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Facoltà di Scienze del Farmaco

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SYNTHESIS OF NEW POLYCYCLIC COMPOUNDS WITH POTENTIAL ANTIMALARIAL AND/OR ANTILEISHMANIAL ACTIVITY

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PhD Thesis of:

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ABSTRACT

Malaria and leishmaniasis are potentially lethal protozoan diseases affecting a huge number of people worldwide, especially in underdeveloped countries. The alarming spread of drug resistance concerning both *Plasmodium* and *Leishmania* parasites makes the search of novel antimalarial and antileishmanial agents an urgent need. Unfortunately, at the moment even the combination therapies are failing in many regions afflicted by the diseases and alternatives are scarcely found. In addition, the available antileishmanial drugs are quite toxic, expensive and very often need monitoring and hospitalization. In the light of this dramatic situation, the discovery of novel effective, safe and affordable molecules is vital.

Thus far, several strategies have been developed to overcome resistance mechanisms; among them, of particular interest are the structural optimization of already known antiprotozoal molecules, the development of hybrid compounds and the search of new chemical scaffolds. Based on these considerations, the aim of the present thesis was the synthesis of different novel sets of molecules, potentially candidates for the treatment of malaria and/or leishmaniasis. On one hand I prepared derivatives of the antiprotozoal agents chloroquine and clofazimine, in order to improve the biological activity and to reduce resistance mechanisms. On the other hand, I explored the potenzialities of new chemical scaffolds, such as indeno[2,1-c]quinolines, to design new antimalarials. Moreover, I evaluated the possibility of creating hybrid molecules, combining moieties with different mechanism of action which could carry out a synergistic effect. In particular, the quinoline nucleus has been combined with different HDACs inhibiting structures to generate antiplasmodial hybrids, whereas aphidicolin (a fungal metabolite with antileishmanial activity) has been condensed with other molecules endowed with antileishmanial activity, such as ethyl 3-chloroacetamidobenzoate and eflornithine.

Biological assays were in general quite encouraging and suggested that these new classes of compounds could be considered as potential leads for the synthesis of new effective antiprotozoal drugs that, in some cases, could hopefully overcome resistance mechanisms.

CONTENTS

1. INTRODUCTION

1.1 Malaria

1.1.1 ***Historical hints and epidemiological data***

Malaria is the most important parasitosis and the second infectious disease in the world in terms of clinical cases and mortality after tubercolosis, involving nearly 207 million cases and 627000 deaths every year, with the highest mortality occurring in Africa and in children under 5 years of age.[1]

The disease is caused by intracellular protozoan parasites of the genus *Plasmodium*, which are transmitted to humans by the bites of female *Anopheles* mosquitoes. There are four main *Plasmodium* species that affect humans: *P. falciparum, P. vivax*, *P. ovale* and *P. malariae*. In addition, a simian parasite, *P. knowlesi*, occasionally infects humans.[2] Among them, *P. falciparum* is the most deadly form whereas *P. vivax* is the most widespread, as it is able to develop at lower temperatures and to survive at higher altitudes and in cooler climates. It has also a dormant liver stage (known as hypnozoite) that enables it to survive during periods when *Anopheles* mosquitoes are not present to continue transmission, such as during winter months.[1]

About 10000 years ago there was the development of the first focuses of *Plasmodium* in Central and Western Africa and, after the migration of people also for commercial purposes, malaria spread in Mesopotamia, South-east Asia and Indian peninsula. Afterwards, it reached also China and Mediterranean areas up to the American shores, becoming in the XIX century a globally widespread disease.[3] Malaria was eliminated from North America, Europe and parts of Asia and South America during the 1950s and 1960s due to a global campaign that relied on the new synthetic insecticide dichlorodiphenyltrichloroethane (DDT) and on new powerful synthetic drugs, such as chloroquine (CQ) and the combination sulfadoxine-pyrimethamine. When the parasites became resistant to these drugs and DDT use was restricted because of environmental and health hazards, malaria returned to many areas and the number of deaths peaked at 1.8 million in 2004.[4]

Nowadays there are 97 countries and territories with ongoing malaria transmission and 7 countries in the prevention of reintroduction phase, making a total of 104 countries and territories in which malaria is presently considered endemic (Fig.1).[1]

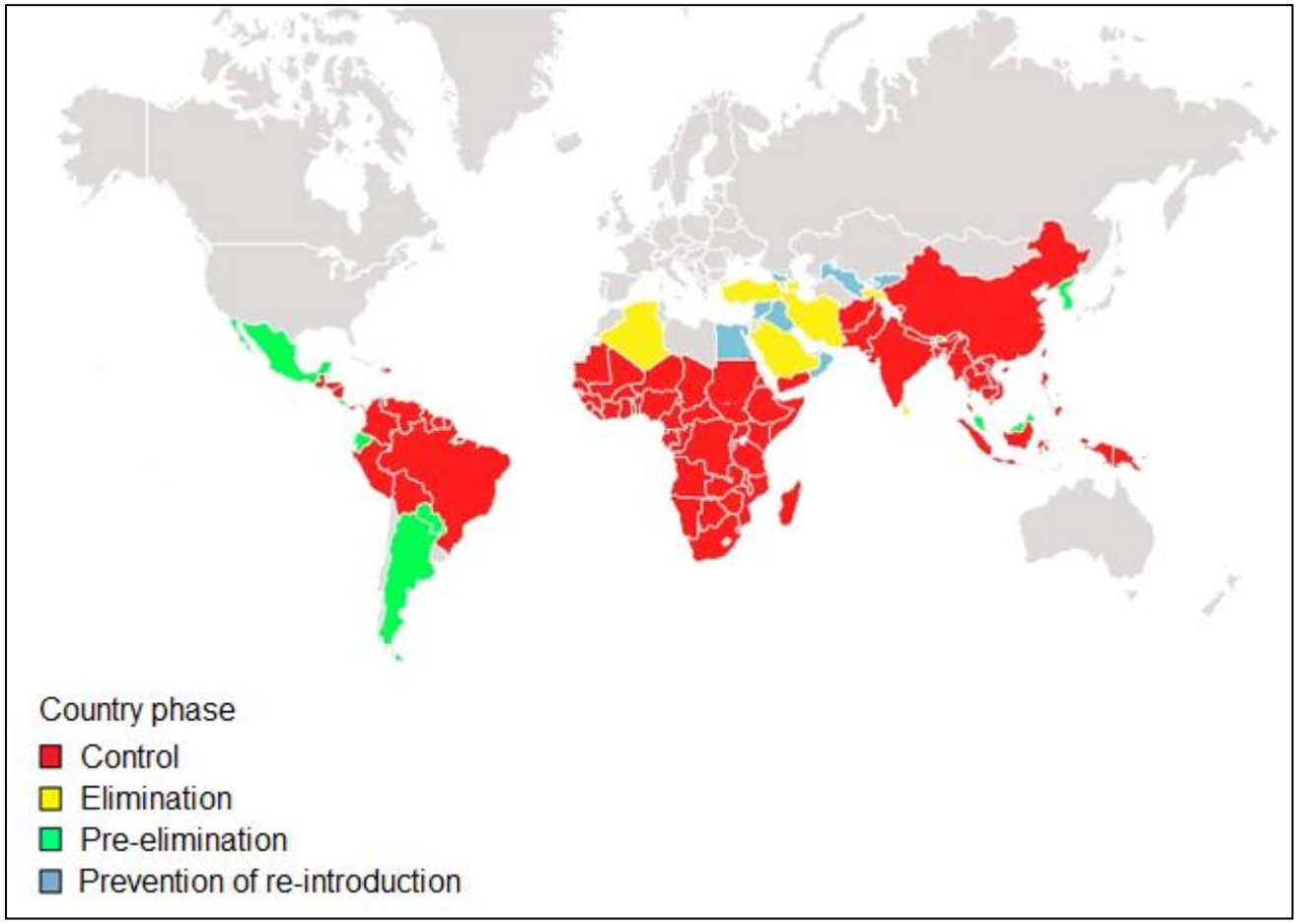


Fig.1 Classification of countries by stage of malaria elimination.[1]

Malaria is mostly present in tropical and sub-tropical regions, such as Sub-Saharan Africa and South-East Asia, as well as Western Pacific and South America. The disease has been eradicated in Europe, except in Azerbaijan, Georgia, Kyrgyzstan, Tajikistan and Turchia, which guess to defeat it within 2015 as reported in the Tashkent Declaration: "The move from malaria control to elimination" (2005).[5]

However, every year thousands of people with malaria arrive in non-endemic countries: case fatality is around 1% overall but varies by age and previous exposure to malaria. This rate is similar

to that seen in endemic countries, but the age profile of deaths is very different. In Africa mortality is highest in young children, but in imported cases it is highest in older patients, especially those over 65 years. If malaria is treated early, with widely available drugs before it becomes severe, death is avoidable and a full recovery almost guaranteed. Late presentation carries a higher risk of death and the management of severe malaria is a medical emergency.[6]

Malaria places a heavy economic burden on many endemic countries, contributing to the cycle of poverty and limiting economic development. For example, Africa alone is estimated to lose at least US$ 12 billion per year in direct losses (e.g. illness, treatment, premature death) and many times more than that in lost economic growth.[7] International disbursements to malaria-endemic countries increased from less than US$ 100 million in 2000 to US$ 1.60 billion in 2011; they were estimated to be US$ 1.94 billion in 2012 and US$ 1.97 billion in 2013 (Fig.2). Increases in international funding have slowed in recent years, falling to an average of 4% per year between 2009 and 2013, compared to average increase of 43% per year between 2005 and 2009. A lower level of funding in 2011 was mainly due to lower levels of disbursements from the Global Fund. The Global Fund is the largest source of funding for malaria control globally; it accounted for 40% of the estimated total disbursed funds in 2011 and 50% in 2013.[1]

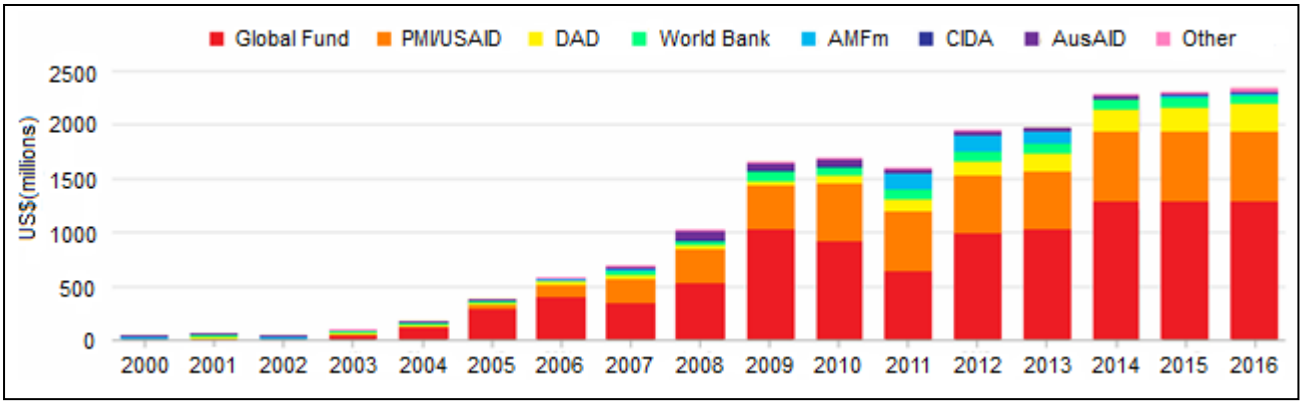


Fig.2 Past and projected international funding for malaria control, 2000-2016.[1]

Global resource requirements for malaria control were estimated in the 2008 Roll Back Malaria (RBM) Global Malaria Action Plan (GMAP) to exceed US$ 5.1 billion per year between 2011 and 2020. In Africa alone, the resource requirements estimated by GMAP were, on average, US$ 2.3 billion per year during the same period. Combining both domestic and international funds, the resources available for malaria control globally were estimated to be US$ 2.5 billion in 2012, leaving a gap of US$ 2.6 billion. Available projections of both domestic and international resources indicate that total funding for malaria control will reach about $US 2.85 billion between 2014 and 2016.[1]

1.1.2 ***Plasmodium life cycle***

P. falciparum has a complex lifecycle involving stages in humans and mosquitoes, with the blood stage of the infection responsible for much of the disease pathology. During this stage the parasite undergoes cycles of growth and division inside the red blood cells (RBCs) of its host.

Female *Anopheles* mosquitoes become infected when they feed on the blood of a human who is carrying mature gametocytes (sexual stage parasites developing within RBCs) (Fig.3). The male and female gametes fuse in the stomach of the mosquito to produce a zygote that undergoes rapid meiosis, then transforms into a motile ookinete. The ookinete migrates through the mosquito midgut wall and matures into an oocyst. Sporozoites (infective stages) develop during a period of 10–22 days, before budding and migrating to the mosquito's salivary glands. When the infected *Anopheles* mosquito bites again a human, it injects the motile sporozoites into the victim's blood stream.

The sporozoites enter the liver and invade hepatocytes. Here they multiply and differentiate to generate many thousands of merozoites. These merozoites invade the host's RBCs to initiate the blood stage of the infection. Each intraerythrocytic asexual cycle takes ~48 h. The intraerythrocytic parasite progresses through the ring and mature trophozoite stages, then divides during the schizont stage, eventually rupturing the host cell to release ~20 daughter merozoites. After a few weeks of asexual cycling in the blood stream some of the intraerythrocytic parasites develop into the highly

specialized gametocyte forms which, when taken up by a feeding mosquito, serve in disease transmission.[8]

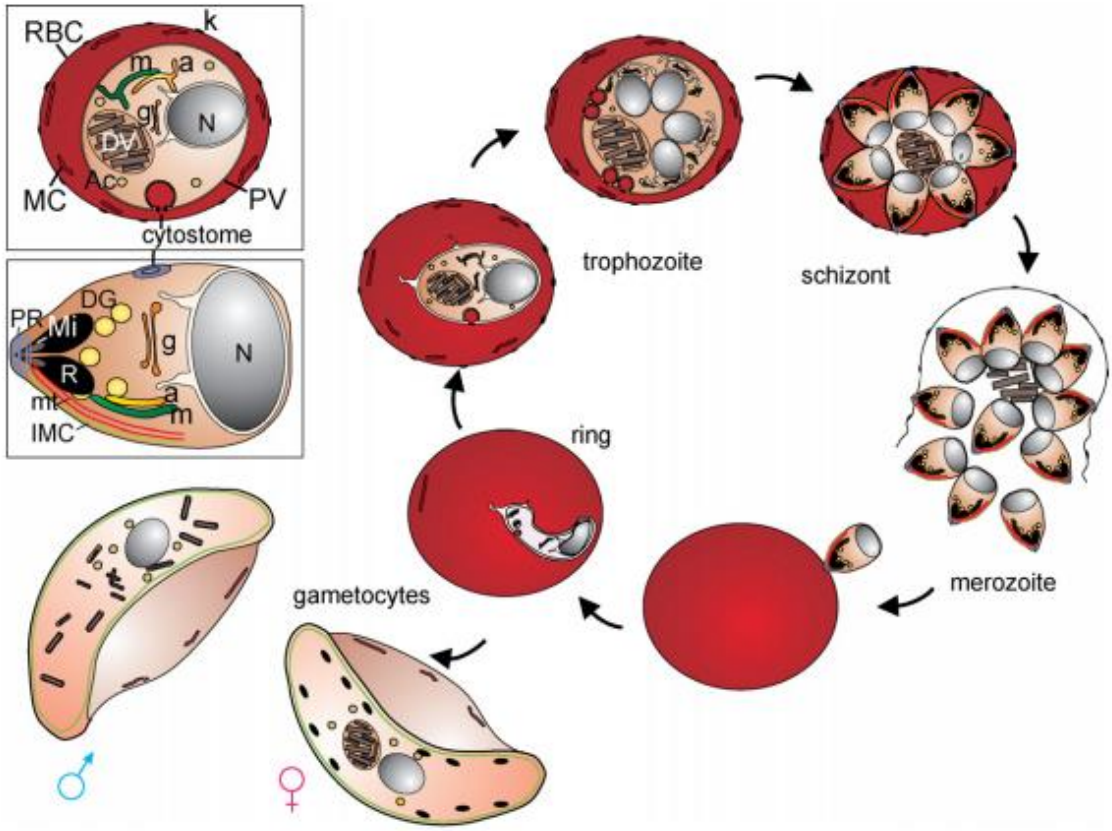


Fig.3 Schematic representation of the intraerythrocytic cycle of *P. falciparum*.[8]

1.1.3 ***Hemozoin synthesis***

During its 48-hour residence within an infected red blood cell, an asexual *Plasmodium* parasite imports up to 80% of host hemoglobin into the acidic digestive vacuole, where the collaborative action of multiple aspartic, cysteine and metal-dependent proteases hydrolyzes the globin polypeptide into diverse small peptides and amino acids (Fig.4).[9] Although during the ring stage some hemoglobin degradation is detectable, the major part of this process takes place during the trophozoite and schizont stages, which perform most of the metabolic activity of the parasite.

Hemoglobin degradation was believed to play a vital role as an amino acids source for malaria parasites because it has been observed that they have a limited capacity to synthesize their own.

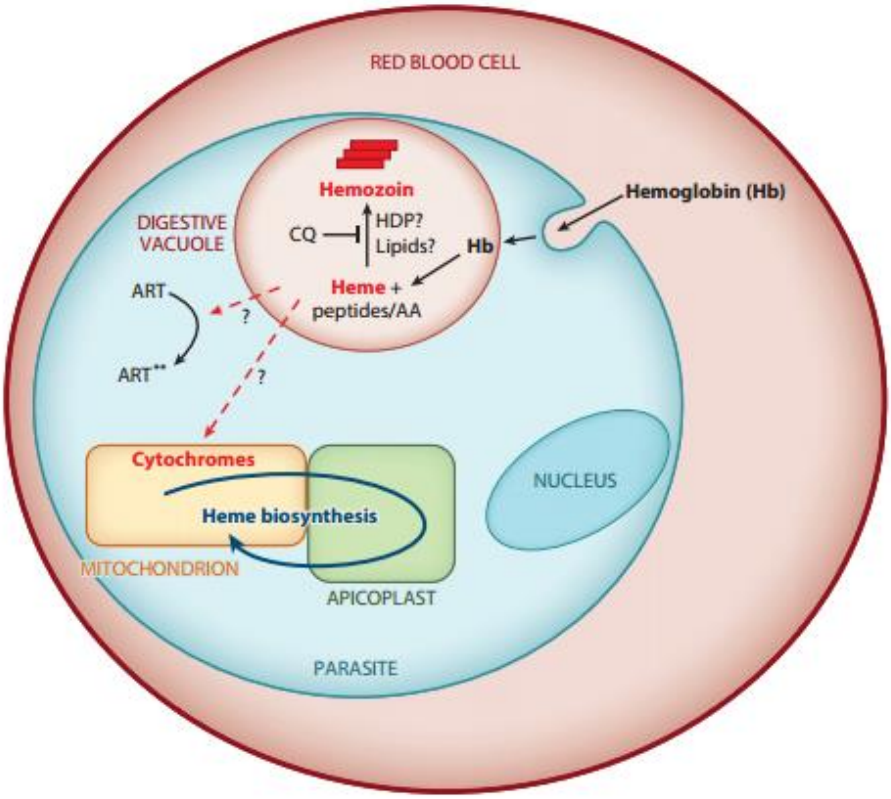


Fig.4 Schematic depiction of heme metabolism within blood-stage *Plasmodium* parasites.[9]

Most of the enzymes implicated in the hemoglobin degradation pathway have been elucidated. Two aspartic proteases (plasmepsins I and II) and a cysteine protease (falcipain) have been isolated and purified from the digestive vacuole of *P. falciparum*. Other enzymes involved are the histoaspartic protease (HAP) and the heme detoxification protein (HDP).[10]

Within the parasite digestive vacuole, the copious heme liberated during hemoglobin proteolysis is expected (and commonly assumed) to be cytotoxic based on its potential to destabilize the vacuolar membrane, to inhibit the function of proteases and other enzymes and to spontaneously generate reactive oxygen molecules, either within this compartment or upon protonation and passive diffusion into the reduced environment of the parasite cytoplasm. However, the cellular mechanisms of heme toxicity to parasites remain poorly defined, but the general ability of the parasite to tolerate and indeed proliferate despite generation of high, local concentrations of heme suggests the presence of protective mechanisms to attenuate heme toxicity.[9]

Rather than degrading heme, *Plasmodium* parasites sequester host heme into crystals of insoluble hemozoin in the digestive vacuole. These crystals, which feature repeating reciprocal dimers of ferric (Fe^{3+}) protoporphyrin IX in which the carboxylate moiety of a propionate group on one monomer coordinates the iron atom in a second monomer and vice versa, are chemically inert. Their formation thus neutralizes the oxidative, cytotoxic properties of unassociated heme *in situ* without a requirement for further chemical processing. Although the structure of hemozoin crystals has been characterized in detail, the specific mechanisms that nucleate and propagate hemozoin crystallization within the digestive vacuole remain unresolved. Distinct hypotheses have proposed that nucleation and growth occur (a) within the digestive vacuole itself or within hemoglobin-containing vesicles en route to the digestive vacuole, (b) within lipid droplets suspended in vacuolar compartments or at the membrane lipid–aqueous interface, or (c) via a spontaneous or protein-assisted process.[9]

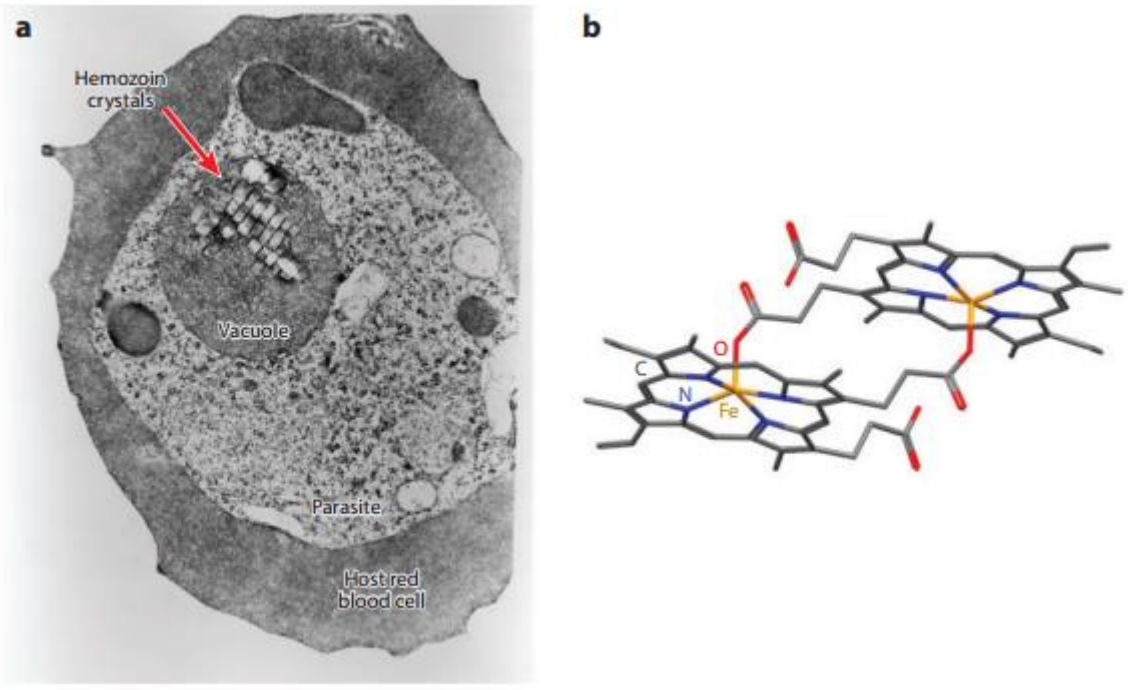


Fig.5 Hemozoin formation. (a) Electron micrograph of an asexual parasite within host red blood cell; (b) Heme-heme reciprocal dimer that forms repeating subunit within hemozoin crystals.[9]

1.1.4 ***Clinical manifestations***

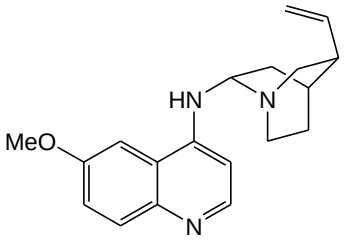
The first symptoms of malaria, common to all the different malaria species, are nonspecific and mimic a flu-like syndrome. The hallmark of malaria is fever. Up to two days before the onset of fever, prodromal symptoms, such as malaise, anorexia, lassitude, dizziness, with a desire to stretch limbs and yawn, headache, backache in the lumbar and sacroiliac region, myalgia, nausea, vomiting and a sense of chillness may be experienced. The fever is usually irregular at first but after some days it tends to become periodic, depending on the synchronized schizogony.[11] The classic symptom of malaria is paroxysm, a cyclical occurrence of sudden coldness followed by shivering and then fever and sweating, occurring every two days (tertian fever) in *P. vivax* and *P. ovale* infections and every three days (quartan fever) for *P. malariae*. *P. falciparum* infection can cause recurrent fever every 36-48 hours or a less pronounced and almost continuous fever.[12] Cyclic fever is the result of the release of protein material, pro-inflammatory cytokines (IL-6, IL-10, IL-12 and TNF-α) and nitric oxide, due to the infected red blood cells lysis.[13]

Most of the severe malaria complications occur in non immune subjects with *P. falciparum* malaria and involve central nervous system (cerebral malaria), pulmonary system (respiratory failure), renal system (acute renal failure) and/or hematopoietic system (severe anemia). These complications can evolve rapidly, indeed severe malaria can lead to death.[11]

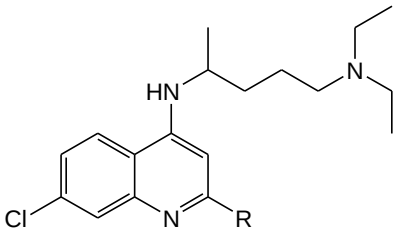
1.1.5 ***Antimalarial therapies***

The cure for malaria originated from Amerindian knowledge when the Spaniards learned to use the bark of the plant named quina (or china) in the 17th century. Quina are species of the Rubiaceae family, especially *Cinchona succirubra* and *Cinchona calisaya* which produce quinine (Table 1). The bark of these plants was introduced to Europe in 1640 by the Jesuits and, until the mid 1800s, the powder prepared from quina bark (Jesuit′s powder) was the most important remedy for the treatment of malaria.[14] Quinine is an effective treatment, especially when given with other drugs for

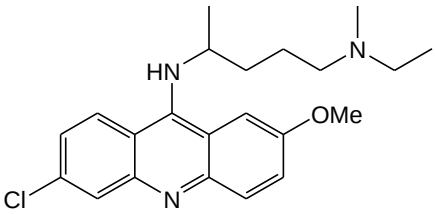
certain types of malaria, but adverse effects ("cinchonism") and a short duration of action make it a suboptimal prophylactic agent. During World War I, countries producing quinine were controlled by anti-German forces. Projected quinine shortages and the need for long-acting prophylactic drugs prompted an effort by German companies to synthesize antimalarial compounds. Consequently, in 1934 the 4-aminoquinoline chloroquine and its derivatives sontoquine and mepacrine were prepared (Table 1). Chloroquine rapidly controlled clinical symptoms of susceptible malaria with minimal toxicity and was useful as a once-weekly prophylactic drug. It has been the first-line treatment of malaria for several years, till early 80s; then, the emergence of chloroquine-resistant strains of *P. falciparum* and *P. vivax* rendered this drug less useful.[15] Other important antimalarial compounds were synthesized during World War I, such as the 8-aminoquinoline primaquine, the biguanide proguanil and mefloquine (Table 1).



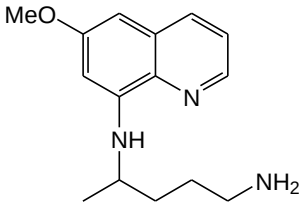
Quinine



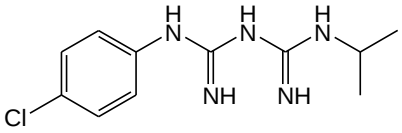
R= H Chloroquine
R= Me Sontoquine



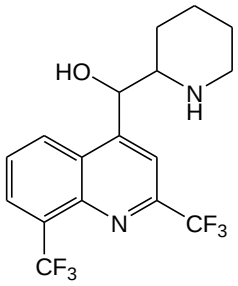
Mepacrine



Primaquine



Proguanile

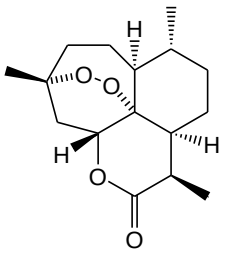


Mefloquine

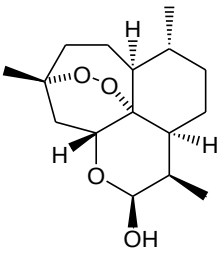
Table 1 First antimalarial agents.

The search for new antiplasmodial agents continued along the whole century with the discovery of a lot of compounds such as atovaquone, amodiaquine, lumefantrine, piperaquine, pyrimethamine, sulfadoxine and tebuquine (Table 2). In early 90s some antibacterial compounds, such as doxycycline, erythromycin and azithromycin (Table 2) were found to have antimalarial effects with a good safety profile, especially for children and pregnant women.

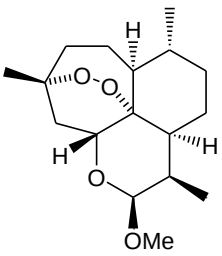
The discovery of artemisinin (Table 2) for malaria therapy by Chinese scientists in the 1970s was one of the greatest discoveries in medicine in the 20th century. The absolute configuration of artemisinin was obtained using anomalous diffraction x-ray crystal analysis in 1976 and it provided further foundation for improvement of the drug. Several derivatives were subsequently produced in China to treat malaria, including artemether and artesunate (Table 2) in 1987, and dihydroartemisinin (DHA) in 1992.[16] The development of resistance to artemisinins by *Plasmodium falciparum* prompted the introduction of the Artemisinin Combination Therapies (ACTs), in which artemisinin is administered in combination with other antimalarial drugs with different mechanism of action. These ACTs are now recommended by WHO as the first-line treatment for uncomplicated malaria. The five ACTs currently recommended by WHO are: artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine and dihydroartemisinin-piperaquine. The choice of the ACT should be based on the therapeutic efficacy of the combination in the country or area of intended use.[1] The most recently developed antiplasmodial compounds are bulaquine and tafenoquine, which are still under clinical trials.[17] Currently, no effective vaccine is available to fight human malaria; however, various antigen formulations are undergoing field trials. In particular, RTS,S/AS01E, a vaccine based on the *P. falciparum* circumsporozoite protein and blood stage parasite proteins, has demonstrated promising results.[17]



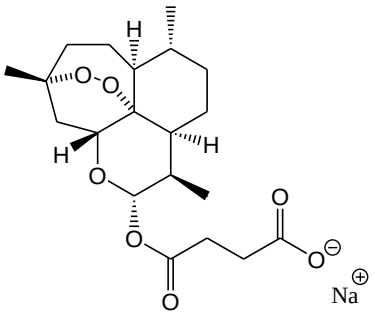
Artemisinin



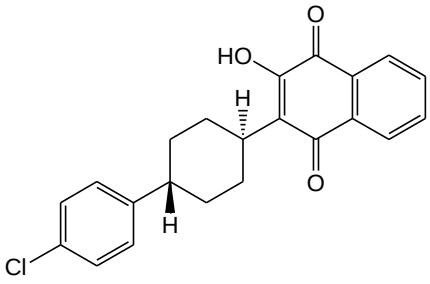
Dihydroartemisinin



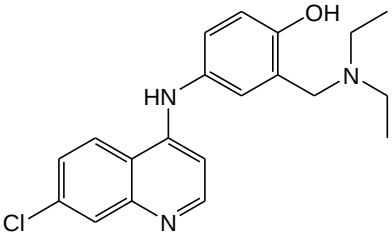
Artemether



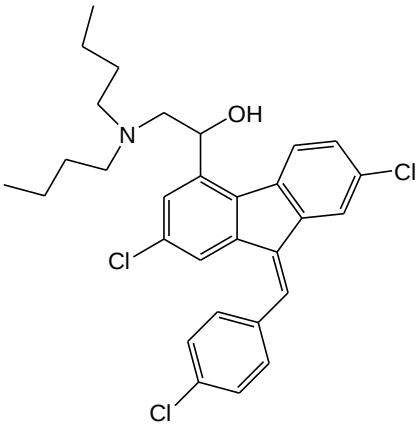
Sodium artesunate



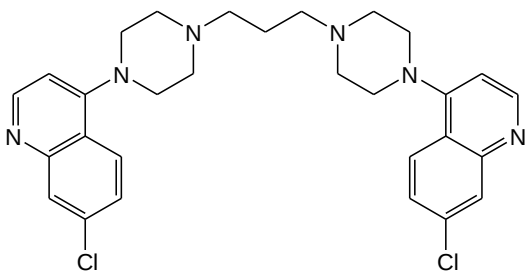
Atovaquone



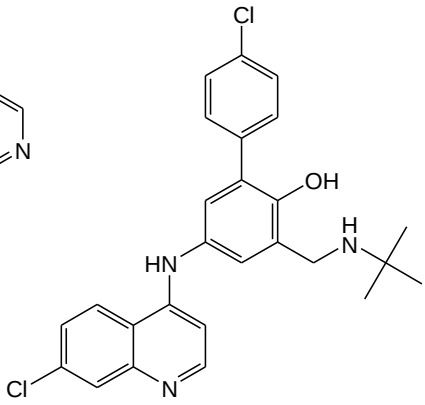
Amodiaquine



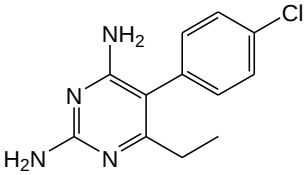
Lumefantrine



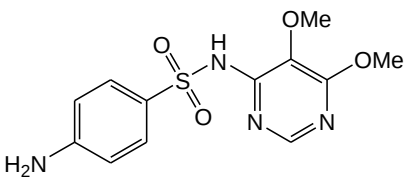
Piperaquine



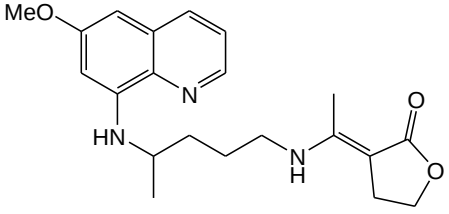
Tebuquine



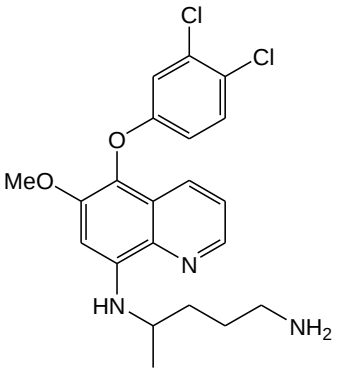
Pyrimethamine



Sulfadoxine



Bulaquine*



Tafenoquine*

Table 2 Most recent antimalarial drugs. *Compounds under clinical trials.

1.2 Leishmaniasis

1.2.1 ***Historical hints and epidemiological data***

Leishmaniasis is a complex of mammalian diseases caused by parasitic protozoans classified as *Leishmania* species (Kinetoplastida, Trypanosomatidae). Natural transmission may be zoonotic or anthroponotic and it occurs usually by the bite of a phlebotomine sandfly species (order Diptera, family Psychodidae; subfamily Phlebotominae) of the genera Phlebotomus (Old World) and Lutzomyia (New World).[18] The cutaneous afflictions of leishmaniasis have been known since antiquity. Descriptions of the cutaneous disease in the Old World are found from the first century AD. New World pottery from Peru and Ecuador dating from AD 400–900 illustrates faces afflicted with a process consistent with leishmaniasis. The first description in English of a lesion resembling leishmaniasis was made in 1756 by Russell, who described the "Aleppo evil" from Syria. In 1885, Cunningham observed organisms in macrophages from lesions of "Delhi boil" in India. A Russian army physician named Borovsky noted the protozoal nature of the organism in 1898 in biopsy specimens from skin lesions. In 1903 Leishman published his identification of the parasite in the spleen of an English private who had died of Dumdum fever in Dum-Dum, India in 1900. Few months later Donovan described identical organisms in a splenic puncture specimen from a living child.[19]

There are three main forms of the disease:

- Visceral leishmaniasis (VL also known as kala-azar) is fatal if left untreated. It is characterized by irregular bouts of fever, weight loss, enlargement of the spleen and liver and anemia. It is highly endemic in the Indian subcontinent and in East Africa. An estimated 200000 to 400000 new cases of VL occur worldwide each year. Over 90% of new cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan.
- Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis and causes ulcers on exposed parts of the body, leaving life-long scars and serious disability. About 95% of

CL cases occur in the Americas, the Mediterranean basin and the Middle East and Central Asia. Over two-third of CL new cases occur in six countries: Afghanistan, Algeria, Brazil, Colombia, Iran (Islamic Republic of) and the Syrian Arab Republic. An estimated 0.7 million to 1.3 million new cases occur worldwide annually.

- Mucocutaneous leishmaniasis leads to partial or total destruction of mucous membranes of the nose, mouth and throat. Almost 90% of mucocutaneous leishmaniasis cases occurs in the Plurinational State of Bolivia, Brazil and Peru.[20]

Anthroponotic cutaneous leishmaniasis (where humans are the major reservoir of the parasite) is predominantly urban and periurban and shows patterns of spatial clustering similar to those of anthroponotic visceral leishmaniasis in South Asia. The disease is usually characterized by large outbreaks in densely populated cities, especially in war and conflicts zones, refugee camps and in settings where there are large-scale migration of populations.[21]

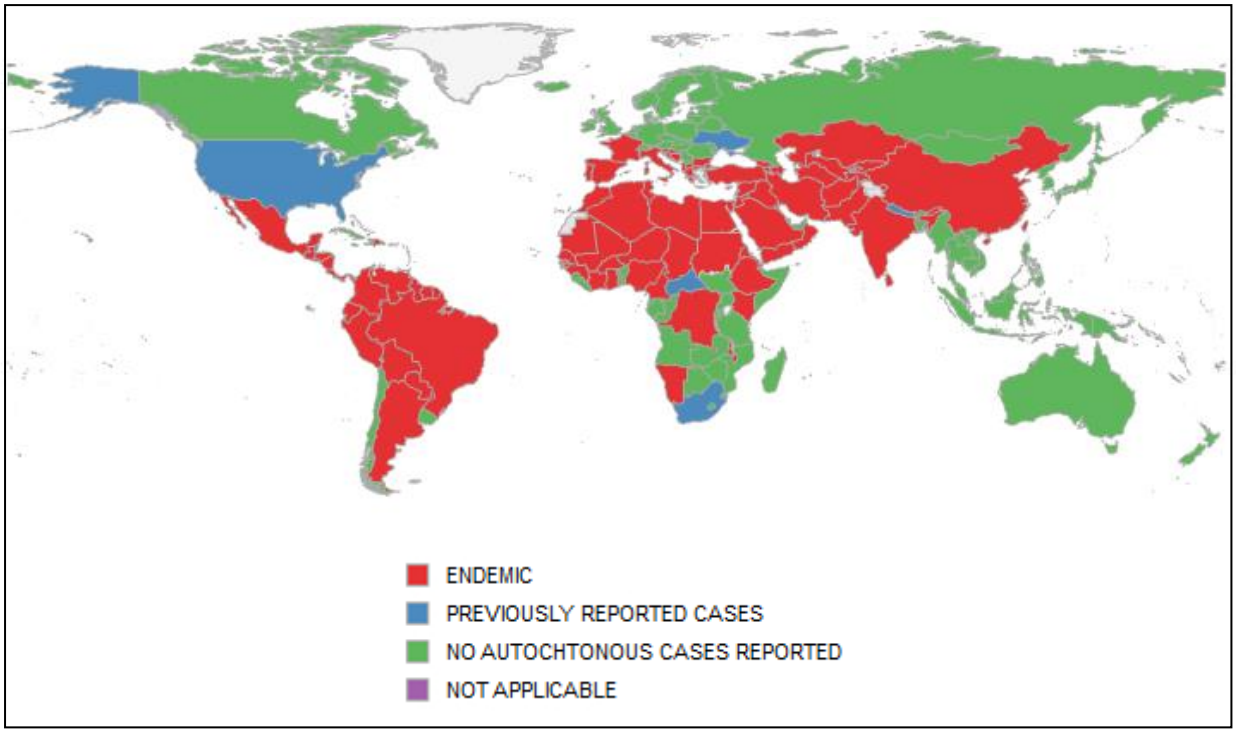


Fig.6 Worldwide distribution of leishmaniasis.[21]

The increased appearance of leishmaniasis in developed countries can be attributed to several factors: increased overseas travel, U.S. Gulf War veterans and the simultaneous encroachment of leishmaniasis into urban areas and of HIV infection into rural areas. In the Mediterranean, where *L. infantum* is endemic, the number of cases of VL in HIV-infected patients has increased dramatically since the mid-1980s. The World Health Organization estimates that 25%-70% of adult VL cases in Southern Europe are related to HIV, while between 2% and 9% of AIDS patients are at risk of experiencing newly acquired or reactivated VL. Preliminary data suggest that *Leishmania* may be a cofactor in the pathogenesis of HIV infection: the lipophosphoglycan (LPG) surface molecule of *L. donovani* has been shown to induce transcription of HIV in $CD4^+$-T cells. The WHO estimates that AIDS increases the risk of VL by 100–1000 times in endemic region.[19]

1.2.2 ***Leishmania life cycle***

The life cycle of *Leishmania* involves alternation between a mammalian host and a phlebotomine sand fly host. In the mammalian host the developmental biology of the parasite is relatively simple and consistent between species: metacyclic promastigotes (infective forms) are introduced into the skin by the bite of the sand fly. These are taken up by macrophages and transformed into intracellular amastigotes (small, immotile and rounded form of the parasite), remaining in this form for the duration of the life cycle in the mammalian host. In contrast, the developmental biology of the parasite in the sand fly host is more complex and less well understood.[22] Outside the vertebrate host, the *Leishmania* life cycle is confined to the digestive tract of sand flies. The precise location differs between subgenera *Leishmania* and *Viannia*. Development in the vector is initiated when female sand flies ingest blood containing macrophages infected with amastigotes. The change in conditions moving from the mammalian host to the sand fly midgut (such as decrease in temperature and increase in pH) triggers morphological transformation and development of the parasite in the vector. The amastigotes transform into procyclic promastigotes (weakly motile forms with a short flagellum beating at the anterior end of the cell). These are the first replicative forms

that proliferate in the early blood meal. Around 48–72 hours later, parasites begin to slow their replication and differentiate into strongly motile long nectomonad promastigotes. They move towards the anterior midgut and later develop into short nectomonad promastigotes, also called leptomonads, which enter another proliferative cycle. Ultimately, *Leishmania* transform into infective metacyclic stages, which are delivered to the vertebrate host during the next blood feeding.[23]

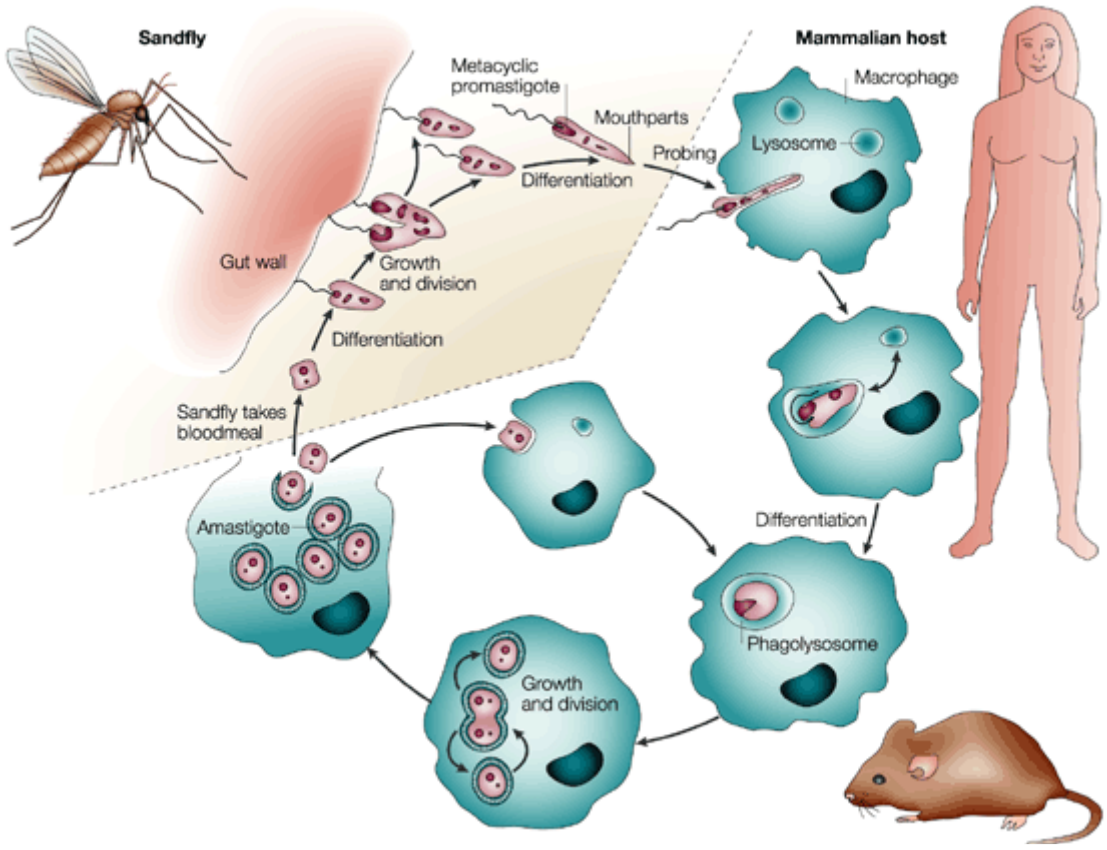


Fig.7 Representation of *Leishmania* life cycle.[24]

1.2.3 ***Pathogenesis***

To initially establish infection, promastigotes enter macrophages silently to evade triggering host responses; progressive intracellular (amastigote) infection depends on the maintenance of macrophages in an inert, deactivated state. At the same time, however, the immunocompetent host

is also equipped and responds with interdigitating non-specific (innate) and antigen-specific (acquired) mechanisms (cell-mediated immunity, delayed-type hypersensitivity). These inflammatory responses mediate disease expression and may (asymptomatic infection, self-healing disease) or may not (non-healing disease) produce the desired clinical end-result. Under optimum conditions, macrophages are eventually activated to a leishmanicidal state largely governed by an intact T-helper cell-type 1 (Th1) response. This complex response revolves around antigen-presenting dendritic cells, responding CD4 T cells, and secretion of pro-inflammatory cytokines, including interleukin 12, interferon and tumor necrosis factor. This same Th1 response also prevents recrudescence of latent, chronic infection. In subclinical infections, host responses are by definition effective and presumably tightly regulated since signs of inflammation are not noticeable. By contrast, inflammation is prominent and underlies pathogenesis in nearly all forms of clinically apparent infection.[25]

1.2.4 ***Clinical manifestations***

Clinical manifestations vary by *Leishmania* species, area of acquisition of infection and host factors. CL is the most common form of leishmaniasis, whereas VL is the most serious one. Typically the patient with VL develops fever, weakness, anorexia, weight loss, pallor, hepatosplenomegaly (usually splenomegaly predominates), lymphadenopathy, and progressive deterioration. Children may also present growth retardation. Late findings include epistaxis, gingival hemorrhage, abdominal distension, edema, and ascites. Occasionally VL may manifest as acute hepatitis, cholecystitis, hemophagocytic syndrome, and Guillain–Barré syndrome. If not treated, VL is almost always fatal. Death is caused by hemorrhage, severe anemia, or secondary infection. HIV-infected patients usually have higher parasite loads and atypical symptoms (e.g., gastrointestinal), especially with low CD4 cell counts. This group of HIV-infected patients responds poorly to treatment and rates of relapse are high, regardless of treatment used.

