines play an important role through interaction with the propionate side chain of FP that explains the importance of substitution at the 4-position (O'Neill *et al.,* 1997). This may also explain the observed decrease in the association constant of **3** with FP at lower pH which could result from at least partial protonation of the propionate groups at lower pH levels, decreasing the electrostatic attraction between the amino and propionate groups (Egan *et al.*, 1997).

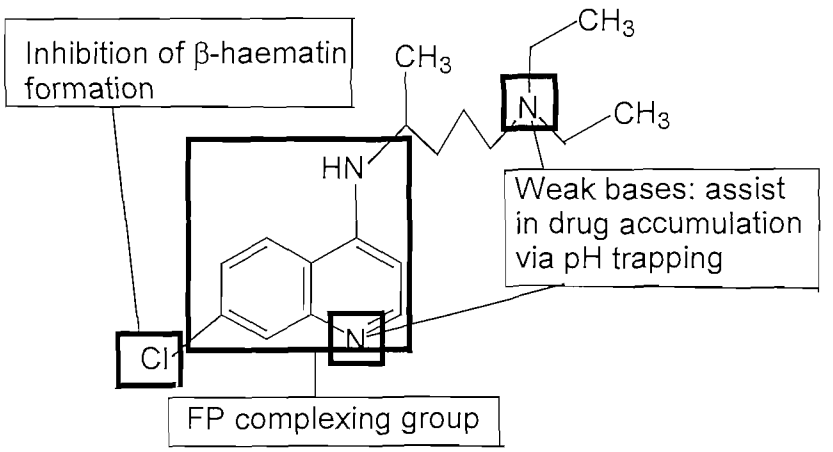


FIGURE 3.2 : Proposed structure-activity relationships in chloroquine based on the work of Egan *et al.* (1997; 2000).

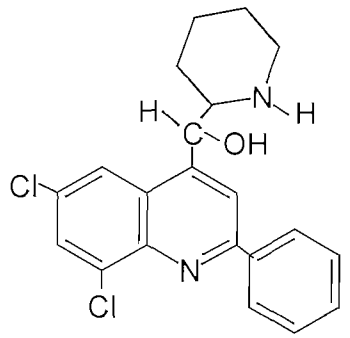
3.2.1 The Nature of the Substituents on the Quinoline Ring

A quinoline-containing compound exhibiting antimalarial activity should possess the following characteristics: (a) must be able to form relatively strong complex with FP, (b) must be able to inhibit β-haematin formation and (c) must contain a basic side chain to assist accumulation at the site of action in the food vacuole as shown in Fig. 3.2 (Egan *et al.*, 2000). Accordingly, the 4-quinoline nucleus alone provides a FP complexing template, which is however, not sufficient for initiation of haemazoin formation. Chemical modification of the positions and nature of the substituents on the quinoline nucleus of chloroquine tends to have a more pronounced impact on biological activity than do modifications of the 4-diethylamino-1-methylbutylamino side chain (Slater, 1993). The antimalarial activity of this class of compounds is greatly influenced by combination of substituents found at C-5, C-7, C-8 and N-1 (Hagen *et al.*, 1990). Thus the 6- and 7-chloro isomers of chloroquine are equally active against *P. gallinaceum* in the chicken, whereas 5- and 8-chloro forms are both about 30-fold less active. The 7-chloro, 7-bromo-, fluoro and trifluoromethyl substituted compounds possess equivalent activity, while the 7-iodo substituted molecule is less active (Thompson and Werbel, 1972). 7-Iodo- and 7-bromo substituted amodiaquines

generally have excellent antiplasmodial activity against *P. falciparum*, while the 7-fluoro amodiaquines are less active than 7-chloro-, 7-iodo or 7-bromo amodiaquines, and most of the 7-methoxy amodiaquines are inactive against both chloroquine-susceptible and -resistant strains of *P. falciparum*, indicating that antiplasmodial activity of the 7-substituted amodiaquines decrease progressively as follows: Cl < I < Br = F = CF_3 < OMe (De *et al.*, 1998). Possession of a halogen such as fluorine or chlorine at position 8 of the quinoline ring system has lead to compounds with high incidences of phototoxicity (Tillotson, 1996).

Normally mono- or disubstitution at the 6-, 7- or 8- position of quinoline nucleus enhances activity relative to the unsubstituted compounds, while substitution at the 2-position appears necessary to retard metabolic oxidation of the quinoline skeleton to the carbostyryl derivatives (Novotny *et al.*, 1974). The introduction of a phenyl group at 2-position was an attempt to increase antimalarial activity (Purcell and Sundaram, 1969). However, a larger number of 2-arylquinoline amino alcohols have proved to be active and curative against *P. berghei* in mice, but most are highly phototoxic, a phenomenon that can be ascribed to the enhanced conjugation by the coplanar aryl group (Patel *et al.*, 1971). Of the 81 related quinoline methanol compounds tested against the Swiss Webster albino mice and Sabouraus agar streaked with *Candida albicans*, 62 were found to be phototoxic (Ison and Davis, 1969). Compounds with a phenyl ring at position 2 should be viewed as a tricyclic ring system with planar features (Rothe and Jacobus, 1968). Such systems have potential photosensitising ability, a phenomenon believed to arise from increased resonance conjugation resulting from the presence of the 2-phenyl group. The phototoxicity of 6,8-dichloro-2-phenyl-α-piperidyl-4-quinoline-methanol (**18**) has produced severe complications in man, and there is a suggestion that in animals the phototoxicity of such compounds is directly related to their antimalarial potencies (Yardley *et al.*, 1971). In the manipulation of the structure of quinine (Brosssi, 1976), replacement of the methoxy group by a hydroxy group or other ether groups had

little effect on the antimalarial activity, while the elimination of the hydroxyl group at C-9 resulted in complete loss of activity. The C-8/N-1 bond seems to be essential for activity.



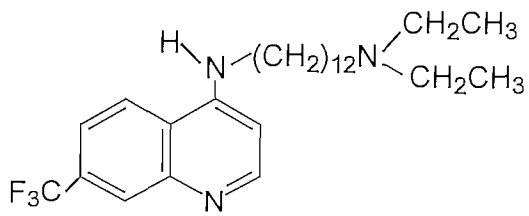
(18)

Recently, a great deal of attention was focussed on the synthesis and evaluation of the activity of the bisquinolines such as the N,N-*bis*(7-chloroquinolin-4-yl) alkane diamines which have shown to possess superior *in vitro* and *in vivo* antimalarial activity, with the (±) *trans*-N^1,N^2-*bis*(7-chloroquinolin-4-yl)cyclohexane1,2-diamine **(16)** possessing a high *in vitro* activity against the chloroquine susceptible and some moderately chloroquine-resistant clones of *P. falciparum* (Basco *et al.*, 1994). Its two enantiomers, the *S,S* and *R,R* show higher levels of inhibition of haem polymerisation than both chloroquine and mefloquine *in vitro* (Ridley *et al.*, 1997). Although the *S,S* enantiomer of **16** is highly active against both the chloroquine-sensitive and choloroquine-resistant *P. falciparum* and other major human malaria parasite, *P. vivax* (with IC_{50} of 10 μM compared with values for chloroquine with 80 μM and mefloquine of 200 μM) when orally administered, and are fast acting with a long lasting effects, toxicological liabilities, particularly phototoxicity and the danger of attendant photocarcinogenicity ruled it out for further development (Ridley *et al.*, 1997). Phototoxicity has precluded further development of the *S,S* enantiomer (Vennerstrom *et al.*, 1998). The phenomenon of phototoxicity manifests itself as severe exfoliation of the skin upon exposure to sunlight (Rosowsky *et al.*, 1971).

Implicated in this process are drugs with significant light absorption in the 280 - 800 nm region (Moore, 1977).

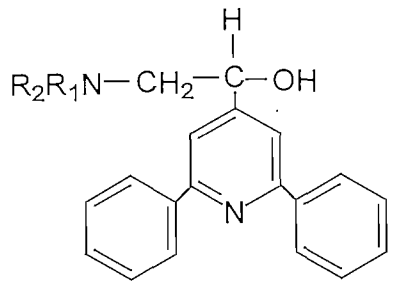
Since its discovery, the quinolinemethanol, mefloquine **(1)**, which is very active against the chloroquine-resistant strain of *P. falciparum* has become the first-choice drug for antimalarial prophylaxis (Palmer *et al.*, 1993). The trifluoromethyl groups attached to the quinoline ring appear to cause **1** to have greater access than chloroquine to identical receptors (Fitch *et al.*, 1979), thus interacting strongly both with ferriprotoporphyrin IX and certain phospholipids (Chevli and Fitch, 1982). The fluorine atom has distinct physical properties such as high ionisation potential, high electronegativity, small size and tightly held non-bonding electron pairs in comparison with chlorine (Smart, 1995), and fluorocarbons often have different physicochemical properties from other halocarbons. Thus, fluorine substitution may alter biological properties of drugs in which they appear (De *et al.*, 1998). The influence of the fluorine atom being a sterically demanding substituent with small van der Waal's radius (1,35 Å) which closely resembles that of hydrogen (1,20Å). Once introduced, the carbon-fluorine bond energy renders the compounds resistant to metabolic transformation (Welch, 1987). As a result of its high electronegativity (4 vs 3,5 for oxygen) it can have pronounced effects on the electron distribution in the molecule, affecting the basicity, or acidity of the neighbouring groups, dipole moments within the molecule and overall reactivity or stability of neighbouring functional groups. However, 7-fluoroamodiaquines are less active than the 7-chloro, 7-iodo or 7-bromo amodiaquines. This is evident from the fact all the 7-fluoroamodiaquines with short diaminoalkane side chains are less active against both the chloroquine-susceptible and chloroquine-resistant *P. falciparum* (De *et al.*, 1998). When the substituent in position 7 of the quinoline ring is either decreased in relative electronegativity or increased in volume, the antimalarial activity is decreased. These structural changes are likely to decrease the affinity for the 2-amino group of guanine in DNA or to provide steric hindrance to intercalation (Hahn *et al.*, 1966; Bass *et al.*, 1971).

Substitution of a $-CF_3$ for a phenyl group at position 2 of the quinoline ring produced only slightly active antimalarials that are moderately phototoxic (Patel *et al.*, 1971), while this group at position 8 resulted in highly active compounds (Ohnmacht *et al.*, 1971; Strube, 1975). Although the $-CF_3$ group is more hydrophobic than the fluorine atom (Smart, 1995), the 7-trifluoromethylaminoquinolines are less active against both the chloroquine-susceptible and chloroquine-resistant *P. falciparum* (De *et al.*, 1998), with an exception of a 7-trifluoromethylamodiaquine **(19),** which is active against the chloroquine-susceptible parasite with IC_{50} of 5 nM and moderately active against the chloroquine-resistant parasites with IC_{50} of 25 nM (Haiti 135 and Indochina strains). Evaluation of the antimalarial activity of the 2,6-*bis*(aryl)-4-pyridinemethanol **(20)** shows that substitution of one $-CF_3$ group with Br results in only slight loss of activity, but a greater loss of activity results if one of the $-CF_3$ is replaced by -Cl and two -Cl groups on one ring results (Markovac *et al.*, 1972).



(19)

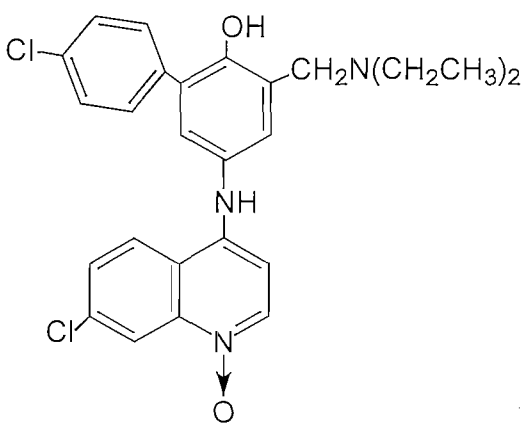
The $-CF_3$ and -Cl groups being electron withdrawing when substituted on aromatic rings should act as acceptors in the formation of charge transfer complexes with DNA and contribute to antimalarial action (Cheng, 1971). The majority of the trifluoromethyl-substituted quinoline derives reviewed by the WRAI contained the trfluoromethyl group at position 2 and 8 (see Table 2.4), with only a few with this group at position 2 and chlorine atoms at C-6 and C-8 of the quinoline. Only one compound contained a trifluoromethyl group at position 7.



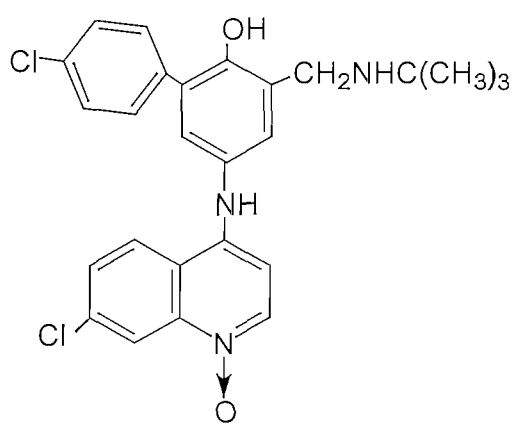
(20)

The oxidation of the quinoline nitrogen of amodiaquine increased the potency some 3 - 4 times against *P. berghei* infection in mice (Werbel *et al.*, 1986). Although amodiaquines with -OCH_3 group at position 6 such as **8** and **11** are active against *P. vivax* as this group is more hydrophobic than chlorine (Hansch and Leo, 1979), most of the 7-methoxy amodiaquine derivatives are inactive against both the chloroquine-susceptible and -resistant *P. falciparum*. For 4-aminoquinolines related to chloroquine, available data suggest that electron-withdrawing functional groups at the 7-position of the quinoline ring are required for activity against both haematin polymerisation and parasite growth and chlorine substitution at position 7 is optimal (Vippagunta *et al.*, 1999). The 7-chloro group introduced on the quinoline ring is responsible for inhibition of haemazoin formation, but probably has little influence on the strength of association with FP (Egan *et al.*, 2000). Thus neither lipophilicity nor hydrophilicity at the 7-position is a significant factor in antiplasmodial activity and electronegativity at the 7-position does not correlate with antiplasmodial activity (De *et al.*, 1998). The hydrochloride salt of (γ-(dibutylamino)-1,3-dichloro-6-(trifluoromethyl)-9-phenanthrenepropanol **(2)** is one of the most effective antimalarial compounds containing a -CF_3 group which compares favourably with **1** as both drugs are effective against the multidrug-resistant *P. falciparum*, including strains that are highly resistant to chloroquine (Cosgriff *et al.*, 1982; Boudreau *et al.*, 1988). However, inspite of the excessive control placed on its administration, mefloquine resistance is emerging at an alarming rate (Nosten *et al.*, 1991). Whereas compound **9** is more than 400-fold cross-resistant with **3**

and **11** is some 720-fold cross resistant with **3** at its SD_{70} value, the derivative of **9** where the oxidation of the quinoline nitrogen has been effected does not exhibit any cross-resistance with **3** or indeed with cycloquanil **(11)** or pyrimethamine, although some cross-resistance with **1** and **8** is observed (Werbel *et al.*, 1986). Similarly, the *tert*- and *des*-N-oxide analogues (5-[(7-chloro-4-quinolinyl)amino]-3-[(alkylamino)methyl]- [1,1'-biphenyl]-2-ols and N^{ω} -oxides) [**(21)** and **(22)**] are cross-resistant with chloroquine and quinine. Both of these compounds are at least 25 times more active as curative agents than the positive control. However, quantitative structure-activity relationship (QSAR) studies of these compounds reveal that in general there appears to be a tendency for the N-oxidation of the quinoline nitrogen to reduce potency.



(21)



(22)

3.2.2 The characteristics of the side chain at the 4-position

The aminoalkyl side chain is a requirement for strong antiplasmodial activity, by probably assisting in drug accumulation in the food vacuole (Egan *et al*, 2000). It also appears to enhance the strength of association with FP in some cases, but this effect does not appear to be essential for its activity. In terms of the older DNA intercalation theory, the diamino side chain attached to the quinoline ring at position 4 bridges the two DNA strands by electrostatic interactions between the diamino nitrogen atoms and the DNA phosphate groups (Bass *et al.*, 1971).

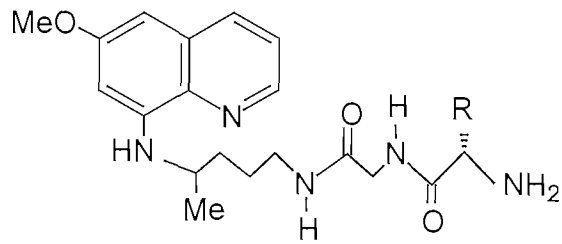
Disubstituted 4-diethylamino-1-methylbutylaminoquinolines tend to exhibit lower activity than chloroquine (Slater, 1993). For aminoquinolines, biological activity is more robust to changes in the basic 4-amino side chain, with activity being approximately equally maintained with between 2 and 5 carbons in the chain. This, however, decreases if this is shortened or lengthened, while branching within the side-chain is also tolerated (Thompson and Werbel, 1972). The tertiary amine-containing compounds in the basic side chain generally give more effective antimalarial compounds. However, toxicity also appeared to increase with greater chain length. Replacement of piperidine moiety with an alkyl chain results in a loss of activity. This can be explained by the weak base theory of drug accumulation, and since the weak base properties of the parent compound resides solely with the piperidine nitrogen atom (pKa = 8,6), then the alkyl derivatives cannot concentrate in the parasite (Slater, 1993). The chloroquine alkyl side chain, especially the aliphatic tertiary nitrogen atom is an important structural determinant in chloroquine drug resistance (Vippagunta *et al.*, 1999). The number of carbon atoms between the two nitrogen atoms in the diaminoalkane side-chain is a major determinant of activity against chloroquine-resistant *P. falciparum* (De *et al.*, 1998). Branching in the side chain may be even more drastic without loss of antimalarial activity while the introduction of sulphur into the side chain permits retention of substantial activity (Thompson and Werbel, 1972).

During the modification of the diaminoalkane side chain of amodiaquines, De *et al.* (1996) noticed that those compounds with side chain containing 2 - 12 carbons were active against the chloroquine-susceptible *P. falciparum* and some against the chloroquine-resistant strain. In all the cases, the terminal diethylamino group was retained in each compound because it is a principal determinant of amodiaquine metabolism by the cytochrome P-450 enzyme of the liver (Jewell *et al.*, 1995). Accordingly, the side-chain length is a critical determinant of amodiaquine action against chloroquine-, mefloquine- and multiple-resistant *P. falciparum* and the efflux molecule responsible for

amodiaquine excretion should have two negative charges spaced approximately 4 - 6 carbons apart (De *et al.*, 1996). However, amopyroquine **(10)**, a structural analogue of **9** where the diethylamino side chain is replaced with a pyrrolidine group is more active than both **3** and **9** against the chloroquine-resistant strain of *P. falciparum* isolates (Raynes *et al.*, 1999). It would appear that sufficiently large changes in diaminoalkyl side chain are responsible for overcoming chloroquine-resistance without having to change to the 4-amino-7-haloquinoline template responsible for FP complexation and inhibition of β-haematin formation (Egan *et al.*,2000). For this reason that changes in the length of the aminoalkyl side chain have little influence on activity against chloroquine-sensitive strains of *P. falciparum* but profound influence on activity against the chloroquine-resistant strains of the parasite. There is also a speculation that the quinoline N-1 atom and the heteroatom (N or O) of the heteroaryl group in most of the active 2-heteroarylquinolines are favourably positioned to form specific hydrogen-bonded complexes with a biological receptor (Strekowsky *et al.*, 1991). A strong conjugation effect of the 4-amino group with the quinoline would increase the stability of such complex by increasing electron density at the quinoline nitrogen atom. It was suggested by these workers that the electron density distribution in the aromatic system of the quinolineamines greatly affect the anti-HIV-1 activity. The more active molecules are those that are electron rich in the quinoline portion of the molecule resulting from stronger resonance effect. This conjugation is noticeable in quinoline ring systems with alkylamino groups at C4.

By contrast, the more sterically demanding dialkylamino group cannot attain a conformation favourable for an efficient overlap of the lone pair of the amino nitrogen with the aromatic π-system of the quinoline. Such sterically hindered derivatives are less electron-rich in the quinoline part of the molecule and show lower anti-HIV-1 activity (Sterkowski *et al.*, 1991). A correlation between the size and shape of the 5'-alkyl substituent of the 4'-hydroxyanilino side chain and the efficacy of these drugs has shown that introduction of large nonpolar substituents such as a N-*tert* butyl or cyclohexyl group substantially decreases antimalarial

activity, while alkyl groups which contained a 3-carbon backbone with no greater than one branch give optimum antimalarial activity (Raynes *et al.*, 1999). In addition, close analogues of chloroquine with a shortened 4-aminoquinoline side chain retain and exhibit better activity against the chloroquine-resistant strains of *P. falciparum*, suggesting that whatever the mechanism(s) is contributing to chloroquine resistance, it is extremely structure specific (Ridley *et al.*, 1996). In an attempt to synthesise new dipeptide derivatives of primaquine that are less able to be transformed into inactive carboxypromaquine, but which can maintain gametocytocidal activity, a number of derivatives of a compound **23** were evaluated and were found to prevent the development of the sporogonic cycle of *P. berghei* at dose levels of 15 and 7,5 mg/kg (Portela *et al.*, 1999). Significantly, the gametocytocidal activity of these derivatives is not related to their rate of peptidase-catalysed hydrolysis to **12**.



(23)

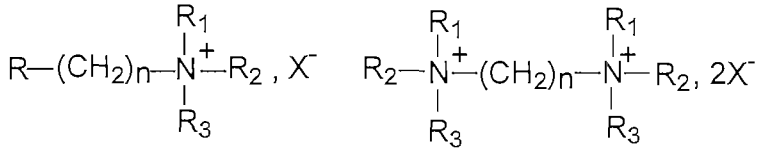
Reasonable changes could be introduced into the Mannich side chain of tebuquine without drastically affecting the biological activity, with both $N(CH_2CH_3)_2$, and $NHC(CH_3)_3$ exhibiting high activity, but analogues with a smaller $N(CH_3)_2$ or larger (NH-adamantyl) substituents also retaining substantial activity (Werbel *et al.*, 1986). In this QSAR study of the substituted phenyl analogues and their N-oxides, increasing antimalarial potency against *P. berghei* in mice was found to be correlated with decreasing size and electron donation of the phenyl ring substituent. The low phototoxicity and curative properties of α–di-*n*-butyl aminomethyl-2-(1-adamantyl)-6,8-dichloroquinolinemethanol suggests

that the substitution of the quinoline nucleus by bulky alkyl group at the 2-position might provide a suitable solution to the problem of phototoxicity (Fugitt and Roberts, 1973). For 2,6-*bis*(aryl)-4-pyridinemethanols similar to **20** with R_2 being a linear alkyl, the descending order of activity with respect to R_2 is as follows: 1-But > 1-Pent > 1-Pr > Et >1-Hex >1-Oct > 1-Het, with the effect of the branching on the alkyl group R_2 (R1 = H) being pronounced. 2-Pent imparts about one dose level more activity than 1-Pent and 4-Hept imparts nearly three dose levels more activity than 1-Hept (Markovac *et al.*, 1972).

Multidimensional analyses have shown that shape, electronegativity, and lipophilicity properties of the compound are related to the *in vitro* antimalarial activity against the human parasite, *P. falciparum*. For a series of basic amides of teicoplanin, some preliminary structure-activity relationships have established some correlations at physiological pH between isoelectric point, lipophilicity and hydrosolubility and antibacterial activity. It has been shown that the combined effect of moderate basicity and coupled with increased lipophilicity at neutral pH have a positive influence on *in vitro* activity (Malabarba *et al.*, 1992). Since all the molecules contained at least one nitrogen atom with various substituents, the activity of the drug is more likely to be related to the nature and geometry of the nitrogen atom substituents rather than to an overall property of the molecules (Calas *et al.*, 1997). It has also been noted that analogues of choline and ethanolamine show that 1-aziridineethanol, DL-2-amino-1,3-propanediol and D- or L-2-amino-1-butanol are the most efficient inhibitors of parasite multiplication, and all similar compounds with a free hydroxyl group exhibit lower potency. 3-amino-1-propanol and 5-amino-1-pentanol are poor inhibitors of *Plasmodium* growth, while where N-alkyl substitutions are possible, diethylaminoethanol is a better inhibitor than dimethylaminoethanol, whereas a rigid two carbon ring structure on the nitrogen such as aziridine-ethanol yields much higher activity and substitution of the N-O aliphatic chain at α of the nitrogen are efficient for analogue activities while substitution on the carbon at β is very deleterious (Vial *et al.*, 1984). On the basis of structure-activity relationship study (Calas *et al.*,

1997) essential parameters required for cationic choline analogues to inhibit *P. falciparum* phospholipid metabolism are electronegativity and lipophilicity. Thus irrespective of the polar head substitution on these agents, increasing the alkyl chain length from 6 to 12 methylene groups always lead to an increase in activity, while for both the mono- and bisquartenary ammonium salts shown below, a moderate increase in lipophilicity around the nitrogen atom is quite beneficial for antimalarial potency (Calas *et al.*, 2000).

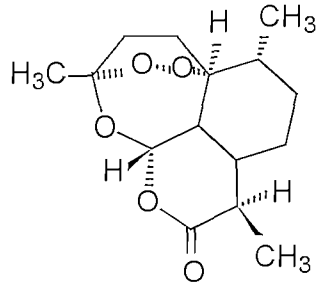
The polyamine of the general structure $RNH(CH_2)_3NH(CH_2)yNH(CH_2)_3$-NHR, tested for antimalarial activity *in vitro* by following the incorporation of [^{3}H]-hypoxanthine into *P. falciparum* have shown that compounds without N-benzyl groups are much less active than the α,ω-dibenzyltetraamine derivatives, whereas compounds with only one N-benzyl group are of intermediate potency.



The central alkyl chain of the α,δ-dibenzyltetraamines could be varied from eight to twelve carbon atoms without significant change in growth inhibitory activity against *P. falciparum in vitro* (Edwards *et al.*, 1991). The terminal aminopropyl chain of these structures can be substituted without any noticeable modification of the inhibitory activity. However, replacement of the benzyl groups with thienylmethyl, cyclohexenylmethyl or *n*-butyl gives less active analogues.

Similarly, the effectiveness of artemisinin **(24)** and its derivatives as novel malarial therapeutic agents is impaired by their (a) poor solubility in either oil or water, (b) high rate of parasite recrudescence after treatment, (c) short plasma half-life and/or (d) poor oral activity (Lin *et al.*, 1997). However, the rapidly increasing urgency for the discovery and isolation of non-traditional antimalarials (Posner *et al.*, 1992) has resulted in the chemical modifications of artemisinin leading to a number of analogues with improved efficacy and increased solubility

in either oil or water. For the dihydroartemisinin derivatives, compounds with -Cl and -Br substituents exhibit better *in vitro* antimalarial activity than artelinic acid, with fluoro (strong electron-withdrawing group) and methoxy (strong electron-donating function) groups showing equal activity with artelinic acid, implying that steric and lipophilicity are the same for all the compounds. Electronic effects may play a role in manipulating the efficacy of this class of compounds (Lin *et al.*, 1997). In teicoplanin derivatives, the conversion of the carboxyl group to an amide generally improves *in vitro* activity and *in vivo* efficacy with the extent of the improvement depending on the ionic and lipophilic character of the resulting derivatives and on the number and structure of sugars (Malabarba *et al.*, 1992).



(24)

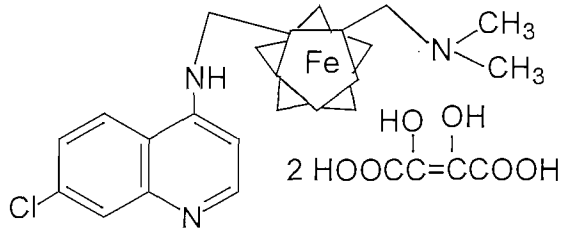
The *bis*(benzyl)polyamine analogues with structure $ArCH_2(NH(CH_2)_3yCH_2Ar$ are markedly cytotoxic to malaria parasites *in vitro* and *in vivo*, with the growth inhibitory activity against *P. falciparum in vitro* increasing as the length of the central methylene chain is lengthened from 4 to 12 (Bitonti *et al.*, 1989). The increasing potency appears to be related to the lipid solubilities of the analogues as well as, possibly the uptake of the compounds into the host erythrocytes. However, bisquinolines with alkyl bridges of three, four or twelve carbon atoms are inactive, while those with bridges of between five and nine carbon atoms are active, with methyl substitution in the bridge improving antimalarial activity (Vennerstrom *et al.*, 1992). In those compounds with a two carbon bridge a decreased conformational mobility seem to increase activity. Amongst the derivatives with a hydrophobic moiety, such as diphenylmethyl, dibenzocycloheptano and α-thienylbenzyl, and dibenzoxepino or benzocycloheptanopyridine show the highest activity and groups such as

chlorobenzyl, *bis*(fluorophenyl) and *bis*(methoxyphenyl) are less active. The deviations exhibited by the two phenyl rings from planarity are essential features of highly active compounds that could be due to the π-hydrogen-π interactions. The activity of the compounds of this nature also depends not only on the presence of the aryl rings in the hydrophobic moiety, but also on relative ring positions. The deviation of the two aryl rings from planarity is an essential feature of highly active compounds. If hydrogen bond donors of P-glycoprotein are set between two deviated aromatic rings in the hydrophobic moieties (π-hydrogen-π interactions), then the enhanced activity of the compounds having nonplanar aryl rings can be due to the π-hydrogen-π interactions. The activity is also affected by the distance between the hydrophobic moiety and the basic nitrogen atom, Nb, of piperazine and this distance should be at least 5 Å.

Molecular modelling of the tebuquine analogues including amodiaquine and chloroquine suggests that inter-nitrogen separation in this class of drugs ranges between 9,3 and 9,8 Å in their isolated diprotonated form and between 7,52 and 10,21 Å in the haem-drug complex (O'Neill *et al.*, 1997). In addition, these studies in an aqueous environment suggest that the 4-hydroxy function in tebuquine allows favourable orientation of the alkylamino function for hydrogen bonding to the carboxylates in haem. The complex is also stabilised by hydrophobic interactions between the quinoline ring and the porphyrin ring system of the haem. Incorporation of a *p*-chlorophenyl function at the 3' position of the amodiaquine and of *tert*-butylamino group improves activity, possibly by enhanced accumulation within the parasite food vacuole and more favourable haem binding. Phenothiazine derivatives inhibit the malarial cysteine protease falcipain and block the hydrolysis of haeglobin, the natural substrate of falcipain (Dominquez *et al.*, 1997). This inhibition may be due to the binding of the phenothiazines to the falcipain active site. Of particular interest is that compounds that do not contain C-2 phenyl groups are less potent, but inhibit falcipain activity and parasite haemoglobin degradation at concentrations similar to those at which the phenothiazines inhibited these processes. Thus those

compounds without the C-2 phenyl ring, the only apparent antimalarial effect is due to the inhibition of falcipain and subsequent blocks in haemoglobin degradation and parasite development.

Findings in the investigation of the potential of the 7-chloro-4-[[2-[(N-dimethylamino)-methyl]ferrocenyl]methyl]amino]quinoline **(25)** were that the compound possesses a highly potent antimalarial activity *in vivo* on mice infected with *P. berghei* N. and *P. yoelii* NS and it is also about 22 times more potent against schizontocides than chloroquine *in vitro* against the drug-resistant strain of *P. falciparum* (Biot *et al.*, 1997). The structure-activity relationship studies demonstrate that the antimalarial activity of this type of compound increases as a result of the presence of ferrocene and the effectiveness of this compound could be ascribed to its ability to penetrate infected cells. It may be necessary to pursue further studies of this compound to determine its usefulness as an antimalarial agent.

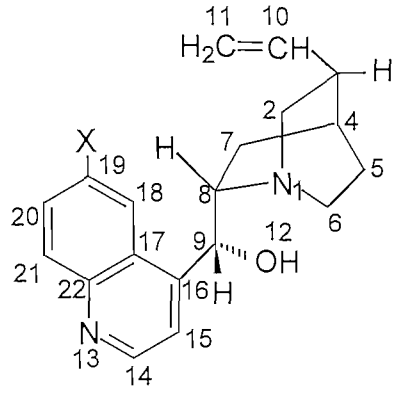


(25)

3.2.3 The Stereochemistry and Antimalarial Activity

The most abundant constituents of the Cinchona bark are the so-called *erythro* isomers: quinine **(8)**, cinchonidine **(8.1)**, cinchonine **(8.2)**, and quinidine **(8.3)** which differ from each other in the absence or presence of the methoxy substituent in the quinoline moiety and/or in the absolute configuration of the two carbon atoms, C(8) and C(9), while the configuration of C(4) and C(3) is the same for all the alkaloids in this group (Oleksyn *et al.*, 1992) (Table 3.2). The

TABLE 3.2: Configuration of Cinchona alkaloids



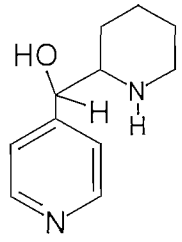
Compound	Absolute configuration		Substituent X
	C(8)	C(9)	
Quinine (**8**)	S	S	OCH_3
Cinchonidine (**8.1**)	S	R	H
Cinchonine (**8.2**)	R	S	H
Quinidine (**8.3**)	R	S	OCH_3
Epicinchonidine (**8.4**)	S	S	H
Epiquinine (**8.5**)	S	S	H
Epicinchonine (**8.6**)	R	R	H
Epiquinidine (**8.7**)	R	R	H

C(9) epimers, epicinchonidine (**8.4**), epiquinine (**8.5**), epicinchonine (**8.6**), and epiquinidine (**8.7**) are practically inactive. This means that the absolute configuration of the C(8)-C(9) pair dictates the antimalarial activity, rather than the configuration of each of these atoms taken separately. It would appear that the conformation of the *erythro* stereoisomers fits the receptor site more closely than the *threo* forms, and the receptor sees the conformation of the racemic mixture to be the same as that of the *erythro* stereoisomers as the combination of

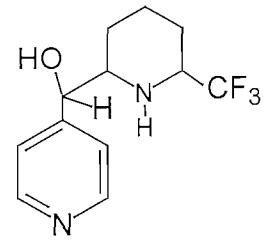
the two isomers is more effective than single alkaloids against *P. falciparum* (Wesche and Black, 1990:158).The *dl-threo* isomer of α-(2-piperidyl)-3,6-*bis*(trifluoromethyl)-9-phenanthrenemethanol has been found to be more active than the *dl-erythro* isomer (Olsen, 1972). However, although difference exists in the dose levels of the *threo* and *erythro* isomers, the *threo* isomer of the amino alcohol α-(2-piperidyl)-3,6-*bis*(trifluoromethyl)-9-phenanthrenemethanol-α-(2-piperidyl)-2,8-*bis*(trifluoromethyl)-4-quinoline-methanol and several α-(2-piperidyl) pyridine-methanols, is usually two to four times more active than its *erythro* isomer, both diastereomers being active antimalarials (Chien and Cheng, 1976).

Although both the *dl-erythro* and *dl-threo* isomers of α-(2-piperidyl)-3,6-*bis*(trifluoromethyl)-9-phenanthrene methanol are highly active, when administered to mice infected with *P. berghei*, the *threo* epimer at a dose of 40 mg/kg gave 5 cures out of 5 infected mice, while the *erythro* isomer gave the 5 cures at 80 mg/kg (Olsen, 1972). The diastereoisomers of 6-bromo-α-[2-(1-methylpiperidyl)]-9-phenanthrenemethanol also exhibit different biological activity, one isomer being active against the *P. berghei*, while the other is either toxic or inactive (Chien and Cheng, 1976). Through a combination of optical resolution and chemical conversion, all the four optical isomers of α-(2-piperidyl)-3,6-bis(trifluoromethyl)-9-phenanthrene methanol and α-(2-piperidyl)-2,8-*bis*(trifluoro-methyl)-4-quinoline methanol were prepared (Carroll and Blackwell, 1974). Against *P. gallinaceum* in chicks, the minimum dosage showing activity was 20 and 160 mg/kg for the *threo* and *erythro* compounds respectively. In addition, by chemical inversion and isolation, it was noticed that the *threo* isomer of α–(2-piperidyl)-4-pyridine methanol **(26)** was more active than the corresponding *erythro* isomer in terms of antimalarial action against the *P. berghei* (LaMontagne *et al.*, 1974). Further chemical modification of this compound produced another active antimalarial, epiroline **(27)**, which incorporates many of the features of **8** (Ash *et al.*,1975). Similarly, the *S,S* enantiomer of the bisquinoline **16**, has demonstrated a superior potency over the *R,R* isomer as a potent inhibitor of both chloroquine-sensitive and chloroquine-resistant parasites (Ridley *et al.*,

1997). Phototoxicity has been the main problem for precluding further development of this drug.

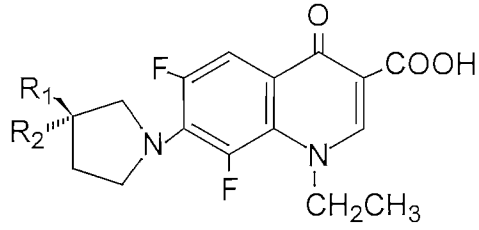


(26)



(27)

While there is no significant difference between the *S*-(-) and *R*-(+) enantiomers of the quinolone at the enzyme or bacterial levels, the *S*-(-) enantiomer **28** does show a consistent trend towards increased potency against the Gram-positive organisms when compared to the *R*-(+) enantiomer **29** (Culbertson *et al.*, 1987).



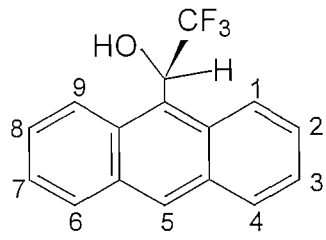
(28): R_1 = $-CH_2NHC_2H_5$ and R_2 = -H

(29): R_1 = -H and R_2 = $-CH_2NHC_2H_5$

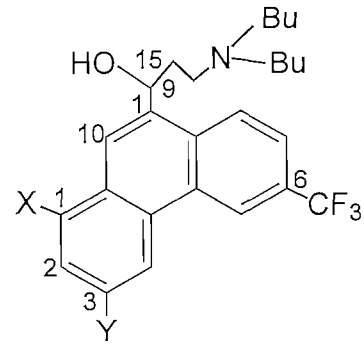
Using the piperidyl compounds of *bis*(trifluoromethyl)phenanthrene and 2,8-*bis*(trifluoromethyl)quinoline methanols, Chien and Cheng (1976) rationalised the difference in activity of the isomers on the minimum N-O distance of the active groups as measured by the Dreiding models. For the active compounds, the minimum distance is 2,5 Å, while the maximum distance is 3,5 Å. Since within a reasonable range, the matching of distance between the active sites of a bioreceptor and the pharmacophore of a drug need not necessarily be exact, any structural isomer having a proper N-O distance to match the receptor should be

active. Isomers sterically favouring the conformation but having a maximum distance of about 5 Å cannot fit the "active site" and are therefore inactive. The Dreiding models indicates that in order to attribute the differences in activities to the N-O internuclear distance, one isomer would have to bind to the active site of the receptor with its aryl group in an axial conformation (the isomer which allows for hydrogen bonding between the piperidine and the carbinol function) and the other isomer, with its aryl group equitorial, would not bind effectively (Chien and Cheng, 1976).

While it is known that stereochemical properties of the molecule plays a role in its biological activity, it is not yet clear why the *erythro* form of the quinolinemethanols should be more active than the *threo* forms, while for the phenanthrenemethanols the opposite is the case. It is also believed that the intermolecular forces responsible for chiral recognition can be diverse and subtle (Camilleri *et al.*, 1994). The well-known chiral resolving reagent represented by compounds **30** and **31** revealed the presence of unusually strong O-H .π-facial hydrogen bonding in the solid state and an asymmetry between the calculated molecular electrostatic potentials (MEPs) of the two π faces attributed to the antiperiplanar (app) interaction with the CF_3 group. Thus the explanation of the different behaviour of the two compounds can be sought from the properties of the π faces that might be sensitive to the orientation of the OH group. In the phenanthrenemethanols the OH hydrogen points towards the dibutylaminopropyl substituent and are nearly parallel to the plane of the phenanthrene ring. The ring then adopts a complementary edge-to-edge dimeric interaction between the pair of enantiomers. This interaction involves contact between apparently electron-deficient aromatic H-1 and a lone pair of electrons on the oxygen atom from the second molecule, slightly staggered to avoid contact between the two H-10 atoms leading to π–π stacking, which is associated with chiral recognition. In this case, shorter range double app interaction should result in C-H...O hydrogen bonding to H-9 (Camilleri *et al.*, 1994).



(30)



(31)

3.3 THE DESIGN OF THE QUINOLINE ANTIMALARIAL DRUGS

As part of the rational design of antimalarial drugs to offset the increasing occurrence of drug-resistant malaria strains, it is imperative that an understanding of the mechanism of action of the drugs themselves and the mechanism involved in the development of drug resistance by the parasites is obtained. It has been noticed that the emergence of multidrug-resistant strains of *Plasmodia* has created a near-desperate situation. The need for new inexpensive antimalarials to circumvent the parasite has become vital (Raynes *et al.*, 1999). In a search for and development of new effective drugs, the following must be taken into account:

a) There must be an understanding of both the structural and thermodynamic factors of the drug molecule that control the binding of such drugs to ferriprotoporphyrin IX (Egan *et al.*, 1997). These factors must be such that they allow for an increased concentration of the drug in the parasite food vacuole due to its weak basic properties as well as its ability to bind to haem released as a result of vacuolar haemoglobin degradation (Egan *et al.*, 1996).

b) Attention should be focussed on the haemoglobin degradation processes. This could involve agents that can inhibit the action of

the plasmepsins and those that would interact with FP to inhibit the formation of haemozoin. The demonstration that potent and relatively selective inhibitors of plasmepsin II also inhibits the growth of *P. falciparum* in cell culture strongly supports the concept that inhibition of plasmepsins may also be a viable strategy for antimalarial therapy (Silva *et al.*, 1996). The nature of the interaction between FP and antimalarial drugs is of considerable relevance to the possible mode of action of such drugs (Adams *et al.*, 1996). An understanding of this phenomenon could also assist in designing active antimalarial agents. The design studies should also take into consideration both the drug interaction with haem as well as the haem-drug complex interaction with the haemozoin polymer (Sullivan *et al.*, 1996). Both free ferriprotoporphyrin IX (FP) and its complex with chloroquine inhibit the proteases that are essential for the degradation of haemoglobin and growth of the parasites (Vader Jagt *et al.*, 1986).

c) The difference in the structure of mammalian and parasite LDH molecules raises the possibility that inhibitor molecules could be designed that would specifically target the parasite enzyme as chloroquine interacts with *Plasmodium falciparum* lactate dehydrogenase (PfLDH) enzyme but not with erythrocyte LDH (Menting *et al.*, 1997).

d) The phospholipid metabolism can also be considered as an attractive target for a new chemotherapeutic approach to malaria since many analogues of N-substituted amino-alcohols with polar head groups are potent inhibitors of parasite multiplication (Vial *et al.*, 1984). Compounds **1** and **3** bind preferentially to acidic phospholipids, but due to the relatively high electrostatic charge of the chloroquine, it binds to an extent less than expected from its hydrophobicity (Shalmiev and Ginsburg, 1993). This is presumably the reason of its inability to intercalate into phospholipid

monolayers, whereas substantial intercalation is observed with quinine and mefloquine. It is also vital in the development of new antimalarial compounds to identify structural and/or physicochemical features of such compounds that greatly enhance the drug's interaction with the high-affinity, low-capacity component and reducing the compound's interaction with the low-affinity, high-capacity component (Hawley *et al.*, 1996). The identification of proteins involved in the mechanisms of action or resistance to chloroquine could point to a means of developing novel antimalarial drugs that could circumvent the problems of chloroquine resistance (Menting *et al.*, 1997).

e) The design of molecules that recognise specific sequence on the DNA double helix would provide new tools to control gene expression and a rational basis for fresh approach to drug development (Hélène, 1998). Double helix DNA can bind to different type of ligands which can be classified in two categories: intercalators which insert their aromatic rings between two adjacent base pairs, and groove binders that bind DNA within either groove of the double helix. Intercalators have limited sequence specificity, because they are interacting only with two base pairs, unless they are linked to a groove-recognition element. DNA topoisomerases regularise the topological state of the DNA during cellular events such as replication, transcription, and recombination, by transient cleavage of the single or double DNA strands, by swivelling, and by religation of the initial bonds. A substance that can stabilise DNA topoisomerase cleavable complex to stop the progression of DNA processes may be useful (Zahir *et al.*, 1996).

f) Since the antimalarial activities of quinoline-type drugs are a function of both the ability of the drug to interfere with the haemozin polymerisation process, and the capacity to accumulate to pharmacologically relevant concentrations at the site of drug action

(Bray *et al.*, 1996; Hawley *et al.*, 1996; 1998), the development of novel compounds with improved capacity to accumulate intracellularly at the site of drug action to produce the effective concentration (Hawley *et al.*, 1998), it is necessary to consider measures that can improve transport of the drug across the cell membrane.

3.4 ADDITIONAL FACTORS FOR CONSIDERATION IN MALARIA CHEMOTHERAPY

3.4.1 The Influence of Calcium Channel Blockers

Verapamil, diltiazem, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), vinblastin and daunomycin, each enhances the accumulation of chloroquine in the acid food vacuoles by the chloroquine-resistant, but not the susceptible parasites (Krogstad *et al.*, 1987). For verapamil to achieve this, it is purported that it can selectively chemosensitise chloroquine-resistant parasites and selectively increase chloroquine accumulation by these isolates, supposedly through inhibition of the chloroquine efflux pump (Krogstad *et al.*, 1987; Martin *et al.*, 1987). The mechanism by which verapamil inhibits efflux of anticancer drug from resistant cancer cells is not completely understood, but may involve its ability to bind to a 150 K to 170 K membrane-associated glycoprotein (Martin *et al.*, 1987). This chemosensitisation also occurs with monodesethylamodiaquine, suggesting that verapamil chemosensitisation for a particular drug is dependent on the general physical features such as lipid solubility and molar refractivity rather than on closely defined structural parameters (Bray *et al.*, 1996). The inhibition of chloroquine release observed after administration of the calcium channel blockers could also be ascribed to the alterations in intracellular calcium that may make it necessary for the release of chloroquine from the resistant parasites (Krogstad *et a.*, 1987). Another suggestion is that, whatever the mechanism(s) contributing to the chloroquine resistance is, it is extremely structure specific (Ridley *et al.*, 1996). This is evidenced by the fact that

compounds with a shortened side chain at the 4-amino group, while they are structurally closely related to chloroquine, they manage to overcome chloroquine resistance to a greater extent than chloroquine itself.

3.4.2 The Role of Combination Antimalarial Chemotherapy

Previous investigations have concluded that the eukaryotic malaria parasite possesses a complex genome that displays great plasticity as exemplified by the remarkable ability of the parasite to adjust (i.e. to develop resistance) to virtually every quinoline-type drug that has been deployed against them (Peters, 1982). This has lead to the conclusion that with increasing usage, correctly or otherwise, of every new antimalarial that becomes available, coupled with the unwarranted dependence placed upon drugs as a means of malaria control, often indeed the sole means, the outlook for today's handful of antimalarials is not promising (White, 1992). It has been suggested that the most important step to limit the further spread of resistance, not only to existing antimalarials, but also to drug that may be developed, is to consider chemotherapy as but one of several measures to be included in an integrated approach to malaria control. The judicious use of drugs in combination is one logical approach, which may be augmented by strict governmental drug control are unfortunately, unrealisable steps that may have to be considered (Peters, 1982). In order to reduce the rate of development of resistance to the major antimalarial agents, it may therefore be necessary to administer most of them in combination. The effectiveness of long-interval suppression of *P. falciparum* by sulphadoxine/pyrimethamine is probably due to the slow elimination of sulphadoxime and pyrimethamine from the blood (Pearlman *et al.*, 1977). Treatment with quinine and tetracyclines gives good cure rate (Meek *et al.*,1986) and triple combination of mefloquine/sulphadoxine/ pyrimethamine (MSP) delays the development of resistance markedly (Merkli and Richle, 1980; Peters and Robinson, 1984). In falciparum malaria, while MSP is effective, it is not better than mefloquine alone (Harinasuta *et al.*, 1985), but there is sound reason to believe that the use of triple combination delays the appearance of mefloquine-resistant strains of *P. falciparum* (White, 1987). The

use of MSP combination in countries where *P. falciparum* is already highly resistant to sulphadoxine-pyrimethamine may be questioned. This delay in the development of resistance to mefloquine treatment is due to the protection offered to mefloquine by pyrimethamine and sulphadoxine. However, acute psychosis following mefloquine administration has been noticed as a serious side-effect in some countries (Björkman, 1980; Stuiver *et al.*, 1989).

However, the problem associated with the use of MSP is that the use of the long-acting sulphonamide, sulphadoxine, carries a significant morbidity from severe allergic reaction, and the pharmacokinetic properties of the three drugs are not well matched, since the elimination of both pyrimethamine ($t_{½}$ of 85 hours) and sulphadoxine ($t_{½}$ of 180 hours) is considerably more rapid than that of mefloquine **(1)** with a $t_{½}$ of 2 to 3 weeks (White, 1985) (See Fig. 3.3). It is evident that after a single dose, sub-therapeutic concentrations of **1** will persist in the blood for months, "unprotected" by either of the compounds (White, 1988). This is the ideal milieu for the development of drug resistance, and it seems for this reason that even strict regulation of **1** use will not prevent the rapid development of mefloquine resistance. It has been commonly proposed that the majority of antimalarial drugs, including chloroquine, should be protected by finding suitable combination partners and the similitude in the biological half-lives of the individual compounds is apparently an important factor in the choice of appropriate combination partner (Fernex, 1979).

Artemisinin-based Combination Therapies (ACTs) are now considered as the best current treatment for uncomplicated falciparum malaria and have the advantages of rapid clinical and parasitological response, improved cure rate, decreased malaria transmission and the potential to delay resistance (South Africa, 2006). The Artemether-lumefantrine fixed combination is the recommended first line treatment for uncomplicated malaria in all malaria transmission areas in South Africa.

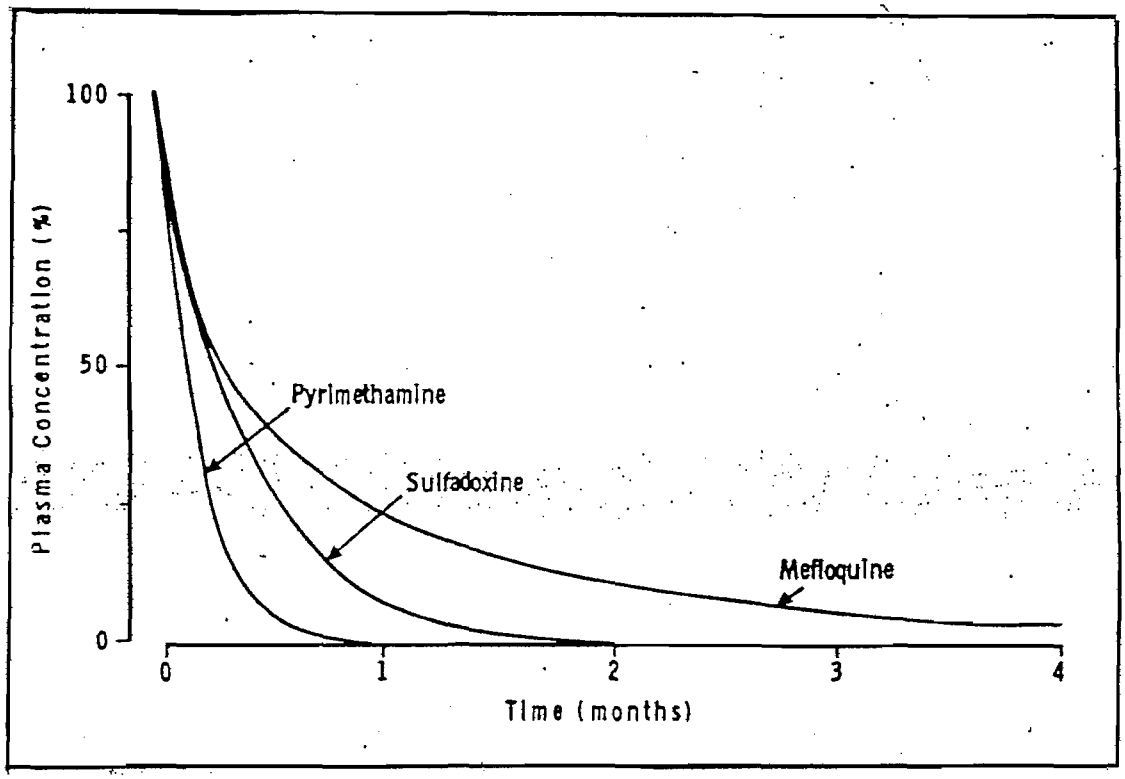


FIGURE 3.3: Plasma concentrations of mefloquine, sulphadoxine and pyrimethamine (expressed as a percentage of peak concentration) following simultaneous administration of the three drugs. Adapted from White, 1988).

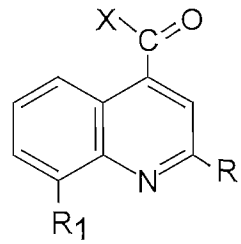
CHAPTER 4

THE CHEMISTRY AND SYNTHESIS OF THE TARGET COMPOUNDS

4.1 INTRODUCTION

The quinoline and 1,2,4-triazino-[5,6b]indole ring systems constitute the scaffolds upon which the synthesis of the compounds with basic structures reflected in Tables 4.1a to 4.1d as **4**, **5**, **6** and **7** shown below and which were used in our investigations are based (see § 1.5). The quinoline group of compounds, include compounds in which positions 2 and/or 8 are substituted with trifluoromethyl groups to constitute series **4.1** to **4.6** which in addition contain either the pyrimidino or 1-ethyl-5-nitro-1*H*-imidazolo functional moieties in position 4 of the quinoline as reflected in Table 4.1a. Each molecule of the other series (**5.1** to **5.10**) consists of two quinoline ring systems with a trifluoromethyl group at position 2 with or without additional trifluoromethyl group at position 8 and either a diaminoalkyl chain or piperidino ring linking the two quinoline rings together (Table 4.1b). The diaminoalkyl chains vary in length from 2 to 6 carbon atoms. The 1,2,4-triazino[5,6b] indole derivatives, on the other hand, have positions 3 and 7 e substituted with trifluoromethyl groups or other functional groups of which the biological activity is being investigated (Tables 4.1c and 4.1d). For this series, the effect on activity of groups such as sulphur, methyl, ethyl and cyclopropyl have been evaluated. The indole moiety has also been extended to form a tetrazole ring system with appropriate substituents at position 3'.

TABLE 4.1a: Structure of the target quinoline compounds



(4)

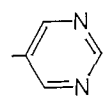
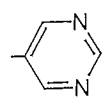
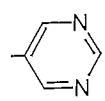
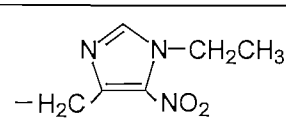
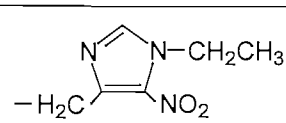
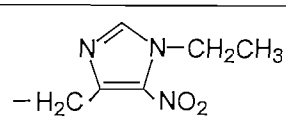
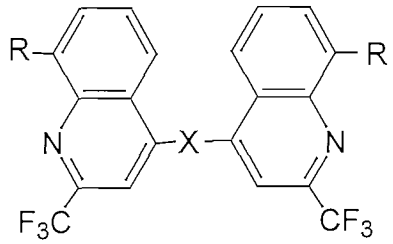
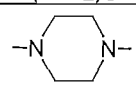
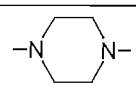
Compound No.	SUBSTITUENTS		
	R	R_1	X
4.1	-H	CF_3	
4.2	$-CF_3$	-H	
4.3	$-CF_3$	$-CF_3$	
4.4	-H	$-CF_3$	
4.5	$-CF_3$	-H	
4.6	$-CF_3$	$-CF_3$	

TABLE 4.1b: Structures of the target bisquinoline compounds



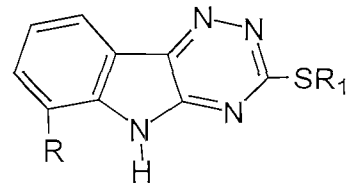
(5)

Compound No.	SUBSTITUENTS	
	R	X
5.1	-H	$-HN(CH_2)_2NH-$
5.2	$-CF_3$	$-HN(CH_2)_2NH-$
5.3	-H	$-HN(CH_2)_3NH-$
5.4	$-CF_3$	$-HN(CH_2)_3NH-$
5.5	-H	$-HN(CH_2)_4NH-$
5.6	$-CF_3$	$-HN(CH_2)_4NH-$
5.7	-H	$-HN(CH_2)_6NH-$
5.8	$-CF_3$	$-HN(CH_2)_6NH-$
5.9	-H	
5.10	$-CF_3$	

4.2 SYNTHETIC APPROACHES TO QUINOLINE COMPOUNDS

The synthesis of quinoline ring systems with or without substituents at each position in the molecule can be modeled along the classical approaches that have been reviewed by Elderfield (1952). These approaches involve chemical reactions that lead to closure of the pyridine ring system either between the γ-carbon atom and the benzene nucleus, between β and γ carbon atoms, between the α and β carbon atoms or between the nitrogen and the α carbon atoms. Some of the more common methods are the Doebner-von Miller, Skraup, Knorr, Pfitzinger, Friedlander's and Doebner pyruvic acid methods and the variations

TABLE 4.1c: Structures of the 1,2,4-triazino[5,6b]indole-3-thione compounds



(6)

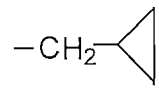
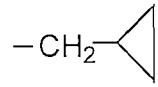
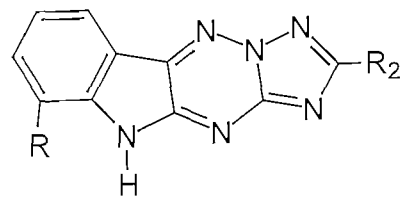
Compound No.	SUBSTITUENTS	
	R	R_1
6.1	-H	$-CH_3$
6.2	$-CF_3$	$-CH_3$
6.3	-H	$-CH_2CH_3$
6.4	$-CF_3$	$-CH_2CH_3$
6.5	-H	
6.6	$-CF_3$	

TABLE 4.1d: Structures of the tetrazole derivatives



(7)

Compound No.	SUBSTITUENTS	
	R	R_1
7.1	-H	H
7.2	$-CF_3$	-H
7.3	H	$-CH_3$
7.4	$-CF_3$	$-CH_3$
7.5	H	$-CF_3$
7.6	$-CF_3$	$-CF_3$
7.7	-H	$-C_6H_4Cl$
7.8	$-CF_3$	$-C_6H_4Cl$

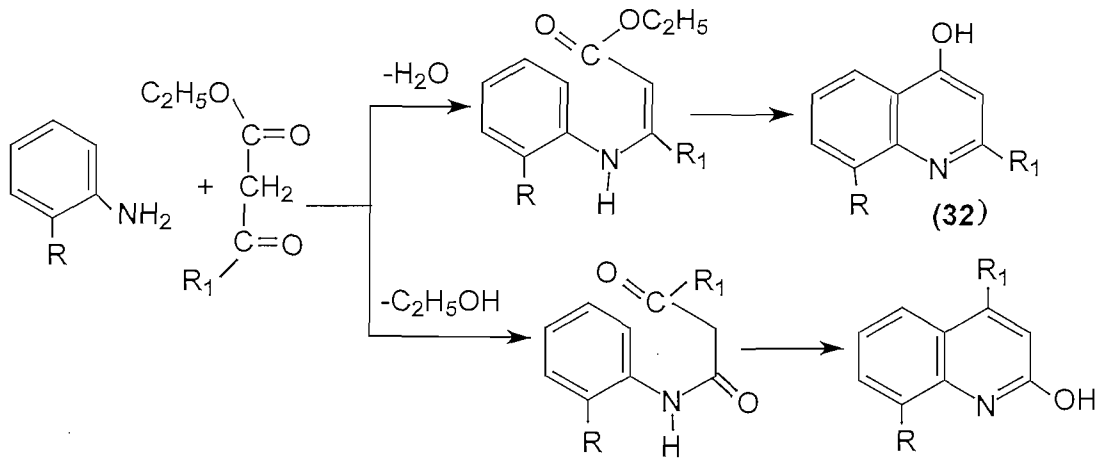
thereof. In addition to these ring closure procedures, quinoline ring systems may be prepared by methods involving the oxidation of polycyclic compounds and other pyrolytic methods. The choice of the synthetic route for the quinoline ring system containing appropriate and desired substituents attached thereto, is governed by the number, the nature and position(s) of substituents desired to be on the quinoline ring. Our investigation in the current studies require a quinoline ring system with a trifluoromethyl group at positions 2 and/or 8 and a position 4 having a carbonyl group for conversion to or linkage with other groups or a reactive functional group such as a halogen for replacement by other moieties. In order to achieve the objectives of the study, the methods of choice are the Doebner-von Miller and Pfitzinger reactions. Derivatives with trifluoromethyl groups at 2 and/or 8 positions and a hydroxyl group at position 4 are conveniently prepared by the former method, while the Pfitzinger reaction is suitable for the synthesis of derivatives with a single trifluoromethyl group at 8 and a carboxylic group at 4 positions.

4.2.1 Doebner-von Miller Method

A β-ketoester such as ethyl acetoacetate when reacted with an aromatic amine forms either a 2-methyl-4-quinolinol or a 4-methyl-2-quinolinol (**32** or **33**), depending on whether the intermediate formed is a β-anilinocrotonate or an acetoacetanilide respectively (Hauser and Reynolds, 1948)(see Scheme 1). The formation of these isomers is due to the fact that the reaction of the amine at the ester group leads to the formation of the acetoacetanilide while the reactions at the β-keto group will lead to the formation of a β-aminocrotonate. Usually, polyphosphoric acid is used as a cyclising agent in these reactions and when used, the product is exclusively the 2-quinolinol (Staskum and Israelstan, 1961; Dey and Joullié, 1965). However, in some acetanilides, low yields of the hydroxyquinolines may be obtained due to the decomposition of the anilides by polyphosphoric acid. When diphenyl ether or mineral oil is used instead of polyphosphoric acid, the reaction leads to the formation of a mixture of both **32** and **33** (Hauser and Reynolds, 1948; Dey and Joullié, 1965; Pinder and Burger,

1968). Temperatures play a role in the formation of the β-anilinocrotonates and acetoacetanilides. At low temperatures the formation of the crotonates is favoured, while temperatures of 130 to 140 °C leads to the production of the anilides. However, both formations are reversible and one form may be converted to the other by heating with equivalent amount of water and traces of an acid at 130 to 140 °C. The anilides can be transformed by heating with ethanol and Drierite. The cyclisation without isolating each of these leads to formation of 2- and 4-quinolinol respectively. Cyclisation by refluxing in Dowtherm of ethyl acetoacetate obtained in high yields from aniline and ethyl acetoacetate, leads to excellent yield of **32** and **33** (Schaefer *et al.*, 1970). In a similar manner, when an amine is treated with diethylethoxymethylene-malonate, compound **32** is formed (Snyder *et al.*, 1947).

Scheme 1

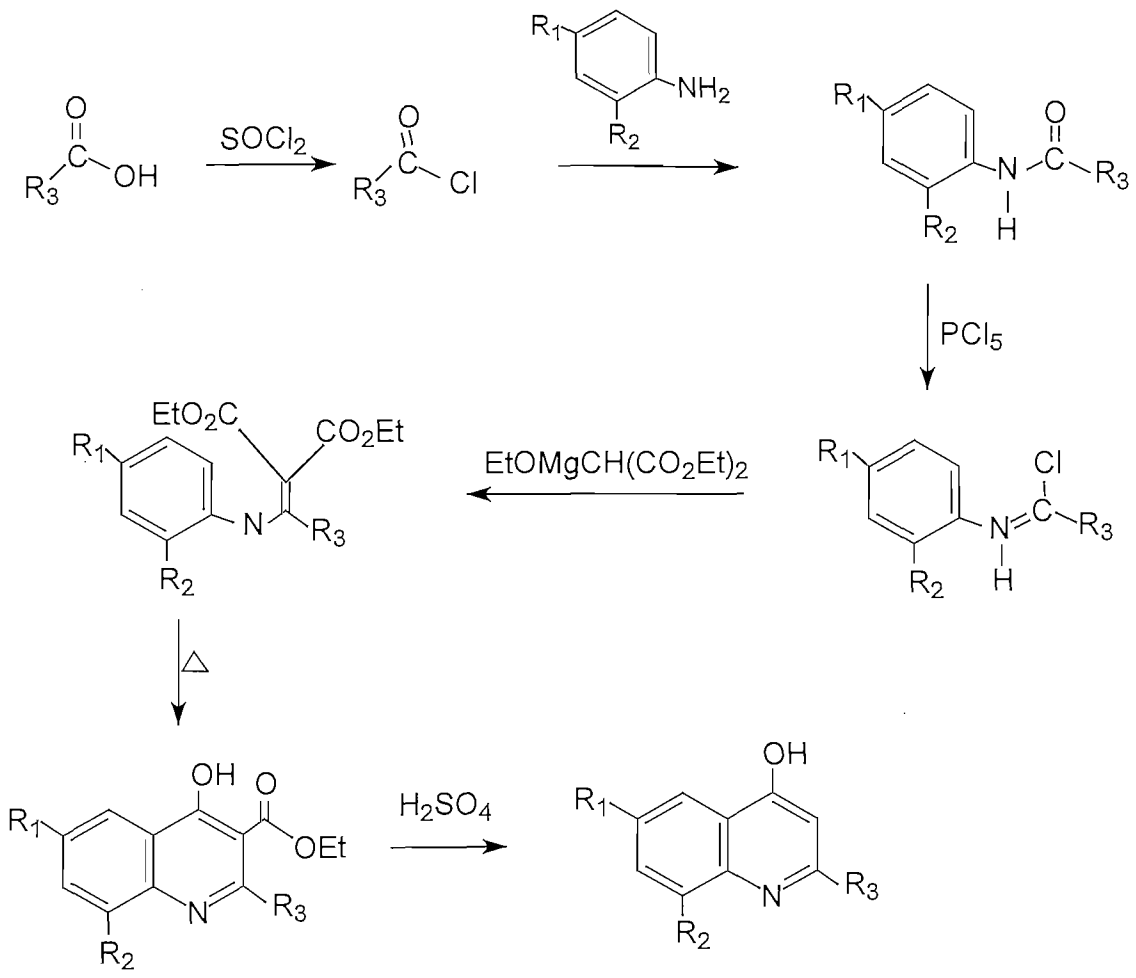


where R = H or CF_3
and R_1 = CH_3 or CF_3

The 4-quinolinols can also be obtained by employing the procedure represented in Scheme 2 (Fugitt and Roberts, 1973) in which the 3-carboethoxy group can be removed by decarboxylation through heating with sulphuric acid under aspirator vacuum. They can also be prepared by treating aniline with ethyl ethoxalylpropionate (Steck *et al.*, 1946; Lisk and Stacy, 1946) or ethyl

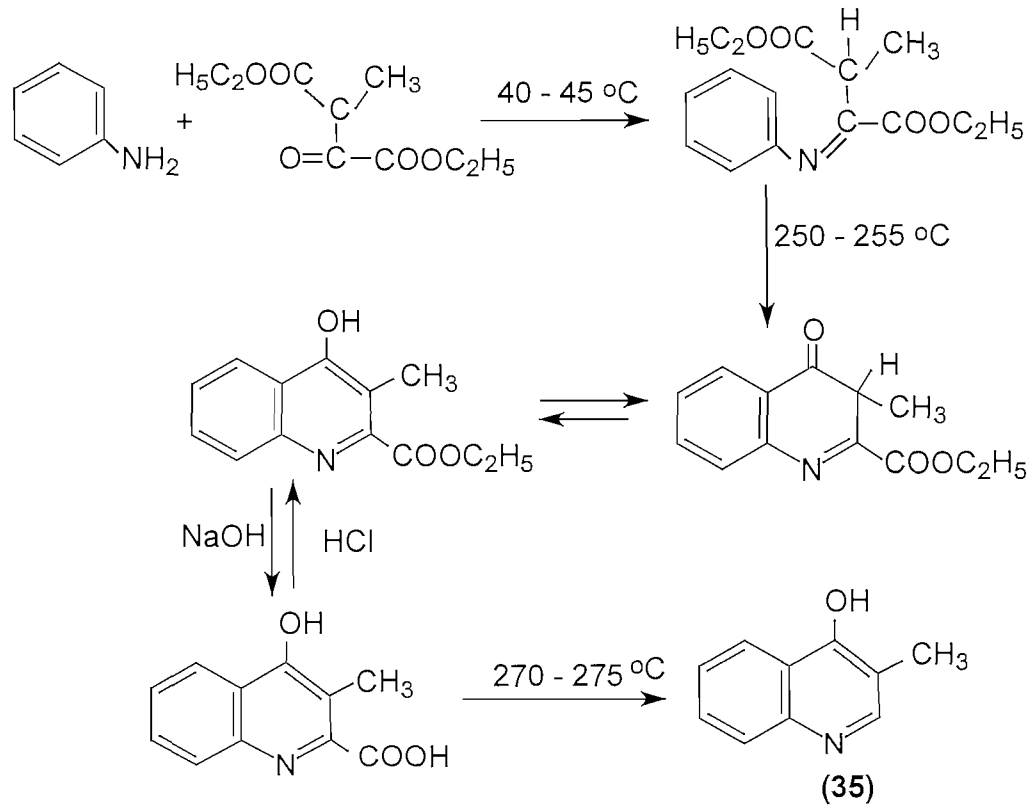
ethoxaldehyde (Riegel *et al.*, 1946). These procedures can be conveniently used to prepare quinoline derivatives with a methyl group at position 3 in yields as high as 90%. The synthesis of the aminoisoquinoline was also reviewed (Van der Goot, 1972).