CL may manifest with one or more skin lesions on the exposed body within several weeks or months after the sand fly bite. New World CL has variable clinical manifestations, ranging from ulcerative skin lesions to destructive mucosal inflammation and is often accompanied with local lymphadenopathy, while Old World CL may present with multiple lesions. The initial lesion is usually a papule, which subsequently progresses to nodule and then to ulcer during the next 1–3 months. Despite the fact that CL is not a lethal disease and many lesions (especially due to Old World species) heal spontaneously over months to years, it is frequently associated with significant morbidity and may affect daily activities and social life when located on the hands and face.

ML occurs in 1–10% of CL cases 1–5 years after healing. Clinical manifestations include chronic nasal congestion and bleeding, ulceration and septal granulomas. ML is not self-cured and may progress and cause permanent or life-threatening complications such as ulcerative destruction of the nose, mouth, pharynx and larynx, nasal septum perforation and facial disfigurement, resulting in social stigma.[26]

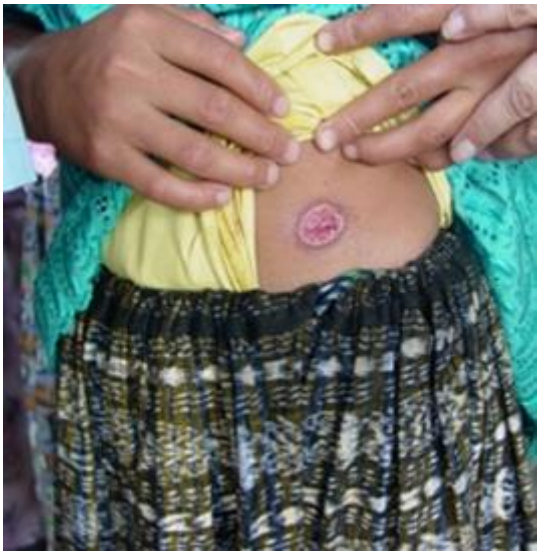
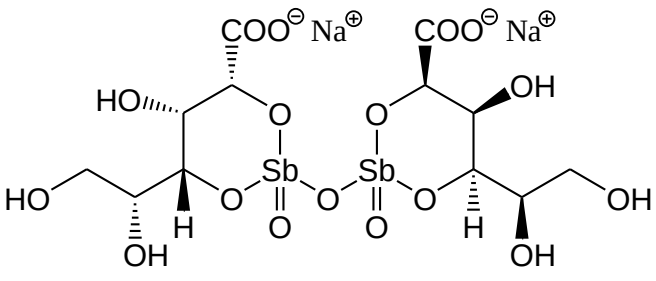


Fig.8 Typical ulcerative skin lesion.[27]

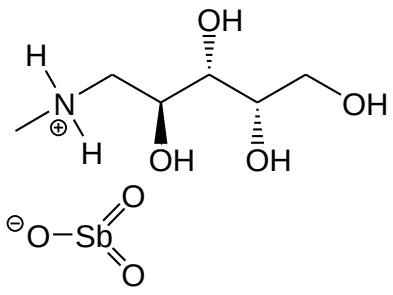
1.2.5 ***Antileishmanial therapies***

The poor knowledge about the disease and the lack of effective health policies are the primary hurdles in the elimination of leishmaniasis.[28] Treatment objectives vary with the form of leishmaniasis; in VL the main objective of treatment is to save the patient's life, as the disease is fatal if untreated. Drugs against VL need to be rapidly effective and safe. High drug efficacy is also essential to prevent relapses. In CL the primary objective of treatment is to reduce the duration and to heal the cutaneous lesion and, for a limited number of species (e.g. *L. braziliensis*), to prevent the occurrence of mucous lesions.[29]

Pentavalent antimonials, in particular sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantim) (Fig.9), are being used in the treatment of leishmaniasis over more than five decades and they are still the first line drugs of choice where resistance is not reported. The pentavalent antimoniate (Sb^{V}) is considered a pro-drug, which is further converted to trivalent antimonite (Sb^{III}), the active form of the drug.[28] Daily injection of 20 mg/kg body weight for 28-30 days have been the standard treatment for VL in most parts of the world. Its major toxicities are cardiac arrhythmias, prolonged QTc interval, ventricular premature beats, ventricular tachycardia, ventricular fibrillation and torsades de pointes. Arthralgia, myalgia and elevated hepatic and pancreatic enzymes are other common adverse events.[30]



Sodium stibogluconate



Meglumine antimoniate

Fig.9 Structures of sodium stibogluconate and meglumine antimoniate.

Increasing refractoriness to Sb^V in India and Nepal led to the adoption of alternative treatment strategies. In these regions the drug of choice is amphotericin B (AmB) (Fig.10), a polyene antifungal drug widely used to treat systemic fungal infections. AmB shows high affinity for ergosterol, the predominant sterol of fungal and leishmanial cell membrane. Despite its high efficacy, AmB causes many adverse effects including infusion reactions, nephrotoxicity, hypokalemia and myocarditis and thus needs close monitoring and hospitalization. To minimize the adverse events of amphotericin B, various lipid formulations have been introduced where deoxycholate is replaced with other lipids leading to less exposure of the free drug to organs. Three formulations have been extensively tested in VL: liposomal amphotericin B (L-AmB: AmBisome), amphotericin B lipid complex (ABL: Albecit) and amphotericin B colloidal dispersion (ABCD: Amphocil). L-AmB is the only approved drug by the US Food and Drug Administration. These formulation are less toxic and show very high efficacy, but their use is limited by the high cost.[28]

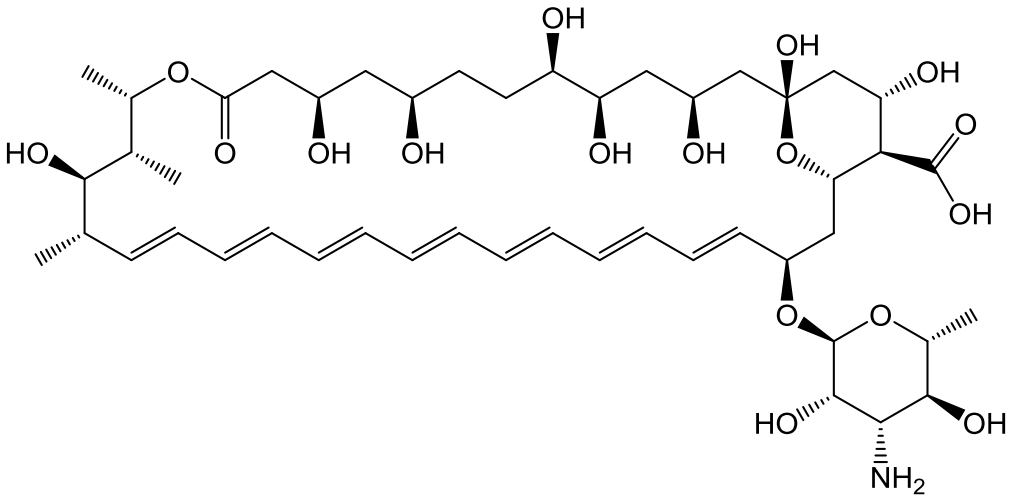


Fig.10 Amphotericin B.

Another compound known to have antileishmanial activity is miltefosine, an anticancer agent that is an alkylphosphocholine (hexadecylphosphocholine). It is the first oral drug used for the treatment of VL and its phase I/II/III trials provoked a storm of protection against VL that was followed by phase IV trial, which also proved its relevance in outpatient setting in those areas where VL is

endemic. Limitations of miltefosine are its relatively high cost, need for monitoring of gastrointestinal side effects, teratogenicity and occasional hepatic toxicity and nephrotoxicity.[30] The exact mode of antileishmanial action is still unclear but it has been found that it causes apoptosis-like processes in *Leishmania donovani*. Miltefosine (Fig.11) also reduces the lipid content in promastigotes membrane and enhances the phosphatidylethanolamine content suggesting a partial inhibition of phosphatidylethanolamine-N-methyltransferase that leads to decreased parasite proliferation.[31]

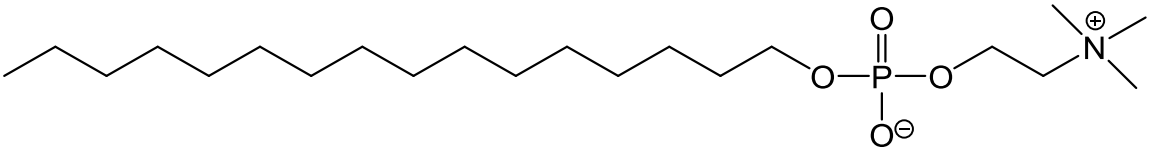


Fig.11 Miltefosine.

Paromomycin (Fig.12) is an aminoglycosidic antibiotic and has both antileishmanial and antibacterial activity. It cures both VL and CL (more effectively) but limited availability restricts its use in endemic regions.[28] Paromomycin is used for the treatment of VL in a parenteral formulation and of CL in both topical and parenteral formulations, but it is a second line drug for the treatment of leishmaniasis as it can cause different important side effects.[30]

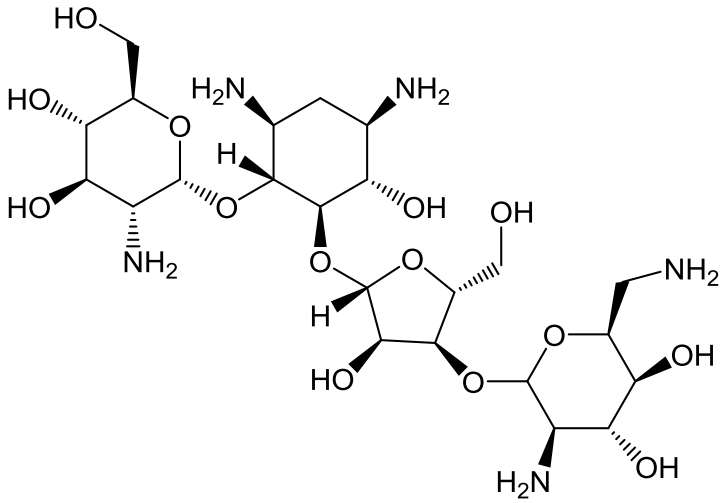
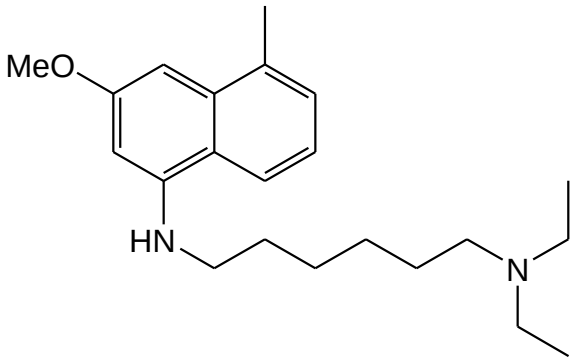
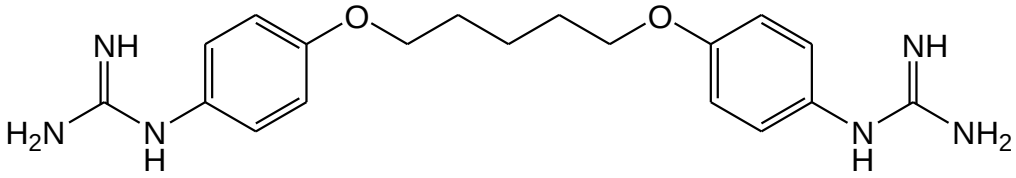


Fig.12 Paromomycin.

Other second choice drugs for the treatment of leishmaniasis are sitamquine and pentamidine. Sitamaquine (Fig.13) is an 8-aminoquinoline under clinical trials for the treatment of VL with the advantage of oral administration. However, despite efficacy, few side effects like vomiting, dyspepsia, cyanosis, nephritic syndrome and glomerulonephritis were also observed. The mechanism of action involves electrostatic interaction between phospholipid anionic polar head groups and positively charged sitamaquine and then with phospholipid acyl chains leading to drug insertion within biological membranes.[28,32] Pentamidine (Fig.13) is an aromatic diamine used in early 1980s for the treatment of refractory VL in India. However, its use has been abandoned for VL due to its serious toxicities such as insulin-dependent diabetes mellitus and declining efficacy.[30]



Sitamaquine



Pentamidine

Fig.13 Structures of sitamaquine and pentamidine.

The growing resistance of the parasite to antileishmanial drugs is particularly worrying and suggests that monotherapy needs to be converted into multidrug therapy, combining compounds with synergistic or additive activity acting at different sites. Advantages are increased efficacy, shorter

duration of therapy and lower dose requirement, thereby reducing chances of toxic side effects and cost, and preventing the emergence of drug resistance.[30]

Currently, there is no effective human vaccine available for any form of leishmaniasis but there are other alternative treatments other than chemotherapy. Thermotherapy exploits the experimental evidence that *Leishmania* parasites do not multiply at temperatures higher than 39 °C *in vitro.* Thermotherapy with radio-frequency waves has been most commonly studied for both Old and New World CL. Clinical trials demonstrated that it was as effective as antimony therapy in treating *L. braziliensis* and *L. mexicana* infections.[30] One of the most interesting approaches being explored is immunotherapy and targeted therapy. The last one acts by blocking essential biochemical or signaling pathways that are indispensable for *Leishmania* growth and survival, whereas immunotherapy involves the use of biological molecules or compounds to modulate immune responses in combination with drugs. Recent studies suggest that these two approaches could be complementary in VL treatment, leading to a synergistic effect.[33]

2. OBJECTIVES

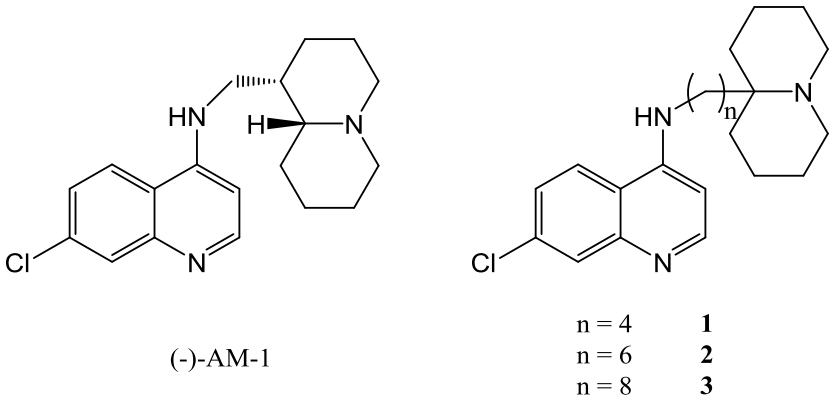
This project is focused on the search and development of new compounds characterized by different chemical scaffolds able to inhibit the growth of *Plasmodium* and/or *Leishmania* parasites and to overcome their mechanisms of resistance. Resistance is the most alarming feature of largely diffused and potentially lethal diseases as malaria and leishmaniasis. Unfortunately, at the moment even the combination therapies are failing in many regions afflicted by the diseases and alternatives are scarcely found. In addition, the available antileishmanial drugs are quite toxic, expensive and very often need monitoring and hospitalization. In the light of this dramatic situation, the discovery of novel effective, safe and affordable molecules is a very urgent need.

The strategies at the base of the project are the structural optimization of already known antiprotozoal molecules, the development of hybrid compounds and the search of new chemical scaffolds. Hybrids' components should be characterized by different biological functions, distinct pharmacophores, reduced toxicity and improved activity.[17] Consequently, my research project is divided into several topics and involves the synthesis of different sets of compounds:

2.1 *4-Aminoquinoline derivatives*

Quinoline-type antimalarials remain an attractive class of compounds because their mechanism of action and resistance are unrelated and resistance is compound-specific and not class-specific. Recently the *N*-(-)-lupinyl-derivative of 7-chloro-4-aminoquinoline (-)-AM-1 (Table 1) showed potent *in vitro* and *in vivo* activity against both chloroquine susceptible and resistant strains of *P. falciparum*. However, (-)-AM-1 is synthesized starting from (-)-lupinine, an expensive alkaloid isolated from *Lupinus luteus* whose worldwide production is not sufficient, at present, for large market purposes.[34,35] To overcome this issue and since the activity of 4-aminoquinoline antimalarials does not seem to be correlated with the chirality of the drug, we decided to prepare

novel not-chiral quinolizidinylalkyl derivatives of 7-chloro-4-aminoquinoline bearing the basic head linked to the 4-N of quinoline through longer alkyl chains (Table 1).



(-)-AM-1

n = 4 **1**
n = 6 **2**
n = 8 **3**

Table 1. Parent compound (-)-AM-1 and synthesized compounds.

2.2 ***Hybrids of 4-aminoquinoline***

Recent studies demonstrated that the histone deacetylases (HDACs) of *P. falciparum* (PfHDACs) have a high homology with the human ones and may be promising targets for new classes of antimalarial drugs.[36] HDACs are Zn-dependent enzymes which play crucial roles in modulating the chromatin structure in eukaryotes through regulating the degree of "packaging/unpackaging" of chromosomal DNA for transcription. The degree of histone acetylation, regulated by the opposing action of histone acetyltransferases and HDACs, is critical for transcriptional control. HDACs also regulate gene expression through the acetylation of other proteins such as transcription factors.[36] Some HDACs inhibitors, among which TSA, apicidin, trapoxin and SAHA (recently approved by FDA for the treatment of cutaneous T-cell lymphoma), showed *in vitro* antimalarial activity also at low concentration.[37] On this basis, we decided to prepare hybrid molecules (Table 2), in which the 4-aminoquinoline nucleus is condensed with Zn^{2+}-chelating moieties, such as hydroxamic acid, typical of the antitumor drug SAHA, and dithiolthiones, recently proved to have HDAC inhibitory activity.[38] The quinoline moiety could facilitate the penetration of the compound inside the *Plasmodium* and, in some case, could allow the obtainment of double target compounds (inhibition of hemozoin polymerization by the aminoquinoline residue and inhibition of HDAC by the side

chain) with improvement of drug efficacy and a reduced resistance onset. The spacer between the two moieties should be basic, in order to recall the side chain of chloroquine and to trap the molecule into the food vacuole of *Plasmodium*. For this reason and on the basis of recent encouraging results of some aromatic 4-aminoquinoline derivatives prepared in our laboratory,[39] I decided to introduce a 2-(piperazin-1-yl)ethyl spacer (compounds **4**, **5**, **6** and **7**) or an aromatic one: in particular, a slightly basic imidazolyl moiety in compound **8** and a completely neutral one in compound **9**.

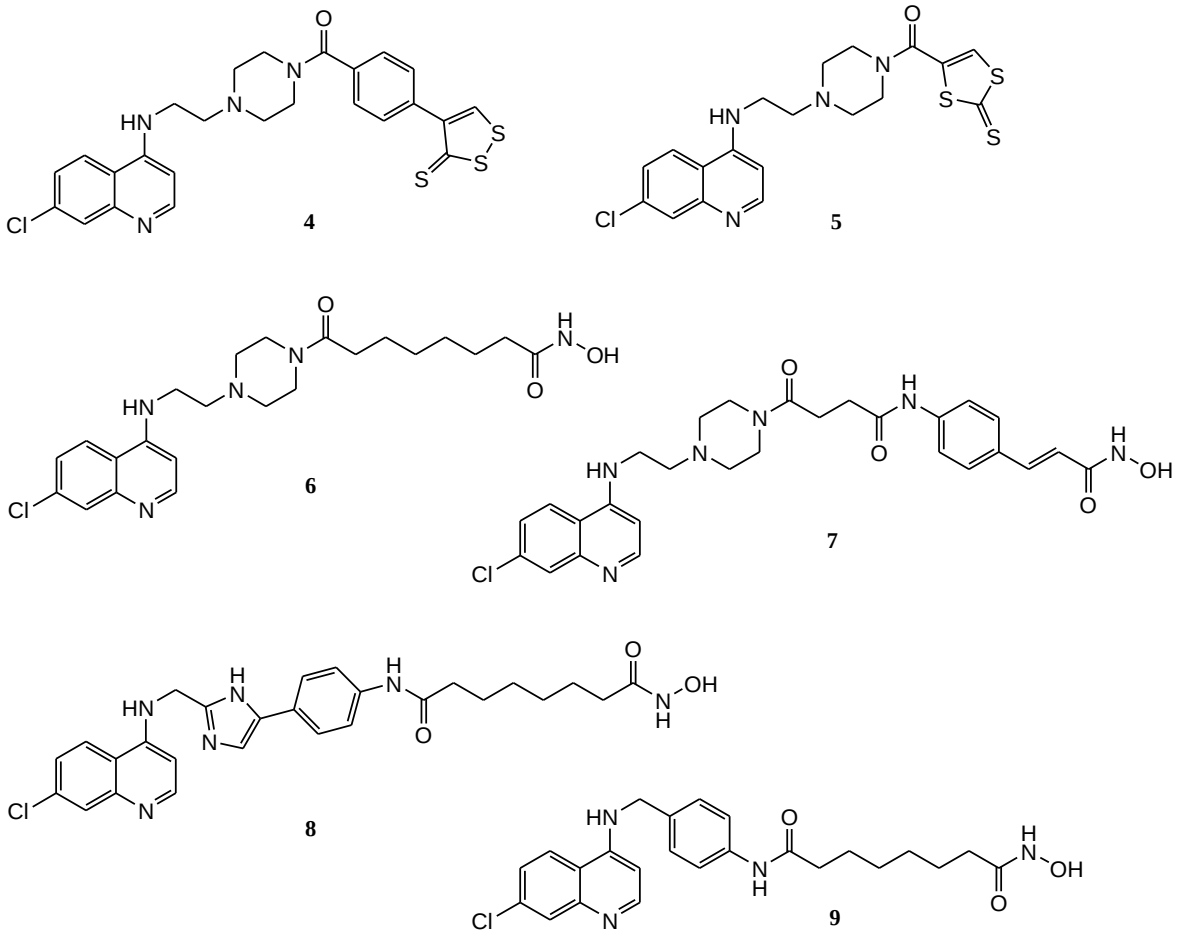


Table 2. Structures of the synthesized compounds.

2.3 ***Indeno[2,1-c]quinolines***

A further approach (whose results may appear somewhat more fortuitous) is represented by the shrewd superimposition in a single molecular scaffold of the structural features responsible of the activity of different antimalarials, or even of non antiplasmodial antimicrobial agents. As *P.*

falciparum and *M. tuberculosis* share enzymatic components of type II fatty acids biosynthetic pathway (FAS-II),[40-42] I found interesting to superimpose in a single molecular scaffold the quinoline ring of chloroquine and the fluorene, that is a substructure of Genz-10850, an inhibitor of FAS-II of both *P. falciparum* and *M. tuberculosis*,[43,44] as well as of the potent antimalarial lumefantrine and also of our 9-lupinyl- and 9-lupinilydenefluorene endowed with high antitubercular activity (MIC= 0.49 μM for the latter)[45,46] (Fig.14).

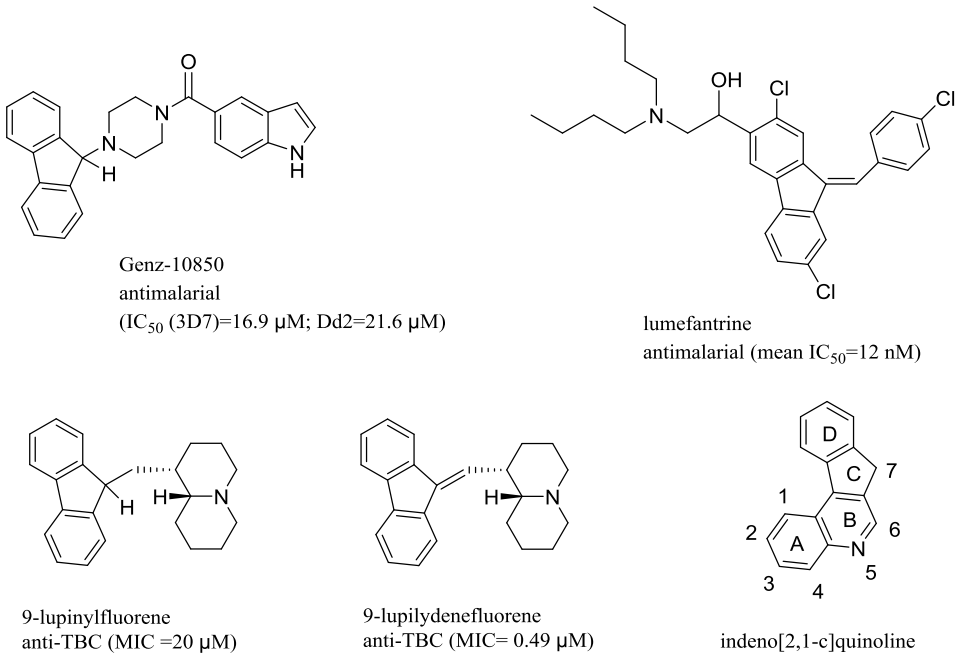
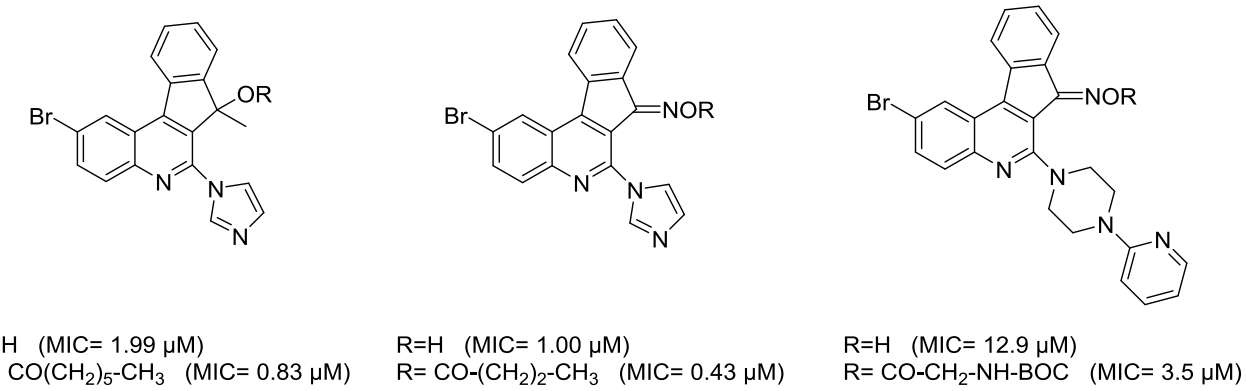


Fig.14 Structures of antimalarial and antitubercular compounds.

The formal overlapping of the two substructures gives rise to the indeno[2,1-c]quinoline ring system (Fig.14).

Derivatives of this ring system have been shown[47] to be good antimycobacterial agents, with MIC in the range of 0.43 to 12.9 μM for the best compounds (and their ester prodrugs) that likely act by targeting the ATP synthase subunit C (Fig. 15).



R=H (MIC= 1.99 μM)
R= $CO(CH_2)_5$-CH_3 (MIC= 0.83 μM)

R=H (MIC= 1.00 μM)
R= CO-$(CH_2)_2$-CH_3 (MIC= 0.43 μM)

R=H (MIC= 12.9 μM)
R= CO-CH_2-NH-BOC (MIC= 3.5 μM)

Figure 15. Structures of indeno[2,1-c]quinoline derivatives endowed with antimycobacterial activity.

As far as we know, none of these indenoquinoline derivatives has been evaluated for antimalarial activity, although during the realization of this project, a paper reporting the study of the *in vitro* antiprotozoal activity of some indeno[2,1-c]quinolines against *L.infantum* and different species of *Tripanosoma* was published by Upadhayaya *et al.*[48] Thus, assuming that the different activities of the above compounds are related to the different substituents decorating the common indenoquinoline scaffold, a set of novel derivatives has been considered to obtain potential antimalarial agents (Table 3).[49]

For this purpose, a chlorine atom has been introduced on ring A either in the position 2, in analogy with the 2-bromine of the above antimycobacterial agents, or in position 3, corresponding to the 7-position of chloroquine. A basic chain in position 6 (ring B) should warrant a reasonable hydrophilicity as well as the molecule concentration in the food vacuole of *Plasmodium*. As first possibility, the basic chain has been linked to the quinoline nucleus through a secondary amino group, that, like the 4-amino group of chloroquine, would allow the resonance with the protonated nitrogen of quinoline. Cyclic basic heads have been preferred to minimize the possibility of metabolic dealkylation as observed for chloroquine. The position 7 (ring C) bears a carbonyl group that could act as hydrogen bond acceptor, but also an oximino group that, viceversa, could act as hydrogen bond donor.[50] Other functional derivatives of the 7-keto group have been considered, like aminoguanidone and 1-imino-4-methylpiperazine. Indeed, the carbonyl group sets up a structural analogy with two kinds of benzophenone derivatives. On one hand, 2,5-

bisacylaminobenzophenones (A) (Fig.16) displayed nanomolar antimalarial activity (IC_{50}= 32 nM for the best compounds) through the inhibition of farnesyl transferase;[51] on the other hand, 3,4-dichloro-4'-trifluoromethylbenzophenone aminoguanidone (B) (Fig.16) was 100% curative when administered at the dose of 80-640 mg/kg in infected animals.[52-54] Unfortunately, this compound resulted somewhat hepatotoxic.

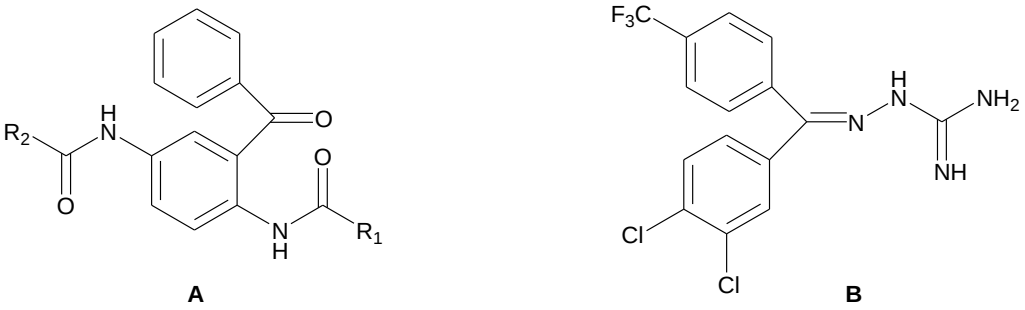
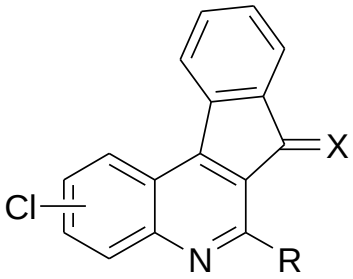


Fig.16 Structures of benzophenone derivatives endowed with antimalarial activity.



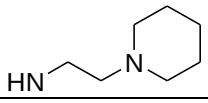
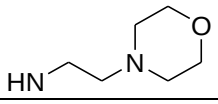
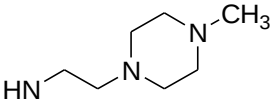
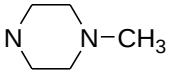
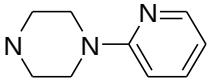
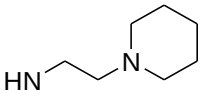
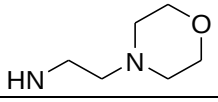
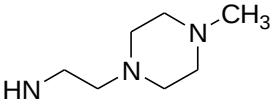
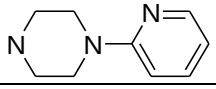
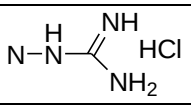
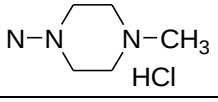
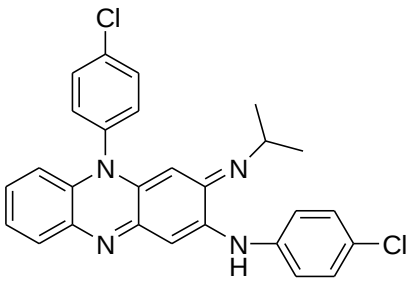
Compound	R	-Cl position	X
10		2	O
11	Id.	3	O
12		3	O
13		2	O
14	Id.	3	O
15		2	O
16		2	O
17		2	O
18		2	NOH
19	Id.	3	NOH
20		3	NOH
21		2	NOH
22	Id.	3	NOH
23		2	NOH
24	Id.	2	N-O-COEt
25		2	NOH
26	OCH_3	2	
27	OCH_3	2	

Table 3. Structures of the synthesized indenoquinolines.

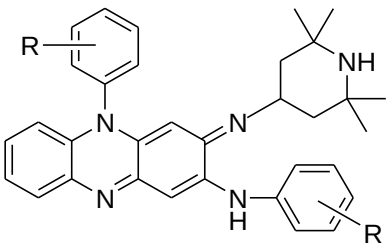
2.4 ***Riminophenazines***

With the aim to explore the antiprotozoal potentialities of new chemical scaffolds, I took into consideration the structure of clofazimine. Clofazimine (Fig.17) is a fat-soluble riminophenazine dye used in combination with rifampicin and dapsone as multidrug therapy for the treatment of leprosy. It is also endowed with a moderate antimalarial and antileishmanial activity, both *in vitro* and *in vivo* (murine models).[55]

I decided to study the effect of the introduction of different side chains containing a bicyclic basic moiety, such as pyrrolizidine or quinolizidine, in this kind of scaffold in order to improve the antiprotozoal activity. This idea started from the evidence that tetramethylpiperidine-substituted phenazines (Fig.17), structurally related to clofazimine, have been described to be endowed with activity against multidrug resistant strains of *P. falciparum* and they seemed to share, at least in part, the same target of chloroquine in malarial parasites. However, it has been demonstrated that the sensitivity of *P. falciparum* to the TMP-phenazines is unaffected by the mechanisms, which confer resistance to the conventional antimalarial agents, including chloroquine.[56]



Clofazimine

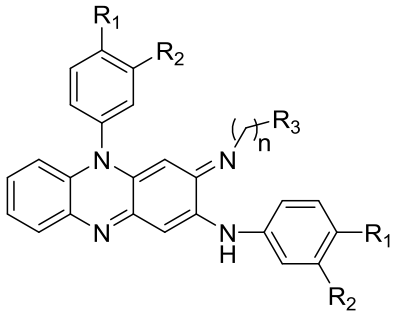


TMP-substituted phenazines

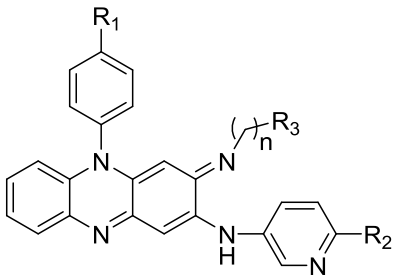
Fig.17 Structures of clofazimine and tetramethylpiperidine-substituted phenazines.

In addition to the quinolizidine moiety, already used for the quinolinic compounds mentioned before, I introduced a pyrrolizidine head linked to the phenazine nucleus through an ethyl chain and a lupininyl group (both previously prepared in the laboratory) with the aim to better understand the role of the bulky basic head and of the length of the linker. To evaluate the role of the steric hindrance of the bicyclic basic heads, the smaller pyrrolidinyl derivative was also studied. To

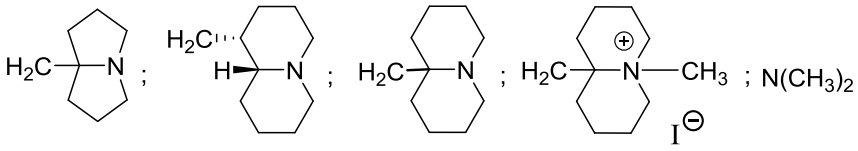
complete the study, I also prepared compounds characterized by the replacement of the aniline moiety in position 2 with an aminopyridine (Table 4, B) or by the quaternarization of the basic nitrogen in the side chain with a methyl group (**36**), to evaluate the contribution of the increment of polarity to the antiprotozoal activity.[57]



A



B



a b c d e

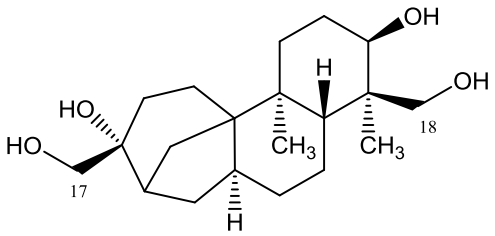
Compound	Series	R_1	R_2	R_3	n
28	A	H	H	a	1
29	A	Cl	H	a	1
30	A	CH_3	H	b	0
31	A	H	H	c	3
32	A	H	H	c	5
33	A	F	H	c	3
34	A	F	H	c	5
35	A	F	Cl	c	5
36	A	H	H	d	3
37	B	H	H	c	3
38	B	H	OCH_3	c	3
39	B	Cl	H	c	3
40	B	Cl	H	e	3

Table 4. Structures of the synthesized phenazines.

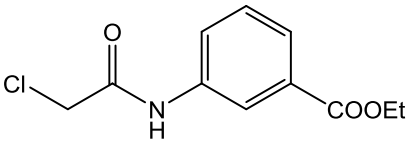
2.5 ***Aphidicolin derivatives***

In the search of new scaffolds with potential antileishmanial activity, I took into consideration the tetracyclic diterpene aphidicolin. Aphidicolin (Fig.18), a fungal metabolite isolated from *Nigrospora sphaerica*, was first described as a highly active drug for inhibiting cell division and synchronizing cell cycles in experimental medicine. Recently, aphidicolin and a series of semisynthetic aphidicolan derivatives have shown antileishmanial activity in the micromolar range.[58] In particular, it was found that the esterification (without acetylation) of C-3, C-18 and C-17 of aphidicolin led to prodrugs with enhanced activity.

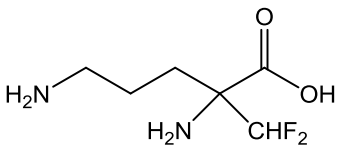
With the aim to explore the potentiality of novel scaffolds as antileishmanial agents, I decided to prepare some derivatives (Table 5) through the condensation of aphidicolin with other molecules endowed with antileishmanial activity, such as ethyl 3-chloroacetamidobenzoate and eflornithine (Fig.18).



Aphidicolin



Ethyl 3-chloroacetamidobenzoate



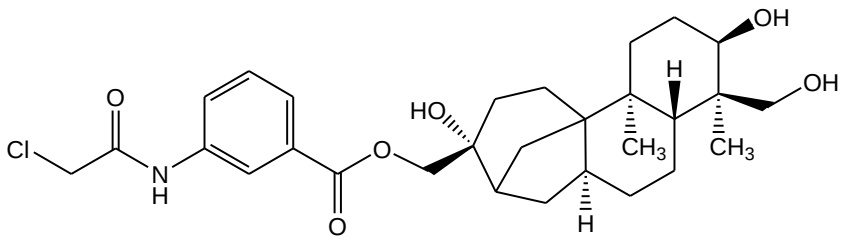
Eflornitine

Fig.18 Moieties used for the hybrids preparation.