Scheme 2



(34)

Scheme 3



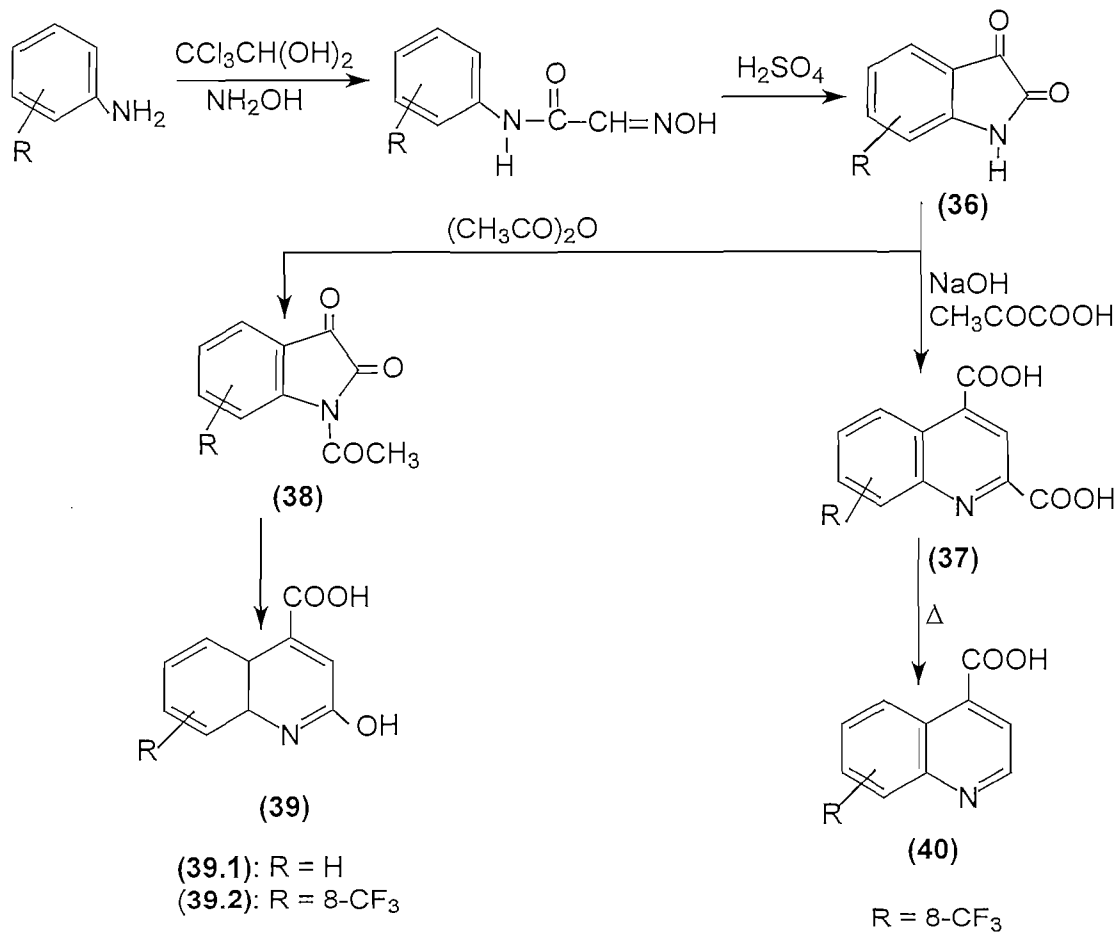
(35)

4.2.2 Pfitzinger Reaction

Isatin or 7-trifluoromethylisatin **(36)** used as a starting material in the Pfitzinger reaction for the preparation of quinoline derivatives is obtained by treating aniline or *o*-aminobenzotrifluoride with chloral hydrate and hydroxylamine, and the resulting isonitrosoacetanilide cyclised with concentrated sulphuric acid (Marvel and Hiers, 1932; Maginnity and Gaulin, 1951) (Scheme 4). The nature of the substituent (R) on the isonitrosoacetanilide plays a significant role in the percentage yield of the isatin (Sadler, 1956). When the isatin is treated with a concentrated sodium hydroxide solution it leads to the formation of isatic acid which when treated with pyruvic acid cyclises to form the quinoline-2,4-dicarboxylic acid **(37)**. The latter, on decarboxylation with boiling nitrobenzene yields the quinoline-4-carboxylic acid **(40)** (Work 1942; Senear *et al.*, 1946; Buchman *et al.*, 1946). Alternatively **36** can be acetylated to form the N-acetylisatin **(38)** that on treatment with sodium hydroxide solution can be

converted into 2-hydroxyquinoline-4-carboxylic acids **(39.1** and **39.2)** (Jacobs *et al.*, 1955). On the other hand, the 4-, 5- and 7-trifluoromethylisatins can be obtained by cyclisation of *o*-aminobenzotrifuoride in an acid (Simet, 1963; Werzel *et al.*, 1973). *m*-Trifluoromethyltoluidine or *p*-nitrotoluene with aminobenzotriflouride and diethylethoxymethylenemalonic ester can be used to prepare the 6- and 8-trifluoromethylquinolines (Gilam and Blume, 1943; Pouterman and Girardet, 1947; Snyder *et al.*, 1947).

Scheme 4

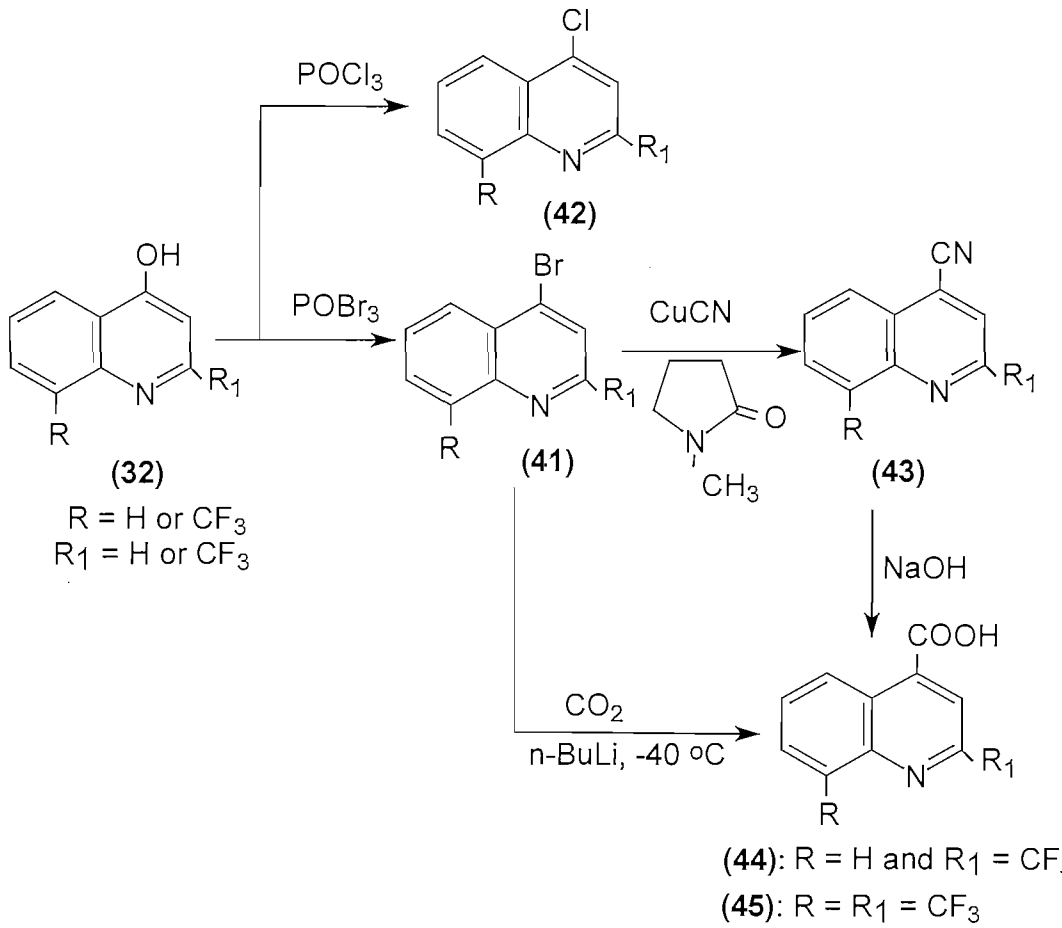


Introduction of a group at position 4 of the quinoline ring may involve replacement of the hydroxyl group on **32** with a halogen by treating the quinolinol with phosphorus oxybromide or oxychloride to form 4-bromo- or 4-

chloroquinoline **(41** and **42)** (Pinder and Burger, 1968; Schaefer *et al.*, 1970). Excess phosphoryl bromide may also be used to produce good yield of 4-bromoquinoline **(41)** (Fugitt and Roberts, 1973). These compounds containing the 4-halo group are versatile intermediates in the preparation of many derivatives since the halogen atom located on the carbon atom which has a low electron density are reactive towards various nucleophiles (Dey and Joullié, 1965). The reactivity of the 2- and 4-halo derivatives of quinolines is considerably greater than that in the 2- and 4-halopyridines, and the reactivity of the 2-halo atom is always greater than that of 4-halo atom in quinoline derivatives (Rowlett and Lutz, 1946). In addition, 4-chloroquinolines with substituents in positions 2 and 3 require longer heating and higher temperature for complete reactions than do the 4-haloquinolines with substituents in the benzene ring only (Drake *et al.*, 1946; Eisch and King, 1981). The 4-haloquinoline on treatment with cuprous cyanide in a Rosenmund-von Braun reaction in N-methylpyrrolidone (Newman and Boden, 1961) or the dimethylformamide (Friedman and Shechter, 1961) leads to the formation of the nitrile **(43).** The reactivity of this reaction is in the order I > Br > Cl > F. On hydrolysis with ethanolic sodium or potassium hydroxide (Pinder and Burger, 1968) or 70% sulphuric acid (Gilman and Spatz, 1941) the cinchonic acid **(44** or **45)** is formed. Alternatively **41** may be treated with *n*-butyllithium at – 35 °C under positive nitrogen pressure, and the resulting lithioquinoline reacted with dry carbon dioxide to give the **44** or **45** (Gilman and Spatz, 1941).

The key quinoline derivatives used in our study are the 2-trifluoromethyl- and 2,8-*bis*(trifluoromethyl)-4-quinolinols [**(32.1)**, where R = H and R_1 = CF_3 and **(32.2)** where R = R_1 = CF_3] which were synthesized according to the reaction pathway similar to Scheme 1 and the 8-trifluoromethylquinoline-4-carboxylic acid [**(40)** where R = 8-CF_3] prepared in accordance with Scheme 4. Since the compounds to be used in the first study were to contain a carbonyl group at position 4 for coupling with other functional groups, this group was introduced through the formation of a carboxylic acid as in Scheme 5. In the preparation of

Scheme 5



intermediates for the synthesis of the 2-trifluoromethylquinolin-4-yl and [2,8-*bis*(trifluoromethyl)quinolin-4-yl]-(pyrimidin-5-yl)methanones (**7.1** and **7.3**) and 2-(1-ethyl-5-nitro-1*H*-imidazol-4-yl)-1-[2-(trifluoromethylquinolin-4-yl]- and [2,8-*bis*(trifluoromethyl)qui-lin-4-yl)ethan-1-ones (**7.4** and **7.6**) we opted for the use of phosphoryl bromide since good yields of bromoquinoline are obtained (Fugitt and Roberts, 1973) and the latter reacts more easily with *n*-butyllithium than the 4-chloroquinoline. However, for the synthesis of the quinoline intermediates of the N,N-*bis*(trifluoromethylquinolin-4-yl)diamino alkanes (**8.1** – **8.10**), the chloro derivative, **42** was used. The reason why this procedure is adopted is that 4-haloquinolines are versatile intermediates as the halogen atom is located on the

carbon atom having a low electron density and therefore reactive towards various nucleophiles (Dey and Joulié, 1965).

As the quinoline compounds required for our investigations must contain one or two trifluoromethyl groups attached to the ring, the influence this group will have on the reactivity of the entire molecule and the ring closure during the synthesis must be taken into account. As a highly electronegative group, the inductive effect of the trifluomethyl group, tends to deactivate the *ortho* and *para* positions of the aromatic ring, thus preventing ring closure (Simet, 1963; Gillespie *et al.*, 1968) when employing the Conrad-Limpach method (Conrad and Limpach, 1887) in the synthesis of quinoline derivatives. Similarly, the Skraup and Knorr methods cannot be utilised as the oxidation of the methyl group on the ring will result in the simultaneous oxidation of the trifluoromethyl group if present. It is also necessary to take extra care when preparing quinoline compounds containing trifluoromethyl group as this group in 2-(trifluoromethyl)aniline, its *para* isomer and heterocyclic analogues exhibit unusual reactivity under basic conditions that may lead to the ionization of the amino group, making the trifluoromethyl group susceptible to hydrolysis and conversion to a carboxylate group or a carbonitrile in an amide ion mediated reaction (Strekowsky *et al.,* 1994). Thus the trifluoromethyl group leads to exclusive reactions of the electronegative β-keto group with the amine, resulting in the formation of mainly **32.1** and **32.2**, although a small quantity of 4–trifluoromethyl-2-quinolinol (**33**) may be formed in accordance with Scheme 1 (Dey and Joullié, 1965), which is easily removed during the precipitation of the desired quinolinol.

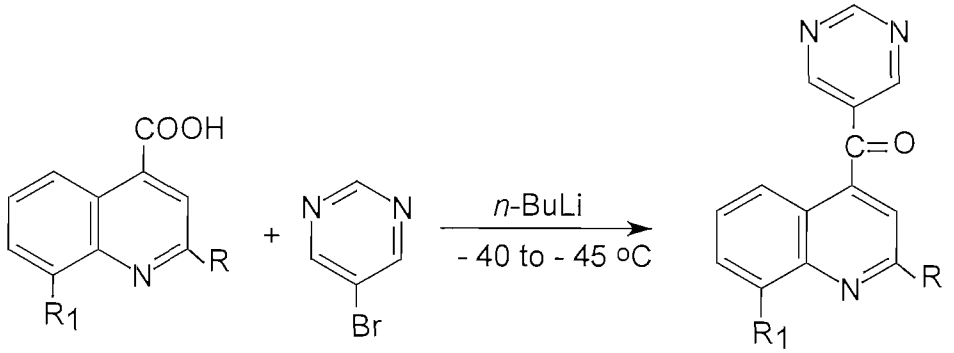
4.3 SYNTHESIS OF TARGET QUINOLINE COMPOUNDS

4.3.1 [2 and 8-trifluoromethyl- and 2,8-bis(trifluoromethyl)quinolin-4-yl]-(pyrimidin-5-yl)methanones

Attachment of the pyrimidine group onto the carbonyl carbon at position 4 of the quinoline ring consists of converting the pyrimidine to a lithium derivative through

treatment of bromopyrimidine with *n*-butyllithium and followed by the condensation reaction between the lithiopyrimidine and any one of the quinoline-4-carboxylic acids at very low temperature as shown in the Scheme 6 below.

Scheme 6



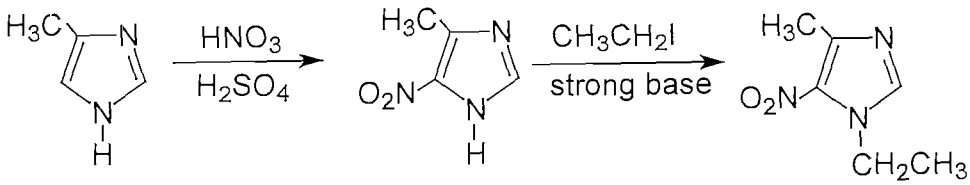
(40): R = H and $R_1 = CF_3$
(44): R = CF_3 and R_1 = H
(45): R = $R_1 = CF_3$

(4.1): R = CF_3; R_1 = H
(4.2): R = H; $R_1 = CF_3$
(4.3): R = $R_1 = CF_3$

4.3.2 2-(1-ethyl-5-nitro-1*H*-imidazol-4-yl)-1-[2-(trifluoromethylquinolin-4-yl], [8-(trifluoro-methyl)quinolin-4-yl] and [2,8-*bis*(trifluoromethyl)quinolin-4-yl)ethan-1-ones

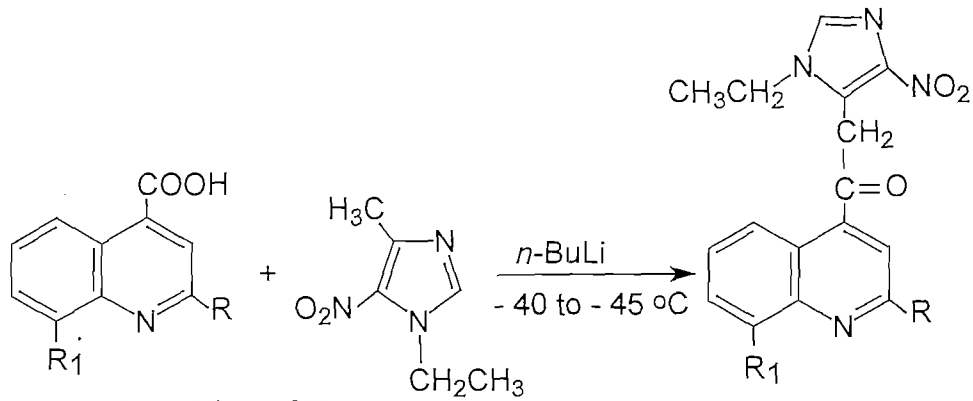
The first step towards the preparation of the above-mentioned target compounds consists of the preparation of N-ethyl-4-methyl-5-nitroimidazole (**46**) through nitrating the 4(5)-methylimidazole and then alkylating the nitro compound with ethylene iodide in the presence of a strong base (Scheme 7). The product, N-ethyl-4-methyl-5-nitroimidazole is finally condensed to a quinoline carboxylic acid using *n*-butyllithium at –40 to –45 °C (Scheme 8).

Scheme 7



(46)

Scheme 8



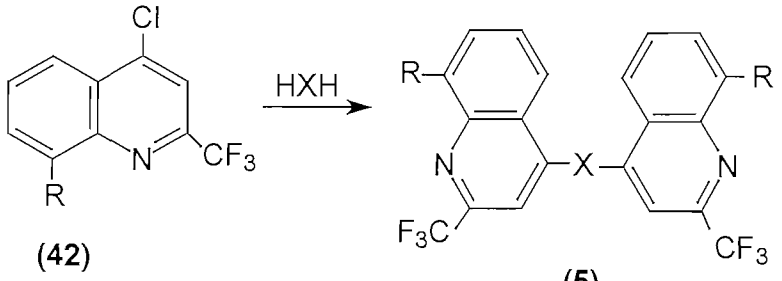
(**40**): R = H and $R_1 = CF_3$
(**44**): R = CF_3 and R_1 = H
(**45**): R = $R_1 = CF_3$

(**4.4**): R = CF_3; R_1 = H
(**4.5**): R = H; $R_1 = CF_3$
(**4.6**): R = $R_1 = CF_3$

4.3.3 N,N-*bis*(trifluoromethylquinolin-4-yl)diaminoalkanes

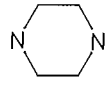
The N,N-*bis*(trifluoromethylquinolin-4-yl)- and *bis*[N,N-*bis*(trifluoromethyl)quinolin -4-yl]diamino alkanes (**8**) are prepared through a displacement reaction at the 4-halo group when the 4-haloquinoline obtained by halogenation with phosphoryl chloride as shown in Scheme 5 is reacted with diaminoalkane or piperazine and triethylamine at a molar concentrations of 2:1:2, and using 2-hydroxyethanol as a refluxing solvent (Scheme 9) (Pearson *et al.*, 1946; Vennerstrom *et al.*, 1998). In view of the difficulty associated with the synthesis of the 4-halo-8-trifluoromethylquinoline derivatives, this series of compounds was not prepared for antimalarial evaluation.

Scheme 9



where R = H or CF_3

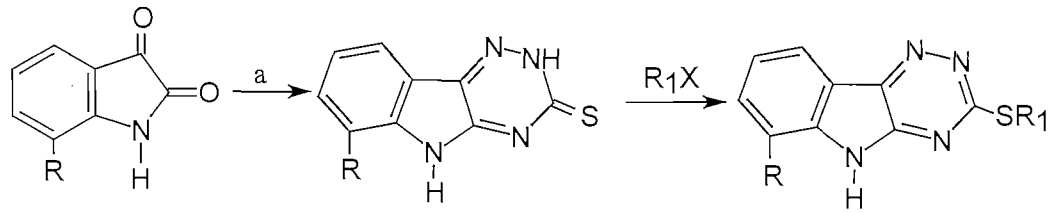
X = $NH(CH_2)_nNH$ and n = 2, 3, 4 or 6

or X = 

4.3.4 The 1,2,4-triazino[5,6b]indole Derivatives

The 1,2,4-triazino[5,6b]indole and their tetrazole derivatives can be prepared by the procedure of Ram (1980) in which compound **36** prepared in accordance with scheme 4 above is condensed with thiocarbazide in a potassium carbonate solution to form the 1,2,4-triazino[5,6b]indole-3-thione derivative (**48**) (Scheme 10). The latter is then alkylated with methyl or ethyl halide or a chlorocyclopropyl-methane to form each of the 3-methylthio, 3-ethylthio and 3- cyclopropylmethyl-thio-1,2,4-triazino[5,6b]indole derivative (**6**). Refluxing **48** or any one of the compounds **6.1** to **6.6** with a mixture of hydrazine hydrate in HCl leads to the formation of 3-hydrazo-1,2,4-triazino[5,6b]indole derivatives (**49**) which on treatment with sodium nitrite in HCl leads to the formation of the 3-azido compound **50**. Either **49** or **50** on reaction with each of formic, acetic, trifluoroacetic or chlorobenzoic acids leads to the series of fused tetrazole compounds, 5*H*-1,2,4-triazolo[1',5',2,3]-1,2,4-triazino[5,6b]indole derivatives (**7.1** to **7.8**) (Scheme 11).

Scheme 10

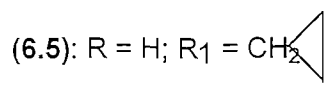


(36)
R = H or CF_3

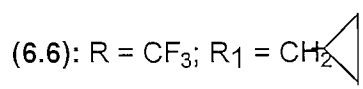
(48.1) R = H
(48.2) R = CF_3

(9)

(6.1): R = H; R_1 = CH_3
(6.2): R = CF_3; R_1 = CH_3
(6.3) : R = H; R_1 = CH_2CH_3
(6.4): R = CF_3; R_1 = CH_2CH_3



(6.5): R = H; R_1 =



(6.6): R = CF_3; R_1 =

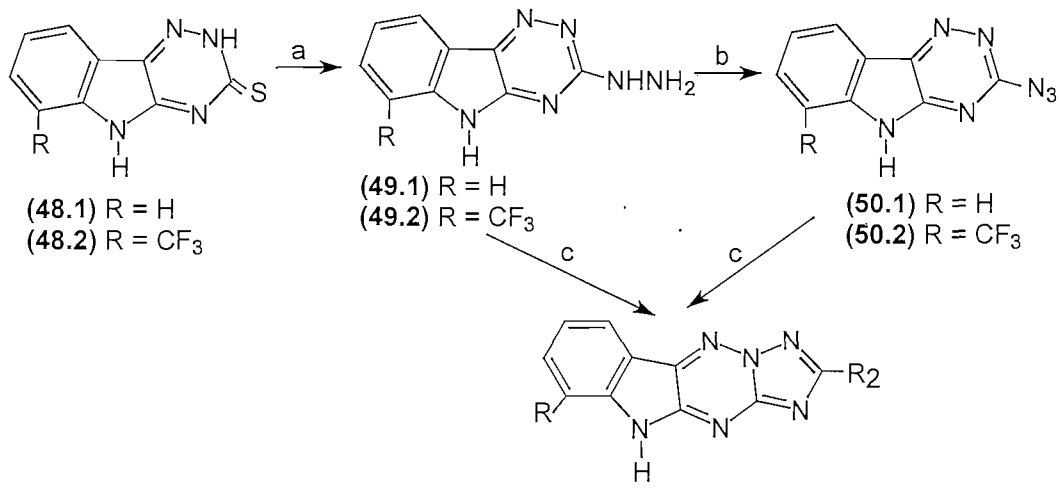
a: $NHCSNHNH_2$

4.4 DETERMINATION OF BIOLOGICAL ACTIVITY

4.4.1 Antimalarial Activity

The *in vitro* antimalarial activity of the new compounds was assessed using the chloroquine-sensitive (D10) and chloroquine-resistant (K1) strains of *P. falciparum.* The chloroquine-resistant K1 is the South African strain of *P. falciparum* that has been isolated from patients who contracted malaria in KwaZulu-Natal. This strain is highly chloroquine resistant. The D10 strains were obtained from a patient in Groote Schuur Hospital. All the *in vitro* tests were carried out in the Department of Pharmacology, Faculty of Medicine, University of Cape Town. Since structure-activity relationships were to be deduced from these investigations, all the compounds were allocated code names in order to prevent analysts and investigators from knowing the structures of the compounds they were investigating.

Scheme 11



(48.1) R = H
(48.2) R = CF_3

(49.1) R = H
(49.2) R = CF_3

(50.1) R = H
(50.2) R = CF_3

(7)

(7.1): R = H; R_2 = H
(7.2): R = CF_3; R_2 = H
(7.3): R = H; R_2 = CH_3
(7.4): R = CF_3; R_2 = CH_3
(7.5): R = H; R_2 = CF_3
(7.6): R = CF_3; R_2 = CF_3
(7.7): R = H; R_2 = C_6H_4Cl
(7.8): R = CF_3; R_2 = C_6H_4Cl

a: N_2H_5OH; b: $NaNO_2$/HCl; c: HCOOH; CF_3COOH; ClC_6H_4COOH

A modification of a known technique (Makler *et al.*, 1993: Makler and Hinrichs, 1993) was used in the assessment of activity of the compounds. This enzymatic colorimetric procedure is based on the observation that parasite lactate dehydrogenase (pLDH) activity is distinguishable from host LDH activity when using the 3-acetyl pyridine adenine dinucleotide (APAD). The LDH enzyme of *P. falciparum* has the ability to rapidly use APAD as a coenzyme in the reaction leading to the formation of pyruvate from lactate. The development of APADH (reduced form of APAD) is thus used as a basis of assay for the rapid detection of the viability of the *P. falciparum* parasites. This procedure differs from the other procedures such as the [G-^{3}H]-hypoxanthine (Desjardins *et al.*, 1979: Webster *et al.*, 1985), Giemsa-stained thick and thin blood films (Rieckmann *et*

al., 1978; Lopez Antuñano and Wernsdorfer, 1979; Wernsdorfer, 1980), candle jar method (Jensen and Trager, 1977), the ^{3}H-leucin uptake (Richards and Williams, 1973), ^{14}C-methionine incorporation into the parasites' protein and lactic acid production (Canfield *et al.*, 1970) and glucose consumption and lactic acid production by the parasites (Cenedella *et al.*, 1970) in that it measures the parasite lactate dehydrogenase (pLDH) activity by using APAD. LDH enzyme of *P. lophurae* can use APAD as a coenzyme, while the LDH activity of *P. falciparum* has been shown to use APAD in the oxidation of lactate (Makler and Hinrichs, 1993).

Parasitaemia is determined by light microscopy using Giemsa-stained thin smears and with light microscopy using the dye, benzothiocarboxypurine. LDH activity is measured spectrophotometrically facilitated by adding a 20:1 mixture of nitroblue tetrazolium (NBT) and phenazine ethosulphate (PES) to the Malstat reagent. As APADH is formed, the NBT is reduced to the blue formazan salt, which can be detected visually and measured at 650 nm (Makler *et al.*, 1993: 739). The parasites' LDH isoenzyme stains intensely whereas the host LDH stains lightly. This difference makes this test a valuable tool in detecting the presence of *P. falciparum* from *in vitro* cultures at parasitaemia levels as low as 0,02% since there is correlation between the level of parasitaemia and the activity of the parasite LDH (Makler and Hinrichs, 1993: 205). The extinction coefficient of APADH is determined spectrophotometrically, or a quantitative interpretation of the parasite growth may be obtained by visual evaluation of the tetrazolium reduction after incubation.

4.4.2 Interaction of the Drugs with DNA

In the past it was believed that the mode of action of quinoline type drugs such as chloroquine was by binding to, intercalating with and stabilising the DNA double helix thus blocking the enzymatic synthesis of the DNA and RNA *in vitro* as well as the biosynthesis of these nucleic acids in susceptible cells (see § 2.2.2). The assessment of the ability of a compound to bind to and intercalate

with the DNA double helix can also be performed *in vitro* by using ethidium bromide-DNA assay studies. Ethidium bromide is an antitrypanosomal agent that is known to bind to and intercalate strongly with DNA (Baguley, 1982; Stewart, 1988). A useful feature of ethidium binding is that its fluorescence is enhanced about 50-fold (depending on the wavelength chosen for the measurement), making it a convenient probe to measure DNA binding of other drug molecules. Confirmation of the intercalative properties of the compounds is conducted through measurements of the displacement of DNA bound ethidium bromide as shown by progressive reduction in the fluorescence as the ethidium bromide is released from its complex with DNA as the drug is added (Olmstead *et al.*, 1975). A bathochromic effect in the wavelength of the ligand in the ultraviolet spectrum is indicative of the intercalation of the compounds with DNA (Mannani *et al.*, 1990). The wavelength absorption band of the ligand that intercalates between the base pairs of DNA undergoes a shift to longer wavelength (Silikas *et al.*, 1996). This shift is believed to be a consequence of an overlap between the π-electrons of the nucleic acid base pairs and the bound ligand chromophore. The intercalative properties of the compounds can also be determined by the thermal denaturation measurements (Panter *et al.*, 1973; Silikas *et al.*, 1996), viscosity studies (Olmstead *et al.*, 1975) or gel electrophoresis (Silikas *et al.*, 1996). The ethidium displacement assay can be used to gain information on the binding site size for a drug, thus forming a method for distinguishing simple intercalating agents from agents that occlude a larger number of base pairs (Baguley, 1982).

Under experimental conditions, the displacement of ethidium from DNA is monitored by recording the changes in the fluorescence when the drugs are added to the ethidium bromide - DNA (calf thymus) complex solution buffered to pH 7 and containing N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulphonate] (HEPES), sodium chloride and EDTA. All the solutions, including the blanks were titrated with microlitre portions of each of the test compounds. The IC_{50}, representing the concentration of the test compounds required to decrease the

fluorescence of DNA-ethidium complex by 50% is determined from the typical titration graphs.

4.4.3 Interaction with Ferriprotoporphyrin IX

It has been pointed out that in aggregated form, the FP serves as a receptor for the majority of the antimalarial drugs. The drugs are thought to coordinate to the FP monomers, blocking the formation of haemozoin and allowing an increased concentration of the haem-antimalarial drug complex to remain in solution resulting in lysis of the parasite (Egan *et al.*, 1994; Dorn *et al.*, 1995) (see § 2.2.1.3). Thus the measurement of the amount of the drug that can complex with the FP serves as indication of the activity of the drug against the parasites.

The experimental procedure consists of titrating solutions of FP in 40% aqueous DMSO and HEPES buffer (pH 7,4) with solutions of the compounds of interest also prepared in aqueous DMSO and HEPES buffer and monitoring the interactions spectrophotometrically. Both the sample and reference solutions are titrated simultaneously with the test compounds.

4.4.4 Cytotoxicity of the Compounds

In order to explore the potential therapeutic implications of these compounds, it may be necessary to evaluate the effect of these compounds on different tumoral and non-tumoral cell lines. The studies can also be used to assess whether the compounds can inhibit the proliferation or growth of tumour cells. Tumour growth or shrinkage is dependent on the proportion of actively proliferating cells in comparison to the dying cells and this may be due to either necrosis (violent cell death) or apoptosis, sometimes referred to as programmed cell death (Efferth *et al.*, 1996; Escribano *et al.*, 2000). Apoptosis may be the primary mechanism of action of anti-cancer activity of several chemotherapeutic compounds (Saki *et al.*, 1999). It is characterized by a two-step formulation of DNA, viz., (a) the cleavage of DNA into 300 and/or 50 kb pair domains by magnesium-dependent nuclease and (b) a facultative step involving internucleosomal degradation into 180 – 200

bp fragments and multiples thereof by a calcium- and magnesium-dependent endonuclease resulting in the classical apoptotic DNA damage (Efferth *et al.*, 1996). The toxicity of compounds on tumour cells is evaluated by examining the intercalative properties of the drugs to the DNA of tumour cells and their inhibitory capacity on topoisomerase I both *in vitro* and in cellular systems (Bailly *et al.*, 1999). Studies to assess the growth inhibitory properties of drugs on proliferating cells may include counting cells that include/exclude a dye after treatment, measuring released ^{51}Cr-labelled protein after cell lysis and measuring incorporation of radioactive nucleotides ([^{3}H]thymidine or [^{125}I]iododeoxyuridine) during cell proliferation (Mosmann, 1983). Viable cells could be measured by using any of several staining methods and colourimetric assay for living cells should utilize a colourless substrate that is modified to a coloured product by the living cell, but not by dead cells or tissue culture medium. The characteristics of some of the dyes that can be used have been reviewed (Skehan *et al.*, 1990). For our purpose, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) has been used as it measures only living cells and can be read on a scanning multiwell spectrophotometer (ELISA)(Mosman, 1983). The standard cultivation conditions consists of cells being grown in suspension culture supplemented with 20% fetal calf serum and maintained in a 5% CO_2 atmosphere at 37 °C.

In this study the assessment of cytotoxicity of the new compounds was conducted against both the tumour and the normal mammalian cells. Human promyelocytic leukemia cells and normal cells were grown as suspension cultures in activated RPMI 16490 media supplemented with 10% decomplemented foetal calf serum. Incubation after treatment with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was then carried out. It is during this period that the viable cells will cleave the tetrazolium ring resulting in the formation of the dark blue formazan crystals. A multiwell ELISA plate reader is used to determine the optical density of the solutions of the crystals. Using a suitable computer programme, calculations of the data and the

corresponding means are done and the results plotted as percentage survival *vs* concentration curves. These curves are then used to determine the IC_{50} of each compound.

To investigate whether the compounds induce apoptosis, internucleosomal DNA fragmentation analysis was conducted according to previous procedures (Efferth *et al.*, 1996; Saeki *et al.*, 1999). Briefly, the procedure consists of allowing leukemic cells to grow under standard cultivation conditions for 10 days. The cell suspension is then treated with propidium iodide and RNase A and after incubation, subjecting the suspension to flow cytometry and recording the fluorescence signals of the cells per histogram. However, it must be pointed out that these measurements of the induction of apoptosis do not provide definite clues as to the specific mechanisms of cytotoxicity of the compounds, i.e. whether through inhibition of enzymes or through DNA damage.

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1 INTRODUCTION

The synthesis of new compounds that elicit envisaged biological activity following rational drug design endeavour is regarded as successful when the relevant characterisation data are in line with expected physicochemical parameters and constants relating to identical molecular structures. For all the new compounds in this study, the data were generated from elemental analysis, ^{1}H and ^{13}C NMR, mass and infrared spectroscopy. The results obtained from these studies are included under the description of method of synthesis of each compound of interest. Comprehensive qualitative data as evidence of the successes achieved in the syntheses of the pyrimidin-5-yl-[2- and 8-(trifluoromethyl)]- and [2,8-*bis*(trifluoromethyl)-quinolin-4-yl]methanones **(4.1 – 4.3)** and the 2-(1-ethyl-5-nitro-*1H*-imidazol-4-yl)-1-[2- and 8-trifluoromethyl- and 2,8-*bis*(trifluoromethyl)-quinolin-4-yl]ethan-1-ones **(4.4 – 4.6)**] outlined in Schemes 8 and 9 are included in the previous reports (Kgokong, 1999; Kgokong and Breytenbach, 2000). On the other hand, complete data obtained during the characterisation of each new compound in the two series of the N,N-*bis*(trifluoromethylquinolin-4-yl)- and *bis*(N,N-*bis*(trifluoromethyl)quino-lin-4-yl]-diamino alkanes **(5.1 – 5.10)** (Scheme 10) and the 1,2,4-triazino[5,6b]indole compounds (**6.1 – 6.6)** and their tetrazole derivatives **(7.1 – 7.8)**] (Schemes 11 and 12) are included under the description of the method of synthesis of each new compound in Chapter 6 in sections 6.1.2 and 6.1.3. The spectra of the synthesised compounds in each of the three series are numbered from 1 to 104 in the § SPECTRA.

Data from elemental analyses of the new compounds for carbon, hydrogen and nitrogen indicate that the experimental values obtained are within 0,4% of the calculated values. Similarly, the alkyl and aromatic or heteroaromatic proton shifts and splitting patterns of each compound are within the expected regions of the ^{1}H NMR and ^{13}C NMR spectra and the carbon atoms from prominent groups such as the trifluoromethyl group in all molecules are clearly reflected by their

splitting into either broad quartets ($^1J_{CF}$ = 300 Hz) and narrow quartets ($^2J_{CF}$ or $^3J_{CF}$ of 27 – 30 Hz). Expected fragmentation sequences and patterns in the electron ionisation mass spectra of the compounds, with appropriate molecular ions where necessary are well exhibited and prominent identifiable groups are also shown in the infrared spectra.

5.2 ANTIMALARIAL ACTIVITY

5.2.1 2- and 8-Trifluoromethyl- and 2,8-*bis*(trifluoromethyl)quinoline derivatives

In the absence of the chloroquine-resistant strain of *P. falciparum* at the time, the assessment of the antimalarial activity of compounds **4.1** to **4.6** was carried out on the chloroquine-sensitive strain (Kgokong and Breytenbach, 1999; Kgokong *et al.*, 2001). The results obtained in that study are indicated as Figure 5.1 and were used to calculate the IC_{50} value of each compound. These are reflected in Table 5.1. Perusal of the data indicates that the new 2- and 8- trifluoromethyl substituted quinoline-type compounds consistently exhibit greater *in vitro* activity against the chloroquine-sensitive strain of the *P. falciparum* than the corresponding compounds with only one trifluoromethyl group at either position 2 or 8 of the ring. Chloroquine sulphate with IC_{50} value of 85 ng/ml was used as positive control. From the data it is clear that both compounds **4.3** and **4.6** with a trifluoromethyl group at both positions 2 and 8 of the quinoline ring, but respectively carrying a pyrimidino and 1-ethyl-5-nitro-1*H*-imidazolo groups at position 4 are slightly more active than compounds **4.1**, **4.2**, **4.4** and **4.5** all of which are substituted with one trifluoromethyl group at position 2 or 8 of the quinoline ring.

Although the quinoline compounds without the trifluoromethyl group are invariably devoid of any antimalarial activity, the new active compounds exhibit much lower *in vitro* activity than mefloquine **(1)** with IC_{50} value of 38,45 ± 2,84 μM (n = 3) under comparable experimental conditions. The role of the alcohol functional group with the associated stereochemical possibilities and the saturated heterocyclic ring system at position 4 of compound **1** as opposed to a

keto group, the lack of stereochemical disposition and unsaturated heterocyclic moieties at position 4 of the new compounds could be responsible for the difference. Additional studies involving the reduction of both the keto group and heterocyclic moieties of these compounds as well as assessment of these compounds and their reduction products on the chloroquine-resistant strains of the *Plasmodium* are necessary to adequately confirm these possibilities.

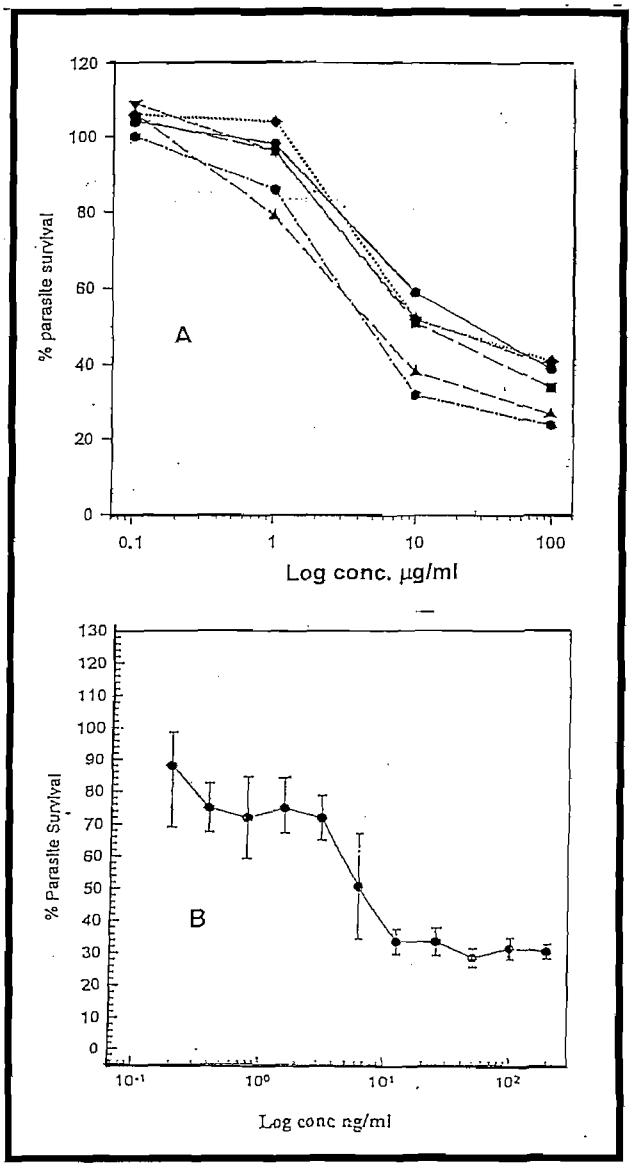
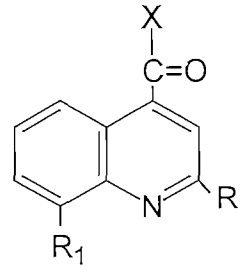
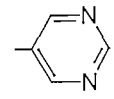
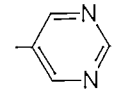
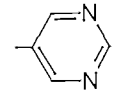
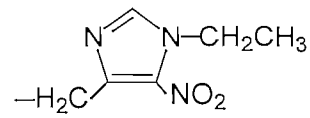
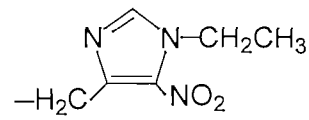
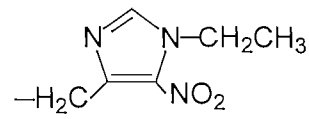


FIGURE 5.1: Effect of the addition of the increasing concentrations of (A) compounds **4.1** (●), **4.2** (■), **4.3** (►), **4.4** (▼) **4.5** (◆) and **4.6** (●) and (B) **3** on the survival of the chloroquine-sensitive strain (D10) of *P. falciparum*.

TABLE 5.1: 2- and 8-Trifluoromethyl- and 2,8-*bis*(trifluoromethyl)quinoline derivatives



Compound	Substituent			IC_{50} (μg/ml)
	R	R_1	X	
4.1	-H	$-CF_3$		11,80
4.2	$-CF_3$	-H		10,50
4.3	$-CF_3$	$-CF_3$		5,20
4.4	-H	$-CF_3$		10,50
4.5	$-CF_3$	-H		10,50
4.6	$-CF_3$	$-CF_3$		4,80
Chloroquine (3)				85 ng/ml

A definite pattern, albeit a small one, is reflected by the data in Table 5.1 with regards to the antimalarial activity of the compounds in this series. Compounds **4.3** and **4.6** both with trifluoromethyl groups at both positions 2 and 8 have a slightly higher *in vitro* activity against the chloroquine-sensitive strain of *P. falciparum* than the mono trifluoromethyl substituted quinolines, with IC_{50} values

of 4,80 and 5,20 μg/ml for the (1-ethyl-5-nitro-1*H*-imidazol-4-yl)-1-ethan-1-one and pyrimidin-5-yl methanone respectively. The mono trifluoromethyl substituted quinolines **(4.1**, **4.2**, **4.4** and **4.5)** have IC_{50} values more or less the same order of magnitude. The low antimalarial activity of the 8-trifluoromethylquinoline derivatives supports earlier findings that the 8-trifluoromethylquinolones show a significant drop of *in vitro* antibacterial activity when compared to the 6,8-difluoroquinolones (Sanchez *et al.*, 1992). However, unlike mefloquine **(1)** to which these compounds are structurally related, these compounds show a much lower *in vitro* antimalarial activity against the chloroquine-sensitive strains of the *P. falciparum*. Compound **1**, which is reported to interact with the membrane phospholipids (Chevli and Fitch, 1982), is very active against the chloroquine-resistant strains. However, due to the unavailability of this strain of *P. falciparum* at the time of this particular study, the activity of the new compounds was not evaluated against this particular strain. In this case the trifluoromethyl group acts as acceptor in the formation of charge-transfer complexes with DNA and thus contribute to antimalarial action (Cheng, 1971).

The study does not show any discernible difference which may be caused by the difference in the nature and size of the functional moiety at position 4 of the quinoline ring as reflected by the *in vitro* data from the pyrimidino and (1-ethyl-5-nitro-1*H*-imidazol-4-yl)-1-ethan-1-one compounds. This could be ascribed to the planarity of both heterocyclic substituents. The antimalarial activity of most quinoline derivatives carrying a piperidine ring is completely abolished when this ring is substituted with a planar pyridine ring (Cheng, 1971). Once introduced on the aromatic ring systems, the high C-F bond energy tends to make the compound resistant to metabolic transformation (Welch, 1987). The fluorine atom seems to cause a tremendous boost in the minimum inhibitory concentration of some quinolone antibacterials, a phenomenon thought to be due the improvement in the drug-gyrase DNA complex binding as well as the increase in cell penetration (Domagala *et al.*, 1986).

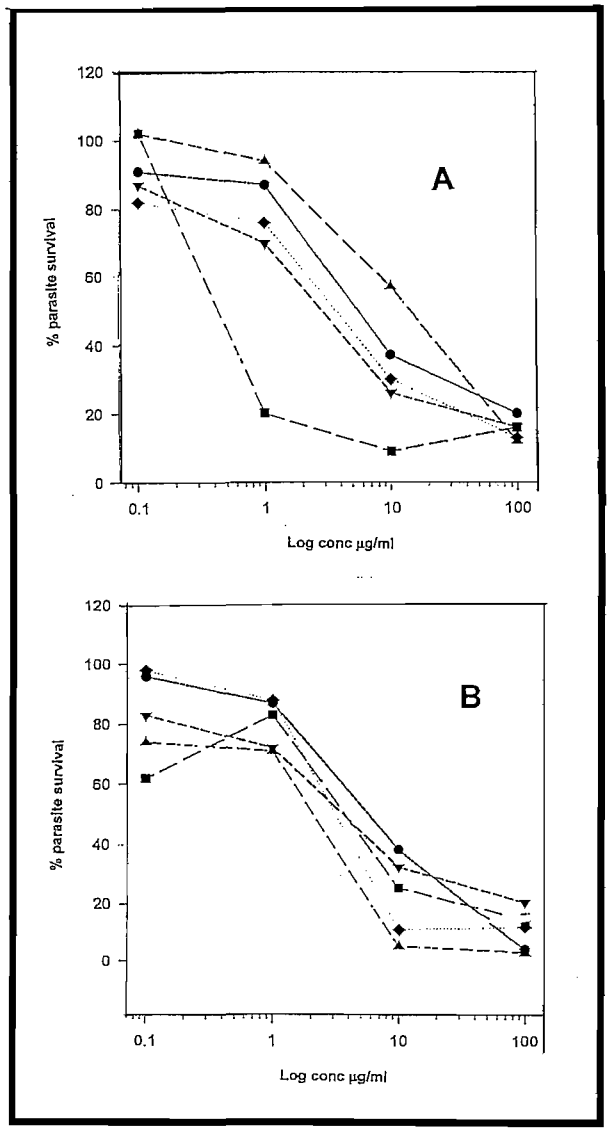


FIGURE 5.2: Dose-response curves in A are for compounds **5.1** (●), **5.2** (■), **5.3** (▲), **5.4** (▼), **5.5** (◆) and in B for **5.6** (●), **5.7** (■), **5.8** (▲), **5.9**(▼) and **5.10** (◆) when the chloroquine-sensitive strain of the *P. falciparum* are exposed to these compounds.

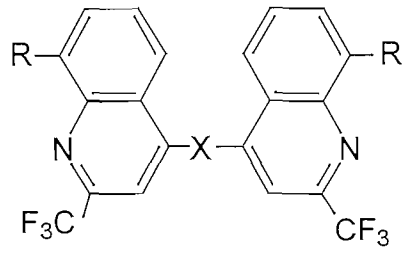
5.2.2 N,N-*Bis*(trifluoromethylquinolin-4-yl)-diamino alkanes

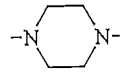
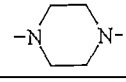
Figures 5.2A and B were use to calculate the IC_{50} values as reflected in Table 5.2, of a series of ten N,N-*bis*(trifluoromethylquinolin-4-yl)- and N,N-*bis*[2,8-*bis*(trifluoromethyl)quinolin-4-yl]diamino alkanes and piperazines compounds **5.1 – 5.10**. These data were obtained from studies where the antimalarial activity of this series of compounds was evaluated against the chloroquine-sensitive (D10)

and chloroquine-resistant (K1) strains of *P. falciparum* using the lactate dehydrogenase activity procedure. The results indicate that against the chloroquine-sensitive strains, the compounds exhibit moderate activity when compared to their activity against the chloroquine- resistant strains of *P. falciparum*. However, when compared to **1** under identical experimental conditions, the compounds are shown to exhibit much lower antimalarial activity. While the activity of the majority of these compounds is essentially of the same order of magnitude, compounds containing the trifluoromethyl groups at both positions 2 and 8 and the diaminoalkyl bridging chains of 2 to 6 carbon atoms are consistently more active than those with a single trifluoromethyl group at position 2 of the quinoline ring.

Compound **5.2** is the most active against the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* with IC_{50} values of 500 and 100 ng/ml respectively. These results are comparable to those of mefloquine under identical experimental conditions. Compounds **5.4**, **5.6** and **5.8** with bridging diaminoalkyl chain of three to six carbon atoms and trifluoromethyl groups at positions 2 and 8 are equally more active than those in which the quinoline ring contains a single trifluoromethyl group at position 2. It was also shown earlier that quinoline compounds with a trifluoromethyl group at position 8 only show initial inhibition followed by stimulation of cell growth in a dose-dependant manner (Kgokong and Wachira, 2001). Compounds **5.9** and **5.10** with the piperazino linking bridge exhibit reduced antimalarial activity than those compounds with a diaminoalkyl bridging chain against the chloroquine-sensitive strains, although compound **5.10** with trifluoromethyl groups at positions 2 and 8 show comparable activity to those with diaminoalkyl bridging chains. It was confirmed earlier (Hahn *et al.*, 1966; Bass *et al.*, 1971) that when the substituent in position 6 of the quinoline ring is either decreased in relative electronegativity, or increased in volume, the antimalarial potency is decreased as these structural changes are likely to decrease the affinity for the 2-amino group of the guanine in the DNA molecule or are likely provide steric hindrance to intercalation. Introduction of one or two fluorine atoms into the phenolic moiety of the aminoquinolines results in a significant decrease in relative electronegativity, or increase in volume, the antimalarial potency is decreased as these structural

TABLE 5.2. The *in vitro* IC_{50} values (μg/ml)of the N,N-*bis*quinolin-4 yl derivatives



Compound	Substituent		IC_{50} (μg/ml)	
	R	X	Chloroquine-sensitive	Chloroquine-resistant
5.1	-H	$-HN(CH_2)_2NH-$	6,00	ND
5.2	$-CF_3$	$-HN(CH_2)_2NH-$	0,50	0,10
5.3	-H	$-HN(CH_2)_3NH-$	13,00	> 100
5.4	$-CF_3$	$-HN(CH_2)_3NH-$	6,00	1,20
5.5	-H	$-HN(CH_2)_4NH-$	4,00	35,30
5.6	$-CF_3$	$-HN(CH_2)_4NH-$	2,10	1,50
5.7	-H	$-HN(CH_2)_6NH-$	3,80	> 100
5.8	$-CF_3$	$-HN(CH_2)_6NH-$	3,30	1,60
5.9	-H		10,70	> 100
5.10	$-CF_3$		12,00	1,02
Mefloquine (1)				0,145
Chloroquine (3)			0,085	113,00

ND = not done

changes are likely to decrease the affinity for the 2-amino group of the guanine in the DNA molecule or are likely provide steric hindrance to intercalation. Introduction of one or two fluorine atoms into the phenolic moiety of the aminoquinolines results in a significant decrease in drug potency in the chloroquine-sensitive isolates, but no real effect on the potency against the chloroquine-resistant isolates in comparison with aminoquinolines (Welch, 1987).

It has been noticed (Raynes *et al.*, 1995) that changes in the length of the diaminoalkyl bridging chain have little influence on activity against chloroquine-sensitive strain of *P. falciparum* but profound influence on activity against the chloroquine-resistant strain of the parasite. The linker chain between the two

quinoline ring systems in the new compounds were limited to six carbon atoms. There is an indication that the optimal chain length for the linker group between the two quinoline rings should be 6 carbons, and that linking through the 8-position is better than through the 6 - position and that a chlorine substituent is not necessary for activity. Similarly, there is speculation that the quinoline N-1 atom and the hetero-atom (N or O) of the hetero-aryl group in most of the active 2-heteroarylquinolines are favourably positioned to form specific hydrogen-bonded complexes with a biological receptor (Strekowsky *et al.*, 1991). A strong conjugation effect of the 4-amino group with the quinoline would increase the stability of such a complex by increasing electron density at the quinoline nitrogen atom. The lack of the activity against the chloroquine-sensitive strain of the *Plasmodium* may be due to the absence of primary amino groups on the piperazine bridge to assist in the accumulation of these compounds in the acid food vacuoles. The presence of the imino hydrogen atoms in these compounds as compared to the newly synthesised compounds in this study, could account for the activity of these compounds. The high potency of the S,S enantiomer of compound 16 as opposed to the R,R enantiomer indicate that the transport factors affecting the compound's ability to accumulate in the parasite's food vacuole may play a role (Ridley *et al.*, 1997; Vennerstrom *et al.*, 1997). These two isomers differ in their abilities to inhibit chloroquine resistant parasite growth. However, these types of bisquinolines have potent activity against the chloroquine-resistant strains of *P. falciparum.* However, it was mentioned in § 3.2.1 that the phototoxicity hampered further development of these isomers.

There is also an observation that the bisquinolines with alkyl bridges of three, four or twelve carbon atoms are inactive, while those with bridges of five and nine carbon atoms are active, with a methyl substituent in the bridge improving the antimalarial activity (Vennerstrom *et al.*, 1992). In addition it was inferred earlier that the aminoalkyl chain is a requirement for strong antiplasmodial activity because of its ability to assist in the accumulation of the drug in the food vacuoles and the larger changes in the diaminoalkyl chains are responsible for overcoming chloroquine resistance, with little or no effect against the chloroquine-sensitive strains.

5.2.3 1,2,4-Triazino[5,6b]indole derivatives

Figures 5.3 to 5.6 represent the percentage viability of the parasites *vs.* logarithmic function of concentrations (mg/ml) of the unsubstituted and the 6-trifluoromethyl-1,2,4-triazino[5,6b]indole and tetrazole analogues that were evaluated for *in vitro* antimalarial activity against the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum.* From these curves, the IC_{50} values of the compounds were estimated and the results summarised in Tables 5.3a and 5.3b, it is clear that the CF_3 group tends to lead to an increased *in vitro* antimalarial activity of the 1,2,4-triazino[5,6b]indole albeit to a smaller degree in some compounds. Analogues without the trifluoromethyl group such as **48.1**, **49.1** and **50.1** which were evaluated simultaneously with others in this study, are all devoid of antimalarial activity even at concentrations as high as 400 μM. The increased activity resulting from the presence of this group could be ascribed to the increased lipophilicity of the compound, as this group is known to be more hydrophobic than even the fluorine atom (Smart, 1995). In this series, the size of the substituent on the thiol group does not have significant effect on *in vitro* antimalarial activity. Replacement of a thiol group by a hydrazino and an azido group in the trifluoromethyl substituted derivatives **50.2** leads to compounds with half the potency of those having alkyl groups attached to the sulphur atom.

On the other hand, the activity of the 6-trifluoromethyl-5*H*-1,2,4-triazolo[1',5',2,3]-1,2,4-triazino[5,6b]indole series **(7.1 – 7.8)** (Table 2) is almost identical to those of the 6-trifluoromethyl-1,2,4- triazino-[5,6b]indole-3-alkylthiols, with the exception of compound 7.4 containing the 6-CF_3 and the 3-CH_3 groups. The latter compound exhibits a fourfold improvement in antimalarial activity against the chloroquine-sensitive strain. However, introduction of a second trifluoromethyl group into the ring particularly at position 3 (compound **7.6**) tends to lead to compounds with reduced or diminished activity. Further consideration of the structures of compounds in this series when taken together with the antimalarial activity they exhibit as measured by the IC_{50} values indicate that compounds without the 6-CF_3 such as **7.3** (R = H and R_1 = CH_3) are the most active against the chloroquine-sensitive strain followed by **7.1** (R = H and R_1 = H).

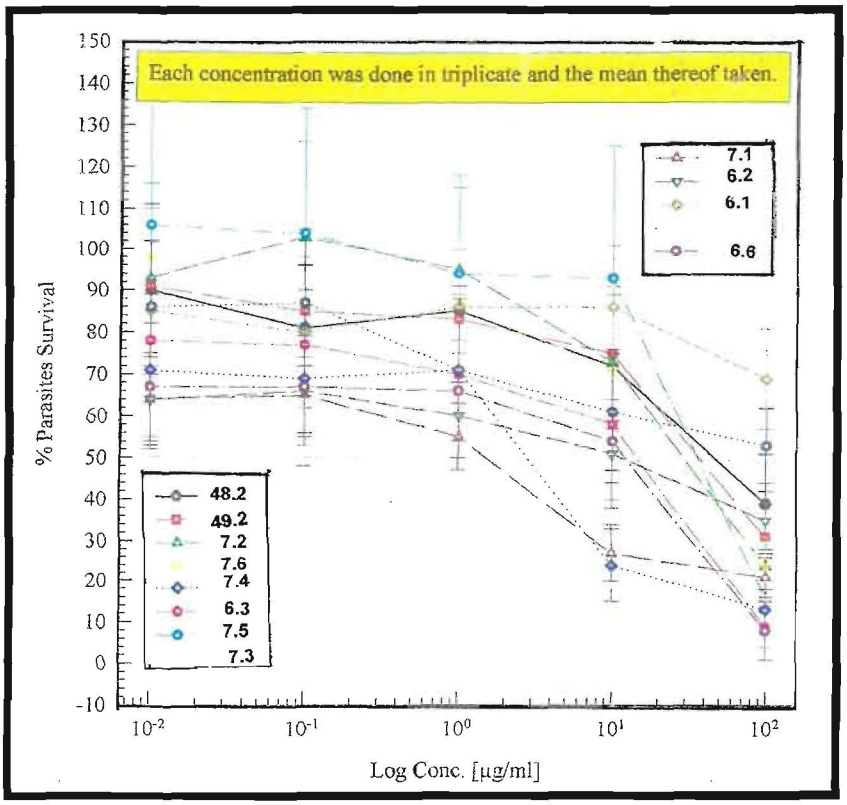
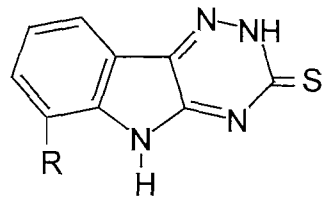


FIGURE 5.3: Dose-response curves obtained when the chloroquine-sensitive strain of *P. falciparum* were treated with the 1,2,4-triazino[5,6b]indole derivatives.

From Figures 5.3 and 5.4 and Table 5.4 it is clear that compound **7.3** exhibits activity against the chloroquine-sensitive strains in the same molar range as the well-established compounds such as **1** and **3** under identical experimental conditions. The structure of this compound indicates that it does not contain a trifluoromethyl, but a methyl group at position 3 of the tetrazole. On the other hand, compound **7.1** is the most active against the chloroquine-resistant strains of *P. falciparum*. Both CF_3 and C_6H_4Cl groups at position 3 without CF_3 at position 7 lead to relatively inactive compounds **7.7** and **7.8**.

The fact that the *P. falciparum* lactate dehydrogenase (PfLDH) activity can be distinguishable from the host LDH (Menting *et al.*, 1997) by using the 3-acetyl pyridine adenine dinucleotide analogue of nicotinamide adenine dinucleotide

TABLE 5.3a: *In vitro* IC_{50} values (μM) of the 7-trifluoromethyl-1,2,4-triazino-[5,6b] indole derivatives



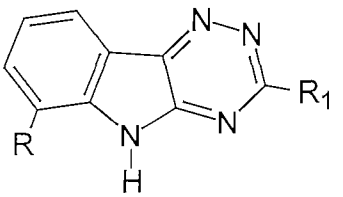
Compound	Substituent	Melting point, °C	IC_{50} (mg/ml)	
			Chloroquine-sensitive	Chloroquine-resistant
48.1	-H	324 - 326, decomposes	> 100	ND
48.2	$-CF_3$	262 - 264	260,00 ± 10	ND

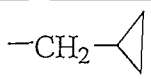
ND = not done

(APAD) has afforded an opportunity for the development of an enzymatic method for the evaluation of antimalarial compounds (Makler *et al.*, 1993).

The compounds selected for screening against the chloroquine-resistant strain of *P. falciparum* show identical but much higher *in vitro* activity than against the chloroquine-sensitive strain, with the exception of compound **7.1** which has IC_{50} value of 48.0 μM which is very close to that of **3** (IC_{50} of 277.5 μM) against this strain. At position 3 the CF_3 group does not lead to any improvement on the *in vitro* antimalarial activity as it does when it is attached at position 6. The 3,6-*bis*(trifluoromethyl)-5*H*-1,2,4-triazolo[1',5',2,3]-1,2,4-triazino[5,6b]indole **(7.6)** has a much lower activity against both the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* than the other 6-CF_3 substituted derivatives (Tables 5.3 and 5.4). As result of a direct relationship between the level of drug accumulation in the parasite food vacuole and antimalarial drug potency, but no simple relationship between accumulation and neither pKa nor lipophilicity (Constantinidis and Satterlee, 1988; Hawley *et al.*, 1996; 1998; Egan *et al.*, 1998), it could be inferred that the bulkiness of the molecule conferred by the second trifluoromethyl group will affect the relative membrane permeability of the molecule, leading to reduced drug accumulation within the parasite food vacuole.

TABLE 5.3b: *In vitro* IC_{50} values (μM) of the 7-trifluoromethyl-1,2,4-triazino-[5,6b]indoles



Compound	R	R_1	Melting point, °C	IC_{50} (μg/ml)	
				Chloroquine-sensitive	Chloroquine-resistant
6.2	$-CF_3$	$-SCH_3$	168 – 170	35,0 ± 5	14,00 ±2
6.4	$-CF_3$	$-SCH_2CH_3$	244 – 246	44,00 ± 8	20,00 ± 5
6.6	$-CF_3$		259 – 260	62,00 ± 10	20,00 ± 2
49.1	-H	$-NHNH_2$	280 - 283	ND	ND
49.2	$-CF_3$	$-NHNH_2$	279 – 281	187,00 ± 10	ND
50.1	-H	$-N_3$	299 – 300, decomposes	> 100	ND
50.2	$-CF_3$	$-N_3$	208 – 213	108,00 ± 10	ND

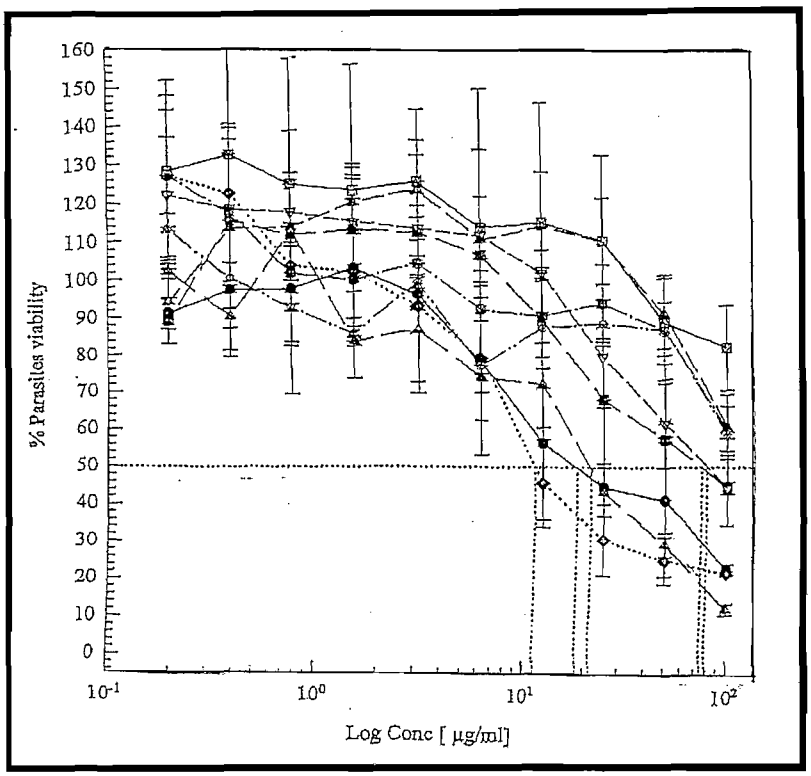
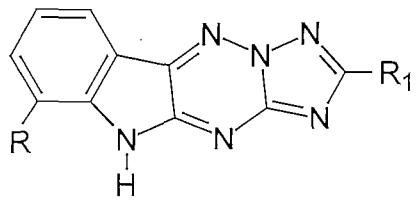


FIGURE 5.4: Dose-response curves obtained when the chloroquine-sensitive strain of *P. falciparum* is exposed to increasing concentrations of compounds **6.2** (●), **6.4** (□), **51** (▲), **7.1** (●), **7.2** (◆), **7.3**(⬟), **7.4** (▲) and **7.5** (■).