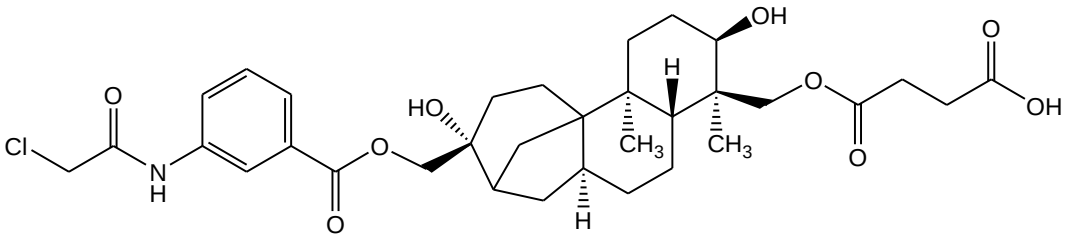
Ethyl 3-chloroacetamidobenzoate (MF29) was found to be an efficient drug on the promastigote stage of three *Leishmania* species (IC_{50}: 0.3-1.8 μM), acting as antimicrotubule agent.[59] Eflornithine or DL-alpha-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase, was discovered to have potent antileishmanial activity.[60] When given as a 2% solution in drinking

water 2 days after infection and continued for 4 days, it suppressed liver parasites by 90% and spleen parasites by 99%. The idea was to obtain hybrid molecules (**41**-**43**; Table 5) where the two components were released in the parasite, exhibiting different mechanisms of action and a synergistic effect.

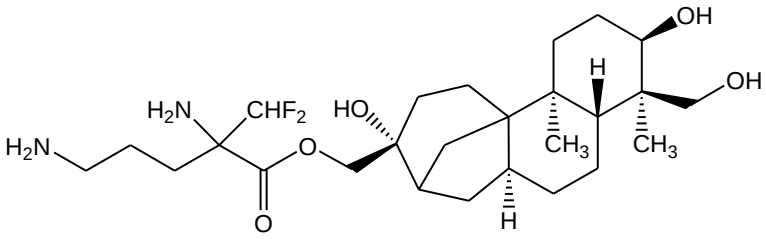
With the aim to evaluate the importance and the influence of the hydrophilicity of these molecules on the antileishmanial activity, I esterified the OH in position 18 of the 3-chloroacetanilido benzoyl derivative with succinic acid (compound **42**).



41



42



43 $2CF_3COOH$

Table 5. Structures of aphidicolin derivatives.

2.6 ***Rosmaricine and derivative***

Another idea took the cue from recent results obtained in our laboratory concerning rosmaricine. Rosmaricine (Fig.19) is an alkaloid obtained through the treatment of carnosic acid (Fig.19), a natural compound contained in the ethanolic extract of *Rosmarinus officinalis*, with ammonia and O_2. Rosmaricine and some of its derivatives were found to be endowed with a modest antiplasmodial activity and I found interesting to introduce a basic head in the structure that could allow to trap the compound inside the food vacuole of *Plasmodium* and, consequently, to improve the activity. Thus, firstly I optimized the complicated extraction process of carnosic acid from rosemary leaves, the synthesis and the purification of rosmaricine. Then, when the procedure was well established, I tried to synthesize the diethylaminopropylamine derivative (**45**) of carnosic acid (Fig.19), using the same synthetic pathway of rosmaricine.

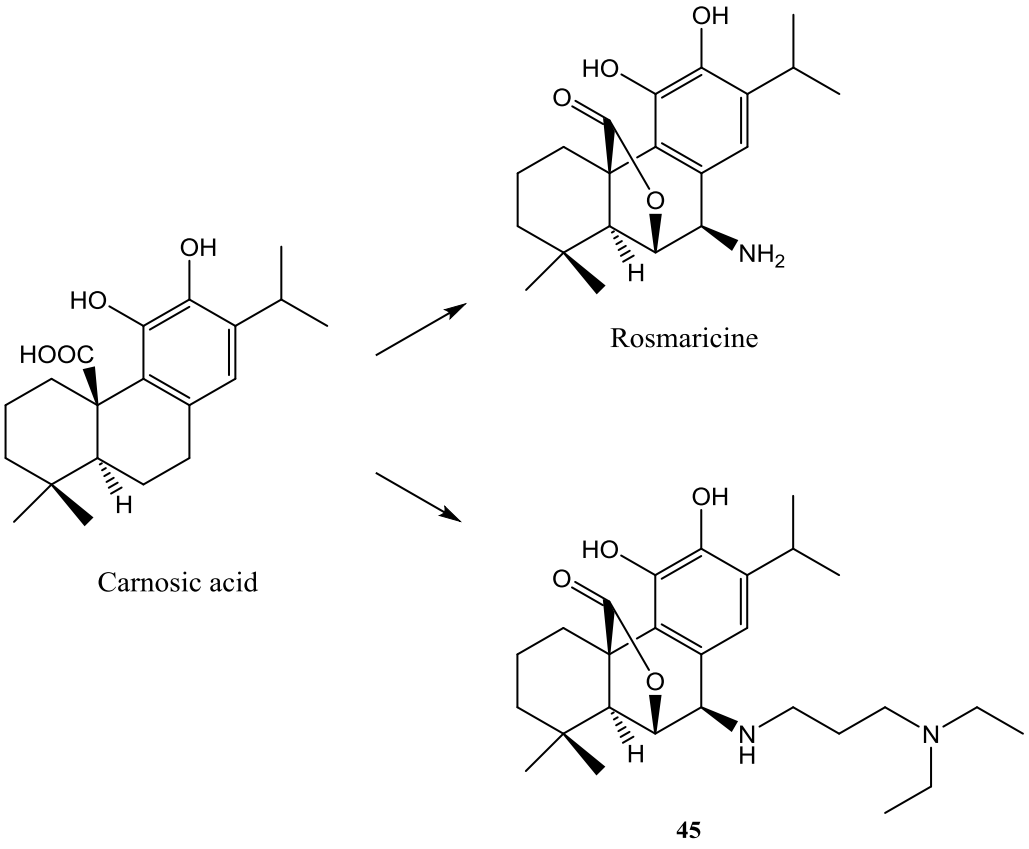
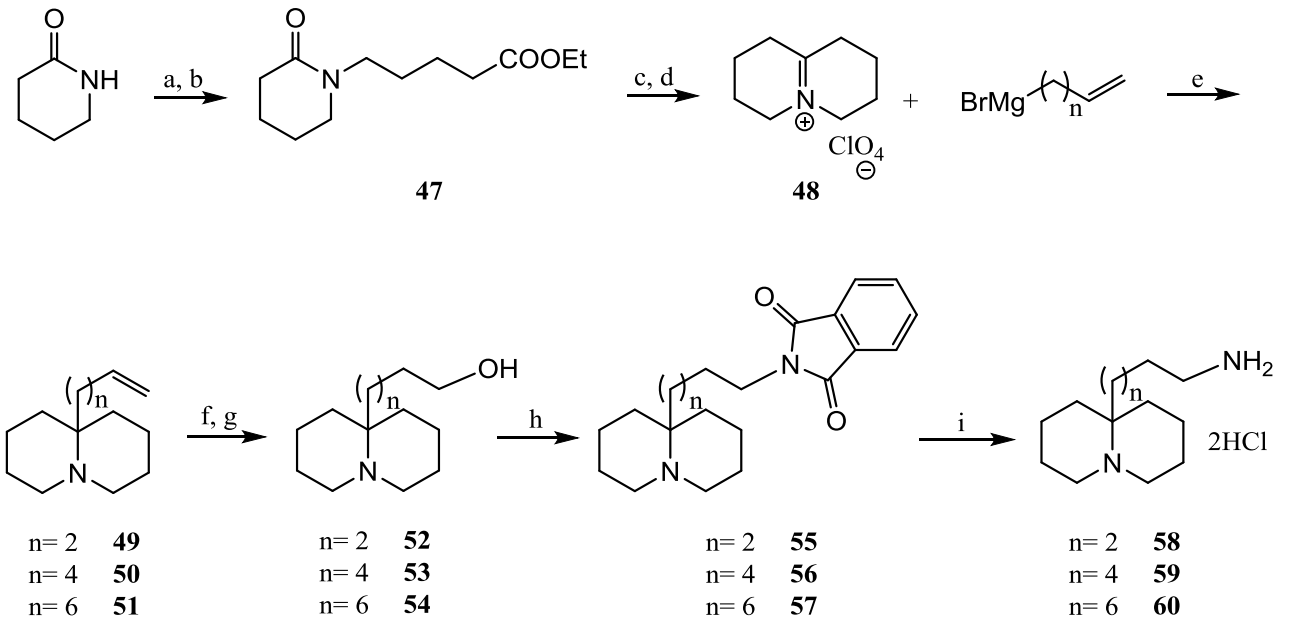


Fig.19 Carnosic acid and derivatives.

3. CHEMISTRY

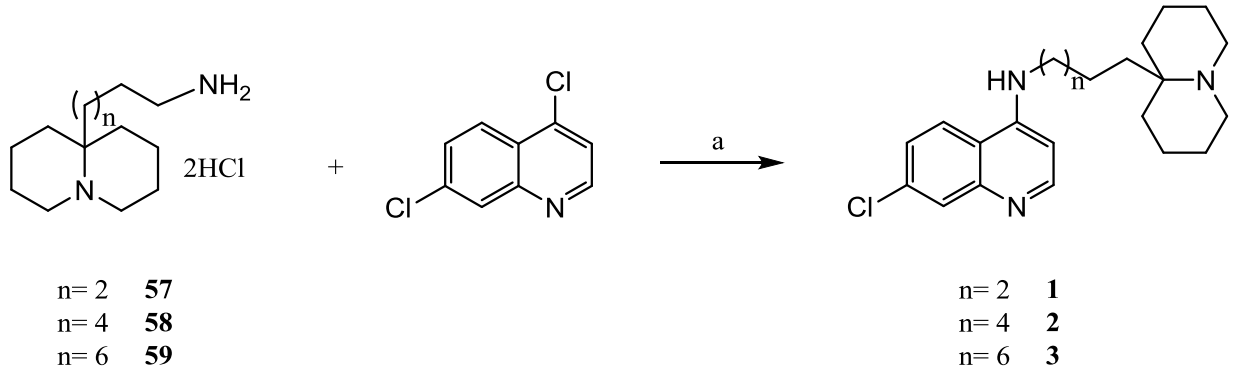
3.1 *4-Aminoquinoline derivatives*

The synthesis of 4-aminoquinoline derivatives required the preliminary preparation of the (octahydro-1H-quinolizin-9a-yl)alkan-1-amines, which were new chemical entities and were obtained in six steps, as indicated in Scheme 1. δ-Valerolactam was reacted firstly with sodium hydride and then with ethyl 5-bromovalerate to generate the corresponding tertiary amide **47**. After treatment with soda lime and distillation, the obtained 2,3,4,6,7,8-hexahydro-1H-quinolizine was converted into the corresponding perchloric salt **48**.[61,62] Compound **48** was treated with the proper Grignard reagent to generate the olefinic quinolizidines (**49-51**),[62,63] which underwent a hydroboration reaction to obtain the alcoholic derivatives (**52-54**).[62] After a Mitsunobu reaction in the presence of phthalimide, DEAD and triphenylphosphine and the following acidic hydrolysis,[64] the desired (octahydro-1H-quinolizin-9a-yl)alkan-1-amine dihydrochloride salts (**58-60**) were obtained.



Scheme 1. Reagents and conditions: a) NaH 60%, dry THF from 0 °C to r.t.; b) ethyl 5-bromovalerate, reflux; c) Soda lime, distillation (160-170 °C); d) $HClO_4$ 70%, EtOH; e) $ZnBr_2$, dry THF, from 50 °C to reflux; f) 1N BH_3 in THF, 2-methyl-2-butene, dry THF, r.t.; g) H_2O, 6N NaOH, H_2O_2 35%, r.t.; h) phthalimide, Ph_3P, DEAD, dry THF, r.t.; i) 6N HCl, reflux.

Final compounds **1-3** were prepared by the condensation of 4,7-dichloroquinoline with the proper (octahydro-1H-quinolizin-9a-yl)alkan-1-amine in the presence of phenol and DIPEA.



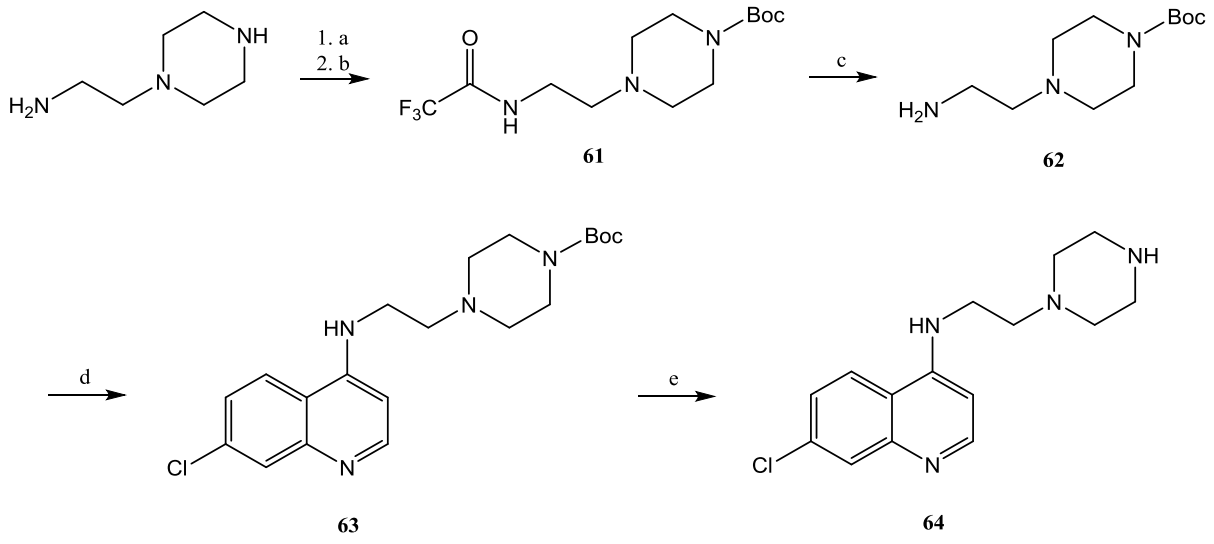
n= 2 **57**
n= 4 **58**
n= 6 **59**

n= 2 **1**
n= 4 **2**
n= 6 **3**

Scheme 2. Reagents and conditions: a) Phenol, DIPEA, 120 °C.

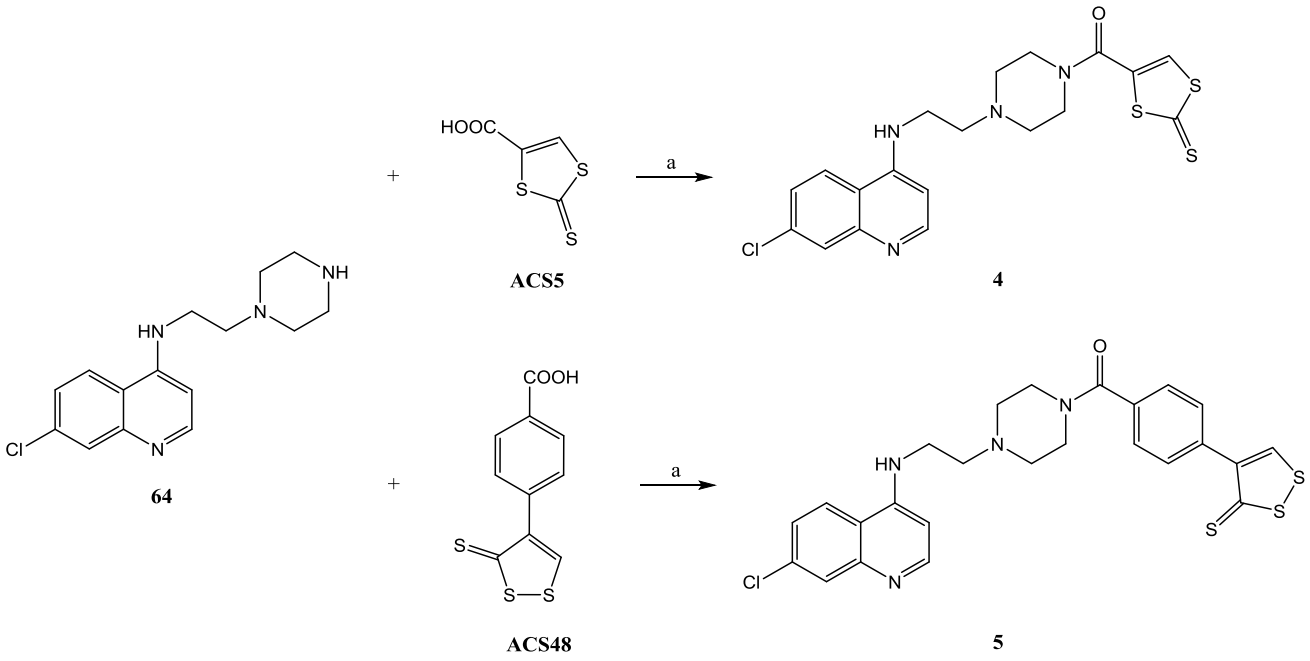
3.2 ***Hybrids of 4-aminoquinoline***

For the synthesis of compounds **4-7**, as first step it was essential the preparation of *tert*-butyl 4-(2-aminoethyl)piperazine-1-carboxylate (**62**). To selectively protect the primary amino group, 2-(piperazin-1-yl)ethanamine was firstly reacted with ethyl trifluoroacetate and then, after the protection of the piperazinyl nitrogen with a Boc group, the alkaline hydrolysis of the trifluoroacetate group provided compound **62**.[65] The primary amine carried out a nucleophilic substitution on 4,7-dichloroquinoline and, after the cleavage of the Boc group, intermediate **64** was obtained (Scheme 3).



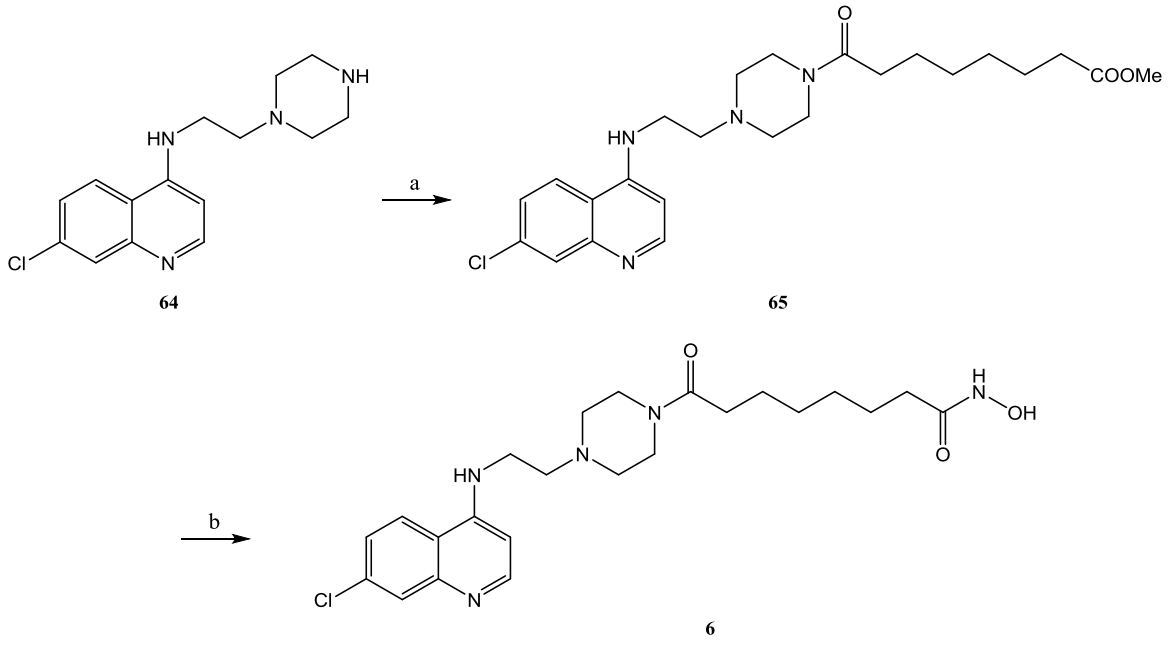
Scheme 3. Reagents and conditions: a) ethyl trifluoroacetate, dry THF, 5 °C; b) Boc_2O, r.t.; c) NaOH 0.2N, MeOH, from 15 to 35 °C; d) DIPEA, 120 °C; e) CF_3COOH, r.t.

The coupling reaction of intermediate **64** with the dithiolthiones **ACS5** and **ACS48**, previously prepared in our laboratory, provided the final compounds **4** and **5** (Scheme 4).



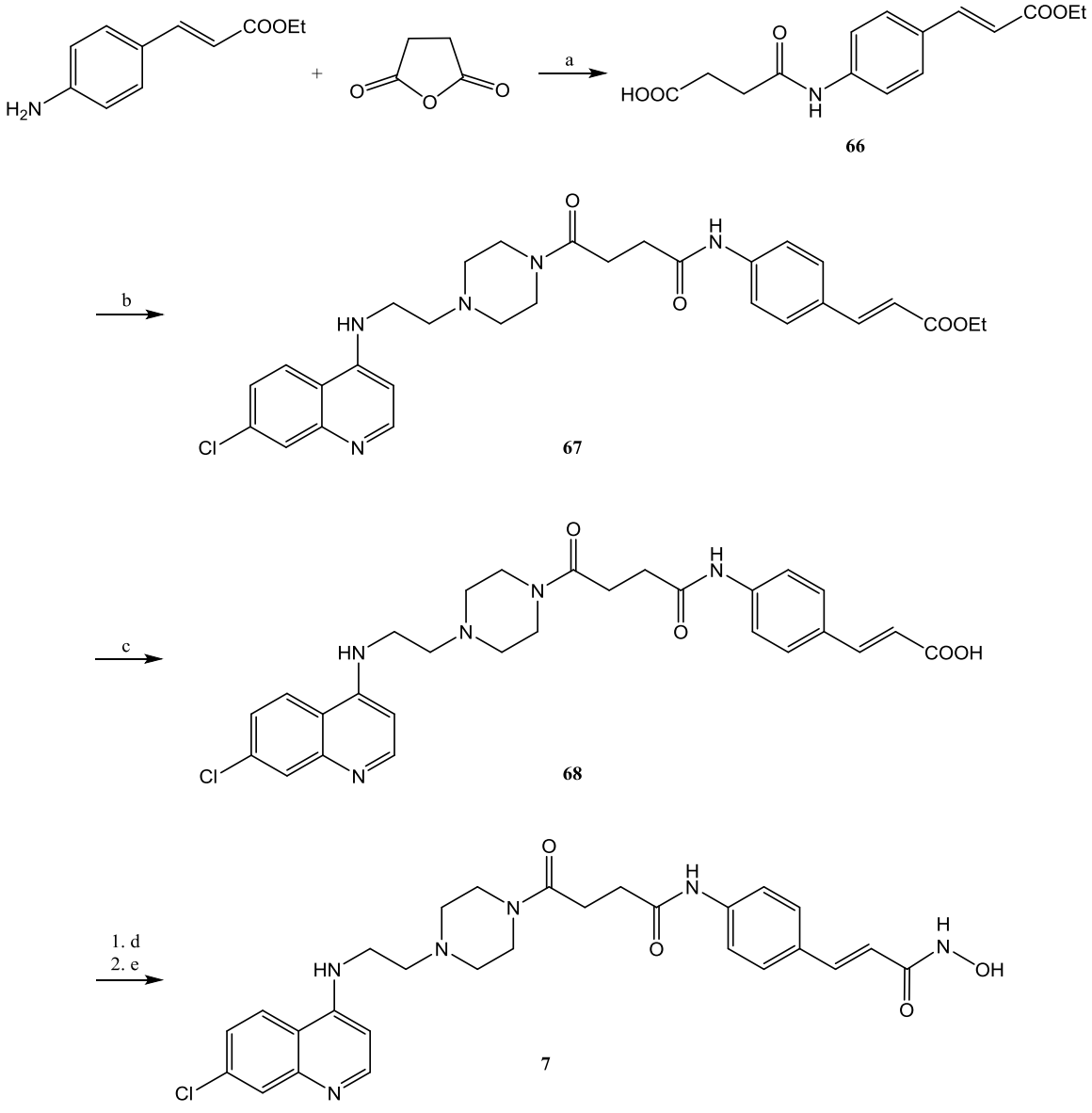
Scheme 4. Reagents and conditions: a) HOBt, DCC, dry DMF, m.w.

The same coupling reaction between intermediate **64** and suberic acid monomethyl ester, followed by the formation of the hydroxamic acid through the reaction with hydroxylamine hydrochloride in alkaline methanol, provided final compound **6** (Scheme 5).



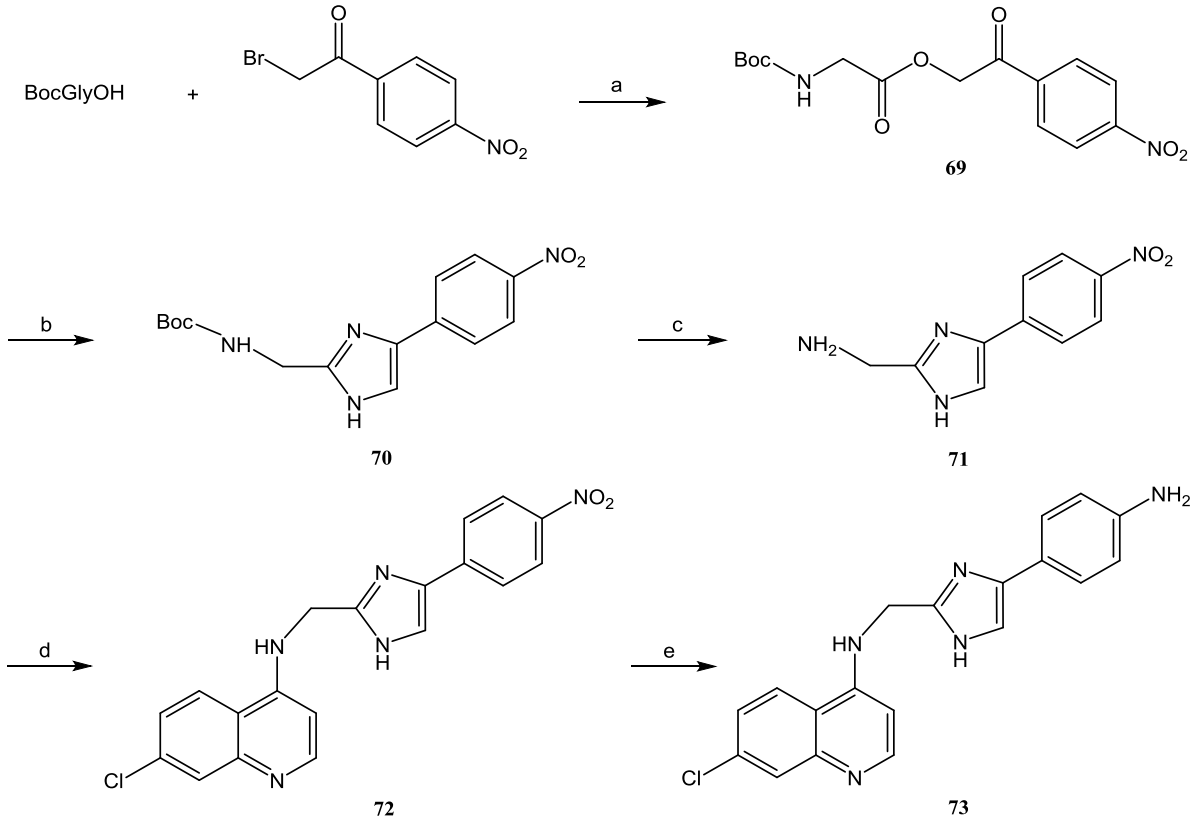
Scheme 5. Reagents and conditions: a) HOBt, DCC, dry DMF, m.w.; b) NH_2OH*HCl, KOH, MeOH, r.t.

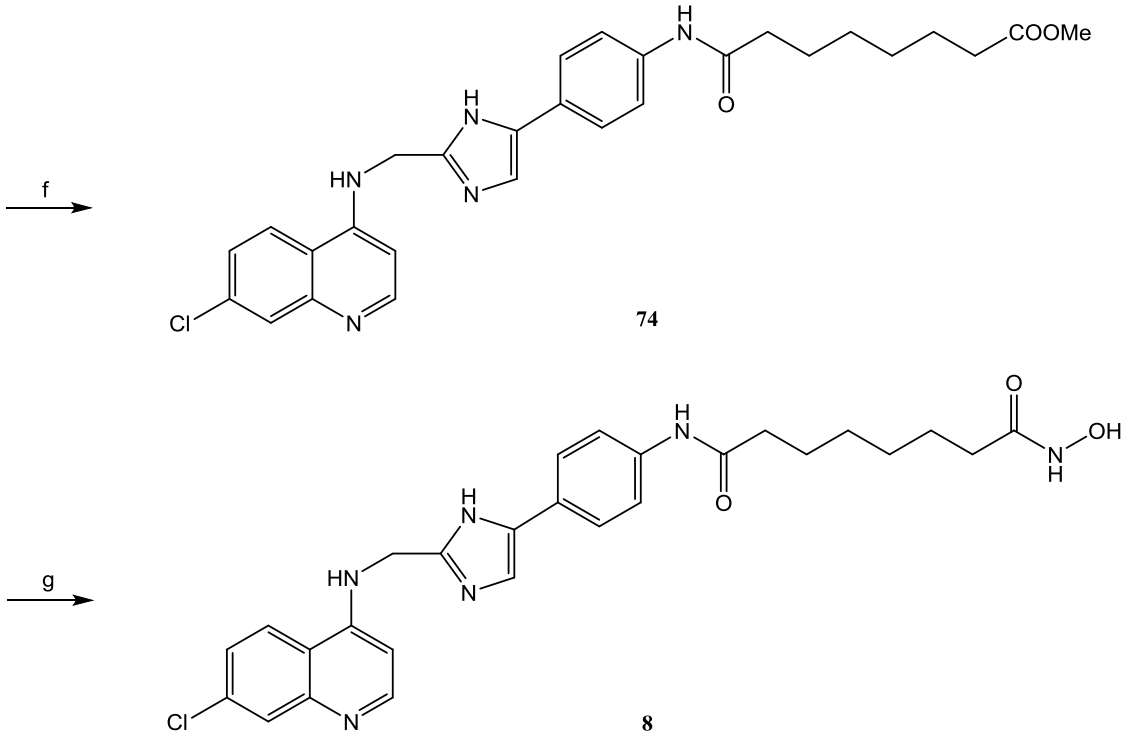
The synthesis of compound **7** required also the preliminary condensation of ethyl 4-aminocinnamate with succinic anhydride in a H_2O/THF solution to provide intermediate **66**, which reacted with intermediate **64** in the presence of coupling reagents. The classic conversion of the ethyl ester (**67**) into hydroxamic acid did not work, probably because the benzylic double bond is a good Michael's acceptor and this is the preferred reaction. Consequently, I found another synthetic route that involved the hydrolysis of the ester into carboxylic acid (**68**) and the following activation of the carbonyl group with ethyl chloroformate,[66] which allowed the attack of the hydroxylamine to obtain the final hydroxamic acid **7** (Scheme 6).



Scheme 6. Reagents and conditions: a) H_2O/THF (2:1), r.t.; b) compound **62**, HOBt, DCC, dry DMF, m.w; c) 1N NaOH, EtOH/THF, 60 °C; d) ethyl chloroformate, TEA, dry THF, r.t.; e) NH_2OH*HCl, KOH, MeOH, r.t.

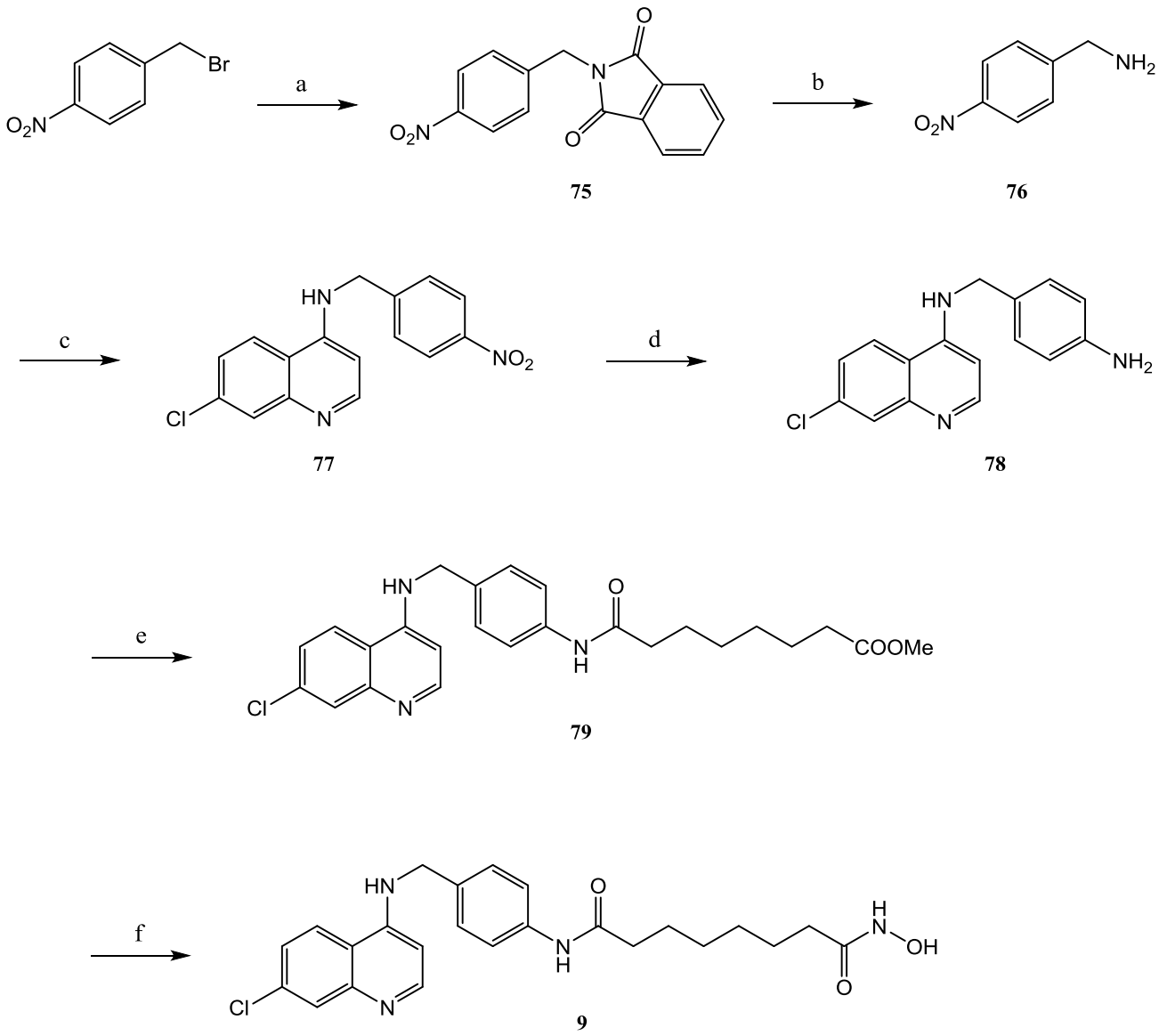
Compound **8** was prepared through a synthetic route of seven steps (Scheme 7). From the reaction between Boc-glycine and 2-bromo-4-nitroacetophenone, intermediate **69** was obtained, which underwent an Ugi reaction with ammonium acetate and xilene to provide intermediate **70**.[67] After the cleavage of the Boc group and the condensation with 4,7-dichloroquinoline, the obtained intermediate **72** was reduced with metallic iron in acetic acid. The amino derivative (**73**) was finally coupled with suberic acid monomethyl ester in a microwave reactor and the ester group was then converted into hydroxamic acid with hydroxylamine hydrochloride in a basic methanolic solution to get the final compound **8**.





Scheme 7. Reagents and conditions: a) EtOH, Cs_2CO_3, dry DMF, r.t.; b) CH_3COONH_4, xilene, 180 °C; c) CF_3COOH, r.t.; d) 4,7-dichloroquinoline, phenol, 130 °C; e) Fe, CH_3COOH, 70 °C; f) suberic acid monomethyl ester, HOBt, DCC, dry DMF, m.w.; g) NH_2OH*HCl, KOH, MeOH, r.t.

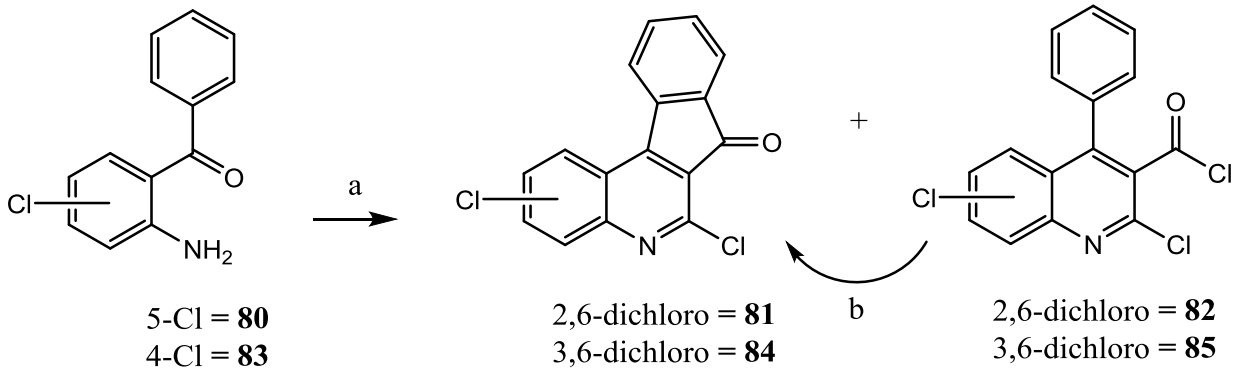
For the synthesis of compound **9**, 4-nitrobenzyl bromide was reacted with phthalimide potassium salt to obtain intermediate **75**, which was treated with hydrazine monohydrate. The amino-derivative **76** carried out a nucleophilic substitution on 4,7-dichloroquinoline in the presence of phenol as both solvent and catalyst. The nitro group was then reduced to amine with metallic iron in acetic acid and the obtained compound **78** underwent a coupling reaction with suberic acid monomethyl ester. The final conversion into hydroxamic acid to get compound **9** were carried out with the same procedure used for compound **8**.



Scheme 7. Reagents and conditions: a) dry DMF, 50 °C; b) hydrazine monohydrate, EtOH/THF, reflux; c) 4,7-dichloroquinoline, phenol, 130 °C; d) Fe, CH_3COOH, 70 °C; e) suberic acid monomethyl ester, HOBt, DCC, dry DMF, m.w.; f) NH_2OH*HCl, KOH, MeOH, r.t.

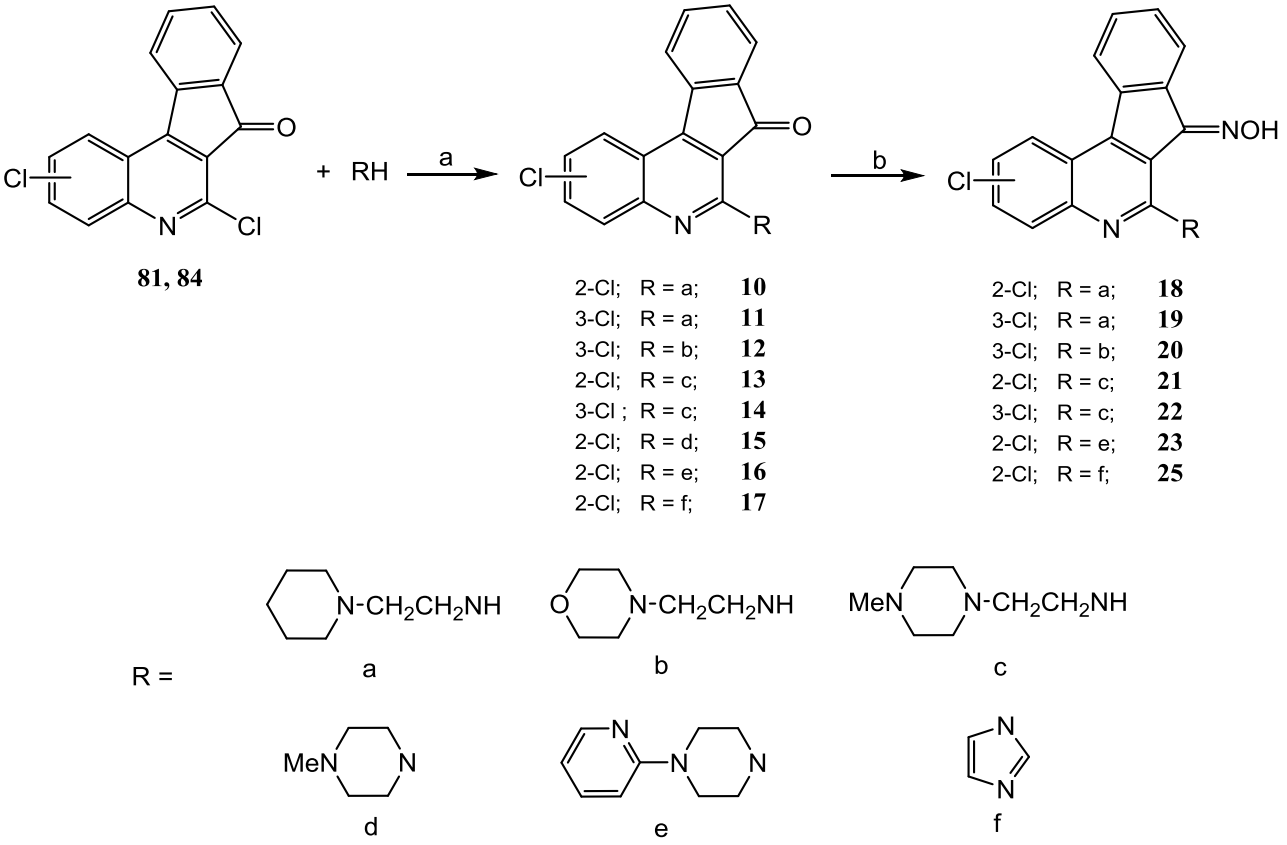
3.3 ***Indeno[2,1-c]quinolines***

The synthesis of the conformationally-constrained indeno[2,1-c]quinolines required the availability of the intermediates 2,6-dichloro-7*H*-indeno[2,1-c]quinolin-7-one **81**[68] and 3,6-dichloro-7*H*-indeno[2,1-c]quinolin-7-one **84**,[68] both obtained, respectively, through the condensation of (2-amino-5-chlorophenyl)(phenyl)methanone **80** or (2-amino-4-chlorophenyl)(phenyl)methanone **83**,[69] with malonic acid and $POCl_3$. This reaction involved the formation of the acyl chloride by-products **82** and **85**, which were subjected to an intramolecular Friedel-Crafts acylation to get the indeno[2,1-c]quinoline nucleus (Scheme 8).



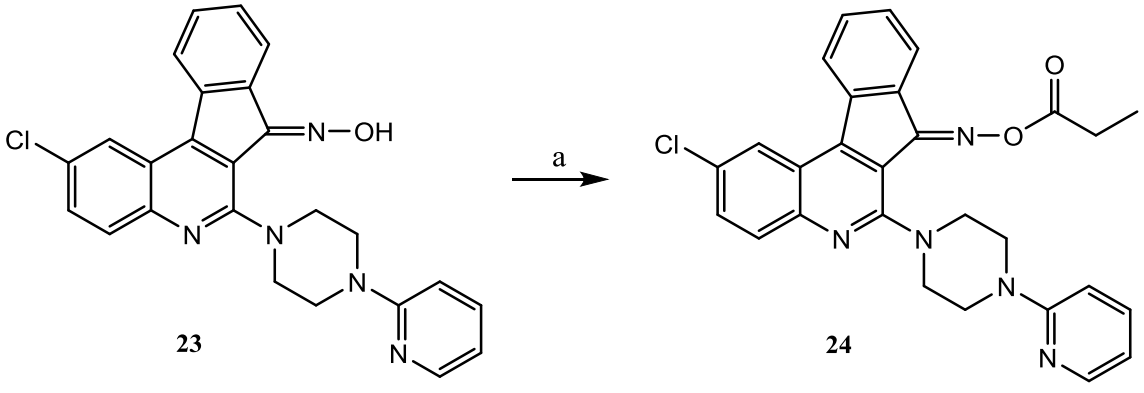
Scheme 8. Reagents and conditions: a) malonic acid, $POCl_3$, reflux; b) $AlCl_3$, dry CH_2Cl_2, from r.t. to reflux.

Compounds **81** and **84** were then reacted with different amines to generate the corresponding 2- or 3-chloro-6-(amino-substituted)-7*H*-indeno[2,1-c]quinolin-7-ones (**10**-**17**), which were converted into the corresponding oximes (**18**-**23** and **25**) with hydroxylamine hydrochloride in a basic hydroalcoholic solution (Scheme 9).



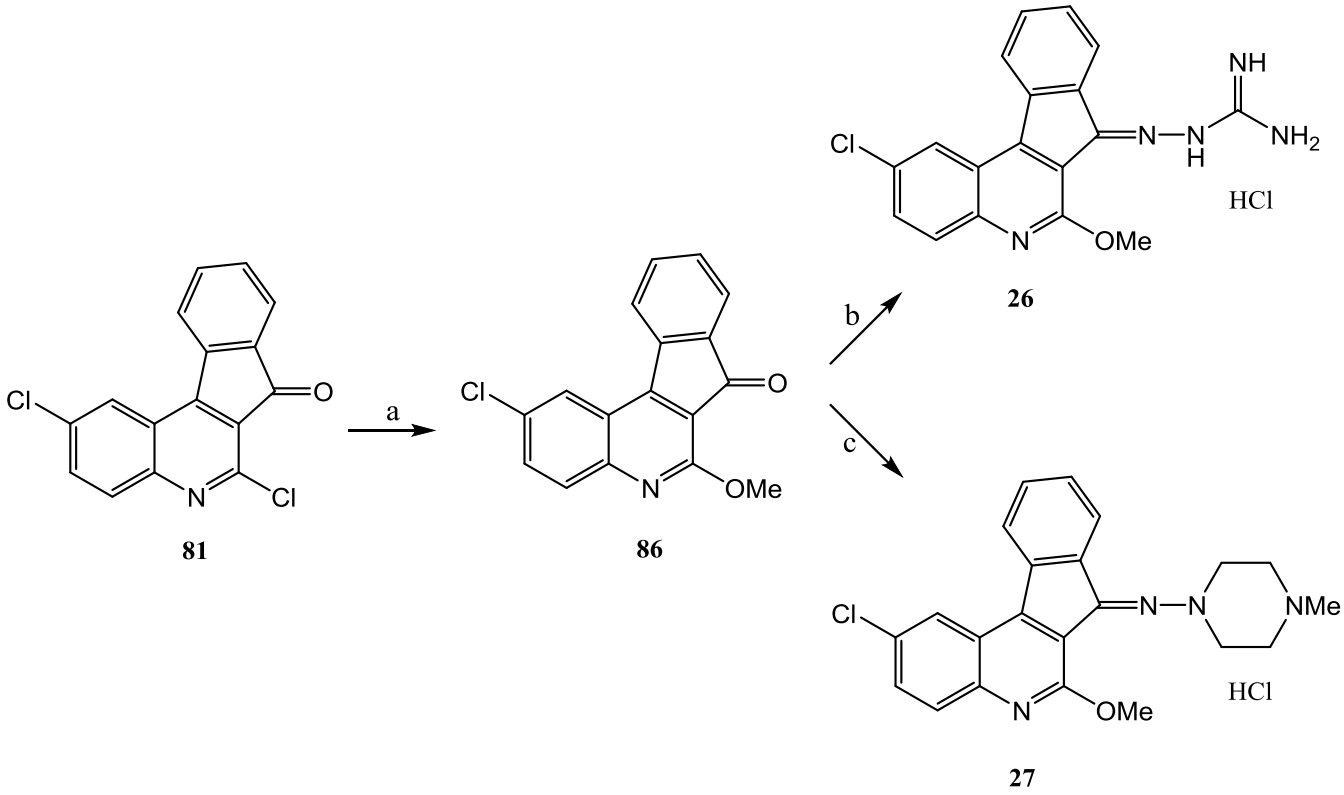
Scheme 9. Reagents and conditions: a) dry pyridine, 100 °C; b) NH_2OH*HCl, NaOH, EtOH/H_2O (2:1), from r.t. to reflux.

The oxime **23** was treated with NaH and propionyl chloride to get 2-chloro-6-(4-(pyridin-2-yl)piperazin-1-yl)-7*H*-indeno[2,1-c]quinolin-7-one O-propionyl oxime **24** (Scheme 10).[70]



Scheme 10. Reagents and conditions: a) NaH, propionyl chloride, DMSO, r.t.

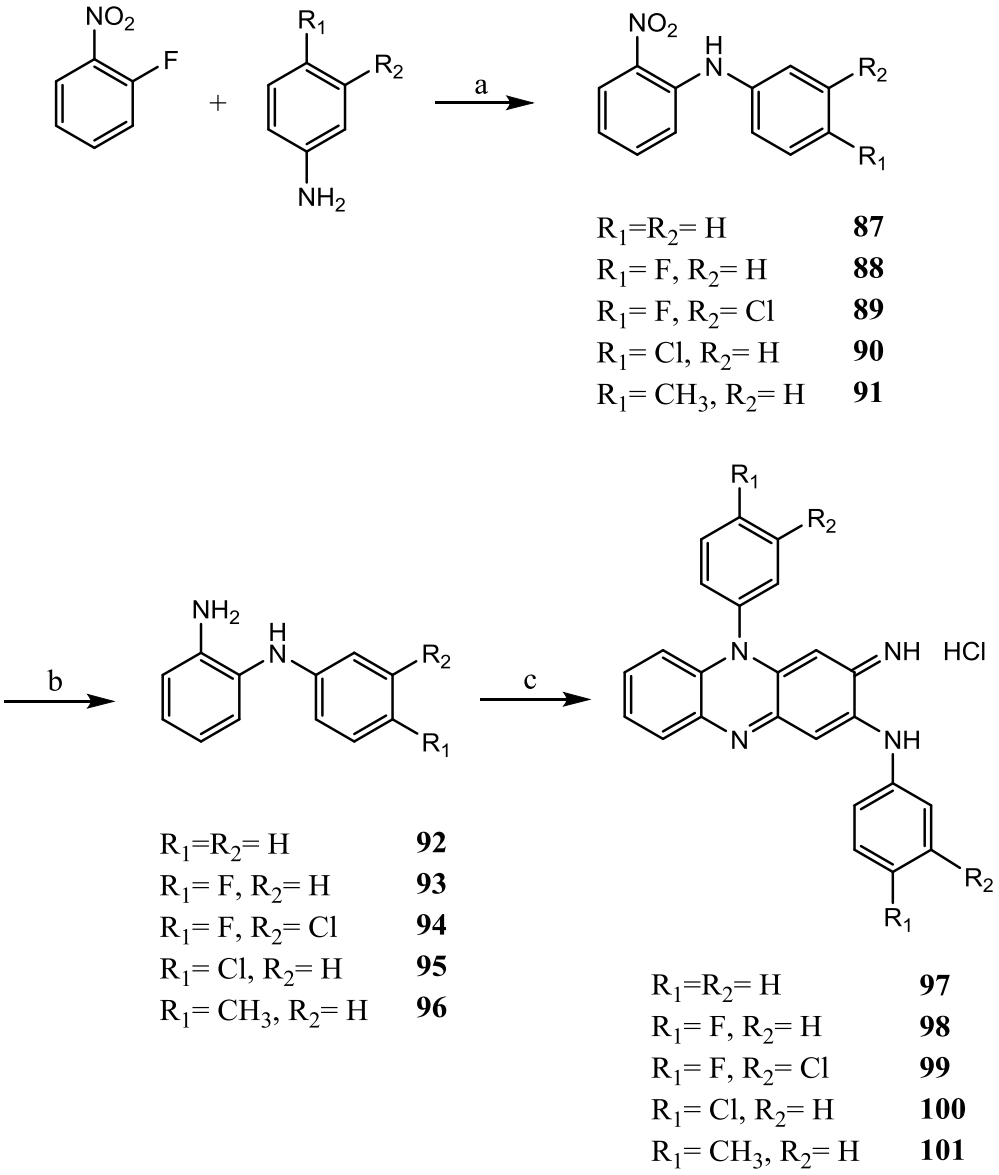
Finally, compound **81** was also converted into the corresponding 6-methoxy derivative **86**, that was then reacted with aminoguanidine hydrochloride or 1-amino-4-methylpiperazine in the presence of an acidic catalyst to obtain, respectively, compounds **26** and **27** (Scheme 11).[71,72]



Scheme 11. Reagents and conditions: a) NaOMe, dry THF/MeOH (2:1), reflux; b) aminoguanidine hydrochloride, AcOH, EtOH, reflux; c) 1-amino-4-methylpiperazine, 1N HCl, EtOH, reflux.

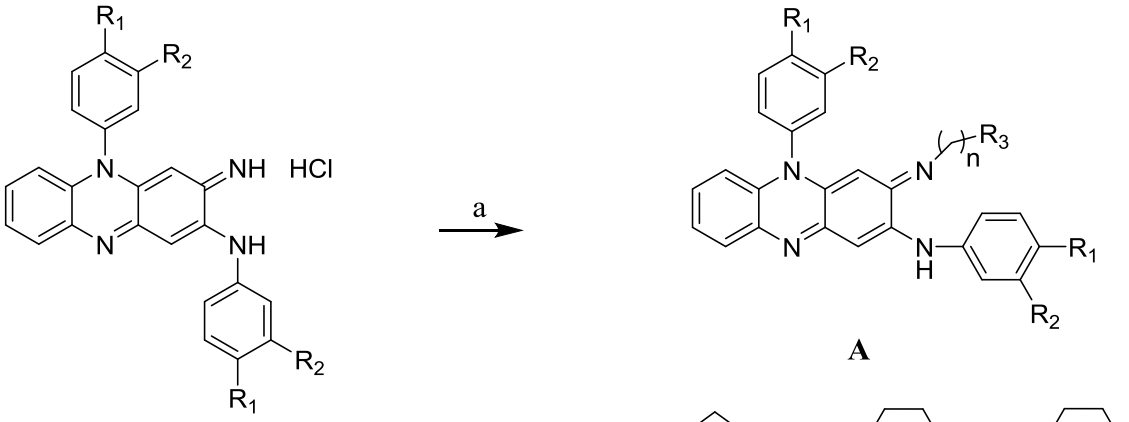
3.4 ***Riminophenazines***

The required iminophenazines hydrochloride **97-101** were prepared by oxidation of the corresponding N^1-(substituted-phenyl)benzene-1,2-diamine (in acid solution) with ferric chloride[73] (Scheme 12). Compounds **93-96** were prepared as reported in the scheme, whereas compound **92** was commercially available.

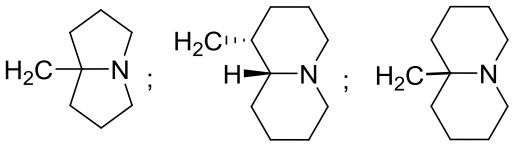


Scheme 12. Reagents and conditions: a) dry pyridine, reflux; b) $SnCl_2$, EtOH/AcOEt (1:1), reflux; c) $FeCl_3*6H_2O$, H_2O, conc. HCl, AcOH, r.t.

Compounds **28**-**35** were synthesized by reacting, in dioxane solution, the 2-[(4-R-phenyl)amino]-10-(4-R-phenyl)-2,10-dihydro-3-iminophenazines hydrochloride (**97**-**101**) with the suitable cyclic alkylamines, **(**(1S,9aR)- or (1R,9aR)-(octahydro-2H-quinolizin-1yl)alkylamines, (hexahydro-1*H*-pyrrolizin-1-yl)alkylamines, (octahydro-1H-quinolizin-9a-yl)alkan-1-amines and 3-(pyrrolidin-1-yl)propylamine in analogy to the previously described riminophenazines[74,75] (Scheme 13).



A



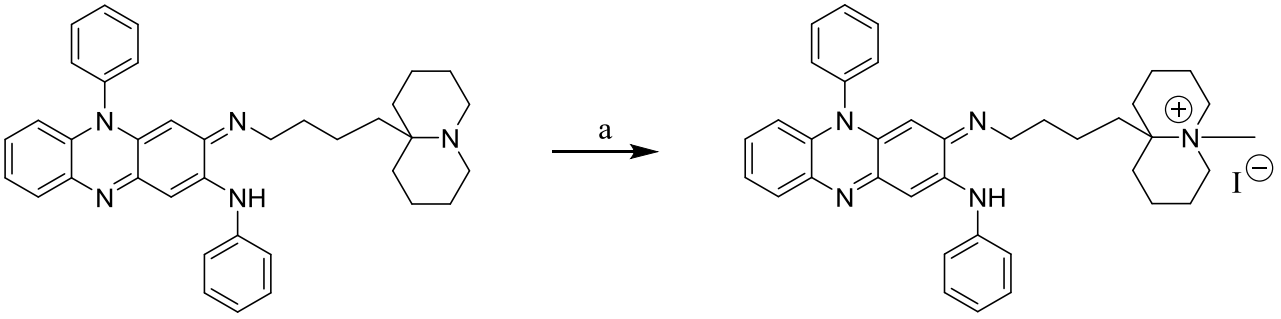
a b c

R_1=R_2= H **97**
R_1= F, R_2= H **98**
R_1= F, R_2= Cl **99**
R_1= Cl, R_2= H **100**
R_1= CH_3, R_2= H **101**

R_1	R_2	R_3	n	Compound
H	H	a	0	**28**
Cl	H	a	0	**29**
CH_3	H	b	0	**30**
H	H	c	3	**31**
H	H	c	5	**32**
F	H	c	3	**33**
F	H	c	5	**34**
F	Cl	c	5	**35**

Scheme 13. Reagents and conditions: a) R_3-$(CH_2)_n$-NH_2, 1,4-dioxane, reflux.

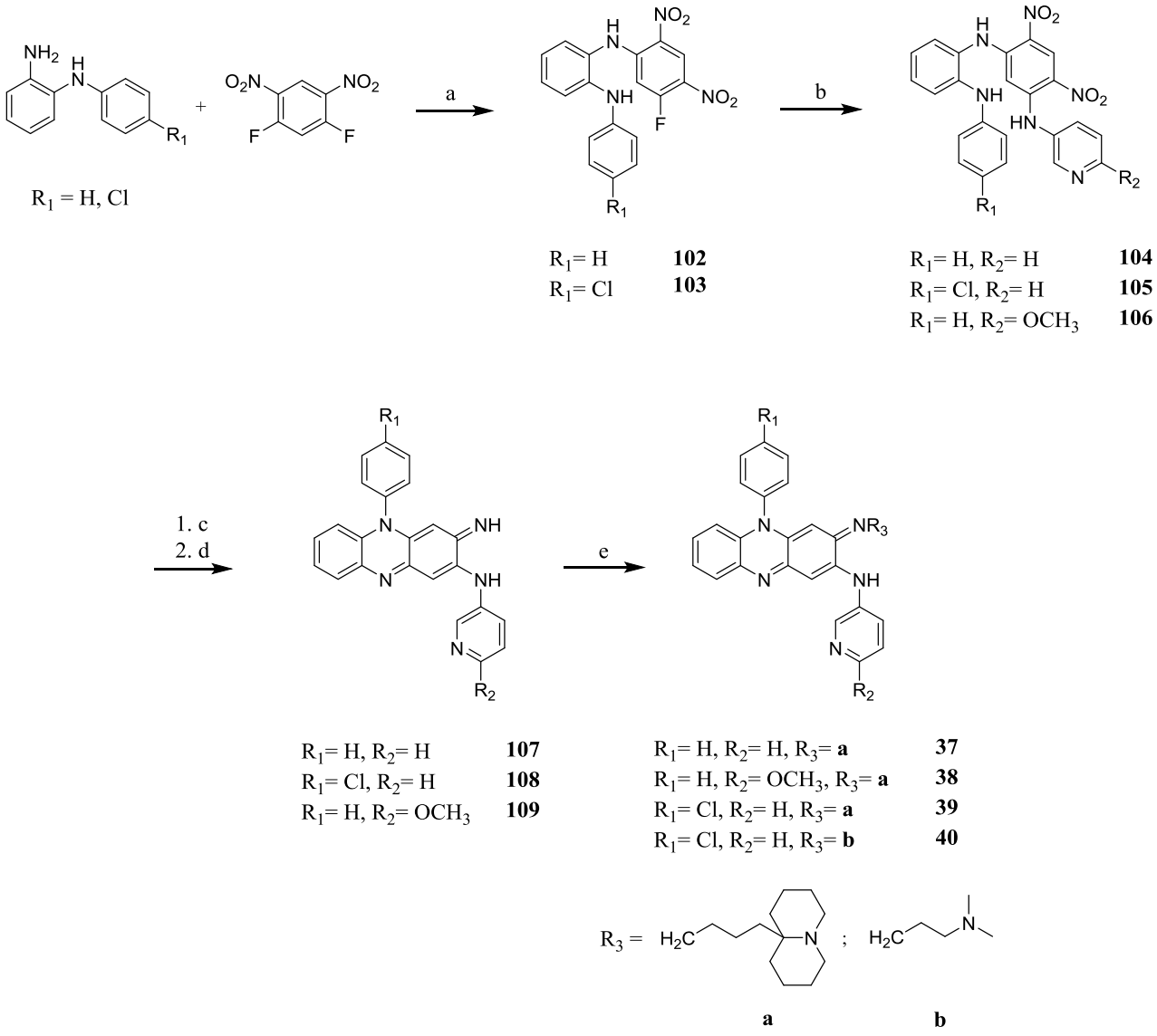
The quaternary ammonium salt **36** was obtained by reacting compound **31** with iodomethane, as shown in Scheme 14.



31 **36**

Scheme 14. Reagents and conditions: a) CH_3I, dry THF, r.t.

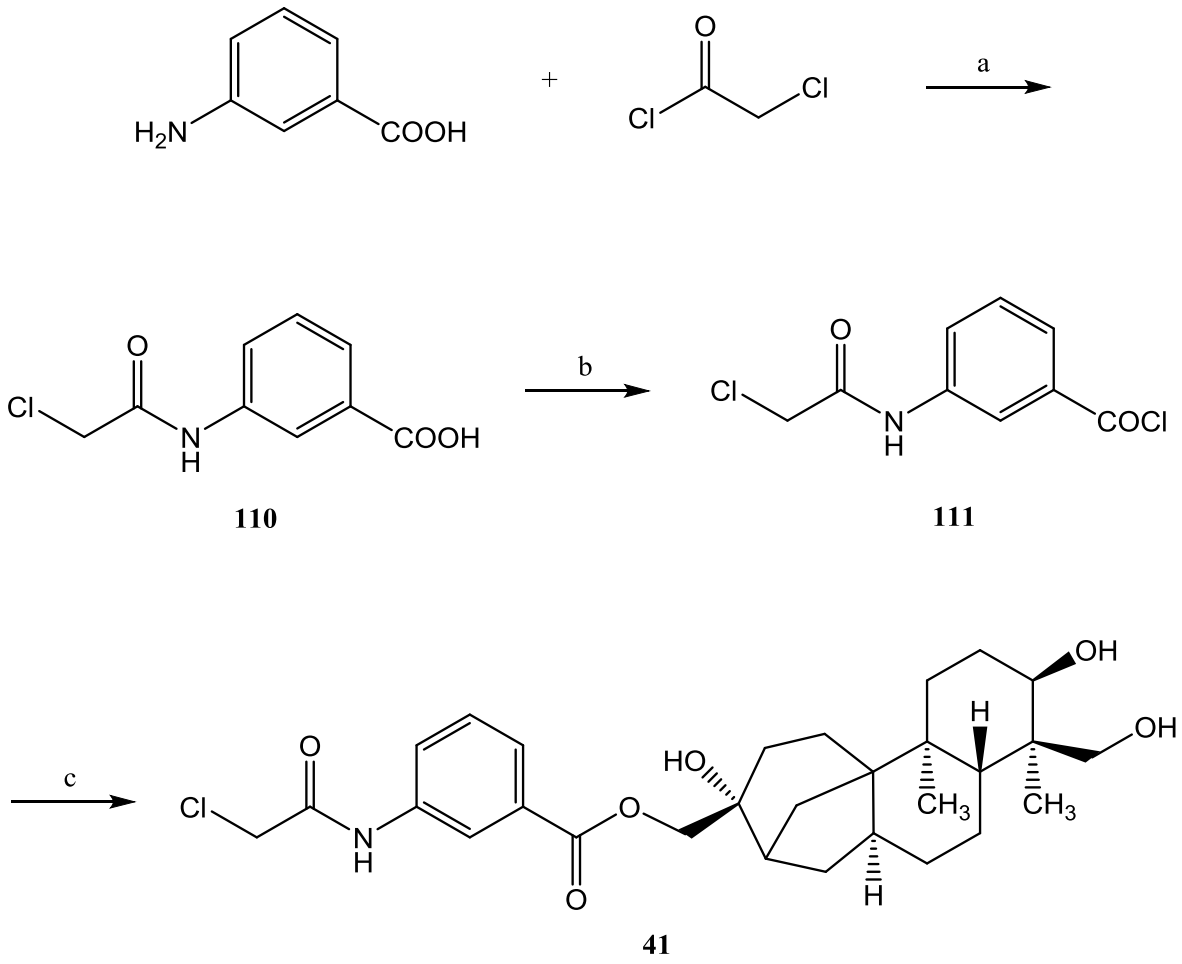
As reported by Zhang *et al.*[76] and by Liu *et al.*,[77] phenylpyridinyliminophenazine compounds (**37-40**) have been prepared in five steps, as indicated in Scheme 15. The suitable substituted N^1-phenylbenzene-1,2-diamines, previously synthesized or commercially available, were condensed with 1,5-difluoro-2,4-dinitrobenzene in the presence of triethylamine (TEA). Nitrocompounds (**102** and **103**) were reacted with pyridin-3-amine or 6-methoxypyridin-3-amine to afford compounds **104-106,** which after reduction with metallic zinc and acetic acid to the corresponding diamino derivatives, underwent a spontaneous oxidative cyclization reaction. The obtained imino compounds (**107-109)** were then reacted with 4-(octahydro-1H-quinolizin-9a-yl)butan-1-amine or with N^1,N^1-dimethylpropane-1,3-diamine to afford the final products (**37**-**40**).



Scheme 15. Reagents and conditions: a) TEA, EtOH, r.t.; b) 3-aminopyridine or 6-methoxypyridin-3-amine, TEA, dry THF, reflux; c) Zn, AcOH, r.t.; d) air, MeOH, r.t.; e) 4-(octahydro-1H-quinolizin-9a-yl)butan-1-amine or N^1,N^1-dimethylpropane-1,3-diamine, 1,4-dioxane, reflux.

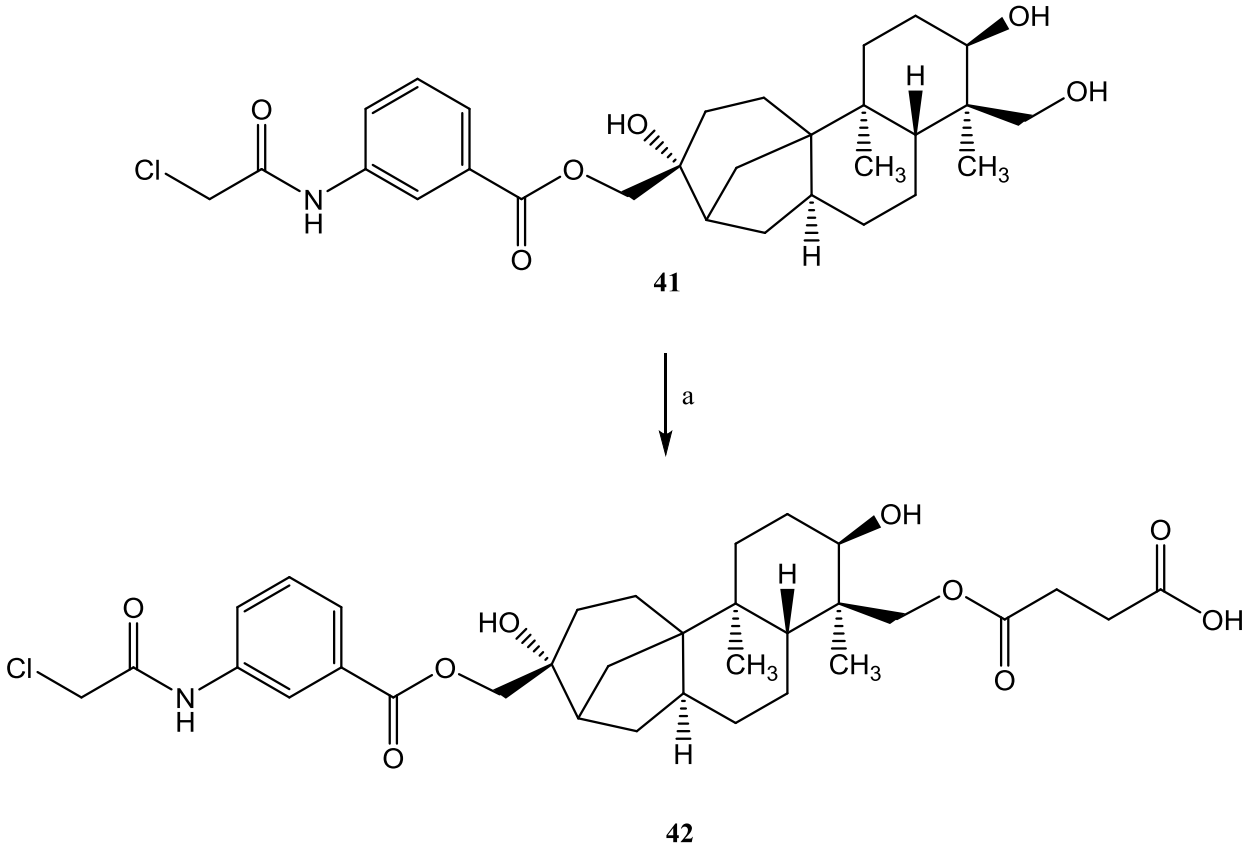
3.5 ***Aphidicolin derivatives***

The synthesis of compound **41** required the previous preparation of intermediate **111**, which was obtained through the conversion of the acid **110** to the corresponding acyl chloride. Compound **110** was prepared reacting 3-aminobenzoic acid with chloroacetyl chloride.[78] Intermediate **111** was reacted with aphidicolin in the presence of pyridine to get the final compound **41** (Scheme 16).



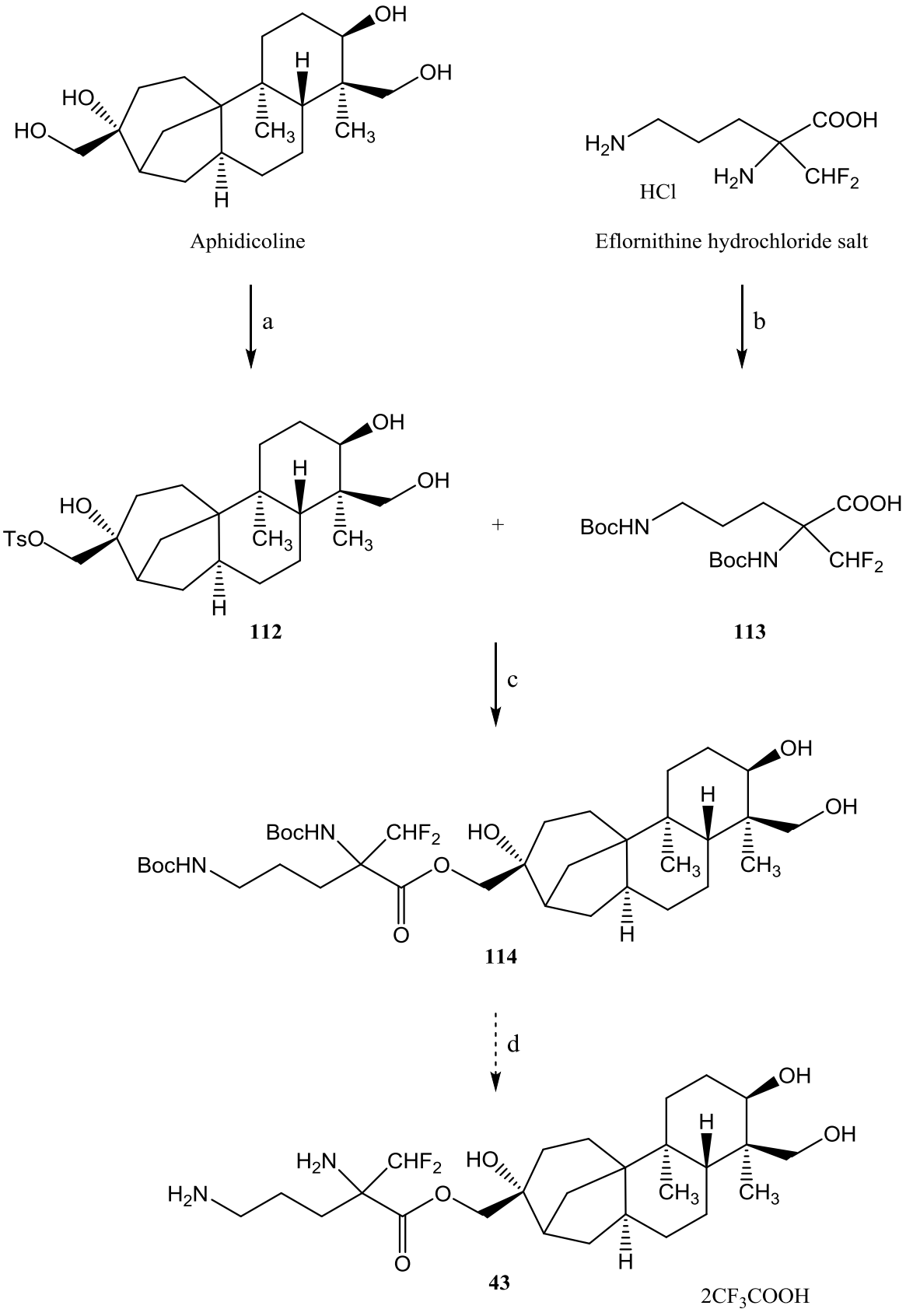
Scheme 16. Reagents and conditions: a) dry DMF, r.t.; b) $SOCl_2$, dry $CHCl_3$, 60 °C; c) dry pyridine, r.t.

Compound **42** was prepared treating compound **41** with succinic anhydride in the presence of pyridine (Scheme 17).[79]



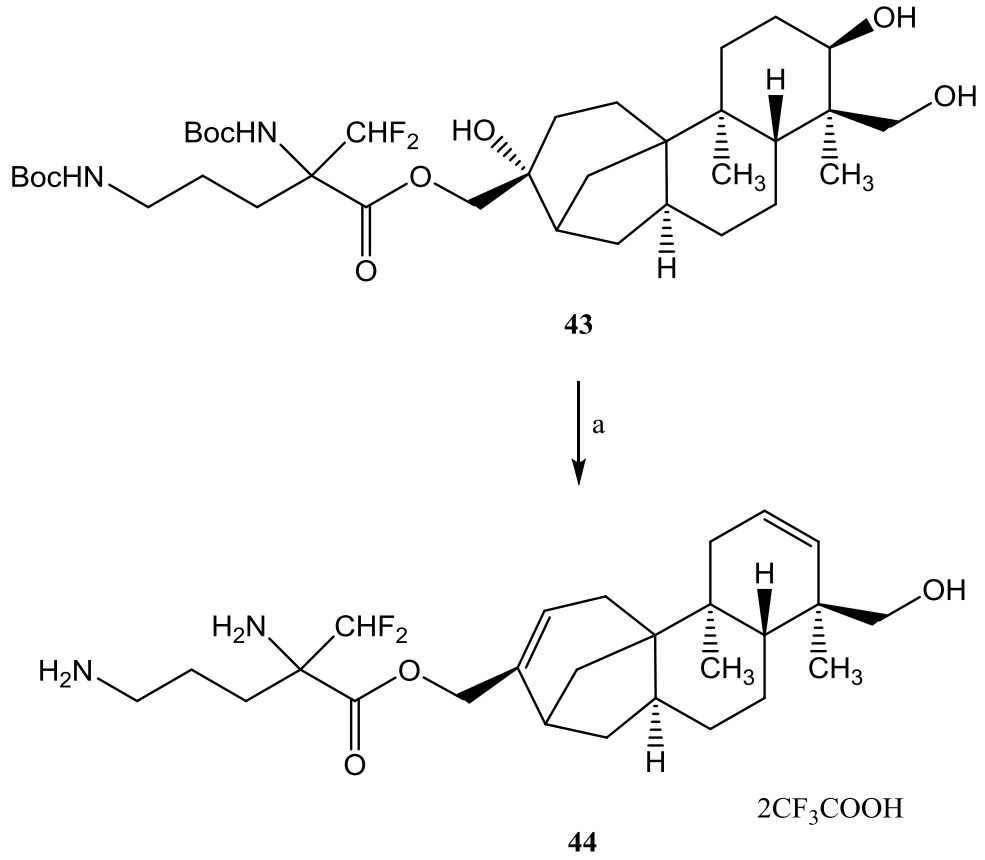
Scheme 17. Reagents and conditions: a) succinic anhydride, dry pyridine, r.t.

For the preparation of compound **43** it was necessary to protect the amino groups of eflornithine with Boc groups[80] and to tosylate the OH in position 17 of aphidicoline.[81] Then the two obtained intermediates were condensed to generate compound **112** which, after the cleavage of Boc groups, should have provided the final compound **43** (Scheme 18).



Scheme 18. Reagents and conditions: a) TsCl, dry pyridine, 4 °C; b) $(Boc)_2O$, DIPEA, MeOH, r.t.; c) DIPEA, dry THF, reflux; d) TFA, dry CH_2Cl_2, r.t.

Unfortunately the last step of the synthesis did not generate the expected compound **43**, but it probably underwent a loss of two molecules of water obtaining compound **44** (Scheme 19), which was confirmed by the mass spectrometry analysis.



Scheme 19. Reagents and conditions: a) TFA, dry $CHCl_3$, r.t.

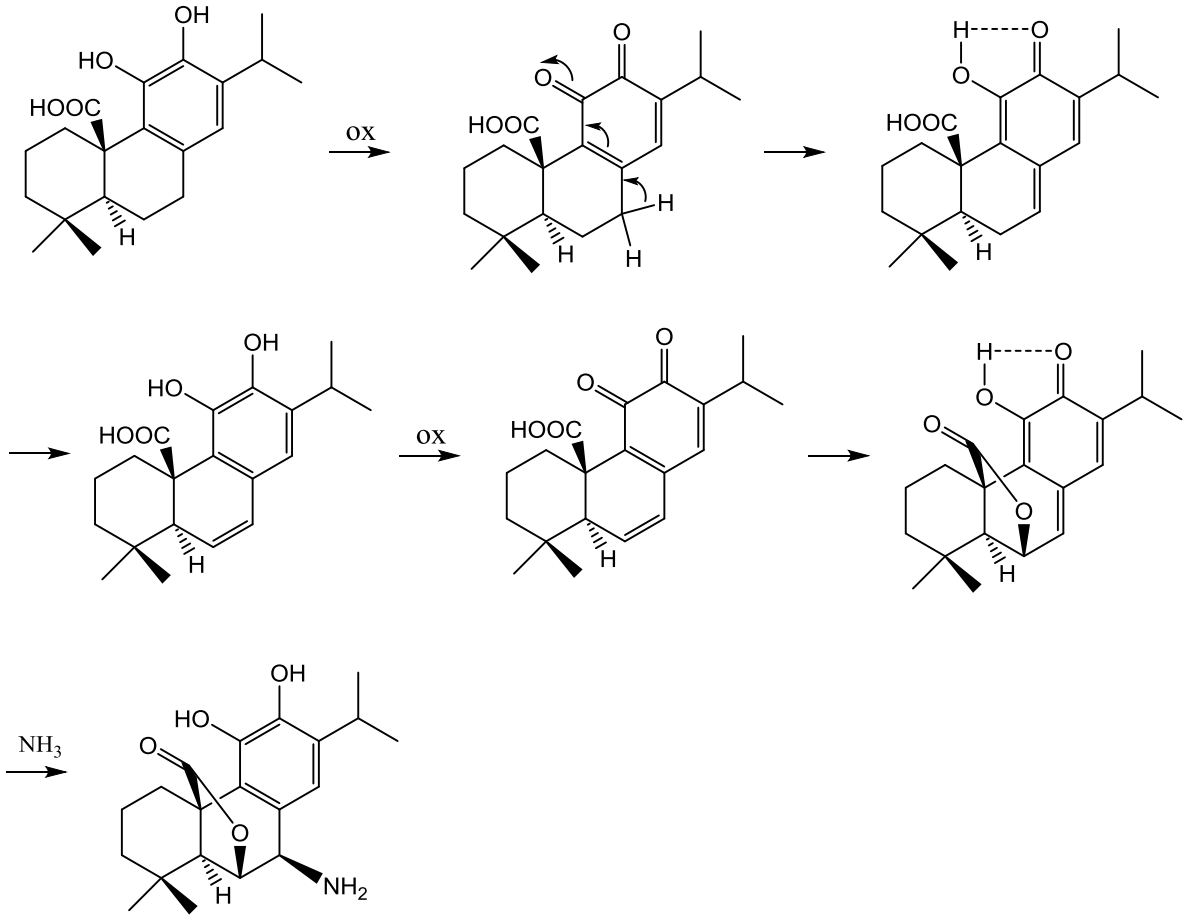
3.6 ***Rosmaricine and derivative***

Before preparing rosmaricine, I had to optimize the extraction of carnosic acid from rosemary leaves, based on the procedure described by A. Boido *et al*.[82] Firstly I tried to follow the reported method, macerating the well-broken leaves in 95% ethanol for two days and repeating the same process twice. Afterwards I thought to shorten this step by using ultrasound sonication, making three cycles of 1 h. Indeed, this modification was helpful and reduced the extraction time from one week to two days.

In addition, after the ultrasound treatment and the filtration of the formed waxes, I introduced also a washing with water, in order to remove all the hydrophilic compounds present in the extract. During this operation, I observed very often the formation of rough emulsions that were solved only with the use of a centrifuge. Moreover, as carnosol (another alkaloid present in rosemary extract) was found to be insoluble in cyclohexane, I introduced a washing with the same solvent after the treatment with water, with the aim to remove the compound from the extract of interest.

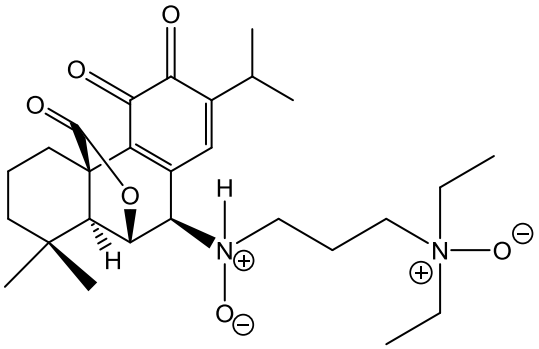
The extract was then treated with ammonia and O_2 to prepare rosmaricine. I tried different reaction conditions in order to evaluate the best parameters and experience demonstrated that a short reaction time and the maintenance of a constant pH value (between 8 and 9) by the addition of three portions of ammonia, provided the best results in terms of yield and purity.

In Scheme 20 is reported the proposed mechanism of rosmaricine formation,[82] which concerns different oxidation and rearrangement steps with the final attack of ammonia to obtain rosmaricine.



Scheme 20. Proposed mechanism of rosmaricine formation.[82]

To prepare rosmaricine derivative (**45**) I followed the same procedure used for rosmaricine, using 3-(diethylamino)propylamine in the place of ammonia. Unfortunately, the high-resolution mass spectrometry analysis (ESI) revealed that the obtained compound was not the expected one. It was probably an oxidation product, both at the cathecolic and at the amino groups level. The hypothetical obtained compound (**46**) is reported below:



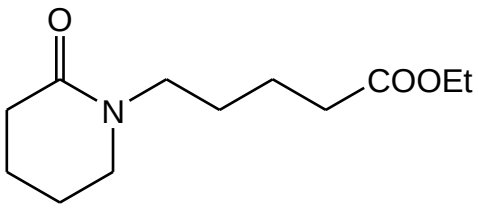
46

4. EXPERIMENTAL SECTION

All commercially available solvents and reagents were used without further purification, unless otherwise stated. CC= flash column chromatography. Mps: Büchi apparatus, uncorrected. ^{1}H-NMR spectra: Varian Mercury 300VX spectrometer; $CDCl_3$, DMSO-d_6, CD_3OD or pyridine-d_5; δ in ppm, J in Hertz. High-resolution mass spectra (HRMS): FT-Orbitrap mass spectrometer in positive electro spray ionization (ESI). Ultrasound sonicator: Eurosonic MU-2.5 l. Centrifuge: HERMLE Z206A. Preparative HPLC: Waters 3000 equipped with an Ascentis™ C18 column, 25 cm x 21.2 mm, 10 μM. Microwave reactor: Biotage® initiator classic.

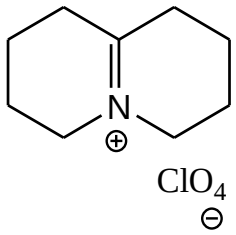
4.1 *4-Aminoquinoline derivatives*

Ethyl 5-(2-oxopiperidin-1-yl)pentanoate (47)



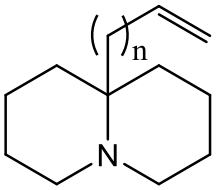
A solution of δ-valerolactam (8 g, 80.7 mmol) in 80 ml of anhydrous THF was added dropwise to an ice-cooled suspension of NaH (3.4 g, 84.0 mmol) in 100 ml of anhydrous THF. After stirring at r.t. for 30 minutes and cooling with an ice-bath, a solution of ethyl 5-bromovalerate (12.8 ml, 80.7 mmol) in 60 ml of anhydrous THF was added dropwise. The mixture was refluxed under nitrogen for 20 h. The cooled resulting mixture was diluted with ethyl acetate and filtered on a celite pad. The filtered mixture was evaporated, diluted with ethyl acetate and washed three times with a saturated solution of aqueous NH_4Cl and two times with brine. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness, resulting in a yellow oil. Yield: 90%. ^{1}H NMR ($CDCl_3$) δ: 4.12 (q, 2H, J= 2.76 Hz); 3.40-3.36 (m, 2H); 3.28-3.25 (m, 2H); 2.41-2.30 (m,4H); 2.08-2.07 (m, 2H); 1.79-1.77 (m, 4H); 1.60-1.58 (m, 2H); 1.24 (t; 3H, J= 2.76 Hz).