TABLE 5.4: *In vitro* IC_{50} values (μM) of the 1',2',4'-triazolo[1',5',2,3]-1,2,4-triazino [5,6b]indole derivatives



Compound	R	R_1	Melting point, °C	IC_{50} μM	
				Chloroquine-sensitive	Chloroquine-resistant
7.1	-H	-H	372 - 374	7,10 ± 1,2	48,0 ± 0,05
7.2	$-CF_3$	-H	236 - 238	40,00 ± 10	ND
7.3	-H	$-CH_3$	401 - 402	36,00 ± 1,30	20,10 ± 1,50
7.4	$-CF_3$	$-CH_3$	241 - 244	86,00 ± 1,20	26,00 ± 2,10
7.5	-H	$-CF_3$	398 - 400	54,00 ± 10	ND
7.6	$-CF_3$	$-CF_3$	263 - 265	142,00 ± 12	ND
7.7	-H	$-C_6H_4Cl$	376 - 378	312,00 ± 32	ND
7.8	$-CF_3$	$-C_6H_4Cl$	286 - 288	46,00 ± 8	ND
3				0,086 ± 0,71	277,5 ± 41,0
1				0,38 ± 0,28	0,81 ± 0,036

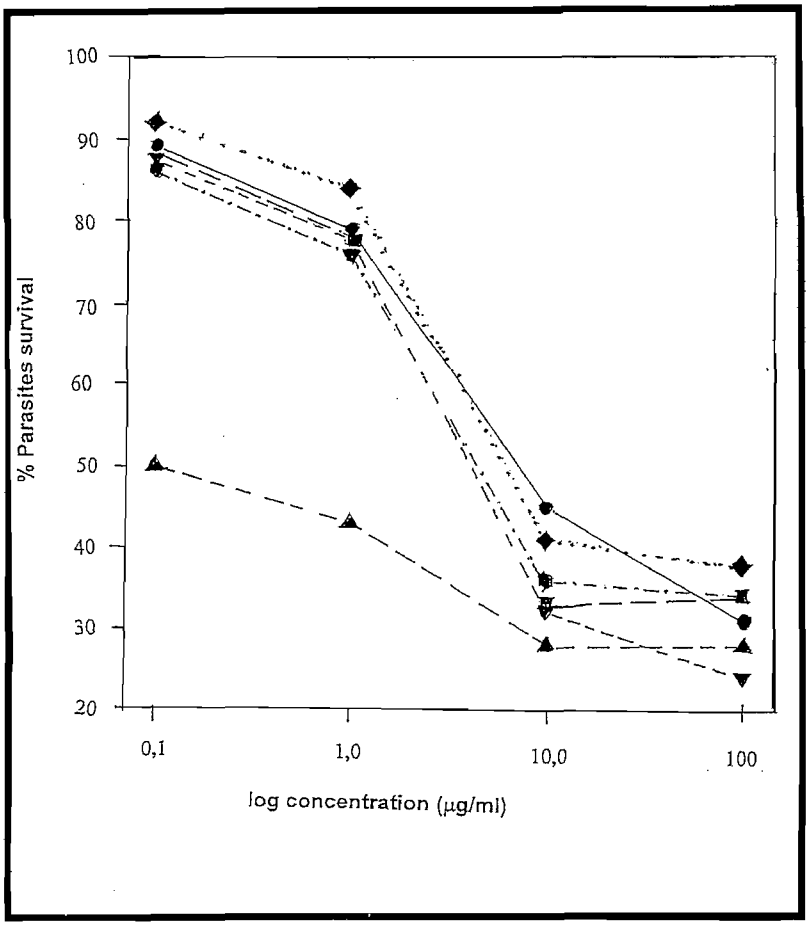


FIGURE 5.5: Dose-response curves from exposure of the chloroquine-resistant strain of *P. falciparun* to increasing concentrations compounds **6.1** (▲),**6.3** (■),**6.4** (●), **7.2** (▼),**7.4** (⬟) and **7.6** (◆),

mechanisms that may include physical interaction of the ketone group of the compound with the DNA phosphate groups.

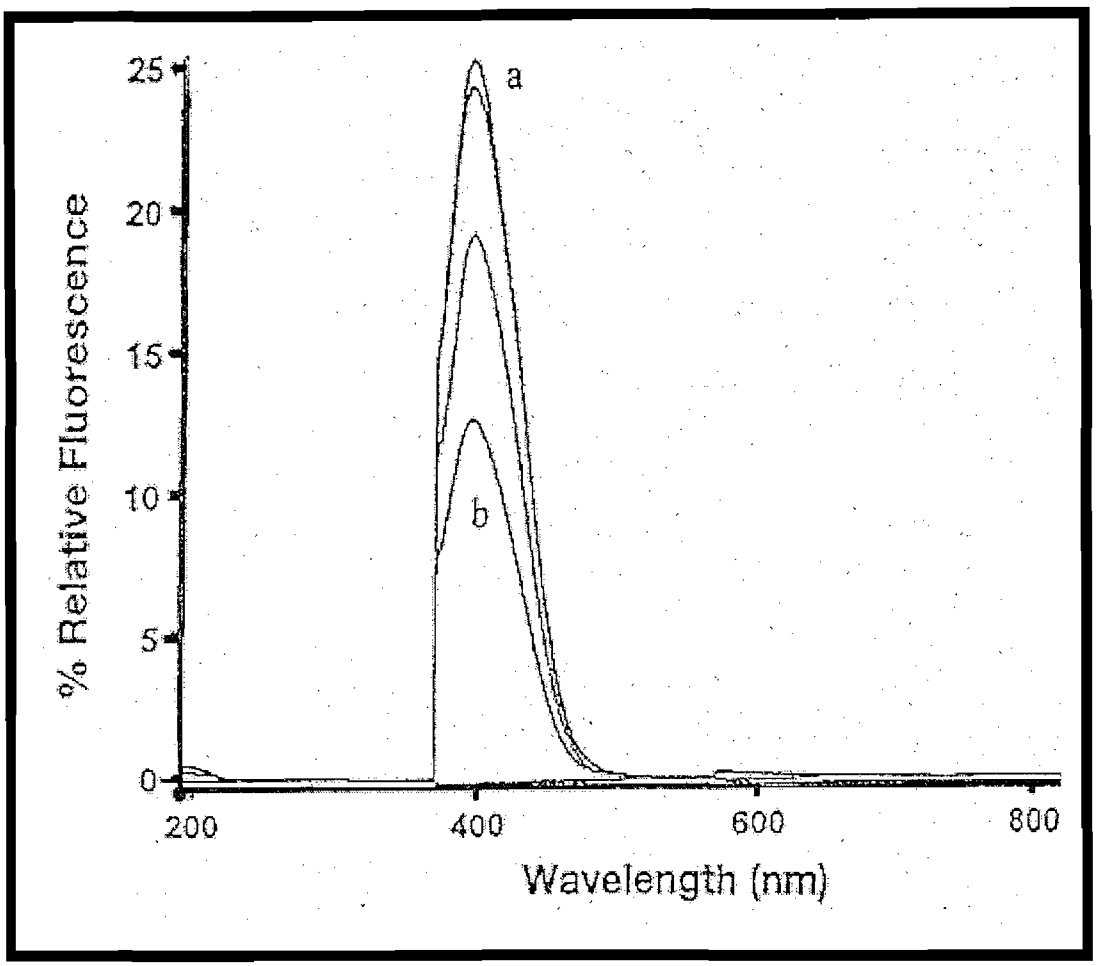


FIGURE 5.7: Ethidium bromide displacement from its complex with DNA by increasing concentration of **4.6**. a: DNA solution saturated with ethidium bromide, and b: increasing concentration of **4.6**.

The pharmacological mechanism of action of these compounds, especially **4.3** to **4.6** appears to be based on the formation of intercalative complexes with DNA. This is shown by their ability to displace ethidium from its complex with DNA and hypochromic and bathochromic shifts shown in the absorption maxima similar to that of quinine as shown in the UV spectra (Figure 5.9). The absorbance peak shifts indicate the formation of a complex between the compound and DNA, while the decrease in the relative fluorescence at higher compound concentration is

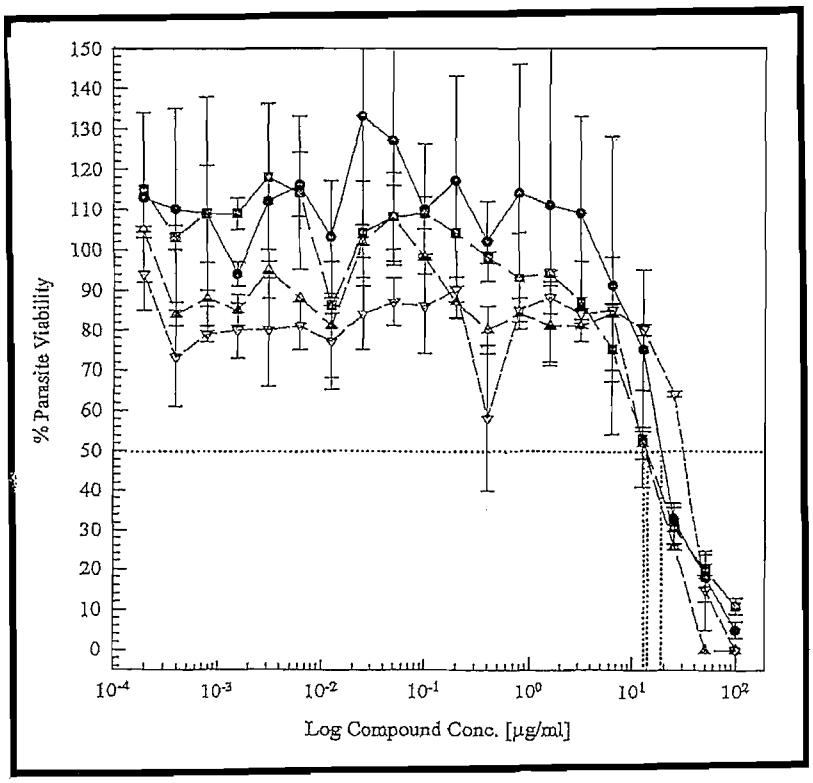


FIGURE 5.6: Dose response curves obtained when the chloroquine-resistant strain of *P. falciparum* was treated with compounds **7.5** (■), **7.6** (●),**7.7** (▲) and solvent control (▽).

5.3 BINDING OF THE COMPOUNDS TO DNA

The binding of substances to DNA is determined by measuring the displacement of DNA bound ethidium bromide. The relative reduction in fluorescence enhancement due to the release of ethidium bromide from DNA complex provides an approximate measure of the binding strength for a variety of substances. As can be observed from Figure 5.7, a 50% reduction in the fluorescence of ethidium bromide/DNA complex was achieved with compound **4.6** and to a similar extent with **4.3** (not shown in the Figure). No discernable reduction is noticed with **4.1** and **4.2**, indicating that these compounds do not bind to DNA to an appreciable extent. Figure 5.9 shows to what extent the new compounds act by intercalation to DNA double helix when their absorption spectra were compared from studies conducted in the presence and absence of excess amount of DNA. Chloroquine was used as standard. Unlike compound **1**, these compounds seem to bind to DNA by intercalation or some other binding

due to the aggregation of the molecules, showing that the molecules of the compound have internal binding property with DNA (Sivaraman *et al.*, 1995).

Ligand rigidity plays a major role in determining sequence specificity, the mode of interaction with DNA and antitumor activity (Ginsburg and Stein, 1991). Thus DNA binding compounds make use of complementary side- chain interactions with AT and GC base pairs to promote selectivity and tight DNA complexation (Ollis *et al.*, 1987). On binding to DNA intramolecular hydrogen bonds break and are replaced by intermolecular hydrogen bonds between the cyclic peptitides and DNA base pairs (Harding, 1992). Binding of the quinoline derivatives to the DNA bases appear to occur predominantly at positions 2, 3 and 4 of the nucleus which are then made available for the new hydrogen-binding partners by the action of the enzyme DNA gyrase (Tillotson, 1996).

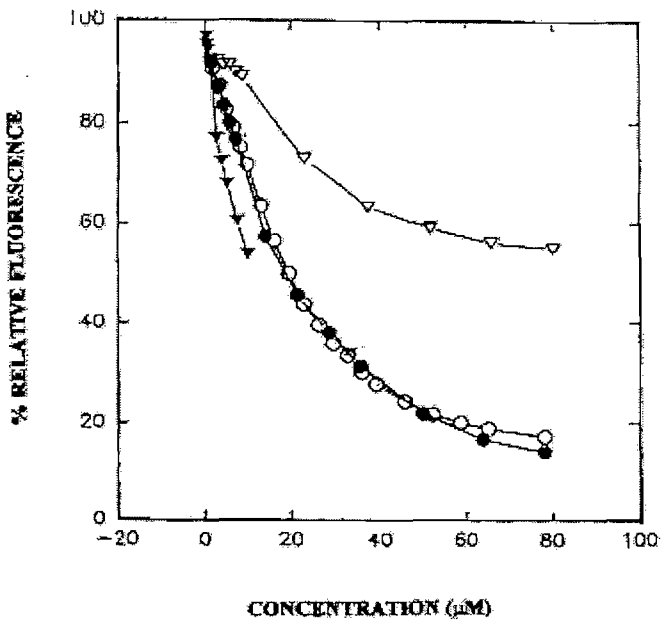


FIGURE 5.8. Ethidium bromide displacement from DNA by compounds **4.6** (▼), **4.3** (●),**4.5** (○) and **4.2** (▽).[DNA] = 3.0 μM and [ethidium bromide] = 1,26 μM. The results are the means of duplicate determinations

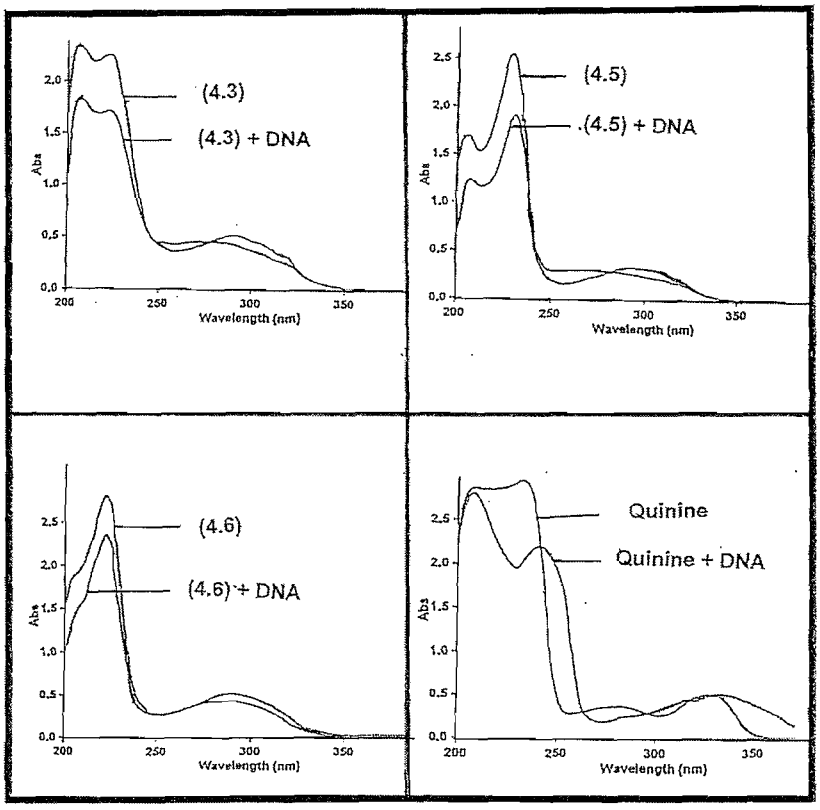


FIGURE 5.9: Effects of DNA on the absorbance of compounds **4.3**, **4.5**, **4.6** and quinine used as a comparator. In each case approximately 0,06M of compound and 5 μM of DNA were used.

The concentration of the drug required to decrease the fluorescence of an ethidium-DNA mixture by 50% is defined as the EC_{50} value. These values can be used as an approximate measure of the binding strength for a wide variety of organic compounds (Baguley, 1982). In this assay, the fluorescence due to DNA-intercalated ethidium is decreased through the addition of increasing concentrations compounds **4.2, 4.3**, **4.5**, and **4.6**. The results from this titration are shown on Figure 5.8. Analyses involving ethidium displacement from DNA provide rapid, readily reproducible measures of drug-DNA binding capacity and require only small amounts of both the drug and DNA (Cain *et al.*, 1978). Scrutiny of Figure 5.8 shows that compound **4.6** binds better to DNA than all the other compounds, while **4.3** and **4.5** seem to have equal binding strengths. This is shown by the fact that this compound achieves a stronger reduction in fluorescence than other compounds. Compound **7.2** binds only moderately to DNA, compounds **4.3** and **4.5** display more or less the same ethidium

displacement capacity but **4.1** and **4.4** do not bind to and intercalate DNA at all as there is no discernable reduction in fluorescence. of the compound and 4 μM of DNA were used.

The decrease in the relative fluorescence at higher compound concentration could be due to the aggregation of molecules, indicating that the molecules of the compound have internal binding property with DNA (Sivaraman *et al.*, 1995). This binding process is through intercalation as shown by the appropriate shifts in absorbance spectra. These shifts indicate the formation of complex between the compound and DNA. Figure 5.9 clearly indicates the bathochromic and hypochromic effect of addition of DNA on the spectra of compounds **4.3, 4.5** and **4.6** as well as quinine, which was used as control.

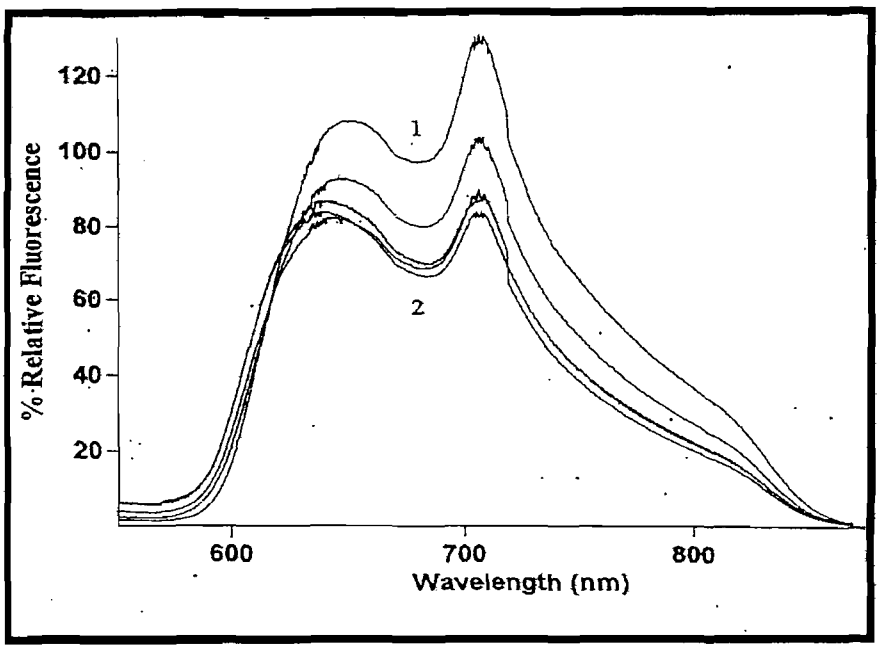


FIGURE 5.10a: Spectrophotometric titration of DNA-ethidium bromide complex with compound **6.1**. Similar results were obtained with other compounds. In each case, 1 is the peak for DNA saturated with ethidium bromide solution and peak 2 is after addition of increasing concentrations of **6.1**.

The results of the spectrophotometric titration of DNA-ethidium bromide complex with 1,2,4-triazino[5,6b] indoles and tetrazoles are shown on Figures 5.10a – 5.10e. As a result of the similar appearance of the spectra it can be concluded

that the compounds displace ethidium from its complex with DNA to a more or less similar extent. However, it will be noticed that Figure 5.10d indicates a sharp drop in %T from 130 to 40 with addition of a drop of compound **7.3,** implying that this compound causes immediate intercalation and precipitation of the DNA. Although the experiments reported here are not sufficient to establish a binding mechanism for these compounds, the slight spectral shifts obtained on the DNA titration support the intercalation into or formation of a complex with the DNA helix as their mode of binding (Panter *et al.*, 1973; Siviraman *et al.*, 1995).

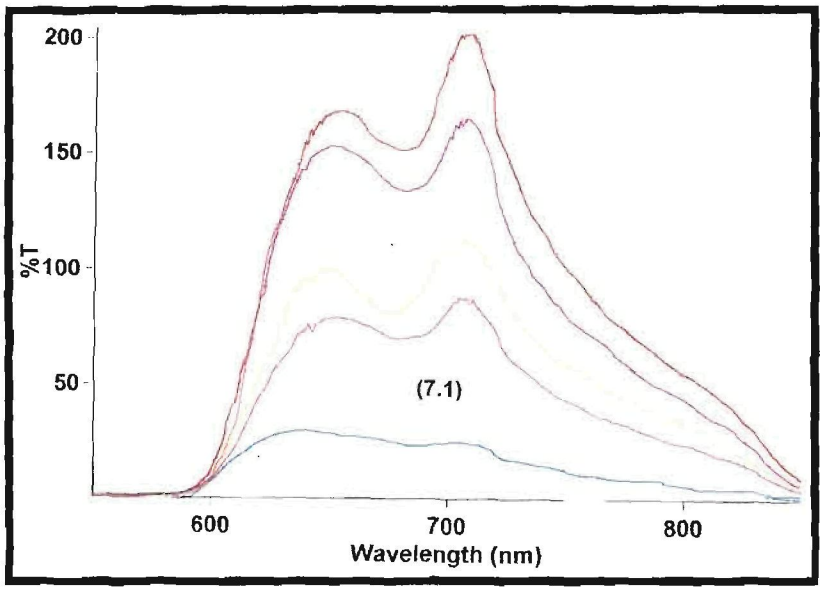


FIGURE 5.10b: Spectrophotometric titration of the DNA-ethidium complex with compound **7.1**.

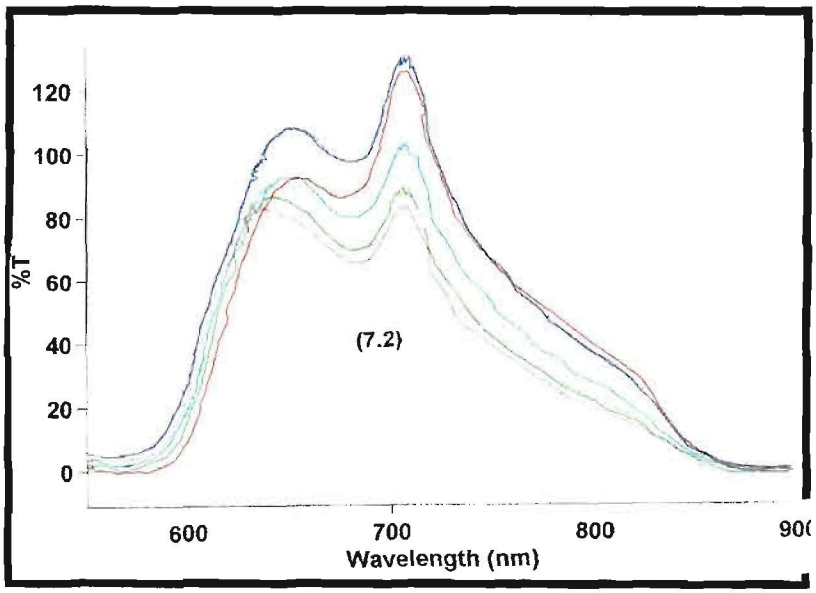


FIGURE 5.10c: Spectrophotometric titration of DNA-ethidium bromide complex with compound **7.2**.

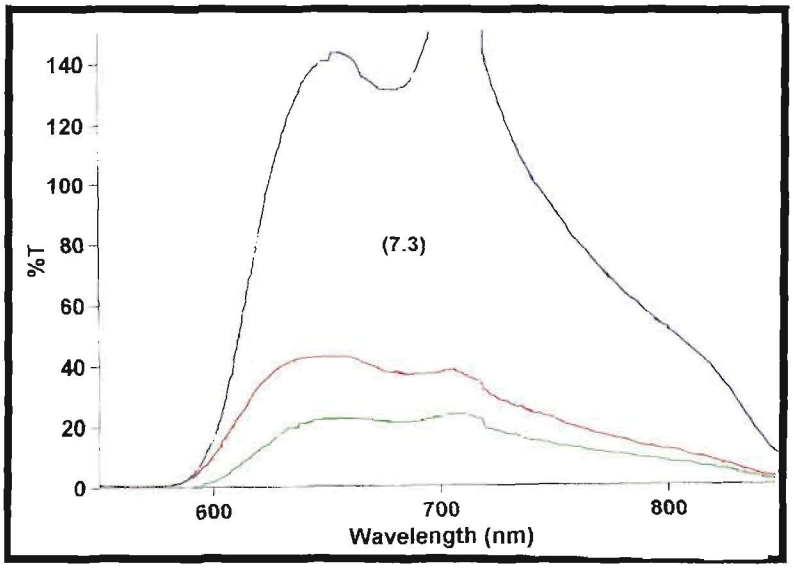


FIGURE 5.10d: Spectrophotometric titration of DNA-ethidium bromide complex with compound **7.3**. A small quantity of the compound leads to a sharp drop in the percentage transmittance and a noticeable precipitation of the complex.

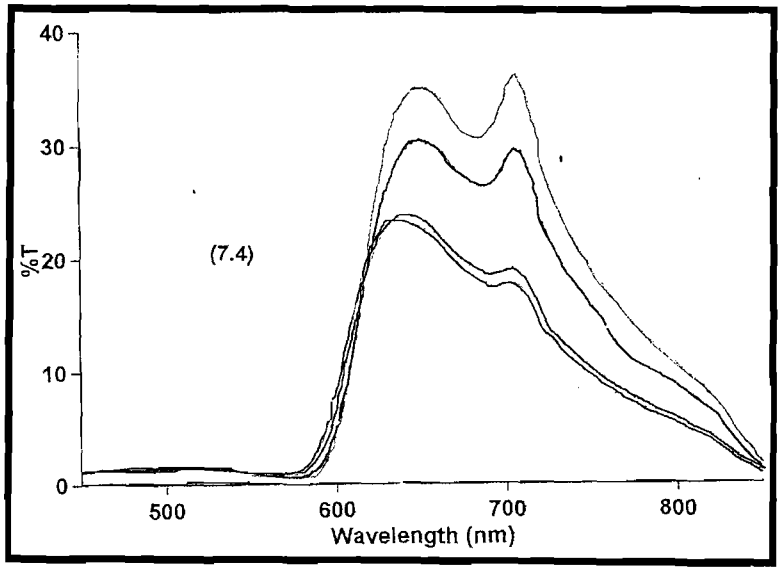


FIGURE 5.10e: Spectrophotometric titration curves of the DNA-ethidium bromide complex with increasing amounts of compound **7.4**.

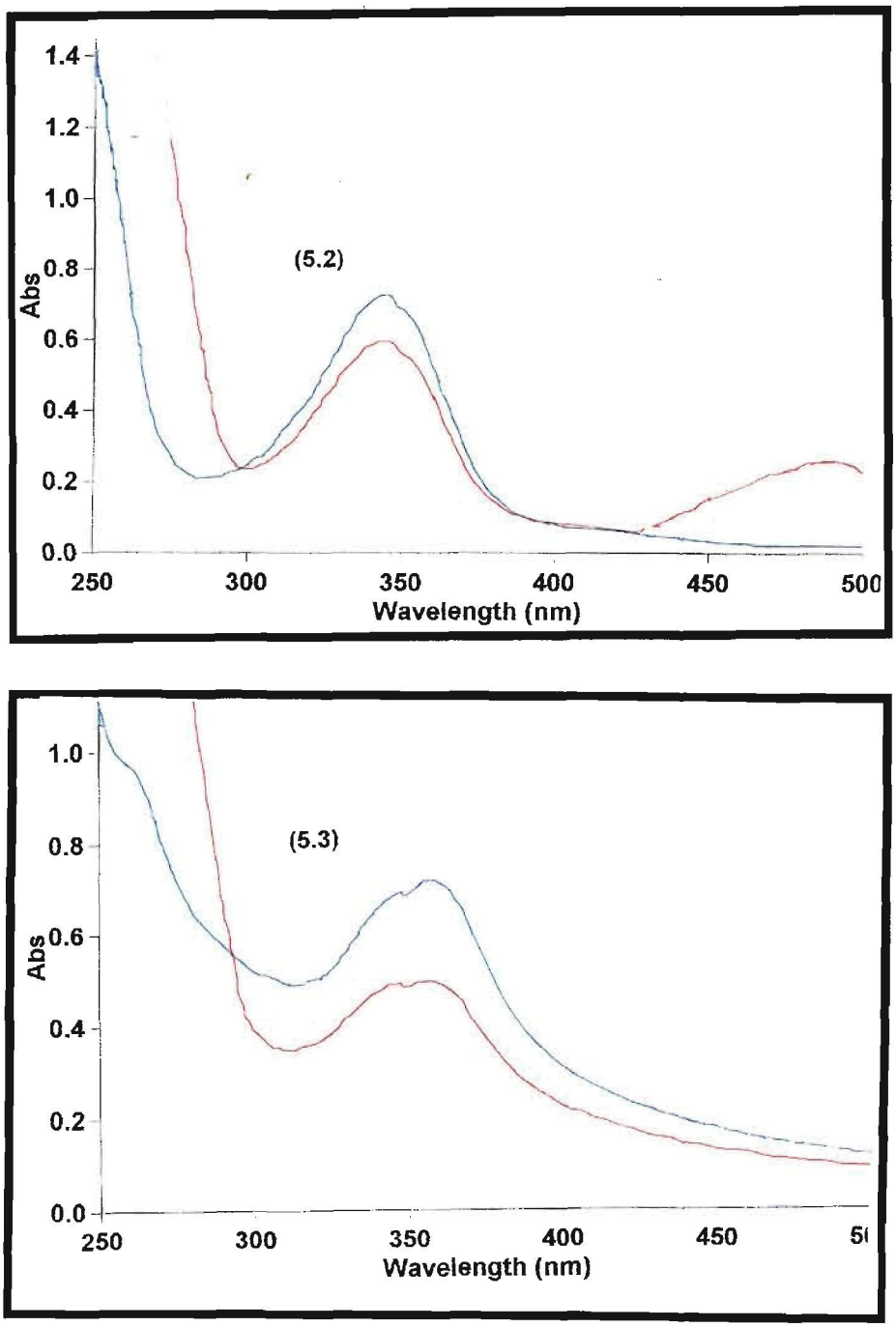
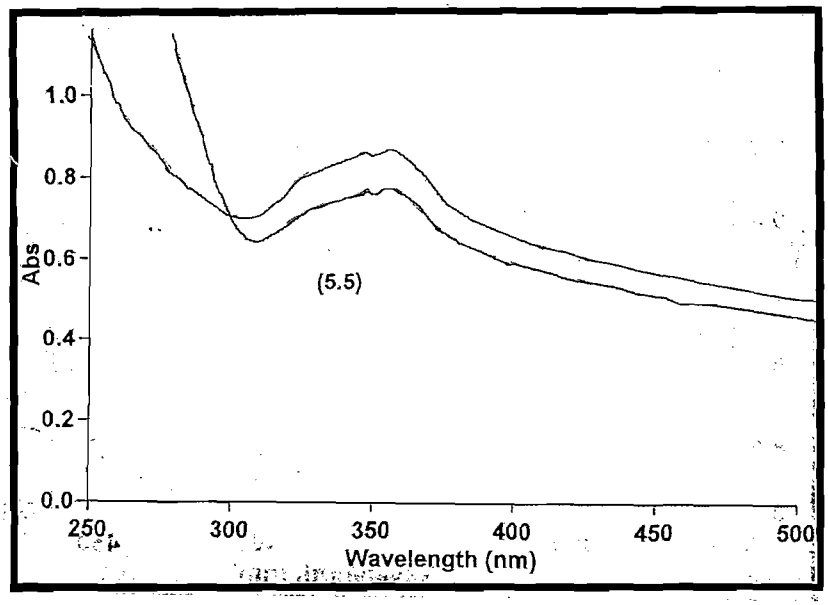


FIGURE 5.11a: Spectrophotometric titration curves of DNA with equimolar concentrations of each of compounds **5.2** and **5.3**.



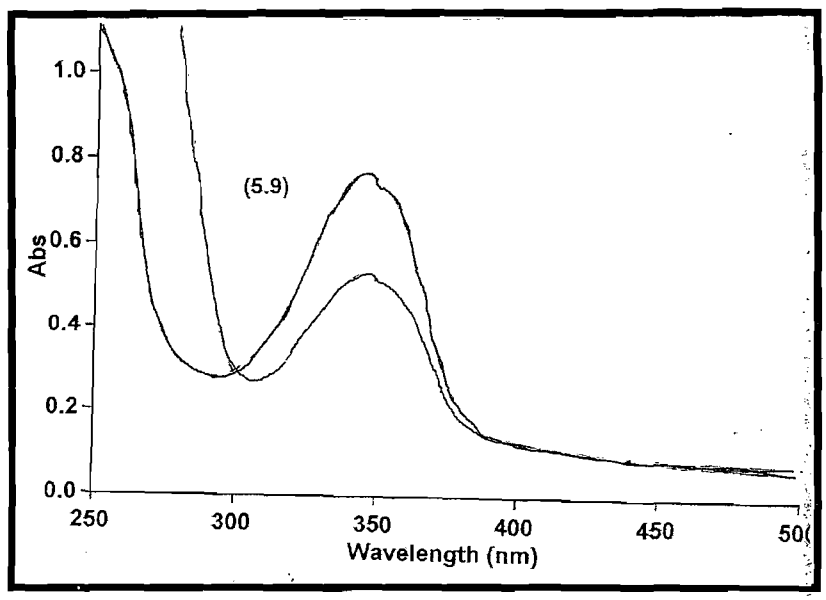


FIGURE 5.11b: Spectrophotometric titration curves of DNA with equimolar concentrations of each of compounds **5.5** and **5.9.**

5.4 INTERACTION WITH FERRIPROTOPORPHYRIN IX

A quinoline ring is known to be a complexing agent with ferriprotoprophyrin IX (FP), and thus participate in the inhibition of the formation of β-haematin (Egan *et al.*, 2000). This property is attributed to the presence of the amino group at the 4-position of the quinoline ring that is thought to interact with the propionate side chains of ferriprotoporphyrin IX (O'Neill *et al.*, 1997). Figures 5.12 to 5.15 represent the results of the spectrophotometric titration of the bisquinolines **(5.1 – 5.10)** while Figure 5.16 represent the titration curves of ferriprotoporphyrin IX with various concentrations of compound **6.1**. Titration with other triazines and tetrazole show similar absorbance patterns. Changes in the absorbance maxima of the Soret band of the UV spectrum is sometimes used as a measure of interaction of the drug with ferriprotoporphyrin IX through the formation of π–π complexes with the induced-shift pattern, upfield or downfield, being different for each drug molecule, leading to different complex structures (Constantinidis and Satterlee, 1988). Spectrophotometric titration of ferriprotoporphyrin IX with these newly synthesised compounds indicate identical patterns in the downward shift of the absorbance maxima of the Soret band of the ferriprotopphyrin IX from 350 to 327 nm, with apparent slight weakening of the bands at 280 and 300 nm as shown in Figure 5.12. While these finding cannot be used to draw conclusions on the structure-activity relationships of these new compounds, compounds **5.1** and **5.10**, both containing a piperazino bridge instead of the diaminoalkyl chain, show very little effect on the absorbance of the ferriprotoporphyrin IX.

Figure 5.12 for compound **6.1** shows a decrease and slight shift in the absorbance of the Soret band of ferriprotoporphyrin IX. It has been noted that this characteristic is distinctly observed with molecular complex formation between the quinoline-type molecules and metalloporphyrins (Hawley *et al.*, 1996). Two processes could be involved here in the behaviour of the compounds with ferriprotoporphyrin IX: either addition of micromolar concentrations of the drugs induces aggregation of ferriprotopor-

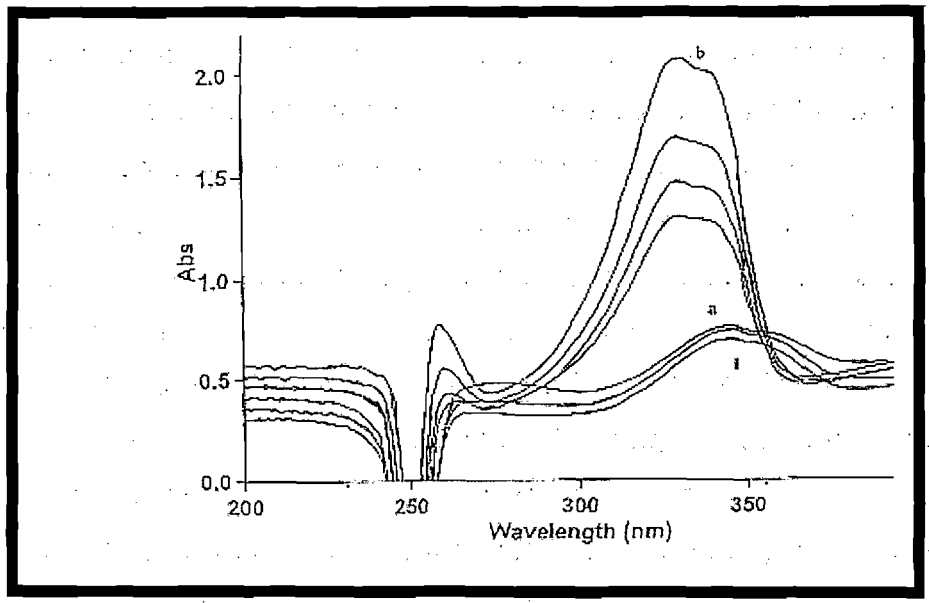


FIGURE 5.12: Spectrophotometric titration of ferriprotoporphyrin IX (1.3 μM) with increasing concentrations of compound **5.2**. In each case 1 is a curve for ferriprotopophyrin IX alone, and *a* to *b* represent the concentrations of compound **5.2** added. At *a* the concentration of this compound is 0.822 μM, while at *b* is 1,37 μM. A reference cell was simultaneously titrated with approximately equal amounts of this compound and the absorbance values corrected for dilution.

phyrin IX, or the changes reflect association of the drugs with ferriprotoporphyrin IX (Egan *et al.*,1997). However, for the compounds under this investigation, it would appear that different structural features of the compounds have no bearing on the manner and extent of their association with ferriprotoporphyrin IX since identical results are obtained. It may be necessary to point out that it is not clear whether the associations of these compound with ferriprotoporphyrin IX is through complex formation between the drug and the ferriprotoporphyrin IX. While large decrease in the absorbance of the Soret band is often an indication of aggregation, equally large decrease can be caused by formation of π-π complexes. For the quinolines to inhibit parasite growth by inhibition of haemazoin formation, it is required that they accumulate and concentrate in the parasitic food vacuoles to levels greater than micromolar levels that are required to inhibit haematin dimerization *in vitro* (Dorn *et al.*,1998). The extent of drug

accumulation at the site of haematin dimerization is also a regulator of antimalarial activity and this may be influenced by the physiological properties of the drug and the proton gradient, which exist between the external environment and the intracellular parasite (Bray *et al.*, 1996).

It may be inferred from the behaviour of these compounds that the low or lack of activity of those compounds with piperazine moiety on the chloroquine-sensitive strain of *Plasmodium falciparum* is due to their inability to associate with and form complexes with ferriprotoporphyrin IX. This could be ascribed to either addition of micromolar concentration of the bisquinolines unable to induce aggregation with ferriprotoporphyrin IX or a reflection of the lack of capacity of these compounds to associate and complex with ferriprotoporphyrin IX, and therefore inhibit the formation of haematin (Ginsburg and Stein, 1991).

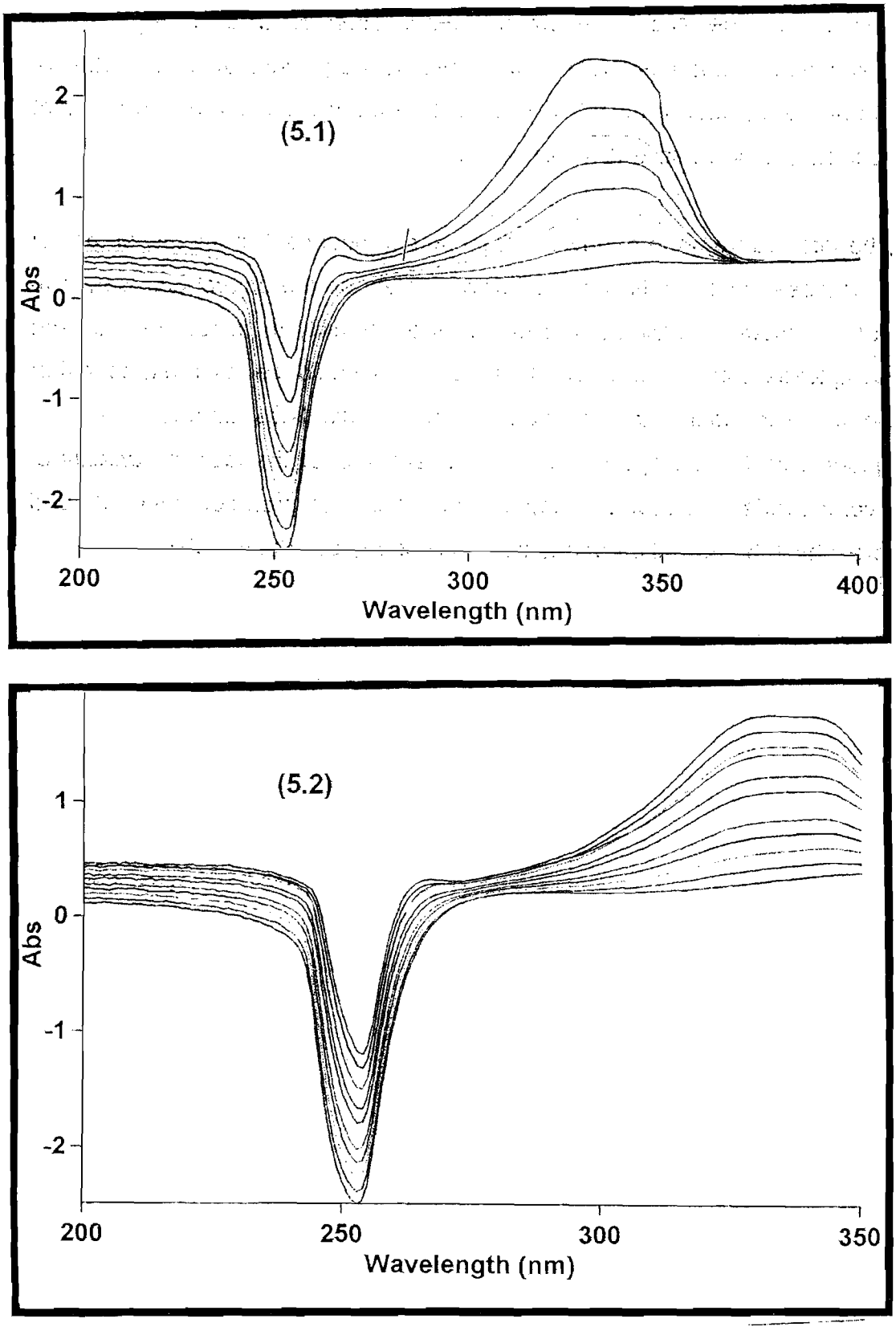


FIGURE 5.13: Spectrophotometric titration of ferriprotoporphyrin IX with increasing concentrations of compounds **5.1** and **5.2.**

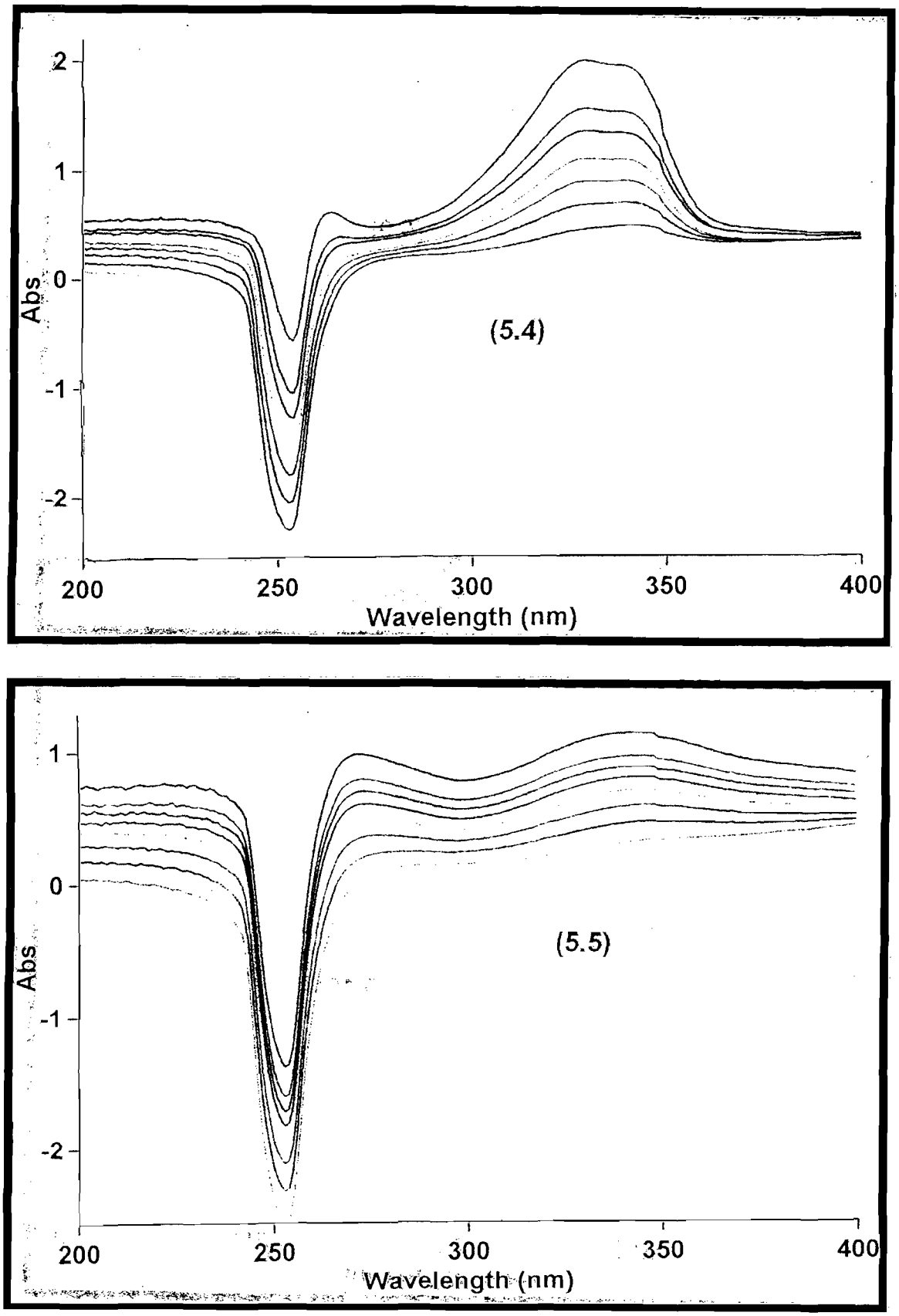


FIGURE 5.14: Spectrophotometric titration of ferriprotoporphyrin IX with increasing concentration of compounds **5.4** and **5.5.**

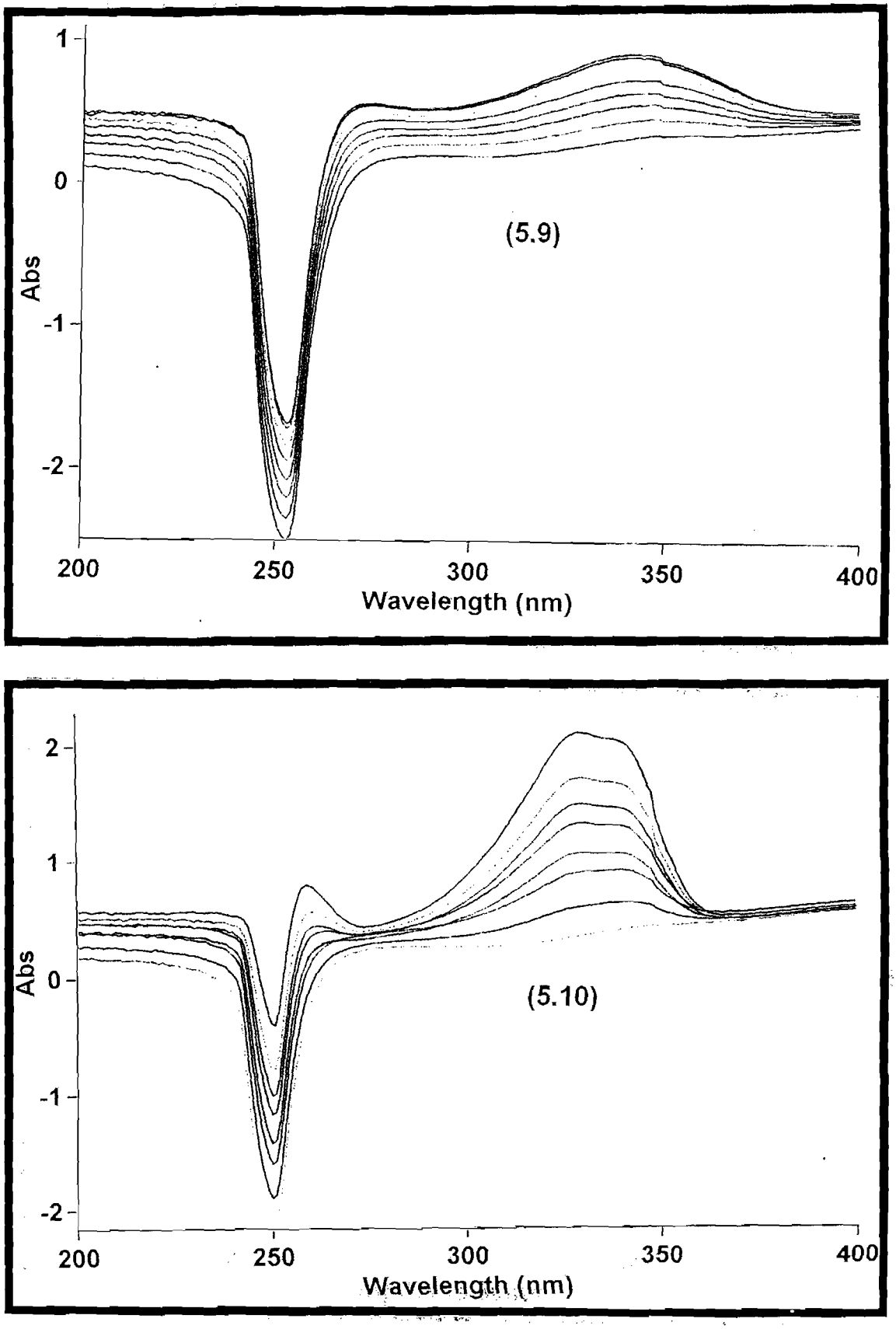


FIGURE 5.15: Spectrophotometric titration of FP with increasing concentrations of compounds **5.9** and **5.10**.

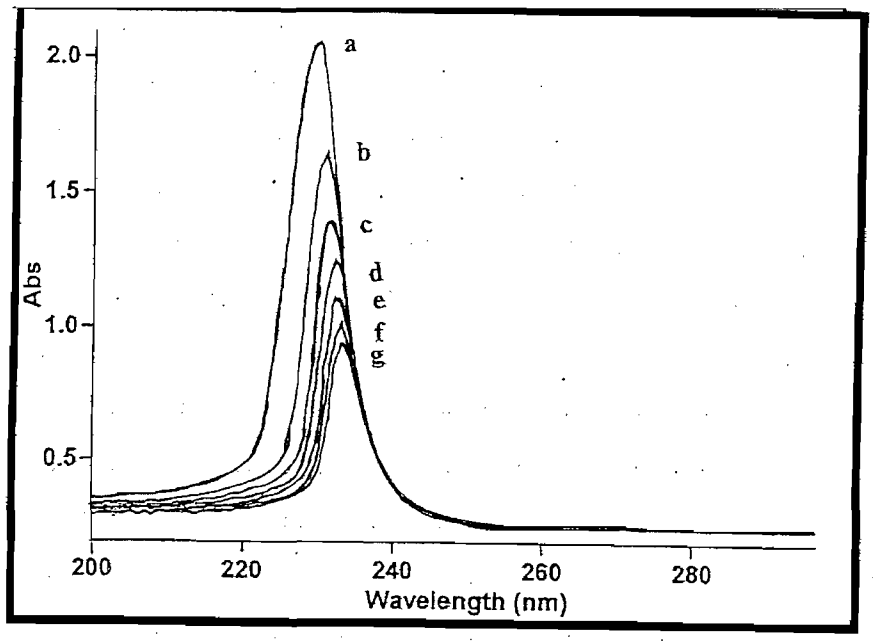


FIGURE 5.16: Spectrophotometric titration of ferriprotoporphyrin IX (1,3 x 10^{-4}M) with compound **6.1**. In each case a is a curve for ferriprotoporphyrin IX, and b to g represent the concentrations of **6.1** at 6,7 x 10^{-7} M; 1,3 x 10^{-6} M; 2,2 x 10^{-6} M; 2,7 x 10^{-6} M; 3,3 x 10^{-6} M and 4,3 x 10^{-6} M added. The reference cell was titrated simultaneously with equimolar amounts of this compound and the absorbance values corrected for dilution.

5.5 CYTOTOXICITY OF THE NEW COMPOUNDS

The newly synthesised quinoline compounds were evaluated for their potential effect against growing tumour cells. Figures 5.17 to 5.20 and Table 5.5 contain the results of the treatment of the human promyelocytic leukaemia cell lines (HL-60) with these compounds. After incubation period of 24 hours in the chosen culture medium, light microscopic inspection of the Giemsa stained cells treated with 20 μM of compound **4.6** reveals that the morphology of the cells has undergone extensive changes, albeit without significant shrinkage (Figure 5.17A). This compound with the two trifluoromethyl groups at positions 2 and 8 and a 2-(1-ethyl-5-nitro-1*H*-imidazol-4-yl)-1-ethan-1-one moiety at position 4, causes extensive chromosomal condensation which is accompanied by nuclear fragmentation and cellular blebbing of the HL-6- cells (Figure 5.17A). The appearance of cells less stained than the untreated cells in a flow cytometrical

DNA histograms is generally accepted as a marker for apoptosis (Efferth *et at.*,1996). Tumour growth and shrinkage are dependent on the proportion of actively proliferating cells in comparison to dying cells, with cell death occurring either by necrosis or by apoptosis, which is commonly referred to as programmed cell death. Evidence presented by Figure 5.18 indicates that compound **4.6** is the only one that induces apoptosis of the cells. Even at concentrations as high as 500 μM, the other compounds such as **4.1 – 4.5** did not affect the morphology of these cells (Figure 5.17B as compared to control cells in Figure 5.17C). Reduced stainability in apoptotic cells can be considered to be due to DNA fragmentation and subsequent diffusion of these fragments out of the cells (Efferth *et al.*, 1996). The results compare favourably with those reported for the induction of apoptosis by epigallocatechin (EGCg) on the human histiocytic lymphoma U937 cells (Figure 5.19) (Saeki *et al.*, 1999). However, it must be clearly pointed out that induction of apoptosis does not provide definite clues as to the specific mechanisms of cytotoxicity, for example, whether it occurs by inhibition of enzymes or by the DNA damage. Preliminary results also indicate that compound **4.5** and to a lesser extent **4.3** and **4.4**, exhibit a dose-dependent cell growth inhibitory activity on normal human and promyelocytic leukaemia cells (Kgokong and Wachira, 2001). In this case **4.5** gives an IC_{50} value of 10 μM. This activity can be ascribed to the ability of these compounds to bind to and intercalate with DNA molecules of the cells.

Cell growth inhibitory studies indicate that compounds **4.3**, **4.5** and **4.6** exhibit dose-dependent cell-growth inhibitory activities on the HL-60 cell lines with compound **4.6** being the most potent as shown by the IC_{50} values of these compounds results in Table 5.5. The calculated IC_{50} value of 10 ± 2,5 μM has been obtained for this compound (Figure 5.20A). The next effective compound is **4.5** with IC_{50} value of 160 ± 15,8 μM (Figure 5.20B). On the other hand compound **4.3** is only moderately active and achieves an IC_{50} value of 540 ± 19,6 μM (Figure 5.20C). Compounds **4.1, 4.2** and **4.4** are relatively inactive

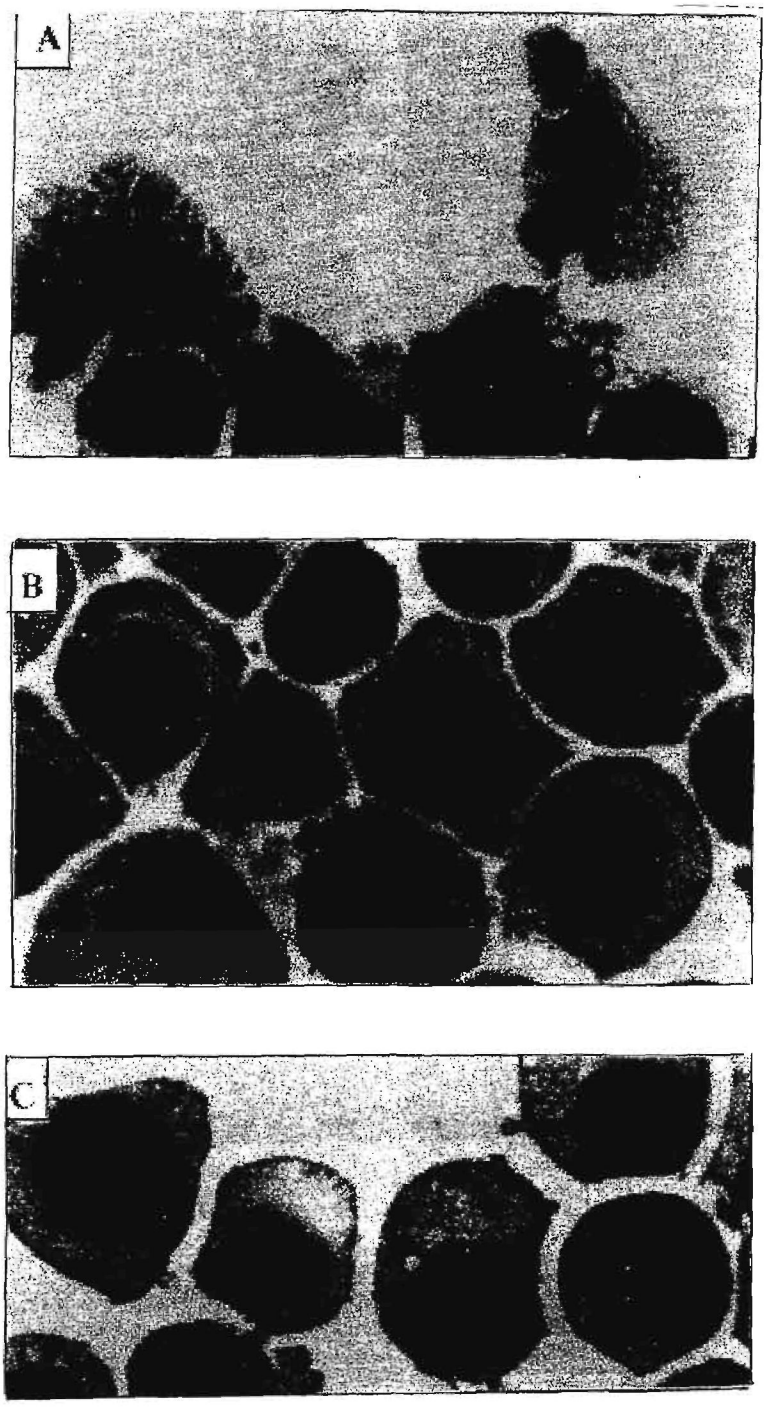


FIGURE 5.17: Morphological appearance of HL-6- cells from culture treated with 20 μM of compound **4.6** (A) and 500 μM of 1 (B) and control cells (C) after 24 hour incubation at 37 °C (magnification x 630).

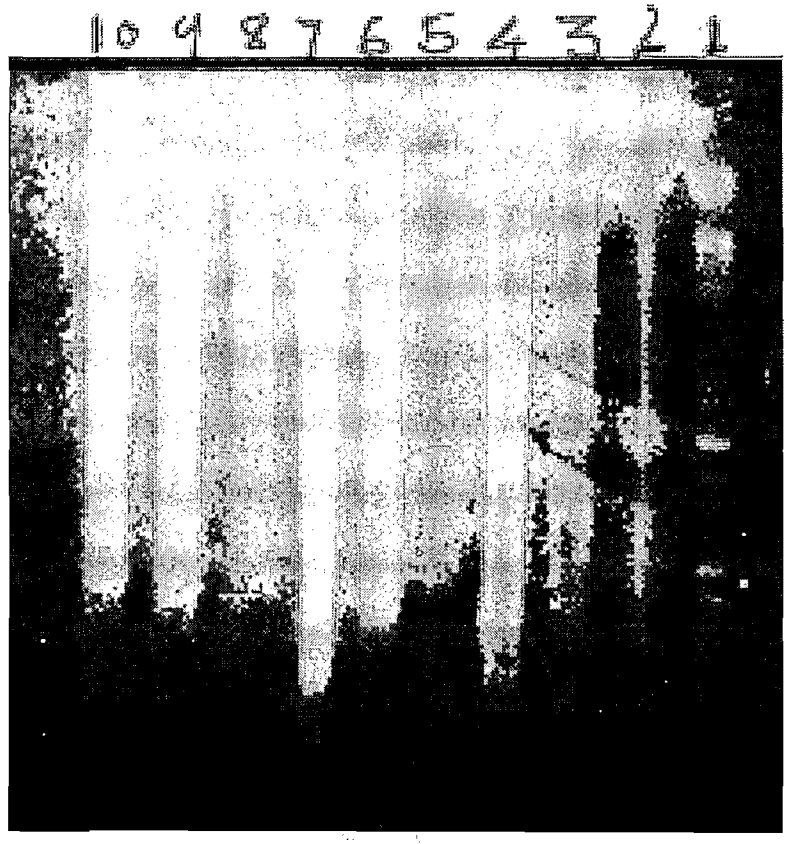


FIGURE 5.18: Induction of apoptosis by 20 μM compound **4.6**. Cells were treated with 10 μM ethanol vehicle and incubated for 3 hours (lanes 2 – 4), 6 hours (lanes 5 – 7) and 24 hours (lanes 8 – 10).

even at concentrations as high as 1,0 mM. Actually compound **4.4** is shown to stimulate growth of the cells in a dose-dependent manner up to 750 ± 50,4 μM as reflected in Figure 5.21A. When the structures of these compounds are considered, it will be noticed that compound **4.6** (with trifluoromethyl groups at positions 2 and 8) and to a lesser extent **4.5** (with a single trifluoromethyl group at position 2) but both with a 2-(1-ethyl-5-nitro-1*H*-imidazol-4-yl)-1-ethan-1-one moiety exhibit significant dose-dependent cell growth inhibitory activity more than the compounds with a pyrimidino group at position 4. The growth-inhibitory activity of **4.6** could be accounted for by its capacity to induce apoptosis as reflected in Figure 5.18, and its ability to bind to DNA by intercalation. Thus the slight growth-inhibitory activity exhibited by **4.3** with a pyrimidin-5-yl moiety could be attributed to the presence of the two trifluoromethyl groups rather than

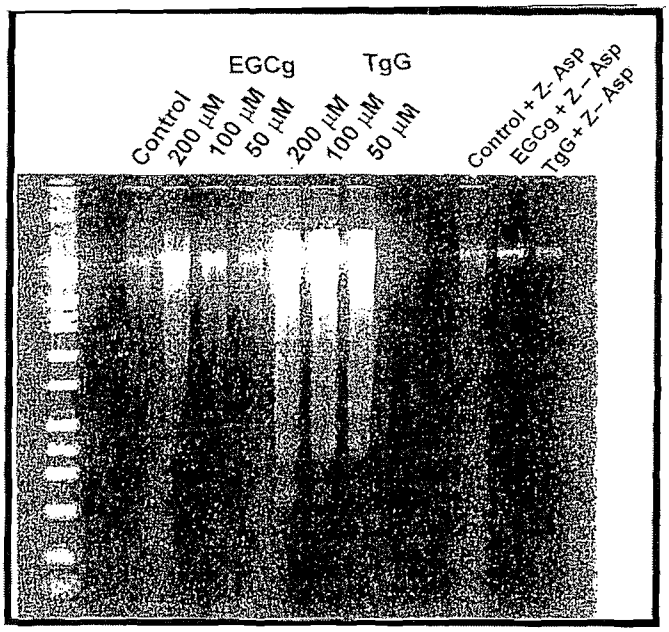


FIGURE 5.19: Concentration-dependent induction of DNA fragmentation by tetragalloyl glucose (TgG) in human cancer WiDr cells. The cells were incubated at 37 °C for 16 h in the presence or absence (control) of epigallocatechin gallate (EGCg) or TgG at a concentration indicated and DNA fragmentation was analysed. Complete inhibition of DNA fragmentation in the cells incubated with EGCg or TgG at 200 μM in the presence of 200 μM caspase inhibitor, A-Asp-CH2-DCB (Z-Asp) is also shown (Saeki *et al.*, 1999).

the pyrimidino group. This enhanced *in vitro* activity of **4.6** which is within the range of clinically useful antitumour drugs, can be ascribed to the increased lipophilicity of the compound resulting from the two trifluoromethyl groups attached to the quinoline ring. However, **4.5** with a 2-CF_3 and a 2-(1-ethyl-5-nitro-1*H*-imidazol-4-yl)-1-ethan-1-one moiety at position 4 seem to be slightly more active than **4.3** with two trifluoromethyl groups at positions 2 and 8 and a pyrimidino group at position 4. Thus the 2-(1-ethyl-5-nitro-1*H*-imidazol-4-yl)-1-ethan-1-one together with a 2-CF_3 group appears to be additional parameters for cytotoxic effect. There is an observation that the overall lipophilicity of the molecule is an additional parameter with great significance for biological activity (Baguley, 1982). While compounds **4.1** and **4.2** seem to have no effect on the growth of the cells, **4.4** carrying a 8-trifluoromethyl and a 2-(1-ethyl-5-nitro-1*H*-

TABLE 5.5: IC_{50} cell viability and cell count (μM) of the compounds

Compound	Viability	Cell count
4.1	> 1000	> 1000
4.2	> 1000	> 1000
4.3	540 ± 19,6	320 ± 15
4.4	> 1000	> 1000
4.5	160 ± 15,8	200 ± 10
4.6	10 ± 2,50	10 ± 3,00

imidazol-4-yl)-1-ethan-1-one groups actually stimulates the growth of the cells. Perhaps the absence of cell growth inhibitory properties exhibited by compounds **4.1**, **4.2** and **4.4** could be attributed to their inability to bind to and intercalate with DNA, a property that can be due to either the lack of weak basic character or the low lipophilicity of the compounds. Both of these properties are necessary for DNA intercalation and antitumour activity (Zahir *et al.*, 1996). Binding of the quinoline derivatives to the DNA bases occurs at positions 2, 3, and 4 of the nucleus that are then make available for new hydrogen-binding partner by the action of the enzyme DNA gyrase (Tillotson, 1996).

The results of the *in vitro* cytotoxicity determination of the new series of bisquinolines against the mammalian cell-line, Chinese hamster ovarian (CHO) cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5- biphenyltetrazolium-bromide (MTT) are reflected in Figure 5.21 and summarised in Table 5.6. From these preliminary results, it is clear that these compounds show a very high selectivity index indicating that the compounds do not affect the normal mammalian cells, while at the same time will act against the proliferating parasites' cells. This is due to the observation that only live cells will actively cleave the MTT, while dead cells will be almost completely negative even immediately after complement-mediated lysis (Mosmann, 1983).

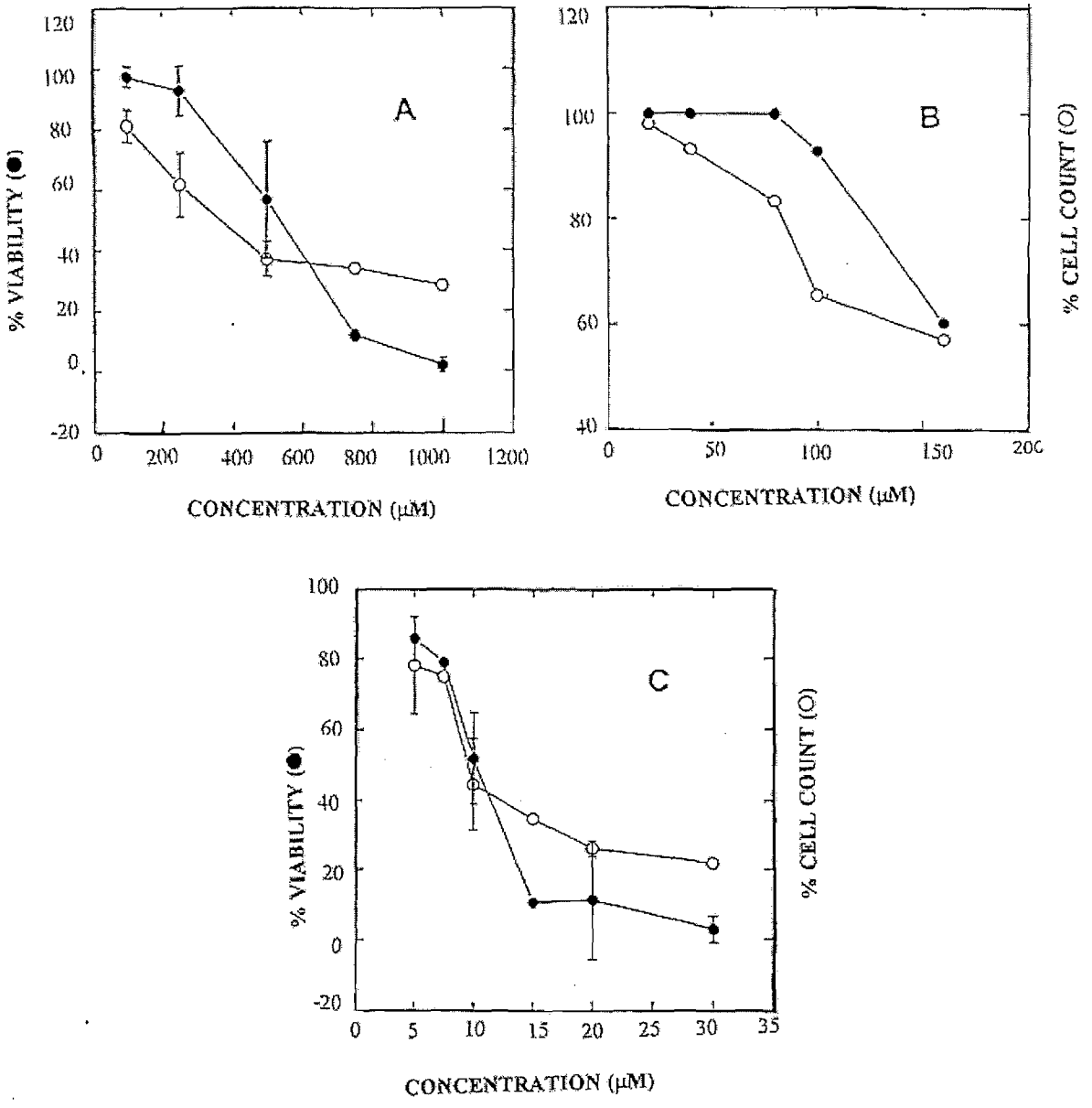


FIGURE 5.20: Effect of the concentrations of compound **4.3** (A), **4.5** (B) and **4.6** (C) on the proliferation of HL-60 cells. The cells were cultured in the presence of increasing concentrations of the compounds for 4 days. The results are the means of triplicate determinations and expressed as percent of controls with ± SD (error bars)

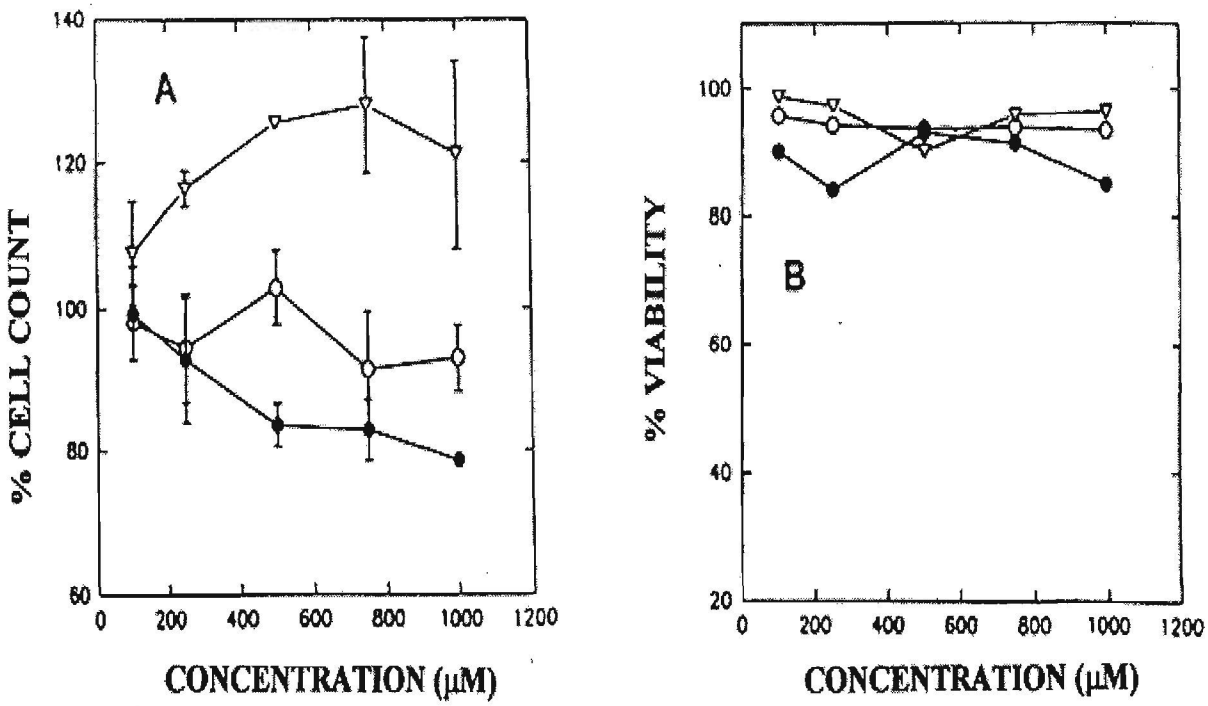


FIGURE 5.21: Effect of concentrations of compounds **4.1** (○), **4.2** (●) and **4.4** (▽) on the proliferation (A) and viability (B) of the HL-60 cells. The cells were cultured in the presence of increasing concentrations of he compounds for 4 days. The results are the means of triplicate determinations and expressed as percent of control with ± SD (error bars).

From these studies, we have shown that [2,8-*bis*(trifluoromethyl)quinolin-4-yl](1-ethyl-5-nitro-1*H*-imidazol-4-yl)-1-ethan-1-one **(4.6)** and to a lesser extent, [2,8-*bis*(trifluoromethyl)quinolin-4-yl](pyrimidin-5-yl)methanone **(4.5)** and [2-trifluoro-methylquinolin-4-yl](1-ethyl-5-nitro-1*H*-imidazol-4-yl)-1-ethan-1-one **(4.2)** cause *in vitro* dose-dependent blockage of the leukemic (HL-60) cell growth. The former appears to achieve its activity by also inducing programmed cell death. Although the experiments reported here are not sufficient to establish a binding mechanism for these compounds, spectral shifts obtained on binding, and the fact that they displace DNA bound ethidium bromide, strongly supports the idea of intercalation as the mode of binding and their possible cell-growth inhibitory activity. The data presented here alone provide a valuable perspective on the binding properties of these compounds without necessary recourse to their absolute binding constants with DNA. On the basis of the IC_{50} values obtained by

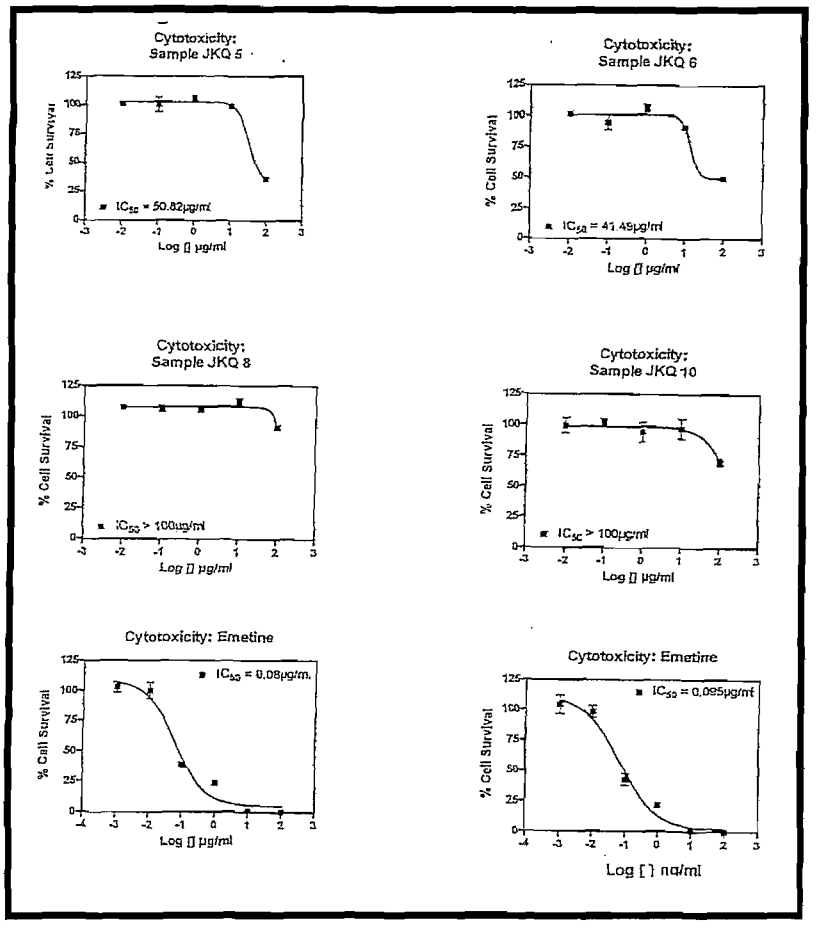


FIGURE 5.22: Comparative cytotoxicity of compounds **5.5** (JKQ5), **5.6** (JKQ6), **5.8** (JKQ8) and **5.10** (JKQ10) and emetine on the Chinese hamster ovarian cell lines

subjecting the new compounds to the evaluation for antimalarial activity against the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*, it can be concluded that the two trifluoromethyl groups on the quinoline ring system are essential for *in vitro* antimalarial activity, particularly against the chloroquine-resistant strains. The type and size or length of the bridging chain groups between the quinoline rings have influence on the activity of this series of compounds. Bridging chains containing primary amino groups seem to be more active than those compounds that lack the imino hydrogen atoms. This is particularly noticeable in compounds used against the chloroquine-sensitive than chloroquine-resistant strains of the *P. falciparum*. Except for N,N-*bis*[2,8-*bis*(triflu-

TABLE 5.6. The *in vitro* IC_{50} values (μg/ml) of the N,N-*bis*quinolin-4 yl derivatives on the chloroquine-resistant strain K1 of *P. falciparum* and CHO cell-line

Compound	Chloroquine-resistant cell line	Chinese hamster ovarian cell line	Selectivity index*
5.4	1,20	50,82	42,00
5.6	1,50	41,49	28,00
5.8	1,60	> 100	> 62,50
5.10	1,00	> 100	> 100
3	113,00		
Emetine		0,09	

*Selectivity index = cytotoxicity IC_{50} value/Antiplasmodial IC_{50} value

oromethyl)quinolin-4-yl]-diamino ethane (**5.2**), the moderately low IC_{50} values obtained for these newly synthesised and screened compounds against both the chloroquine-sensitive and chloroquine-resistant strains of the *P. falciparum in vitro* suggest that the compounds are not sufficiently potent enough to warrant further investigation. However, these compounds exhibit higher *in vitro* activity against the chloroquine-resistant strains. Unfortunately the results of the interaction of these new compounds with ferriprotoporphyrin IX cannot be used to assess the structure-activity relationships since identical shift patterns are observed in the UV spectra. The new compounds are highly selective in their action against the plasmodial cells and no serious cytotoxicity is observed against the Chinese hamster ovarian cell lines in the preliminary investigation using MTT.

5.6 POSSIBLE MODE OF ANTIMALARIAL DRUG ACTION

In spite of the uncertainty regarding the mode of action of antimalarial drugs, the new compounds were subjected to some analytical procedures to assess their intercalative ability with deoxyribonucleic acid (DNA) through ethidium bromide displacement studies and their capacity to complex with ferriprotoporphyrin IX.

(a) **DNA studies**: Figures 5.7 and 5.9 reflect the ability of the [2- and 8-trifluorome-thyl-] and [2,8-*bis*(trifluoromethyl-quinolin-4-yl](pyri-midin-5-yl)methanones and 2-(1-ethyl-5-nitro-1*H*-imidazol-4-yl)-1-ethan-1-ones to displace ethidium bromide from its complex with DNA, and changes in the UV absorbance of the new compounds associated with the addition of DNA to the solutions of these compounds. Quinine, known to exert its antimalarial activity by intercalating with DNA was used as a reference standard. Identical shifts in the absorbance were noted. Similarly, the Figures 5.10a to 5.10e indicate the new 1,2,4-triazines and tetrazoles interact and intercalate with DNA. The addition of small mounts of these compounds leads to a profound decrease in the percentage transmittance as was observed with compound **7.3**. Figures 5.11(1) to 5.11 (4) indicate that the N,N-*bis*(trifluoromethyl-quinolin-4-yl)- and N,N-*bis*[*bis*(trifluoromethyl)-quinolin-4-yl]diamino-alkanes **(5.1** to **5.10)** intercalate with the DNA double helix as evidence by the shift to lower wavelengths of the absorbance bands. It was inferred in § 2.2.2 that such behaviour of chemical compounds is indicative of the intercalative properties of such compounds. The conclusion is supported by the previous findings such as the comparative DNA binding properties of the indocarbazole antitumor drug, NB-506 and its derivative, in which its N-formylamino group has been replaced by a more hydrophilic group, N-*bis*(hydroxymethyl)-(methylamino (Figure 5.24) and Figure 5.25. In both cases of Figure 5.24, the main absorption band centred at 305 is shifted to 315 nm, indicating that binding of drugs to DNA induces well-defined bathochromic and hypochromic effects (Bailly *et al.*, 1999). Acraflavine has been known to exhibit this characteristic when treated with DNA (Figure 5.25) (Sivaran *et al.*, 1995; Dheyongera, 2000). Similarly, 1-(2-dimethylamino)-9(10H)-thioacridone was found to exhibit similar spectral characteristics when treated with DNA (Figure 5.26) (Dheyongera *et al.*, 2005). The latter compound was subject to identical experimental conditions and examined in the same laboratory as our compounds.

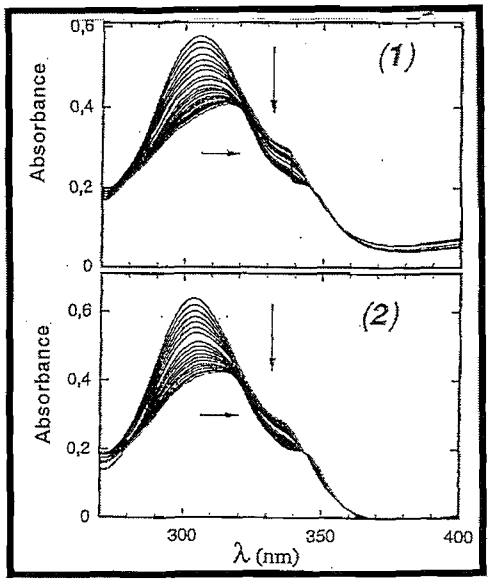


FIGURE 5.24: Absorption titration for the interaction of the antitumor compound NB-506 (1) and its N-*bis*(hydroxymethyl)- methylamino derivative (2) with calf thymus DNA. The figure contains the absorption spectrum of the free drug, intermediate spectra, and final spectra of the drug-DNA complexes, in which the ligands have been sequestered completely by the DNA (Bailly *et al.*, 1999).

(b) **Complex formation with ferriprotoporphyrin**: Figures. 5.11 to 5.15 indicate that all the N,N-*bis*(trifluoromethylquinolin-4-yl) and N,N-*bis*[*bis*(trifluoromethylquinolin-4-yl]diaminoalkanes and piperazine derivatives (**5.1** to **5.10**) interact with ferriprotoporphyrin IX to more or less same extent as evidenced by identical titration curves obtained. In each case the maximum absorbance of ferriprotoporphyrin IX at 328 shifts slightly to the right and downwards with each addition of the compounds of interest. This behaviour is also exhibited by the tetrazoles as shown in Figure 5.16. The data are also identical to those shown by Figure 5.27 obtained in the investigation of the polymerisation of haematin (Inada and Shibata, 1962). However, these studies cannot be used to build structure-activity relationship profiles of

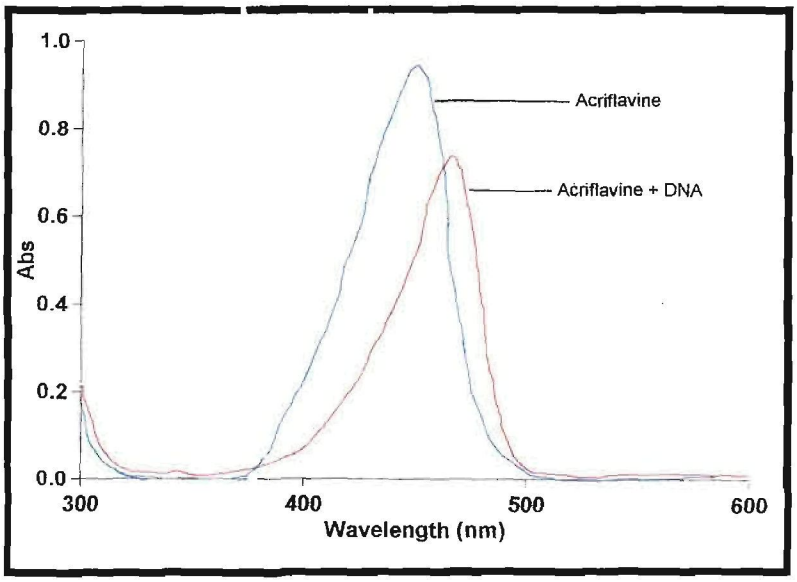


FIGURE 5.25: UV-visible absorption spectrum of acriflavine showing typical hypochromism and a shift of absorption maximum to longer wavelength in the presence of DNA (Dheyongera, 2000).

compounds as the absorbance *vs* concentration curves are more or less identical for all the compounds. More or less identical results were obtained in the titration of ferriprotoporphyrin IX with chloroquine (Figure 5.28), 9-epiquinine (Figure 5.29) and 4-amino-7-chloroquinoline (Figure 5.30) and in the saturated solution of ferriprotoporphyrin IX with increasing concentrations of sodium acetate (Figure 5.31).

5.7 CONCLUSION

The results of the characterisation studies to confirm the structures of the newly synthesised compounds indicate that:

- The elemental analysis results in cases where it was conducted are in good agreement with and within 15% of the calculated values calculated for each compound and with the stated molecular weight of that compound,
- The mass spectral data indicate that the molecular ions and the fragmentation patterns are those of the envisaged compounds,

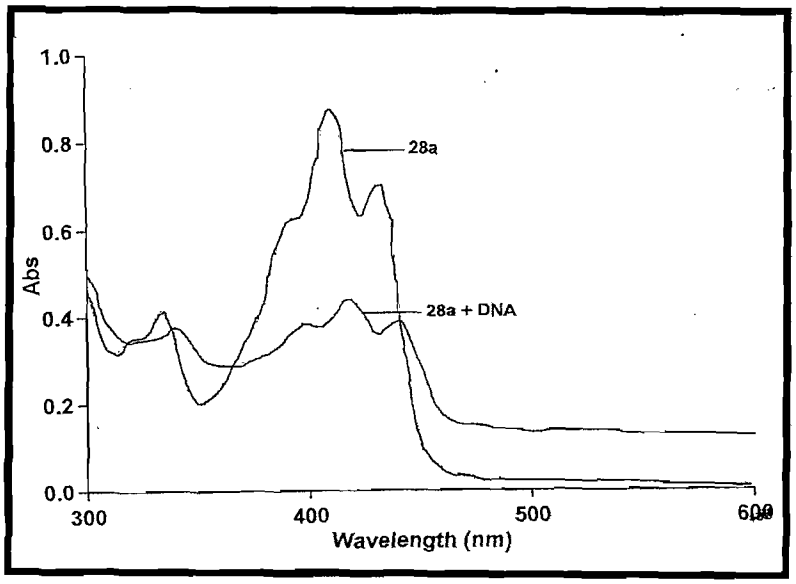


FIGURE 5.26: UV absorption spectrum showing the bathochromic effect of DNA addition on the spectrum of 1-(2-dimethylamino-ethylamino)-9(10*H*)-thioacridone (Dheyongera *et al.*, 2005).

- ^{1}H-NMR spectral analyses show the expected number of protons, multiplicities and couplings,

- ^{13}C NMR spectral analyses indicate chemical shifts that are consistent with the number of carbon atoms in the proposed structures. Those compounds containing trifluoromethyl groups are shown by apparent splitting of the carbon peaks into either broad quartets (200 – 300 Hz) for the carbon to which the fluorine atoms are attached and narrow quartets (30 – 32 Hz) for all adjacent carbon atoms.
- Infrared spectrophotometric analyses provided useful information in the determination of the structures of the new compounds containing chromophores that are useful in the determination of the structure and which have absorption frequencies that are structure dependent in an interpretable manner.

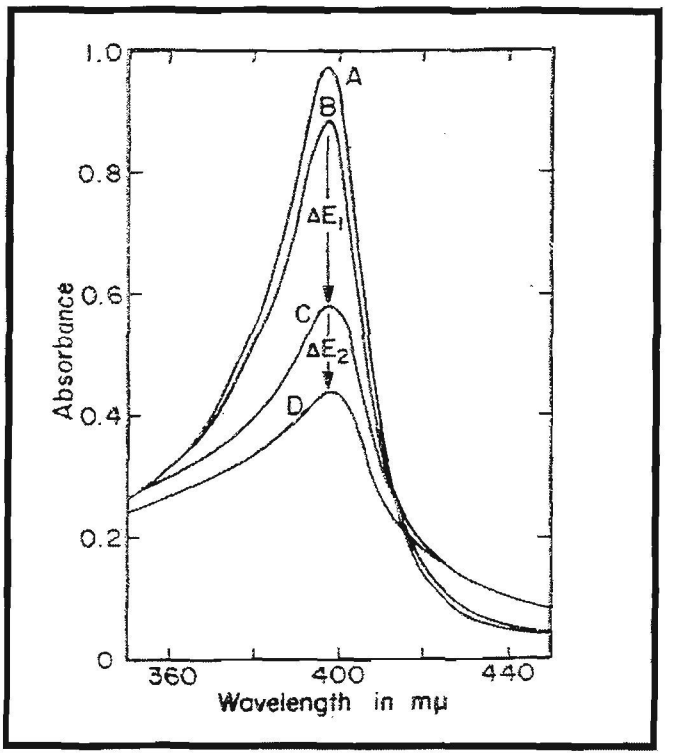


FIGURE 5.27: The Soret bands of aqueous solution of haematin during polymerisation. Curve A is the spectrum of monomeric haematin at pH 6.8, B represents the spectrum of the dimeric form of haematin and C and D represent spectra of haematin after polymerisation. If polymerisation was not occurring curves B top D will be identical to curve A (Inada and Shibata, 1962).

In spite of the inherent limitations in the study, the following conclusion can be drawn from the outcomes of these investigations on the antimalarial activity and cytotoxicity of the newly synthesised compounds:

(a) A trifluoromethyl group substituted onto the quinoline ring and benzene portion of the 1,2,4-triazine[5,6b]- and 1,2,4-triazolo[1',5',2,3]-1,2,4-triazino[5,6b]indole derivatives render these compounds to be active against the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* as depicted in Figures 5.3 and 5.4 and Tables 5.3 and 5.4. Identical compounds without this substituent are all devoid of activity However, when two trifluoromethyl groups are substituted in one molecule of the tetrazole, the antimalarial activity is reduced. This could be ascribed to the bulkiness of the molecule, making it incapable of entering and

accumulating in the food vacuoles where activity takes place. In the case of the quinoline-containing compounds (series **4.1** to **4.6** and **5.1** to **5.10**), the activity is more pronounced when the trifluoromethyl group is substituted at positions 2 and 8 of the quinoline ring as shown in Figures. 5.1 and 5.2 and Tables 5.1 and 5.2. The behavious of tehse compounds is particularly evident against the chloroquine-resistant than the chloroquine-sensitive strains of *Plasmodium falciparum* as shown by Table 5.2. Unfortunately compounds **4.1** to **4.6** were not screened against the chloroquine-resistant strain of the *Plasmodium*.

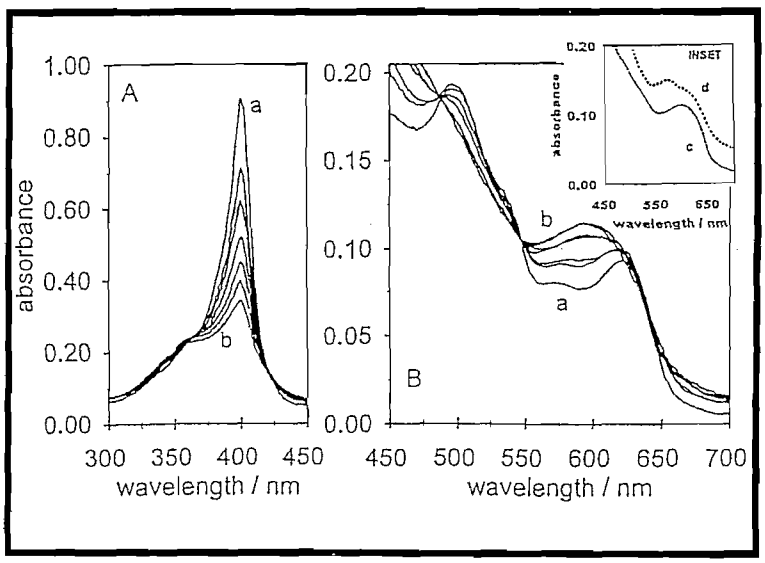


FIGURE 5.28: Spectroscopic changes observed when ferriprotoporphyrin IX (FP) is titrated with chloroquine. (A) indicates the changes in the Soret band of FP and (B) indicates the changes in the visible region of the spectrum of FP. In each case a represents FP in the absence of chloroquine, while b is the FP with large molar excess of chloroquine. Conditions: apparent pH 7.5, 25 °C, 40% DMSO and 0.020 M HEPES buffer.

It is expected that they would have exhibited results identical to those in Table 5.2. Quinoline-type compounds with a trifluoromethyl group only at position 2 or 8 are less active, but the 8-trifluoromethylquinolines such as **4.4** also exhibit cell growth-stimulatory properties in a dose-dependent manner particularly below 1 and above 40 μg/ml towards both malaria

parasites and the leukaemia cells (see Figure 5.20A). For the 1,2,4-triazino-[5,6b]-indole-3-thione (**48.1** and **48.2**), the sulphur atom does not have significant effect on activity, but when alkyl groups are attached to the sulphur atoms significant improvement in activity in the order: methyl > ethyl > cyclopropyl (compounds **6.2**, **6.4** and **6.6**) is noticed (Table 5.3). Similarly, substituents at position 3 of the 5*H*-1,2,4-triazolo[1'5',2,3b]-1,2,4-triazine[5,6b]indole derivatives indicate some improvement in activity in the order H > CH_3 > CF_3 > C_6H_4Cl (Table 5.4).

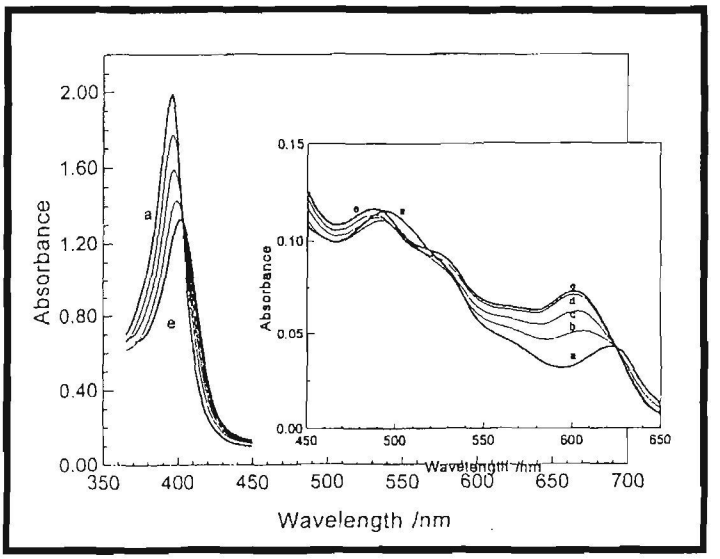


FIGURE 5.29: *Spectroscopic changes, corrected for dilution* accompanying the addition of 9-epiquinine to a 12,7 μM solution of heme-octapeptide at pH 6.25 and 25 °C. a: Heme-octapeptide without 9-epiquininine, b: with 1.65, c: 8.37 and e: 51.9 mM (Marques *et al.*, 1996).

(b) The effect of the difference in the size and structure of heterocyclic moieties such pyrimidine and 1-ethyl-5-nitro-1*H*-imidazole attached to the carbonyl carbon at position 4 on the antimalarial activity of these new compounds is not discernible (Table 5.1 and Figure 5.1). Compounds containing these show more or less identical activity. However, compounds with a 2-(1-ethyl-5-nitro-1*H*-imidazol-4-yl)ethan-1-one moiety exhibits increased antimalarial activity and cell growth inhibitory properties

of the drug against leukemia cells than identical compounds with pyrimidino group.

(c) Bisquinoline compounds with diaminoalkyl chains or piperazino groups bridging the two quinoline rings that are substituted with trifluoromethyl groups at positions 2 and 8 of the quinoline ring and a bridging diaminoalkyl chain containing two carbon atoms exhibit slightly higher activity than compounds with larger linking chains (Table 5.2). This is more evident against the chloroquine-resistant than the chloroquine-sensitive strains of the parasites. It would appear that the absence of replaceable amino hydrogen atoms in the piperazine compounds make these compounds less active (see § 3.2 and 3.2.2). Except for the N,N-*bis*(2-trifluoromethylquinolin-4-yl)diamino propane (**5.3**), all the compounds in this series exhibit a 2-fold increase in activity against the chloroquine-resistant than chloroquine-sensitive strains. This is particularly so for the 2,8-*bis*(trifluoromethyl)quinoline series. *Bis*[N,N-2,8-*bis*-(trifluoromethyl)-quinolin-4-yl]piperazine (**5.10**) is about ten-fold more active against the chloroquine-resistant than against the chloroquine-sensitive strains. This phenomenon can be ascribed to the presence of the two trifluoromethyl groups in the quinoline ring. The limited number of samples in the study did not afford us the opportunity to assess the role played by the size and nature of the diaminoalkyl bridging chain in the activity of the compounds.

(d) The new compounds seem to undergo intercalation with DNA double helix or complex formation with ferriprotoporphyrin IX as a possible mode of antimalarial action (Figures. 5.7 to 5.11).

(e) [2,8-*Bis*(trifluoromethyl)quinolin-4-yl]-2-(1-ethyl-5-nitro-1*H*-imidazol-4-yl)-1-ethan-1-one (**5.6**) has high cell growth inhibitory activity against the human promyelocytic leukaemia cells and induces apoptosis of these cells. Normal human cells were not affected.

(f) The high selectivity indices exhibited by the N,N-*bis*(trifluoromethyl)-and N,N-*bis*[*bis*(trifluoromethyl)quinolin-4-yl)diaminoalkanes reflected in Table 5.6 and Figure 5.22 indicate that the compounds affect only the parasite cells.

(g) The low antimalarial activity of these compounds could be explained

on the basis of lack of weak basic characters or the rigidity of the molecules, both of which are necessary for either DNA intercalation or haem polymerisation. Since the overall lipophilicity of the molecules is an additional parameter with great significance for biological activity (Baguley, 1982), the low activity of these compounds, in particular **4.1** to **4.5** could be attributed to this property. It has been suggested that the antimalarial activity arise from the weak basic properties of the drug, and as the drug accumulates in the acidic food vacuole, its tertiary amino group and the heterocyclic nitrogen in the quinoline ring become fully protonated (Ginsburg and Stein, 1991).

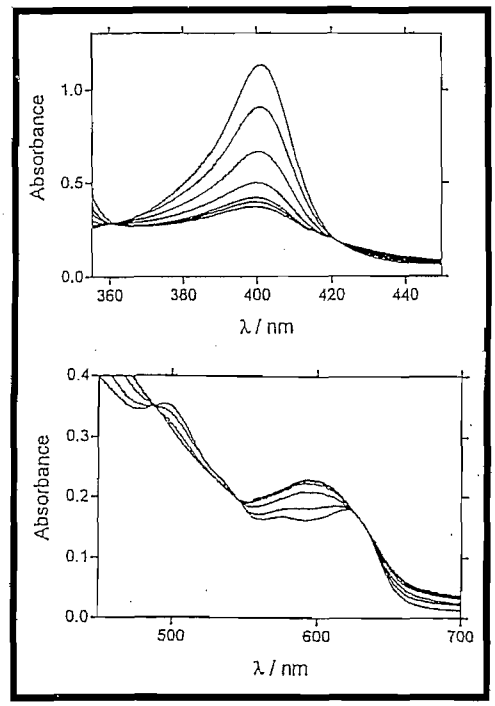


FIGURE 5.30: Visible spectra of ferriprotoporphyrin IX in 40% aqueous DMSO, pH 7.5, titrated with increasing concentrations of 4-amino-7-chloroquine. In the upper panel the highest concentration of the quinoline corresponds to the weakest spectrum, while in the lower panel, the highest concentration of the quinoline corresponds to the spectrum which lies at the bottom of the series at 500 nm and at the top at 600 nm (Egan *et al.*, 2000).

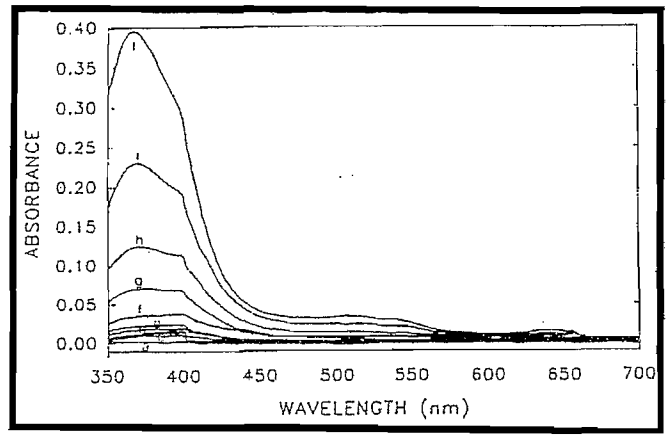


FIGURE 5.31: Visible spectra of saturated solutions of haematin in (a) 0, 2.0, (c) 2.5, (d) 3.0, (e) 3.5, (f) 4.0, (h) 5.0, (i) 5.5) and (j) 6.0 M acetate at pH 5.0 and 23 °C.

Weak bases are protonated rapidly in the acid vesicles because of their high dissociation constants, raising the intravesicular pH by means of the consumption of hydrogen ions (Krogstad and Schlesinger, 1987; Egan *et al.*, 1998), which is an ideal condition for the influx of antimalarial agents into the food vacuole. The low activity of compounds such as **5.9** and **5.10** with a piperidine moiety could be due to this fact. Although the bonding of the quinoline to ferriprotoporphyrin IX is a necessary, but not a requirement for antimalarial activity (Egan *et al.*, 1998), for molecules to bind to haem under prevailing conditions of the food vacuoles of the malaria parasite, they should maintain their bonding at pH 5,3. This would suggest bonding to the haem either via a mechanism other than coordination to the iron centre of the protoporphyrin IX such as π–π complexation or coordination via the Lewis base atom with a relatively low pKa value (Marques *et al.*, 1996; Adams, *et al.*, 1996). Absence of tertiary amino groups in these compounds could account for their low *in vitro* antimalarial activity.

5.8 FUTURE STUDIES AND APPLICATIONS

The findings from these investigations provide a rationale for further studies to investigate and confirm the following:

1) The mechanism of action of antimalarial drugs: the inhibition of the haemazoin polymerisation and ferriprotoporphyrin IX-drug complex

formation as opposed to the DNA binding and intercalating hypothesis as possible mechanism of action of quinoline-type drugs, the explanation why mefloquine-like drugs bind to membrane phospholipids to exert their effects, and the mode of action of the sesquiterpine lactones such artemisin and derivatives.

2) Development of resistance against antimalarial drugs:

3) The role of the nature of substituents at position 4 of the quinoline ring on the activity of the drug molecule with respect to the accumulation in the food vacuoles.

4) The nature and position of the substituents on the quinoline ring on activity against the chloroquine-sensitive and chloroquine-resistant strains of the Plasmodia, particularly the trifluoromethyl group and chlorine atom.

5) The antiviral activity of the 1,2,4-triazino[5,6b]indole and 1,2,4-triazolo[2,3b]-1,2,4-triazino[5,6b] indole derivatives particularly against the *Hepes simplex.* These compounds exhibit structural similarity to the current antiviral drugs.

6) The antitumor activity of quinoline-type compounds substituted with a trifluoromethyl group at positions 2 and 8 and other appropriate substituents at position 4.

7) It is necessary to confirm the role played by the nature and size of the functional groups at position 4 of the quinoline ring on antimalarial activity.

8) The basicity or acidity of the compounds need to be confirmed in order to establish the driving force behind the accumulation of these compounds in the food vacuoles which are regarded as the loci of activity of antimalarial drugs.

CHAPTER 6

EXPERIMENTAL SECTION

6.1 SYNTHESES

Melting points were determined in open capillary tubes on a Büchi B-545 apparatus and are uncorrected. IR spectra were recorded in KBr on a Perkin-Elmer Paragon 1000 FT-IR spectrophotometer. ^{1}H NMR spectra were taken on a GEMINI 2000BB spectrometer at 300 MHz. Chemical shifts are reported in δ (p.p.m) relative to internal trimethylsilane in DMSO-d_6 or as specified for some compounds. ^{13}C NMR spectra were recorded on the GEMINI 2000BB spectrometer at 75 MHz. Electron ionisation mass spectra as m/z (% relative intensity) values were carried out on a Turbomass GC-Mass spectrometer. TLC was carried out on pre-coated silica gel $60F_{254}$ analytical plates and the resulting chromatograms visualised under UV light (254 nm).

6.1.1 2- and 8-Trifluoromethyl- and 2,8-*bis*(trifluoromethyl)quinoline derivatives [(4.1) – (4.6)]

The synthesis and characterisation of this series of compounds was carried out in the previous studies (Kgokong, 1999; Kgokong and Breytenbach, 2000).

6.1.2 N,N-*Bis* (trifluoromethylquinolin-4-yl)diamino alkanes

General methods for the synthesis of the trifluoromethylquinolin-4yl diamino alkanes

2-Trifluoromethyl- or 2,8-*bis*(trifluoromethyl)-4-chloroquinoline (0.01 mole), diaminoalkane (including piperazine) (0.005 mole) and triethylamine (0.01 mole) were refluxed in 2-hydroxyethanol (20 ml) for 4 hours under a slight positive nitrogen pressure. The mixture was cooled and shaken with ethyl acetate (10 ml) and water (10 ml). The solid that formed was filtered and washed with ethyl acetate and water and dried.

N,N-bis(2-trifluoromethylquinolin-4-yl)diamino ethane (***5.1***): Yield 27%. Recrystallisation solvent: DMF, M.p. 286 °C (Found: C, 58.6; H, 3.7; N, 12.3. $C_{22}H_{16}N_4F_6$ requires C, 58.7; H, 3.6; N, 12.4). ^{1}H N.M.R. (Spectrum 1) δ: 2.7, t, CH_2; 3.3, t, CH_2; 3.9, bs, NH; 7.8, t , H 6; 7.9, t, H 7; 8.0, s, H 3; 8.2, d, J 6 Hz, H 5; 8.4, d, J 6 Hz, H 8. ^{13}C N.M.R.(Spectrum 2) δ: 46.1, CH_2; 109.5, C 3; 119.0, C 4a; 121.9, bq, $^1J_{CF}$ 213 Hz, 2-CF_3; 124.8, C 5; 126.0, C 6; 129.6, C 8; 130.4, C 7; 143.2, C 8a; 147.3, q, $^2J_{CF}$ 32 Hz, C 2; 152.2, C 4. E.i. mass spectrum (Spectrum 3) *m/z*: 450 (M, 12%), 226 (72), 225 (100), 197 (15), 128 (7). ν_{max} (Spectrum 4) (KBr): 3298s, 1581s, 1356s, 1295s, 1180s, 1128s.

N,N-bis[2,8-bis(trifluoromethyl)quinolin-4-yl]diamino ethane (***5.2***):Yield 33%. Recrystallisation solvent: ethanol. M.p. 222 °C. (Found: C, 50.8; H, 2.9; N, 9.6. $C_{24}H_{14}N_4F_{12}$ requires C, 49.2; H, 2.4; N, 9.6). ^{1}H N.M.R. (Spectrum 5) δ: 2.6, t, CH_2; 2.8, t, CH_2; 3.5, bs, NH; 3.8, bs, NH; 7.0, t, H 6; 8.1, s, H 3; 8.3, d, J 6 Hz, H 5; 8.5, d, J 6 Hz, H 5. ^{13}C N.M.R. (Spectrum 6) δ: 43.6, CH_2; 94.3, CH_2; 117.4, C 3; 118.5, C 4a; 119.5, bq, $^1J_{CF}$ 213 Hz, 2-CF_3; 124.6, q, $^2J_{CF}$ 32 Hz, C 8); 125.9, bq, $^1J_{CF}$ 213 Hz, 8-CF_3; 126.1, C 7; 129.1, C 6; 133.1, C 5; 144.0, C 8a; 148.2, q, $^2J_{CF}$ 32 Hz, C 2); 152.5, C 4. E.i. mass spectrum (Spectrum 7) *m/z*: 586 (M, 54%), 567 (27), 293 (100), 273 (25), 224 (33). ν_{max} (Spectrum 8) (KBr): 3368m, 1594s, 1443m, 1313s, 1136s.

N,N-bis(2-trifluoromethylquinolin-4-yl)diamino propane (**5.3**): Yield 17%. Recrystallisation solvent: methanol. M.p. 180 °C. (Found: C, 58.2; H, 4.0; N, 11.4. $C_{23}H_{18}N_4F_6$ requires C, 59.5; H, 3.9; N, 12.1). ^{1}H N.M.R. (Spectrum 9) δ: 2.1, q, CH_2; 3.4, q, CH_2; 3.9, q, CH_2; 4.4, bs, NH; 7.7, t, H 6; 7.8, t, H 7; 8.0, s, H 3; 8.2, d, J 6 Hz, H 5; 8.3, d, J 6 Hz, H 8. ^{13}C N.M.R. (Spectrum 10) δ: 46.2, CH_2; 62.8, CH_2; 108.9, C 3; 117.6, C 4a; 119.3, bq, $^1J_{CF}$ 213 Hz, 2-CF_3; 124.8, C 5; 125.9, C 6; 129.5, C 8; 130.3, C 7; 144.3, C 8a; 146.5, q, $^2J_{CF}$ 32 Hz, C 2); 152.1, C 4. E.i. mass spectrum (Spectrum 11) *m/z*: 464 (M, 69%), 252 (18), 239 (100), 213 (63). ν_{max} (Spectrum 12) (KBr): 3445w, 1580s, 1305m, 1132s, 1014w.

*N,N-bis[2,8-bis(trifluoromethyl)quinolin-4-yl]diamino propane (***5.4***):* Yield 34%. Recrystallisation solvent: DMF. M.p. 240 °C. (Found C, 50.9; H, 2.8; N, 9.2. $C_{25}H_{16}N_4F_{12}$ requires C, 50.0; H, 2.7; N, 9.3). ^{1}H N.M.R. (Spectrum 13) δ: 1.8, t, CH_2; 2.5, t, CH_2; 3.1, t, CH_2; 3.3, bs, NH; 7.1, t, H 6; 8.2, s, H 3; 8.3, d, J 6 Hz, H 7; 8.5, d, J 6 Hz, H 5. ^{13}C N.M.R. (Spectrum 14) δ: 46.6, CH_2; 62.7, CH_2; 110.2, C 3; 118.6, C 4a; 119.5, bq, $^1J_{CF}$ 213 Hz, 2-CF_3; 124.4, q, $^2J_{CF}$ 32 Hz, C 8; 125.9, bq, $^1J_{CF}$ 213 Hz, 8-CF_3; 126.3, C 7; 129.6, C 6; 133.1, C 5; 143.9, C 8a; 146.5, q, $^2J_{CF}$ 32 Hz, C 2; 152.4, C 4. E.i. mass spectrum (Spectrum 15) *m/z:* 600 (M, 82%), 343 (61), 319 (21), 307 (66), 293 (43), 280 (100), 273 (20), 265 (60), 224 (21), 196 (33), 176 (30), 57 (20), 45 (65). ν_{max} (Spectrum 16) (KBr): 3467m, 2924w, 1595s, 1312s, 1151s, 952m.

*N,N-bis(2-trifluoromethylquinolin-4-yl)diamino butane (***5.5***):*Yield 31%. Recrystallisation solvent: DMF. M.p. 219 °C. (Found C, 59.8; H, 4.6; N, 11.1. $C_{24}H_{20}N_4F_6$ requires C, 60.2; H, 4.2; N, 11.7). ^{1}H N.M.R. (Spectrum 17) δ: 3.3, bs, NH; 3.6, d, J 4,6 Hz, CH_2; 3.9, m, CH_2; 7.1, t, H 6; 7.4, t, H 7; 7.8, s, H 3; 8.3, d, J 6 Hz, H 7; 8.5, d, J 6 Hz, H 5. ^{13}C N.M.R. (Spectrum 18) δ: 26.8, CH_2; 44.8, CH_2; 109.7, C 3; 118.6, C 4a; 119.5, bq, $^1J_{CF}$ 213 Hz, 2-CF_3; 124.9, C 5; 127.8, C 6; 130.4, C 7; 132.6, C 8; 143.8, C 8a; 147.1, q, $^2J_{CF}$ 32 Hz, C 2; 152.3, C 4. E.i. mass spectrum (Spectrum 19) *m/z*: 478 (M, 53%), 266 (48), 239 (90), 237 (49), 225 (100), 213 (85), 205 (25). ν_{max} (spectrum 20) (KBr): 3386s, 1652s, 1407s, 1001m, 831m.

N,N-bis[2,8-bis(trifluoromethyl)quinolin-4-yl]diamino butane (**5.6**)*:* Yield 22%. Recrystallisation solvent: DMF. M.p.242 °C. (Found: 51.0; 3.2; N, 8.1. $C_{26}H_{18}N_4F_{12}$ requires C, 50.8; H, 3.0; N, 9.1). ^{1}H N.M.R (Spectrum 21) δ (acetone): 3.0, bs, NH; 3.6, m, CH_2; 6.9, t, H 6; 8.1, s, H 3; 8.4, d, J 6 Hz, H 7; 8.6, d, J 6 Hz, H 5. ^{13}C N.M.R. (Spectrum 22) δ: 26.9, CH_2; 44.1, CH_2; 110.3, C 3; 119.4, C 4a; 122.9, bq, $^1J_{CF}$ 213 Hz, 2-CF_3; 124.3, q, $^2J_{CF}$, 32 Hz, C 8; 125.7, bq, $^1J_{CF}$ 213 Hz, 8-CF_3; 126.4, C 7; 129.6, C 6; 133.4, C 5; 144.8, C 8a; 146.1, q, $^2J_{CF}$ 32 Hz, C 2; 153.9, C 4. E.i. mass spectrum (Spectrum 23) *m/z* : 614 (M, 19%), 573 (23), 382 (24), 334 (37), 314 (24), 307 (43), 293 (54),

281 (49), 267 (27), 224 (24), 97 (24), 69 (33), 57 (69), 44 (100), 28 (54). ν_{max} (Spectrum 24) (KBr): 2942w, 1592m, 1312s, 1138s, 950m, 764m.

N,N-bis(2-trifluoromethylquinolin-4-yl)diamino hexane (**5.7**)*:* Yield 32%. Recrystallisation solvent: DMF. M.p. 145 °C. (Found: C, 61.7, H, 5.0; N, 10.9. $C_{26}H_{24}N_4F_6$ requires C, 61.6; H, 4.8; N, 11.1). ^{1}H N.M.R. (Spectrum 25) δ 1.4, bs, NH; 1.7, bs, NH; 3.4, m, CH_2; 6.9, t, H 6; 7.5, t, H 7; 7.7, s, H 3; 7.9, d, J 6 Hz, H 5; 8.3 d, J 6 Hz, H 8. ^{13}C N.M.R. (Spectrum 26) δ: 26.4, CH_2; 30.8, CH_2; 42.5, CH_2; 118.9, C 3; 122.0, bq, $^1J_{CF}$ 213 Hz, 2-CF_3; 123.2, C 5; 125.9, C 4a; 129.6, C 6; 130.4, C 7; 133.5, C 8; 144.3, C 8a; 148.0, q, $^2J_{CF}$ 32 Hz, C 2; 152.1, C 4. E.i. mass spectrum (Spectrum 27) *m/z* : 506 (M, 17%), 486 (18), 281 (62), 239 (100), 205 (22), 44 (16). ν_{max} (Spectrum 28) (KBr): 3449m, 2931m, 1586s, 1289m, 1128s, 937m.

N,N-bis[2,8-bis(trifluoromethyl)quinolin-4-yl]diamino hexane (**5.8**)*:* Yield 29%. Recrystallisation solvent: DMF. M.p. 45 °C. (Found: C, 52.5; H, 3.6; N, 8.5. $C_{28}H_{22}N_4F_{12}$ requires C, 52.3; H, 3.5; N, 8.7). ^{1}H N.M.R.(Spectrum 29) δ: 1.3, t, CH_2; 1.4, t, CH_2; 3.8, t, CH_2; 3.9, t, CH_2; 4.5, bs, NH; 7.2, t, H 6; 7.7, s, H 3; 8.0, d, J 6 Hz, H 7; 8.8, d, J 6 Hz, H 5. ^{13}C N.M.R. Spectrum 30) δ: 26.1, CH_2; 42.4, CH_2; 65.2, CH_2; 110.2, C 3; 118.5, C 4a; 119.4, bq, $^1J_{CF}$ 213 Hz, 2-CF_3; 124.3, q, $^2J_{CF}$ 32 Hz, C 8; 125.2, bq, $^1J_{CF}$ 213 Hz, 8-CF_3; 128.8, C 7; 130.2, C 6; 133.4, C 5; 143.9, C 8a; 147.9, q, $^2J_{CF}$ 32 Hz, C 2; 152.4, C 4. E.i. mass spectrum (Spectrum 31) *m/z* : 642 (M, 18%), 362 (16), 349 (46), 307 (100), 293 (77), 273 (23), 224 (30), 44 (44). ν_{max} (Spectrum 32) (KBr): 3423w, 2938m, 1593s, 1446w, 1173s.

N,N-bis(2-trifluoromethylquinolin-4-yl) piperazine (**5.9**)*:* Yield 44%. Recrystallisation solvent: methanol. M.p. 310 °C. (Found: C, 60.0; H, 3.8; N, 11.7. $C_{24}H_{18}N_4F_6$ requires C, 60.5; H, 3.8; N, 11.8). ^{1}H N.M.R. (Spectrum 33) δ: 3.2, t, CH_2; 3.3, t, CH_2; 7.1, t, H 6; 7.2, t, H 7; 7.4, s, H 3; 7.9, d, J 6 Hz, H 5; 8.1, d, J 6 Hz, H 8. ^{13}C N.M.R. (Spectrum 34) δ: 25.7, CH_2; 42.5, CH_2; 65.3, CH_2; 109.7, C 3; 118.4, C 4a; 120.2, bq, $^1J_{CF}$ 213 Hz, 2-CF_3; 124.5, C 5; 125.7, C 6; 128.0, C 8; 132.8, C 7; 142.8, C 8a; 146.4, q, $^2J_{CF}$ 32 Hz, C 2;

151.4, C 4. E.i. mass spectrum (Spectrum 35) *m/z*: 476 (M, 67%), 251 (100), 224 (44), 89 (18), 45 (34). ν_{max} (Spectrum 36) (KBr): 3386w, 2934w, 1583s, 1419s, 1303m, 1242m, 1111s.

N,N-bis[2,8-bis(trifluoromethyl)quinolin-4-yl]piperazine (**5.10**): Yield 30%. Recrystallisation solvent: DMF. M.p. 350 °C (Found: C, 51.5; H, 2.6; N, 9.4. $C_{26}H_{16}N_4F_{12}$ requires C, 51.0; H, 2.6; N, 9.2). ^{1}H N.M.R.(Spectrum 37) δ: 3.1, t, CH_2; 3.3, t, CH_2; 7.1, t, H 6; 7.8, s, H 3; 8.2, d, J 6 Hz, H 7; 8.5, d, J 6 Hz, H 5. ^{13}C N.M.R. (Spectrum 38) δ: 26.8, CH_2; 43.0, CH_2; 65.5, CH_2; 109.7, C 3; 118.3, C 4a; 119.5, bq, $^1J_{CF}$ 213 Hz, 2-CF_3; 124.3, q, $^2J_{CF}$ 32 Hz, C 8; 125.6, bq, $^1J_{CF}$ 213 Hz, 8-CF_3; 126.8, C 7; 129.5, C 6; 132.9, C 5; 144.0, C 8a; 147.8, q, $^2J_{CF}$ 32 Hz, C 2; 152.5, C 4. E.i. mass spectrum (Spectrum 39) *m/z*: 612 (M, 52%), 319 (100), 292 (38), 223 (42), 44 (22). ν_{max} (Spectrum 40) (KBr): 3423w, 2938m, 1593s, 1314s, 1137s, 765m.

6.1.2 1,2,4-Triazino[5,6b]indole derivatives

A mixture of isatin or 7-trifluoromethylisatin (21.5 g: 0.1 mole), thiosemicarbazide (9.1 g: 0.1 mole) and K_2CO_3 (56.7 g: 0.15 mole) in 500 ml of water was refluxed with stirring for 3 hours. On cooling the mixture was filtered and precipitated by acidification with acetic acid. The solid was washed with water and dried. A sample was recrystallised from DMF.

1,2,4-Triazino5,6b]indole-3-thione (**48.1**):

Yield 15,20g (80,0%). M.p. 324 – 326 °C, with decomposition. Elemental analysis not done. ^{1}H N.M.R. (Spectrum 41) δ: 7.3 t, H5; 7,5 d, H4; 7,7 t, H6; 8.3 d, H7. ^{13}C N.M.R. (Spectrum 42) δ: 111,5; 118,0; 122,0; 130,0; 140,0 141,0; 140,6; 166,5; 180,6. E.i. mass spectrum (Spectrum 43) *m/z* (%): 201 (20), 188 (56), 172 (100), 146 (98), 129 (18), 102 (35), 98 (20), 45 (30). ν_{max} (Spectrum 44) (KBr): 2916, 1694, 1601s, 1158 s.

Trifluoromethyl-1,2,4-triazino[5,6b]indole-3-thione **(48.2):**

Yield 10.8 g (40%). M.p. 262 - 264 °C. (Found: C, 43.04; H, 1.13; N, 21.89. $C_9H_5N_4F_3S$ requires C, 41.84; H, 1.95; N, 21.71). ^1H N.M.R. (Spectrum 45) δ: 7.1, H5; 7.6 d, J 6 Hz, H 6; 7.9 d, J 8 Hz, H 4; 9.6 s, NH. ^{13}C N.M.R. (Spectrum 46) δ: 115.8 q, $^2J_{CF}$ 32 Hz, C7; 119.6; 120.8; 122.3; 125,6, bq, $^1J_{CF}$ 300 Hz, CF_3; 127.3; 140.6; 147.9; 149.9; 156.2. E.i. mass spectrum (Spectrum 47) *m/z* (%): M^+ 260 (100), 222 (46), 193 (24), 152 (47), 60 (51), 43 (33), 28 (29). ν_{max} (Spectrum 48) (KBr): 3430m, 3048s, 1602m, 1515s, 1407m, 1318s, 1182s, 1113s.

A general procedure for the synthesis of 3-alkylthio-1,2,4-triazino[5,6b]indoles: To a solution of **48.1** or **48.2** (0.01 mole) in aqueous sodium hydroxide (4%), alkyl halide (0.01 mole) was added during 2 - 5 min with stirring. The mixture was stirred for 1 hr and the precipitate filtered and washed with water and dried.

3-Methylthio-7-trifluoromethyl-1,2,4-triazino[5,6b]indole **(6.2)**
Yield 46%. Recrystallisation solvent: methanol. M.p. 168 - 170 °C. (Found: C, 42.,32; H, 2.93; N, 20.34. $C_{10}H_7N_4F_3S$ requires C, 44,10; H, 2,59; N, 20,59). ^1H N.M.R. (Spectrum 49) δ: 1.6, s, CH_3; 7.2, t, H 5; 7.8, d, J 6 Hz, H 6), 8.3, d, *J* 6 Hz, H 4; 9.8, bs, NH. ^{13}C N.M.R. (Spectrum 50) δ: 51.7, CH_3; 115.6, q, $^2J_{CF}$ 32 Hz, C 7; 119.4; 122.5; 124.3; 125.7, bq, $^1J_{CF}$ 300 Hz, CF_3;127.4; 142.4; 149.7; 154.7; 163.4. E.i. mass spectrum (Spectrum 51) *m/z* (%): M^+ 272 (100), 256 (19), 241 (18), 220 (33), 177 (20). ν_{max} (Spectrum 52) (KBr): 3415s, 3068m, 2926m, 1596s, 1411m, 1319s, 1175s.

3-Ethylthio-7-trifluoromethyl-1,2,4-triazino[5,6b]indole (**6.4**)
Yield 88%. Recrystallisation solvent: methanol. M.p. 244 - 246 °C. (Found: C, 45.97; H, 2.98; N, 20.03. $C_{11}H_9N_4F_3S$ requires C, 46.13; H, 3.17; N, 19.58). ^1H N.M.R. (Spectrum 53) δ: 1.4, t, 3H, CH_3; 2.3,q, *J* 7.4 Hz, 2H, CH_2; 7.6, t, H 5; 7.9, d, *J* 6 Hz, H 4; 8.6, d, *J* 6 Hz, H 6. ^{13}C N.M.R. (Spectrum 54) δ: 14.5, CH_3; 49.0, CH_2; 113.1, q, $^2J_{CF}$ = 32 Hz; 119.9; 122.3; 124.7; 125.6, bq, $^1J_{CF}$ 300 Hz, CF_3); 127.3; 140.0; 147.9; 155.8; 166.9. E.i. mass spectrum

(Spectrum 55) *m/z* (%): M^+ 286 (100), 242 (8), 222 (10), 177 (6), 69 (2), 44 (20). ν_{max} (Spectrum 56) (KBr): 3410s, 1563s, 1413s, 1320s, 1182s, 1121s.

3-Cyclopropylmethylthio-7-trifluoromethyl-1,2,4-triazino[5,6b]indole **(6.6)**
Yield 55 %. Recrystallisation solvent: DMF. M.p. 259 - 260 °C. (Found: C, 49.45; H, 4.06; N, 18.67. $C_{13}H_{11}N_4F_3S$ requires C, 49.97; H, 3.55; N, 17.95. ^{1}H N.M.R. (Spectrum 57) δ: 1.9, d, *J* 8.2 Hz, 2H, CH_2); 2.5,q, 2H, CH_2; 3.4, m, 4H, CH; 7.3, t, H 5; 7.8, d, *J* 6 Hz, H 4; 8.1, d, J 6 Hz, H 6; 8.3, s, 1H, NH). ^{13}C N.M.R. (Spectrum 58) δ: 14.7 (CH_2); 25.6 (CH_2); 53.5 (CH); 112.7, bq, $^2J_{CF}$ 32 Hz; 118.7; 122.5; 124.3; 124.8; 125.6, bq, $^1J_{CF}$ 300 Hz, CF_3; 137.2; 139.5; 161.0. E.i. mass spectrum (Spectrum 59) *m/z* (%): M^+ 312 (60), 309 (30), 270 (100), 242 (21), 222 (68). ν_{max} (Spectrum 60) (KBr): 3460s, 1597s, 1413m, 1320m, 1161s.

3-Hydrazino-1,2,4-triazino-and 3-Hydrazino-7-trifluoromethyl-1,2,4-triazino-[5,6b]indoles **(49.1 and 49.2)**:
A mixture of 6-trifluoromethyl-1,2,4-triazino[5,6b]indole-3-thione (2.70 g : 0.01 mole) and 10 ml of hydrazine hydrate (98%) was refluxed for 4 hrs. On cooling alight yellow solid separated and was washed with water, methanol and recrystallized from DMF.

3-Hydrazino-1,2,4-triazino[5,6b]indole (**49.1**): Yield 7,6 g (65%). M.p. 280 – 283 °C. Elemental analysis not done. ^{1}H N.M.R. (Spectrum 61) δ: 3.4 bs, NH, 7.4 t, H5, 7.5 d, H4, 7.7 t, H6, 8.3 d, H7. ^{13}C N.M.R. (Spectrum 62) δ: 112, 118, 122, 130, 140, 147, 156, 168. E.i. mass spectrum (Spectrum 63) m/z (%): 200 (55), 155 (40), 129 (56), 103 (100), 76 (60), 45 (50). ν_{max} (Spectrum 64) (KBr): 2925 bs, 1678s, 1614s, 1195m.

3-Hydrazino-7-trifluoromethyl-1,2,4-triazino[5,6b]indole (**49.2**):
Yield:1,40 g (52 %). M.p. 279 – 281 °C. (Found: C, 44.97; H, 2.75; N, 30.86. $C_{10}H_7N_6F_3$ requires 44.76; H, 2.63; N, 31.35). ^{1}H N.M.R. (Spectrum 65) δ: 2.5, t, 3H, NH; 3.3, d, *J* 8 Hz, 2H, NH_2; 7.4, t, H 5; 7.8, d, J 6 Hz, H 4; 8.4, d, J 6 Hz, H 6; 8.8, s, 1H, NH. ^{13}C N.M.R. (Spectrum 66) δ: 113.6, q, $^2J_{CF}$ 32 Hz;

117. 2; 119.8; 122,4; 124.3; 124.6; 125.8, bq, $^1J_{CF}$ 300 Hz, CF_3; 127.4; 139.8; 147.8; 168.5. E.i. mass spectrum (Spectrum 67) *m/z* (%): M^+ 268 (100), 204 (34), 177 (35), 44 (11), 28 (8). ν_{max} (Spectrum 68) (KBr): 3270s, 1612s, 1518s, 1402m, 1310s, 1176s, 1110s.

3-Azido-7-trifluoromethyl-1,2,4-triazino[5,6b]indole **(50.2)**: Refluxing of 0,1 mole of 49.2 with sodium nitrite in hydrochloric acid and the precipitating solid filtered and washed with water. The solid was crystallised from boiling DMF. Yield 2,7 g (56%). M.p 208 – 213 °C. Elemental analysis not done. ^{1}H N.M.R. (Spectrum 69) δ: 7.6 t, H5; 8.05 d, H4; 8.45 d, H6. ^{13}C N.M.R. (Spectrum 70) δ: 104.3; 115.98 q, $^2J_{CF}$ 32 Hz, C7; 122.1; 123.0; 133.5, bq, $^1J_{CF}$ 300 Hz, CF_3; 136.4; 140.7; 145.8; 148.2. E.i. mass spectrum (Spectrum 71) m/z (%): M+ 279 (60), 228 (30), 203 (32), 177 (15), 151 (100), 144 (14), 69 (30). ν_{max} (Spectrum 72) (KBr): 2887m, 1601s, 1424m, 1153s.

General procedure for the synthesis of the 5H-1,2,4-triazolo[2,3b]-1,2,4-triazino[5,6b]indole derivatives

2-Hydrazino-1,2,4-triazino[5,6b]indole derivative **(49.1** or **49.2)** (0.01 mole) was refluxed in 10 ml of each of formic, acetic, trifluoroacetic and chlorobenzoic acid for 2 hrs. The solution was precipitated by addition of water, the product filtered and dried.

5H-1,2,4-Triazolo[2,3b]-1,2,4-triazino[5,6b]indole **(7.1)**

Yield 78%. Recrystallisation solvent: DMF. Mp 372 – 374 °C. (Found C, 56.97; H, 3.07; N, 39.83. $C_{10}H_6N_6$ requires C, 57.12; H, 2.88; N, 40.00). ^{1}H N.M.R. (Spectrum 73) δ: 7.3, t, H 6; 7.4, d, *J* 6 Hz, H 4; 7.7, t, H 5; 8.1, d, *J* 6 Hz, H 7; 9.5, s, H, H 1; 12.2, br s, 1H, NH. ^{13}C N.M.R. (Spectrum 74) δ: 112.5; 116.0; 122.4; 133.6; 136.5; 145.8; 151.0; 155.3; 158.3; 168.1. E.i. mass spectrum (Spectrum 75) *m/z* (%): M^+ 210 (100), 155 (58), 128 (79), 103 (91), 76 (63), 50 (26), 44 (34), 28 (20). ν_{max} (Spectrum 76) (KBr): 2634w, 1611s, 1452m.

7-Trifluoromethyl-5H-1,2,4-triazolo[2,3b]-1,2,4-triazino[5,6b]indole (**7.2**)

Yield 58 %. Recrystallisation solvent: ethanol. M.p. 236 – 238 °C. (Found: 47.56; H, 1.75; N, 30.43. $C_{11}H_5N_6F_3$ requires C, 47.47; H, 1.81; N, 30.22). ^{1}H N.M.R. (Spectrum 77) δ: 7.5, t, H 5; 8.0, d, *J* 6 Hz, H 4; 8.5, d, *J* 6 Hz, H 6; 9.6, s, 1H, H 1; 12.6, bs, 1H, NH; ^{13}C N.M.R. (Spectrum 78) δ: 115.8, q, $^2J_{CF}$ 32 Hz; 117.2; 120.9; 126.1, bq, $^1J_{CF}$ 300 Hz, CF_3; 128.7; 135.6; 147.8; 148.1; 153.4; 162.1. E.i. mass spectrum (Spectrum 79) *m/z* (%): M^+ 278 (100), 258 (52), 223 (26), 203 (56), 177 (34), 170 (26), 151 (91), 75 (25), 57 (6), 29 (16). ν_{max} (Spectrum 80) (KBr): 3105m, 1710m, 1619s, 1533m, 1317s, 1124s, 740m.