1,2,3,4,5,6,7,8,9-Octahydroquinolizinium perchlorate (48)



Compound **47** (16.5 g, 72,6 mmol) was ground with powdered soda lime (24.8 g) until an intimate mixture was obtained. The mixture was placed in a 250 ml round bottom flask fitted with a short-path distillation apparatus. The system was purged with N_2 under vacuum and the flask heated slowly. Vigorous gas evolution occurred, followed by distillation of a mixture of ethanol and water (80°-105°C) and then organic material (160-170°C). After distillation had stopped, the two-phase distillate was diluted with *tert*-butyl-methyl-ether and washed with brine for three times. The aqueous layer was saturated with K_2CO_3 and washed three times again with t*ert*-butyl-methyl ether. The collected organic layers were dried with anhydrous Na_2SO_4 and evaporated to dryness. The resulting yellow oil was poured slowly into an ice-cooled mixture of 8 ml of 70% perchloric acid and 100 ml of ethanol. Storage in the freezer overnight followed by filtration gave a light pink solid. Yield: 33%. ^{1}H NMR (DMSO-d_6) δ: 3.60-3.58 (m, 4H); 2.69-2.49 (m, 4H); 2.49-2.48 (m, 4H); 1.86-1.62 (m, 4H).

9a-(Alkyl-enyl)-octahydro-1H-quinolizines (49-51)



n= 2 **49**
n= 4 **50**
n= 6 **51**

General procedure: A solution of n-bromo-1-alkene (17.3 mmol) in 5 ml of anhydrous THF was added dropwise to a mixture of granular magnesium (17.3 mmol) and a catalytic amount of zinc bromide in 9 ml of anhydrous THF. After the addition of a iodine crystal to trigger the reaction, the mixture was stirred for 4 h at 50 °C under nitrogen. When the Grignard reagent was completely formed, 30 ml of anhydrous THF and 1,2,3,4,5,6,7,8,9-octahydroquinolizinium perchlorate (8.65 mmol) were charged and the resulting mixture was refluxed for 20 h. After cooling, the mixture was diluted with 10 ml of a saturated solution of aqueous NH_4Cl and acidified with 6N HCl. The aqueous layer was separated and extracted three times with diethyl ether, then alkalized and saturated with K_2CO_3 and extracted six times with diethyl ether. The collected organic layers were dried with anhydrous Na_2SO_4 and evaporated to dryness to give an orange oil, which was directly used for the next step.

9a-(But-3-enyl)-octahydro-1H-quinolizine (49)

Yield: 89%. ^{1}H NMR ($CDCl_3$) δ: 5.92-5.78 (m, 1H); 5.00-4.91 (m, 2H); 2.63-2.55 (m, 2H); 2.44-2.26 (m, 2H); 1.92-1.23 (m, 13H); 1.22-1.11 (m, 3H).

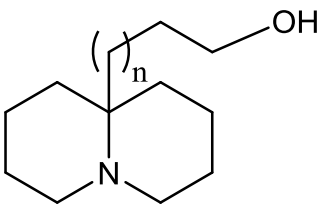
9a-(Hex-5-enyl)-octahydro-1H-quinolizine (50)

Yield: 63%. ^{1}H NMR ($CDCl_3$) δ: 5.92-5.78 (m, 1H); 5.02-4.91 (m, 2H); 2.63-2.55 (m, 2H); 2.44-2.30 (m, 2H); 2.18-2.01 (m, 2H); 1.92-1.11 (m, 18H).

9a-(Oct-7-enyl)-octahydro-1H-quinolizine (51)

Yield: 54%. ^{1}H NMR ($CDCl_3$) δ: 5.82-5.77 (m, 1H); 5.03-4.91 (m, 2H); 2.92-2.39 (m, 6H); 2.26-1.20 (m, 22H).

ω-(Octahydro-1H-quinolizin-9a-yl)alkan-1-ols (52-54)



n= 2 **52**
n= 4 **53**
n= 6 **54**

General procedure: A solution of 9a-(alkyl-enyl)-octahydro-1H-quinolizine (9.3 mmol) in 40 ml of anhydrous THF was added dropwise during 30 minutes to an ice-cooled mixture of 1M borane tetrahydrofurane complex solution (37.2 mmol) and 2-methyl-2-butene (74.5 mmol) diluted with 7 ml of anhydrous THF. The mixture was stirred at r.t. for 4.5 h and then diluted with 11 ml of H_2O, 37 ml of aqueous 6N NaOH and 9.2 ml of aqueous H_2O_2 (35%). The resulting mixture was stirred at r.t. for 1 h and the aqueous layer was saturated with Na_2CO_3, separated from the organic solution and extracted three times with diethyl ether. The collected organic layers were evaporated and the resulting oil was diluted with diethyl ether and extracted three times with aqueous 6N HCl. The aqueous layer was alkalized and saturated with Na_2CO_3 and extracted several times with diethyl ether. The organic layer, dried with anhydrous Na_2SO_4 and evaporated to dryness, gave a crude oil which was purified by CC (neutral alumina, grade IV; petroleum ether/diethyl ether in gradient).

4-(Octahydro-1H-quinolizin-9a-yl)butan-1-ol (52)

CC (petroleum ether/diethyl ether in gradient); the obtained oil crystallized spontaneously into a white solid, which was rinsed with petroleum ether/diethyl ether (1:1). Yield: 80%. Mp 92.4-93.2 °C. ^{1}H NMR (DMSO-d_6) δ: 4.33 (br s, 1H, collapsed with D_2O); 3.40-3.30 (m, 2H); 2.50-2.44 (m, 2H); 2.28-2.24 (m, 2H); 1.52-1.32 (m, 14H); 1.12-0.96 (m, 4H).

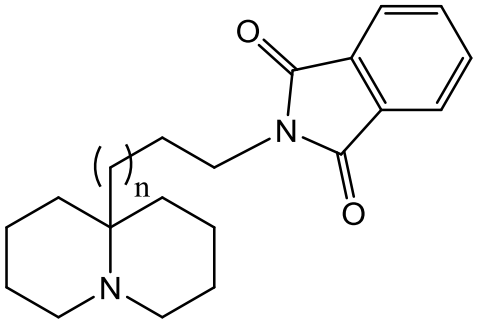
6-(Octahydro-1H-quinolizin-9a-yl)hexan-1-ol (53)

CC (petroleum ether/diethyl ether in gradient); a light yellow oil was obtained. Yield: 74%. ^{1}H NMR (DMSO-d_6) δ: 4.31 (br s, 1H, collapsed with D_2O); 3.39-3.30 (m, 3H); 2.51-2.44 (m, 3H); 2.28-2.25 (m, 2H); 1.50-1.38 (m, 16H); 1.27-1.00 (m, 4H).

8-(Octahydro-1H-quinolizin-9a-yl)octan-1-ol (54)

CC (petroleum ether/diethyl ether in gradient); a light yellow oil was obtained. Yield: 74%. ^{1}H NMR ($CDCl_3$) δ: 3.66-3.62 (m, 2H); 2.64-2.55 (m, 2H); 2.42-2.38 (m, 2H); 1.69-1.08 (m, 26H). 1H is not visible, probably for the interaction with the solvent.

2-(ω-(Octahydro-1H-quinolizin-9a-yl)alkyl)isoindoline-1,3-diones (55-57)



n= 2 **55**
n= 4 **56**
n= 6 **57**

General procedure: Diethylazodicarbossilate (16.7 mmol) diluted with 6.5 ml of anhydrous THF were added dropwise to an ice-cooled mixture of 8-(octahydro-1H-quinolizin-9a-yl)alkan-1-ol (8.0 mmol), phthalimide (16.0 mmol), triphenylphosphine (16.0 mmol) and 24 ml of anhydrous THF. The resulting mixture was stirred at r.t. for 20 h. The solvent was evaporated and the crude orange oil was purified by CC (silica gel; CH_2Cl_2/MeOH/conc. NH_3 in gradient).

2-(4-(Octahydro-1H-quinolizin-9a-yl)butyl)isoindoline-1,3-dione (55)

A yellow oil was obtained. Yield: 95%. ^{1}H NMR ($CDCl_3$) δ: 7.83-7.71 (m, 4H); 3.69-3.47 (m, 2H); 2.58-2.42 (m, 4H); 1.59-1.17 (m, 18H).

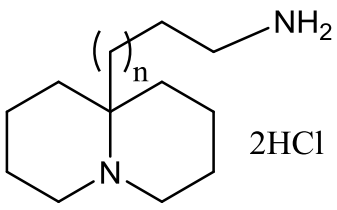
2-(6-(Octahydro-1H-quinolizin-9a-yl)hexyl)isoindoline-1,3-dione (56)

An orange oil was obtained. Yield: 98%. ^{1}H NMR ($CDCl_3$) δ: 7.85-7.80 (m, 2H); 7.73-7.69 (m, 2H); 3.68 (t, 2H, J= 7.42 Hz); 2.64-2.59 (m, 2H); 2.56-2.40 (m, 2H); 1.68-1.00 (m, 22H).

2-(8-(Octahydro-1H-quinolizin-9a-yl)octyl)isoindoline-1,3-dione (57)

An orange oil was obtained. Yield: 96%. ^{1}H NMR ($CDCl_3$) δ: 7.85-7.81 (m, 2H); 7.73-7.69 (m, 2H); 3.70-3.65 (m, 2H); 3.48-3.47 (m, 2H); 2.66-2.52 (m, 4H); 1.67-1.09 (m, 24H).

ω-(Octahydro-1H-quinolizin-9a-yl)alkan-1-amines dihydrochloride (58-60)



n= 2 **58**
n= 4 **59**
n= 6 **60**

General procedure: 2-(n-(Octahydro-1H-quinolizin-9a-yl)alkyl)isoindoline-1,3-dione (5.9 mmol) were dissolved in 20 ml of aqueous 6N HCl and refluxed for 20 h. The solution was stored in the freezer overnight and the precipitated phthalic acid was filtered and washed with a small amount of cold water. The filtered aqueous solution was evaporated under vacuum and the resulting amorphous solid was crystallized and rinsed with different solvents.

4-(Octahydro-1H-quinolizin-9a-yl)butan-1-amine dihydrochloride (58)

Solid was crystallized with a mixture of ethanol/diethyl ether (9.5:0.5) and rinsed with diethyl ether to give a white solid. Yield: 88%. Mp 221.0-222.7 °C. ^{1}H NMR (DMSO-d_6) δ: 10.45 (br s, 1H, collapsed with D_2O); 8.08 (br s, 3H, collapsed with D_2O); 3.10-3.02 (m, 2H); 2.89-2.78 (m, 4H); 1.95-1.03 (m, 18H).

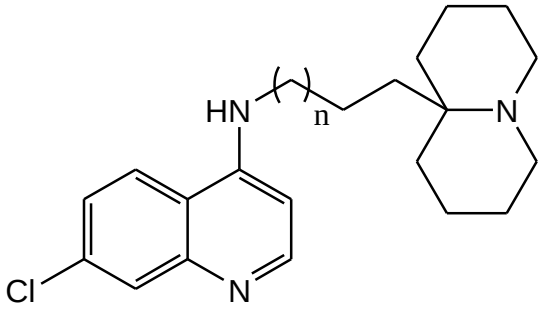
6-(Octahydro-1H-quinolizin-9a-yl)hexan-1-amine dihydrochloride (59)

Solid was crystallized with a mixture of ethanol/diethyl ether (1:1) and rinsed with diethyl ether to give a white solid. Yield: 59%. Mp 184-185 °C. ^{1}H NMR (DMSO-d_6) δ: 10.56 (br s, 1H, collapsed with D_2O); 8.10 (br s, 3H, collapsed with D_2O); 3.32-3.10 (m, 2H); 2.88-2.85 (m, 2H); 2.72-2.49 (m, 2H); 1.97-1.12 (m, 22H).

8-(Octahydro-1H-quinolizin-9a-yl)octan-1-amine dihydrochloride (60)

Solid was crystallized with diethyl ether and rinsed with a mixture of diethyl ether/ethanol (9:1) to obtain an hazel hygroscopic solid. Yield: 84%. ^{1}H NMR (DMSO-d_6) *δ:* 10.27 (br s, 2H, collapsed with D_2O); 7.99 (br s, 2H, collapsed with D_2O); 3.45-2.72 (m, 8H); 1.93-1.01 (m, 24H).

7-Chloro-N-(ω-(octahydro-1H-quinolizin-9a-yl)alkyl)quinolin-4-amines (1-2)



n= 2 **1**
n= 4 **2**

General procedure: A mixture of suitable n-(octahydro-1H-quinolizin-9a-yl)alkan-1-amine dihydrochloride (0.88 mmol), 4,7-dichloroquinoline (0.97 mmol), phenol (4.41 mmol) and DIPEA (1.76 mmol) was heated under a N_2 atmosphere for 5 h at 130 °C. After cooling, the mixture was diluted with CH_2Cl_2 and the resulting organic layer washed three times with 2N NaOH solution and then with brine. After driing with anhydrous Na_2SO_4, the solvent was evaporated to dryness. The crude solid was purified by CC (silica gel; different eluents and conditions as indicated for each compound).

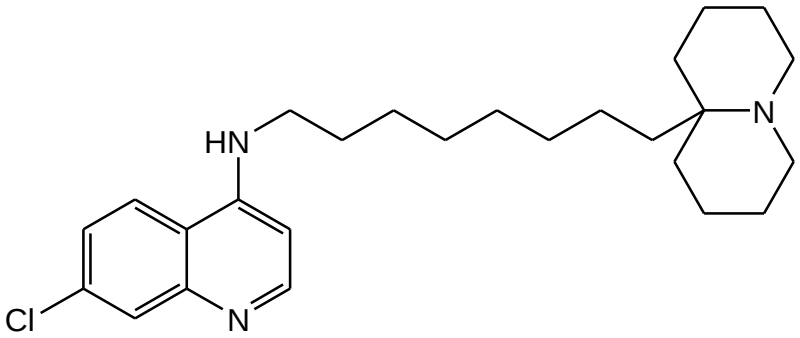
7-Chloro-N-(4-(octahydro-1H-quinolizin-9a-yl)butyl)quinolin-4-amine (1)

CC (CH_2Cl_2/MeOH; 94:6); solid washed with petroleum ether. Yield: 64%. Mp 157.8-159.3 °C. ^{1}H NMR ($CDCl_3$) δ: 8.54 (d, 1H, *J*= 5.50 Hz); 7.95 (d, 1H, *J*= 2.20 Hz); 7.65 (d, 1H, *J*= 8.80 Hz); 7.36 (dd, 1H, *J*= 2.20, 8.80 Hz); 6.43 (d, 1H, *J*= 5.50 Hz); 4.96 (br s, 1H, collapsed with D_2O); 3.37-3.31 (m, 2H); 2.64-2.42 (m, 4H); 1.84-1.19 (m, 18H).

7-Chloro-N-(6-(octahydro-1H-quinolizin-9a-yl)hexyl)quinolin–4–amine (2)

CC (CH_2Cl_2/MeOH; 92:8); amorphous solid crystallized and washed with diethyl ether. Yield: 46%. Mp 144.6-146.0 °C. ^{1}H NMR ($CDCl_3$) δ: 8.53 (d, 1H, *J*= 5.22 Hz); 7.96 (d, 1H, *J*= 2.20 Hz); 7.65 (d, 1H, *J*= 8.80 Hz); 7.36 (dd, 1H, *J*= 2.20, 8.80 Hz); 6.42 (d, 1H, *J*= 5.22 Hz); 4.96 (br s, 1H, collapsed with D_2O); 3.35-3.29 (m, 2H); 2.65-2.41 (m, 4H); 1.82-1.12 (m, 22H).

7-Chloro-N-(8-(octahydro-1H-quinolizin-9a-yl)octyl)quinolin-4-amine (3)

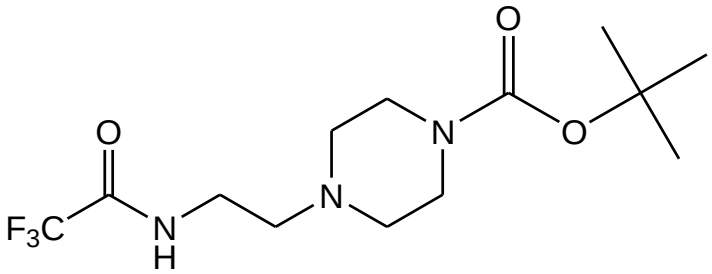


After converting compound **60** into the corresponding free base, a mixture of this amine (170 mg, 0.64 mmol), 4,7-dichloroquinoline (153 mg, 0.77 mmol) and phenol (301 mg, 3.20 mmol) was heated under a N_2 atmosphere for 3 h at 130 °C.[4] After cooling, the mixture was diluted with CH_2Cl_2 and the resulting organic layer washed two times with 2N NaOH solution and then with brine, dried with anhydrous Na_2SO_4 and evaporated to dryness. The crude solid was purified by CC (silica gel; CH_2Cl_2/MeOH; 94:6). The amorphous resulting solid was crystallized and washed with diethyl ether. Yield: 55%. Mp 116.8-118.3 °C.

The product was converted into the corresponding dihydrocloride salt with 1N HCl in ethanol. Mp: 250-252 °C (dec.) ^{1}H NMR (DMSO-d_6) δ: 10.20 (br s, 2H); 9.62 (br s, 1H); 8.70 (d, 1H, J= 8.80 Hz); 8.52 (d, 1H, J= 7.15 Hz); 8.06 (s, 1H); 7.76 (d, 1H, J= 8.80 Hz); 6.85 (d, 1H, J= 7.15 Hz); 3.52-3.49 (m, 2H); 3.40-3.35 (m, 2H); 3.09-2.86 (m, 2H); 1.91-1.05 (m, 26H).

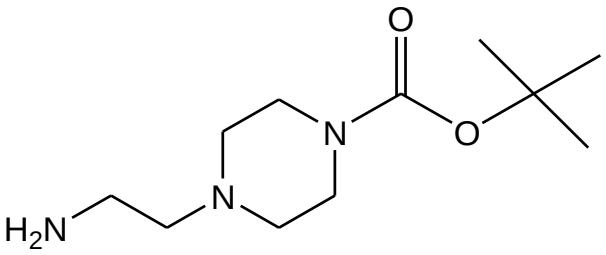
4.2 ***Hybrids of 4-aminoquinoline***

tert-Butyl 4-(2-(2,2,2-trifluoroacetamido)ethyl)piperazine-1-carboxylate (61)



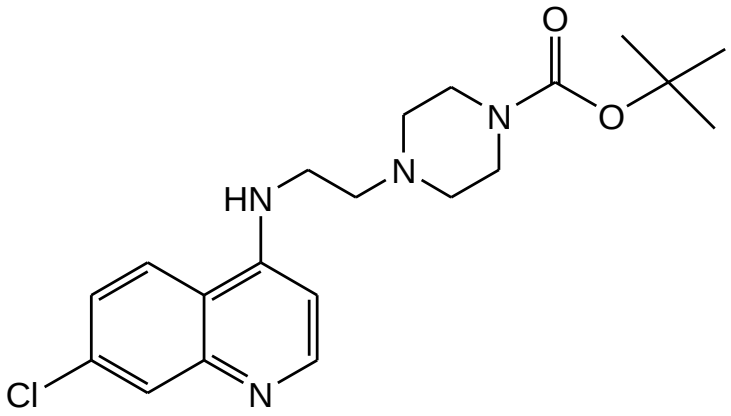
Ethyl trifluoroacetate (2.6 g, 0.02 mmol) was added dropwise to an ice-cooled solution of 1-(2-aminoethyl)piperazine (2.4 g, 0.02 mmol) and 2.5 ml of anhydrous THF. After stirring for 45 minutes at r.t. and under a N_2 atmosphere, the mixture was cooled with an ice-bath and $(Boc)_2O$ (4 g, 0.02 mmol) dissolved in 5 ml of anhydrous THF was added dropwise. The obtained solution was stirred for 24 h at r.t. The solvent was evaporated and the obtained residue was rinsed with ethyl acetate and washed with brine. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a brown oil that crystallized with diethyl ether. After washing with a mixture of diethyl ether/petroleum ether (1:1), a slightly pink solid was obtained. Yield: 85%. Mp 98.0-99.1°C. ^{1}H NMR ($CDCl_3$) δ: 7.02 (br s, 1H, collapsed with D_2O); 3.49-3.42 (m, 6H); 2.58 (t, 2H, J= 6.05 Hz); 2.44 (t, 4H, J= 6.05 Hz); 1.46 (s, 9H).

tert-Butyl 4-(2-aminoethyl)piperazine-1-carboxylate (62)



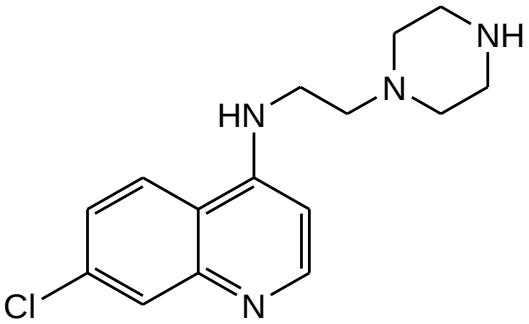
40 ml of 0.2 N NaOH were added drowise to an ice-cooled solution of compound **61** (2.36 g, 7.25 mmol) and 30 ml of methanol, keeping the internal temperature below 15 °C. Once the addition was over, the mixture was warmed to 35 °C and stirred for 48 h. The solvent was evaporated and the aqueous phase was saturated with K_2CO_3 and extracted with chloroform. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a yellow oil that was purified by CC (CH_2Cl_2/MeOH/ conc. NH_3 in gradient); the product eluted with 10% of MeOH and 1% of NH_3. A yellow oil was obtained. Yield: 94%. ^{1}H NMR ($CDCl_3$) δ: 3.42 (t, 4H, J= 4.95 Hz); 2.79 (t, 2H, J= 6.05 Hz); 2.45-2.37 (m, 6H); 1.65 (br s, 2H, collapsed with D_2O); 1.45 (s, 9H).

tert-Butyl 4-(2-((7-chloroquinolin-4-yl)amino)ethyl)piperazine-1-carboxylate (63)



4,7-Dichloroquinoline (380 mg, 1.92 mmol) was added to a solution of compound **62** (400 mg, 1.74 mmol), DIPEA (0.5 ml, 3.49 mmol) and phenol (820 mg, 8.72 mmol). The mixture was stirred at 120 °C under nitrogen for 4.5 h and then diluted with CH_2Cl_2 and washed with 2 N NaOH and brine. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a yellow oil that was purified by CC (CH_2Cl_2/MeOH in gradient); the product eluted with 6% of MeOH. A white solid was obtained. Yield: 73%. Mp 149.0-150.0 °C. ^{1}H NMR ($CDCl_3$) δ: 8.53 (d, 1H, *J*= 5.50 Hz); 7.96 (d, 1H, *J*= 2.20 Hz); 7.65 (d, 1H, *J*= 8.80 Hz); 7.37 (dd, 1H, *J*= 2.20, 8.80 Hz); 6.37 (d, 1H, *J*= 5.50 Hz); 5.89 (br s, 1H, collapsed with D_2O); 3.48 (t, 4H, *J*= 4.68 Hz); 3.38-3.32 (m, 2H); 2.79 (t, 2H, *J*= 6.05 Hz); 2.48 (t, 4H, *J*= 4.68 Hz); 1.46 (s, 9H).

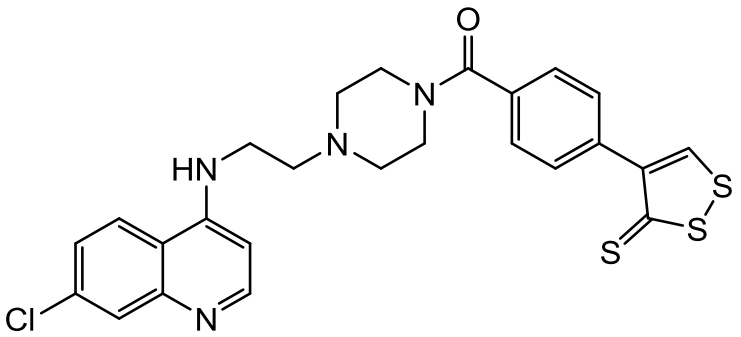
7-Chloro-N-(2-(piperazin-1-yl)ethyl)quinolin-4-amine (64)



A solution of compound **63** (500 mg, 1.28 mmol) and 2.5 ml of CF_3COOH was refluxed for 4.5 h. The mixture was then diluted with CH_2Cl_2 and water and alkalized with solid K_2CO_3. The obtained white precipitate was filtered and washed with water and diethyl ether. The filtrate was extracted several times with CH_2Cl_2 and the organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The obtained residue was recrystallized with CH_2Cl_2 and rinsed with diethyl ether to obtain a white solid. Yield: 83%. Mp 146.8-148.1 °C. ^{1}H NMR ($CDCl_3$) δ: 8.53 (d, 1H, *J*= 5.20 Hz); 7.96 (d, 1H, *J*= 2.20 Hz); 7.65 (d, 1H, *J*= 8.80 Hz); 7.38 (dd, 1H, *J*= 2.20, 8.80 Hz); 6.37 (d, 1H, *J*= 5.20 Hz); 6.00 (br s, 1H, collapsed with D_2O); 3.37-3.29 (m, 2H); 2.98-2.94 (m, 4H); 2.79-2.73 (m, 2H); 2.57-2.53 (m, 4H).

1H is not visible, probably for the interaction with the solvent.

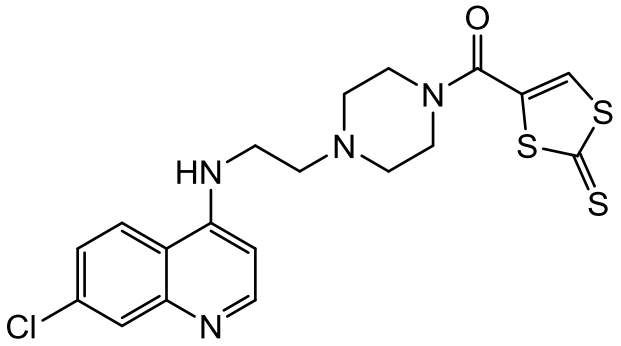
(4-(2-(7-Chloroquinolin-4-ylamino)ethyl)piperazin-1-yl)(4-(3-thioxo-3H-1,2-dithiol-4-yl)phenyl)methanone (4)



Compound **64** (150 mg, 0.52 mmol) was dissolved in 0.8 ml of anhydrous DMF and charged into a 0.5-2 ml microwave vial. To the solution HOBt (80 mg, 0.52 mmol), DCC (110 mg, 0.52 mmol) and 4-(3-thioxo-3H-1,2-dithiol-4-yl)benzoic acid (130 mg, 0.52 mmol) were added. The mixture was stirred in a microwave reactor at 55 °C for 1 h. After the filtration of the obtained DCU, the solvent was evaporated and the obtained residue was diluted with CH_2Cl_2 and washed with a saturated solution of $NaHCO_3$, water and brine. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness and the obtained residue was purified by CC (CH_2Cl_2/MeOH in gradient), using the Flash Chromatography Purification System Biotage SP-1; the product eluted with 3.5% of MeOH. After washing with diethyl ether, an orange solid was obtained. Yield: 79%. Mp 101.0-103.0 °C. ^{1}H NMR ($CDCl_3$) δ: 8.54 (d, 1H, *J*= 5.50 Hz); 8.44 (s, 1H); 7.96 (d, 1H, *J*= 2.20 Hz); 7.67 (d, 1H, *J*= 8.80 Hz); 7.69 (d, 2H, *J*= 8.25 Hz); 7.48 (d, 2H, *J*= 8.25 Hz); 7.39 (dd, 1H, *J*= 2.20, J= 9.08 Hz); 6.39 (d, 1H, *J*= 5.50 Hz); 5.82 (br s, 1H, collapsed with D_2O); 3.85-3.81 (m, 2H); 3.59-3.56 (m, 2H); 3.39-3.33 (m, 2H); 2.84-2.80 (m, 2H); 2.67-2.50 (m, 4H).

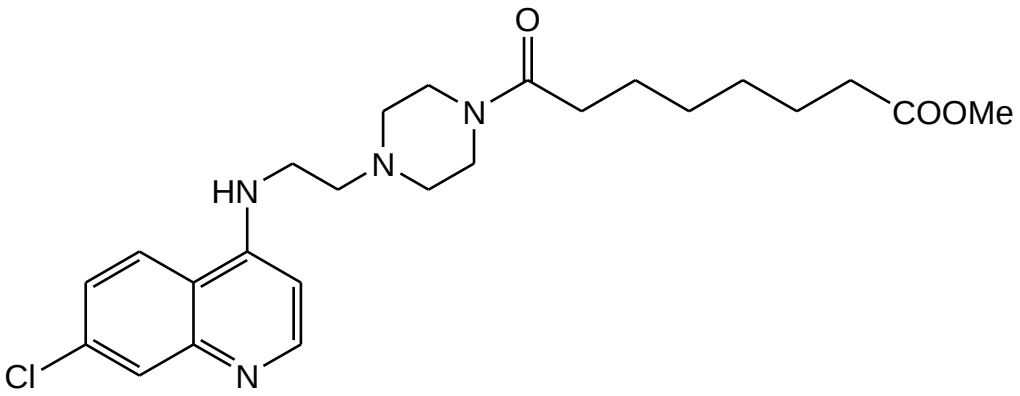
(4-(2-(7-Chloroquinolin-4-ylamino)ethyl)piperazin-1-yl)(2-thioxo-1,3-dithiol-4-yl)methanone

(5)



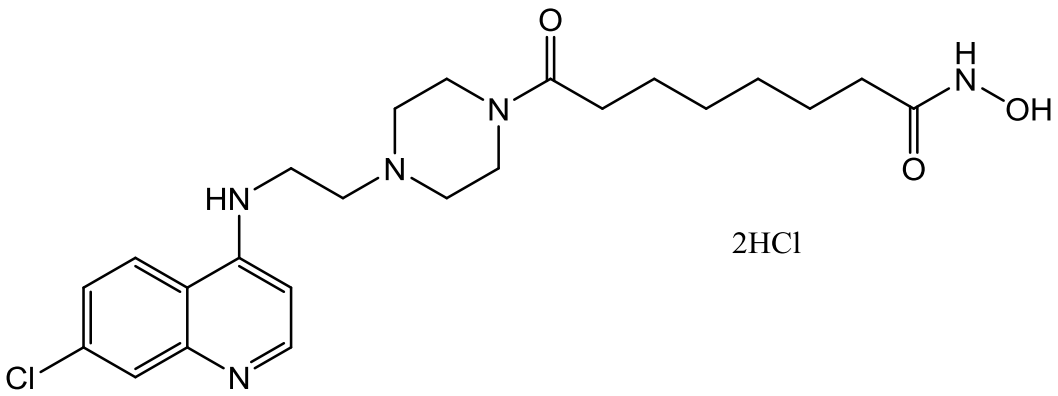
Compound **64** (150 mg, 0.52 mmol) was dissolved in 0.8 ml of anhydrous DMF and charged into a 0.5-2 ml microwave vial. To the solution HOBt (80 mg, 0.52 mmol), DCC (110 mg, 0.52 mmol) and 2-thioxo-1,3-dithiole-4-carboxylic acid (90 mg, 0.52 mmol) were added. The mixture was stirred in a microwave reactor at 55 °C for 1 h. After the filtration of the obtained DCU, the solvent was evaporated and the obtained residue was diluted with CH_2Cl_2 and washed with a saturated solution of $NaHCO_3$, water and brine. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The obtained residue was washed with diethyl ether and then purified by CC (CH_2Cl_2/MeOH in gradient); the product eluted with 5% of MeOH. After washing with diethyl ether, a yellow solid was obtained. Yield: 51%. Mp 191.9-193.7 °C. ^{1}H NMR (DMSO-d_6) δ: 8.38 (d, 1H, *J*= 5.22 Hz); 8.21 (d, 1H, *J*= 9.08 Hz); 7.87 (s, 1H); 7.77 (d, 1H, *J*= 2.20 Hz); 7.44 (dd, 1H, *J*= 2.20, 9.08 Hz); 7.20 (br s, 1H, collapsed with D_2O); 6.49 (d, 1H, *J*= 5.22 Hz); 3.64-3.57 (m, 4H); 3.42-3.38 (m, 2H); 2.70-2.60 (m, 2H); 2.52-2.45 (m, 4H).

Methyl 8-(4-(2-(7-chloroquinolin-4-ylamino)ethyl)piperazin-1-yl)-8-oxooctanoate (65)



Compound **64** (260 mg, 0.89 mmol) was dissolved in 1 ml of anhydrous DMF and charged into a 0.5-2 ml microwave vial. To the solution HOBt (140 mg, 0.89 mmol), DCC (180 mg, 0.89 mmol) and suberic acid monomethylester (0.16 ml, 0.89 mmol) were added. The mixture was stirred in a microwave reactor at 55 °C for 1 h. After the filtration of the DCU, the solvent was evaporated and the obtained residue was diluted with CH_2Cl_2 and washed with 2 N NaOH and brine. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a residue that was purified by CC (CH_2Cl_2/MeOH in gradient), using the Flash Chromatography Purification System Biotage SP-1; the product eluted with 4% of MeOH. A yellow amorphous solid was obtained. Yield: 79%. ^{1}H NMR ($CDCl_3$) δ: 8.53 (d, 1H, *J*= 5.50 Hz); 7.96 (d, 1H, *J*= 2.20 Hz); 7.65 (d,1H, *J*= 9.08 Hz); 7.37 (dd, 1H, *J*= 2.20, 9.08 Hz); 6.38 (d, 1H, *J*= 5.50 Hz); 5.84 (br s, 1H, collapsed with D_2O); 3.65 (s, 3H); 3.54-3.49 (m, 4H); 3.38-3.31 (m, 2H); 2.82-2.79 (m, 2H); 2.57-2.49 (m, 4H); 2.35-2.24 (m, 4H); 1.65-1.58 (m, 4H); 1.39-1.32 (m, 4H).

8-(4-(2-(7-Chloroquinolin-4-ylamino)ethyl)piperazin-1-yl)-N-hydroxy-8-oxooctanamide (6)

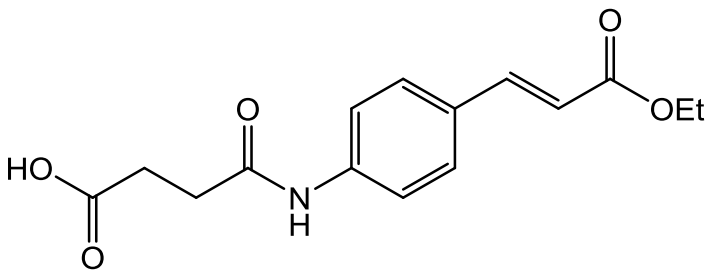


A solution of KOH (760 mg, 13.53 mmol) in 4.5 ml of methanol was added to an ice-cooled suspension of hydroxylamine hydrochloride (940 mg, 13.53 mmol) in 2.4 ml of methanol. After stirring for few minutes, the precipitated KCl was filtered off and the filtrate was charged into a flask containing compound **65** (310 mg, 0.68 mmol) and KOH (570 mg, 1.02 mmol). The mixture was refluxed for 4 h. The solvent was evaporated and the obtained residue was diluted with 15 ml of water. After acidification with glacial acetic acid (pH= 6), the aqueous phase was extracted with a mixture of CH_2Cl_2/MeOH.

The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain an amorphous solid that was crystallized and rinsed with diethyl ether. A pale yellow solid was obtained. Yield: 51%. Mp 58.2-60.0 °C.

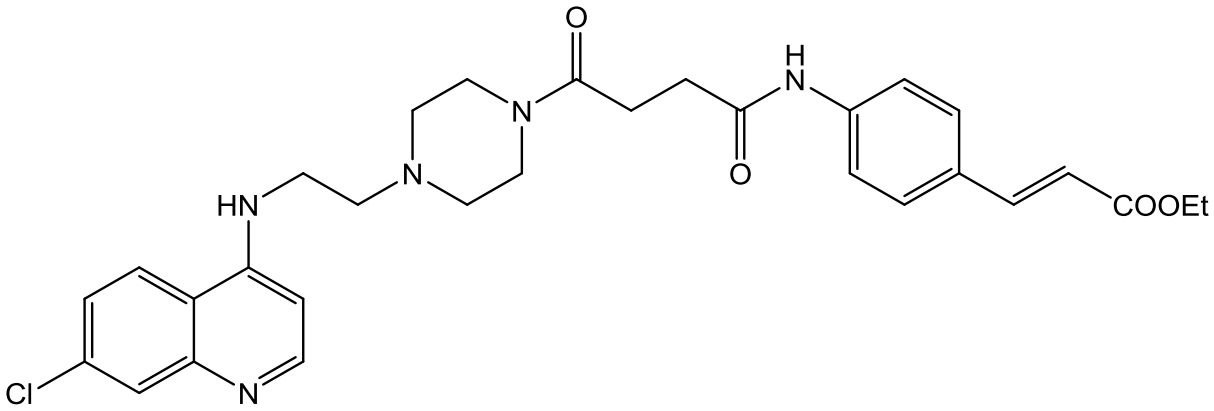
The product was converted into the corresponding dihydrochloride salt with 1N HCl in ethanol. Mp 213.6-215.2 °C. ^{1}H NMR (DMSO-d_6) δ: 11.49 (br s, 1H, collapsed with D_2O); 10.35 (br s, 1H, collpased with D_2O); 9.78 (br s, 1H, collapsed with D_2O); 8.81 (d, 1H, *J*= 9.37 Hz); 8.66 (d, 1H, *J*= 6.61 Hz); 8.11 (s, 1H); 7.79 (d, 1H, *J*= 9.37 Hz); 7.03 (d, 1H, *J*= 6.61 Hz); 4.45 (br s, 1H, collapsed with D_2O); 4.10-3.98 (m, 2H); 3.70-3.35 (m, 7H, 1H collapsed with D_2O); 3.20-2.87 (m, 4H); 2.39-2.35 (m, 2H); 1.95-1.89 (m, 2H); 1.52-1.42 (m, 3H); 1.24-1.18 (m, 3H).

(*E*)-4-((4-(3-Ethoxy-3-oxoprop-1-en-1-yl)phenyl)amino)-4-oxobutanoic acid (66)



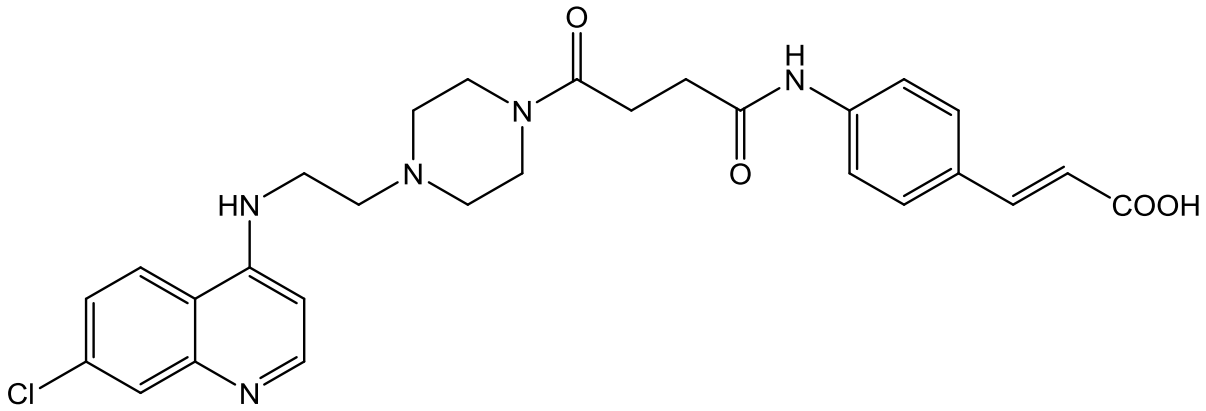
A solution of ethyl (E)-3-(4-aminophenyl)acrylate (500 mg, 2.62 mmol) and succinic anhydride (968 mg, 9.68 mmol) in a mixture of H_2O/THF (2:1) was stirred at r.t. for 40 minutes. The obtained precipitate was filtered, washed several times with water and then with diethyl ether. A white solid was obtained. Yield: 83%. Mp 178.1-180.2 °C. ^{1}H NMR (DMSO-d_6) δ: 12.13 (s, 1H, collapsed with D_2O); 10.16 (s, 1H, collapsed with D_2O); 7.70-7.52 (m, 4H); 7.56 (d, 1H, *J*=15.96 Hz); 6.49 (d, 1H, *J*= 15.96 Hz); 4.21-4.13 (m, 2H); 2.53 (q, 4H, *J*= 7.15 Hz); 1.23 (t, 3H, *J*= 7.15 Hz).

Ethyl (*E*)-3-(4-(4-(4-(2-((7-chloroquinolin-4-yl)amino)ethyl)piperazin-1-yl)-4-oxobutanamido)phenyl)acrylate (67)



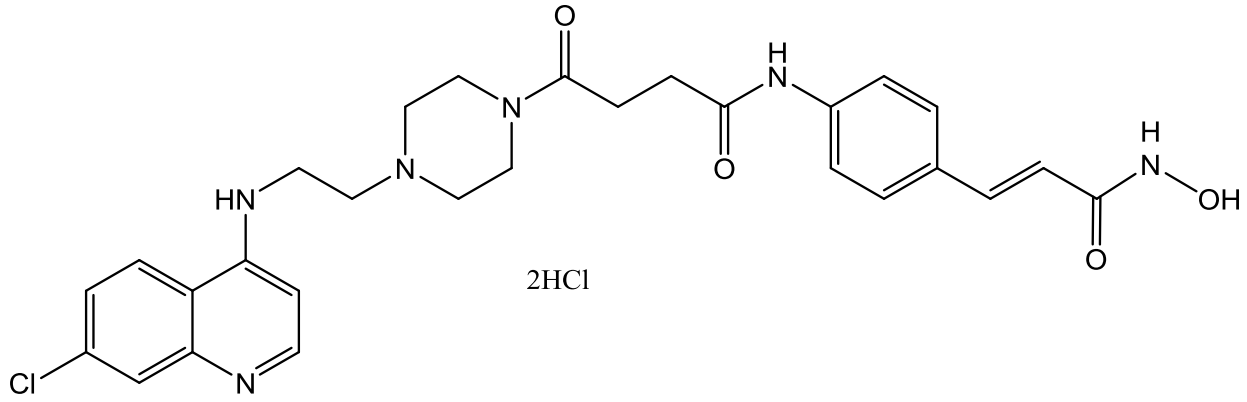
Compound **64** (200 mg, 0.69 mmol) was dissolved in 1 ml of anhydrous DMF and charged into a 0.5-2 ml microwave vial. To the solution HOBt (104 mg, 0.69 mmol), DCC (142 mg, 0.69 mmol) and compound **66** (200 mg, 0.69 mmol) were added. The mixture was stirred in a microwave reactor at 55 °C for 1 h. After the filtration of the DCU, the solvent was evaporated and the obtained residue was diluted with CH_2Cl_2 and washed with water, a solution of $NaHCO_3$ at 5% (w/w) and brine. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain an oily residue that was crystallized and rinsed with diethyl ether. The obtained solid residue was purified by CC (silica gel; CH_2Cl_2/MeOH in gradient); the product eluted with 4% of MeOH. The fraction containing the desired product was rinsed with a mixture of diethyl ether/MeOH (9:1) to provide a white solid. Yield: 67%. Mp 192.2-193.4 °C. ^{1}H NMR (DMSO-d_6) δ: 10.15 (s, 1H, collapsed with D_2O); 8.39 (d, 1H, *J*= 5.20 Hz); 8.21 (d, 1H, *J*= 9.07 Hz); 7.77 (s, 1H); 7.67-3.62 (m, 4H); 7.56 (d, 1H, *J*= 15.96 Hz); 7.45 (d, 1H, *J*= 9.07 Hz); 7.19 (br s, 1H, collapsed with D_2O); 6.49-6.45 (m, 2H); 4.16 (q, 2H, *J*= 7.15 Hz); 3.52-3.38 (m, 6H); 2.64-2.58 (m, 6H); 2.43-2.39 (m, 4H); 1.24 (t, 3H, *J*= 7.15 Hz).