3-Methyl-5H-1,2,4-triazolo[2,3b]-1,2,4-triazono[5,6b]indole **(7.3)**

Yield 76%. Recrystallisation solvent: DMF. Mp 401 – 402 °C. (Found: C, 59.47; H, 4.03; N, 36.86. $C_{11}H_8N_6$ requires C, 58.90; H, 3.60; N, 37.50). ^{1}H N.M.R. (Spectrum 81) δ: 2.7, s, CH_3; 7.3, t, H 5; 7.4, d, *J* 6 Hz, H 4; 7.7, t, H 5; 8.1, d, *J* 6 Hz, H 7; 12.1, br s, NH. ^{13}C N.M.R. (Spectrum 82) δ: 13.5, CH_3; 115.6; 118.8; 121.5; 125.6; 131.5; 140,1;, 146.7; 148.1; 156.1; 158.0. E.i. mass spectrum (Spectrum 83) *m/z* (%): M^+ 224 (100), 155 (46), 143 (3), 128 (71), 103 (88), 101 (22), 84 (8), 76 (53) 57 (22), 50 (24), 44 (66), 28 (34). ν_{max} (Spectrum 84) (KBr): 3054 m, 2943m, 1612s, 1521s, 1463m, 1379m, 1297m, 1081m, 745m.

3-Methyl-7-trifluoromethyl-5H-1,2,4-triazolo[2,3b]-1,2,4-triazino[5,6b]indole ***(7.4)***

Yield 47 %. Recrystallisation solvent: methanol. M.p. 241 – 244 °C. Found: 48.74; H, 2.98; N, 29.08. $C_{12}H_8N_6F_3$ requires C, 49.13; H, 2.75; N, 28.67). ^{1}H N.M.R. (Spectrum 85) δ: 2.8, s, 1H, CH_3; 7.5, t, H 5; 8.0, d, *J* 6 Hz, H 4; 8.4, d, *J* 6 Hz, H 6; 12.6, bs, 1H, NH. ^{13}C N.M.R. (Spectrum 86) δ: 9.8, CH_3; 114.3, q, $^2J_{CF}$ 30 Hz; 118.4; 120.5; 121.8; 125.1, bq,$^1J_{CF}$ 300 Hz, CF_3; 129.6; 141.8; 144.3; 148.3; 155.6; 165.1. E.i. mass spectrum (Spectrum 87) *m/z* (%): M^+ 292 (100), 151 (8), 141 (2), 125 (2), 57 (7), 44 (17), 28 (11). ν_{max} (Spectrum 88) (KBr): 3372m, 1618s, 1512s, 1456m, 1315s, 1117s, 753m.

3-Trifluoromethyl-5H-1,2,4-triazolo[2,3b]-1,2,4-triazino[5,6b]indole **(7.5)**

Yield 92%. Recrystallisation solvent: DMF. Mp 398 – 400 °C. (Found: C, 46.97; H, 1.91; N, 31.21. $C_{11}H_5N_6F_3$ requires C, 47.47; H, 1.81; N, 30.22). ^1H N.M.R. (Spectrum 89) δ: 7.3, t, H 5; 7.5, d, *J* 6 Hz, H 4; 7.8, t, H 6; 8.3, d, *J* 6 Hz, H 7; 12.6, br s, NH. ^{13}C N.M.R.(Spectrum 90) δ: 115.9; 117.2; 118.3; 121.8; 128.3, br q, $^1J_{CF}$ 300 Hz, CF_3; 130.8 140.3; 147.2; 148.7; 155.1, q, $^2J_{CF}$ 30 Hz; 156.2. E.i. mass spectrum (Spectrum 91) *m/z* (%): M^+ 278 (100), 200 (54), 155 (34), 143 (8), 128 (68), 103 (94), 76 (52). ν_{max} (Spectrum 92) (KBR): 3079m, 2979m, 1677m, 1616s, 1385m, 1195s, 751m.

3,7-Bis(trifluoromethyl)-5H-1,2,4-triazolo[2,3b]-1,2,4-triazino[5,6b]indole (**7.6**)

Yield 34 %. Recrystallisation solvent: ethanol. M.p. 263 – 265 °C. (Found: C, 40.87; H, 0.97; N, 24.67. $C_{12}H_4N_6F_6$ requires C, 41.61; H, 1.17; N, 24.28). ^1H N.M.R. Spectrum 93) δ: 7.5, t, H 5; 7.9, d, *J* 6 Hz, H 4; 8.4, d, *J* 6 Hz, H 6. ^{13}C N.M.R. (Spectrum 94) δ: 115,1,q, $^2J_{CF}$ 30 Hz; 118.4; 121.3; 122.8; 126.9, bq, $^1J_{CF}$ 300 Hz, CF_3; 128.6, bq, $^1J_{CF}$ 300 Hz, CF_3; 131.5; 140.2;, 148.2; 148.3; 155.9, q, $^2J_{CF}$ 30 Hz; 157.6. E.i. mass spectrum (Spectrum 95) *m/z* (%): [M-1] 345 (3), 286 (4), 268 (11), 253 (12), 205 (14), 177 (9), 128 (5), 97 (8), 57 (22), 44 (100). ν_{max} (Spectrum 96) (KBr): 3233m, 2925m, 1679s, 1655s, 1406m, 1322m, 1195s

3-(2-Chlorophenyl)-5H-1,2,4-triazolo[2,3b]-1,2,4-triazino[5,6b]indole **(7.7)**

Yield 55%. Recrystallisation solvent: DMF. Mp 376 – 378 °C. (Found: C, 61,07; H, 2.27; N, 25.89. $C_{16}H_9N_6Cl$ requires C, 59.89; H, 2.83; N, 26.22). ^1H N.M.R. (Spectrum 97) δ: 7.2, m,) H-arom; 7.3, t, H 5; 7.4, d, *J* 6 Hz, H 4; 7.7, t, H 6; 8.1, d, *J* 6 Hz, H 7; 12.2, s, 1H, NH. ^{13}C N.M.R. (Spectrum 98) δ: 112.4; 117.9; 122.1; 123.1; 127.9; 130.0; 131.8; 132.1; 133.6; 136.5; 140.7; 145.8; 155.3; 156.5; 168.3; 198.1. E.i. mass spectrum (Spectrum 99) *m/z* (%): M+ 320 (50), 209 (100), 155 (60), 112 (50), 101 (80), 85 (20), 45 (25). ν_{max} (Spectrum 100) (KBr): = 2625m, 1610s, 1520s, 1204m, 1151w, 753m.

3-(2-Chlorophenyl)-7-trifluoromethyl-5H-1,2,4-triazolo[2,3b]-1,2,4-triazino[5,6b]indole (**7.8**)

Yield 48 %. Recrystallisation solvent: DMF. M.p. 286 - 288 °C. (Found: C, 52.76; H, 1.93; N, 22.56. $C_{17}H_8N_6F_3Cl$ requires C, 52.50; H, 2.08; N, 21.63). ^{1}H N.M.R. (Spectrum 101) δ: 7.3, t, 1H arom); 7.5, t,), H 5; 8.1, d, *J* 6 Hz, H 4; 8.3, d, *J* 6 Hz, H 6; 10.3, bs, NH. ^{13}C N.M.R. (Spectrum 102) δ: 116.3, q, $^2J_{CF}$ 30 Hz; 121.2; 122.6; 126.6, bq, $^1J_{CF}$ 300 Hz, CF_3; 127.9; 128.0; 130.2; 131.3; 131.8; 132.1; 135.6; 148.2; 155.3; 157.5; 169.6. E.i. mass spectrum (Spectrum 103) *m/z* (%): M^+ 388 (5), 357 (22), 333 (70), 278 (87), 268 (42), 177 (24), 151 (58), 139 (30), 111 (31), 97 (44), 85 (49), 69 (51), 57 (100), 45 (41). ν_{max} (Spectrum 104) (KBr): 3248w, 2921m, 1612s, 1315m, 1195s.

6.2 THE EVALUATION OF THE ANTIMALARIAL ACTIVITY

6.2.1 The Preparation of Compound suspensions

The test compounds were dissolved in 10% methanol to give a 2 mg/ml stock solution. All the compounds formed a milky suspension in this solvent system and were tested as such. Chloroquine (CQ) was used as the reference drug in all experiments. The stock solutions of the compounds were stored at – 20 °C until use. A full dose response was performed with a starting concentration of 100 μg/ml, which was serially diluted 2-fold in complete culture medium (RPMI 1640 containing 25 mmol// HEPES buffer, 20 μg/ml gentamycin, 27 mmol $NaHCO_3$, and 10% normal type A human serum) to give 10 concentrations ranging from 0.01 to 100 μg/ml. Chloroquine was tested at a starting concentration of 1000 ng/ml using the same dilution technique. The highest concentration of solvents to which the parasites were exposed to had no measurable effect on the parasite viability.

6.2.2 The Preparation of the Parasite Inocula

The parasite inocula used in the experiments consisted of isolates of the chloroquine-sensitive (D10) and chloroquine-resistant (K1) strains of *P. falciparum* obtained from Groote Schuur Hospital. These were used when the tropozoite stage parasitaemia was adjusted to at least 2 % with normal type A

human red blood cells (2% hematocrit). The parasite culture was suspended in complete tissue culture medium (RPMI 1640 containing 25 mmol// HEPES buffer, 20 μg/ml gentamycin, 27 mmol $NaHCO_3$) and 10% normal type A human serum. 25 μl of the drug suspension to contain from 0,01 to 100 μg/ml of the drugs were added in triplicate at 0.2 ml//well into 96-well flat-bottom microtitre plates, leaving one row for positive control (no drug containing parasitised red blood cells) and one row for background control (no parasites but only red blood cells). Incubation was carried out at 37 °C in a humidified atmosphere of 6% CO_2, 3 % O_2 and 91% N_2 for 48 hours.

6.2.3 The Harvesting and the Parasite Lactate Dehydrogenase Assay

After the incubation period, the parasite lactate dehydrogenase (pLDH) was determined. 10 μl from each well of the suspended culture was transferred into another 96 well, flat bottom microtiter plate that contained 100 μg of the Malstat reagent. The plates were left in the dark for 0.5 – 1.0 hour. At the end of this period, the reduction of APAD to APADH was followed kinetically. To each well 25 μl of a 20:1 mixture of nitroblue tetrazolium (NBT) and phenazine ethosulphate (PES) was added and the reduction of the tetrazolium to the blue formazan salt was followed for 10 minutes at 650 nm. Finally, the blue formazan product was evaluated after addition of 30 μl of 2N H_2SO_4 by end-point analysis at 650 nm using the Anthos Labotec HT2 model 1.06 (Anthos Labotec Instruments, Salzburg, Austria). The 50% inhibitory concentration (IC_{50}) values were obtained using a non-linear dose-response curve fitting analyses *via* GraphPad Prism v 4.0 software.

6.2.4 Compounds Interaction with Ferriprotoporphyrin IX

Stock solutions were prepared by dissolving between 6 to 8 mg of accurately weighed ferriprotoporphyrin IX (Sigma-Aldrich Chemie, Steinheim, Germany) in 10 ml AR grade DMSO (Sigma-Aldrich Chemie). These stock solutions were stored in the dark. Aqueous-DMSO (40% v/v) solutions of FP were prepared daily by mixing 20 μl of the FP stock solution with 4 ml DMSO and 1.0 ml of 0. 2 mM HEPES buffer (pH 7,4), and making up to 10 ml with deionised water. Solutions of the compounds of interest were prepared by

dissolving them in 0.02M HEPES and 40% DMSO to obtain final concentrations of about 2 mM. The ferriprotoporphyrin IX - compounds interactions were monitored by spectrophotometric titration of both sample and reference solutions in a thermostated cell holders using a Cary 100 Conc UV/VIS Spectrophotometer (Varian Australia (Pty) Ltd., Mulgrave Victoria, Australia) at 25 °C and measuring the absorbance of the Soret band at 230 nm. A reference cell was titrated simultaneously with the compounds. The compounds seem to give similar reaction with Ferriprotoporphyrin IX.

6.2.5 The Binding of the Compounds with the DNA

In binding studies performed according to previous procedure (Stewart, 1988), ethidium bromide was added to 3 ml buffer solution [2 mM N-hydroxyethyl-piperazine-N'-2-ethylsulphonic acid (HEPES), 8 mM NaCl and 0,05 mM EDTA, pH 7] to obtain a final concentration of 1,26 μM of ethidium in a 1 cm pathlength cuvette and a fluorescence spectrophotometer reading adjusted to between 6 and 8. DNA (calf thymus; Sigma- Aldrich Chemie, Steinhein, Germany) (3 μM) was added to the solution and the enhancement of fluorescence recorded to obtain a near saturation of ethidium bromide with DNA. The optimal amount of DNA determined by this method was used in the competition experiments. The test compounds were added in microlitre portions via a syringe while the temperature was maintained at 25 °C during the assays. Titration were performed in duplicate and EC_{50} values, representing the concentration of the compounds needed to decrease the DNA-bound ethidium fluorescence by 50% were determined and recorded. From the values obtained % reduction in relative fluorescence vs. concentration curves of the drugs plots were prepared. Solutions of the test compounds and quinine sulphate were prepared each in 7,5 mM NaH_2PO_4, 1 mM EDTA, pH 7 to obtain a final concentration of 0,06 mM and their UV absorption spectra in the presence and absence of salmon testis DNA (3,0 mM) was monitored at 280 to 500 nm with a Cary UV/VIS spectrophotometer (Varian Australia (Pty) Ltd., Mulgrave Victoria, Australia).

6.3 ASSESSMENT OF CYTOTOXICITY OF THE NEW COMPOUNDS

6.3.1 Cytotoxicity against Human Promyelocytic Leukaemia Cell Lines

A cytotoxicity assay based on a published procedure (Rosowsky *et al.*, 1988; Mannani *et al.*, 1990) that uses a colour reaction to measure the number of viable cells was used. Human promyelocytic leukemia (HL-60) cells (American Type Culture Collection, Rockville, MD) were grown as a suspension culture and activated in RPMI 1640 medium supplemented with 10% decomplemented foetal calf serum (FCS) (Highveld Biologicals, South Africa), penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone (0,5 μl/ml), L-glutamin and sodium hydrogen carbonate and maintained at 37 °C. Cells in the exponential growth phase were used in all experiments. Compounds were prepared in dimethyl sulphoxide (DMSO) at a concentration of 10 μM and stored as stock solution at -20 °C in the dark. Serial addition of aliqouts of each compound in RPMI 1640 medium containing 10% decomplemented FCS were added in individual wells of a 96-well tissue culture plate, leaving one row for control (no drug) and one row for blank (no cells). The cells were diluted to 1,2 x 10^5 cells/ml and 50 μl of the cell suspension was added to each well containing various concentrations (from 5 to 1000 μM) of the drugs except in the blank row. Incubations were carried out at 37 °C in a humidified atmosphere with 5% CO_2 - 95% air for 48 hours.

After a period of incubation, 10 μl of a 5 mg/ml solution of tetrazolium salt dissolved in 10 mM sodium phosphate buffer/ 150 mM NaCl, pH 7,4 and sterile-filtered, was added to each well. During the ensuing 3 hour incubation period, the mitochondrial dehydrogenases in the viable cells cleaved the tetrazolium ring resulting in the formation of dark blue formazan crystals which were dissolved by adding 100 μl of 0,04 N HCl in *i*-PrOH to each well with thorough mixing. The optical density of each well was then measured daily at 570 nm on an ELISA plate reader. The plates were read within 1 hour following the addition of the HCl and *i*-PrOH. Using a Coulter Z1 counter (Counter Electronics Ltd., UK), the number of cells in each sample was determined and % viability calculated from:

$$\% \text{ viability} = N_1/N_2 \times 100$$

where N_1 is the number of live cells in the compound treated culture, and N_2 is the number of live cells in the control culture. All the determinations were done in triplicate. Aggregate values obtained were used to prepare the % cell count and % cell viability vs. concentrations of the drugs curves. From these plots, the concentrations of the drugs causing a 50% decrease in the number of viable cells compared to the control (IC_{50} values) were read.

6.3.1.1 Detection of Apoptosis

The effect of the compounds on internucleosomal DNA fragmentation was analysed according to a published method with some modifications (Efferth *et al.*, 1996). Briefly, control and experimental cultures were harvested by centrifugation and washed twice in ice-cold PBS, pH 7,4. The cell pellets were suspended in 0,5 ml of 10 mM Tris-HCl buffer (pH 7,6), 0,5 % Triton X-100, and 1,0 mM EDTA by gentle pipetting. The lysates were clarified by centrifugation at 15 000 rpm for 10 minutes and the pellets containing high molecular mass DNA and cellular debris discarded. The supernatants were treated with 0,5 mg/ml RNase A (Sigma, St Louis, MO) at 37 °C for 3 hours followed by proteinase K (Boeringer Mannheim, Germany) at 0,5 mg/ml for a further 2 hours at 37 °C with gentle agitation. The DNA in the supernatant was finally extracted three times with equal volumes of phenol:chloroform mixture (1:1) and precipitated in the presence of 0,3 M sodium acetate, pH 5,2, with equal volume of ice-cold isopropanol. The DNA was spun, washed with 70 % ethanol, air-dried, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8,0). The DNA was quantitated by a Cary 100 UV/VIS spectrophotometer (Varian Australia (Pty) Ltd., Mulgrave Victoria, Australia) and fractionated on 2% agarose gels in the presence of 0,5 μg/ml ethidium bromide after 3, 6 and 24 hours.

6.3.1.2 Light Microscopy

Cells from the test culture after treatment with the 10 μM of the drugs were centrifuged at 500 rpm for 10 minutes and placed onto glass slides by

cytospin (Shandon), and fixed with absolute methanol prior to staining with "Hemacolor rapid staining set for blood smears" as recommended by the manufacturers (Merck Darmstd, Germany. The effect of the compounds on the morphology of the cells was evaluated using the Zeiss Axiolab microscope with magnification of 630 to which a MC 100 spot camera (all from Zeiss, Germany) is attached.

6.3.2 Cytotoxicity against Chinese Hamster Ovarian Cell lines

The mammalian cell line, Chinese hamster Ovarian (CHO) obtained from the Department of Pharmacology, Faculty of Medicine, University of Cape Town were grown in RPMI 1640 supplemented with 50 μM 2-mercaptoethanol and 5 – 10% foetal bovine serum in a 6% CO_2 atmosphere, were used for the study. *Stock solutions of compounds exhibiting highest activity against the* chloroquine-resistant strains of *P. falciparum,* the compounds were prepared to a 2 mg/ml in 10% methanol and stored at –20 °C until use. All the compounds formed milky suspensions. The initial concentration of emetine (Sigma-Aldrich Chemie, Steinheim, Germany) used as a standard, was 100 μg/ml and was serially diluted in complete medium to 10-fold dilutions to give 6 concentrations ranging from 0.001 to 100 μg/ml. Similarly, the test compounds were diluted from the stock solutions to form concentrations from 0.01 to 100 μg/ml. The highest concentration of the solvent to which the cells were exposed to did not have a measurable effect on the cell viability.

3-(4,5-dimethilthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich Chemie, Germany) was dissolved in phosphate-buffered saline at 5 mg/ml and filtered to sterilise and remove a small amount of insoluble residue present to form a stock solution. At regular intervals this stock solution (10 μl per 100 μl) was added to all the wells of the 96-well flat bottom trays, each of which contains 1 x 10^6 cells to which 25 μl of the drug suspensions varying in concentrations from 0.01 to 100 μg/ml was added. One row contained the cells and the emetine standard ranging in concentration from 0.01 to 100 μg/ml. The plates were incubated at 37 °C for 4 hours. 100 μl of acid-isopropanol (0.04 N HCl in isopropanol) was added to all the wells and mixed

thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, the plates were read at 650 nm using the Anthos Labotec.HT2 model 1.06 MicroELISA reader linked to a computer with GraphPad Prism v.4 software. The 50% inhibitory concentration (IC_{50}) values were obtained from the full dose-response curves using a non-linear dose response curve fitting analysis. The plates were normally read within 1 hour of adding the isopropanol.

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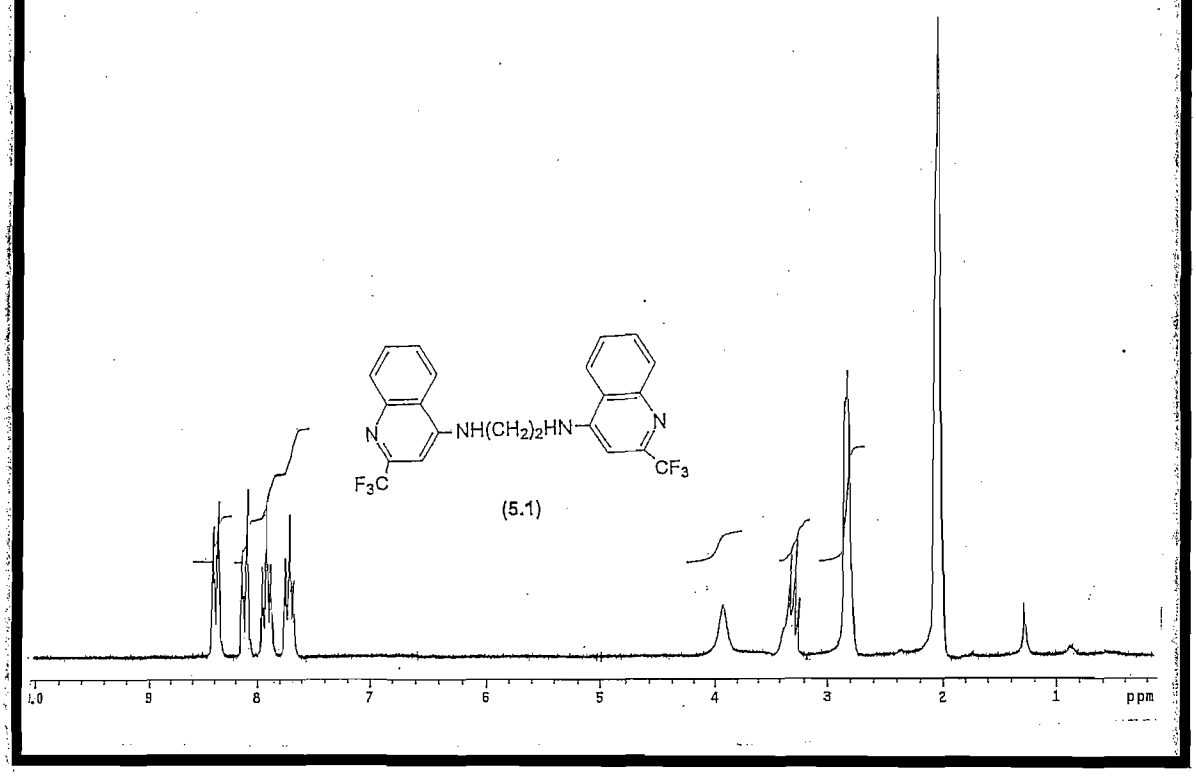

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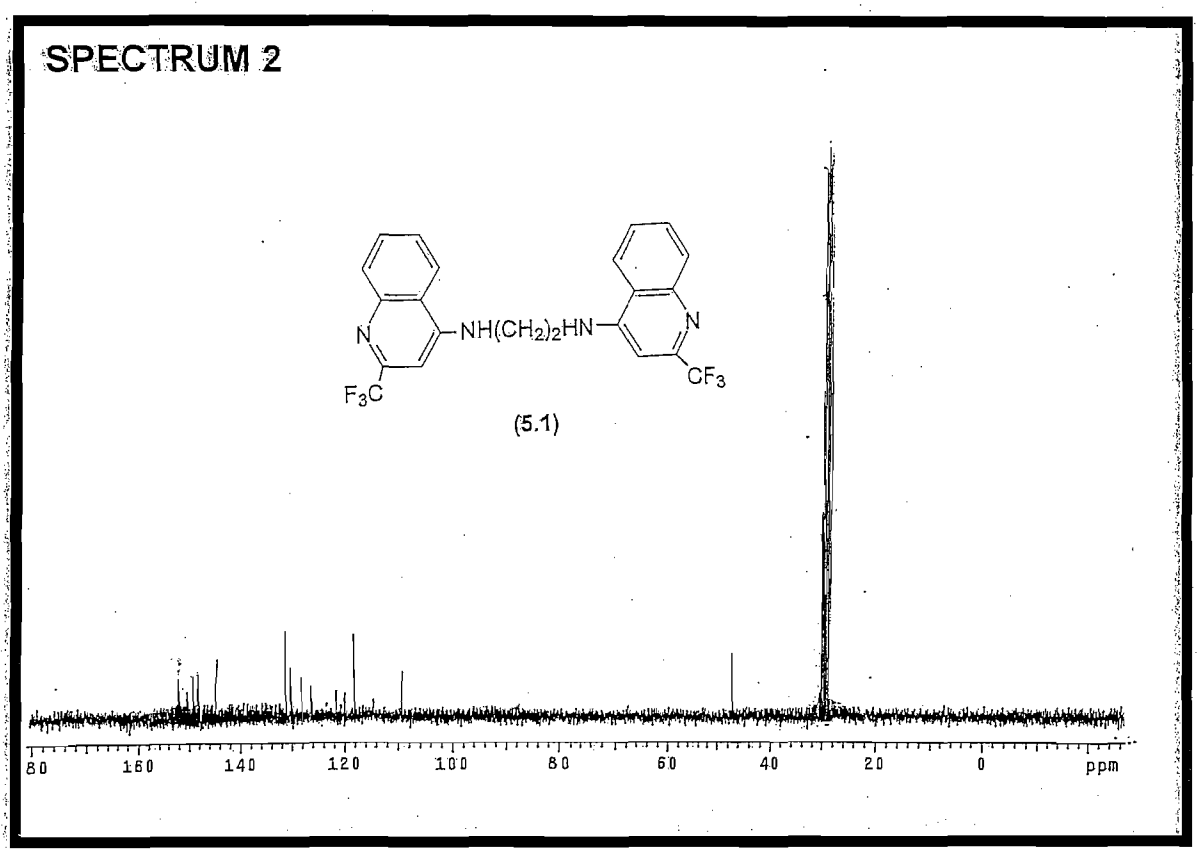

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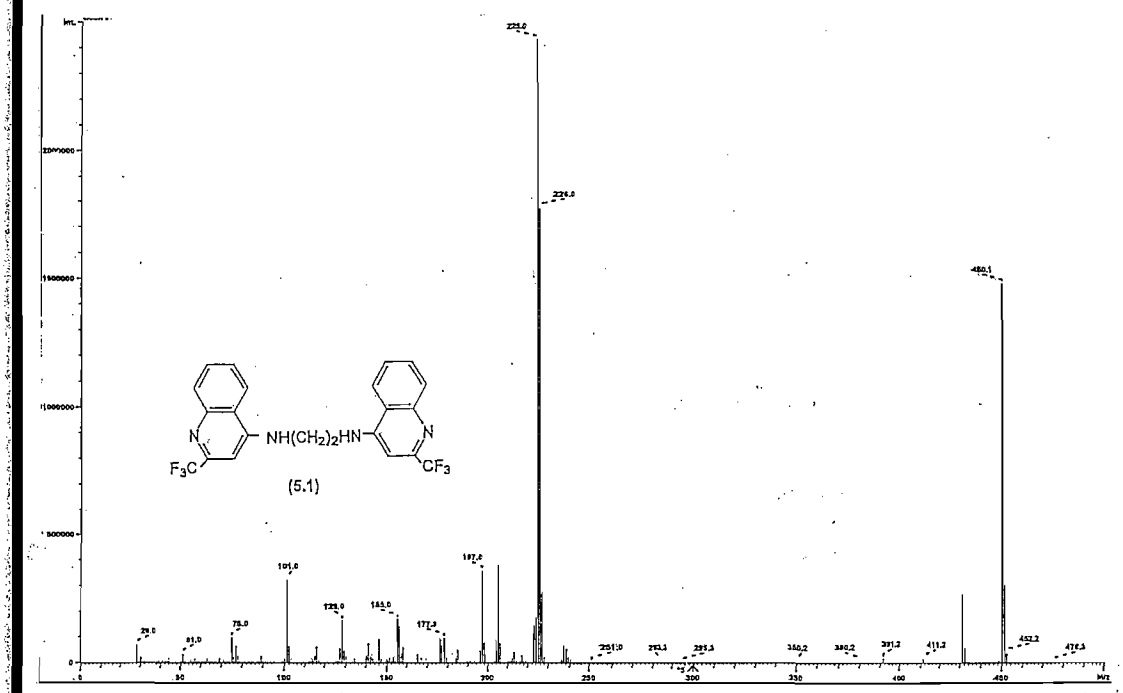

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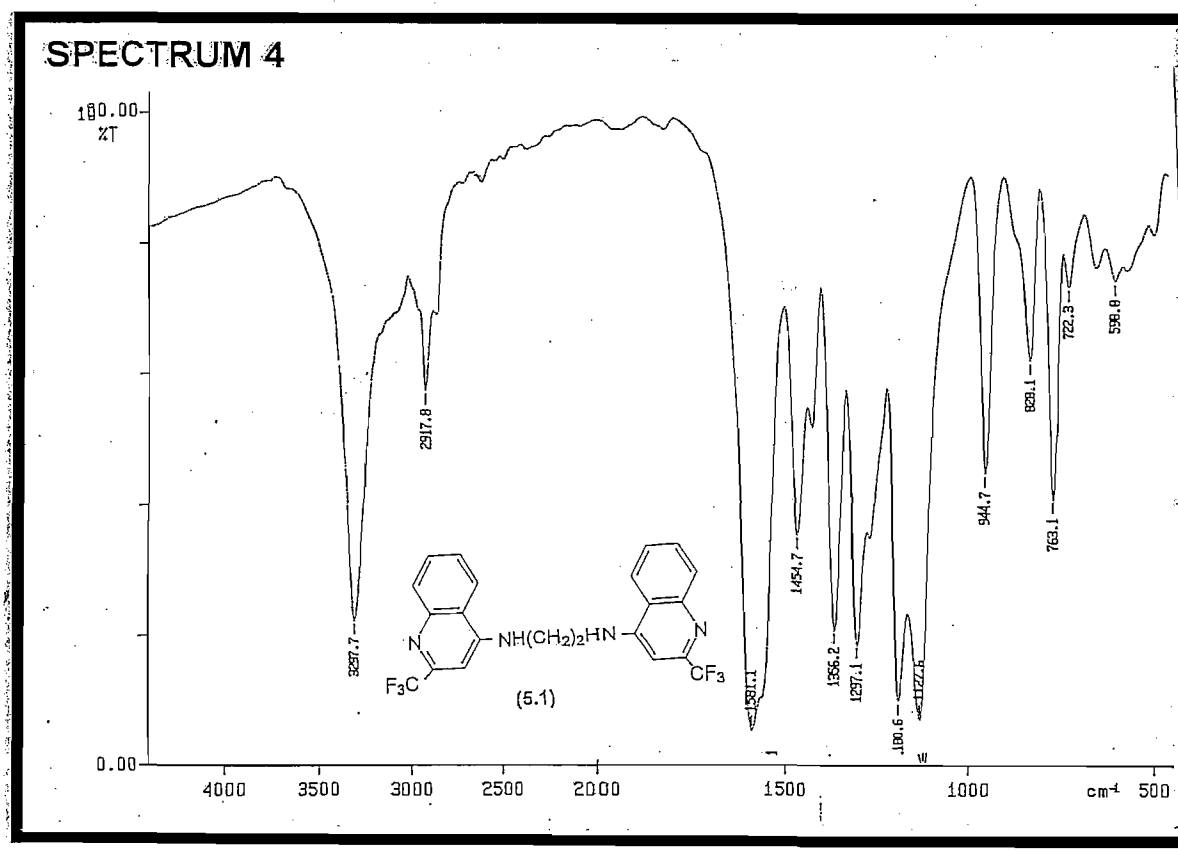

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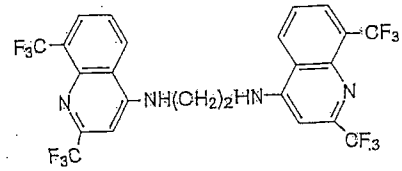

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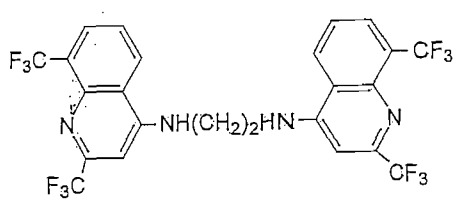

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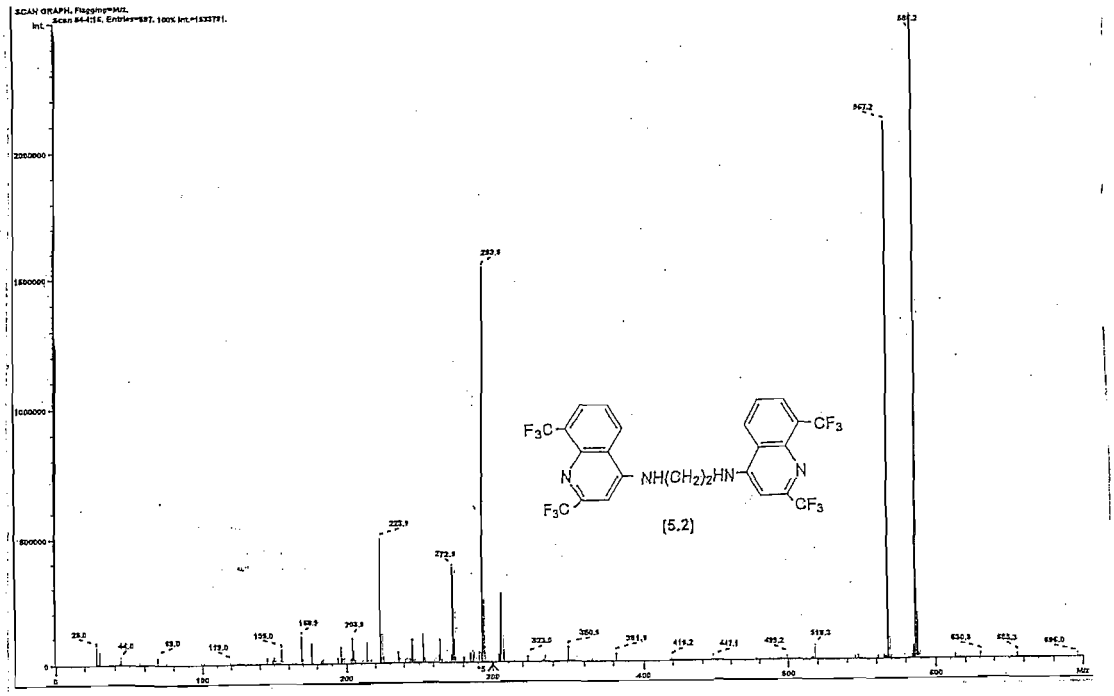

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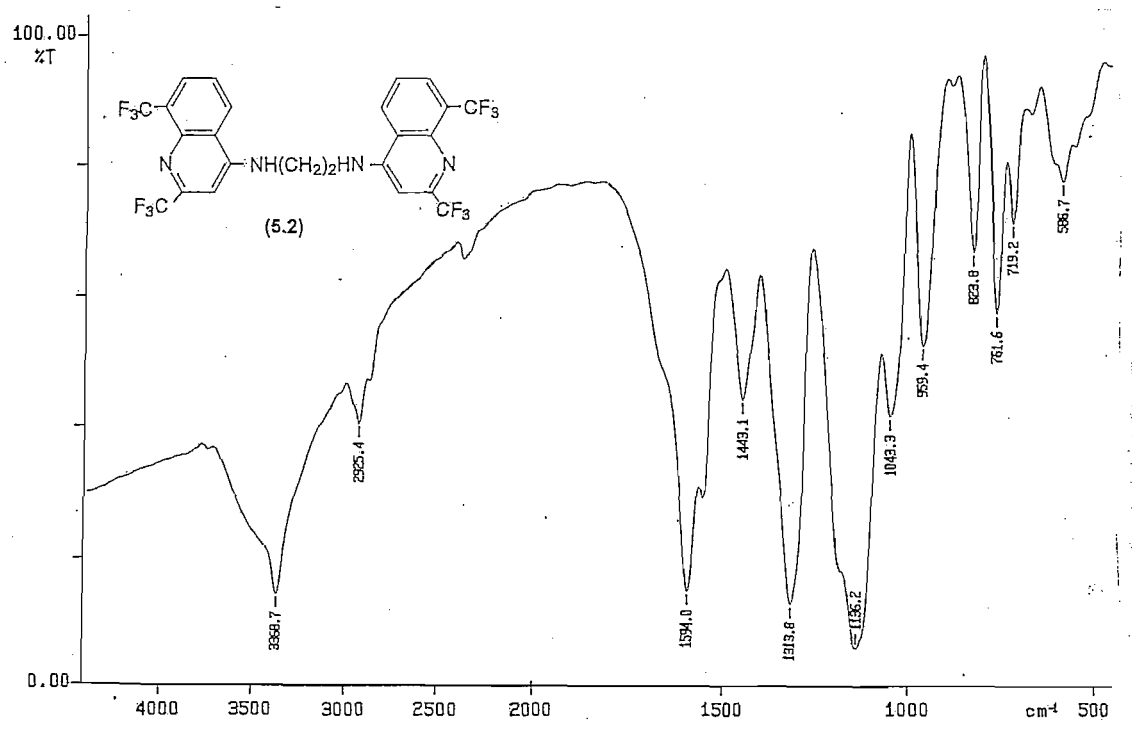

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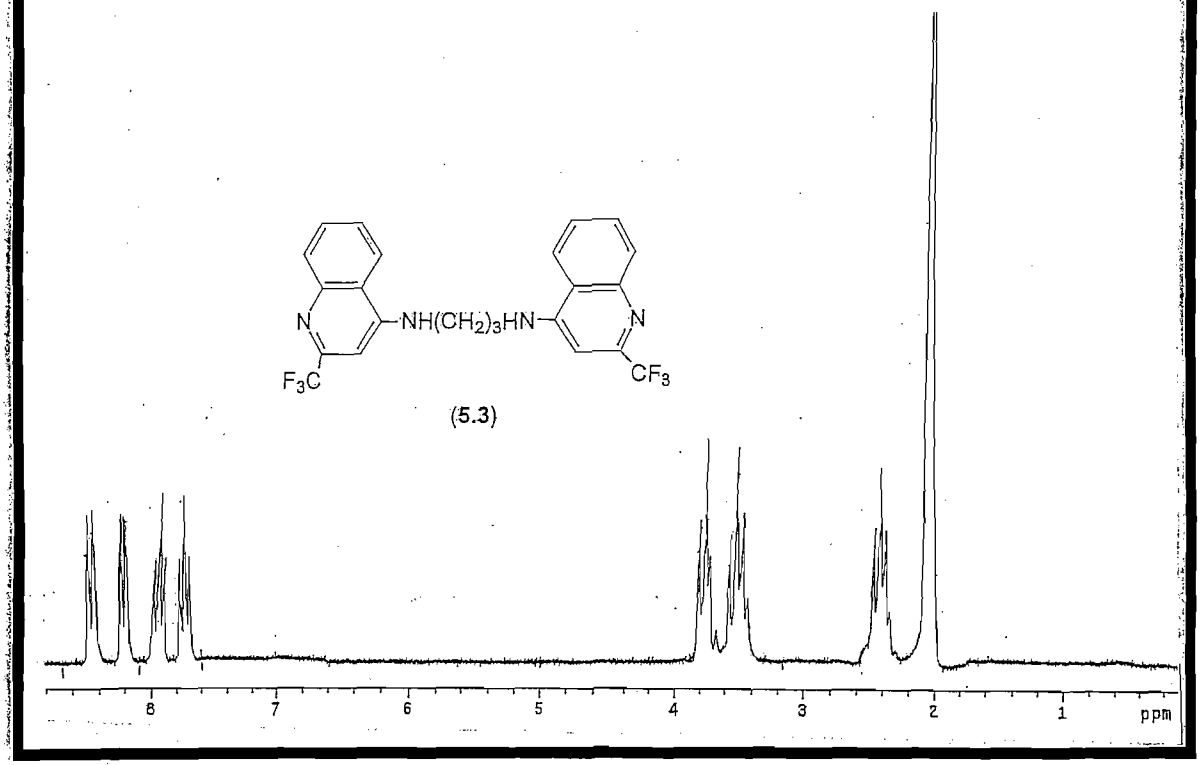

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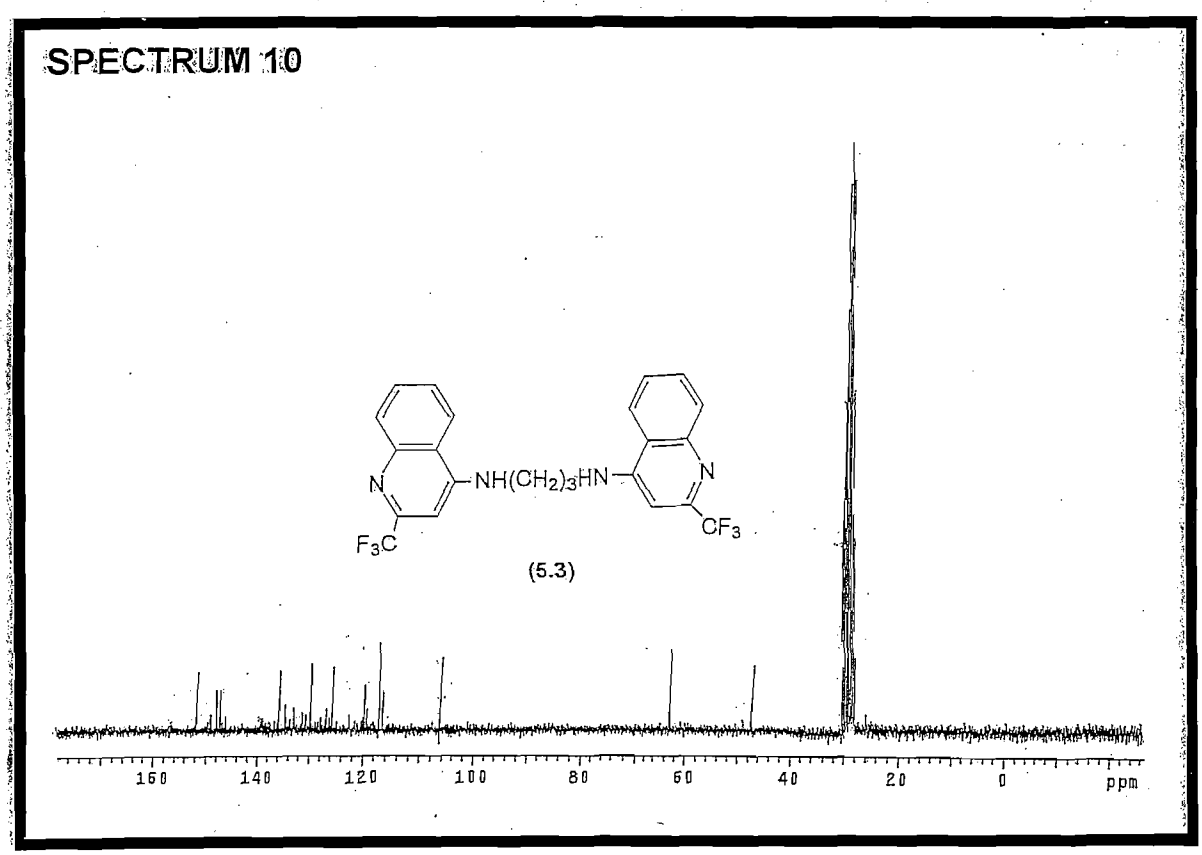

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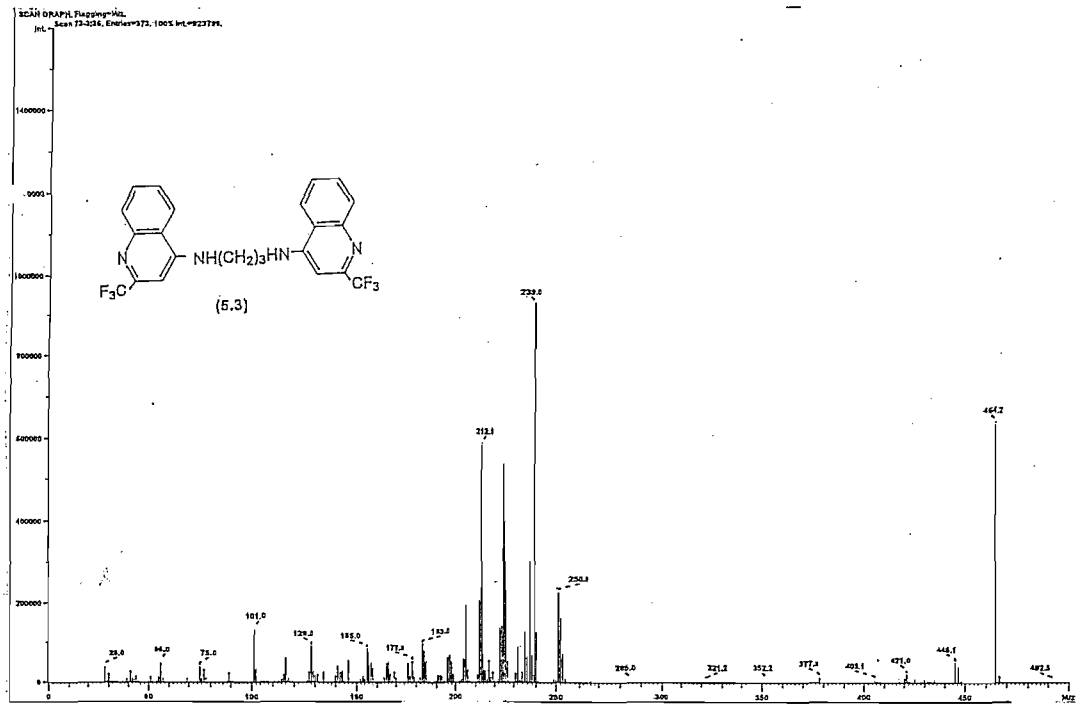

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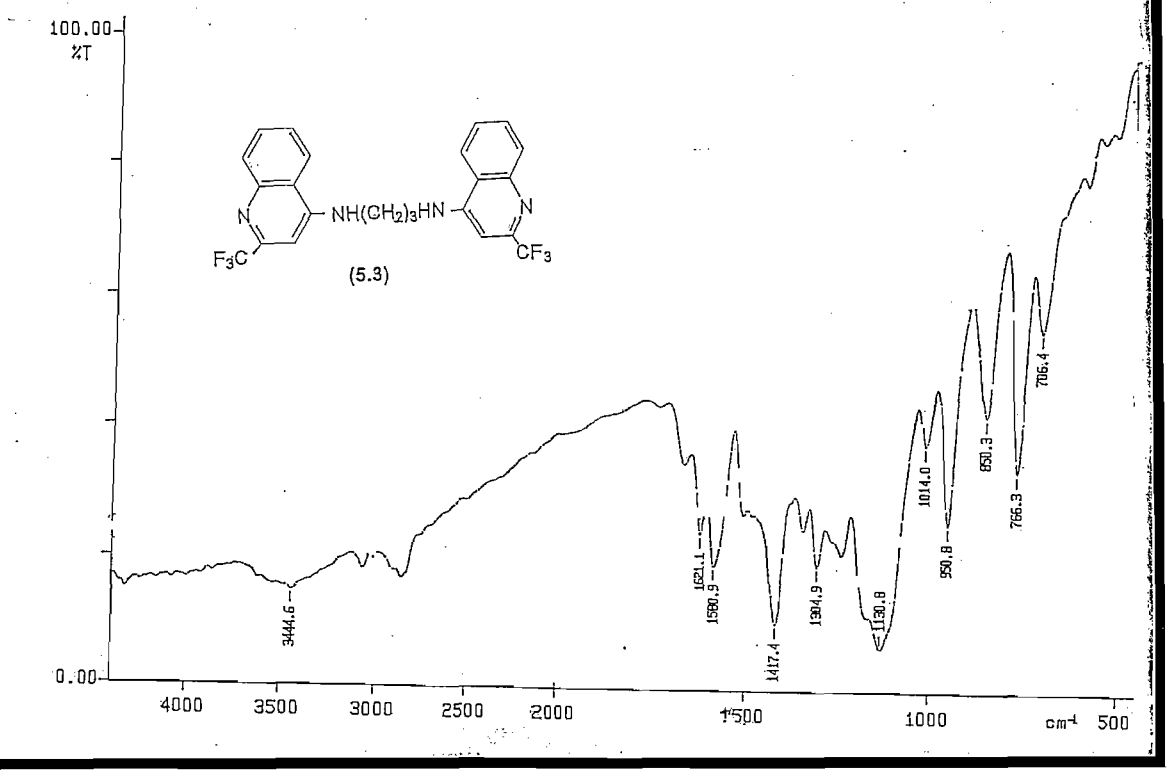

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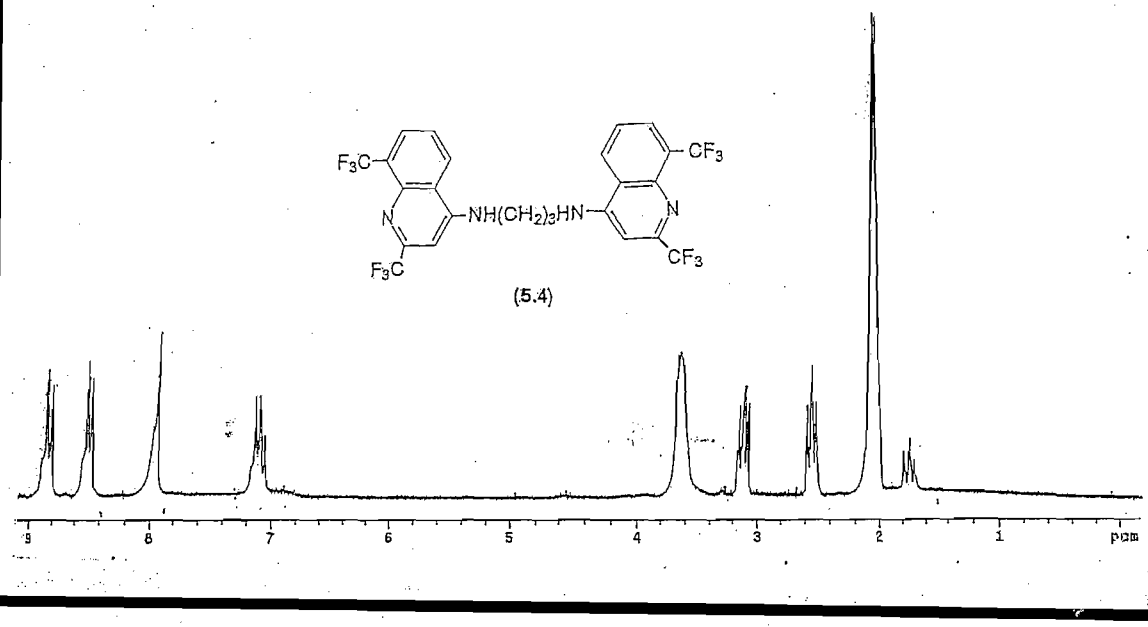

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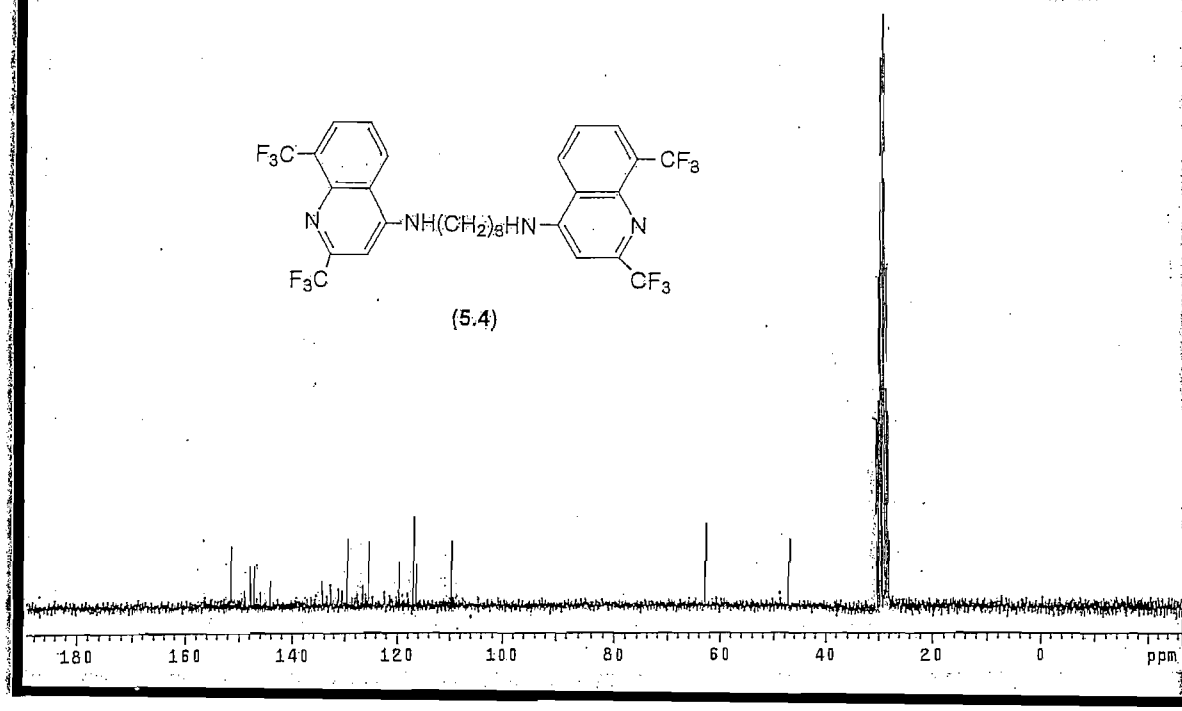

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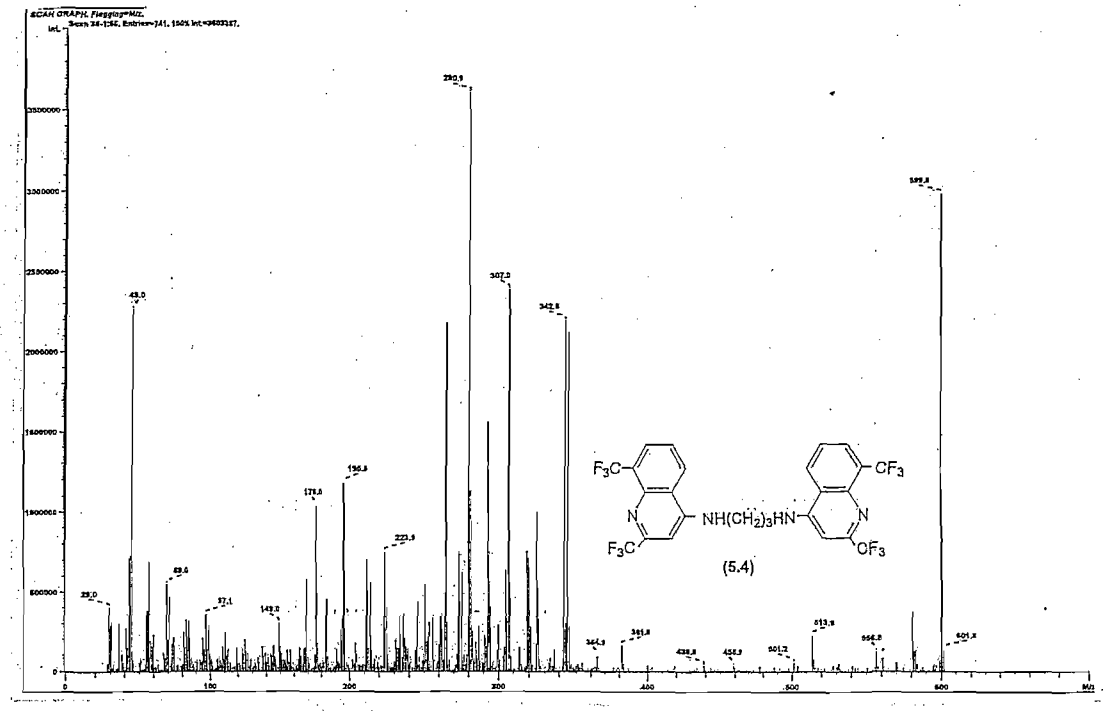

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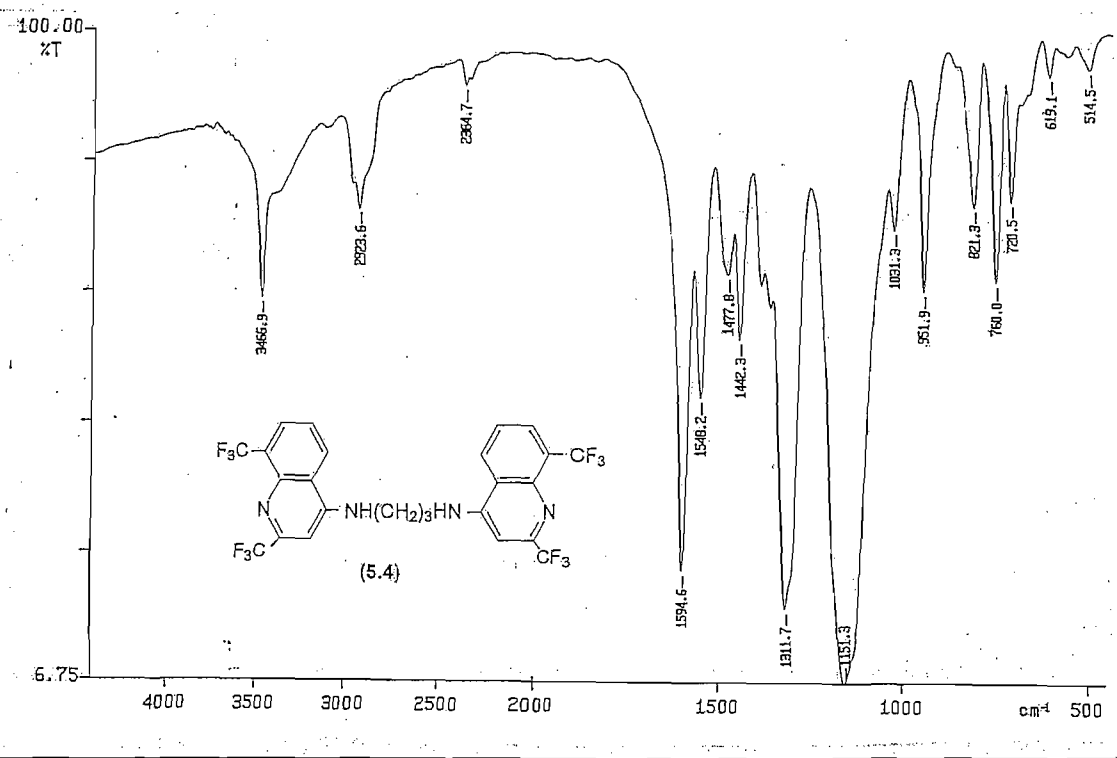

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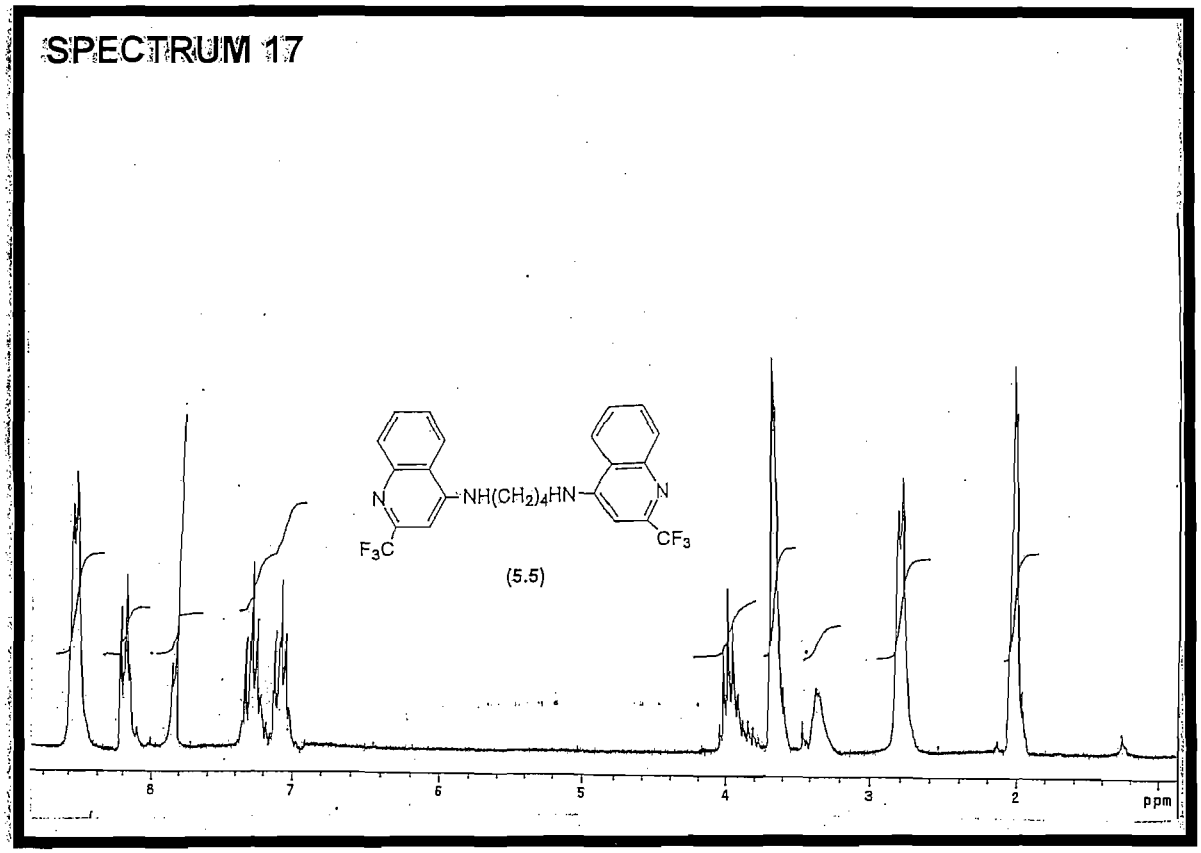

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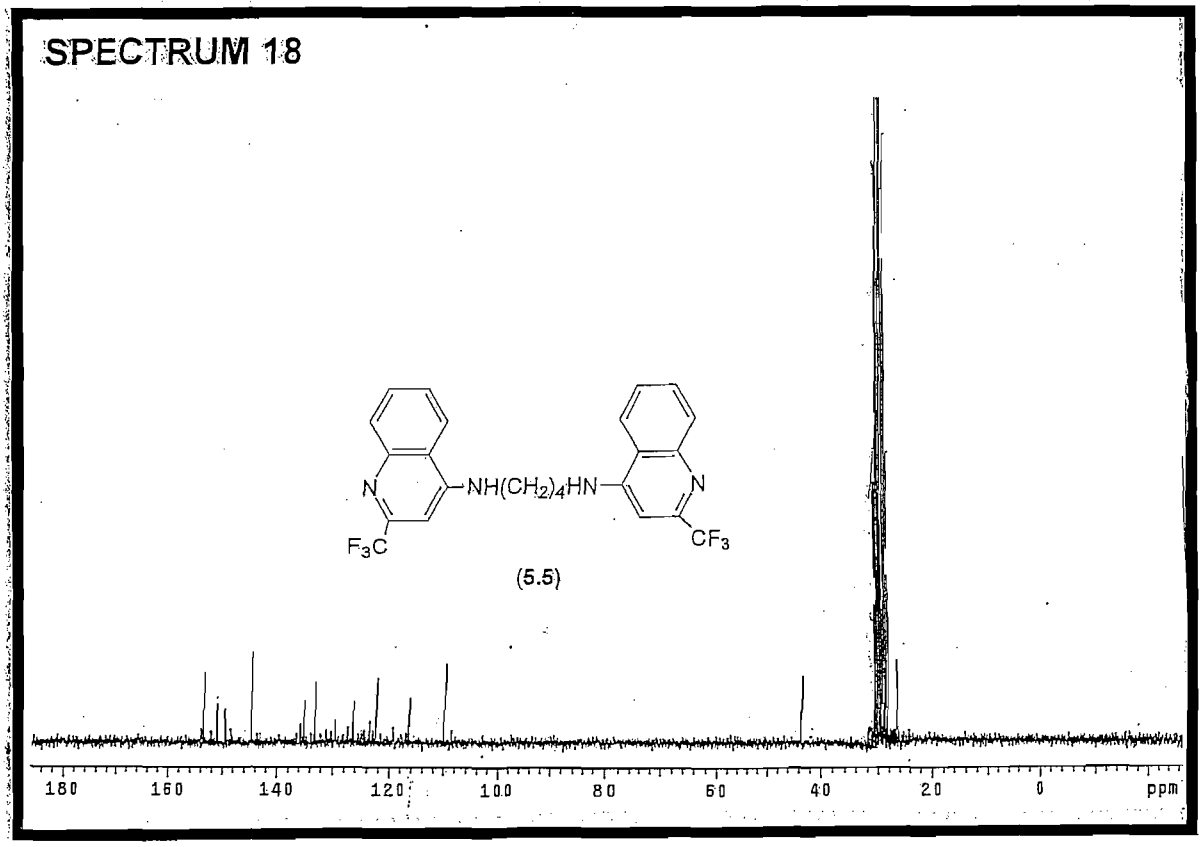

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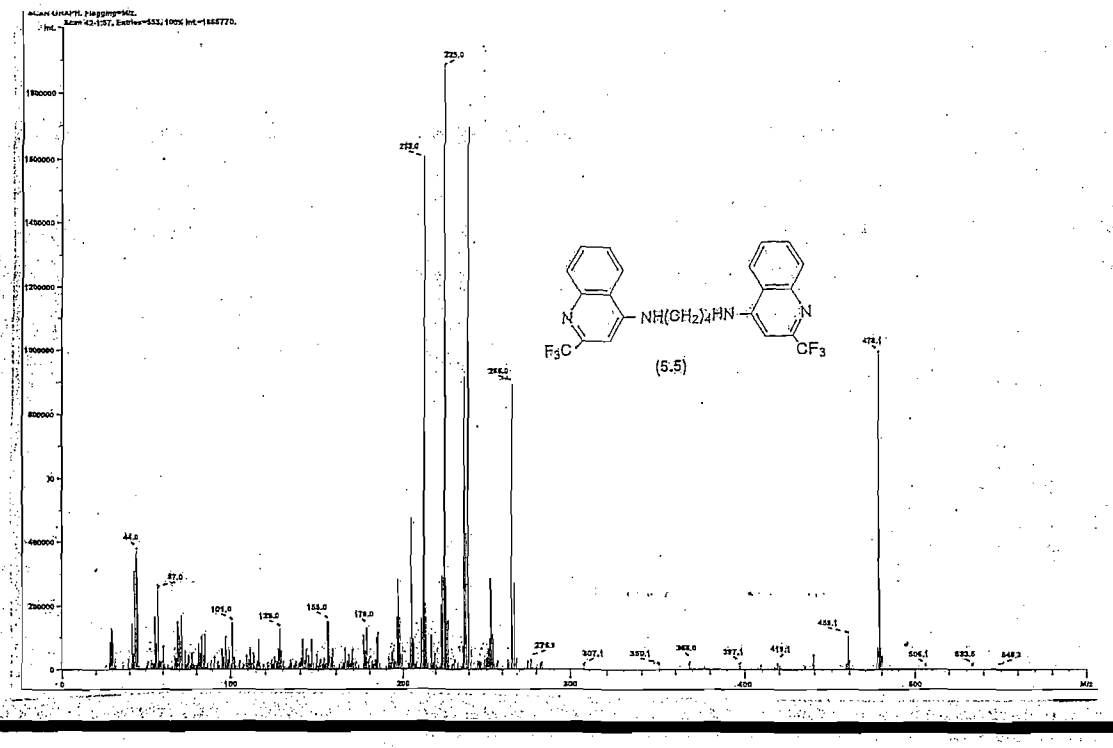

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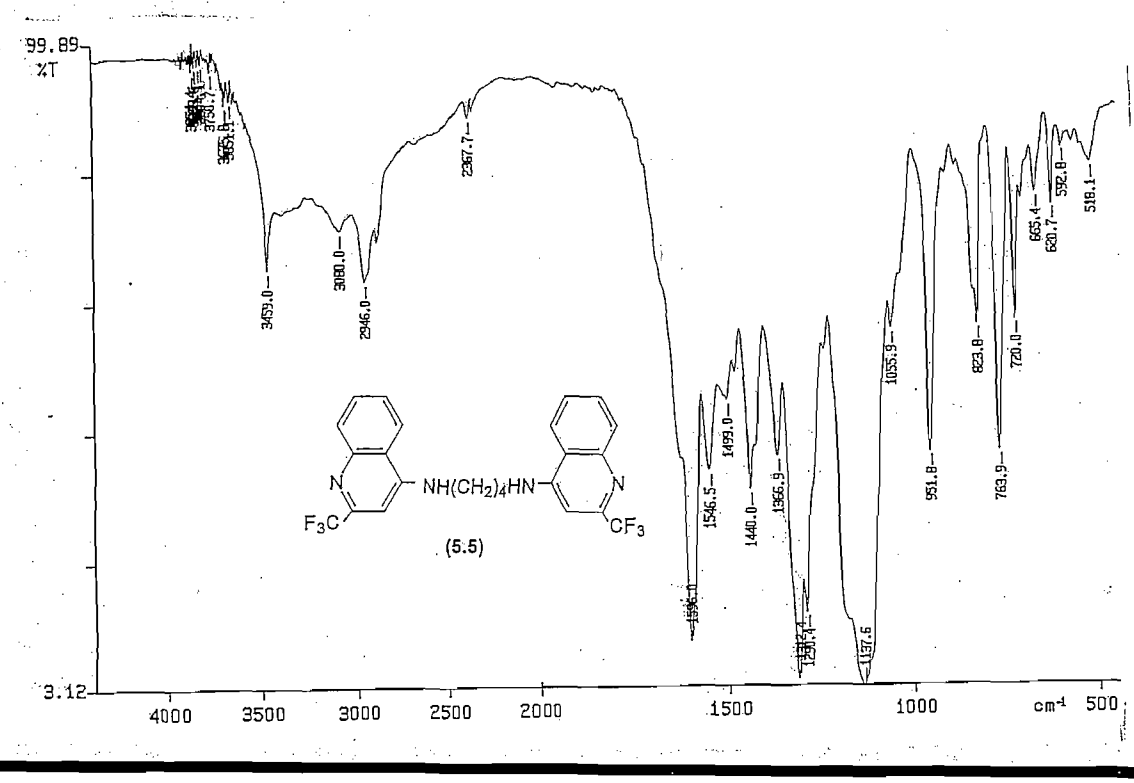

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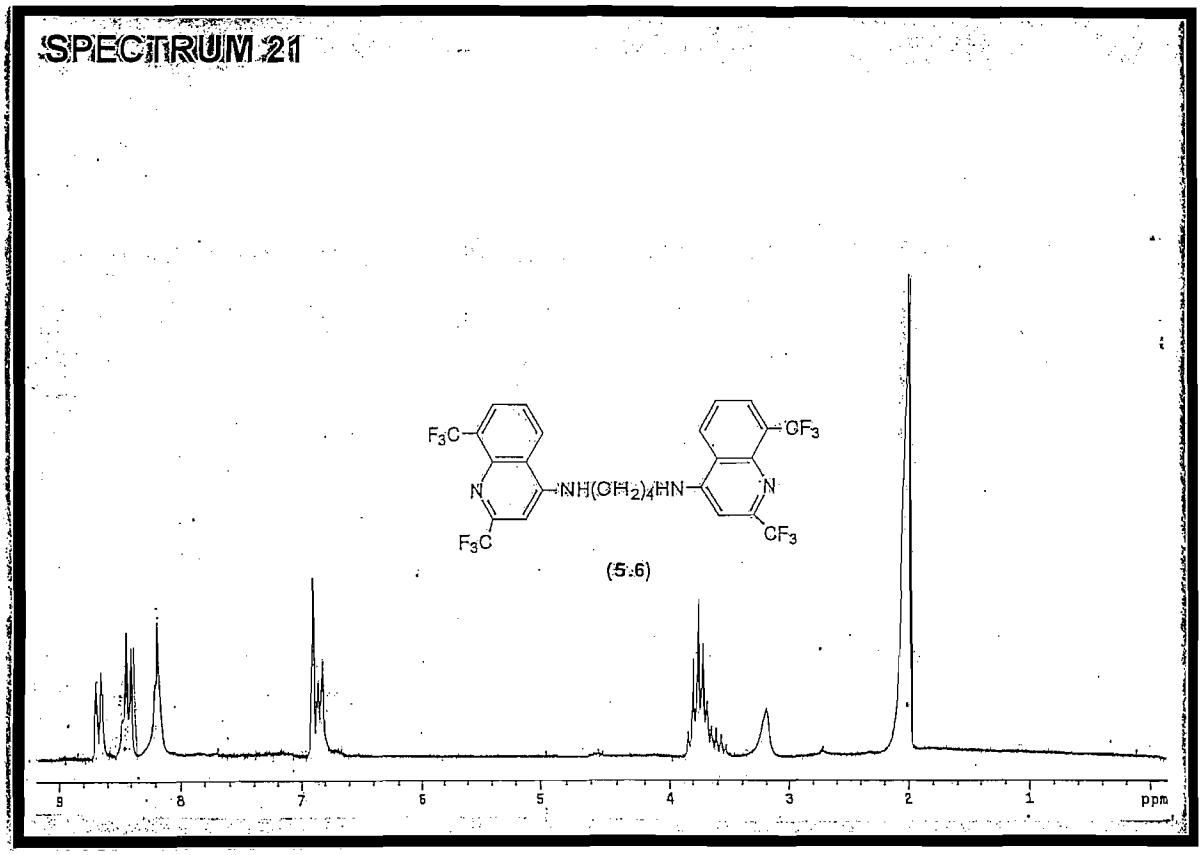

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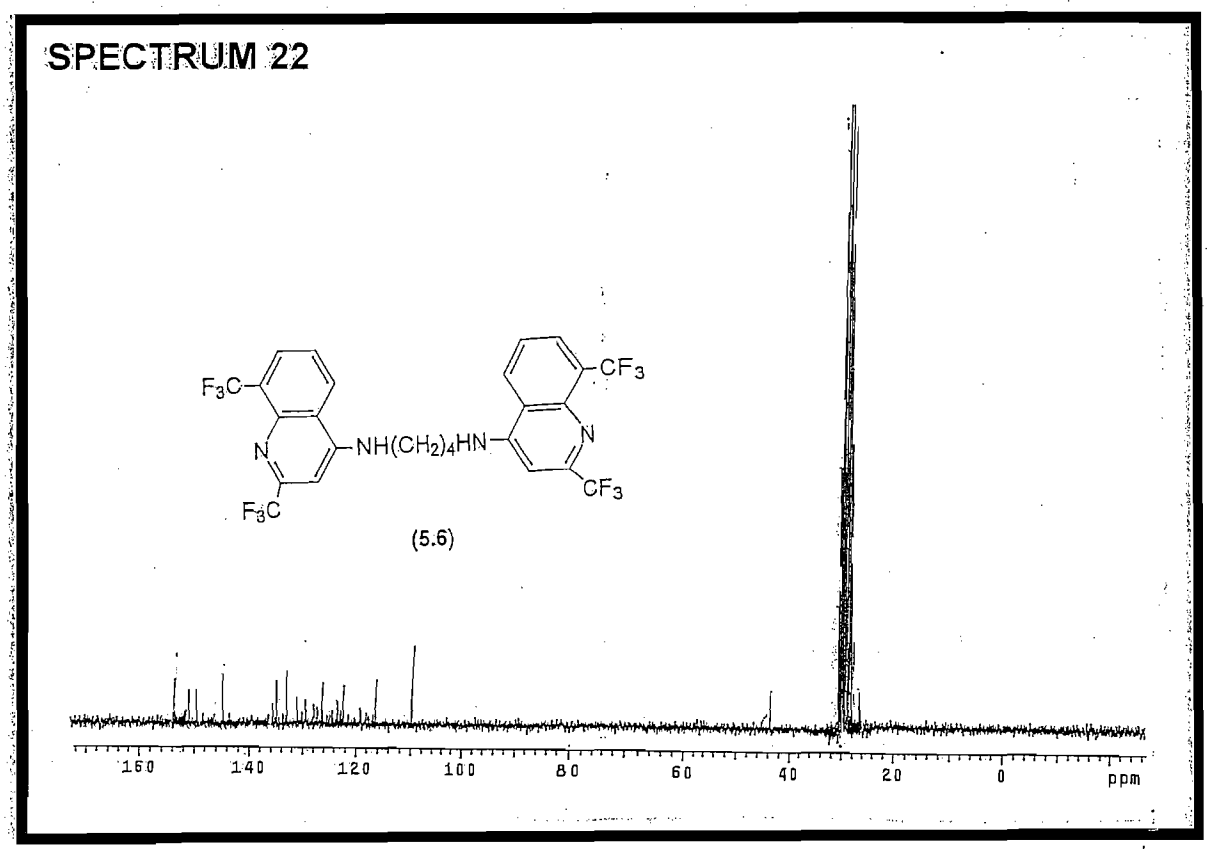

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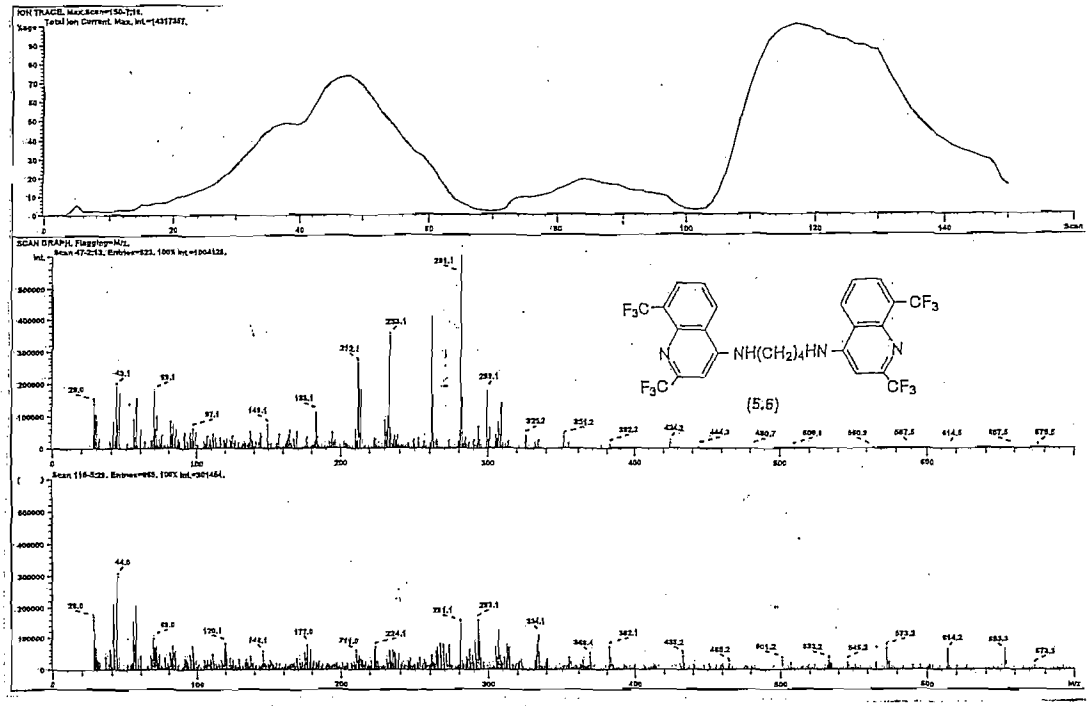
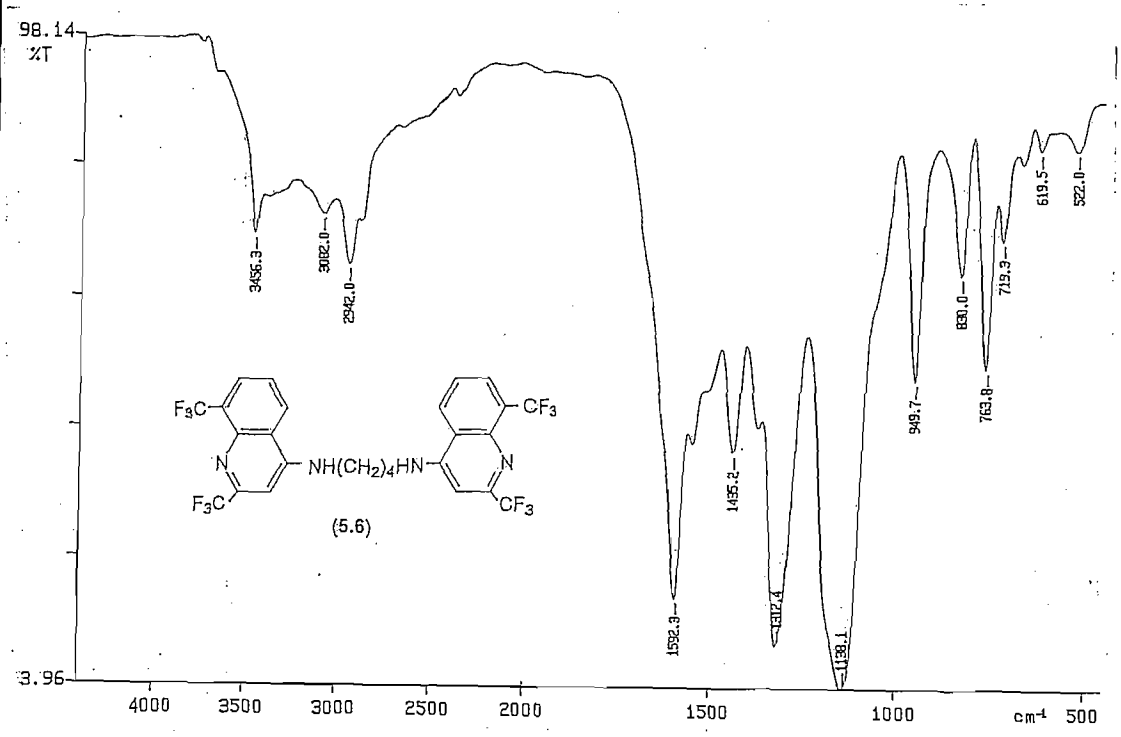
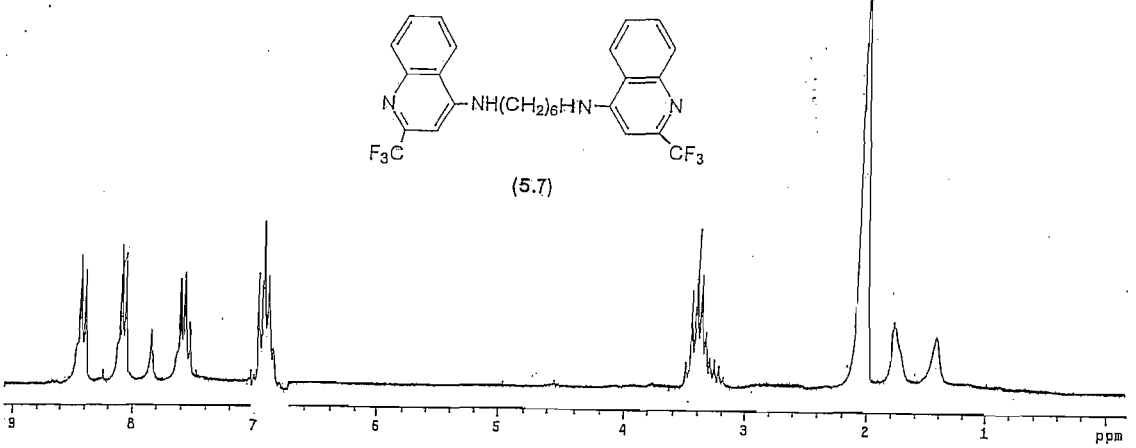


SPECTRUM 24

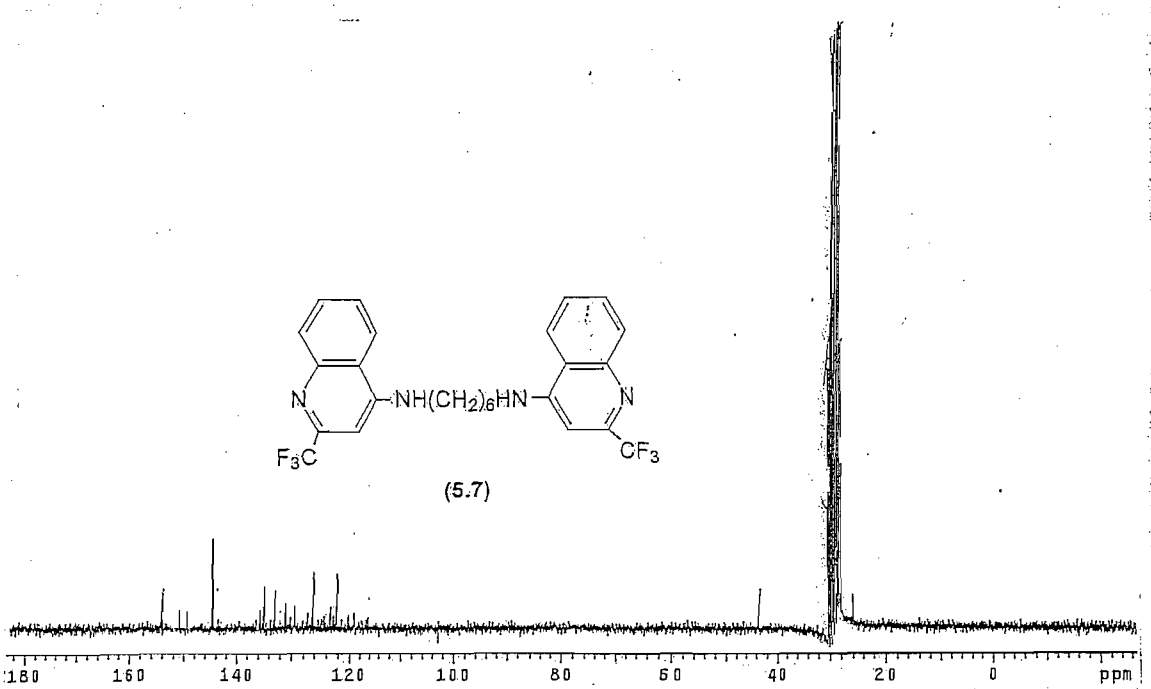


SPECTRUM 25



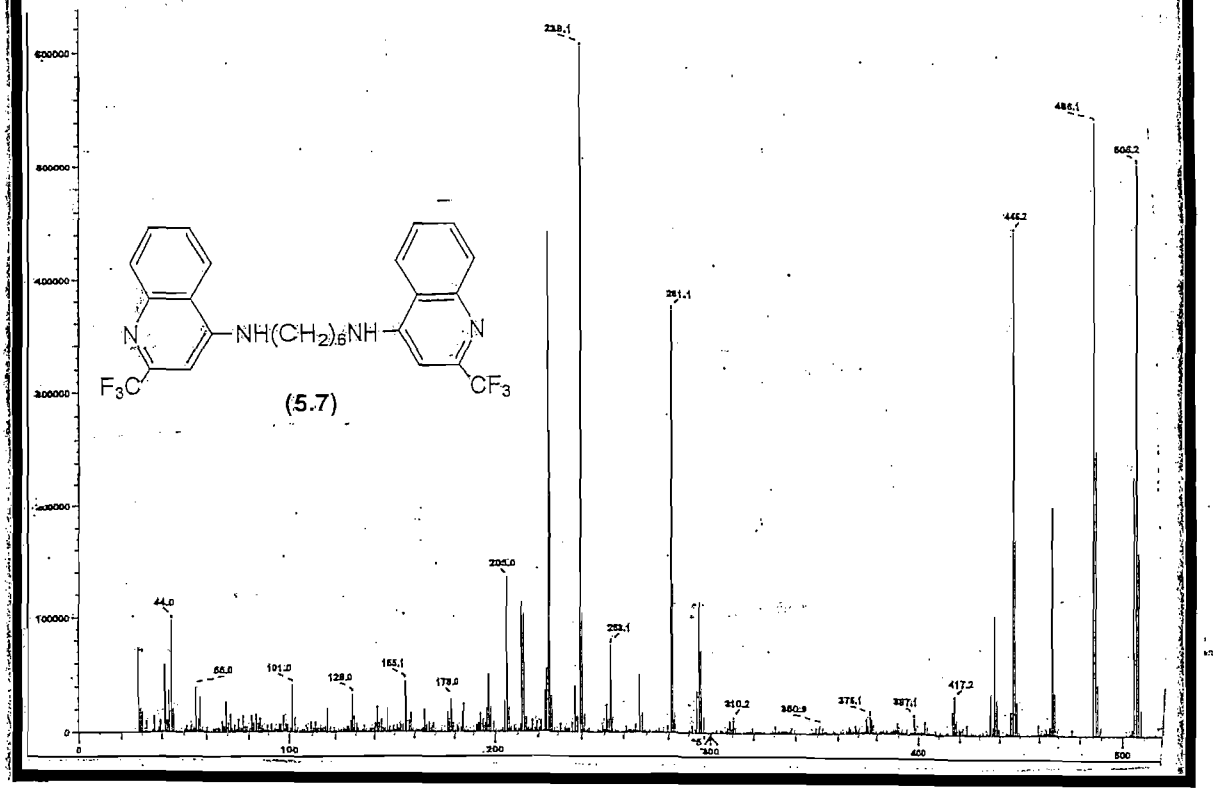
(5.7)

SPECTRUM 26



(5.7)

SPECTRUM 27



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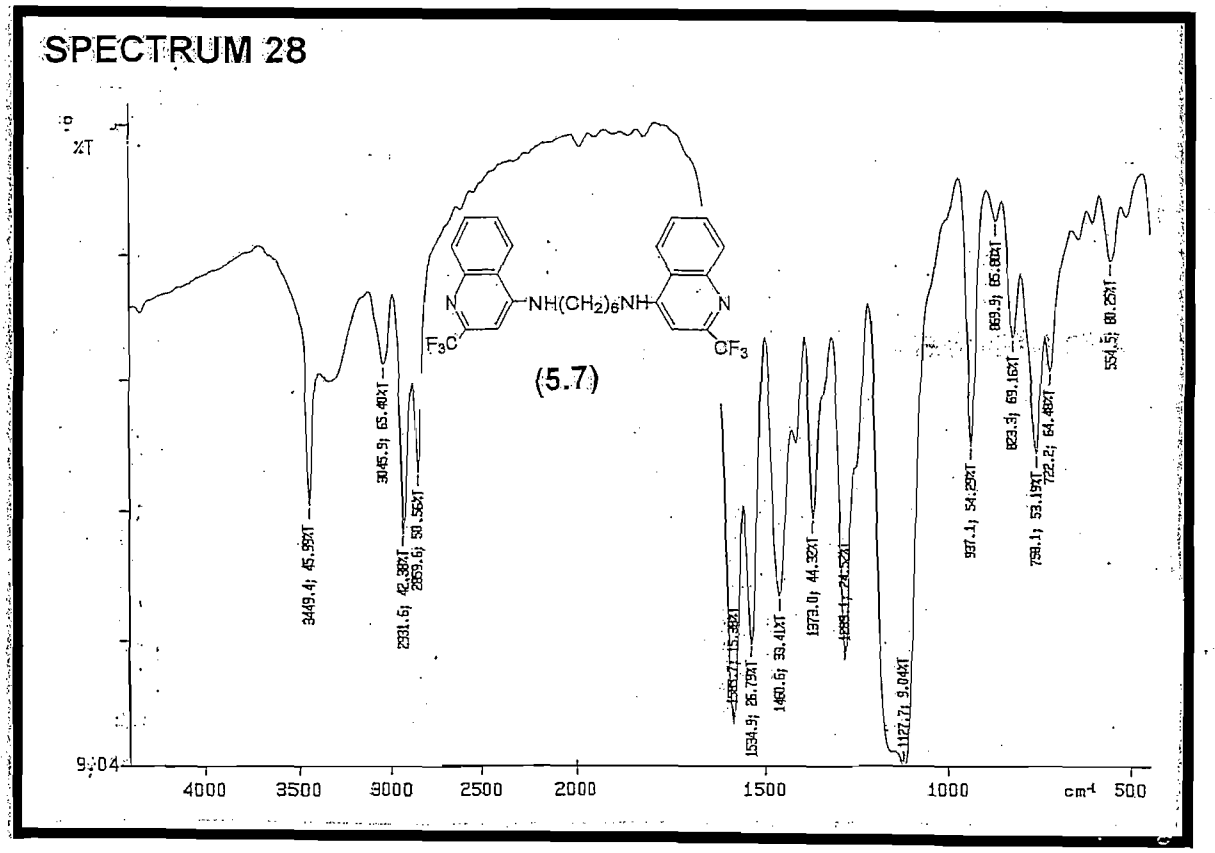

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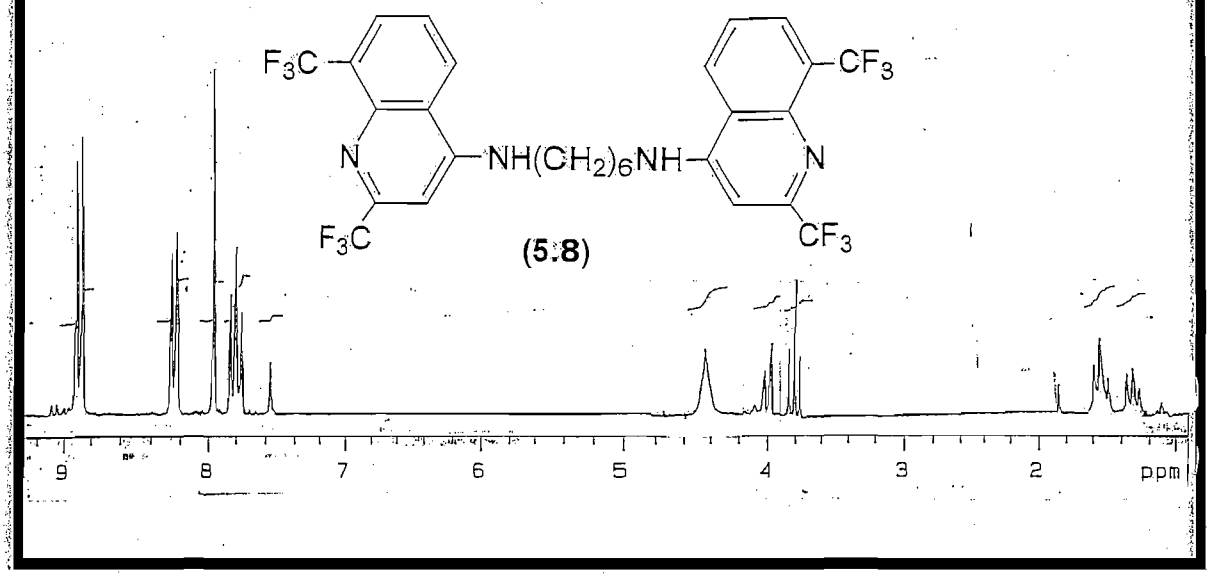
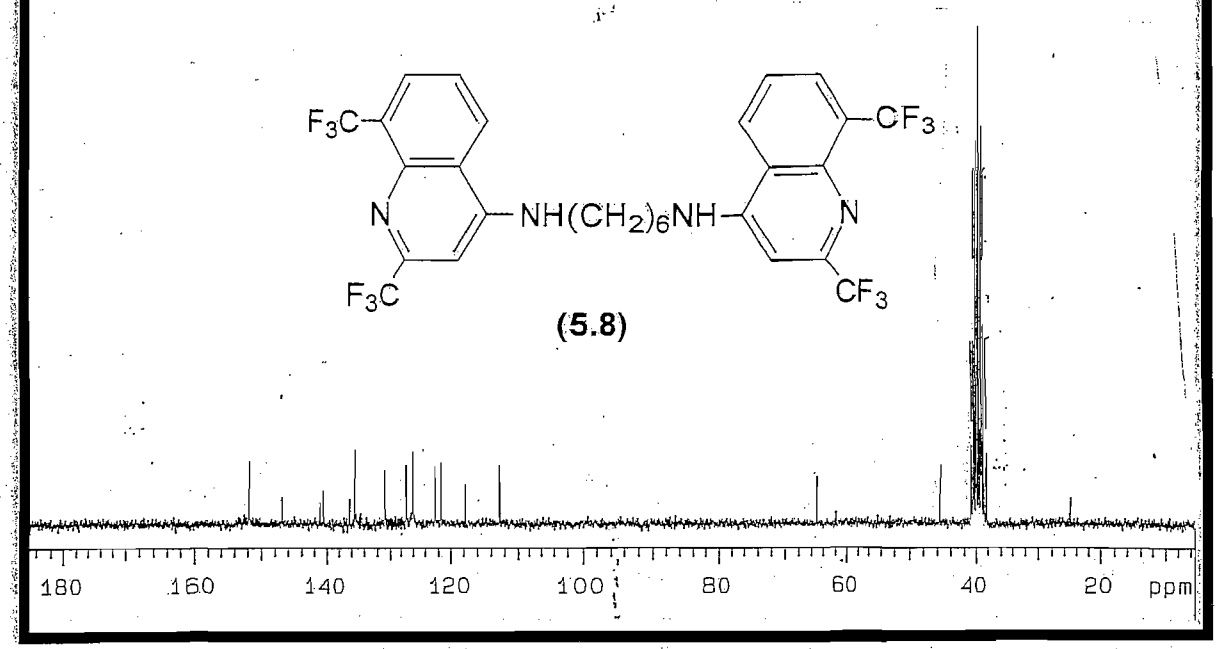
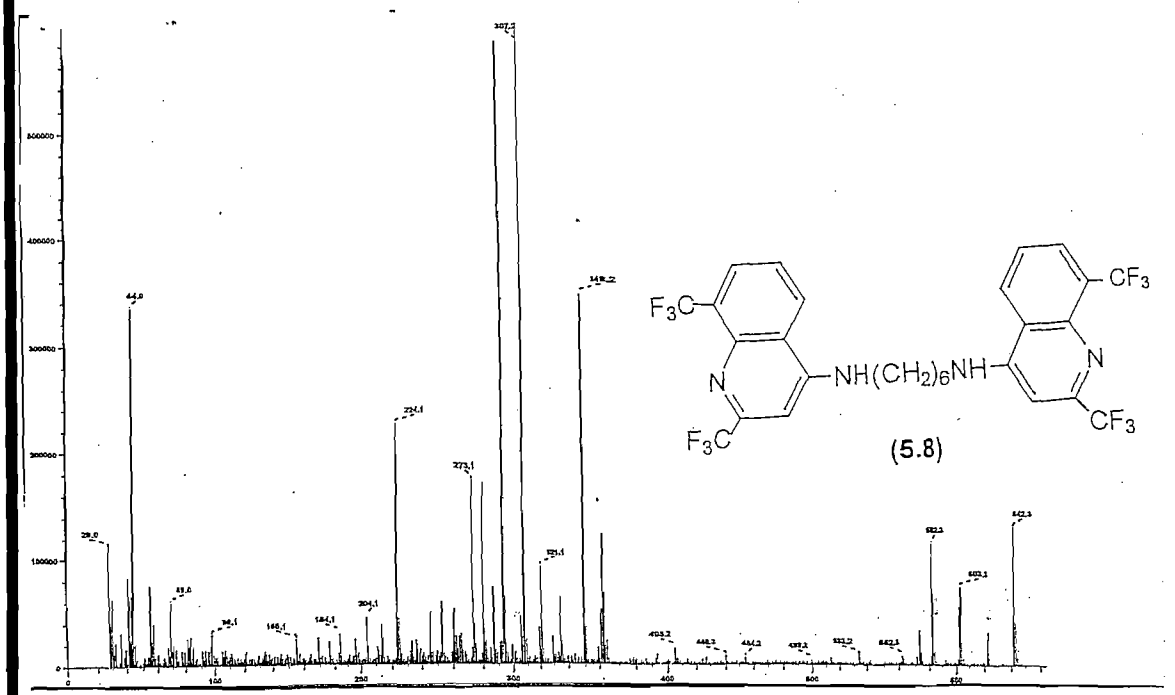


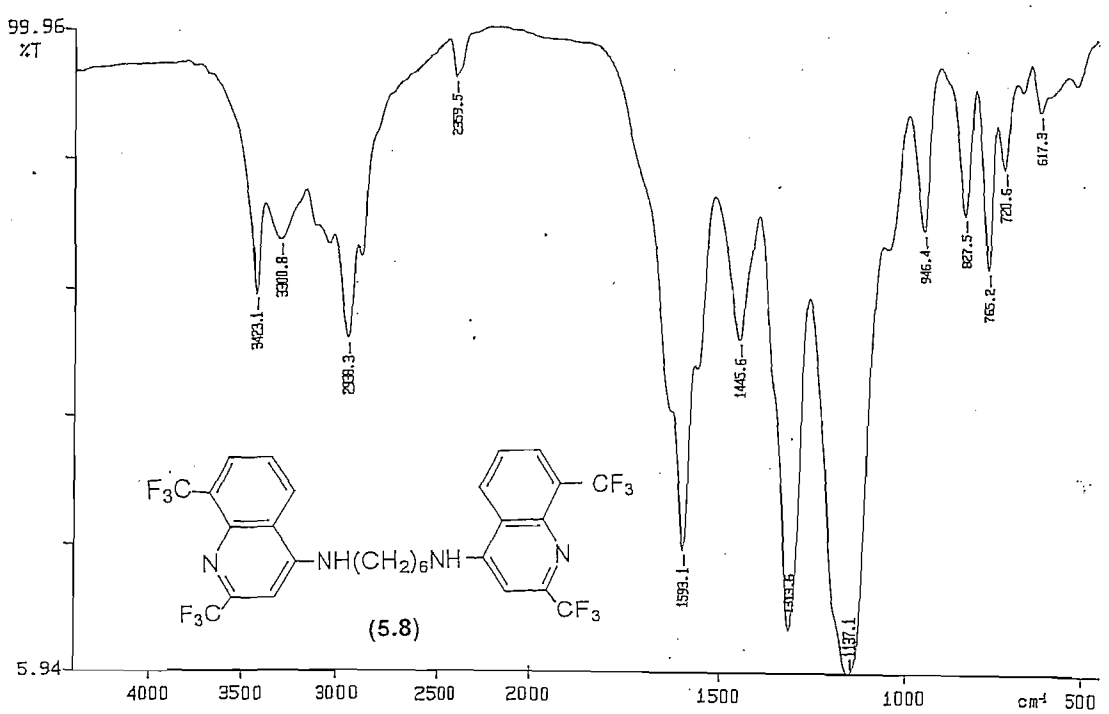
SPECTRUM 30



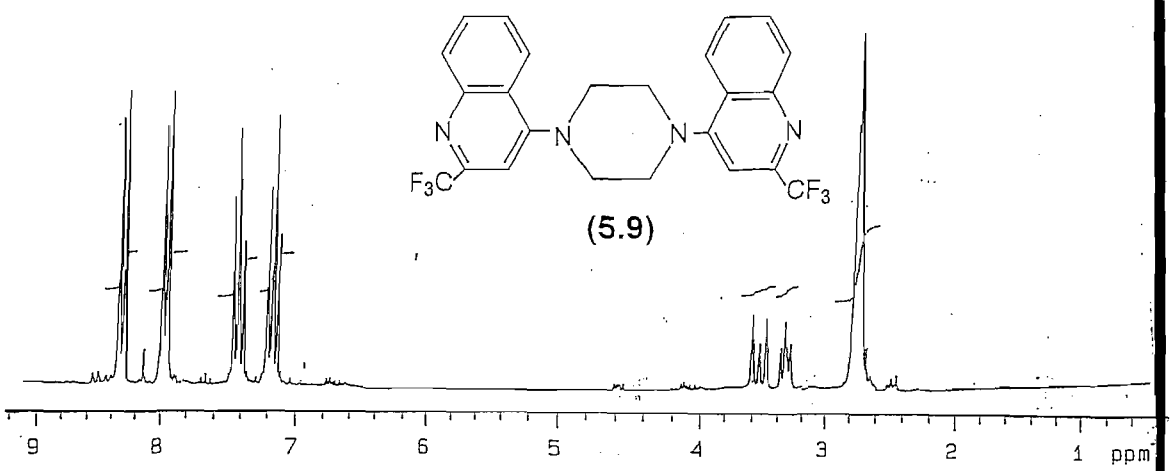
SPECTRUM 31



SPECTRUM 32



SPECTRUM 33



(5.9)

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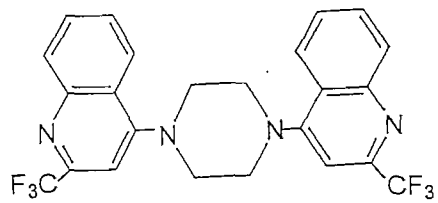
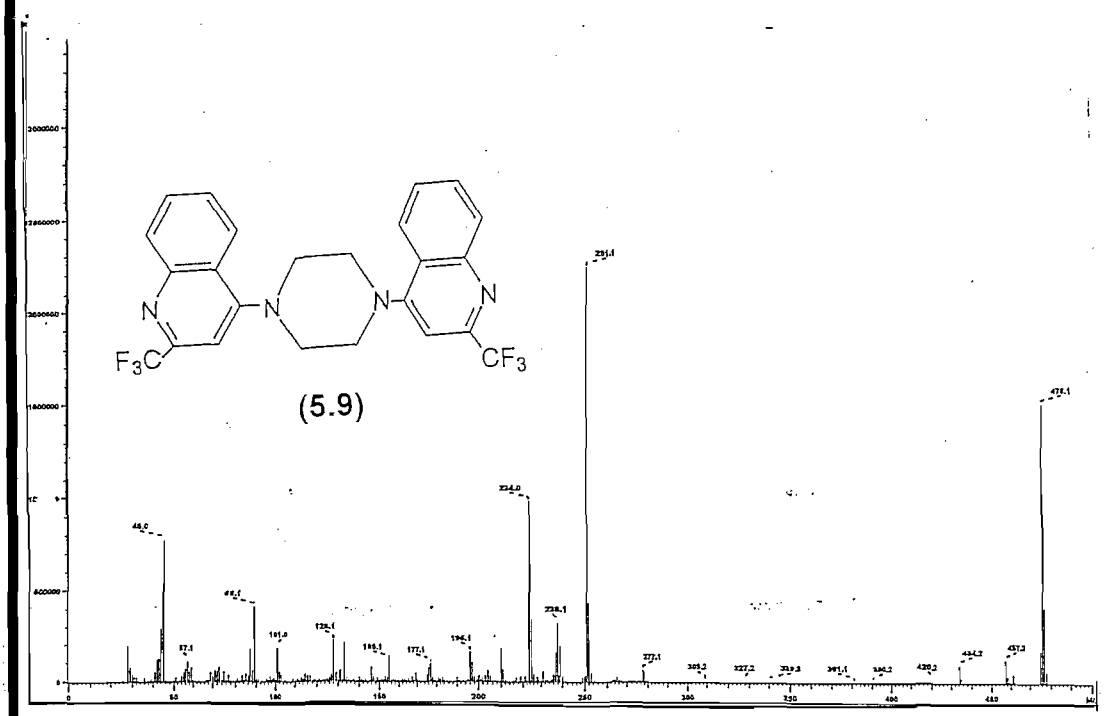
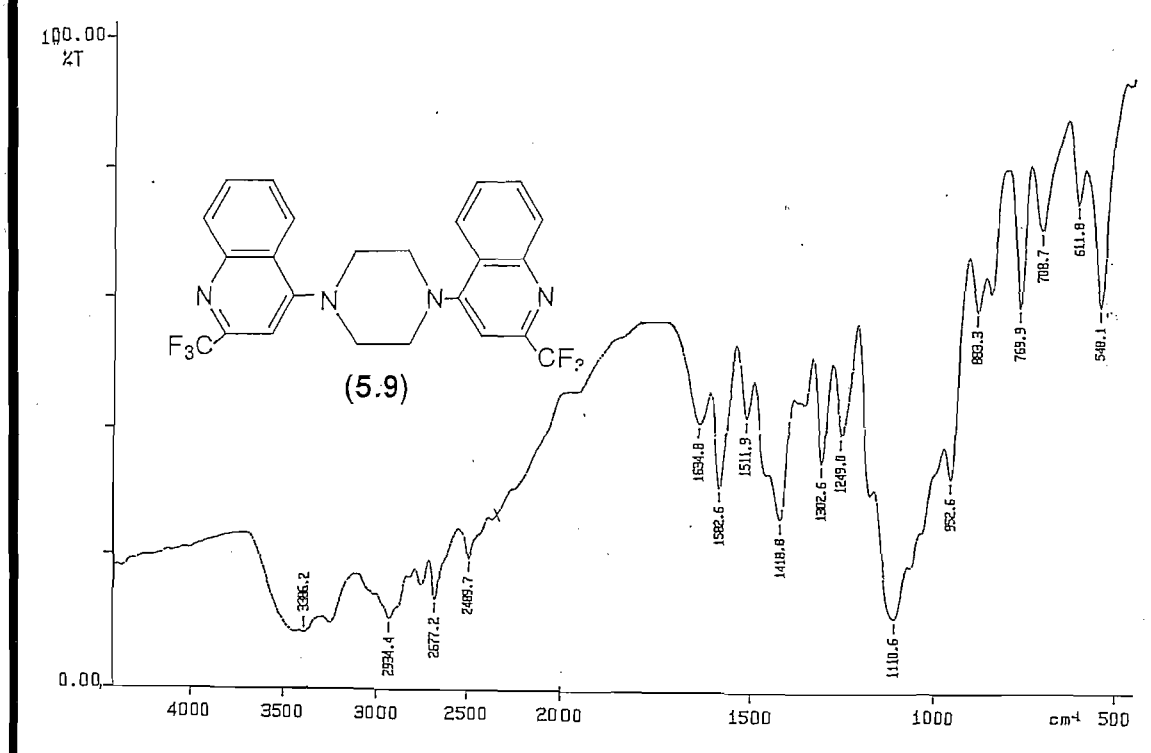


(5.9)

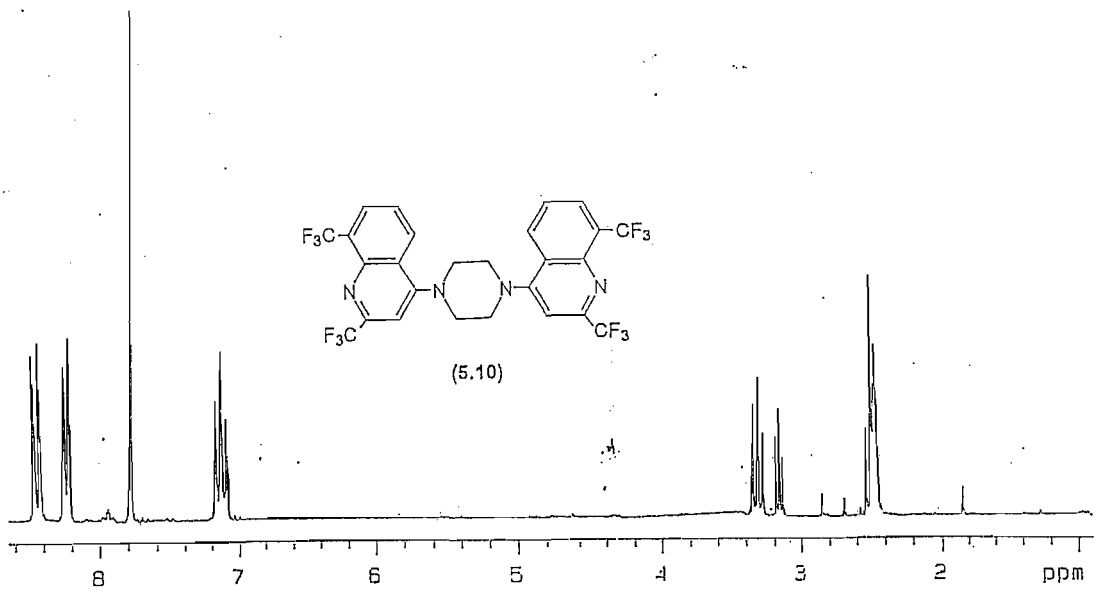
SPECTRUM 35



SPECTRUM 36

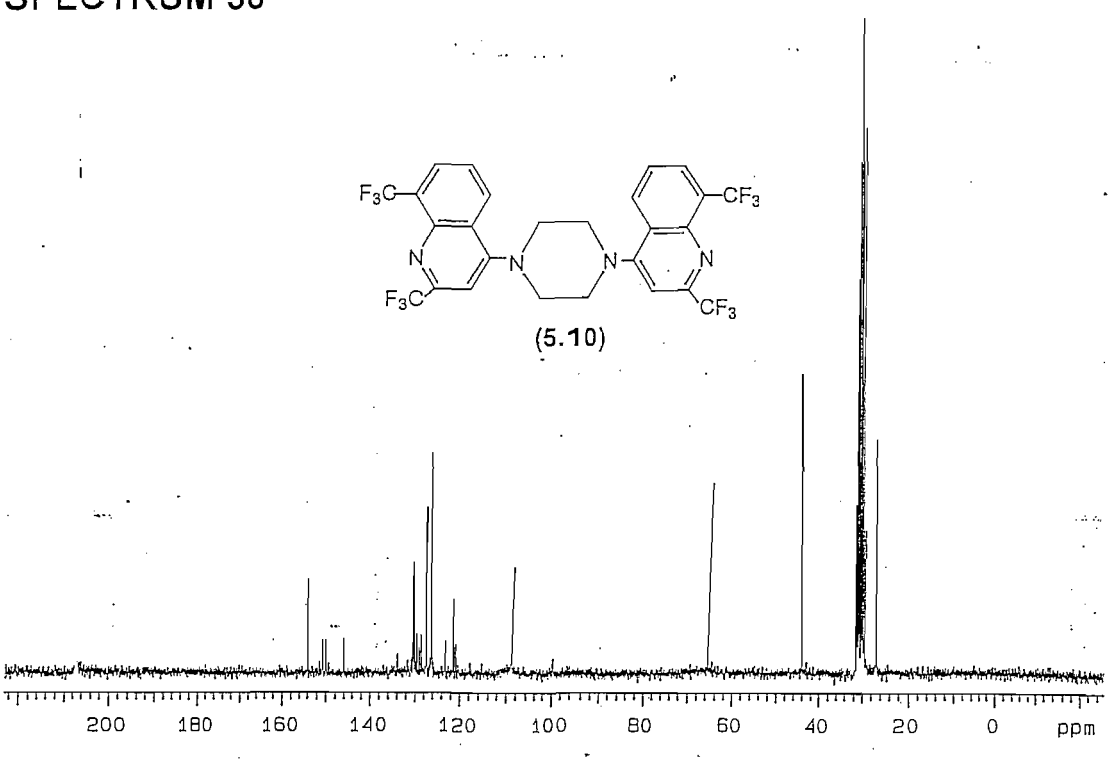


SPECTRUM 37



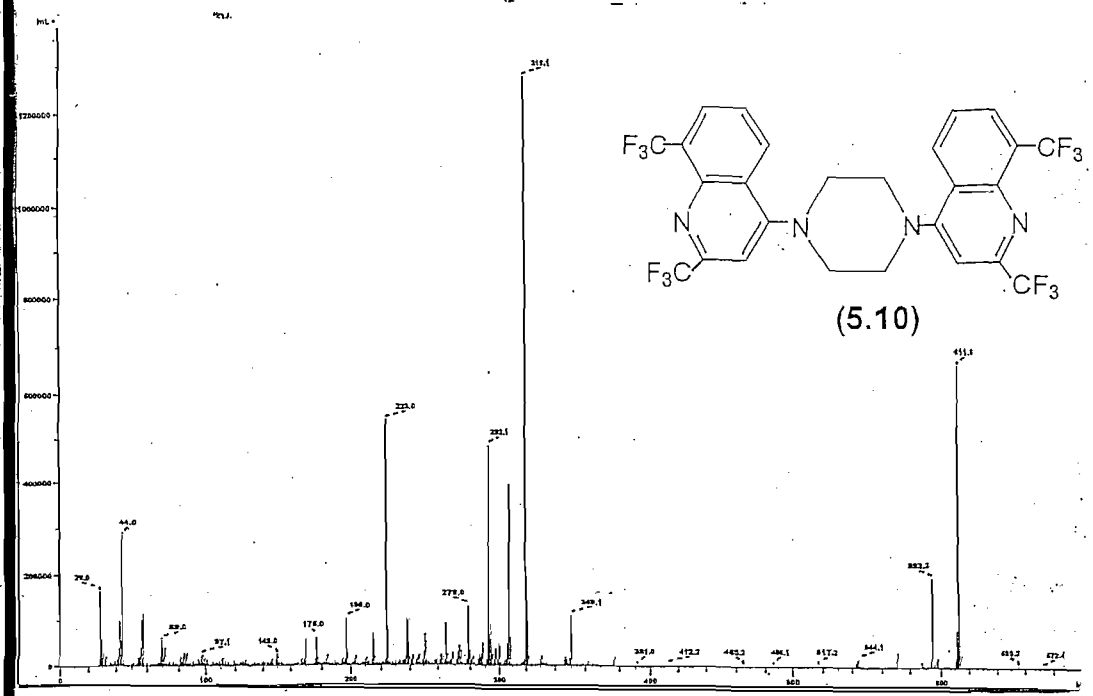
(5.10)

SPECTRUM 38



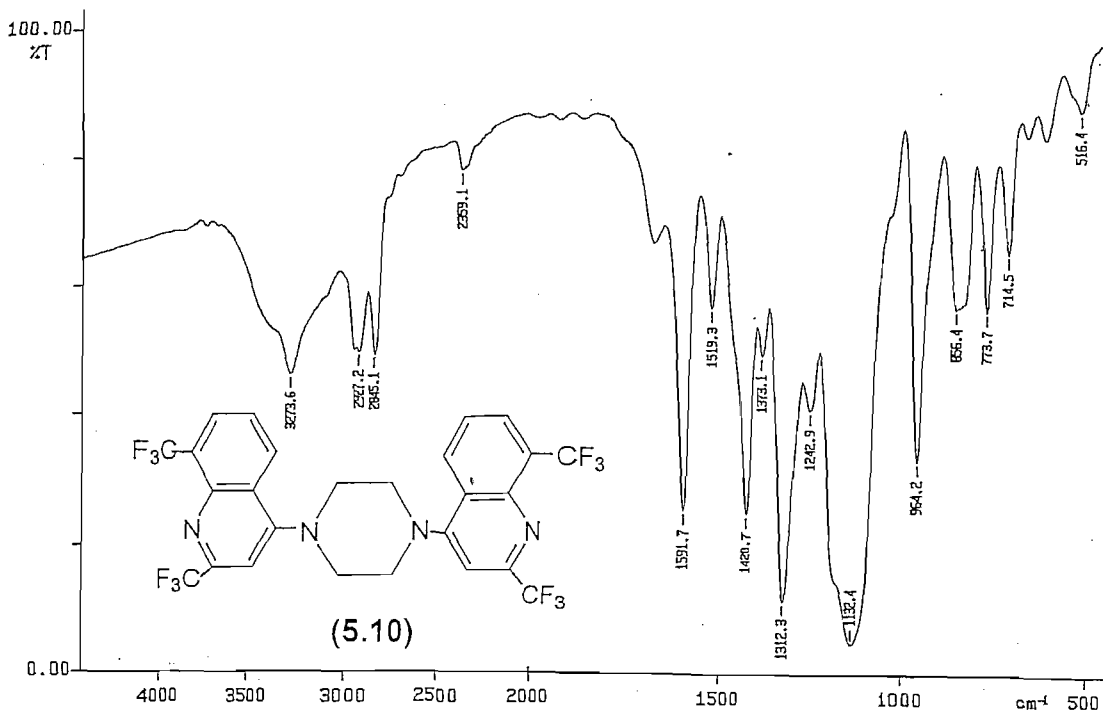
(5.10)

SPECTRUM 39



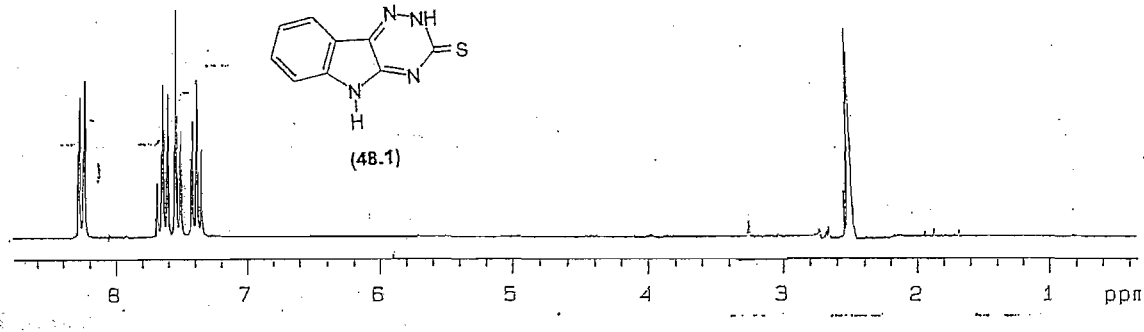
(5.10)

SPECTRUM 40



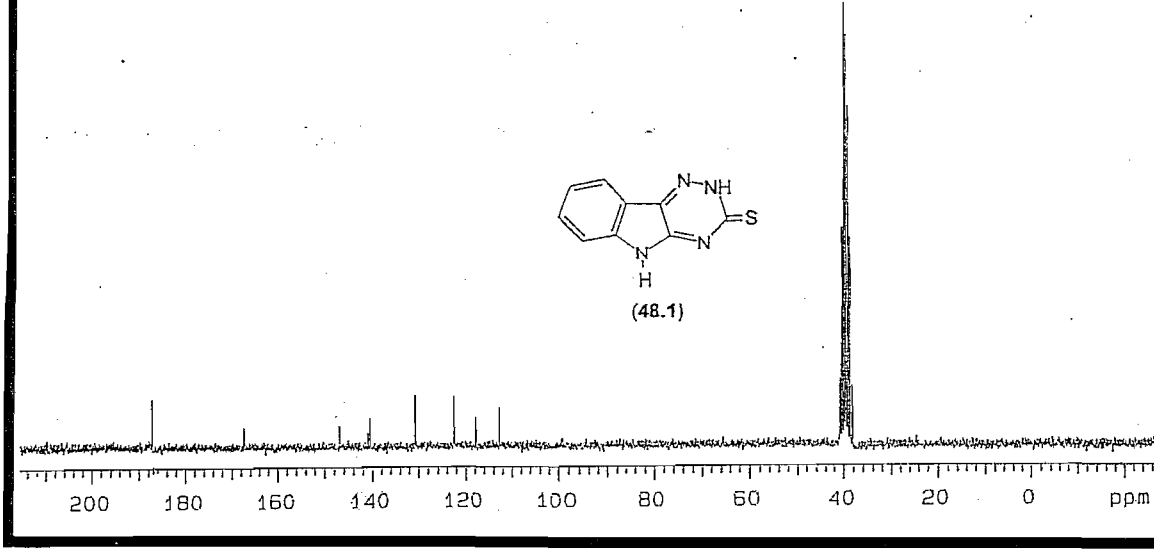
(5.10)

SPECTRUM 41



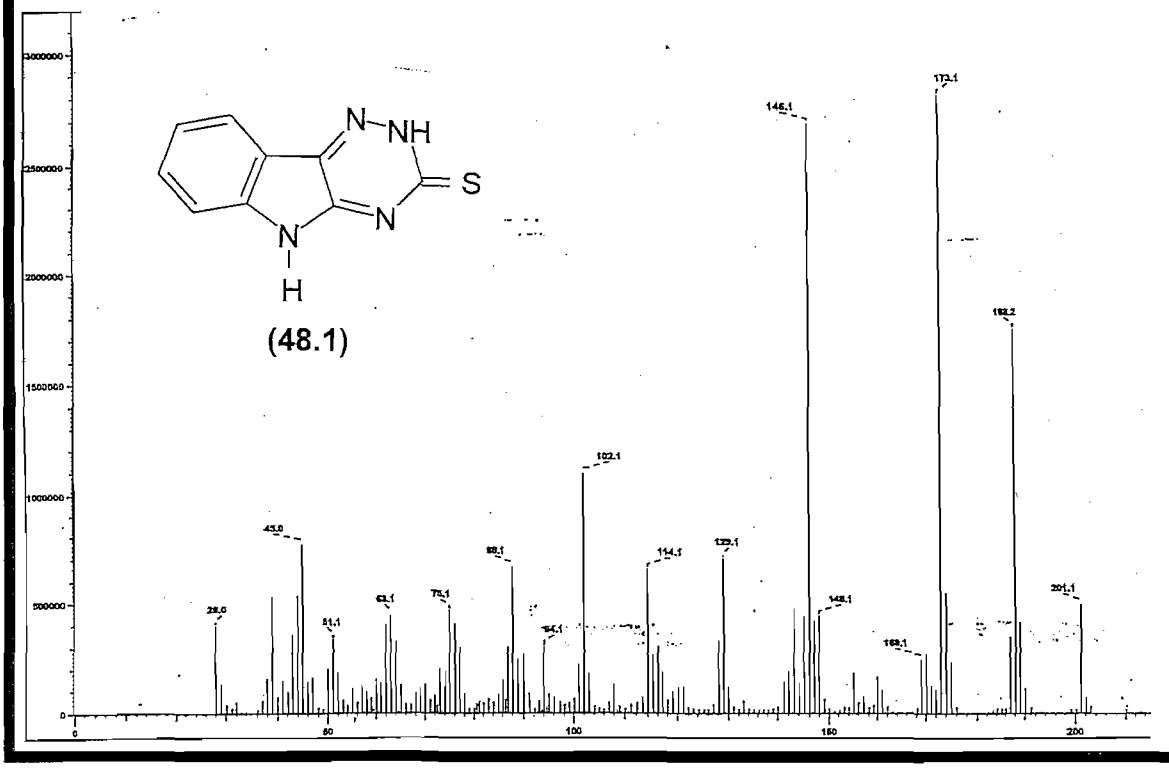
(48.1)

SPECTRUM 42

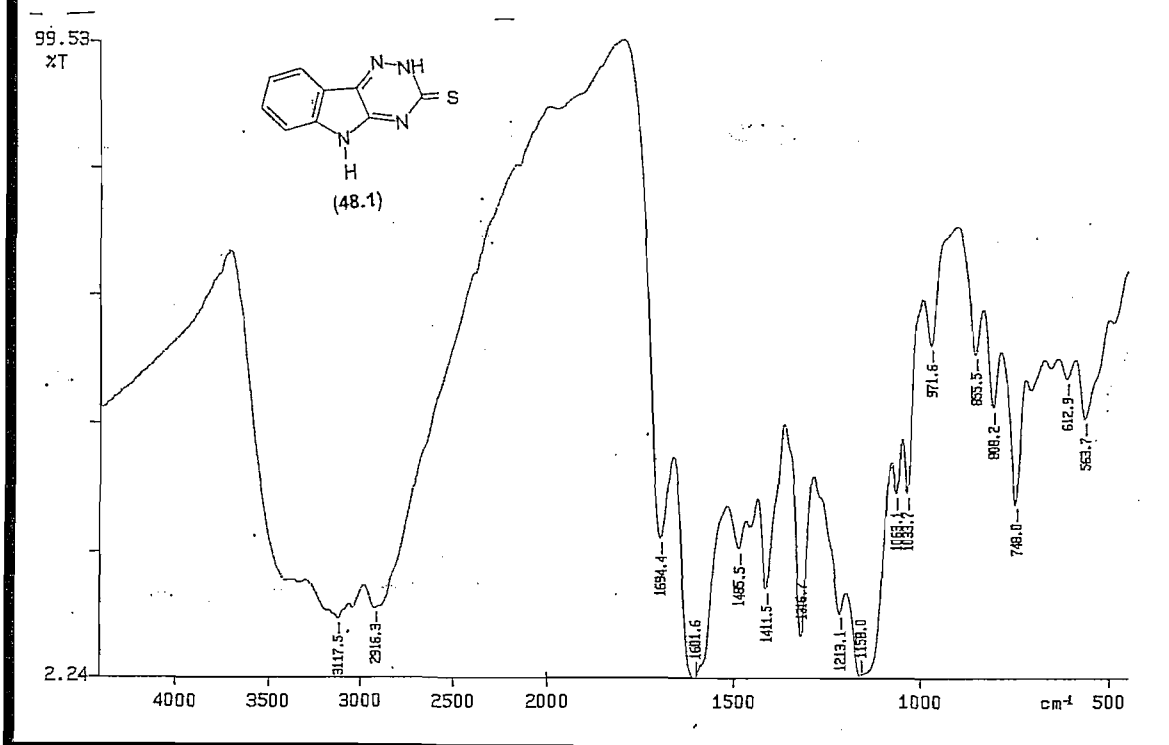


(48.1)

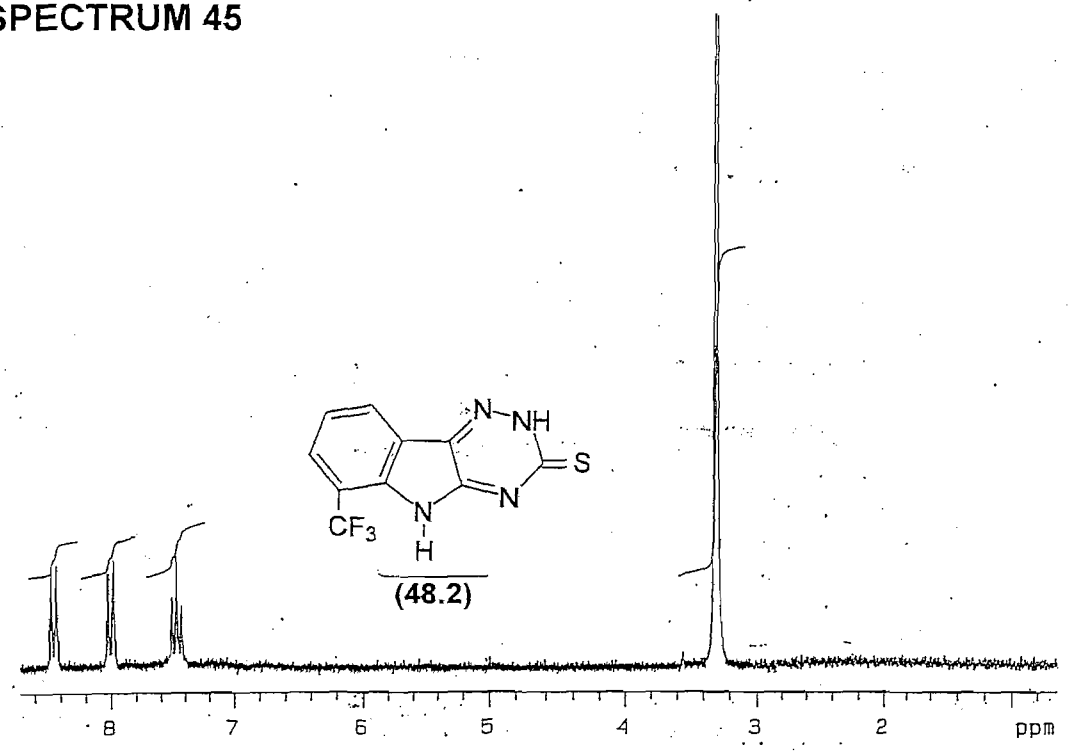
SPECTRUM 43



SPECTRUM 44



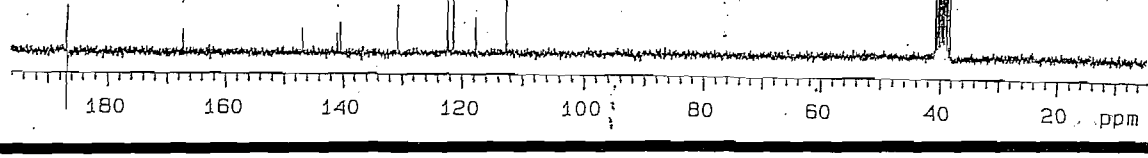
SPECTRUM 45



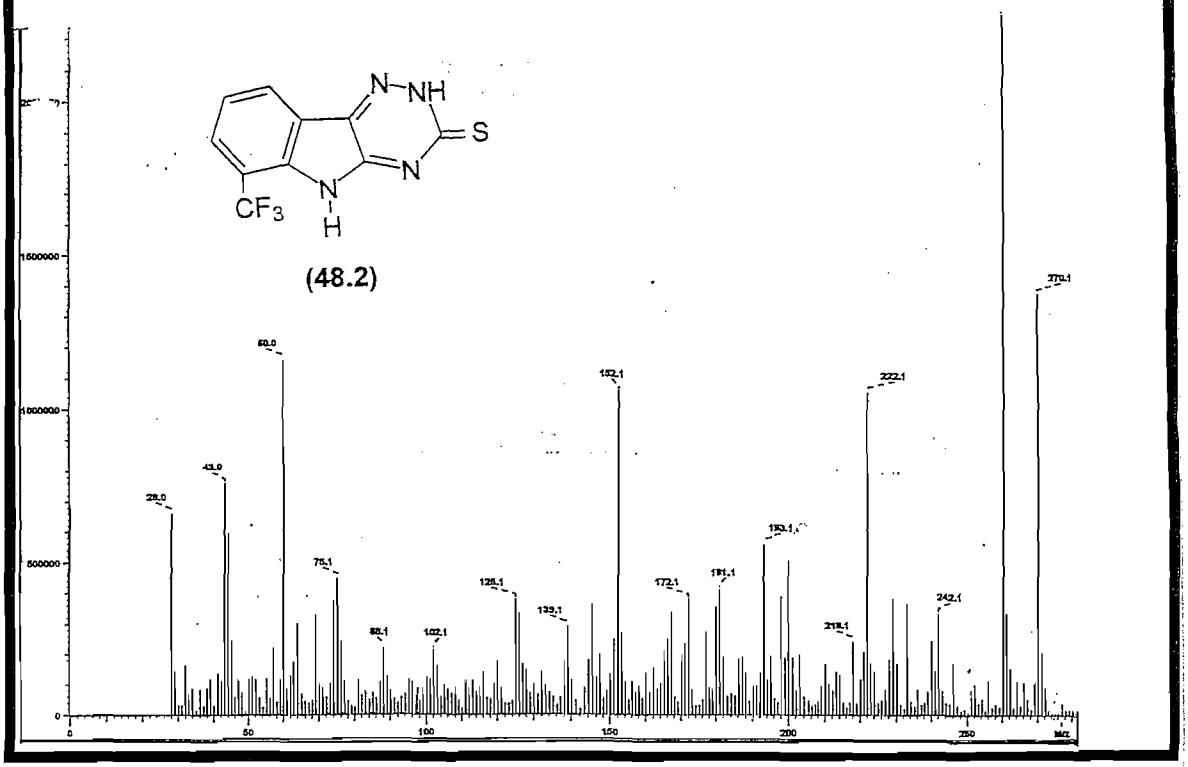
(48.2)