(*E*)-3-(4-(4-(4-(2-((7-Chloroquinolin-4-yl)amino)ethyl)piperazin-1-yl)-4-oxobutanamido)phenyl)acrylic acid (68)



1 N NaOH (0.4 ml) was added to a solution of compound **67** (200 mg, 0.36 mmol) in EtOH/THF (1:1) and the mixture was stirred at 60 °C for 2 h. After cooling to r.t., 0.5 N HCl was added until pH= 6 and a copious precipitate formed. It was filtered and washed with water to obtain a white solid. Yield: 79%. Mp 202.1-204.5 °C. ^{1}H NMR (DMSO-d_6) δ: 12.20 (br s, 1H, collapsed with D_2O); 10.14 (s, 1H, collapsed with D_2O); 8.41-8.38 (m, 1H); 8.21 (d, 1H, *J*= 8.80 Hz); 7.78-7.76 (m, 1H); 7.61-7.40 (m, 5H); 7.21 (br s, 1H, collapsed with D_2O); 6.52-6.49 (m, 1H); 6.38 (d, 1H, *J*= 15.96 Hz); 3.56-3.08 (m, 8H); 2.63-2.20 (m, 8H).

(*E*)-4-(4-(2-((7-Chloroquinolin-4-yl)amino)ethyl)piperazin-1-yl)-N-(4-(3-(hydroxyamino)-3-oxoprop-1-en-1-yl)phenyl)-4-oxobutanamide (7)

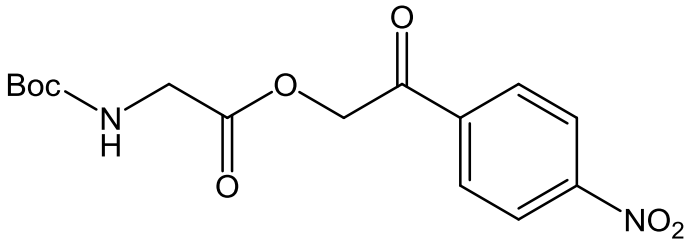


Potassium hydroxide (19.5 mg, 0.28 mmol) was added to an ice-cooled solution of hydroxylamine hydrochloride (16 mg, 0.28 mmol) in 0.5 ml of methanol. The mixture was stirred at 0 °C for 1 h. Meanwhile, ethyl chloroformate (23.5 mg, 0.21 mmol) was added to a suspension of compound **68** (75 mg, 0.14 mmol) and TEA (39 µl, 0.28 mmol) in 1.5 ml of anhydrous THF. The mixture was stirred at r.t. for 1.5 h. The hydroxylamine suspension was filtered to remove the formed KCl and the filtrate was charged into the flask containing the activated carboxylic acid. The mixture was stirred at r.t. for 3 h, then the unreacted compound **68** was filtered off. The filtrate was evaporated to obtain a residue that, after diluition with water, was filtered and washed with water. The solid was suspended in water and alkalized with 2 N NaOH, obtaining a colloidal suspension. 0.5 N HCl was added very slowly and when pH= 8 was reached, the formation of a precipitate was observed. It was filtered and washed with water to collect a cream-coloured solid that was purified by C-18 HPLC (phase A: water + 0.1% TFA; phase B: MeOH + 0.1% TFA), with the following elution method:

Time (min.)	% A	% B	Flow (ml/min.)
0	86	14	0
1	86	14	14
21	0	100	14
26	0	100	14
30	86	14	14
34	86	14	14
35	86	14	0

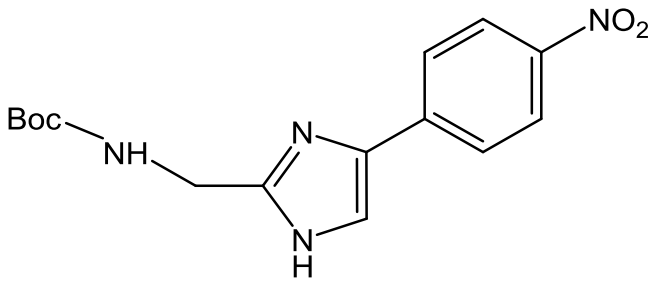
The obtained product was converted into the corresponding dihydrochloride salt with 1 N HCl in EtOH to get a white solid. Yield: 12%. Mp 172.5-175.1 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 10.10 (br s, 1H, collapsed with D_2O); 8.30-8.27 (m, 2H); 7.59-7.42 (m, 6H); 6.45-6.38 (m, 3H); 3.55-3.10 (m, 10H); 2.60-2.23 (m, 6H).

2-(4-Nitrophenyl)-2-oxoethyl (tert-butoxycarbonyl)glycinate (69)



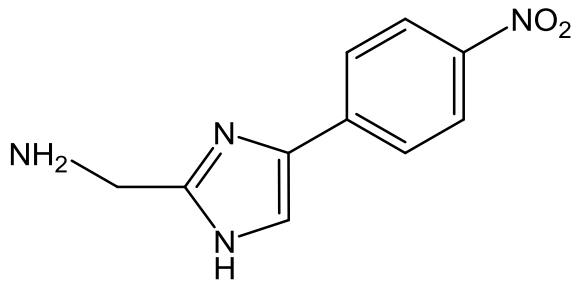
A mixture of Boc-glycine (2.9 g, 16.40 mmol), cesium carbonate (2.7 g, 8.30 mmol) and 50 ml of ethanol was stirred under argon at r.t. for 30 minutes. Then the solvent was evaporated and the residue diluted with 50 ml of anhydrous DMF. 2-Bromo-4-nitroacetophenone (4.0 g, 16.40 mmol) was added and the mixture stirred under argon at r.t. for 2 h. The solvent was evaporated and the residue diluted with ethyl acetate, observing the formation of a precipitate of CsBr that was filtered and washed with ethyl acetate. The filtrate was evaporated and the obtained residue was purified by CC (silica gel; cyclohexane/ethyl acetate in gradient), using the Flash Chromatography Purification System Biotage SP-1; the product eluted with 11% of ethyl acetate. The fraction containing the desired product was rinsed with a mixture of diethyl ether/ petroleum ether (1:1) to collect a cream-coloured solid. Yield: 68%. Mp 113.9-116.9 °C. ^{1}H NMR ($CDCl_3$) δ: 8.34 (dd, 2H, J= 1.93, 8.50 Hz); 8.07 (dd, 2H, J= 1.93, 8.50 Hz); 5.41 (s, 2H); 5.05 (br s, 1H, collapsed with D_2O); 4.11 (d, 2H, J= 5.78 Hz); 1.45 (s, 9H).

tert-Butyl ((4-(4-nitrophenyl)-1H-imidazol-2-yl)methyl)carbamate (70)



A mixture of compound **69** (3.6 g, 10.64 mmol), ammonium acetate (5.2 g, 135.80 mmol) and 80 ml of xylene was stirred under nitrogen at 180 °C for 4 h, removing the forming water with a Dean-Stark apparatus. The mixture was diluted with ethyl acetate and washed with water, a solution of $NaHCO_3$ at 5% (w/w) and brine. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a residue that was purified by CC (silica gel; cyclohexane/ethyl acetate in gradient), using the Flash Chromatography Purification System Biotage SP-1; the product eluted with 20% of ethyl acetate. The fraction containing the desired product was rinsed with a mixture of diethyl ether/ petroleum ether (3:7) to collect a reddish solid. Yield: 52%. Mp 204.9-206.9 °C. ^{1}H NMR (DMSO-d_6) δ: 12.17 (br s, 1H, collapsed with D_2O); 8.18 (d, 2H, J= 8.80 Hz); 7.98 (d, 2H, J= 8.80 Hz); 7.83 (s, 1H); 7.31 (br s, 1H, collapsed with D_2O); 4.18 (d, 2H, J= 5.77 Hz); 1.39 (s, 9H).

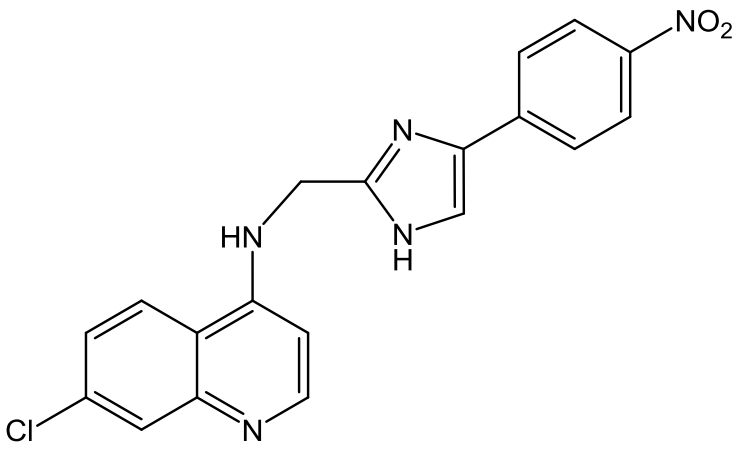
(4-(4-Nitrophenyl)-1H-imidazol-2-yl)methanamine (71)



Compound **70** (1.6 g, 5.03 mmol) was stirred in 4 ml of TFA at r.t. for 1 h. A mixture of diethyl ether/ petroleum ether (1:1) was added and the formation of a yellow precipitate was observed. It was filtered, washed with diethyl ether/MeOH (8:2) and dissolved in water. After alkalization with K_2CO_3, water was evaporated and the obtained residue was purified by flash CC (silica gel; CH_2Cl_2/MeOH/conc. NH_3, 10:1:0.1). the fraction containing the product was rinsed with a mixture of diethyl ether/petroleum ether (7:3) to obtain a brown solid. Yield: 80%. Mp 193.3-194.9 °C. ^{1}H NMR (DMSO-d_6) δ: 8.19 (dd, 2H, *J*= 2.20, 8.80 Hz); 7.97 (dd, 2H, *J*= 2.20, 8.80 Hz); 7.81 (s, 1H); 3.75 (d, 2H, *J*= 2.47Hz).

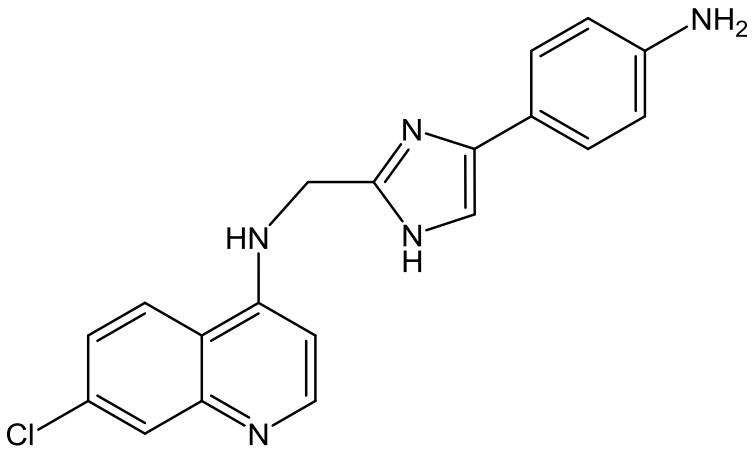
3H are not visible, probably for the interaction with the solvent.

7-Chloro-N-((4-(4-nitrophenyl)-1H-imidazol-2-yl)methyl)quinolin-4-amine (72)



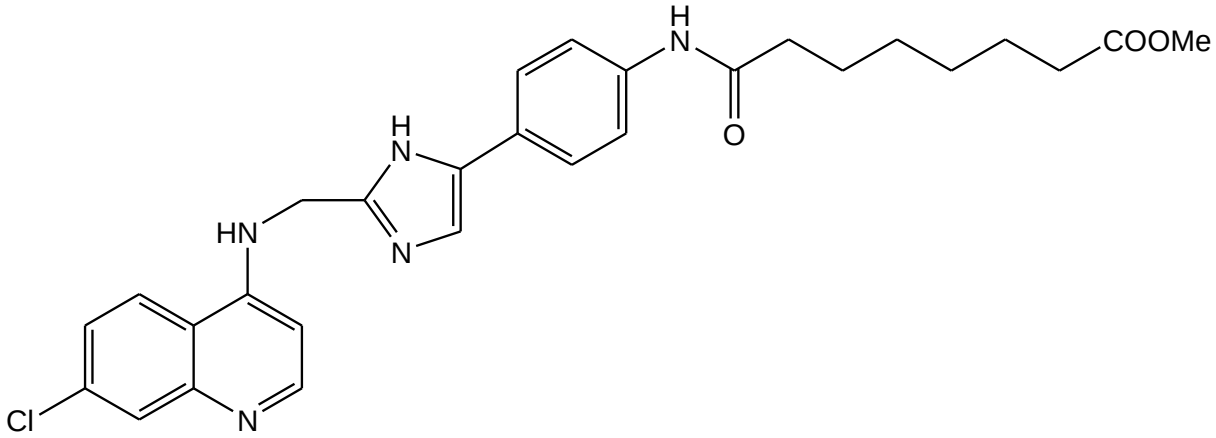
A mixture of compound **71** (750 mg, 3.44 mmol), 4,7-dichloroquinoline (819 mg, 4.13 mmol) and phenol (2.1 g, 17.20 mmol) was stirred under nitrogen at 130 °C for 3 h. Then it was put into a 250 ml flask and diluted with a mixture of CH_2Cl_2/MeOH (9:1) and 2 N NaOH. The obtained precipitate was filtered and washed with water and diethyl ether to collect a mustard-coloured solid. Yield: 92%. Mp 270.6-273.4 °C. ^{1}H NMR (DMSO-d_6) δ: 12.41 (br s, 1H, collapsed with D_2O); 8.41-8.30 (m, 2H); 8.21-8.18 (m, 2H); 8.03-7.96 (m, 3H); 7.89 (s, 1H); 7.82 (s, 1H); 7.53-7.50 (m, 1H); 6.48 (s, 1H); 4.60 (s, 2H).

N-((4-(4-Aminophenyl)-1H-imidazol-2-yl)methyl)-7-chloroquinolin-4-amine (73)



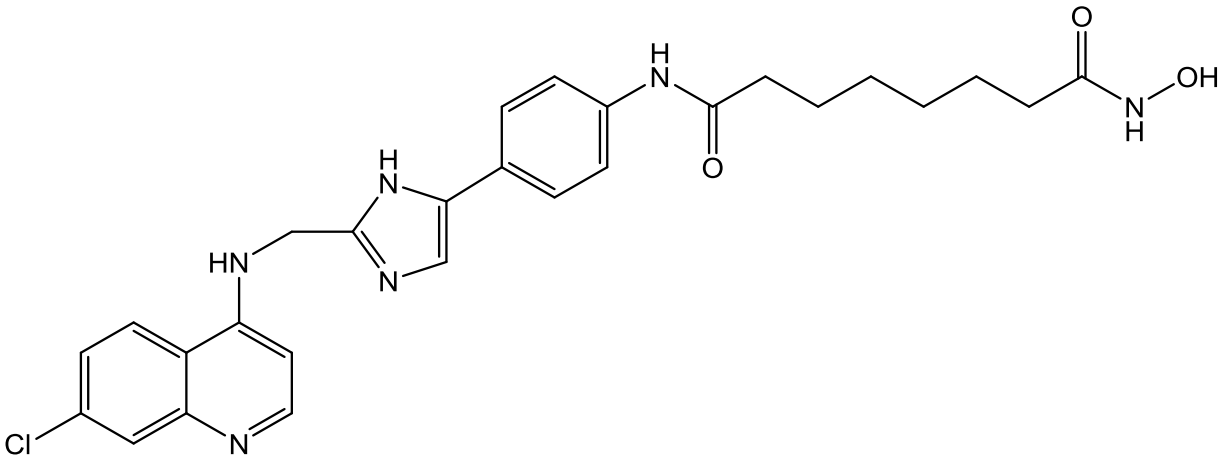
A mixture of compound **72** (600 mg, 1.58 mmol), metallic iron (441 mg, 7.90 mmol) and 15 ml of glacial acetic acid was stirred at r.t. for 1.5 h. The solvent was evaporated and the obtained residue was diluted with a mixture of ethyl acetate/MeOH (9:1). After cooling with an ice-bath, 50 ml of water was added and the mixture was alkalized with K_2CO_3. The two layers were separated and the organic one was washed with water and brine and then dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a residue that was purified by CC (silica gel; CH_2Cl_2/MeOH in gradient), using the Flash Chromatography Purification System Biotage SP-1; the product eluted with 7% of MeOH. The fraction containing the desired product was rinsed with diethyl ether/petroleum ether (3:7) to obtain a nut-brown solid. Yield: 67%. Mp 152.3-156.5 °C. ^{1}H NMR (DMSO-d_6) δ: 11.80 (s, 1H, collapsed with D_2O); 8.36 (d, 1H, J= 5.50 Hz); 8.31 (d, 1H, J= 9.08 Hz); 7.90 (s, 1H, collapsed with D_2O); 7.79 (d, 1H, J= 2.20); 7.47 (dd, 1H, J= 2.20, 9,08 Hz); 7.36 (d, 2H, J= 8.25 Hz); 7.13 (s, 1H); 6.56-6.52 (m, 3H); 5.00 (br s, 2H, collapsed with D_2O); 4.50 (d, 2H, J= 5.50 Hz).

Methyl 8-(4-(2-((7-chloroquinolin-4-ylamino)methyl)-1H-imidazol-5-yl)phenylamino)-8-oxooctanoate (74)



Compound **73** (286 mg, 0.82 mmol) was dissolved in 2 ml of anhydrous DMF and charged into a microwave vial. To the solution HOBt (124 mg, 0.82 mmol), DCC (169 mg, 0.82 mmol) and suberic acid monomethylester (0.15 ml, 0.82 mmol) were added. The mixture was stirred in a microwave reactor at 58 °C for 1 h. After the filtration of the DCU, the solvent was evaporated and the obtained residue was diluted with CH_2Cl_2 and washed with water, then with a solution of $NaHCO_3$ at 5% (w/w) and finally with brine. In the organic layer a precipitate formed and it was filtered, washed with water and then purified by CC (silica gel; CH_2Cl_2/MeOH in gradient), using the Flash Chromatography Purification System Biotage SP-1; the product eluted with 8% of MeOH. A white solid was obtained. Yield: 68%. Mp 103.1-104.2 °C. ^{1}H NMR (DMSO-d_6) δ: 12.05 (br s, 1H, collapsed with D_2O); 9.82 (s, 1H); 8.38 (d, 1H, J= 5.20 Hz); 8.32 (d, 1H, J= 8.80 Hz); 7.96 (br s, 1H, collapsed with D_2O); 7.80 (s, 1H); 7.62-7.48 (m, 4H); 7.39 (s, 1H); 6.51 (d, 1H, J= 5.50 Hz); 4.55 (d, 2H, J= 5.20 Hz); 3.56 (s, 3H); 2.30-2.25 (m, 4H); 1.59-1.48 (m, 4H); 1.30-1.25 (m, 4H). 1H is not visible, probably for the interaction with the solvent.

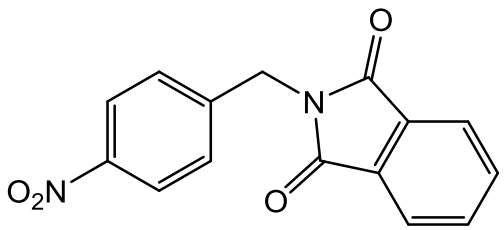
N^1-(4-(2-(((7-Chloroquinolin-4-yl)amino)methyl)-1H-imidazol-5-yl)phenyl)-N8-hydroxyoctanediamide (8)



Potassium hydroxide (432 mg, 7.70 mmol) was added to an ice-cooled solution of hydroxylamine hydrochloride (535 mg, 7.70 mmol) in 1.4 ml of methanol. The mixture was stirred at 0 °C for 10 minutes. The hydroxylamine suspension was filtered to remove the formed KCl and the filtrate was charged into a flask containing compound **74** (200 mg, 0.39 mmol) and further KOH (32 mg, 0.57 mmol). The mixture was stirred at r.t. for 4 h and then the solvent was evaporated. The obtained residue was diluted with a small amount of water and 0.1 N HCl was added until the formation of a colloidal suspension. After the addition of CH_2Cl_2, the obtained precipitate was filtered and washed with CH_2Cl_2 and diethyl ether. A white solid was obtained. Yield: 61%. Mp 157.2-158.8 °C.

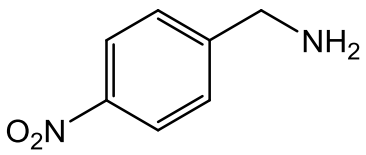
The product was converted into the corresponding dihydrochloride salt with 1 N HCl in ethanol. Mp 218.8-220.5 °C. ^{1}H NMR (DMSO-d_6) δ: 10.35 (s, 1H, collapsed with D_2O); 10.20 (br s, 2H, collapsed with D_2O); 8.76-8.68 (m, 2H); 8.14 (s, 1H); 8.00 (s, 1H); 7.85-7.70 (m, 5H); 7.04 (d, 1H, *J*= 7.15 Hz); 5.24-5.19 (m, 2H); 2.33-2.28 (m, 2H); 1.97-1.91 (m, 2H); 1.60-1.43 (m, 4H); 1.32-1.21 (m, 4H).

2-(4-Nitrobenzyl)isoindoline-1,3-dione (75)



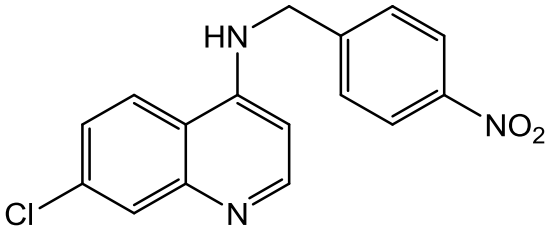
A suspension of 1-(bromomethyl)-4-nitrobenzene (2 g, 9.26 mmol), potassium phthalimide (1.7 g, 9.26 mmol) and 27 ml of anhydrous DMF was stirred under nitrogen at 50 °C for 12 h. The mixture was poured into cold water and the obtained white precipitate was filtered and washed with water and ethanol. The solid was purified by CC (silica gel; CH_2Cl_2), using the Flash Chromatography Purification System Biotage SP-1. The fraction containing the desired product was rinsed with diethyl ether to obtain a white solid. Yield: 72%. Mp 172.2-173.5 °C. ^{1}H NMR ($CDCl_3$) δ: 8.18 (dd, 2H, *J*= 2.20, 8.80 Hz); 7.90-7.87 (m, 2H); 7.77-7.72 (m, 2H); 7.49 (dd, 2H, *J*= 2.20, 8.80 Hz); 8.92 (s, 2H).

(4-Nitrophenyl)methanamine (76)



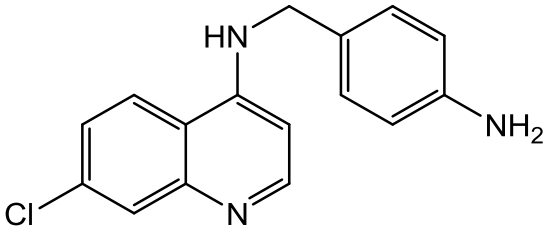
Hydrazine hydrate (0.43 ml, 8.86 mmol) was added to a suspension of compound **75** (500 mg, 1.77 mmol) in 70 ml of EtOH/THF (9:1) and the mixture was refluxed under nitrogen for 24 h. After cooling with an ice-bath, 39 ml of a 1 M solution of oxalic acid were slowly added. The obtained mixture was refluxed for 30 minutes and then, after cooling to r.t., it was alkalized with 2 N NaOH (pH= 12). The formed white precipitate was filtered and washed with water. The filtrate was extracted with CH_2Cl_2 and the organic layer was washed with brine, dried with anhydrous Na_2SO_4 and evaporated to dryness. An orange oil was obtained, which was directly used in the next step without further purification. ^{1}H NMR ($CDCl_3$) δ: 8.19 (dd, 2H, *J*= 1.95, 7.15 Hz); 7.50 (dd, 2H, *J*= 1.95, 7.15 Hz); 4.02 (s, 2H). 2H are not visible, probably for the interaction with the solvent.

7-Chloro-N-(4-nitrobenzyl)quinolin-4-amine (77)



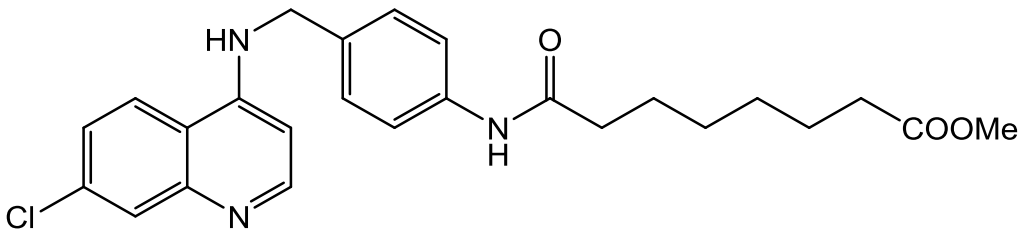
A mixture of compoun **76** (217 mg, 1.43 mmol), 4,7-dichloroquinoline (311 mg, 1.57 mmol) and phenol (1.3 g, 14.24 mmol) was stirred under nitrogen at 130 °C for 3.5 h and then diluted with CH_2Cl_2/MeOH (9.5:0.5). After washing with 2 N NaOH, water and brine, the organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The obtained residue was purified by CC (silica gel; CH_2Cl_2/MeOH in gradient), using the Flash Chromatography Purification System Biotage SP-1; the product eluted with 2.7% of MeOH. The fraction containing the desired product was rinsed with diethyl ether to obtain a cream-coloured solid. Yield: 59%. Mp 209.8-210.9 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 8.37-8.29 (m, 2H); 8.20-8.15 (m, 3H, 1H collapsed with D_2O); 7.80 (d, 1H, *J*= 1.43 Hz); 7.62 (d, 2H, *J*= 9.15 Hz); 7.47 (dd, 1H, *J*= 1.43, 8.80 Hz); 6.31 (d, 1H, *J*= 6.05 Hz); 4.68 (d, 2H, *J*= 6.05 Hz).

N-(4-Aminobenzyl)-7-chloroquinolin-4-amine (78)



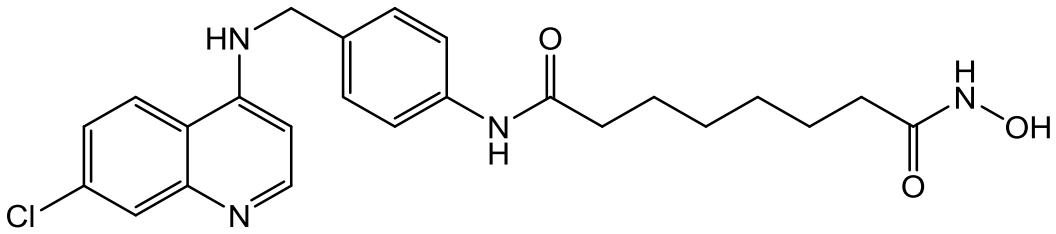
A mixture of compound **77** (776 mg, 2.47 mmol), metallic iron (691 mg, 12.37 mmol) and 10 ml of acetic acid was stirred vigorously at 70 °C for 1.5 h. The solvent was evaporated and the residue was diluted with CH_2Cl_2/MeOH (9:1) and water and the mixture was neutralized with $NaHCO_3$. The two layers were separated and the organic one was washed with water and brine, dried with anhydrous Na_2SO_4 and evaporated to dryness. The obtained residue was rinsed with petroleum ether and diethyl ether to generate a white solid. Yield: 66%. Mp 143.5-145.1 °C. ^{1}H NMR (DMSO-d_6) δ: 8.36-8.29 (m, 2H); 7.85 (t, 1H, *J*= 6.05 Hz, collapsed with D_2O); 7.77 (d, 1H, *J*= 1.43 Hz); 7.51-7.45 (m, 1H); 7.02 (dd, 2H, *J*= 1.95, 8.80 Hz); 6.49 (dd, 2H, *J*= 1.95, 8.80 Hz); 6.38 (d, 1H, *J*= 6.05 Hz); 4.97 (br s, 2H, collapsed with D_2O); 4.30 (d, 2H, *J*= 6.05 Hz).

Methyl 8-((4-(((7-chloroquinolin-4-yl)amino)methyl)phenyl)amino)-8-oxooctanoate (79)



Compound **78** (200 mg, 0.71 mmol) was dissolved in 0.8 ml of anhydrous DMF and charged into a 0.5-2 ml microwave vial. To the solution HOBt (107 mg, 0.71 mmol), DCC (145 mg, 0.71 mmol) and suberic acid monomethylester (0.13 ml, 0.71 mmol) were added. The mixture was stirred in a microwave reactor at 55 °C for 1 h. After the filtration of the DCU, the solvent was evaporated and the residue was diluted with 2 N NaOH. The obtained precipitate was filtered, washed with water and then purified by CC (silica gel; CH_2Cl_2/MeOH in gradient); the product eluted with 2% of MeOH. The fraction containing the desired product was rinsed with diethyl ether/CH_2Cl_2 (9:1) to obtain a white solid. Yield: 56%. Mp 166.4-167.0 °C. ^{1}H NMR ($CDCl_3$) δ: 8.52 (d, 1H, *J*= 6.20 Hz); 7.97 (d, 1H, *J*= 2.20 Hz); 7.70 (d, 1H, *J*= 9.15 Hz); 7.55 (d, 2H, *J*= 8.80 Hz); 7.42 (br s, 1H, collapsed with D_2O); 7.38-7.29 (m, 3H); 6.42 (d, 1H, *J*= 6.20 Hz); 5.37 (br s, 1H, collapsed with D_2O); 4.47 (d, 2H, *J*= 6.20 Hz); 3.65 (s, 3H); 2.38-2.25 (m, 4H); 1.78-1.60 (m, 4H); 1.42-1.36 (m, 4H).

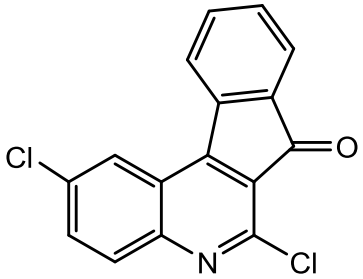
N^1-(4-(((7-Chloroquinolin-4-yl)amino)methyl)phenyl)-N8-hydroxyoctanediamide (9)



Potassium hydroxide (544 mg, 9.69 mmol) was added to an ice-cooled solution of hydroxylamine hydrochloride (674 mg, 9.69 mmol) in 1.7 ml of methanol. The mixture was stirred at 0 °C for 10 minutes. The hydroxylamine suspension was filtered to remove the formed KCl and the filtrate was charged into a flask containing compound **79** (220 mg, 0.48 mmol) and further KOH (41 mg, 0.73 mmol). The mixture was stirred at r.t. for 5 h and then the solvent was evaporated. The obtained residue was diluted with a small amount of water and acetic acid was added until the formation of a precipitate (pH= 5). It was filtered and washed with water to obtain a white solid. Yield: 92%. Mp 173.1-174.4 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 10.35 (s, 1H, collapsed with D_2O); 9.93 (s, 1H, collapsed with D_2O); 9.67 (br s, 1H, collapsed with D_2O); 8.63 (d, 1H, *J*= 7.95 Hz); 8.42 (d, 1H, *J*= 6.05 Hz); 7.98 (s, 1H); 7.71 (d, 1H, *J*= 7.95 Hz); 7.57 (d, 2H, *J*= 8.80 Hz); 7.32 (d, 2H, *J*= 8.80 Hz); 6.68 (d, 1H, *J*= 6.05 Hz); 4.61 (s, 2H); 2.35-2.21 (m, 2H); 1.98-1.92 (m, 2H); 1.59-1.45 (m, 4H); 1.32-1.21 (m, 4H). 1H is not visible, probably for the interaction with the solvent.

4.3 ***Indeno[2,1-c]quinolines***

2,6-Dichloro-7H-indeno[2,1-c]quinolin-7-one (81)

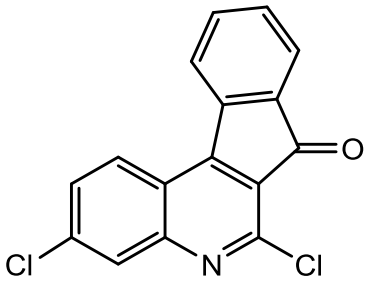


Method A: A mixture of malonic acid (2.9 g; 27.6 mmol), (2-amino-5-chlorophenyl)(phenyl)methanone (**80**; 3.2 g; 13.8 mmol) and $POCl_3$ (48 ml) was refluxed for 4 h under nitrogen. After cooling, the mixture was slowly poured into water and crushed ice, under a vigorous agitation. After the addition of CH_2Cl_2 (400 ml), the formed insoluble yellow solid was collected through filtration and washed successively with CH_2Cl_2, methanol, ethyl acetate and n-hexane. The aqueous phase was extracted with CH_2Cl_2 and the organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The obtained solid residue was washed with CH_2Cl_2, methanol, ethyl acetate and n-hexane. The two crude solids were joined and submitted to a digestion with CH_2Cl_2 to get yellow crystals of the desired product (**81)**. Yield: 43%. Mp 283.2-283.6 °C (dec). ^{1}H NMR (TFA-d) δ: 9.05 (d, 1H, *J*= 3.85 Hz): 8.58 (t, 1H, *J*= 6.60 Hz); 8.33 (d, 2H, *J*= 3.85 Hz); 8.16 (t, 1H, *J*= 6.60 Hz); 8.02-7.96 (m, 2H).

The organic washing solvents of the two solids, containing the by-product 2,6-dichloro-4-phenylquinoline-3-carbonyl chloride (**82**), were joined and evaporated to dryness and purified by CC (silica gel; cyclohexane/ethyl acetate in gradient). The obtained residue was rinsed with petroleum ether to get white crystals. Yield: 26%. Mp 168.2-169.9 °C. ^{1}H NMR ($CDCl_3$) δ: 8.05 (d, 1H, *J*= 9.08 Hz); 7.77 (dd, 1H, *J*= 2.48, 9.08 Hz); 7.60-7.56 (m, 4H); 7.40-7.38 (m, 2H).

Method B: To an ice-cooled solution of 2,6-dichloro-4-phenylquinoline-3-carbonyl chloride (**82**; 1.2 g; 3.6 mmol) in 24 ml of anhydrous CH_2Cl_2, $AlCl_3$ (1.2 g; 8.9 mmol) was added and the mixture was stirred under nitrogen at r.t. for 3.5 h and then was refluxed for 30 minutes. After cooling, the mixture was poured into water and crushed ice and the obtained precipitate was filtered and washed with methanol, ethyl acetate and n-hexane to get a yellow solid. Yield: 93%. Mp 283.0-283.5 °C (dec). ^{1}H NMR (TFA-d) δ: 9.05 (d, 1H, *J*= 3.85 Hz): 8.58 (t, 1H, *J*= 6.6 Hz); 8.33 (d, 2H, *J*= 4.68 Hz); 8.16 (t, 1H, *J*= 6.32 Hz); 8.02-7.96 (m, 2H).

3,6-Dichloro-7H-indeno[2,1-c]quinolin-7-one (84)



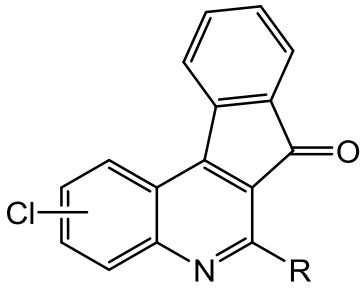
Method A: A mixture of malonic acid (671 mg; 6.45 mmol), (2-amino-4-chlorophenyl)(phenyl)methanone (**83**; 1 g; 4.31 mmol) and $POCl_3$ (20 ml) was refluxed for 4 h under nitrogen. After cooling, the mixture was slowly poured into water and crushed ice under a vigorous agitation. The obtained precipitate was filtered and washed with water. The filtrate was extracted with CH_2Cl_2 and the organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The two solid residues were mixed and purified by CC (silica gel; cyclohexane/ethyl acetate in gradient). The obtained residue was rinsed with CH_2Cl_2, methanol and ethyl acetate to get 255 mg of yellow crystals of the desired product (**84**). Yield: 35%. Mp 247.6-248.0 °C. ^{1}H HMR (DMSO-d_6) δ: 8.74 (d, 1H, J= 9.08 Hz); 8.62 (d, 1H, J= 7.43 Hz); 8.08 (d, 1H, J= 2.2 Hz); 7.79-7.74 (m, 3H); 7.72-7.63 (m, 1H).

The fraction containing the by-product 2,7-dichloro-4-phenylquinoline-3-carbonyl chloride (**85**) was evaporated and washed with petroleum ether to give white crystals. Yield: 20%. Mp 97.8-99.3 °C. ^{1}H NMR ($CDCl_3$) δ: 8.11 (d, 1H, J= 2.2 Hz); 7.59-7.55 (m, 4H); 7.50 (dd, 1H, J= 2.2, 9.08 Hz); 7.39-7.36 (m, 2H).

Method B: To an ice-cooled solution of 3,6-dichloro-4-phenylquinoline-3-carbonyl chloride (**85**; 500 mg; 1.5 mmol) in 6 ml of anhydrous CH_2Cl_2, $AlCl_3$ (495 mg; 3.7 mmol) was added and the mixture was stirred under nitrogen at r.t. for 4 h and then was refluxed for 30 minutes. After cooling, the mixture was poured into water and crushed ice and the obtained precipitate was filtered

and washed with CH_2Cl_2, methanol, ethyl acetate and n-hexane to get a yellow solid. Yield: 79%. Mp 247.1-248.0 °C (dec). ^{1}H NMR (DMSO-d_6) δ: 8.74 (d, 1H, J= 9.08 Hz); 8.62 (d, 1H, J= 7.43 Hz); 8.08 (d, 1H, J= 2.2 Hz); 7.79-7.74 (m, 3H); 7.72-7.63 (m, 1H).

2- or 3-Chloro-6-(amino-substituted)-7H-indeno[2,1-c]quinolin-7-ones (10-17)



General procedure: a suspension of the indenoquinoline **81** or **84** (1.67 mmol) and the appropriated amine (3.33 mmol) in 7 ml of dry pyridine was heated at 100 °C for 20 h. The reaction mixture was poured into iced water (150-200 ml) and when it was possible, the precipitate was filtered, otherwise it was extracted with ethyl acetate or dichloromethane. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The residue was purified by CC (silica gel; CH_2Cl_2/MeOH or cyclohexane/ethyl acetate in gradient as indicated for each compound).

2-Chloro-6-(2-(piperidin-1-yl)ethylamino)-7*H*-indeno[2,1-c]quinolin-7-one (10)

CC (CH_2Cl_2/MeOH in gradient up to 98:2); the residue was washed with hot ethanol and then with diethyl ether to obtain a red solid. Yield: 52%. Mp 169.7-170.5 °C. ^{1}H NMR ($CDCl_3$) δ: 8.13 (d, 1H, *J*= 2.20 Hz); 7.95 (d, 1H, *J*= 7.42 Hz); 7.68-7.53 (m, 4H); 7.51-7.40 (m, 2H, 1H collapsed with D_2O); 3.83-3.79 (m, 2H); 2.75-2.60 (m, 6H); 1.74-1.70 (m, 4H); 1.54-1.50 (m, 2H). HRMS (ESI) m/z Calcd for $C_{23}H_{23}ClN_3O$ $[M+H]^+$: 392.15297; found: 392.15238.

3-Chloro-6-(2-(piperidin-1-yl)ethylamino)-7*H*-indeno[2,1-c]quinolin-7-one (11)

CC (CH_2Cl_2/MeOH in gradient up to 97:3); the residue was rinsed with a mixture of petroleum ether/diethyl ether (6:4) to obtain a brick-red solid. Yield: 40%. Mp 161.0-163.5 °C. ^{1}H NMR ($CDCl_3$) δ: 8.11 (d, 1H, *J*= 9.07 Hz); 7.94 (d, 1H, *J*= 7.42 Hz); 7.68-7.66 (m, 2H); 7.53 (t, 1H, *J*= 7.42 Hz); 7.42 (m, 2H, 1H collapsed with D_2O); 7.21 (d, 1H, *J*= 9.07 Hz); 3.84-3.82 (m, 2H); 2.76

(m, 2H); 2.63 (m, 4H); 1.74 (m, 4H); 1.51 (m, 2H). HRMS (ESI) m/z Calcd for $C_{23}H_{23}ClN_3O$ $[M+H]^+$: 392.15297; found: 392.15237.

3-Chloro-6-(2-morpholinoethylamino)-7*H*-indeno[2,1-c]quinolin-7-one (12)

CC (CH_2Cl_2/MeOH in gradient up to 98:2); the residue was rinsed with a mixture of petroleum ether/diethyl ether (8:2) to obtain a red solid. Yield: 44%. Mp 174.5-175.8 °C. ^{1}H NMR ($CDCl_3$) δ: 8.13 (d, 1H, *J*= 8.80 Hz); 7.95 (d, 1H, *J*= 7.42 Hz); 7.70-7.67 (m, 2H); 7.55 (t, 1H, *J*= 6.60 Hz); 7.46-7.41 (m, 2H, 1H collapsed with D_2O); 7.22 (dd, 1H, *J*= 1.92, 8.80 Hz); 3.85 (m, 6H); 2.80-2.68 (m, 6H). HRMS (ESI) m/z Calcd for $C_{22}H_{21}ClN_3O_2$ $[M+H]^+$: 394.13223; found: 394.13168.

The product was converted into the corresponding dihydrochloride salt (yellow solid) with 1 N HCl in ethanol. Mp 267.6-269.8 °C (dec).

2-Chloro-6-(2-(4-methylpiperazin-1-yl)ethylamino)-7*H*-indeno[2,1-c]quinolin-7-one (13)

CC (CH_2Cl_2/MeOH in gradient up to 90:10); the residue was recrystallized with diethyl ether and rinsed with petroleum ether to obtain a red solid. Yield: 52%. Mp 175.9-177.7 °C. ^{1}H NMR ($CDCl_3$) δ: 8.13 (d, 1H, *J*= 2.20 Hz); 7.95 (d, 1H, *J*= 7.42 Hz); 7.68-7.50 (m, 4H); 7.45-7.36 (m, 2H, 1H collapsed with D_2O); 3.78-3.72 (m, 2H); 2.74-2.65 (m, 10H); 2.39 (s, 3H). HRMS (ESI) m/z Calcd for $C_{22}H_{24}ClN_4O$ $[M+H]^+$: 407.16386; found: 407.16327.