SPECTRUM 46

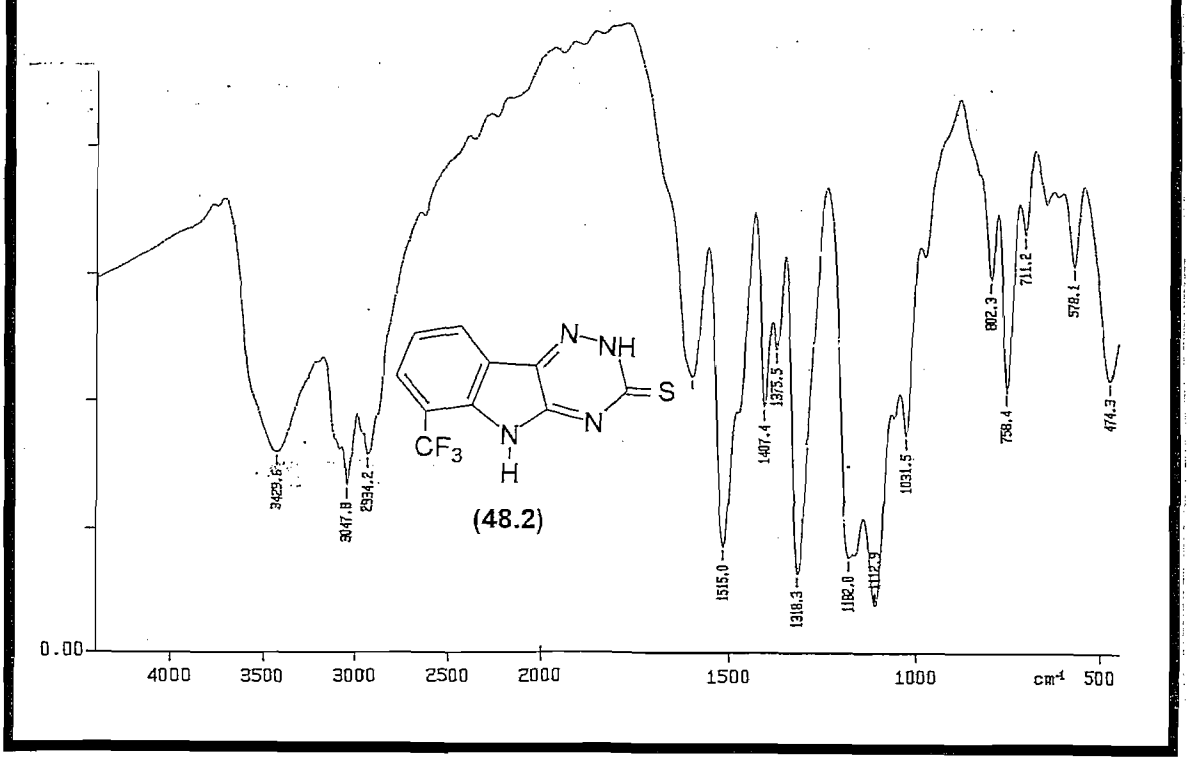
(48.2)



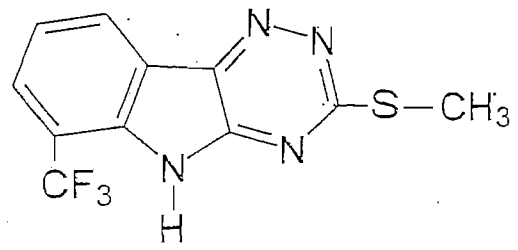
SPECTRUM 47



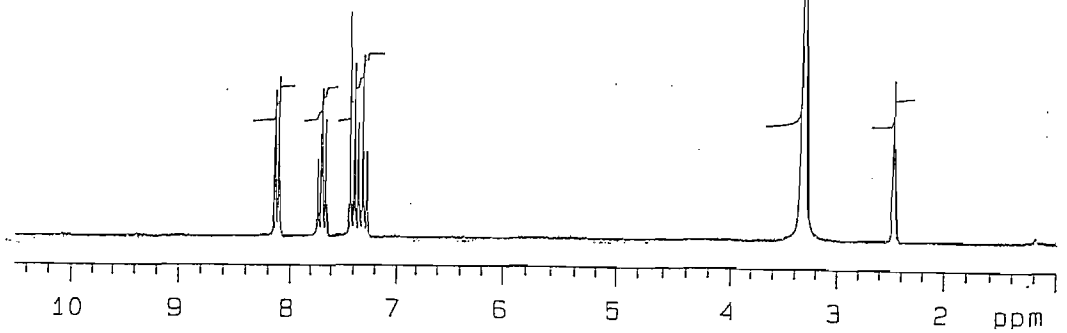
SPECTRUM 48



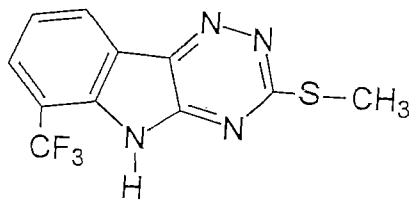
SPECTRUM 49



(6.2)

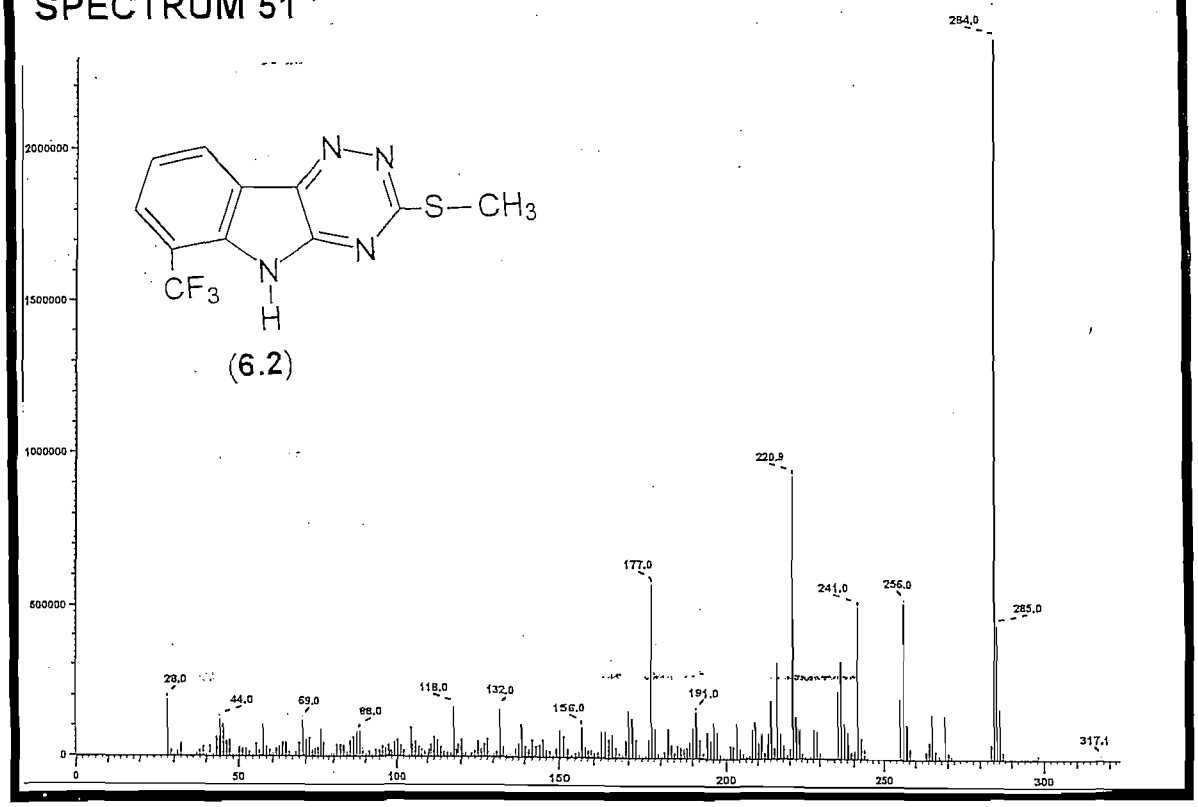


SPECTRUM 50



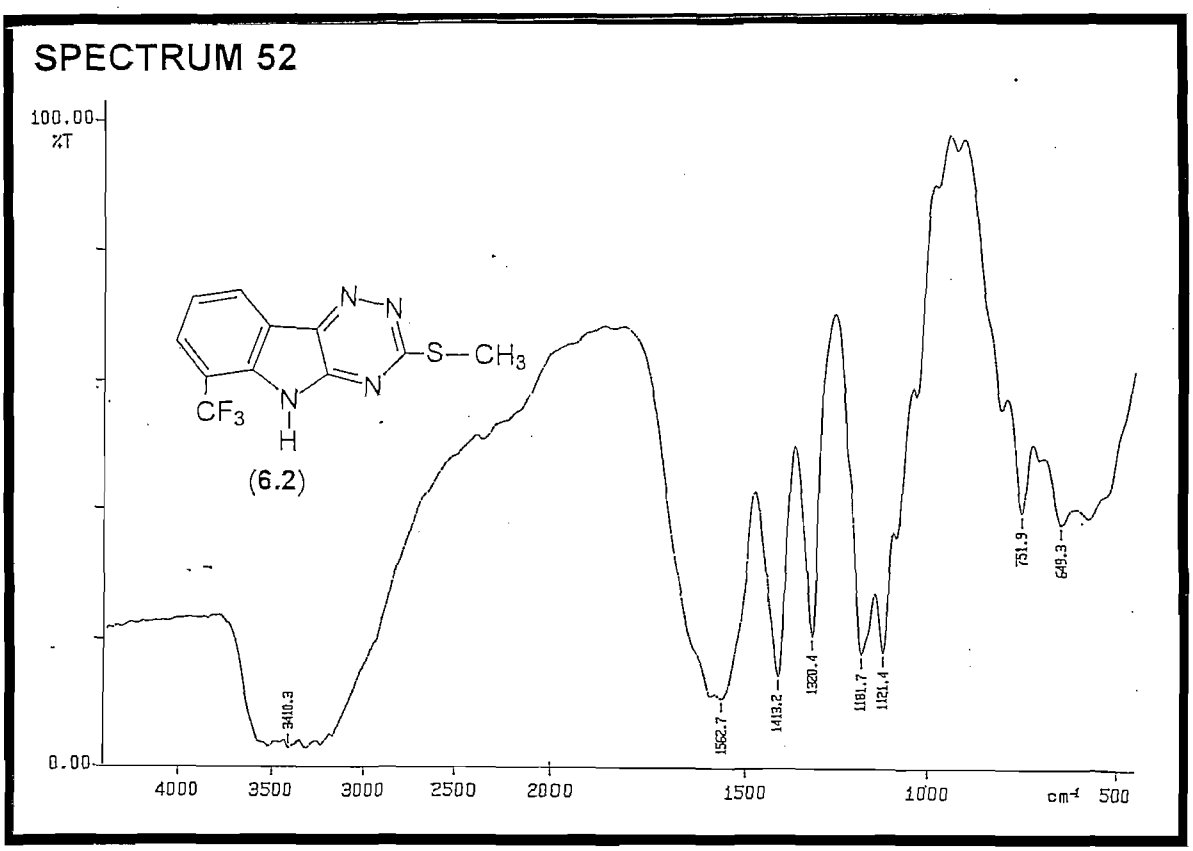
(6.2)

SPECTRUM 51



(6.2)

SPECTRUM 52



(6.2)

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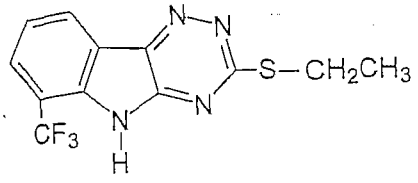
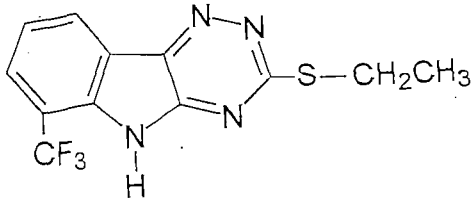


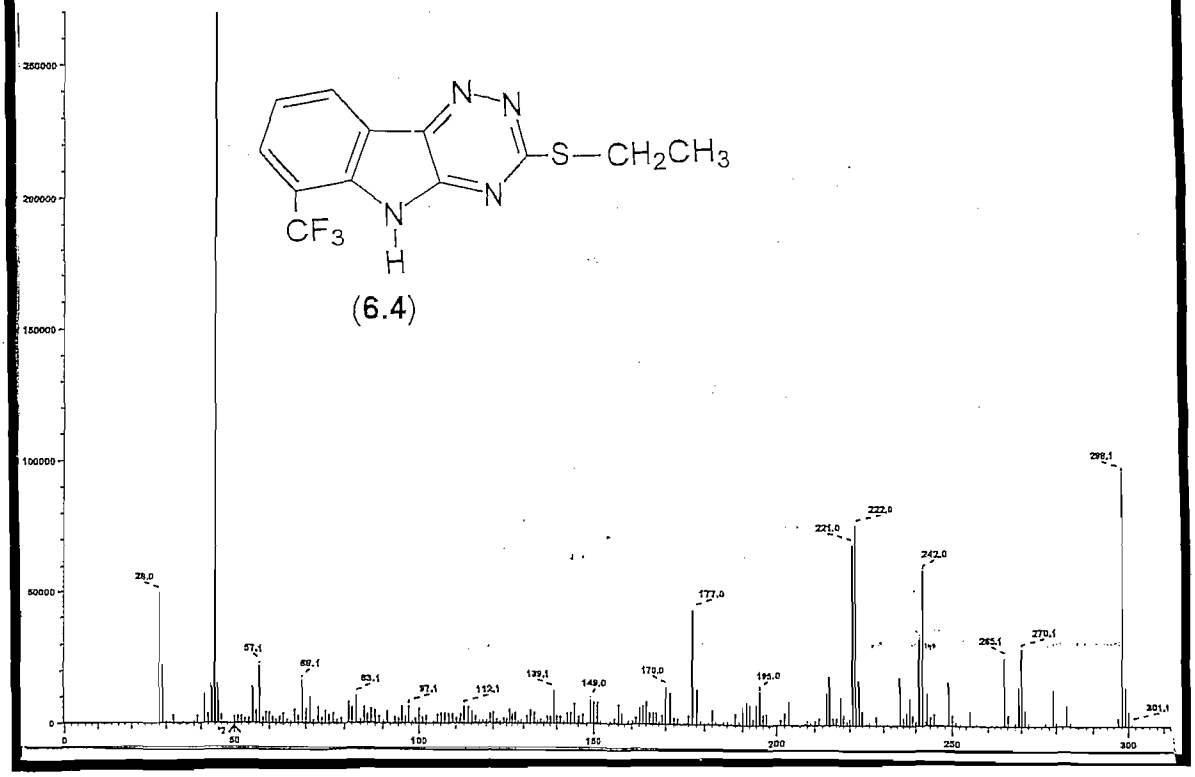
(6.4)

SPECTRUM 54

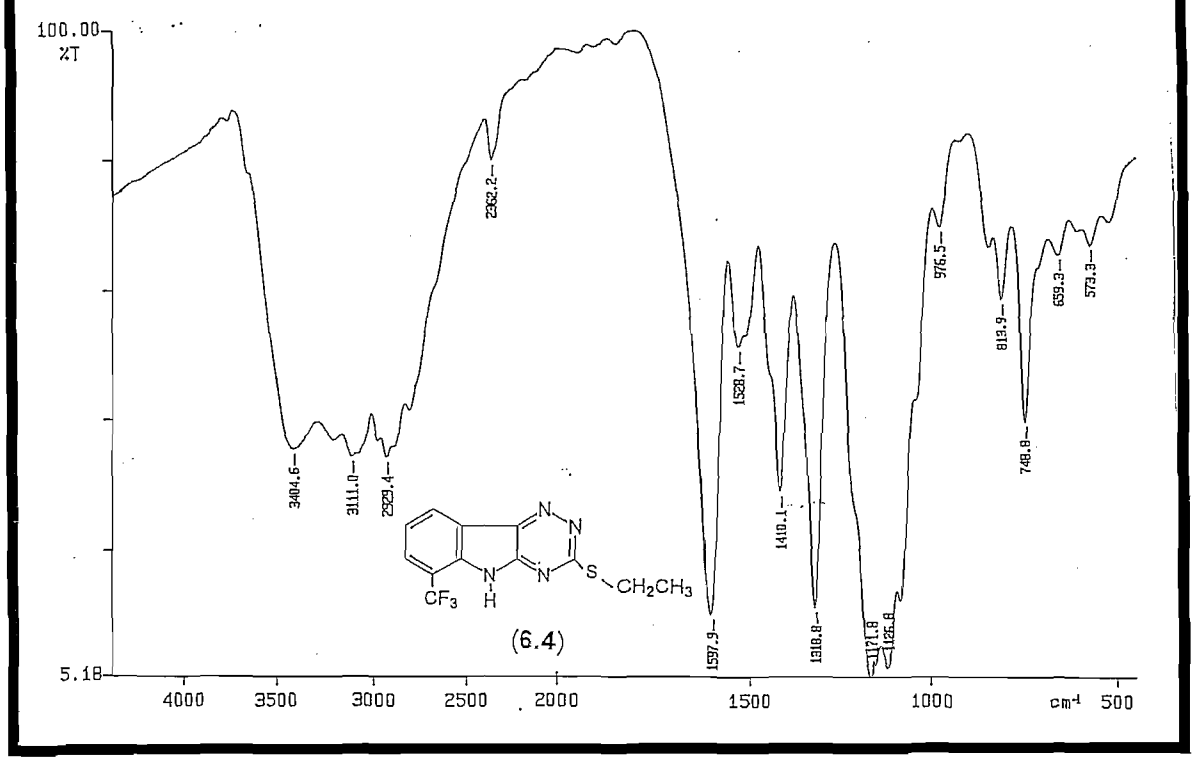


(6.4)

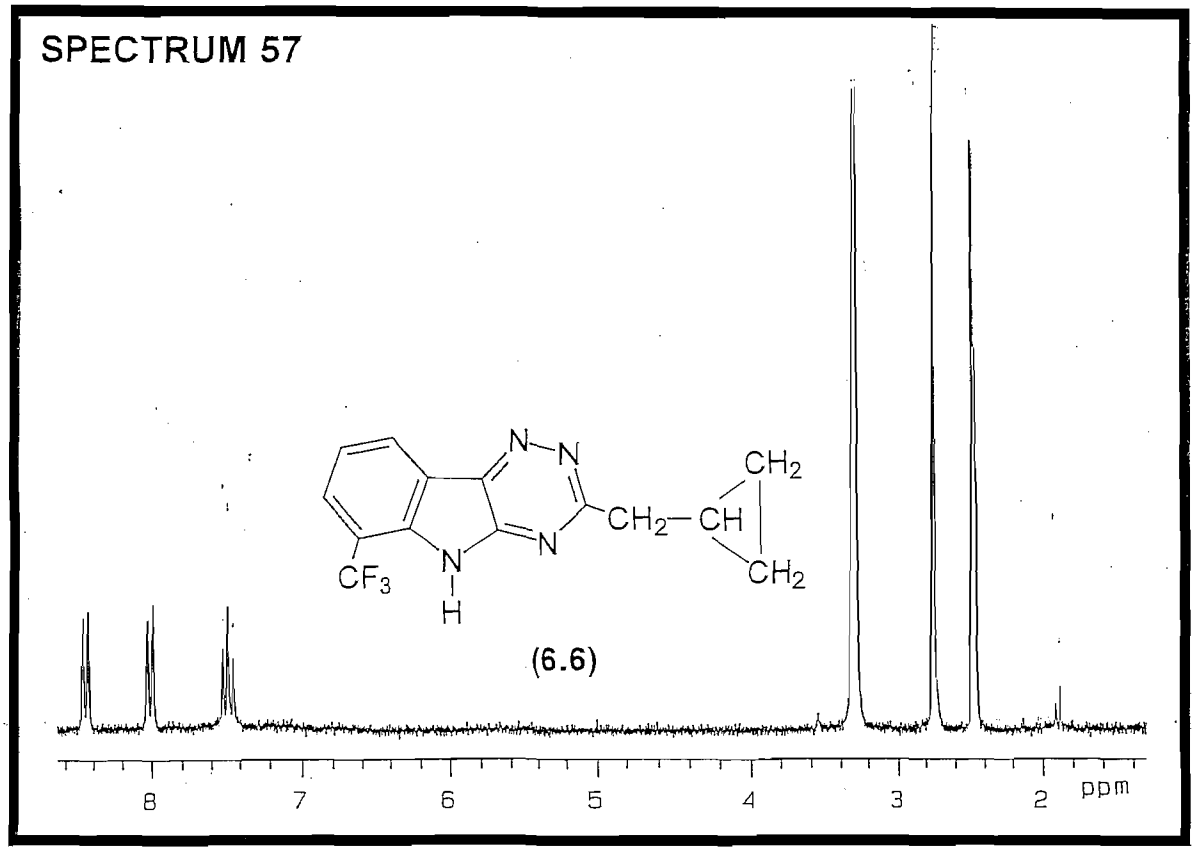
SPECTRUM 55



SPECTRUM 56

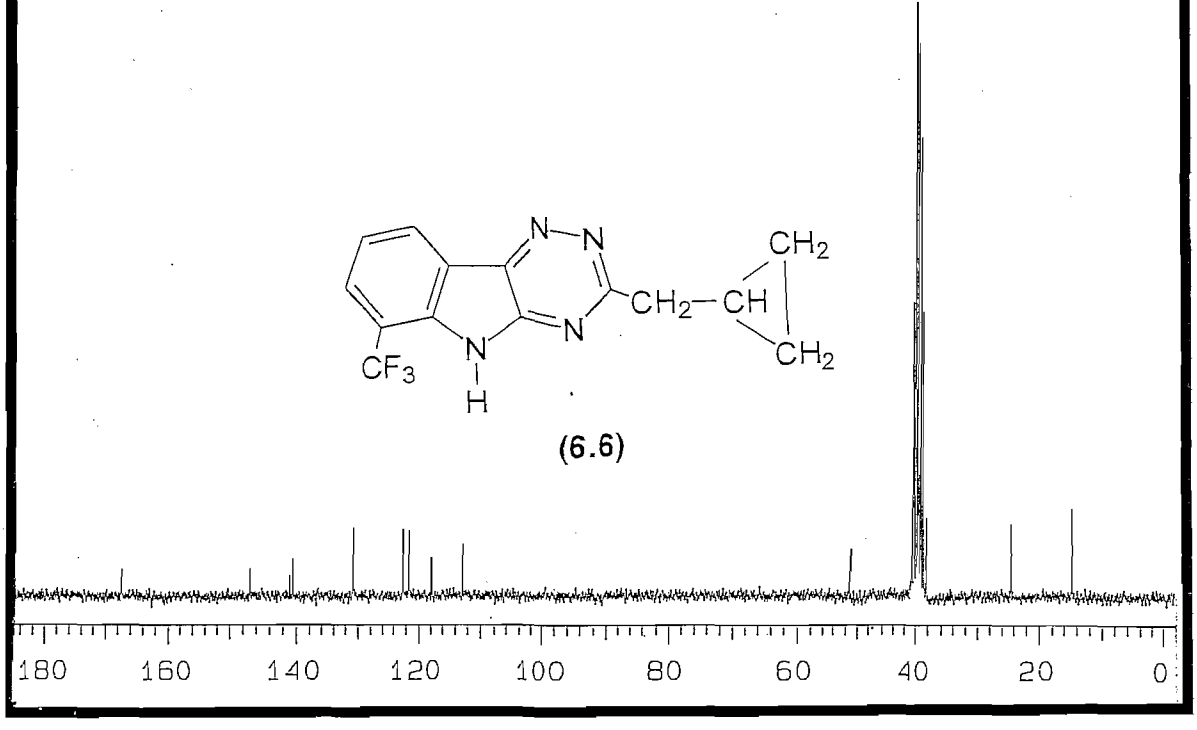


SPECTRUM 57



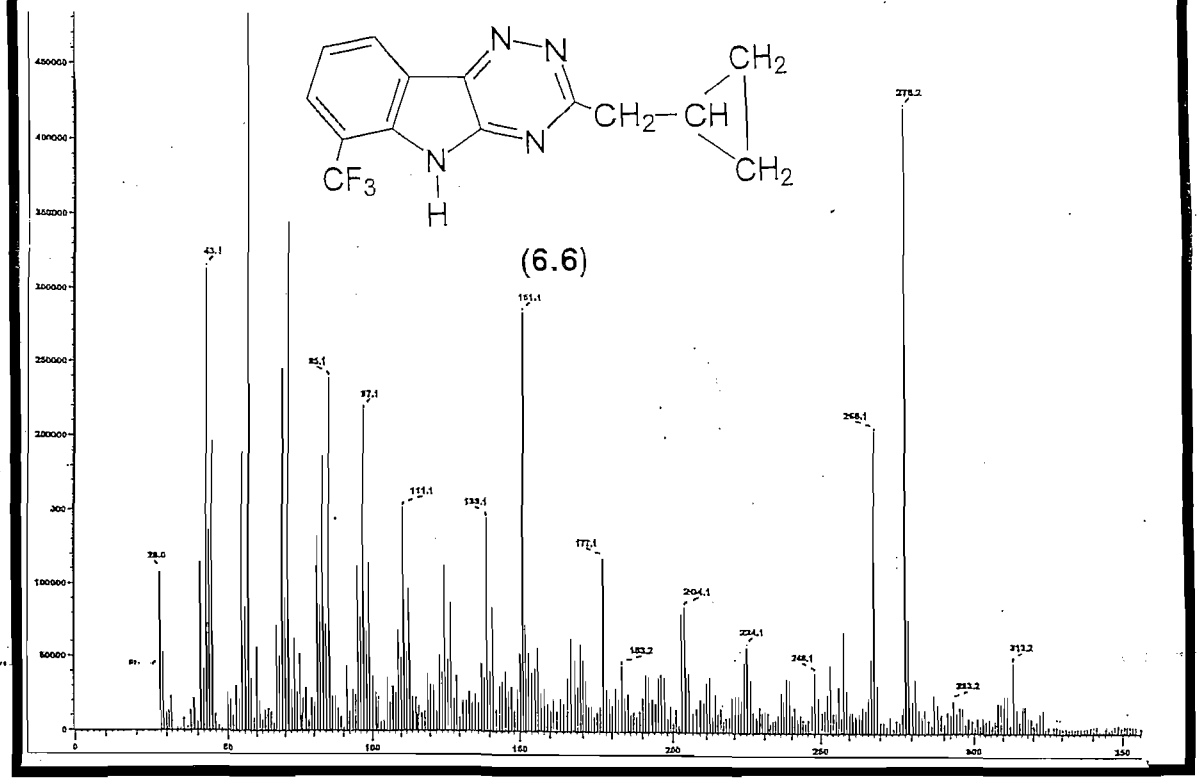
(6.6)

SPECTRUM 58

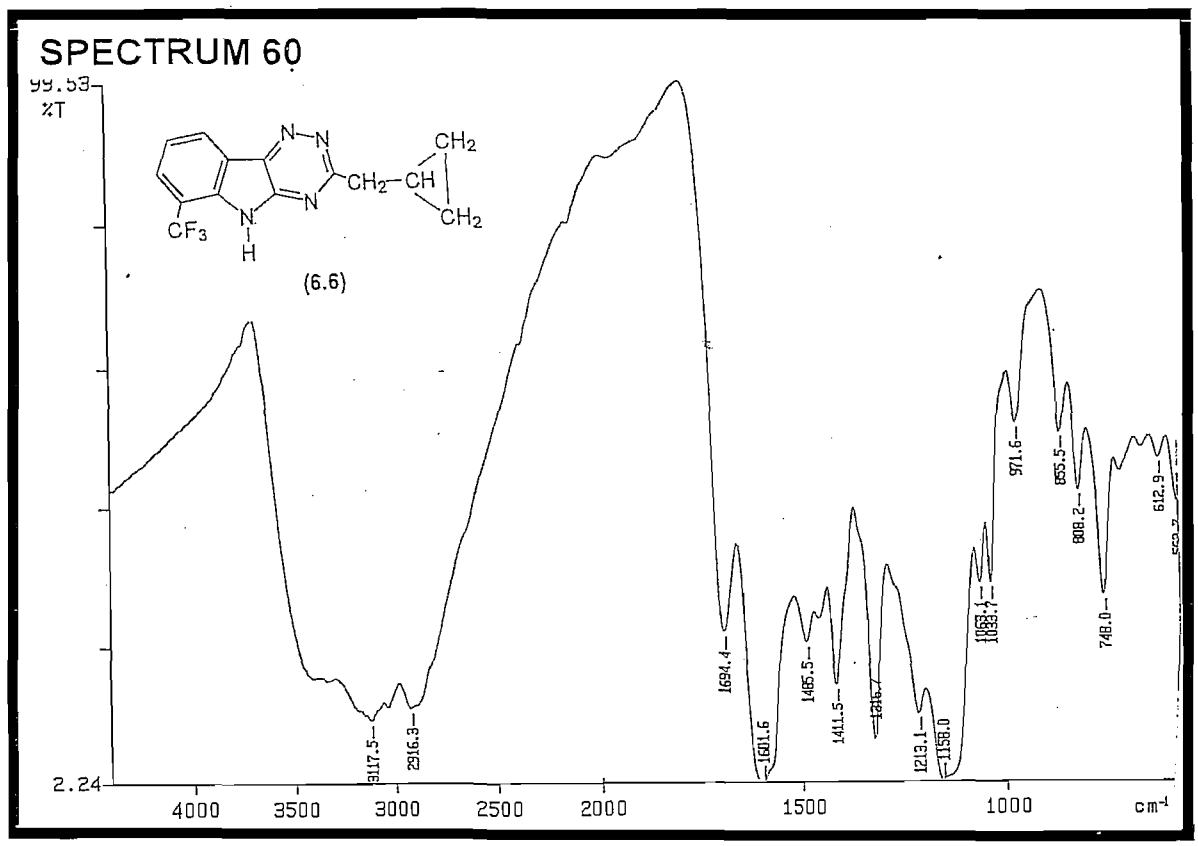


(6.6)

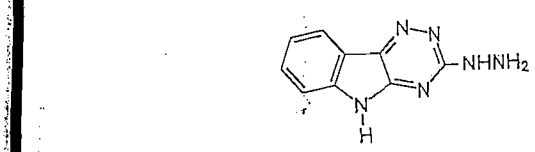
SPECTRUM 59



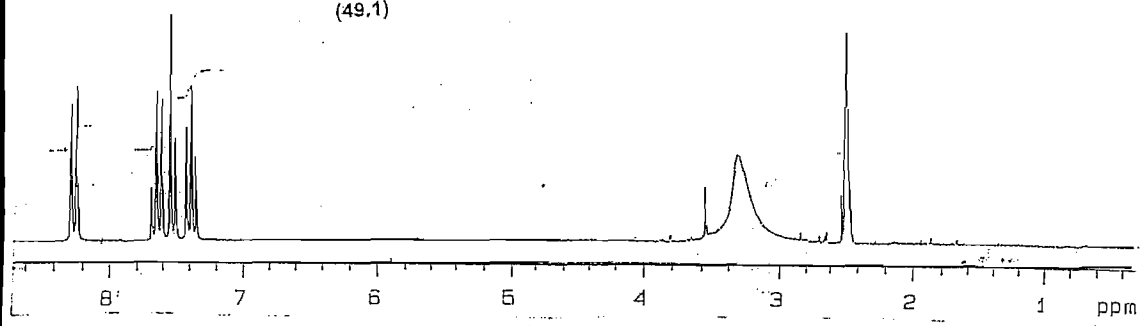
SPECTRUM 60



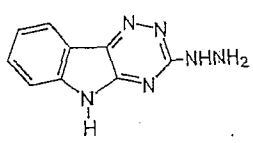
SPECTRUM 61



(49.1)

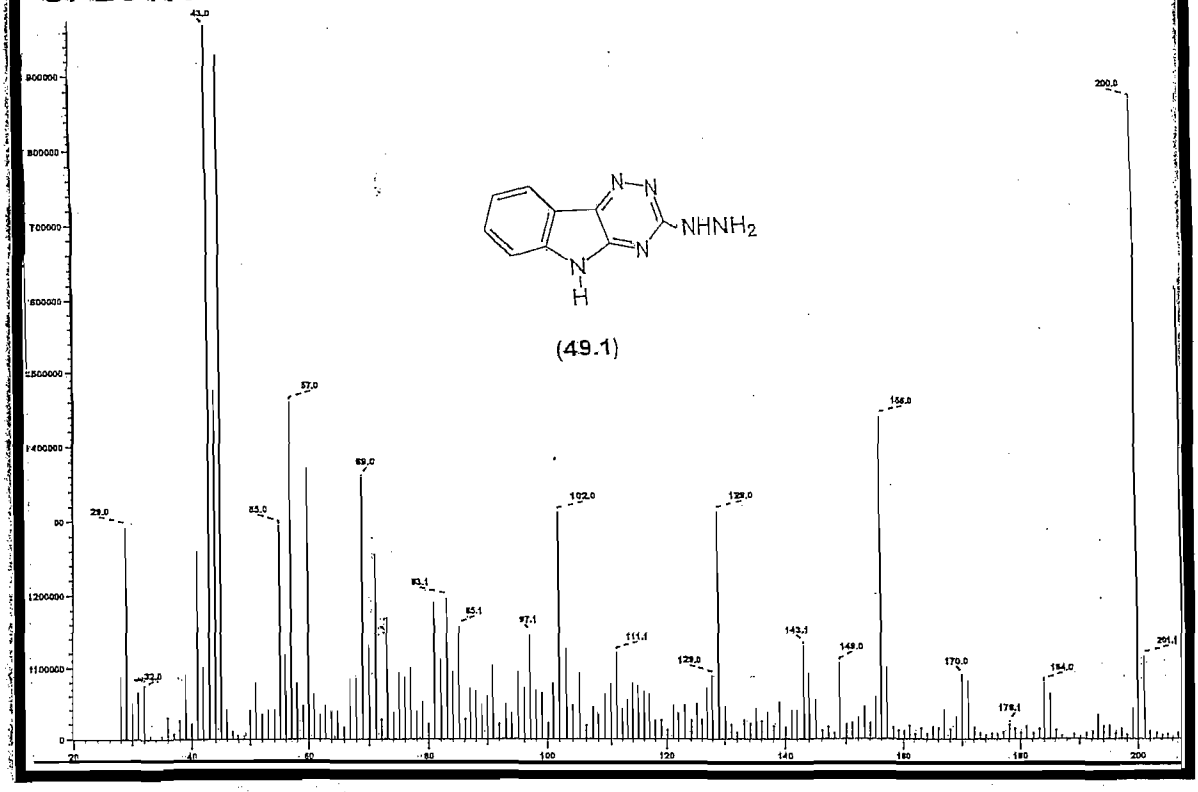


SPECTRUM 62

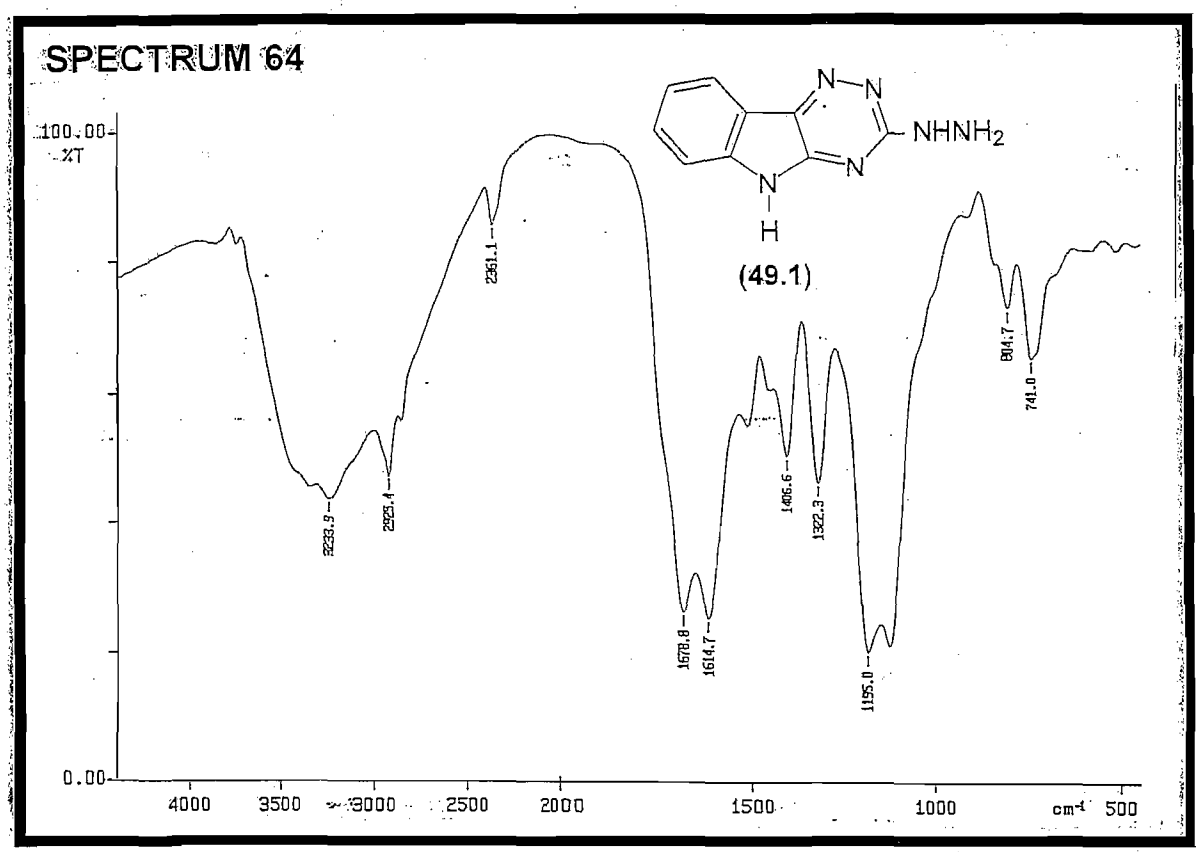


(49.1)

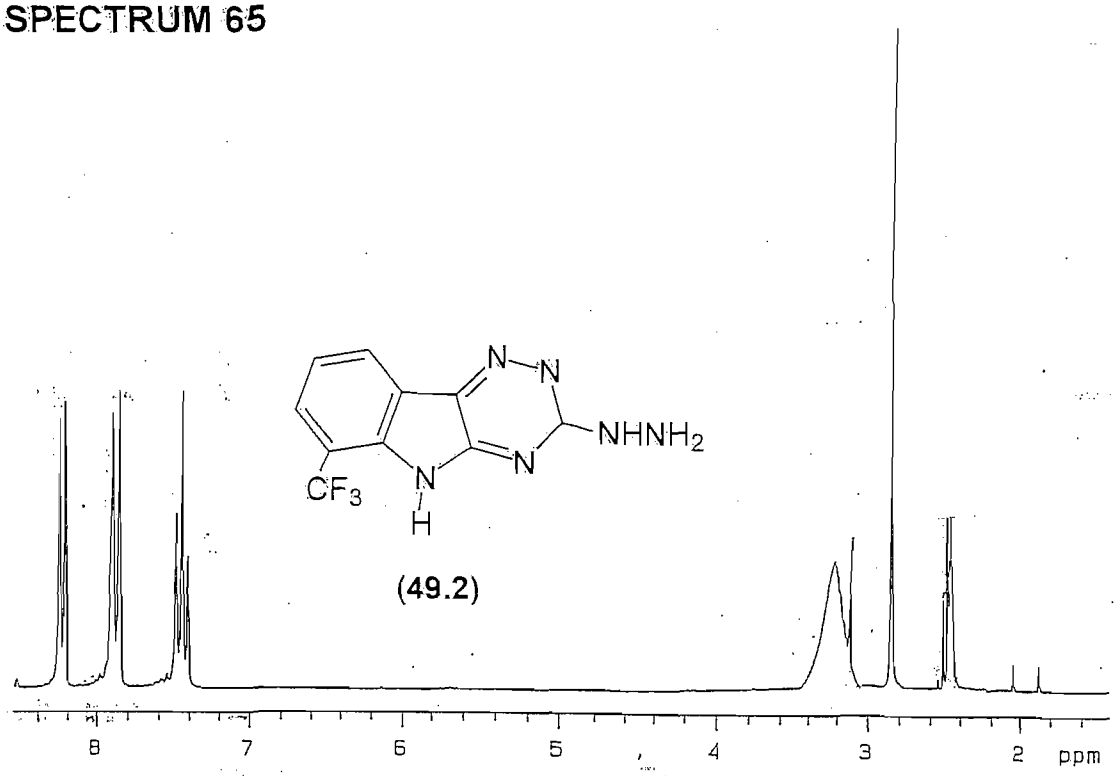
SPECTRUM 63



SPECTRUM 64

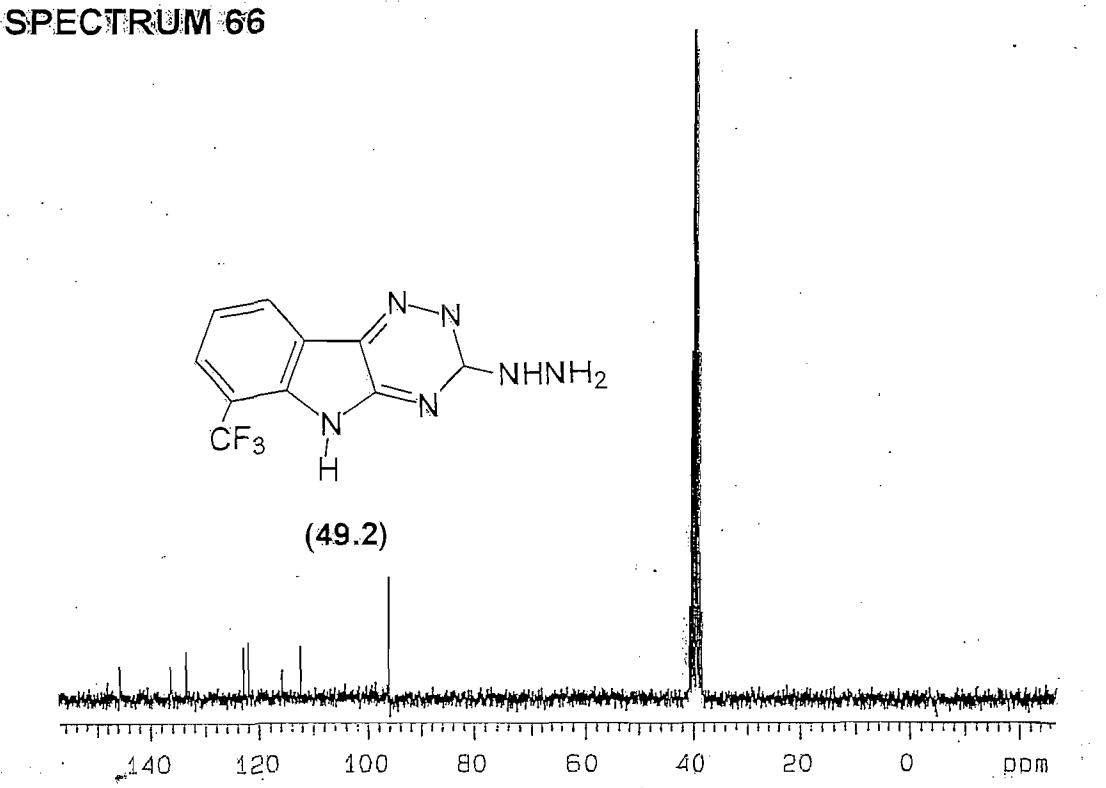


SPECTRUM 65



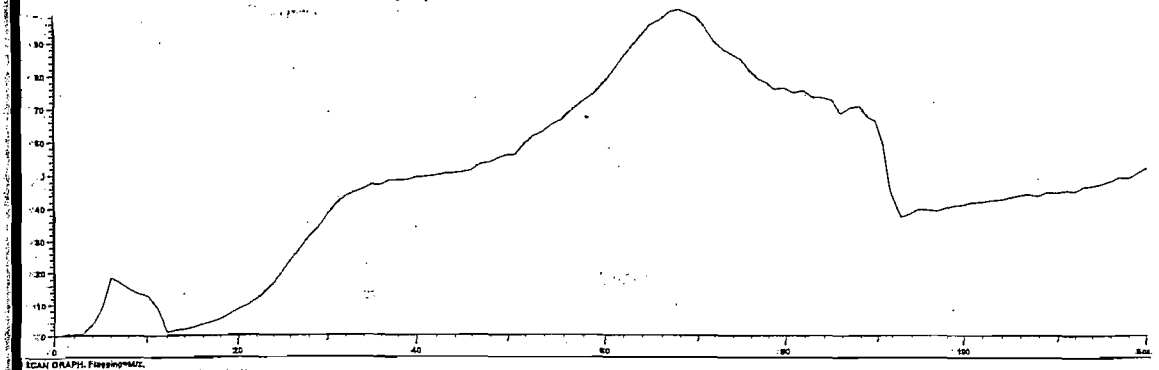
(49.2)

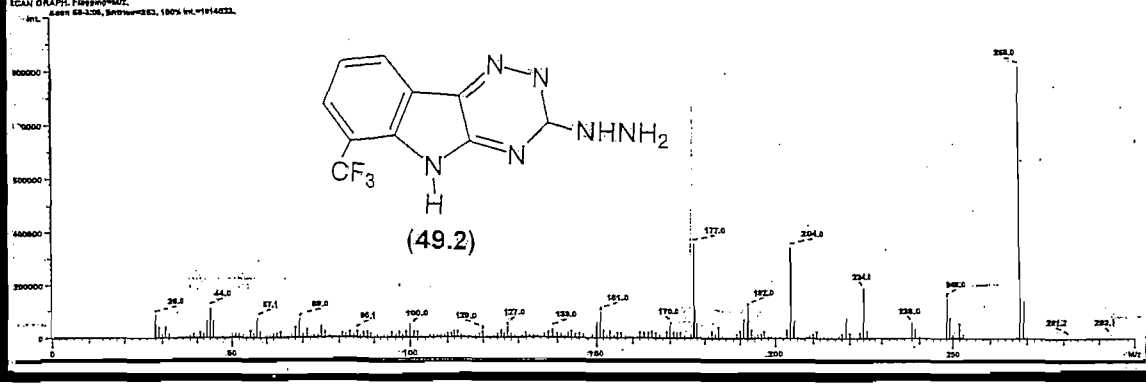
SPECTRUM 66



(49.2)

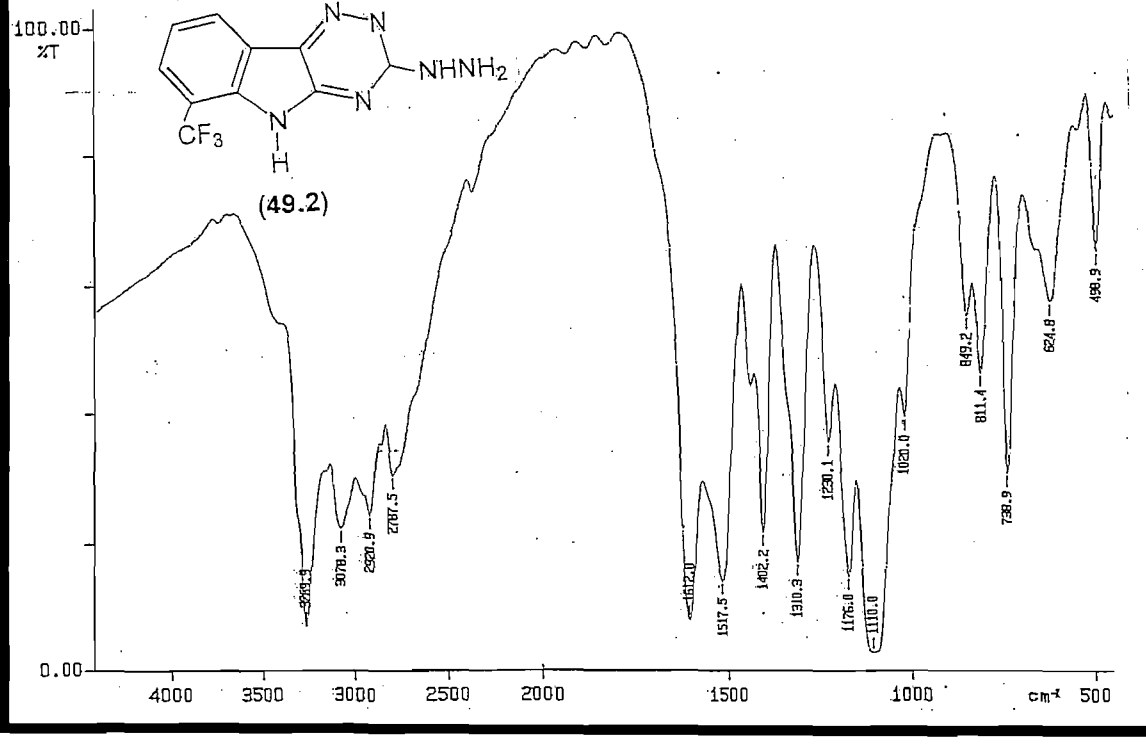
SPECTRUM 67





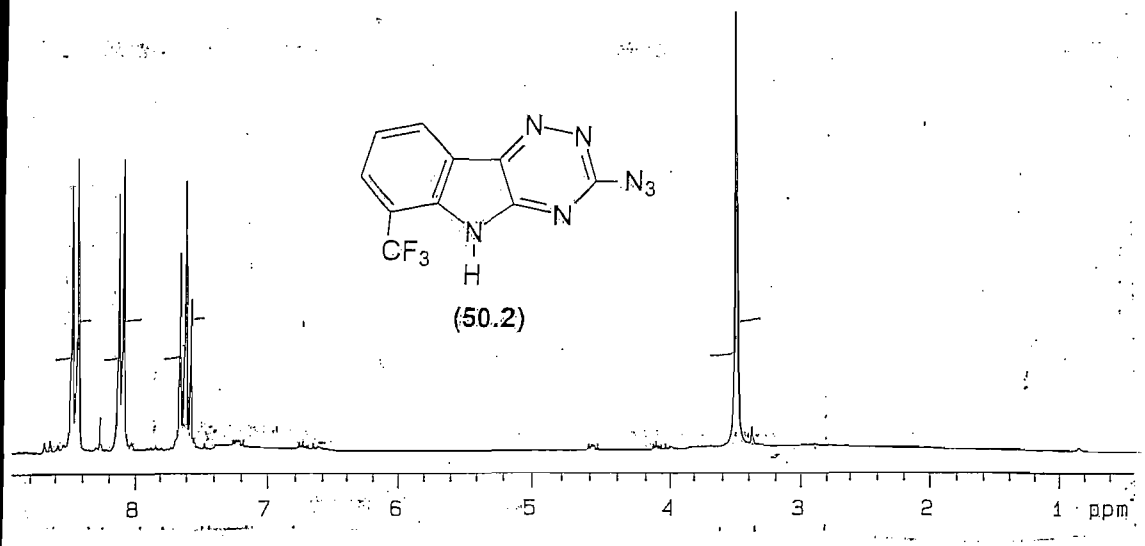
(49.2)

SPECTRUM 68



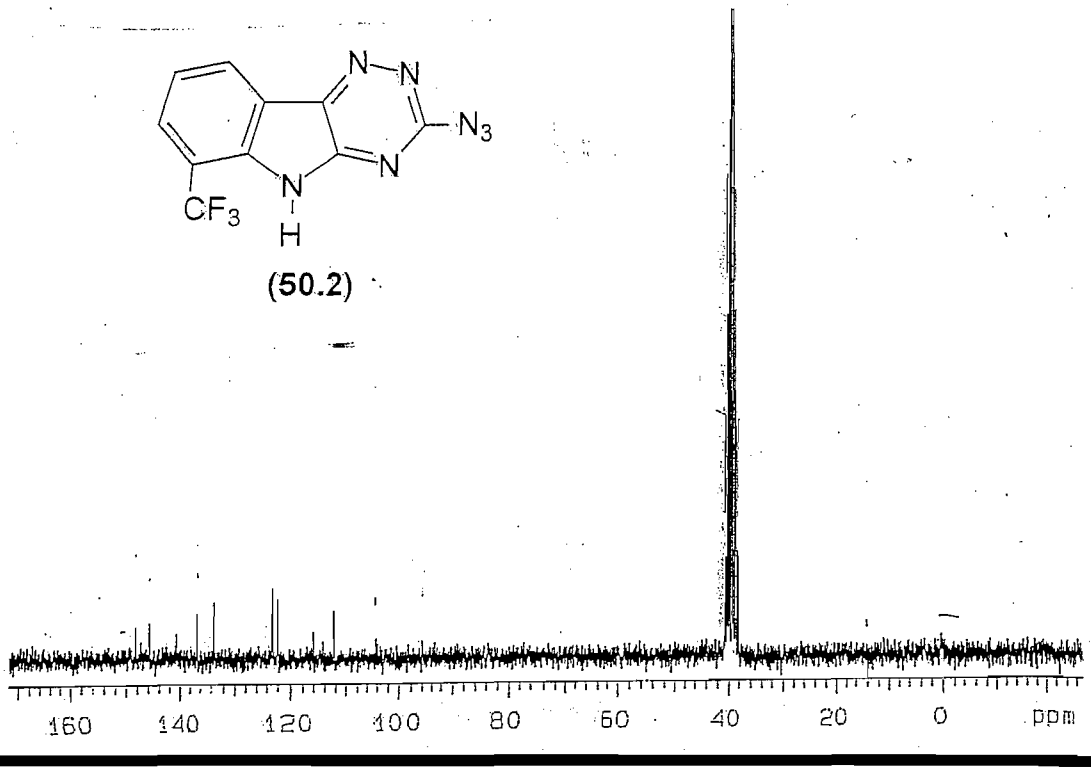
(49.2)

SPECTRUM 69



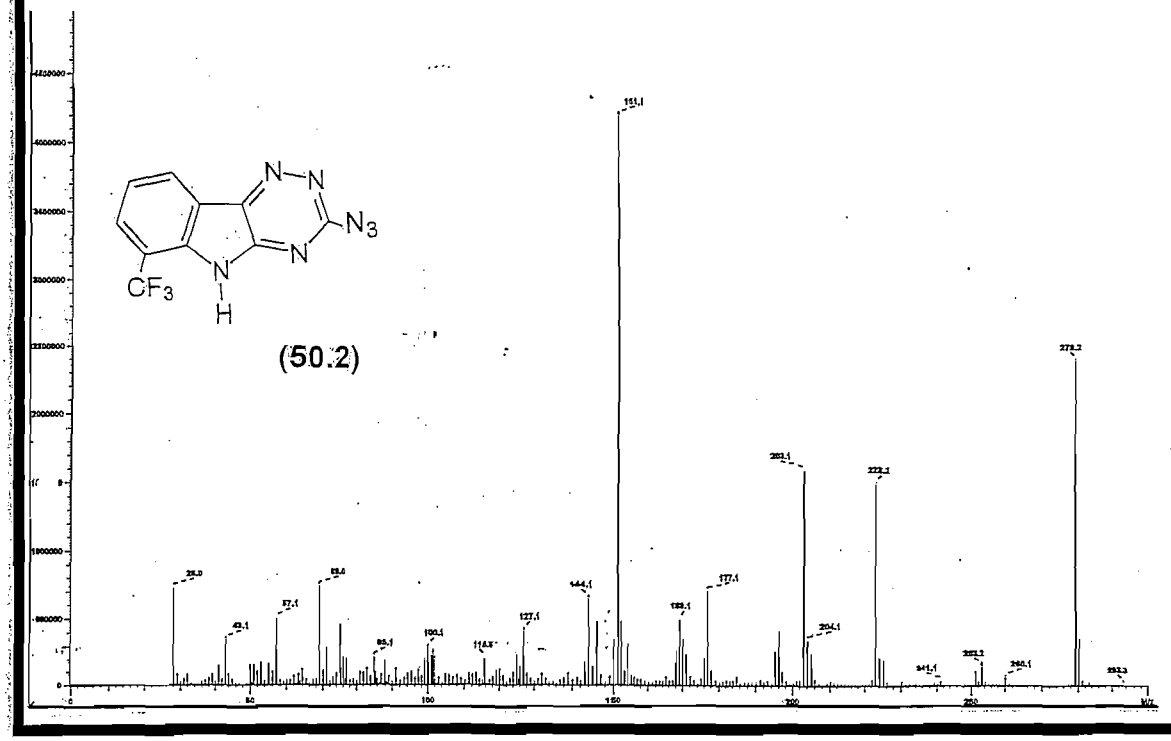
(50.2)

SPECTRUM 70

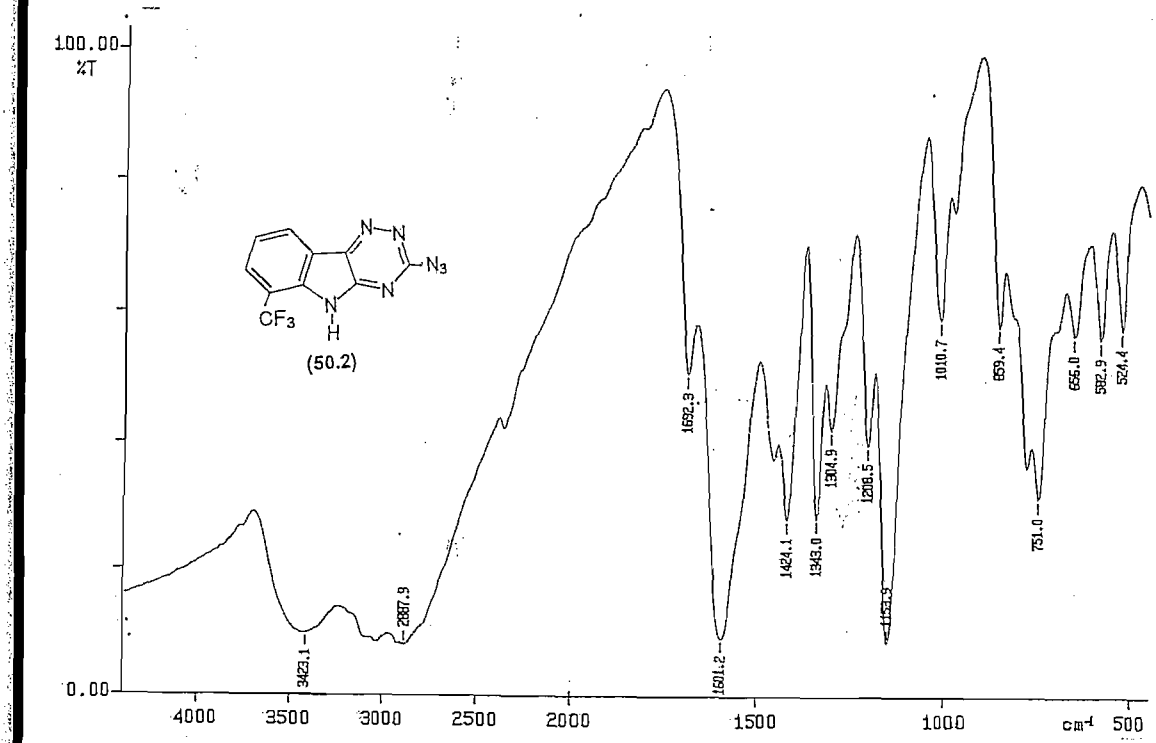


(50.2)

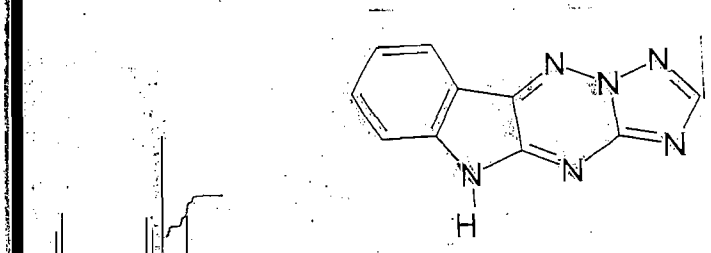
SPECTRUM 71



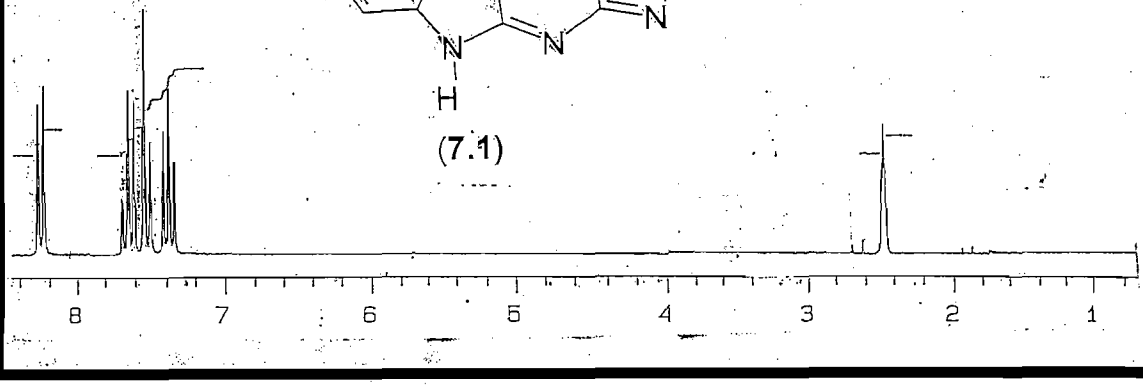
SPECTRUM 72



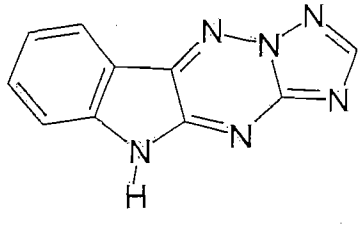
SPECTRUM 73



(7.1)

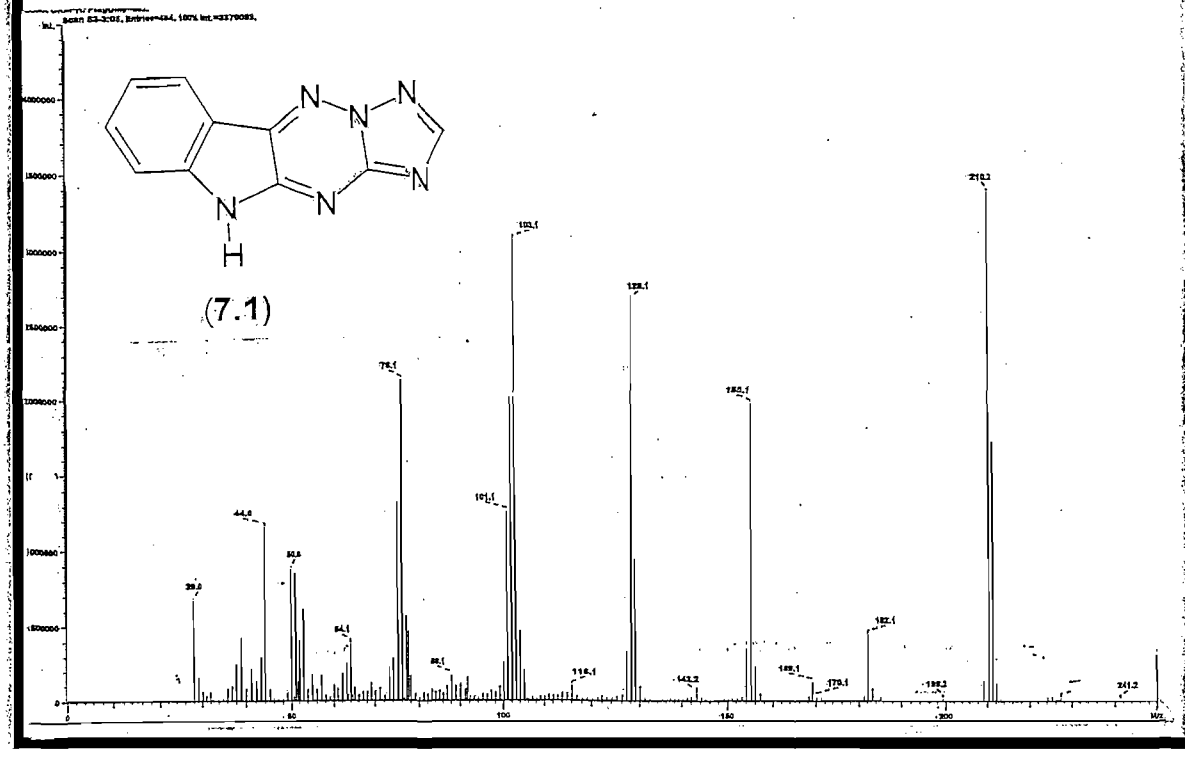


SPECTRUM 74

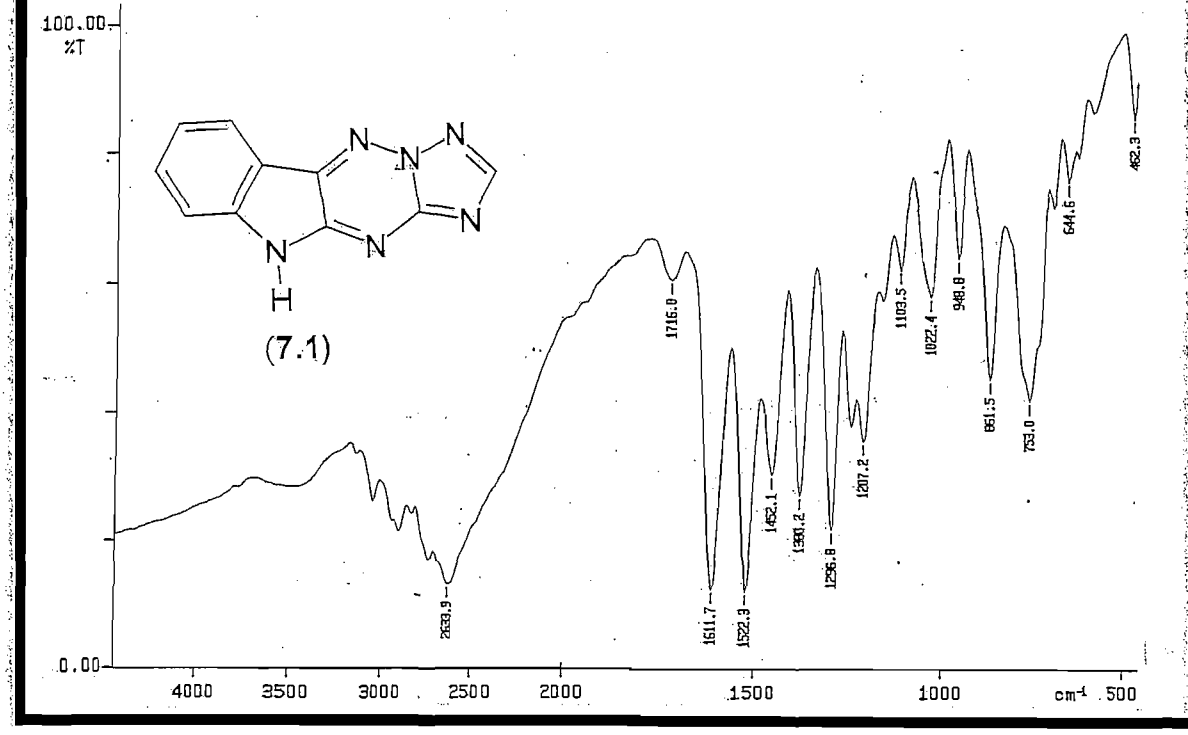


(7.1)

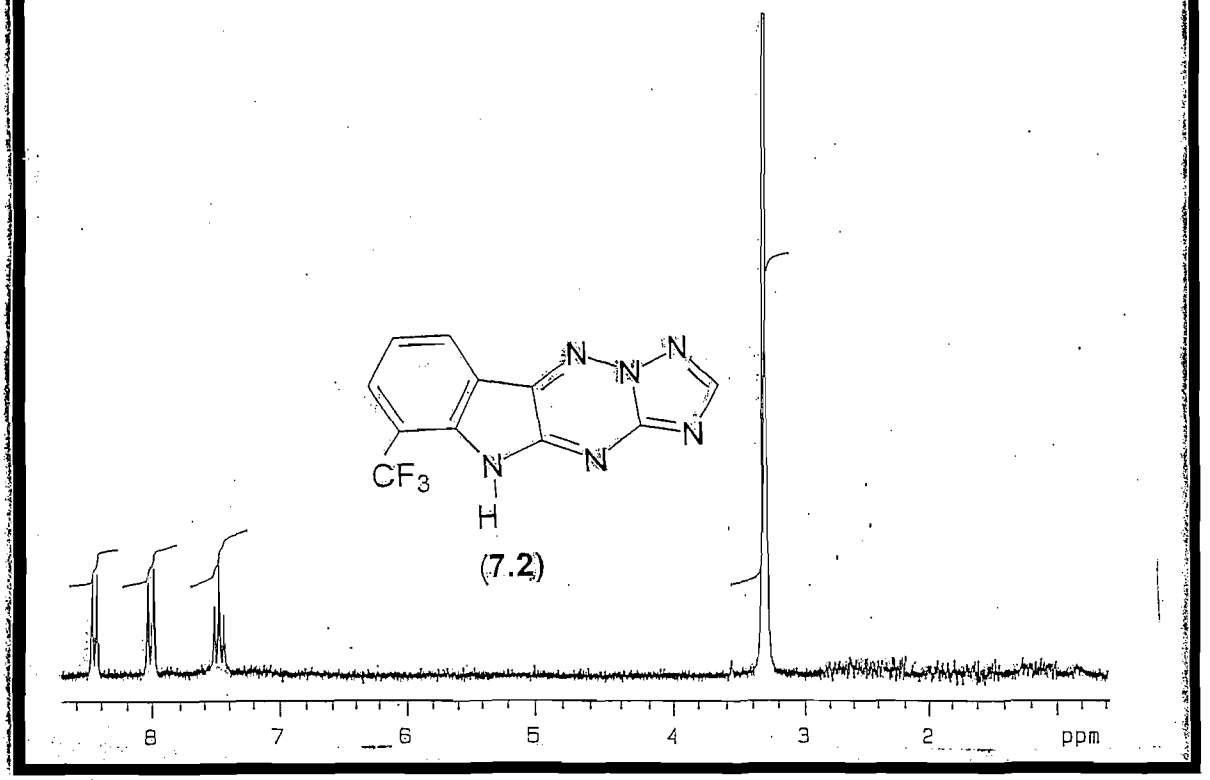
SPECTRUM 75



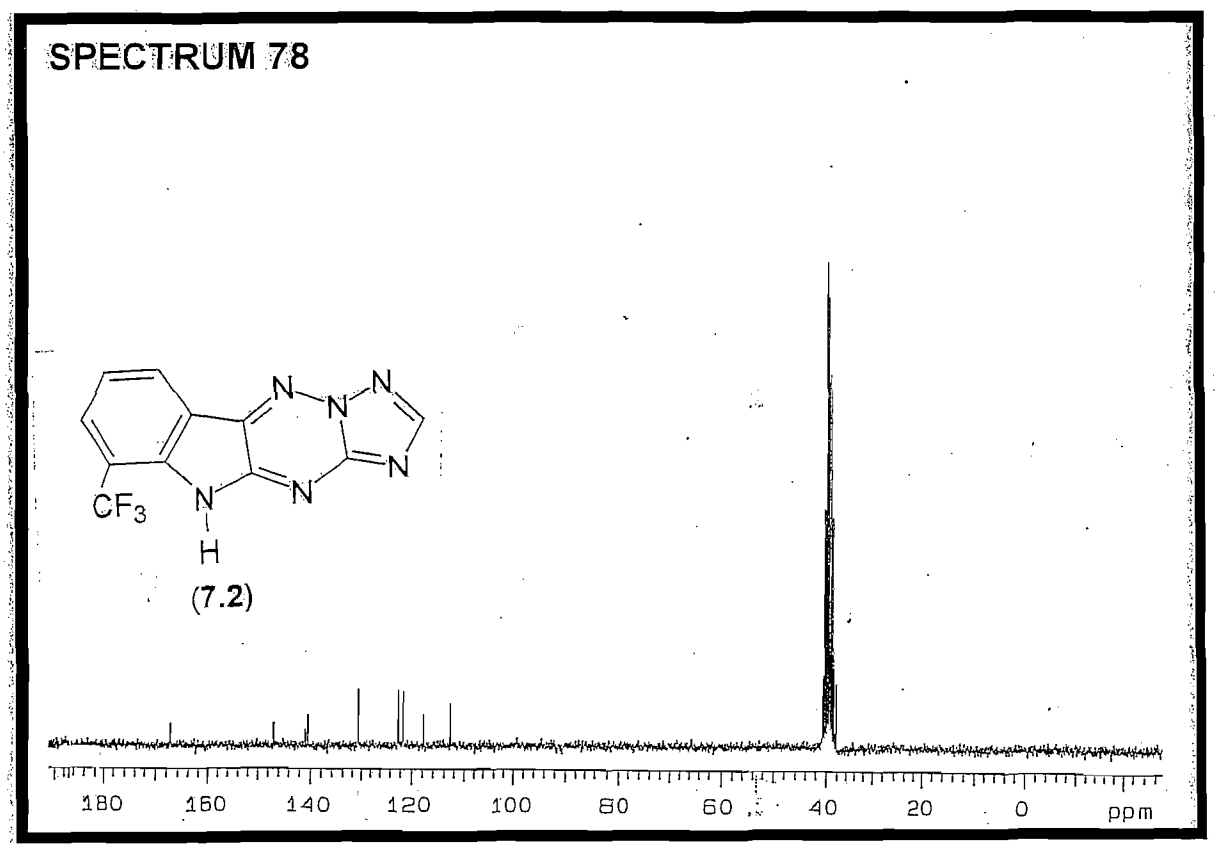
SPECTRUM 76



SPECTRUM 77



SPECTRUM 78



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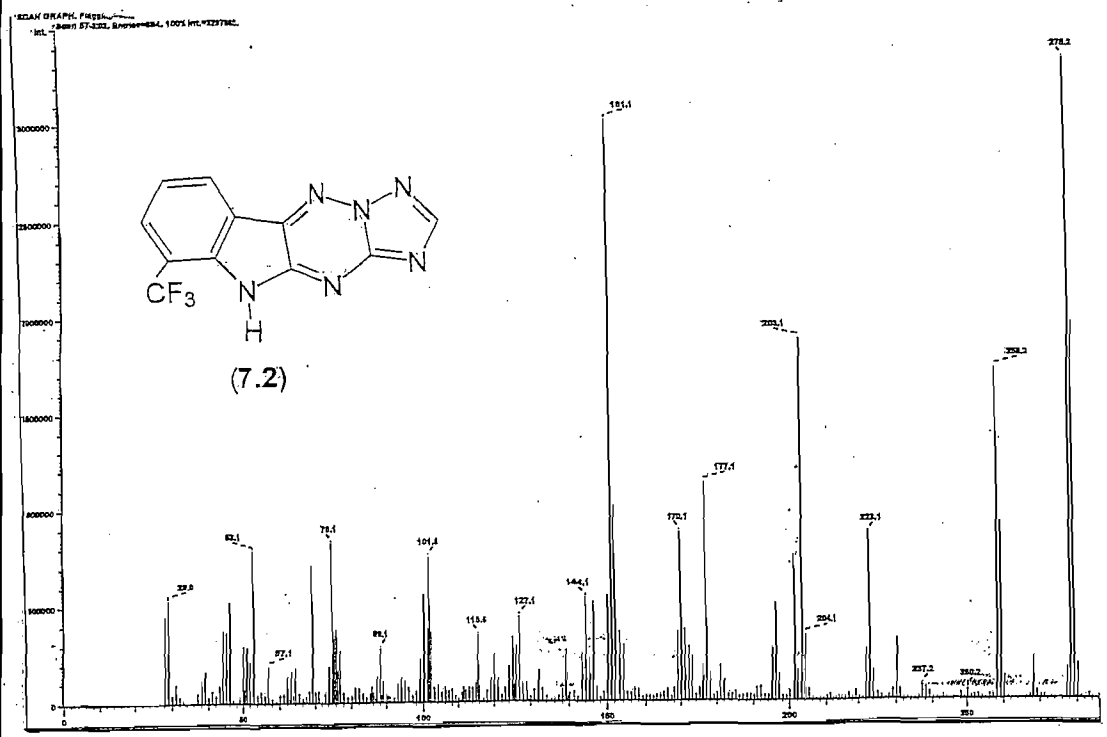

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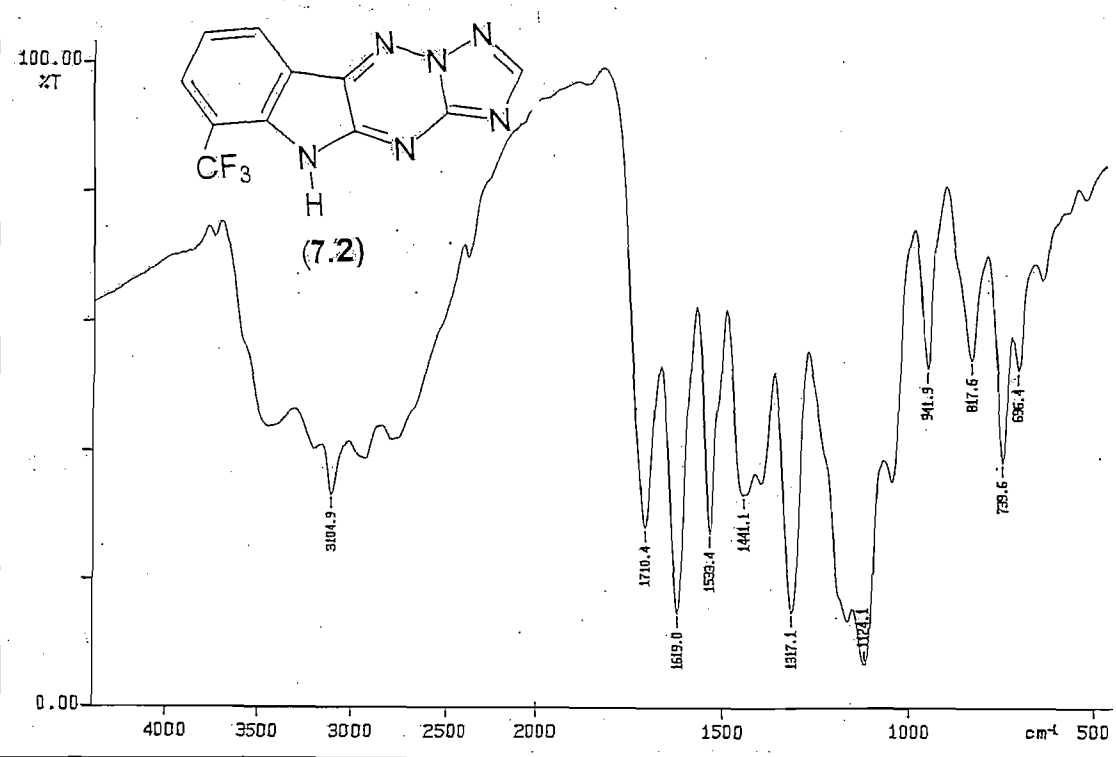

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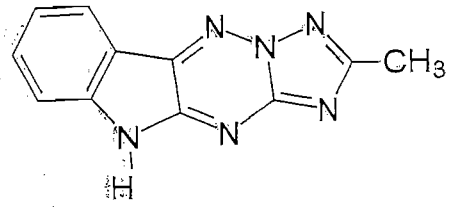
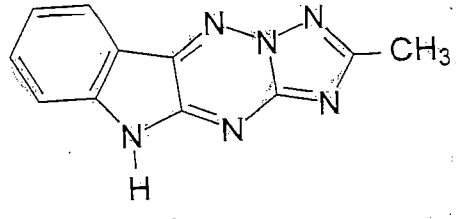


(7.3)

SPECTRUM 82



(7.3)

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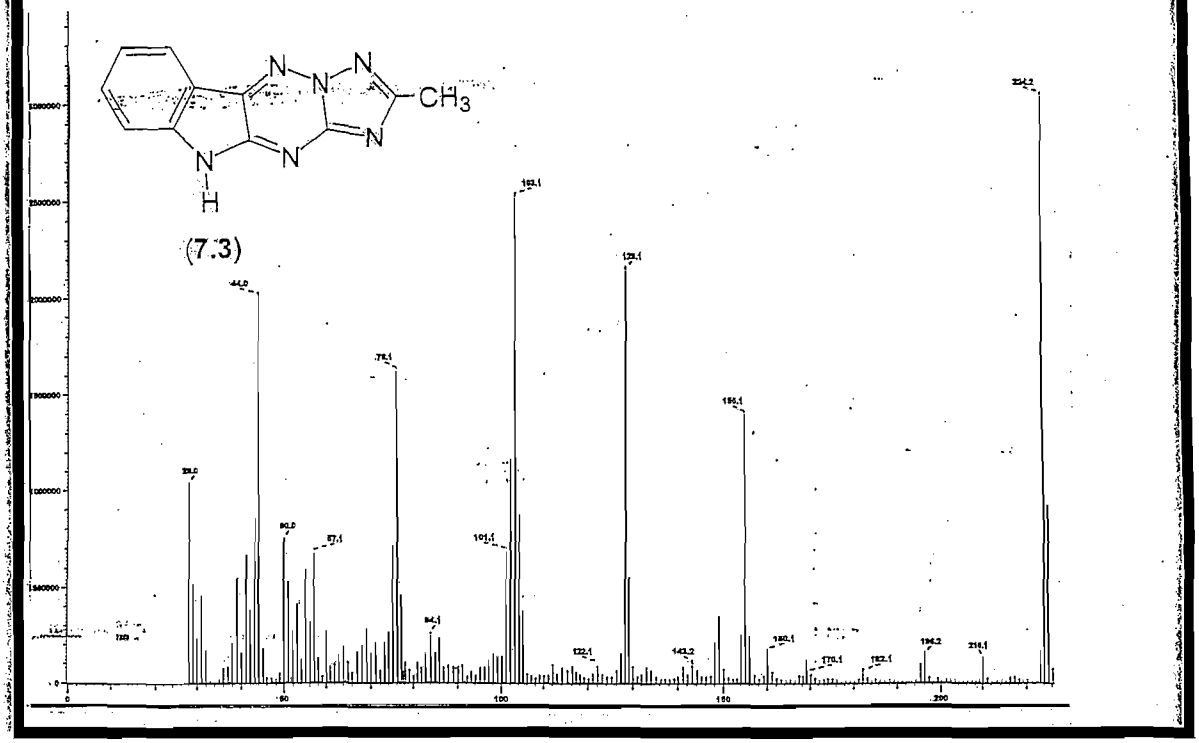

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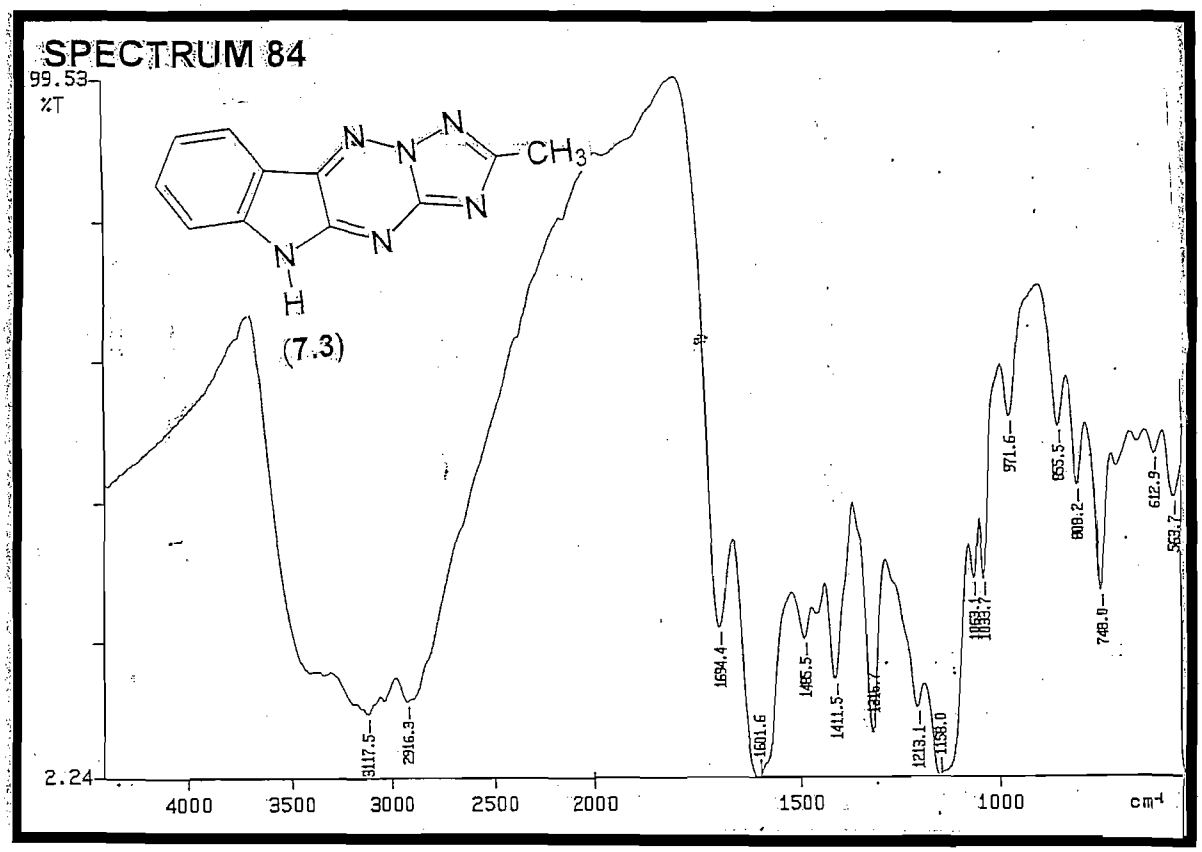

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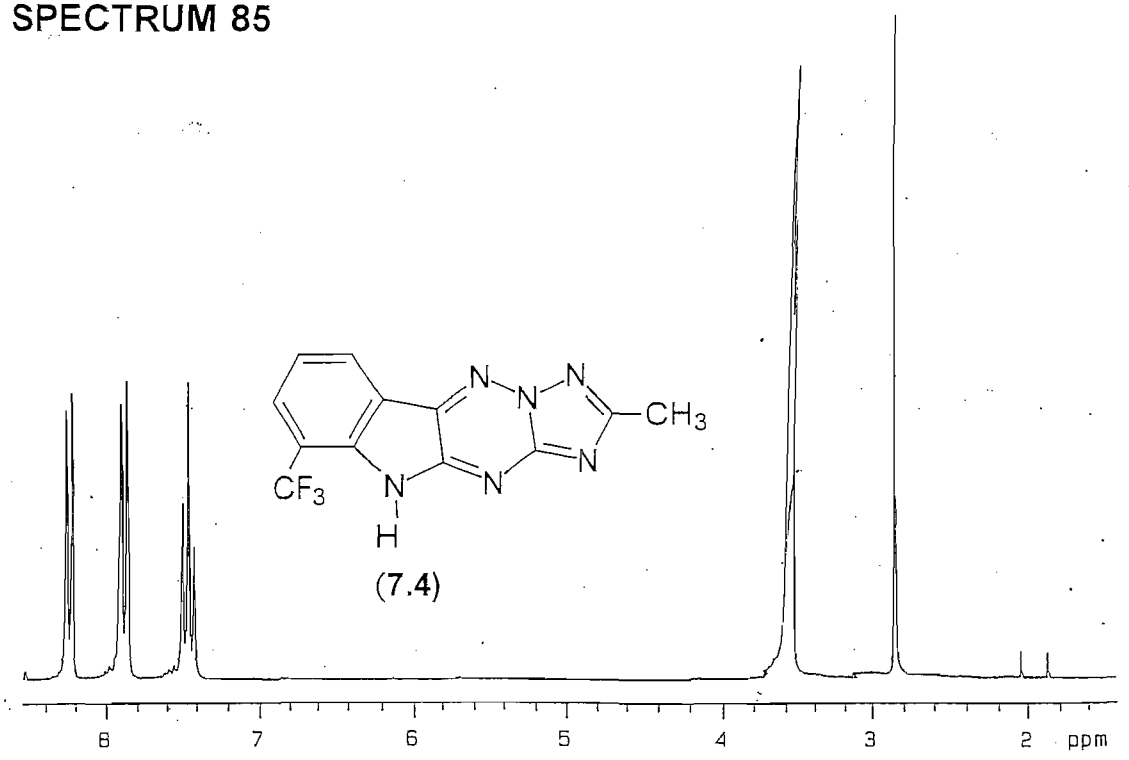

(7.4)

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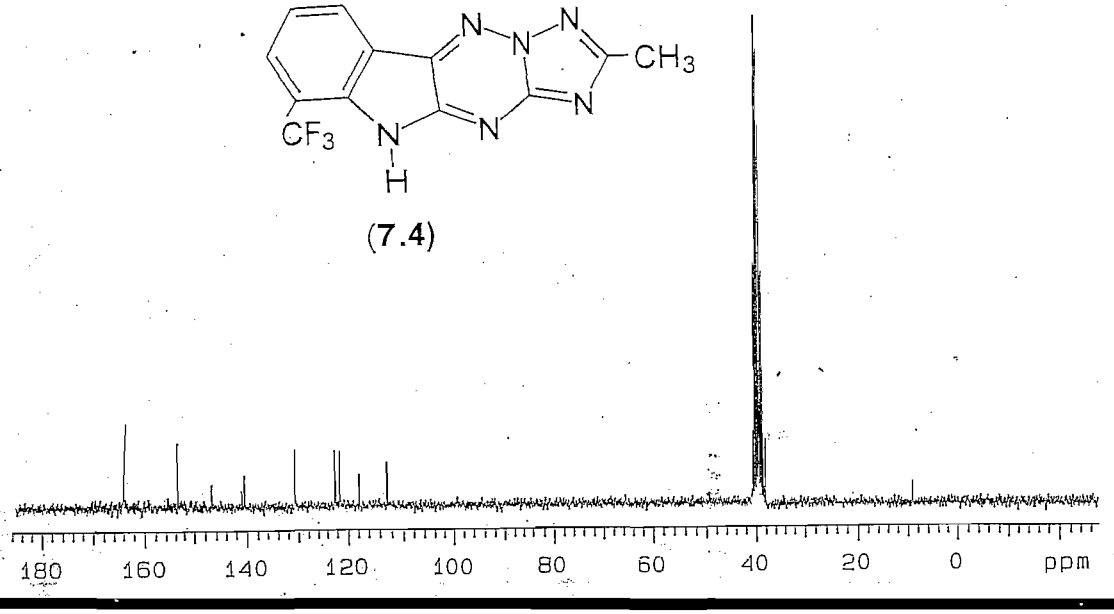

(7.4)

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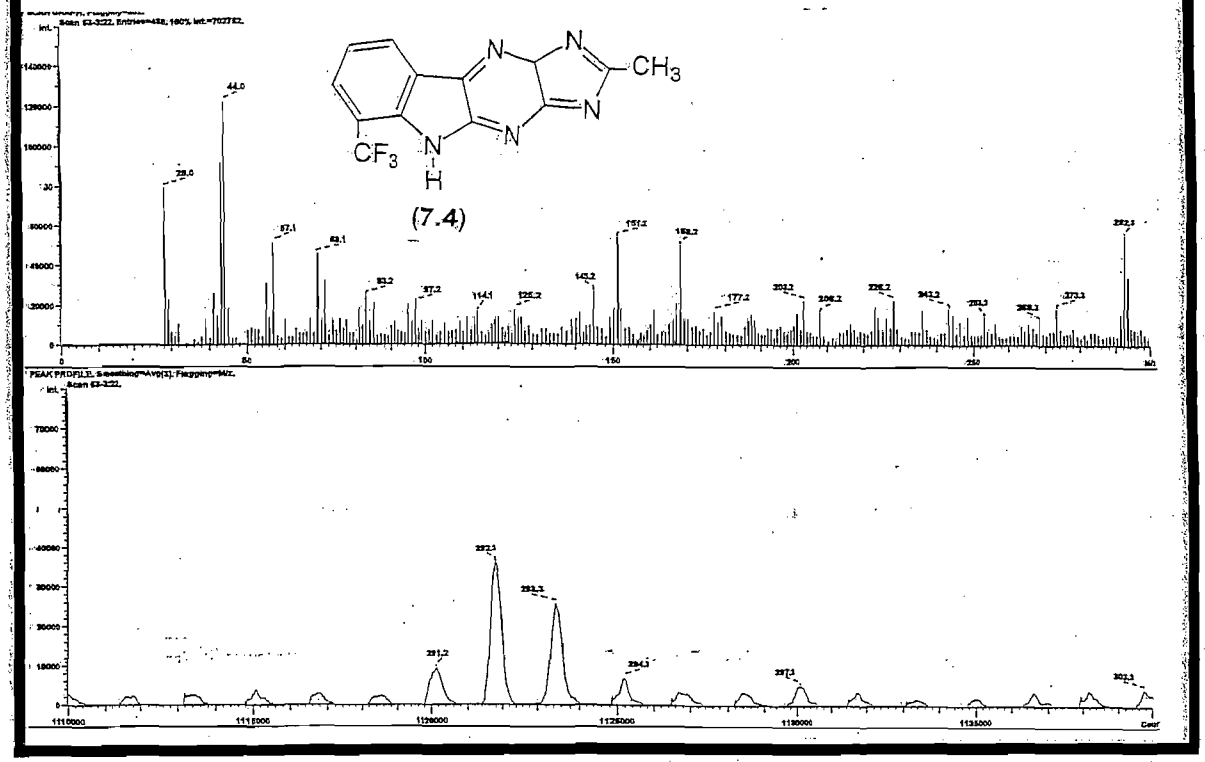
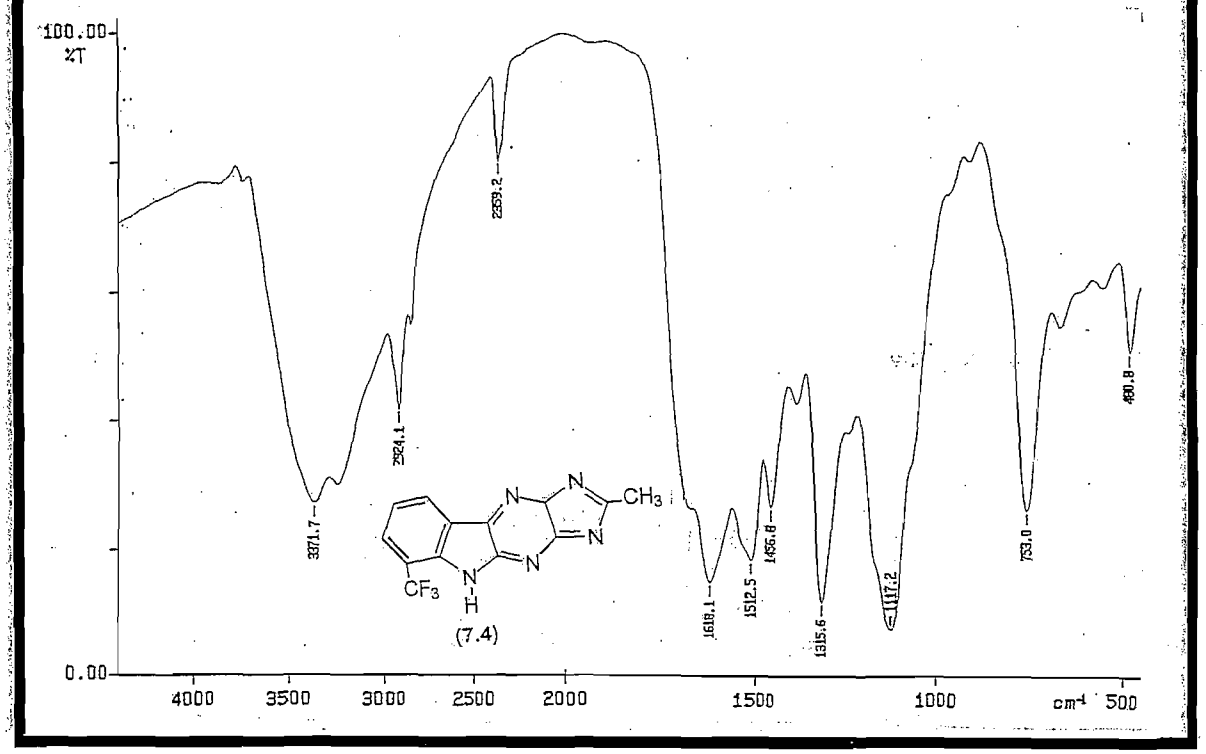
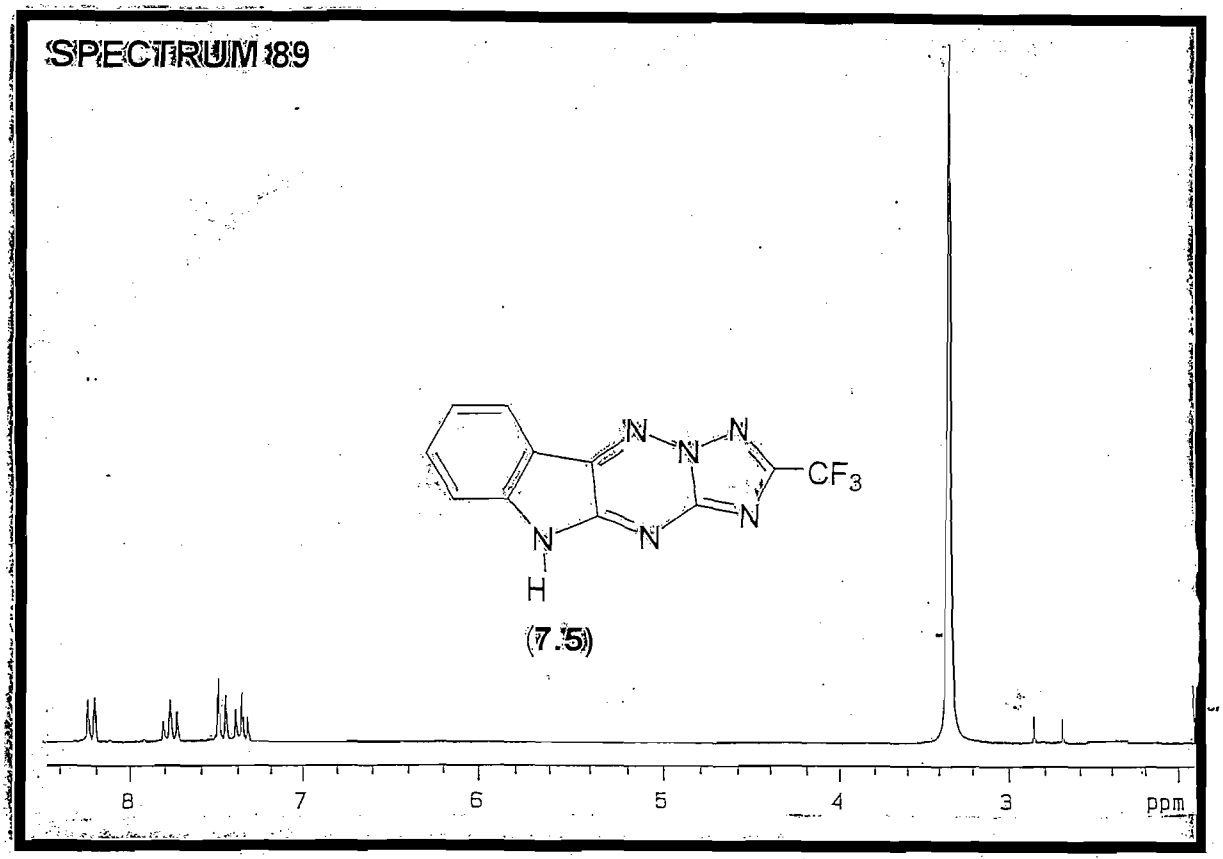


SPECTRUM 88



SPECTRUM 89



(7.5)

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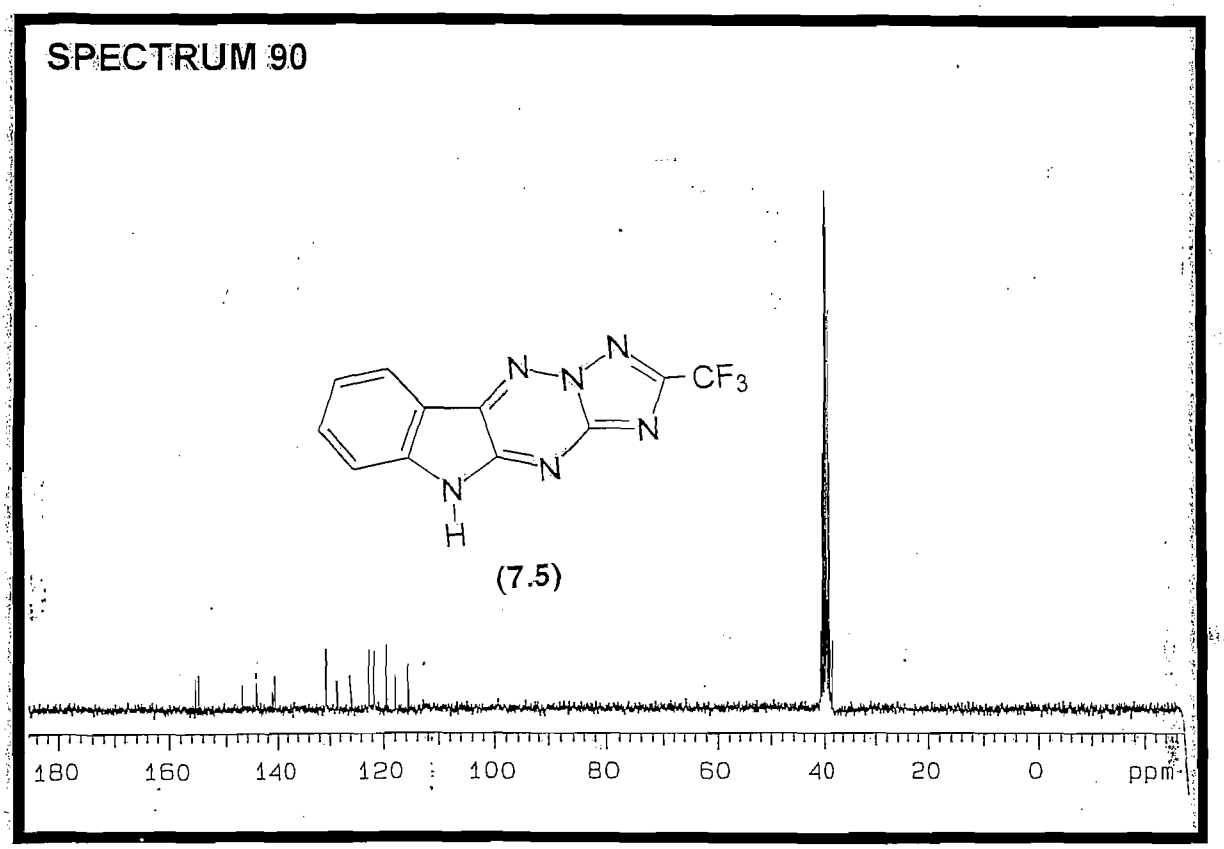
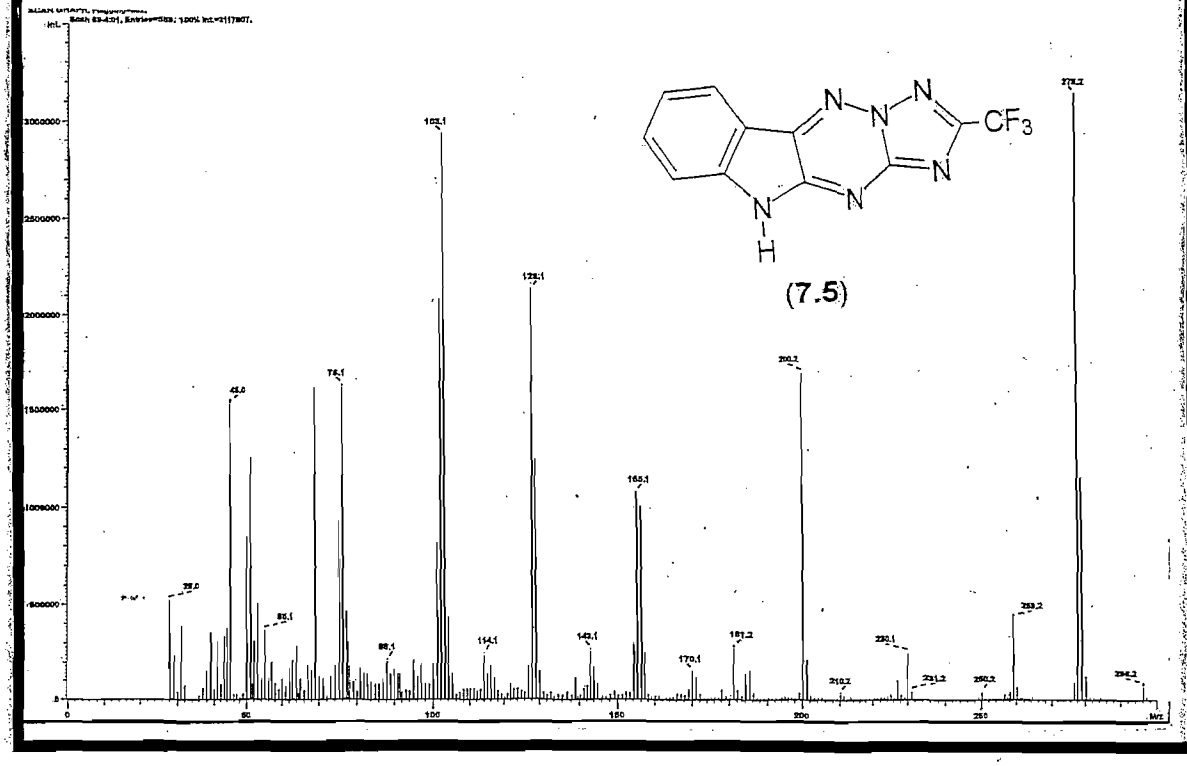
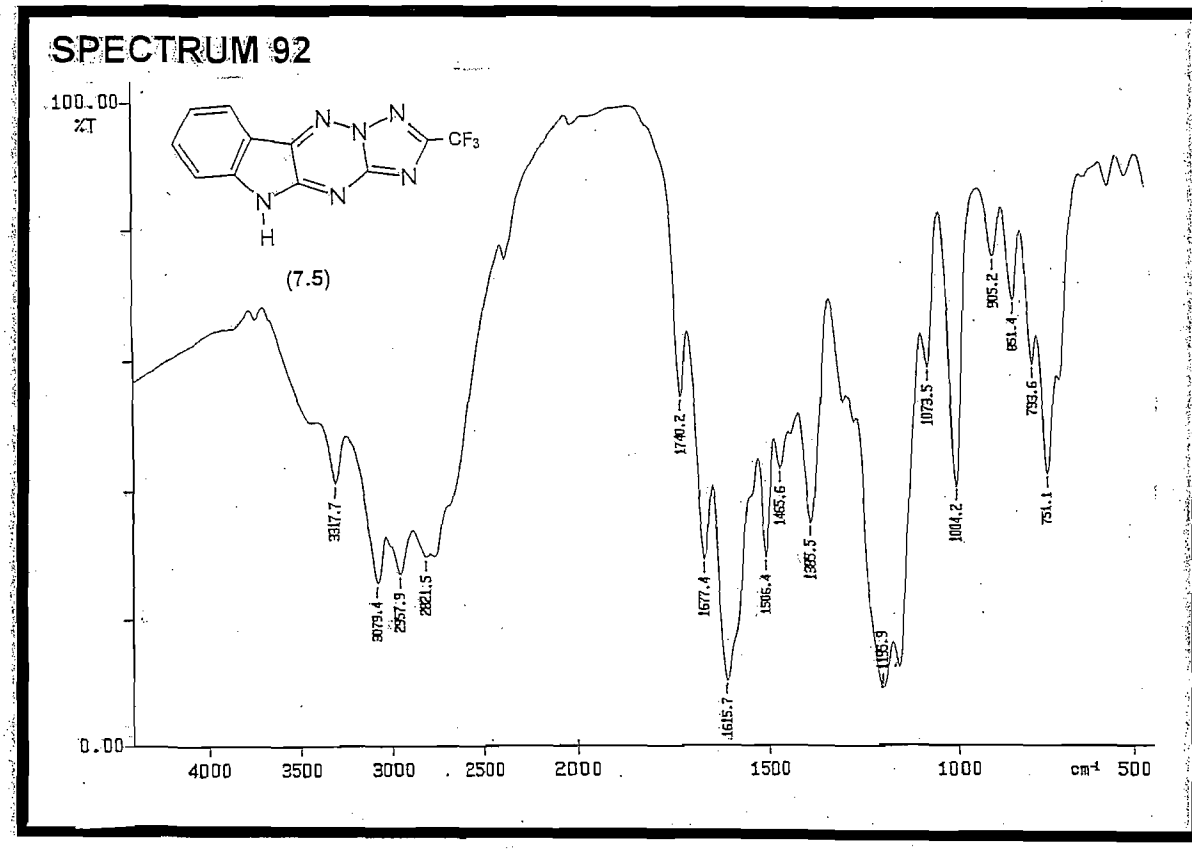


(7.5)

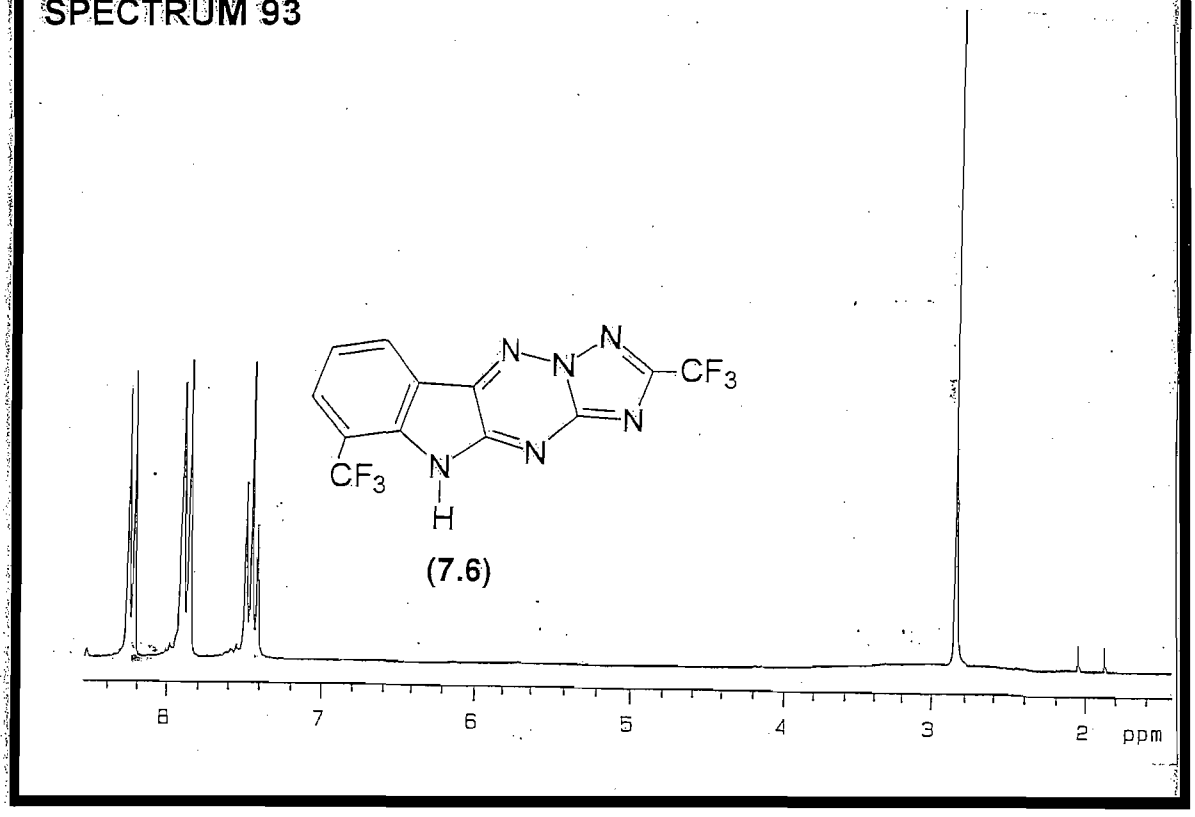
SPECTRUM 91



SPECTRUM 92

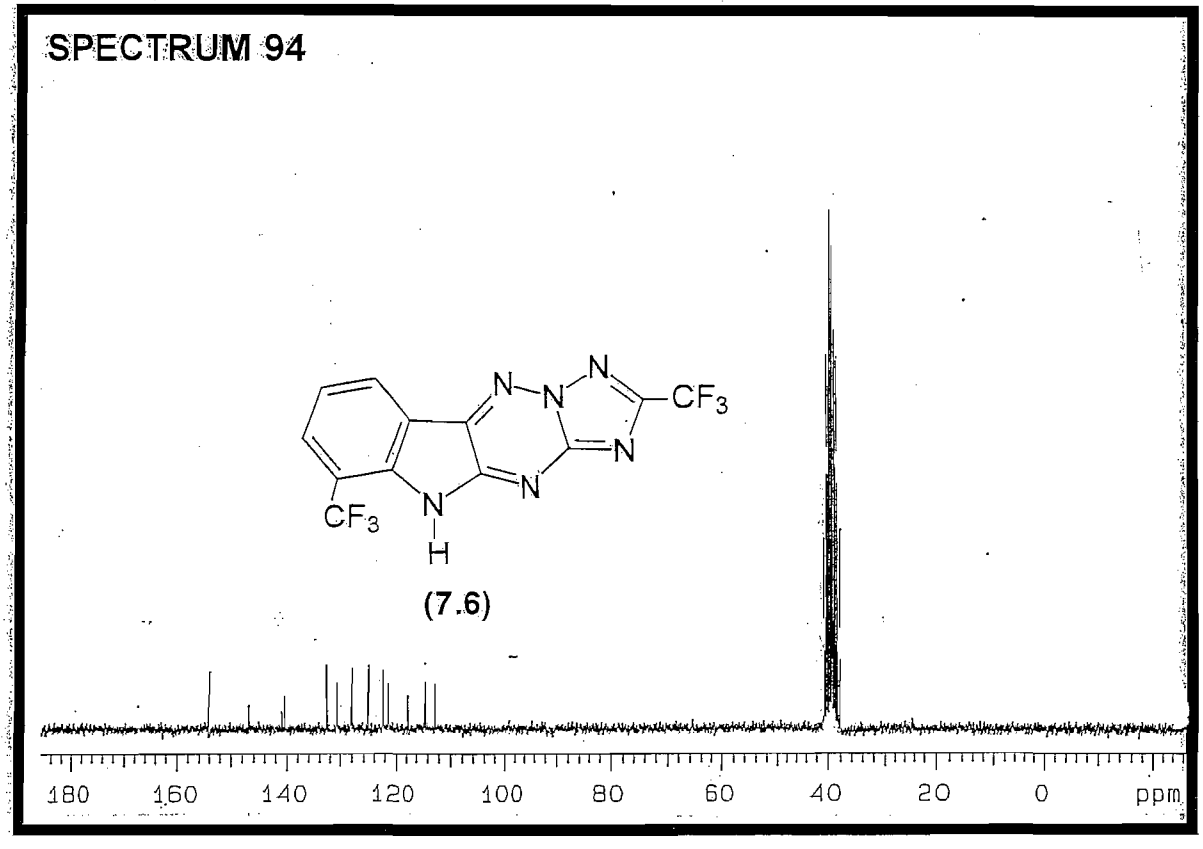


SPECTRUM 93



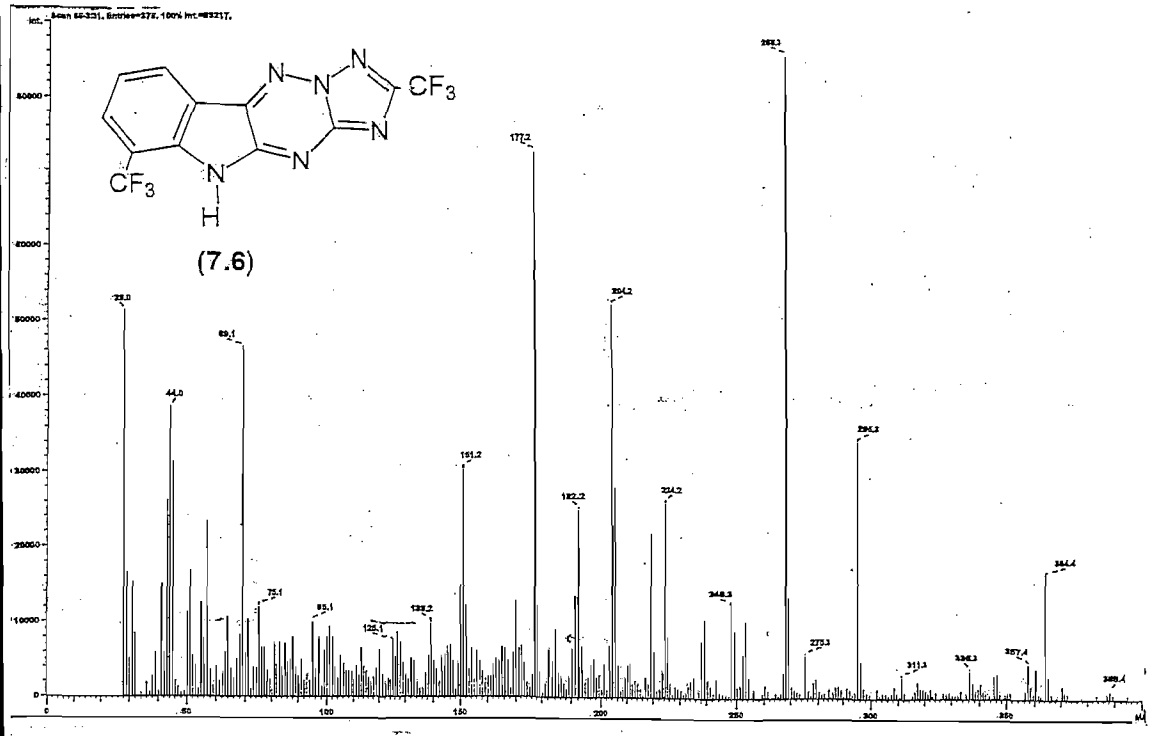
(7.6)

SPECTRUM 94

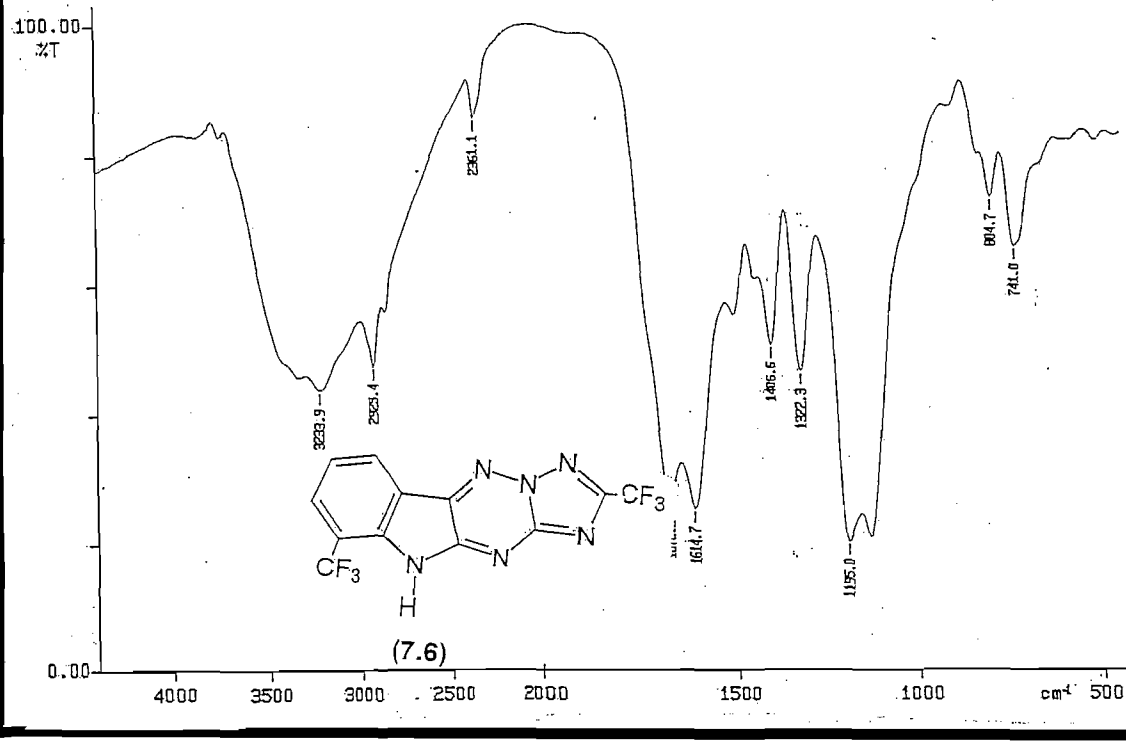


(7.6)

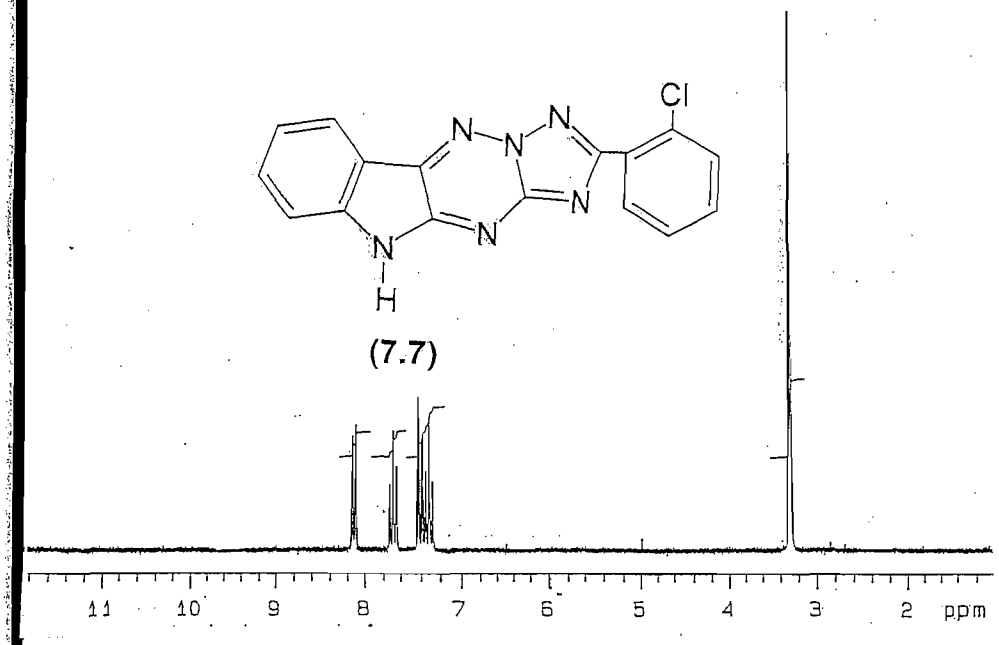
SPECTRUM 95



SPECTRUM 96

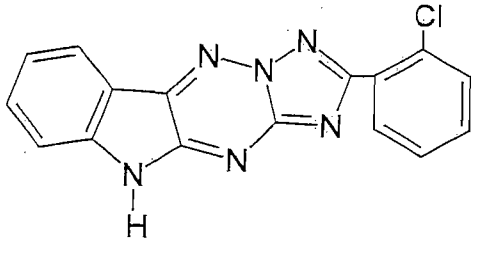


SPECTRUM 97



(7.7)

SPECTRUM 98



(7.7)

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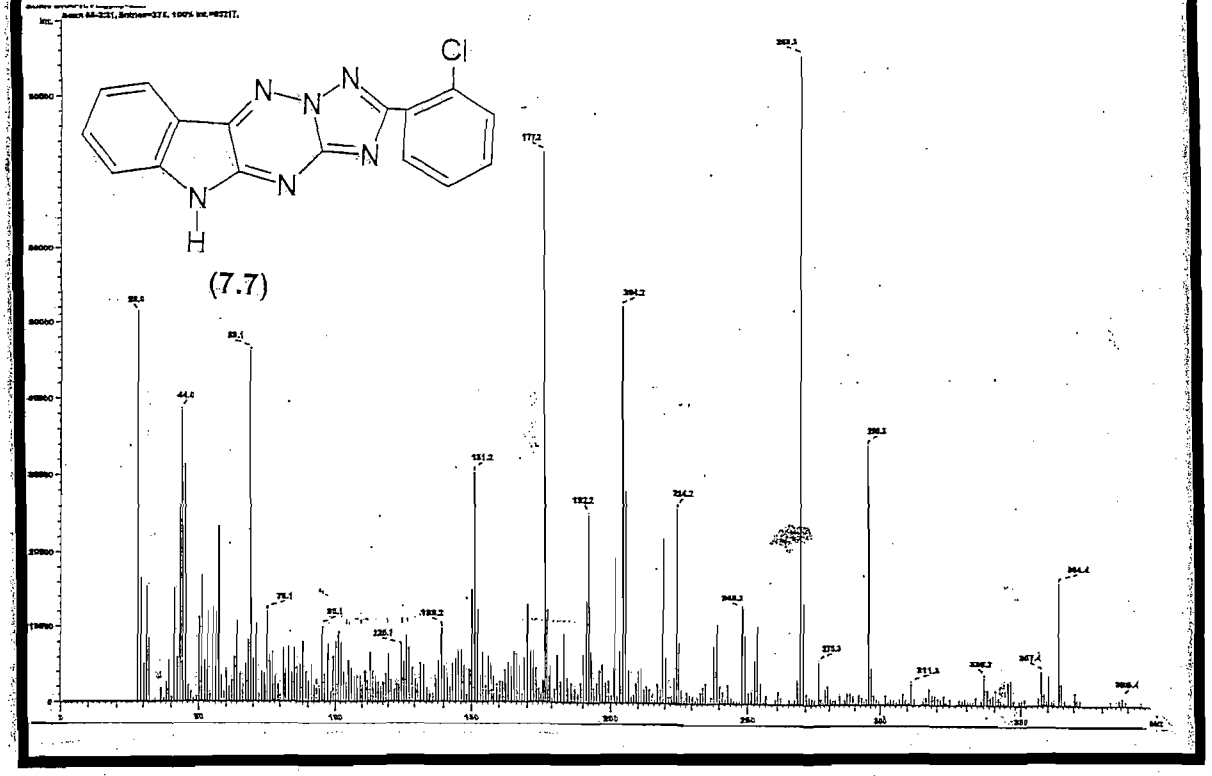

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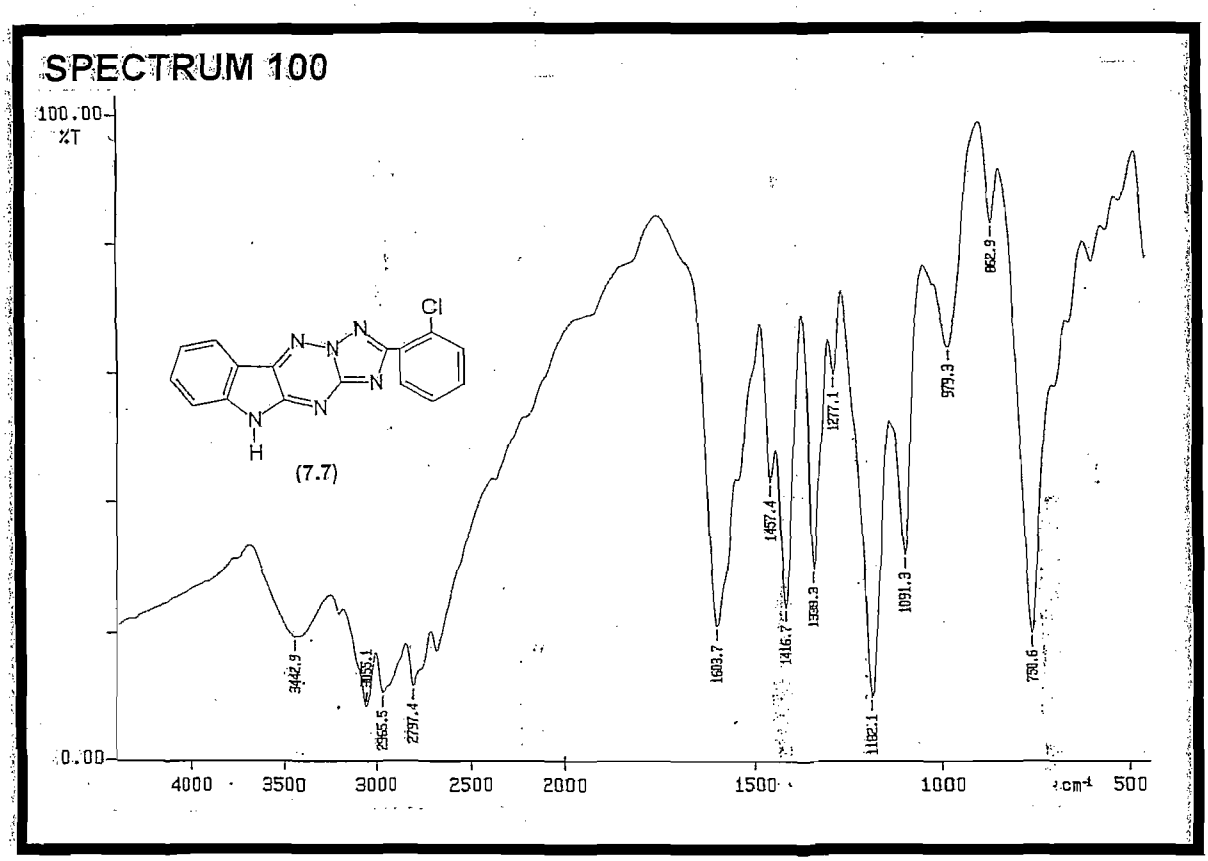

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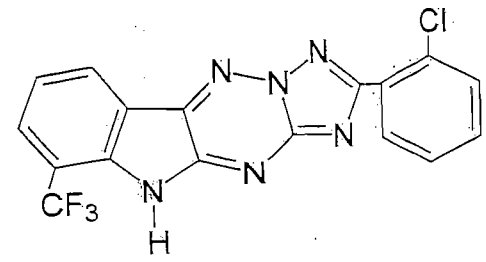

(7.8)

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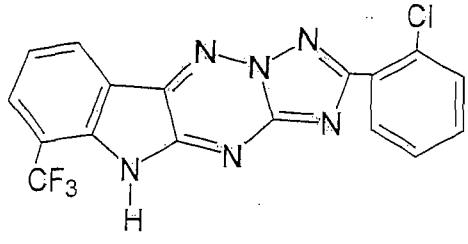

(7.8)

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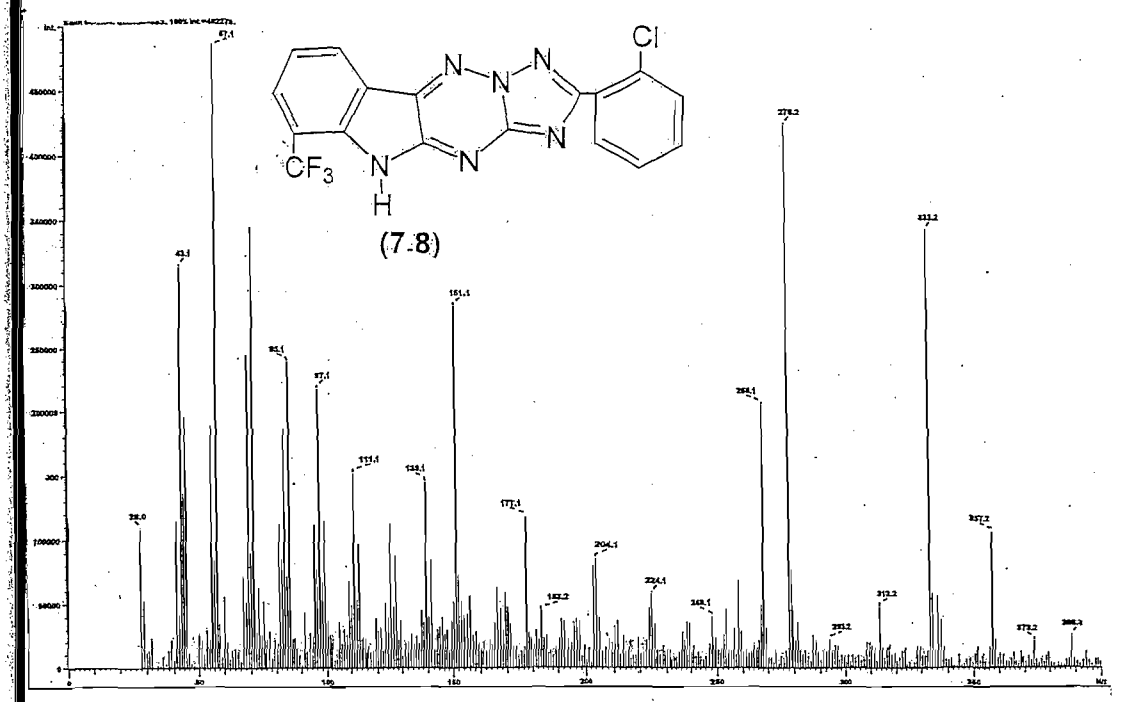
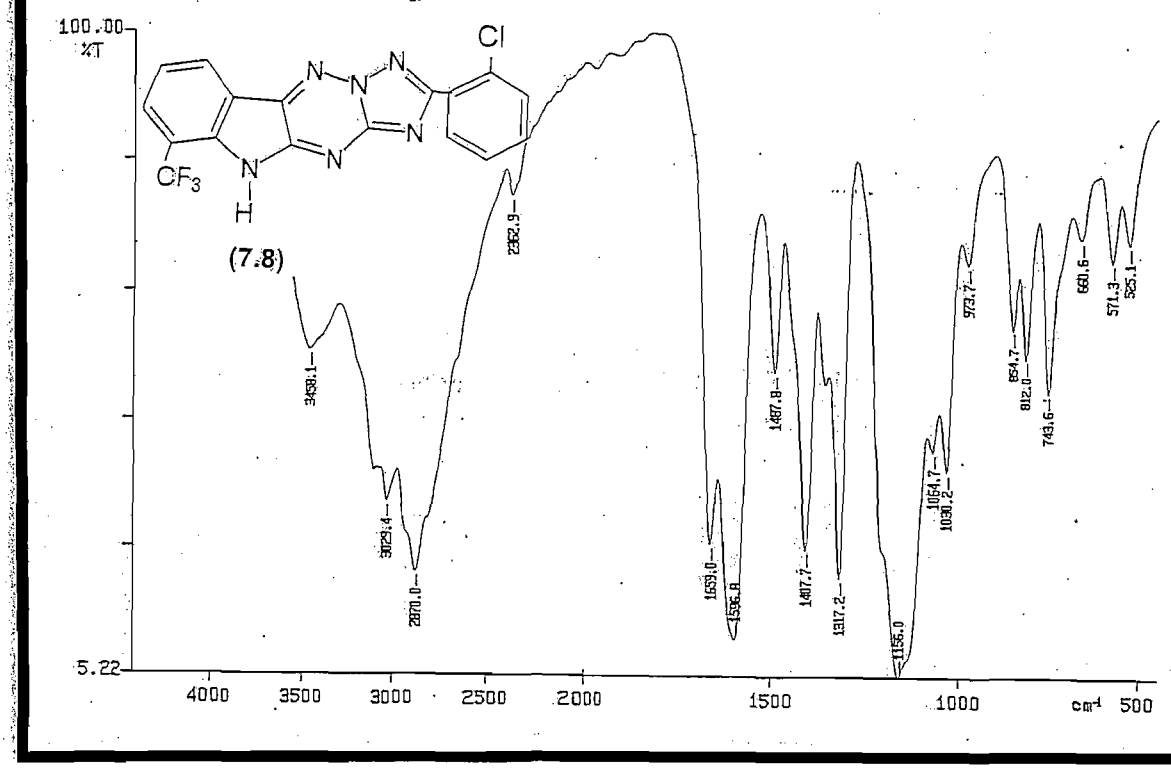


(7.8)

SPECTRUM 104



(7.8)

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