The product was converted into the corresponding trihydrochloride salt (yellow solid) with 1 N HCl in ethanol. Mp 246.6-248.0 °C (dec.).

3-Chloro-6-(2-(4-methylpiperazin-1-yl)ethylamino)-7*H*-indeno[2,1-c]quinolin-7-one (14)

CC (CH_2Cl_2/MeOH in gradient); the residue was rinsed with petroleum ether to obtain a dark orange solid. Yield: 48%. Mp 141.7-142.3 °C. ^{1}H NMR ($CDCl_3$) δ: 8.10 (d, 1H, J= 8.80 Hz); 7.94 (d, 1H, *J*= 7.15 Hz); 7.69-7.60 (m, 2H); 7.54 (t, 1H, *J*= 7.15 Hz); 7.45-7.40 (m, 2H, 1H collapsed

withD_2O); 7.20 (dd, 1H, *J*= 1.93, 8.80 Hz); 3.78-3.72 (m, 2H); 2.72-2.68 (m, 10 H); 2.34 (s, 3H). HRMS (ESI) m/z Calcd for $C_{22}H_{24}ClN_4O$ $[M+H]^+$: 407.16386; found: 407.16330.

The product was converted into the corresponding trihydrochloride salt (yellow solid)with 1 N HCl in ethanol. Mp 243.8-245.0 °C (dec).

2-Chloro-6-(4-methylpiperazin-1-yl)-7*H*-indeno[2,1-c]quinolin-7-one (15)

Compound **15** was obtained as a by-product of the reaction to generate the 2-chloro-6-(2-(4-methylpiperazin-1-yl)ethylamino)-7H-indeno[2,1-c]quinolin-7-one (**13**), because 2-(4-methylpiperazin-1-yl)ethanamine used was impure of 1-methylpiperazine. CC (CH_2Cl_2/MeOH in gradient up to 95:5); the residue was rinsed with diethyl ether to obtain a red solid. Yield: 6%. Mp 170.0-171.3 °C. ^{1}H NMR ($CDCl_3$) δ: 8.29 (s, 1H); 8.02 (d, 1H, *J*= 7.43 Hz); 7.72-7.67 (m, 2H); 7.61-7.56 (m, 2H); 7.45 (t, 1H, *J*= 7.43 Hz); 3.73-3.70 (m, 4H); 2.69-2.66 (m, 4H); 2.40 (s, 3H). HRMS (ESI) m/z Calcd for $C_{21}H_{18}ClN_3O$ $[M+H]^+$: 364.12166; found: 364.12094.

The product was converted into the corresponding dihydrochloride salt (brick red solid) with 1 N HCl in ethanol. Mp 259.0-260.5 °C (dec).

2-Chloro-6-(4-(pyridin-2-yl)piperazin-1-yl)-7*H*-indeno[2,1-c]quinolin-7-one (16)

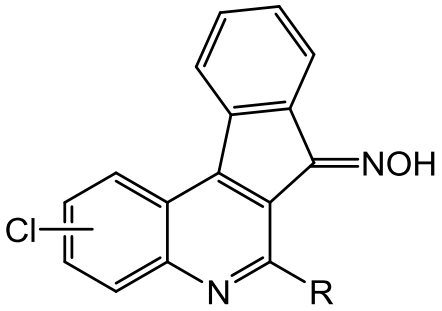
CC (cyclohexane/ethyl acetate in gradient up to 6:4); the residue was rinsed with petroleum ether to obtain a red solid. Yield: 91%. Mp 180.2-181.5 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 8.31 (s, 1H); 8.24 (d, 1H, *J*= 4.68 Hz); 8.04 (d, 1H, *J*= 7.42 Hz); 7.76-7.70 (m, 2H); 7.63-7.45 (m, 4H); 6.72 (d, 1H, *J*= 8.8 Hz); 6.70 (t, 1H, *J*= 5.77 Hz); 3.90-3.86 (m, 4H); 3.74-3.71 (m, 4H). . HRMS (ESI) m/z Calcd for $C_{25}H_{20}ClN_4O$ $[M+H]^+$: 427.13256; found: 427.13168.

The product was converted into the corresponding dihydrochloride salt (red solid) with 1 N HCl in ethanol. Mp 192.3-193.2 °C (dec).

2-Chloro-6-(1*H*-imidazol-1-yl)-7*H*-indeno[2,1-c]quinolin-7-one (17)

The residue was rinsed with a mixture of diethyl ether/methanol (9:1) and then with hot diethyl ether to obtain a dark yellow solid. Yield: 71%. Mp 262.3-263.6 °C (dec). HRMS (ESI) m/z Calcd for $C_{19}H_{11}ClN_3O$ $[M+H]^+$: 332.05906; found: 332.05893.

2- or 3-Chloro-6-(amino-substituted)-7*H*-indeno[2,1-c]quinolin-7-one oximes (18-25)



General procedure: Hydroxylamine hydrochloride (1.58 mmol) and sodium hydroxide (2.65 mmol) were added to an ice-cooled suspension of the proper ketone compound (0.53 mmol) in 10 ml of a mixture of ethanol/H_2O (2:1). After stirring for 15 minutes at r.t., the mixture was refluxed for 2 h. The ethanol was evaporated and the alkaline aqueous solution was neutralized with 1N HCl. When possible, the obtained suspension was filtered and the aqueous solution was extracted with CH_2Cl_2 and the organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The crude residue was purified as indicated for each compound.

2-Chloro-6-(2-(piperidin-1-yl)ethylamino)-7*H*-indeno[2,1-c]quinolin-7-one oxime (18)

The residue was purified by C-18 HPLC (phase A (H_2O/CH_3CN 97:3 + 0.1 TFA)/phase B (H_2O/CH_3CN 70:30 + 0.1 TFA) (70:30). The product was converted into the corresponding dihydrochloride salt to get a yellow solid. Yield: 35%. Mp 251.0-252.5 °C (dec). ^{1}H NMR (DMSO-d_6) (**18*2HCl**) δ: 10.33 (br s, 1H, collapsed with D_2O); 8.51-8.44 (m, 3H); 8.16-8.15 (m, 2H, 1H collapsed with D_2O); 7.77 (d, 1H, J= 7.98 Hz); 7.64 (m, 2H); 4.48 (br s, 2H, collapsed with D_2O); 4.23-4.22 (m, 2H); 3.55-3.54 (m, 2H); 3.42-3.41 (m, 2H); 3.02-3.01 (m, 2H); 1.79-1.43 (m, 6H). HRMS (ESI) m/z Calcd for $C_{23}H_{24}ClN_4O$ $[M+H]^+$: 407.16386; found: 407.16329.

3-Chloro-6-(2-(piperidin-1-yl)ethylamino)-7*H*-indeno[2,1-c]quinolin-7-one oxime (19)

The residue was purified by CC (C-18; CH_3CN/H_2O/TFA; 30:70:0.1) and the product was converted into the corresponding dihydrochloride salt to get a yellow solid. Yield: 16%. Mp 225.0-230.0 °C (dec). ^{1}H NMR (DMSO-d_6) (**19*2HCl**) δ: 10.03 (br s, 1H, collapsed with D_2O); 8.62 (d, 1H, *J*= 8.52 Hz); 8.48 (m, 2H); 7.98 (br s, 1H, collapsed with D_2O); 7.66-7.61 (m, 3H); 7.44 (d, 1H, *J*= 8.52 Hz); 4.44 (br s, 2H, collapsed with D_2O); 4.14 (m, 2H); 3.59-3.56 (m, 2H); 3.40 (m, 2H); 3.02-3.00 (m, 2H); 1.79-1.43 (m, 6H). HRMS (ESI) m/z Calcd for $C_{23}H_{24}ClN_4O$ $[M+H]^+$: 407.16386; found: 407.16327.

3-Chloro-6-(2-morpholinoethylamino)-7*H*-indeno[2,1-c]quinolin-7-one oxime (20)

The residue was purified by CC (ethyl acetate/MeOH 90:10) and C-18 HPLC (phase A (H_2O/CH_3CN 97:3 + 0.1 TFA)/phase B (H_2O/CH_3CN 70:30 + 0.1 TFA) (70:30). The product was converted into the corresponding dihydrochloride salt to get a yellow solid. Yield: 33%. Mp 230.0-231.0 °C (dec). ^{1}H NMR (DMSO-d_6) (**20*2HCl**) δ: 11.01 (br s, 1H, collapsed with D_2O); 8.63 (d, 1H, *J*= 8.25 Hz); 8.48 (m, 2H); 8.21 (br s, 2H, 1 H collapsed with D_2O); 7.64 (m, 2H); 7.48 (d, 1H, *J*= 8.25 Hz); 4.43-4.24 (m, 6H, 2H collapsed with D_2O); 3.90 (m, 3H); 3.51-3.40 (m, 5H).

1H is not visible, probably for the interaction with the solvent. HRMS (ESI) m/z Calcd for $C_{22}H_{22}ClN_4O_2$ $[M+H]^+$: 409.14313; found: 409.14258.

2-Chloro-6-(2-(4-methylpiperazin-1-yl)ethylamino)-7*H*-indeno[2,1-c]quinolin-7-one oxime (21)

The residue was purified by CC (ethyl acetate/MeOH/conc. NH_3 in gradient up to 89:10:1). The product was converted into the corresponding trihydrochloride salt to get a yellow solid. Yield: 49%. Mp 221.0-222.8 °C (dec). ^{1}H NMR (DMSO-d_6) (**21*3HCl**) δ: 11.25 (br s, 1H, collapsed with D_2O); 8.56-8.49 (m, 3H); 7.99-7.96 (m, 2H, 1H collapsed with D_2O); 7.76-7.64 (m, 3H); 4.45 (br s,

3H, collapsed with D_2O); 4.06-4.05 (m, 2H); 3.58-3.57 (m, 4H); 3.37-3.28 (m, 6H); 2.83 (s, 3H). HRMS (ESI) m/z Calcd for $C_{22}H_{25}ClN_5O$ $[M+H]^+$: 422.17476; found: 422.17422.

3-Chloro-6-(2-(4-methylpiperazin-1-yl)ethylamino)-7*H*-indeno[2,1-c]quinolin-7-one oxime (22)

The residue was purified by CC (ethyl acetate/MeOH/conc. NH_3 in gradient up to 89:10:1). The product was converted into the corresponding trihydrochloride salt and digested with hot MeOH to get a yellow solid. Yield: 47%. Mp 224.2-226.0 °C (dec). ^{1}H NMR (DMSO-d_6) (**22*3HCl**) δ: 11.57 (br s, 1H, collapsed with D_2O); 8.64 (d, 1H, *J*= 9.08 Hz); 8.48 (m, 2H); 8.36 (m, 1H); 8.20 (m, 1H, collapsed with D_2O); 7.67 (m, 2H); 7.52-7.49 (m, 1H); 5.01 (br s, 3H, collapsed with D_2O); 4.22 (m, 2H); 3.72-7.62 (m, 4H); 3.41 (m, 6H); 2.84 (s, 3H). HRMS (ESI) m/z Calcd for $C_{22}H_{25}ClN_5O$ $[M+H]^+$: 422.17476; found: 422.17422.

2-Chloro-6-(4-(pyridin-2-yl)piperazin-1-yl)-7*H*-indeno[2,1-c]quinolin-7-one oxime (23)

The residue was rinsed with diethyl ether to obtain a yellow solid. Yield: 87%. Mp 224.2-225.5 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 8.60 (d, 1H, *J*= 2.48 Hz); 8.56 (d, 1H, *J*= 8.00 Hz); 8.49 (d, 1H, *J*= 8.00 Hz); 8.15-8.14 (m, 1H); 7.84 (d, 1H, *J*= 9.07); 7.72-7.55 (m, 4H); 6.89 (d, 1H, *J*= 9.07 Hz); 6.69-6.65 (m, 1H); 3.70 (m, 4H); 3.59 (m, 4H).

1H is not visible, probably for the interaction with the solvent. HRMS (ESI) m/z Calcd for $C_{25}H_{21}ClN_5O$ $[M+H]^+$: 442.14346; found: 442.14282.

The product was converted into the corresponding dihydrochloride salt (green solid) with 1 N HCl in ethanol. Mp 228.2-231.4 °C (dec).

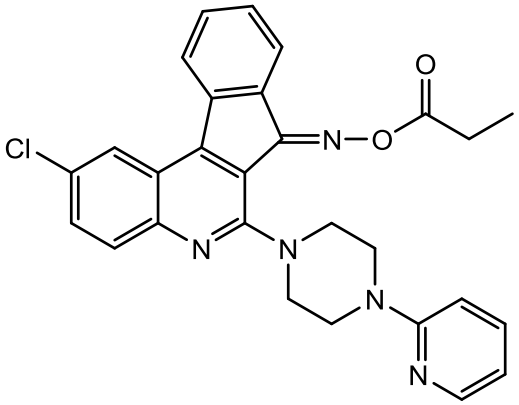
2-Chloro-6-(1H-imidazol-1-yl)-7*H*-indeno[2,1-c]quinolin-7-one oxime (25)

The formed precipitate was filtered and washed with water. The residue was then digested with MeOH to obtain a green-yellow solid. Yield: 48%. Mp 271.0-273.0 °C (dec.). ^{1}H NMR (DMSO-d_6)

δ: 9.54 (m, 1H); 8.91 (m, 1H); 8.77 (m, 1H); 8.57 (m, 1H); 8.20 (m, 2H); 8.03 (m, 1H); 7.81-7.74 (m, 3H).

1H is not visible, probably for the interaction with the solvent. HRMS (ESI) m/z Calcd for $C_{19}H_{12}ClN_4O$ $[M+H]^+$: 347.06996; found: 347.06996.

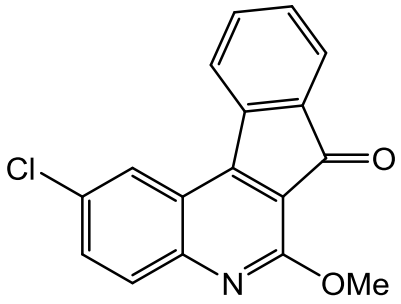
2-Chloro-6-(4-(pyridin-2-yl)piperazin-1-yl)-7*H*-indeno[2,1-c]quinolin-7-one O-propionyl oxime (24)



To an ice-cooled suspension of compound **23** (150 mg; 0.33 mmol) in 10 ml of DMSO, NaH (32 mg; 1.33 mmol) was added. After stirring for 30 min., propionyl chloride (114 µl; 1.33 mmol) was added and the mixture was stirred at r.t. under nitrogen for 15 h. Finally, the reaction mixture was poured into 100 ml of iced water and extracted with CH_2Cl_2. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The crude product was washed with n-hexane and diethyl ether to obtain an orange solid. Yield: 80%. Mp 173.1-175.2 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 8.42 (d, 2H, *J*= 7.97 Hz); 8.22 (d, 2H, *J*= 4.68 Hz); 7.85 (d, 1H, *J*= 9.08 Hz); 7.63 (dd, 2H, *J*= 1.1, 5.78); 7.60-7.47 (m, 2H); 6.75 (d, 1H, *J*= 9.08 Hz); 6.62 (dd, 1H, *J*= 4.68 Hz); 3.90-3.86 (m, 4H); 3.74-3.71 (m, 4H); 2.75-2.67 (m, 2H); 1.35 (t, 3H, *J*= 7.7 Hz). HRMS (ESI) m/z Calcd for $C_{28}H_{24}ClN_5O_2$ $[M+H]^+$: 498.16968; found: 498.16891.

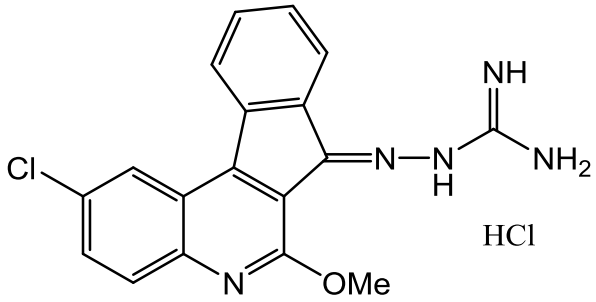
The product was converted into the corresponding dihydrochloride salt (light green solid) with 1 N HCl in ethanol. Mp 199.0-205.0 °C (dec).

2-Chloro-6-methoxy-7*H*-indeno[2,1-c]quinolin-7-one (86)



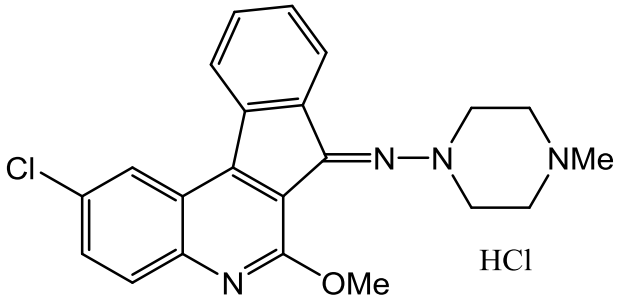
To a suspension of compound **81** (500 mg; 1.70 mmol), in 50 ml of a mixture of anhydrous THF/MeOH (2:1), sodium methoxide (897 mg; 6.00 mmol) was added and the mixture was refluxed under nitrogen for 5 h. The solvent was evaporated and the crude residue was diluted with 250 ml of CH_2Cl_2 and washed first with water and then with brine. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The crude product was rinsed with diethyl ether to obtain a yellow solid. Yield: 87%. Mp 222.2-223.1 °C (dec). ^{1}H NMR ($CDCl_3$) δ: 8.30 (d, 1H, J= 1.93 Hz); 8.00 (d, 1H, J= 6.88 Hz); 7.81 (d, 1H, J= 9.07 Hz); 7.75 (d, 1H, J= 1.93 Hz); 7.73-7.57 (m, 2H); 7.50-7.46 (m, 1H); 4.20 (s, 3H).

2-(2-Chloro-6-methoxy-7*H*-indeno[2,1-c]quinolin-7-ylidene)hydrazine-1-carboximidamide hydrochloride (26)



Aminoguanidine hydrochloride (48 mg; 0.44 mmol) was added to a suspension of compound **86** (130 mg; 0.44 mmol) in 20 ml of ethanol. After the addition of a catalytic amount of acetic acid, the mixture was refluxed for 24 h and then the solvent was evaporated to dryness. The crude residue was digested with hot ethanol and then rinsed with ethyl acetate to obtain an orange solid. Yield: 37%. Mp 236.0-237.0 °C. ^{1}H NMR (DMSO-d_6) δ: 11.61 (br s, 1H, collapsed with D_2O); 8.66-8.38 (m, 3H, 1H collapsed with D_2O); 7.89-7.64 (m, 7H, 2H collapsed with D_2O); 4.15 (s, 3H). HRMS (ESI) m/z Calcd for $C_{18}H_{15}ClN_5O$ $[M+H]^+$: 352.09651; found: 352.09595.

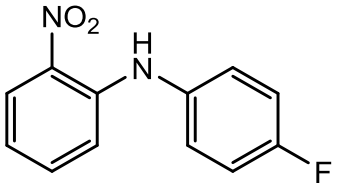
N-(2-Chloro-6-methoxy-7*H*-indeno[2,1-c]quinolin-7-ylidene)-4-methylpiperazin-1-amine hydrochloride (27)



Compound **86** (180 mg; 0.61 mmol) was added to a solution of 1-amino-4-methylpiperazine (110 µl; 0.91 mmol) and 1 N HCl (0.9 ml) in 20 ml of ethanol. The mixture was refluxed for 20 h and then the solvent was evaporated to dryness. The crude residue was digested and rinsed with ethyl acetate to generate an orange solid. Yield: 88%. Mp 201.7-203.5 °C (dec). ^{1}H NMR (DMSO-d_6) δ: 11.02 (br s, 1H, collapsed with D_2O); 8.60 (s, 1H); 8.50 (d, 1H, *J*= 7.70 Hz); 8.29 (d, 1H, *J*= 7.70 Hz); 7.87 (d, 1H, *J*= 9.08 Hz); 7.77 (d, 1H, *J*= 9.08 Hz); 7.67-7.63 (m, 2H); 4.09 (s, 3H); 3.51-3.43 (m, 8H); 2.85 (s, 3H). HRMS (ESI) m/z Calcd for $C_{22}H_{21}ClN_4O$ $[M+H]^+$: 393.14821; found: 393.14755.

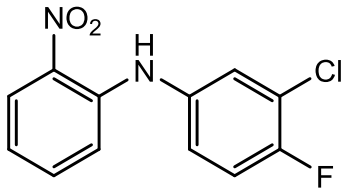
4.4 ***Riminophenazines***

N-(4-Fluorophenyl)-2-nitroaniline (88)



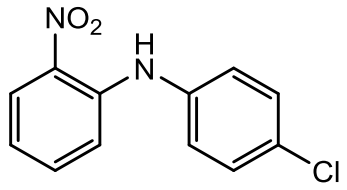
A mixture of 1-fluoro-2-nitrobenzene (3.0 ml, 28.35 mmol), 4-fluoroaniline (3.7 ml, 38.84 mmol) and 3.4 ml of pyridine was stirred under nitrogen at 120 °C for 6 h. After cooling, the mixture was diluted with water, alkalized with 6 N NaOH and extracted with CH_2Cl_2. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a residue that was purified by CC (silica gel; cyclohexane/AcOEt in gradient); the product eluted with 6% of ethyl acetate. The fraction containing the desired product was rinsed with petroleum ether to collect an orange solid. Yield: 65%. Mp 81.5-82.6 °C. ^{1}H NMR ($CDCl_3$) δ: 9.40 (br s, 1H, collapsed with D_2O); 8.22-8.19 (dd, 1H, *J*= 1.46, 9.08 Hz); 7.38-7.33 (m, 1H); 7.27-7.23 (m, 2H); 7.15-7.03 (m, 3H); 6.79-6.74 (t, 1H, *J*= 7.62 Hz).

3-Chloro-4-fluoro-N-(2-nitrophenyl)aniline (89)



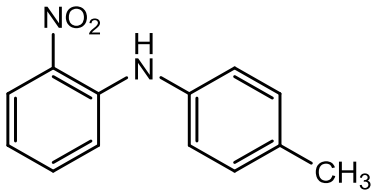
A mixture of 1-fluoro-2-nitrobenzene (3.0 ml, 28.35 mmol), 3-chloro-4-fluoroaniline (5.7 g, 38.84 mmol) and 3.4 ml of pyridine was stirred under nitorgen at 120 °C for 6 h. After cooling, the mixture was diluted with water, alkalized with 6 N NaOH and extracted with CH_2Cl_2. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a residue that was purified by CC (silica gel; cyclohexane/AcOEt in gradient); the product eluted with 6% of ethyl acetate. The fraction containing the desired product was rinsed with petroleum ether to collect an orange solid. Mp 114.0-115.1 °C. Another fraction containing the desired product mixed with an impurity was purified by a further CC (silica gel; cyclohexane/AcOEt in gradient), using the Flash Chromatography Purification System Biotage SP-1; the product eluted with 7% of ethyl acetate. The obtained product was rinsed with petroleum ether to collect an orange solid. Mp 114.2-115.3 °C. Total yield: 47%. ^{1}H NMR ($CDCl_3$) δ: 9.34 (s, 1H); 8.21 (dd, 1H; J= 1.65, 8.80 Hz); 7.43-7.34 (m, 2H); 7.19-7.08 (m, 2H); 6.85-6.80 (m, 2H).

N-(4-Chlorophenyl)-2-nitroaniline (90)



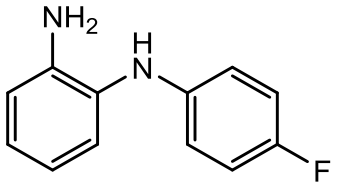
A mixture of 1-fluoro-2-nitrobenzene (2.5 ml, 20.00 mmol), 4-chloroaniline (3.5 g, 27.00 mmol) and 3.0 ml of pyridine was stirred under nitorgen at 120 °C for 8 h. After cooling, the mixture was diluted with water, alkalized with 6 N NaOH and extracted with CH_2Cl_2. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a residue that was purified by CC (silica gel; cyclohexane/AcOEt in gradient); the product eluted with 6% of ethyl acetate. The fraction containing the desired product was rinsed with diethyl ether to collect an orange solid. Yield: 42%. Mp 143.0-145.3 °C. ^{1}H-NMR ($CDCl_3$) δ: 9.40 (s, 1H); 8.21 (dd, 1H, J= 1.38, 8.53 Hz); 7.43-7.37 (m, 3H); 7.25-7.19 (m, 3H); 6.83-6.80 (m, 1H).

2-Nitro-N-(*p*-tolyl)aniline (91)



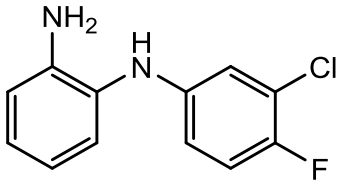
A mixture of 1-fluoro-2-nitrobenzene (0.7 ml, 7.08 mmol), 4-chloroaniline (1.0 g, 9.70 mmol) and 1.0 ml of pyridine was stirred under nitorgen at 120 °C for 6 h. After cooling, the mixture was diluted with water, alkalized with 6 N NaOH and extracted with CH_2Cl_2. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a residue that was purified by CC (silica gel; cyclohexane/AcOEt in gradient), using the Flash Chromatography Purification System Biotage SP-1; the product eluted with 5% of ethyl acetate. The fraction containing the desired product was rinsed with ethanol to collect an orange solid. Yield: 81%. Mp 66.2-67.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 9.45 (br s, 1H, collapsed with D_2O); 8.20 (dd, 1H, *J*= 1.43 Hz); 7.36 (t, 1H, *J*= 8.80 Hz); 7.27-7.12 (m, 5H); 6.77 (t, 1H, *J*= 8.80 Hz); 2.38 (s, 3H).

N^1-(4-Fluorophenyl)benzene-1,2-diamine (93)



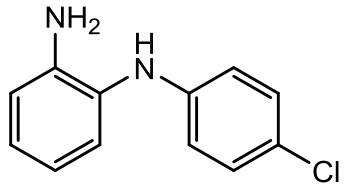
$SnCl_2$*$2H_2O$ (7.8 g, 8.60 mmol) was added portionwise to a solution of compound **88** (2 g, 8.60 mmol) in 34 ml of EtOH/AcOEt (1:1). The mixture was refluxed for 6 h and then the solvent was evaporated. The obtained residue was diluted with water and alkalized with 6 N NaOH. The formed precipitate was filtered and washed with ethyl acetate and then the filtrate was separated and the aqueous layer extracted with ethyl acetate. The collected organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain an oily residue which crystallized with diethyl ether/petroleum ether (7:3). A pale pink solid was obtained. Yield: 86%. Mp 63.3-64.1 °C. H NMR ($CDCl_3$) δ: 7.42-7.38 (m, 4H); 6.70-6.68 (m, 4H); 5.10 (s, 1H, collapsed with D_2O); 3.74 (s, 2H, collapsed with D_2O).

N^1-(3-Chloro-4-fluorophenyl)benzene-1,2-diamine (94)



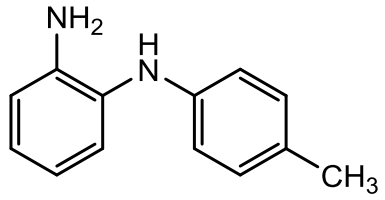
$SnCl_2$*$2H_2O$ (3.4 g, 15.00 mmol) was added portionwise to a solution of compound **89** (1 g, 3.75 mmol) in 14 ml of EtOH/AcOEt (1:1). The mixture was refluxed for 8 h and then the solvent was evaporated. The obtained residue was diluted with water and alkalized with 6 N NaOH. The formed precipitate was filtered and washed with ethyl acetate and then the filtrate was separated and the aqueous layer extracted with ethyl acetate. The collected organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The obtained residue was rinsed with petroleum ether/diethyl ether (9:1) to collect a cream-coloured solid. Yield: 86%. Mp 101.2-102.6 °C. ^{1}H NMR ($CDCl_3$) δ: 7.07-6.94 (m, 3H); 6.82-6.70 (m, 3H); 6.58-6.53 (m, 1H); 5.12 (s, 1H, collapsed with D_2O); 3.73 (br s, 2H, collapsed with D_2O).

N^1-(4-Chlorophenyl)benzene-1,2-diamine (95)



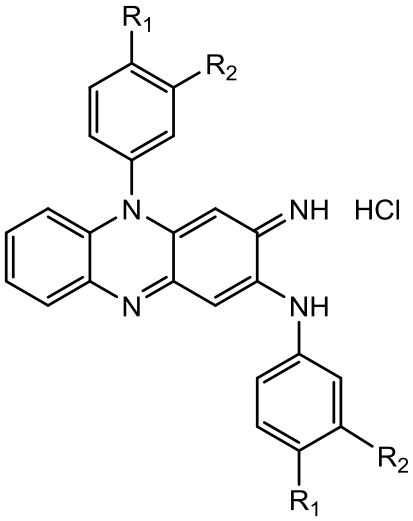
Compound **90** (1.4 g, 5.6 mmoli), Pd/C 10% (280 mg, 2.63 mmol) and 35 ml of ethanol were charged into an hydrogenation flask and treated with H_2 at atmospheric pressure for 6 h. Pd/C was filtered on a celite pad and the filtrate was evaporated to collect a brown solid, which was directly used in the next step without further purification. Yield: 87%. Mp 95.0-98.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.05-6.93 (m, 4H); 6.84-6.78 (m, 2H); 6.67 (d, 2H, J= 8.25 Hz); 5.25 (s, 1H); 4.10 (s, 2H).

N^1-(*p*-Tolyl)benzene-1,2-diamine (96)



A mixture of compound **91** (700 mg, 3.00 mmol), metallic iron (837 mg, 15.00 mmol) and 10 ml of acetic acid was stirred at 70 °C for 45 minutes. After cooling, the solvent was evaporated and the residue was diluted with 2 N NaOH and CH_2Cl_2. The formed $Fe(OH)_2$ was filtered and the filtrate was separated. The aqueous phase was extracted with CH_2Cl_2 and the organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The obtained residue was rinsed with petroleum ether to collect a brown solid. Yield: 76%. Mp 71.0-74.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.11-7.02 (m, 4H); 6.83-6.75 (m, 4H); 4.09 (br s, 2H, collapsed with D_2O); 2.27 (s, 3H). 1H is not visible, probably for the interaction with the solvent.

N,5-*bis*(Substituted phenyl)-3-imino-3,5-dihydrophenazin-2-amines hydrochloride (75-101)



<u>*General procedure*</u>: A solution of $FeCl_3$*$6H_2O$ (9.25 mmol) in 13.4 ml of water and 0.33 ml of concentrated HCl was added to a solution of the proper N^1-(substituted phenyl)benzene-1,2-diamine **92-96** (3.34 mmol) and 7 ml of acetic acid. The mixture was stirred at r.t. for 4 h and then the obtained precipitate was filtered and washed with water and absolute ethanol.

3-Imino-N,5-diphenyl-3,5-dihydrophenazin-2-amine hydrochloride (97)

A dark brown solid was obtained. Yield: 93%. Mp 300.0-302.0 °C (dec.). ^{1}H NMR in $CDCl_3$ δ: 9.91 (s, 1H, collapsed with D_2O); 8.12 (d, 1H, *J*= 8.25 Hz); 7.81-7.67 (m, 3H); 7.64-7.42 (m, 5H); 7.39-7.26 (m, 3H); 7.20-7.15 (m, 2H); 7.03 (d, 1H, *J*= 8.25 Hz); 6.34-6.27 (m, 2H).

1H is not visible, probably for the interaction with the solvent.

N,5-*bis*(4-Fluorophenyl)-3-imino-3,5-dihydrophenazin-2-amine hydrochloride (98)

A dark violet solid was obtained. Yield: 90%. Mp 307.0-309.0 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 9.70 (s, 1H, collapsed with D_2O); 9.36 (s, 2H, collapsed with D_2O); 8.24-8.14 (m, 1H); 7.82-7.60 (m, 6H); 7.51-7.46 (m, 2H); 7.38-7.25 (m, 2H); 7.19 (s, 1H); 7.06-7.03 (m, 1H); 6.20 (s, 1H).

N,5-*bis*(3-Chloro-4-fluorophenyl)-3-imino-3,5-dihydrophenazin-2-amine hydrochloride (99)

Solid rinsed with diethyl ether to collect a dark violet solid. Yield: 85%. Mp > 300 °C. ^{1}H NMR (DMSO-d_6) δ: 9.72 (s, 1H, collapsed with D_2O); 9.33 (s, 2H, collapsed with D_2O); 8.20-8.14 (m, 2H); 8.03-8.00 (m, 1H); 7.97-7.63 (m, 3H); 7.58-7.49 (m, 3H); 7.32-7.18 (m, 2H); 6.24 (s, 1H).

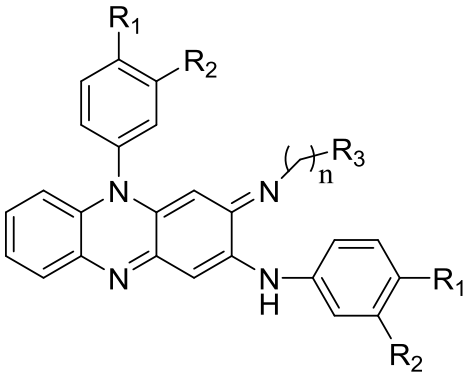
N,5-*bis*(4-Chlorophenyl)-3-imino-3,5-dihydrophenazin-2-amine hydrochloride (100)

A dark brown solid was obtained. Yield: 86%. Mp >300 °C. ^{1}H-NMR (DMSO-d_6) δ: 9.24 (s, 1H); 8.19 (m, 2H); 7.98 (d, 2H, *J*= 6.33 Hz); 7.84-7.82 (m, 1H); 7.79-7.73 (m, 4H); 7.59-7.49 (m, 2H); 7.45 (d, 2H, *J*= 6.33 Hz); 7.37 (s, 1H); 7.16-7.14 (m, 1H); 6.20 (s, 1H).

3-Imino-N,5-di-*p*-tolyl-3,5-dihydrophenazin-2-amine hydrochloride (101)

The residue was purified by CC (silica gel; CH_2Cl_2/MeOH/ conc. NH_3 in gradient); the product eluted with the 6% of MeOH. A dark brown solid was obtained. Yield: 70%. Mp >300 °C. ^{1}H-NMR (DMSO-d_6) δ: 9.42 (br s, 3H, collapsed with D_2O); 8.05-8.03 (m, 1H); 7.64-7.55 (m, 6H); 7.36-7.29 (m, 4H); 7.12 (s, 1H); 6.92-6.89 (m, 1H); 6.08 (s, 1H); 2.52 (s, 3H); 2.35 (s, 3H).

3-[ω-(Azacycloalkyl)alkyl]imino)-N,5-diphenyl-3,5-dihydrophenazin-2-amines (Series A: 28-35)



General procedure: Differently substituted phenazine hydrochloride (**97-101**; 1.6 mmol) and the proper amine (1.6 mmol) were dissolved in 7 ml of 1,4-dioxane and refluxed for 5 h. After cooling, the solvent was evaporated and the obtained mixture diluted with H_2O and aqueous 6N NaOH and extracted several times with diethyl ether. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The crude product was purified by CC (CH_2Cl_2/MeOH/conc. NH_3 in gradient) and the purified fraction was treated with the indicated solvent.

3-[(Hexahydro-1*H*-pyrrolizin-7a-yl)methylimino]-N,5-diphenyl-3,5-dihydrophenazin-2-amine (28)

Solid rinsed with petroleum ether to obtain brick-red crystals. Yield: 45%. Mp 160.0-162.0 °C (dec.). ^{1}H NMR ($CDCl_3$) δ: 7.75-7.63 (m, 4H); 7.54 (d, 2H, J= 7.70 Hz); 7.37-7.30 (m, 4H); 7.23-7.02 (m, 4H); 6.51 (d, 1H, J= 7.70 Hz); 5.32 (s, 1H); 3.60 (s, 2H); 3.27 (s, 2H); 2.84-2.80 (m, 2H); 2.07-1.91 (m, 5H); 1.79-1.73 (m, 3H). 1H is not visible, probably for the interaction with the solvent. HRMS (ESI) m/z calcd for $C_{32}H_{32}N_5$ $[M+H]^+$: 486.26577; found: 486.26483. The obtained solid was converted into the corresponding dihydrochloride salt with 1N HCl in ethanol. Mp 189.0-191.5 °C (dec.).

N,5-*bis*(4-Chlorophenyl)-3-[(hexahydro-1*H*-pyrrolizin-7a-yl)methylimino]-3,5-dihydrophenazin-2-amine (29)

Solid rinsed with diethyl ether to give brick-red crystals. Yield: 11%. Mp 168.4-169.4 °C (dec.). ^{1}H NMR ($CDCl_3$) δ: 8.62 (br s, 1H, collapsed with D_2O); 7.68 (d, 3H, *J*= 7.70 Hz); 7.32-7.20 (m, 6H); 7.18-7.09 (m, 2H); 6.86 (s, 1H); 6.41 (d, 1H, *J*= 7.98 Hz); 5.28 (s, 1H); 3.20-3.09 (m, 4H); 2.69 (s, 2H); 1.97-1.67 (m, 6H); 1.65-1.61 (m, 2H). HRMS (ESI) m/z calcd for $C_{32}H_{30}N_5Cl_2$ $[M+H]^+$: 554.18783; found: 554.18695. The obtained solid was converted into the corresponding dihydrochloride salt with 1N HCl in ethanol. Mp 199.0-202.0 °C (dec.).

3-{[(1S,9aR)-(Octahydro-2H-quinolizin-1-yl)methyl]imino}-2-(4-tolylamino)-5-(4-tolyl)-3,5-dihydrophenazine (30)

Solid rinsed with petroleum ether to collect a dark red solid. Yield: 61%. Mp 173.0-175.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.75-7.63 (m, 1H); 7.50-7.45 (m, 2H); 7.42-7.32 (m, 8H); 6.83 (s, 1H); 6.47-6.45 (m, 1H); 5.37 (s, 1H); 3.40-3.34 (m, 2H); 2.85-2.78 (m, 2H); 2.53 (s, 3H); 2.35 (s, 3H); 2.10-1.92 (m, 4H); 1.87-1.65 (m, 3H); 1.54-1.36 (m, 7H). 1H is not visible, probably for the interaction with the solvent. HRMS (ESI) m/z calcd for $C_{36}H_{40}N_5$ $[M+H]^+$: 542.32837. Found: 542.32715.

The product was converted into the corresponding dihydrocloride salt with 1N HCl in ethanol. Mp: 235-237 °C (dec.).

^{1}H-NMR ($DMSO\text{-}d_6$) δ: 10.59 (br s, 1H, collapsed with D_2O); 10.48 (br s, 1H, collapsed with D_2O); 9.75 (br s, 1H, collapsed with D_2O); 8.17 (d, 1H, *J*= 7.43 Hz); 7.78-7.60 (m, 6H); 7.39-7.20 (m, 5H); 7.02 (d, 1H, *J*= 7.43 Hz); 5.81 (s, 1H); 3.93-3.91 (m, 1H); 3.24-3.07 (m, 7H); 2.58 (s, 3H); 2.35 (s, 3H); 1.95-1.79 (m, 4H); 1.53-1.38 (m, 5H); 0.86-0.84 (m, 1H).

3-[4-(Octahydro-1*H*-quinolizin-9a-yl)butylimino]-N,5-diphenyl-3,5-dihydrophenazin-2-amine (31)

Solid rinsed with a mixture of diethyl ether/petroleum ether (1:1) to give dark red crystals. Yield: 37%. Mp 157.9-158.7 °C. ^{1}H NMR ($CDCl_3$) δ: 7.75-7.63 (m, 4H); 7.37-7.33 (m, 6H); 7.18-7.09 (m, 3H); 6.93 (s, 1H); 6.50-6.47 (m, 1H); 5.27 (s, 1H); 3.16-3.11 (m, 2H); 2.59-2.54 (m, 2H); 2.43-2.40 (m, 2H); 1.67-1.14 (m, 18H). 1H is not visible, probably for the interaction with the solvent. HRMS (ESI) *m/z* Calcd for $C_{37}H_{42}N_5$ $[M+H]^+$: 556.34402; found: 556.34283.

The product was converted into the corresponding dihydrochloride salt with 1N HCl in ethanol. Mp 260-261 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 10.28 (br s, 1H, collapsed with D_2O); 10.15-10.07 (m, 2H, collapsed with D_2O); 8.18 (d, 1H, J= 8.25 Hz); 7.92-7.84 (m, 3H); 7.82-7.72 (m, 4H); 7.54-7.45 (m, 4H); 7.31 (s, 1H); 7.28-7.23 (m, 1H); 7.05 (d, 1H, J= 8.25 Hz); 5.73 (s, 1H); 3.20-3.07 (m, 4H); 2.92-2.89 (m, 2H); 1.88-1.13 (m, 18H).

3-[6-(Octahydro-1*H*-quinolizin-9a-yl)hexylimino]-N,5-diphenyl-3,5-dihydrophenazin-2-amine (32)

Solid rinsed with petroleum ether and few drops of diethyl ether to give dark red crystals. Yield: 52%. Mp 129.2-130.6 °C. ^{1}H NMR ($CDCl_3$) δ: 7.75-7.70 (m, 4H); 7.66-7.33 (m, 6H); 7.19-7.06 (m, 3H); 6.92 (s, 1H); 6.48 (d, 1H, J= 7.98 Hz); 5.28 (s, 1H); 3.15-3.11 (m, 2H); 2.64-2.60 (m, 2H); 2.57-2.44 (m, 2H); 1.61-1.11 (m, 22H). 1H is not visible, probably for the interaction with the solvent. HRMS (ESI) *m/z* Calcd for $C_{39}H_{46}N_5$ $[M+H]^+$: 584.37532; found: 584.37421.

The product was converted into the corresponding dihydrochloride salt with 1N HCl in ethanol. Mp 207.3-209.0 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 10.26-10.03 (m, 3H, collapsed with D_2O); 8.19 (d, 1H, J= 8.53 Hz); 7.90-7.84 (m, 3H); 7.75-7.73 (m, 4H); 7.54-7.45 (m, 4H); 7.31-7.25 (m, 2H); 7.05 (d, 1H, J= 8.53 Hz); 5.72 (s, 1H); 4.98-3.08 (m, 4H); 2.90-2.72 (m, 2H); 1.90-1.10 (m, 22H).

N,5-*bis*(4-Fluorophenyl)-3-[4-(octahydro-1*H*-quinolizin-9a-yl)butylimino]-3,5-dihydrophenazin-2-amine (33)

Solid recrystallized with a mixture of diethyl ether/petroleum ether (1:1) and rinsed with diethyl ether to give dark brown crystals. Yield: 35%. Mp 135.3-137.0 °C. ^{1}H NMR ($CDCl_3$) δ: 7.70 (m, 1H); 7.42-7.05 (m, 8H); 6.75 (s, 1H); 6.48 (m, 1H); 5.26 (s, 1H); 3.16 (m, 2H); 2.68-2.47 (m, 4H); 1.61-1.19 (m, 18H). 1H is not visible, probably for the interaction with the solvent. HRMS (ESI) *m/z* Calcd for $C_{37}H_{40}N_5F_2$ $[M+H]^+$: 592.32518; found: 592.32410.

The product was converted into the corresponding dihydrochloride salt with 1N HCl in ethanol. Mp 214-215 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 10.23-10.05 (m, 3H, collapsed with D_2O); 8.19 (m, 1H); 7.75-7.10 (m, 12H); 5.76 (s, 1H); 3.27-2.94 (m, 6H); 1.72-1.22 (m, 18H).

N,5-*bis*(4-Fluorophenyl)-3-[6-(octahydro-1*H*-quinolizin-9a-yl)hexylimino]-3,5-dihydrophenazin-2-amine (34)

Solid rinsed with a mixture of diethyl ether/petroleum ether (1:1) to give dark red crystals. Yield: 23%. Mp 128.5-130.0 °C. ^{1}H NMR ($CDCl_3$) δ: 7.69 (m, 1H); 7.46-6.91 (m, 10H); 6.73 (s, 1H); 6.48 (m, 1H); 5.26 (s, 1H); 3.44-3.13 (m, 2H); 2.63-2.46 (m, 4H); 1.62-1.12 (m, 22H). 1H is not visible, probably for the interaction with the solvent. HRMS (ESI) *m/z* Calcd for $C_{39}H_{44}N_5F_2$ $[M+H]^+$: 620.35648; found: 620.35535.

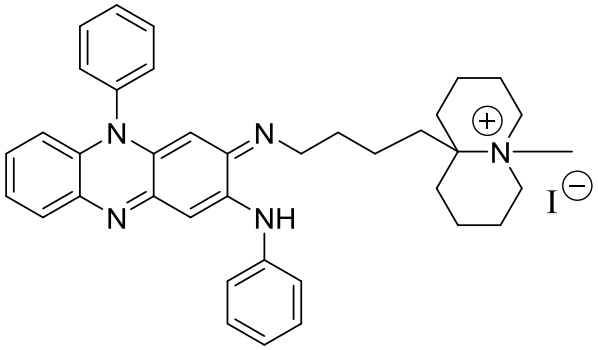
The product was converted into the corresponding dihydrochloride salt with 1N HCl in ethanol. Mp 213-214 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 10.18 (br s, 1H, collapsed with D_2O); 9.96 (m, 2H, collapsed with D_2O); 8.18 (d, 1H, J= 7.70 Hz); 7.82-7.74 (m, 6H); 7.50-7.45 (m, 2H); 7.39-7.33 (m, 2H); 7.19 (s, 1H); 7.10 (d, 1H, J= 7.70 Hz); 5.72 (s, 1H); 3.41-3.14 (m, 4H); 3.10-2.94 (m, 2H); 1.79-1.01 (m, 22H).

N,5-*bis*(3-Chloro-4-fluorophenyl)-3-[6-(octahydro-1*H*-quinolizin-9a-yl)hexylimino]-3,5-dihydrophenazin-2-amine (35)

Solid rinsed with diethyl ether to give brick-red crystals. Yield: 25%. Mp 108.2-109.4 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 8.76 (br s, 1H, collapsed with D_2O); 7.99-7.88 (m, 1H); 7.85-7.82 (m, 1H); 7.63-7.59 (m, 3H); 7.45-7.42 (m, 2H); 7.22 (m, 2H); 6.61 (s, 1H); 6.53 (m, 1H); 5.18 (s, 1H); 3.50-3.32 (m, 2H); 2.18-2.72 (m, 2H); 2.32 (m, 2H); 1.50-1.05 (m, 22H). HRMS (ESI) *m/z* Calcd for $C_{39}H_{42}N_5Cl_2F_2$ $[M+H]^+$: 688.27853; found: 688.27771.

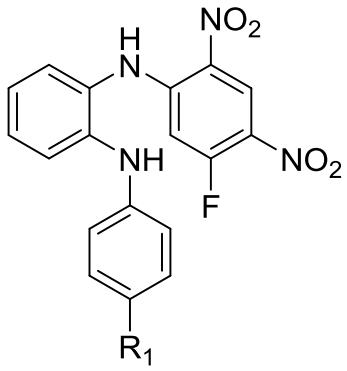
The product was converted into the corresponding dihydrochloride salt with 1N HCl in ethanol. Mp 245-247 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 10.23-9.97 (m, 3H, collapsed with D_2O); 8.21-8.01 (m, 2H); 7.97 (d, 1H, J= 8.80 Hz); 7.84-7.75 (m, 3H); 7.64-7.48 (m, 3H); 7.31-7.21 (m, 1H); 7.19 (d, 1H, J= 8.80 Hz); 5.77 (s, 1H); 3.58-3.42 (m, 4H); 3.38-2.94 (m, 4H); 1.84-1.02 (m, 20H).

5-Methyl-9a-{4-[10-phenyl-3-(phenylamino)phenazin-2(10*H*)-ylideneamino]butyl}-decahydroquinolizinium iodide (36)



Iodomethane (4 µl, 0.05 mmol) was added to a solution of compound **31 (**30 mg, 0.05 mmol) in 1.5 ml of anhydrous THF and the mixture was stirred at r.t. for 72 h. The obtained precipitate was filtered and washed with a mixture of THF/diethyl ether (1:1) to provide a dark red solid. Yield: 65%. Mp 191.0-192.5 °C. ^{1}H NMR ($CDCl_3$) δ: 7.77-7.69 (m, 4H); 7.36-7.25 (m, 4H); 7.11-6.99 (m, 6H); 6.56-6.40 (m, 1H); 5.30 (s, 1H); 4.02-3.47 (m, 4H); 3.27 (s, 3H); 3.15-3.07 (m, 2H); 2.10-1.20 (m, 18H). 1H is not visible, probably for the interaction with the solvent. HRMS (ESI) *m/z* Calcd for $C_{38}H_{44}N_5$ $[M+H]^+$: 570.35967; found: 570.35858.

N^1-(5-Fluoro-2,4-dinitrophenyl)-N^2-(4-substitutedphenyl)benzene-1,2-diamines (102, 103)



General procedure: 1,5-Difluoro-2,4-dinitrobenzene (10.86 mmol) and TEA (10.94 mmol) were added to a solution of the proper N^1-((4-substituted)phenyl)benzene-1,2-diamine (10.86 mmol) in 40 ml of ethanol. The mixture was stirred at r.t. for 1.5 h. The formed suspension was filtered and the resulting solid washed with ethanol.

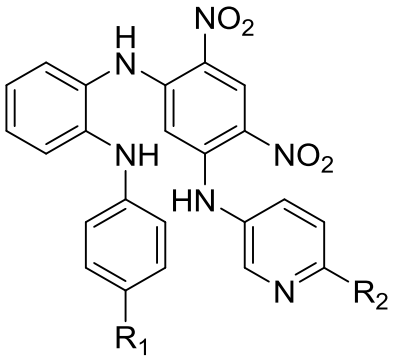
N^1-(5-Fluoro-2,4-dinitrophenyl)-N^2-phenylbenzene-1,2-diamine (102)

Yield: 93%. Mp 163.6-164.7 °C. ^{1}H-NMR ($CDCl_3$) δ: 9.60 (br s, 1H, collapsed with D_2O); 9.13 (d, 1H, *J*= 7.70 Hz); 7.36-7.24 (m, 5H); 7.07-6.99 (m, 4H); 6.65 (d, 1H, *J*= 13.20 Hz); 5.65 (br s, 1H, collapsed with D_2O).

N^1-(4-Chlorophenyl)-N^2-(5-fluoro-2,4-dinitrophenyl)benzene-1,2-diamine (103)

Yield: 94%. Mp 211.7-214.0 °C. ^{1}H NMR ($CDCl_3$) δ: 9.6 (br s, 1 H, collapsed with D_2O); 9.14 (d, 1 H, *J*= 7.70 Hz); 7.36-7.21 (m, 5 H); 7.10-7.04 (m, 1 H); 6.97-6.93 (m, 2 H); 6.67-6.63 (m, 1 H); 5.63 (br s, 1 H, collapsed with D_2O).

N^1-(Substituted phenyl)-N^2-[2,4-dinitro-5-(substituted pyridin-3-ylamino)phenyl]benzene-1,2-diamines (104-106)



General procedure: Pyridine-3-amine (5.38 mmol) and TEA (4.89 mmol) were added to a solution of the proper N^1-(5-fluoro-2,4-dinitrophenyl)-N^2-((4-substitutedphenyl)benzene)-1,2-diamine **102, 103** (4.89 mmol) in 18 ml of THF and the mixture was refluxed for 20 h. The formed precipitate was filtered and washed with THF and CH_2Cl_2.

N^1-[2,4-Dinitro-5-(pyridin-3-ylamino)phenyl]-N^2-phenylbenzene-1,2-diamine (104)

Yield: 88%. Mp 213.4-215.2 °C. ^{1}H-NMR (DMSO-d_6) δ: 9.70 (br s, 1H, collapsed with D_2O); 9.48 (br s, 1H, collapsed with D_2O); 8.99 (s, 1H); 8.40-8.37 (m, 2H); 7.62-7.60 (m, 2H); 7.36-7.32 (m, 1H); 7.20-7.10 (m, 5H); 6.85-6.82 (m, 4H); 5.96 (s, 1H).

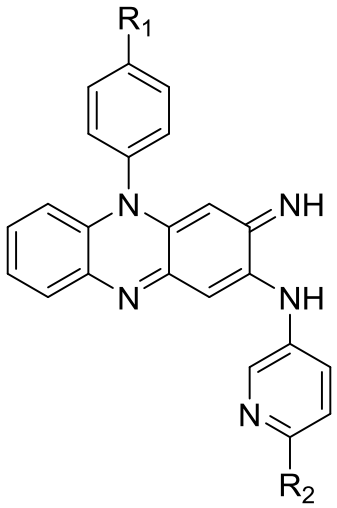
N^1-(4-Chlorophenyl)-N^2-[2,4-dinitro-5-(pyridin-3-ylamino)phenyl]benzene-1,2-diamine (105)

Yield: 94%. Mp 211.7-214.0 °C. ^{1}H NMR ($CDCl_3$) δ: 9.72 (s, 1H, collapsed with D_2O); 9.49 (s, 1H, collapsed with D_2O); 9.01 (s, 1H); 8.41 (m, 2 H); 7.77 (s, 1H, collapsed with D_2O); 7.61 (m, 1H); 7.38-7.15 (m, 6H); 6.90-6.80 (m, 3 H); 5.94 (s, 1H).

N^1-[5-(6-Methoxypyridin-3-ylamino)-2,4-dinitrophenyl]-N^2-phenylbenzene-1,2-diamine (106)

Yield: 85%. Mp 189.9-191.3 °C. ^{1}H-NMR (DMSO-d_6) δ: 9.59 (s, 1 H, collapsed with D_2O); 9.43 (s, 1 H, collapsed with D_2O); 8.98 (s, 1 H); 7.95 (d, 1H, *J*= 2.47 Hz); 7.61 (s, 1H); 7.51 (dd; 1H, *J*= 2.47; 8.80 Hz); 7.20-7.10 (m, 5H); 6.85-6.76 (m, 5H); 5.77 (s, 1H); 3.34 (s, 3H).

3-Imino-N-(substituted pyridin-3-yl)-5-(substituted phenyl)-3,5-dihydrophenazin-2-amines (107-109)



General procedure: Zinc powder (36.16 mmol) was added portion wise into a suspension of the proper N^1-(substituted phenyl)-N^2-[2,4-dinitro-5-(substituted pyridin-3-ylamino)phenyl]benzene-1,2-diamine **104-106** (1.81 mmol) in 10 ml of glacial acetic acid cooled with an ice-water bath. The mixture was stirred at r.t. until the color turned to light green and then filtered and washed with glacial acetic acid and methanol. The filtrate was concentrated and the residue was treated with water and alkalized with conc. NH_3. The formed precipitate was filtered, washed with water and then dissolved in methanol. The solution was stirred under air overnight. The formed solid was filtered and washed with methanol and diethyl ether. The crude product was directly used for the next step.

3-Imino-5-phenyl-N-(pyridin-3-yl)-3,5-dihydrophenazin-2-amine (107)

Yield: 84%. Mp 206.3-209.5 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 9.90 (br s, 2H, collapsed with D_2O); 8.55 (s, 1H); 8.28 (d, 1H, *J*= 2.80 Hz); 7.80-7.63 (m, 5H); 7.57 (d, 2H, *J*= 7.15 Hz); 7.42-7.38 (m, 1H); 7.25-7.18 (m, 2H); 6.65 (s, 1H); 6.42 (d, 1H, *J*= 8.80 Hz); 5.42 (s, 1H).

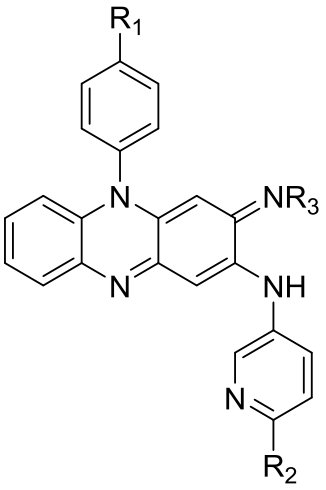
5-(4-Chlorophenyl)-3-imino-N-(pyridin-3-yl)-3,5-dihydrophenazin-2-amine (108)

Yield: 42%. Mp 190.0-194.0 °C. ^{1}H NMR (DMSO-d_6) δ: 9.91 (br s, 2H, collapsed with D_2O); 8.54 (s, 1H); 2.28 (d, 1H, *J*= 2.80 Hz); 7.89-7.78 (m, 3H); 7.62-7.58 (m, 3H); 7.41-7.39 (m, 1H); 7.22-7.20 (m, 2H); 6.64 (s, 1H); 6.51-6.43 (m, 1H); 5.42 (s, 1H).

3-Imino-N-(6-methoxypyridin-3-yl)-5-phenyl-3,5-dihydrophenazin-2-amine (109)

Yield: 49%. Mp 191.2-193.0 °C. ^{1}H NMR (DMSO-d_6) δ: 9.12 (br s, 2H, collapsed with D_2O); 8.15 (d, 1H, *J*= 2.75 Hz); 7.79-7.66 (m, 4H); 7.59-7.56 (m, 1H); 7.47 (d, 2H, *J*= 7.15 Hz); 7.18-7.15 (m, 2H); 6.89 (d, 1H, *J*= 8.53 Hz); 6.39-6.37 (m, 1H); 6.36 (s, 1H); 5.31 (s, 1H); 3.86 (s, 3H).

N-(Substituted pyridin-3-yl)-3-(aminoalkyl)-5-substituted phenyl-3,5-dihydrophenazin-2-amines (series B: 37-40)



General procedure: The proper phenazine **107-109** (0.56 mmol) and the 4-(octahydro-1H-quinolizin-9a-yl)butan-1-amine or N',N'-dimethylpropane-1,3-diamine (1.12 mmol) were dissolved in 5 ml of 1,4-dioxane and refluxed for 24 h (compounds **37** and **38**) or 4 h (compounds **39** and **40**). After cooling, the solvent was evaporated and the obtained mixture diluted with CH_2Cl_2 and washed three times with water. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The crude product was purified as indicated.

3-{[4-(Octahydro-1*H*-quinolizin-9a-yl)butyl]imino}-5-phenyl-N-(pyridin-3-yl)-3,5-dihydrophenazin-2-amine dihydrochloride (37)

The amorphous residue was digested with diethyl ether. The filtrate was evaporated and washed with a mixture of petroleum ether/diethyl ether (1:1). The solid was converted into the corresponding dihydrochloride salt with 1N HCl in ethanol. Yield: 38%. Mp 218.0-219.8 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 10.75 (s, 1H, collapsed with D_2O); 10.22 (s, 1H, collapsed with D_2O); 10.05 (s, 1H, collapsed with D_2O); 8.52 (d, 1H, J= 5.28 Hz); 8.35-8.26 (m, 2H); 7.93-7.67 (m, 9H);

7.12-7.10 (m, 1H); 5.76 (s, 1H); 3.19-3.06 (m, 4H); 2.91-2.88 (m, 2H); 1.89-1.56 (m, 14H); 1.22-1.18 (m, 4H). HRMS (ESI) *m/z* Calcd for $C_{36}H_{41}N_6$ $[M+H]^+$: 557.33927; found: 557.33838.

N-(6-Methoxypyridin-3-yl)-3-{[4-(octahydro-1*H*-quinolizin-9a-yl)butyl]imino}-5-phenyl-3,5-dihydrophenazin-2-amine dihydrochloride (38)

The amorphous residue was purified by CC (neutral alumina, grade IV; CH_2Cl_2/MeOH in gradient). The obtained solid was converted into the corresponding dihydrochloride salt with 1N HCl in ethanol. Yield: 24%. Mp 217.6-219.0 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 10.28-10.22 (m, 3H, collapsed with D_2O); 8.24-8.18 (m, 2H); 7.92-7.71 (m, 6H); 7.21-6.92 (m, 5H); 5.72 (s, 1H); 3.91 (s, 3H); 3.19-2.88 (m, 6H); 1.89-1.55 (m, 14H); 1.34-1.22 (m, 4H). HRMS (ESI) *m/z* Calcd for $C_{37}H_{43}N_6O$ $[M+H]^+$: 587.34984; found: 587.34906.

5-(4-Chlorophenyl)-3-{[4-(octahydro-1*H*-quinolizin-9a-yl)butyl]imino}-N-(pyridin-3-yl)-3,5-dihydrophenazin-2-amine trihydrochloride (39)

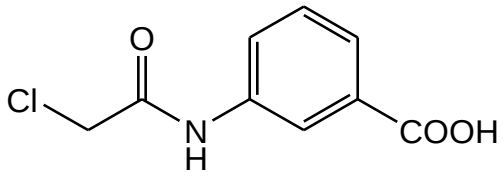
The amorphous residue was purified by CC (silica gel; CH_2Cl_2/MeOH in gradient). The obtained solid was converted into the corresponding trihydrochloride salt with 1N HCl in ethanol. Yield: 86%. Mp 228.0-231.0 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 10.74 (s, 1H, collapsed with D_2O); 10.22-10.13 (m, 2H, collapsed with D_2O); 8.83 (s, 1H); 8.53-8.51 (m, 1H); 8.27-8.24 (m, 2H); 8.05-7.97 (m, 3H); 7.85-7.77 (m, 4H); 7.69 (s, 1H); 7.14-7.11 (m, 1H); 5.79-5.77 (m, 1H); 3.44-3.39 (m, 2H); 3.28-3.07 (m, 2H); 2.91-2.87 (m, 2H); 1.91-1.01 (m, 18H). 1H is not visible, probably for the interaction with the solvent. HRMS (ESI) *m/z* Calcd for $C_{36}H_{40}N_6Cl$ $[M+H]^+$: 591.30030; found: 591.29919.

N^1-[10-(4-Chlorophenyl)-3-(pyridin-3-ylamino)phenazin-2(10*H*)-ylidene]-N^3,N^3-dimethylpropane-1,3-diamine (40)

The amorphous residue was purified by CC (silica gel; CH_2Cl_2/MeOH in gradient). The obtained dark red oil was crystallized with a mixture of petroleum ether/diethyl ether (95:5) to give a dark red solid. Yield: 45%. Mp 115.5-117.5 °C. ^{1}H NMR (DMSO-d_6) δ: 8.60-8.58 (m, 1H); 8.35-8.33 (m, 1H); 7.80-7.60 (m, 4H); 7.32-7.28 (m, 3H); 7.23-7.15 (m, 2H); 6.86 (s, 1H); 6.49-6.46 (m, 1H); 5.29 (s, 1H); 3.20 (t, 2H, *J*= 6.83 Hz); 2.36 (t, 2H, *J*= 6.83 Hz); 2.22 (s, 6H); 1.82 (q, 2H, *J*= 7.15 Hz). 1H is not visible, probably for the interaction with the solvent. HRMS (ESI) *m/z* Calcd for $C_{28}H_{28}N_6Cl$ $[M+H]^+$: 483.20640; found: 483.20581.

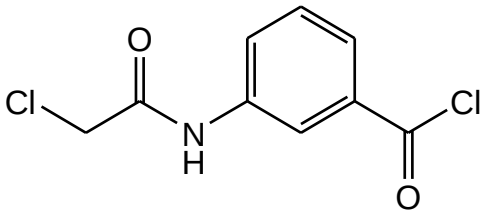
4.5 ***Aphidicolin derivatives***

3-(2-Chloroacetamido)benzoic acid (110)



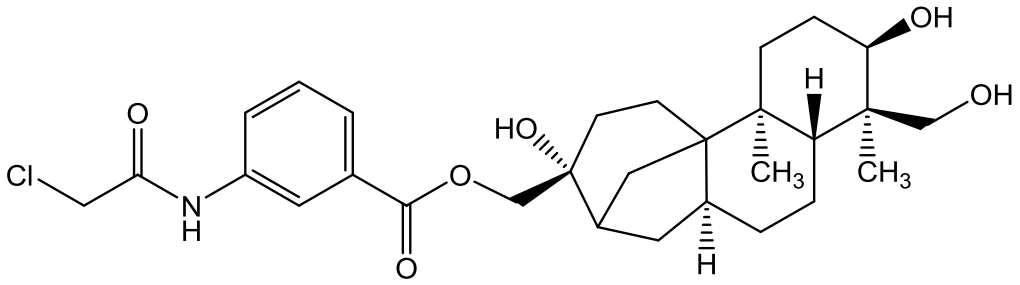
Chloroacetyl chloride (0.58 ml, 7.29 mmol) was added dropwise to a solution of 3-aminobenzoic acid (1 g, 7.29 mmol) in 5 ml of anhydrous DMF. The mixture was stirred under nitrogen at r.t. for 20 h and then poured into 50 ml of iced water. The obtained white precipitate was filtered and washed twice with water and twice with petroleum ether. A white solid was obtained. Yield: 92%. Mp 225.5-226.6 °C. ^{1}H NMR (DMSO-d_6) δ: 10.48 (s, 1H, collapsed with D_2O); 8.21 (s, 1H); 7.79 (d, 1H, J= 7.15 Hz); 7.64 (d, 1H, J= 7.15 Hz); 7.43 (t, 1H, J= 7.15 Hz); 4.22 (s, 2H). 1H is not visible, probably for the interaction with the solvent.

3-(2-Chloroacetamido)benzoyl chloride (111)



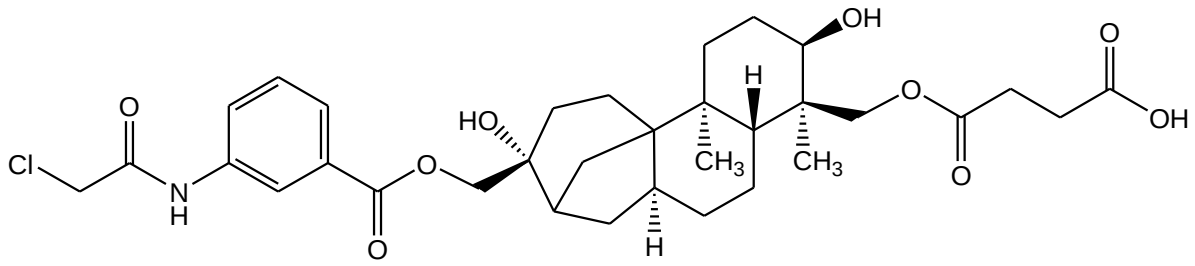
Compound **110** (300 mg, 1.40 mmol) was added to a solution of 1.5 ml of $SOCl_2$ and 0.5 ml of anhydrous chloroform heated at 60 °C. The solution was stirred under nitrogen at 60 °C for 4.5 h and then diluted with 3 ml of petroleum ether. The obtained precipitate was filtered and washed with petroleum ether to obtain a cream-coloured solid. Yield: 75%. Mp 97.8-100.2 °C. ^{1}H NMR (DMSO-d_6) δ: 10.54 (s, 1H, collapsed with D_2O); 8.21 (s, 1H); 7.80 (d, 1H, *J*= 7.15 Hz); 7.63 (d, 1H, *J*= 7.15 Hz); 7.43 (t, 1H, *J*= 7.15 Hz); 4.24 (s, 2H).

(3,9-Dihydroxy-4-(hydroxymethyl)-4,11b-dimethyltetradecahydro-8,11a-methanocyclohepta[a]naphthalen-9-yl)methyl 3-(2-chloroacetamido)benzoate (41)



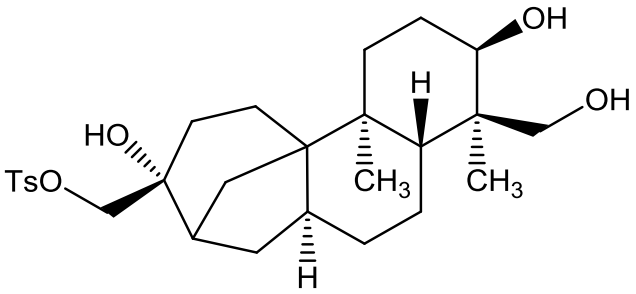
Compound **111** (58 mg, 0.25 mmol) was added dropwise to an ice-cooled solution of aphidicolin (65 mg, 0.19 mmol) in 1 ml of anhydrous pyridine. The mixture was stirred at r.t. for 2.5 h and then diluted with few drops of water, in order to quench the unreacted acyl chloride. Pyridine was evaporated and the obtained residue was diluted with water and ethyl acetate, acidified with 1 N HCl (pH= 1-2) and extracted with ethyl acetate. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a residue that was purified by CC (silica gel; CH_2Cl_2/MeOH in gradient); the product eluted with 2% of MeOH. The fraction containing the desired product and a by-product was purified again by another CC in the same conditions. The fractions containing the purified product were gathered up, crystallized and rinsed with diethyl ether to provide a white solid. Yield: 21%. Mp 129.2-131.0 °C. ^{1}H NMR (pyridine-d_5) δ: 11.62 (s, 1H, collapsed with D_2O); 8.79 (s, 1H); 8.46 (d, 1H, *J*= 7.15 Hz); 8.18 (d, 1H, *J*= 7.15 Hz); 7.57 (t, 1H, *J*= 7.15 Hz); 4.78-4.63 (m, 3H); 4.17 (s, 1H); 4.06-4.04 (m, 1H); 3.86-3.84 (m, 1H); 3.11-3.08 (m, 2H); 2.77-2.52 (m, 2H); 2.48-2.17 (m, 2H); 2.07-1.80 (m, 6H); 1.63-1.30 (m, 7H); 1.21 (s, 3H); 1.17-1.14 (m, 2H); 0.98 (s, 3H). 3H are not visible, probably for the interaction with the solvent.

4-((9-(((3-(2-Chloroacetamido)benzoyl)oxy)methyl)-3,9-dihydroxy-4,11b-dimethyltetradecahydro-8,11a-methanocyclohepta[a]naphthalen-4-yl)methoxy)-4-oxobutanoic acid (42)



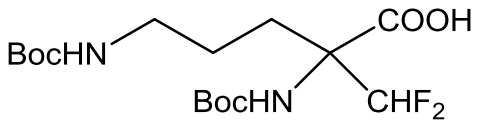
A solution of compound **41** (10 mg, 18.72 mmol), 0.3 ml of anhydrous pyridine and succinic anhydride (2 mg, 18.72 mmol) was stirred at r.t. for 20 h. The mixture was diluted with few drops of water and acidified with H_2SO_4 at 30% (pH= 2). After a first extraction with ethyl acetate to isolate unreacted **41**, the aqueous layer was saturated with NaCl and extracted with CH_2Cl_2. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a residue that was rinsed by settling with diethyl ether. An amorphous solid was obtained. Yield: 50%. ^{1}H NMR (pyridine-d_5) δ: 13.30 (s, 1H, collapsed with D_2O); 9.56 (s, 1H, collapsed with D_2O); 8.92 (s, 1H); 8.22-8.18 (m, 1H); 7.82-7.80 (m, 1H); 7.65-7.63 (m, 1H); 4.38-4.23 (m, 2H); 3.75 (s, 1H); 3.63-3.61 (m, 1H); 3.45-3.37 (m, 2H); 3.20-3.16 (m, 1H); 2.98 (s, 2H); 2.65-2.62 (m, 1H); 2.38-1.84 (m, 4H); 1.79-1.37 (m, 6H); 1.28-0.86 (m, 8H); 0.81 (s, 3H); 0.71-0.69 (m, 2H); 0.58 (s, 3H). 2H are not visible, probably for the interaction with the solvent.

((3R,4R,4aR,6aS,9R,11bS)-3,9-Dihydroxy-4-(hydroxymethyl)-4,11b-dimethyltetradecahydro-8,11a-methanocyclohepta[a]naphthalen-9-yl)methyl 4-methylbenzenesulfonate (112)



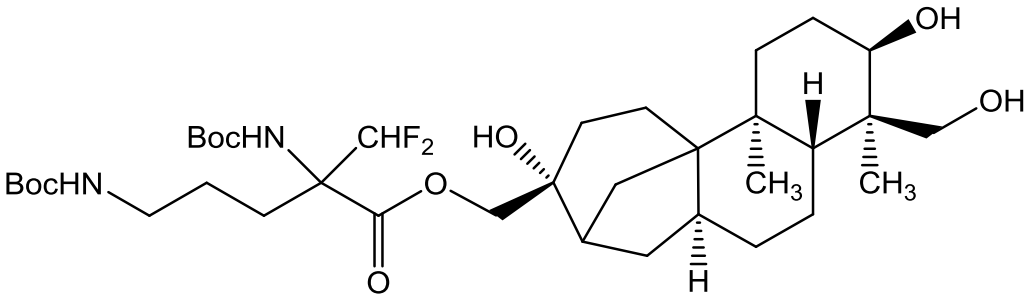
Tosyl chloride (52 mg, 0.27 mmol) was added dropwise during 20 minutes to an ice-cooled solution of aphidicolin (60 mg, 0.18 mmol) in 2.2 ml of anhydrous pyridine. The mixture was stirred at 0 °C for 2 h and then left in the fridge for 18 h. After warming to r.t., 90 µl of water were added to quench the unreacted tosyl chloride and then the solvent was evaporated. The residue was diluted with water and extracted with ethyl acetate. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a residue that was purified by CC (silica gel; cyclohexane/ethyl acetate in gradient); the product eluted with 40% of ethyl acetate. The fraction containing the desired product was rinsed with diethyl ether to give a white solid. Yield: 56%. Mp 132.5-134.5 °C. ^{1}H NMR ($CDCl_3$) δ: 7.78 (d, 2H, J= 8.80 Hz); 7.36 (d, 2H, J= 8.80 Hz); 3.90 (d, 1H, J= 9.15 Hz); 3.81 (d, 1H, J= 9.15 Hz); 3.68 (s, 1H); 3.49-3.33 (m, 2H); 2.48 (s, 3H); 2.42-2.38 (m, 1H); 2.15-1.64 (m, 12H, 3H collapsed with D_2O); 1.54-1.21 (m, 7H); 1.01-0.98 (m, 4H); 0.80-0.68 (m, 1H); 0.68 (s, 3H).

2,5-Bis((tert-butoxycarbonyl)amino)-2-(difluoromethyl)pentanoic acid (113)



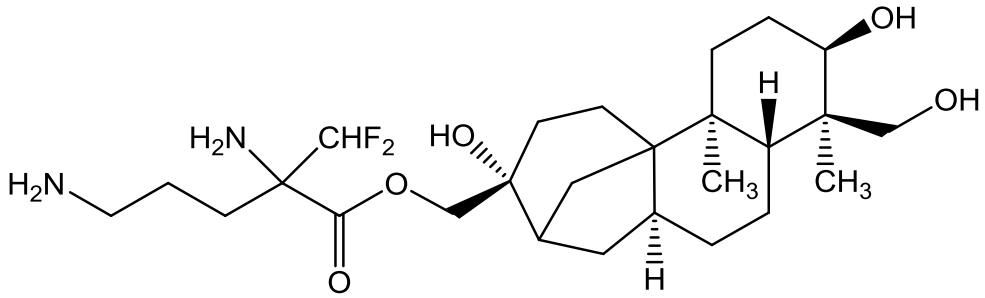
Boc anhydride (1.25 g, 5.17mmol) was dissolved in 3.3 ml of methanol and added dropwise to a solution of eflornithine hydrochloride (250 mg, 1.14 mmol) in 22 ml of methanol. After the addition of DIPEA (2 ml, 11.43 mmol), the mixture was stirred at r.t. for 5 h. Further Boc anhydride (498 mg, 2.28 mmol) was charged and the mixture stirred again at r.t. for 22 h. the solvent was evaporated and the residue diluted with 25 ml of water, acidified with acetic acid (pH= 4-5) and extracted with ethyl acetate. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a residue that crystallized spontaneously in the fridge during the night. The solid was rinsed with petroleum ether to give white crystals. The filtrate was evaporated to obtain an oily residue that was purified by CC (silica gel; CH_2Cl_2/MeOH in gradient); the product eluted with 4% of MeOH. A white solid was obtained. Yield: 66%. Mp 144.3-146.2 °C. ^{1}H NMR (DMSO-d_6) δ: 7.29 (br s, 1H, collapsed with D_2O); 6.78 (br s, 1H, collapsed with D_2O); 6.25 (t, 1H, J= 57.3 Hz); 2.90-2.81 (m, 2H); 1.79-1.68 (m, 2H); 1.43-1.28 (m, 20H). 1H is not visible, probably for the interaction with the solvent.

((3R,4R,4aR,6aS,9R,11bS)-3,9-Dihydroxy-4-(hydroxymethyl)-4,11b-dimethyltetradecahydro-8,11a-methanocyclohepta[a]naphthalen-9-yl)methyl 2,5-bis((tert-butoxycarbonyl)amino)-2-(difluoromethyl)pentanoate (114)



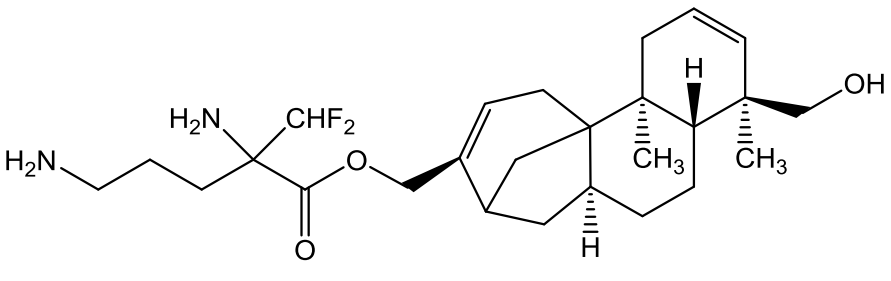
Compound **112** (32 mg, 0.07 mmol) was added to a solution of compound **113** (25 mg, 0.07 mmol) and DIPEA (50 µl, 0.29 mmol) in anhydrous THF. The mixture was refluxed for 18 h and then the solvent was evaporated. The residue was diluted with CH_2Cl_2 and washed with 0.1 N HCl, water and brine. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to obtain a residue that was purified by CC (silica gel; CH_2Cl_2/MeOH in gradient); the product eluted with 1% of MeOH. A yellow amorphous solid was obtained. Yield: 43%. ^{1}H NMR ($CDCl_3$) δ: 5.88 (t, 1H, *J*= 57.3 Hz); 4.85 (br s, 1H, collapsed with D_2O); 4.18-4.05 (m, 2H); 3.66 (s, 1H); 3.47 (d, 1H, *J*= 9.15 Hz); 3.37 (d, 1H, *J*= 9.15 Hz); 3.18 (s, 1H, collapsed with D_2O); 3.12-3.08 (m, 2H); 2.43-2.38 (m, 2H, 1H collapsed with D_2O); 2.13-1.64 (m, 16H); 1.55-1.20 (m, 20H); 0.98-0.88 (m, 7H); 0.69 (s, 3H). 2H are not visible, probably for the interaction with the solvent.

((3R,4R,4aR,6aS,9R,11bS)-3,9-Dihydroxy-4-(hydroxymethyl)-4,11b-dimethyltetradecahydro-8,11a-methanocyclohepta[a]naphthalen-9-yl)methyl 2,5-diamino-2-(difluoromethyl)pentanoate (43)



A solution of compound **114** (25 mg, 0.04 mmol) and TFA (27 µl, 0.36 mmol) in 0.5 ml of CH_2Cl_2 was stirred at r.t. for 2 h. Further TFA (27 µl, 0.36mmol) was added and after 1 h another addition was made (50 µl, 0.65 mmol). The mixture was stirred for further 20 h at r.t. The solvent was evaporated and the residue diluted with ethanol. The obtained white precipitate was filtered and the filtrate, which contained the desired product, was evaporated to dryness. The residue was crystallized and rinsed with diethyl ether to obtain a nut-brown solid.

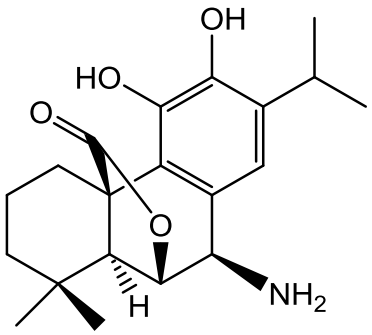
The NMR analysis and the mass spectrometry assay revealed that the obtained compound was not the expected one. Indeed, it underwent a loss of two molecules of water to generate the following compound (**44**). Yield (referred to compound **44**): 68%. Mp 139.0-143.0 °C. ^{1}H NMR (CD_3OD) δ: 6.47 (t, 1H, *J*= 57.3 Hz); 5.02 (s, 1H); 4.35-4.11 (m, 3H); 3.52-3.41 (m, 1H); 3.01 (t, 2H, *J*= 6.55 Hz); 2.23-1.59 (m, 13H); 1.51-1.30 (m, 6H); 1.15-0.98 (m, 8H). 7H are not visible, probably for the interaction with the solvent.



44 $2CF_3COOH$

4.6 *Rosmaricine and derivative*

Rosmaricine

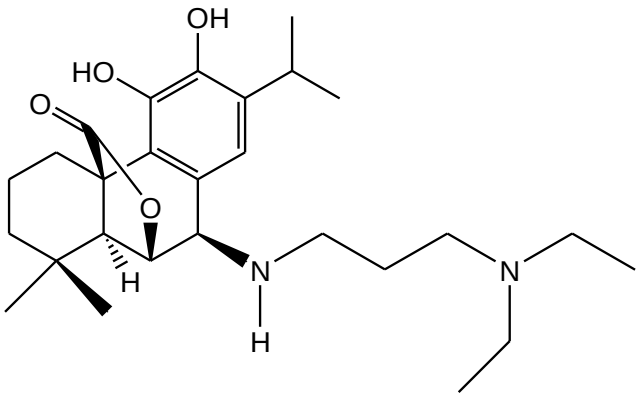


150 g of rosemary dry leaves were divided into three 500 ml flasks and each portion was suspended in 200 ml of ethanol and kept in an ultrasound sonicator for 1 h (from 25 to 60 °C). After cooling, the mixture was filtered on a Buchner funnel and washed with ethanol. The filtrate was evaporated and stored in the absence of light. The residual leaves were treated twice with the same procedure. All the filtrates were gathered, diluted with ethanol and concentrated at 100 ml of solvent volume. The mixture was filtered again to remove the most waxes as possible and the filtrate was evaporated. The obtained residue was diluted with ethyl acetate and washed several times with water. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness and the rough solid was washed several times with cyclohexane.

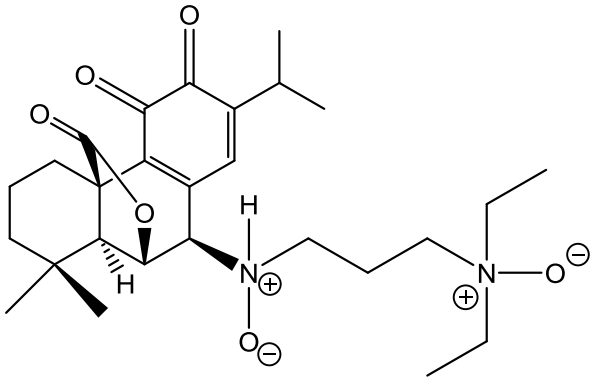
Assuming that 150 g of rosemary leaves contained 1.5% of carnosic acid,[82] the dry extract was diluted with 150 ml of ethanol, treated with 33% NH_3 (1.8 ml) and stirred at r.t. for 24 h in contact with air. Further 33% NH_3 was added after 19 h (0.3 ml) and after 21 h (0.45 ml) in order to keep the pH constant (8-9). The mixture was evaporated, diluted with $CHCl_3$ and extracted with 5% H_2SO_4. The aqueous layer was charged into a flask, cooled with an ice-bath and alkalized with NH_3 till pH= 9. The formed precipitate was filtered and washed with water to collect 90 mg of a nut-brown solid, which corresponded to the desired product. The filtrate was extracted with $CHCl_3$ and the organic layer was then extracted with 20% HCl. The aqueous layer was alkalized again with

NH_3 till pH= 9 and extracted with $CHCl_3$. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to collect an amorphous solid that was crystallized with diethyl ether/petroleum ether (9.5:0.5). Finally crystals were rinsed with diethyl ether to obtain 233 mg of a nut-brown solid corresponding to the desired product. Yield (referred to dry leaves): 0.22%. Mp 198.2-202.5 °C. ^{1}H-NMR (DMSO-d_6) δ: 8.05 (br s, 2H, collapsed with D_2O); 6.77 (s, 1H); 4.37 (d, 1H, *J*= 2.47 Hz); 3.72 (d, 1H, *J*= 2.47 Hz); 3.30 (br s, 2H, collapsed with D_2O); 3.26-3.09 (m, 2H); 1.84-1.70 (m, 1H); 1.60-1.18 (m, 5H); 1.11 (d, 3H, *J*= 6.88 Hz); 1.07 (d, 3H, *J*= 6.88 Hz); 0.94 (s, 3H); 0.77 (s, 3H).

Reaction of the alcoholic rosmary extract with diethylaminopropylamine



Assuming that 150 g of rosemary leaves contained 1.5% of carnosic acid,[82] the dry extract was diluted with 200 ml of ethanol, treated with 5 ml of 3-(diethylamino)propylamine and stirred at r.t. for 24 h in contact with air. The mixture was evaporated, diluted with $CHCl_3$ and extracted with 5% H_2SO_4. The aqueous layer was charged into a flask, cooled with an ice-bath and alkalized with NH_3 till pH= 9. The mixture was extracted with $CHCl_3$ and the organic layer was then extracted with 20% HCl. As the desired product did not pass in the aqueous phase, the organic layer was alkalized with NH_3, washed with water and dried with anhydrous Na_2SO_4. After evaporation of the solvent, the residue was washed with diethyl ether and petroleum ether and the rough brown solid was purified by CC (silica gel, CH_2Cl_2/MeOH in gradient); the product eluted with 4% of MeOH. The fraction containing the desired product was rinsed with petroleum ether/diethyl ether (8:2) to collect a reddish solid. The high resolution mass spectrometry assay (ESI) revealed that the obtained compound was not the expected one. It was probably an oxidation product, both at the cathecolic and at the amino groups level. The hypothetical obtained compound (**46**) is reported below, but it couldn't be established for sure. Yield (referred to compound **46**): 2.1%. Mp 120.0-122.5 °C (dec.). ^{1}H-NMR ($CDCl_3$) δ: 8.77 (br s, 1H, collapsed with D_2O); 6.24 (s, 1H); 4.56 (d, 1H, J= 8.25 Hz); 3.63-3.05 (m, 6H); 2.85-2.81 (m, 1H); 2.74-2.69 (m, 1H); 2.62-2.59 (m, 1H); 2.32-2.31 (m, 2H); 2.09-1.98 (m, 3H); 1.59-1.54 (m, 2H); 1.47-1.25 (m, 9H); 1.08-1.06 (d, 3H, J= 6.60 Hz); 1.00-0.85 (m, 9H). HRMS (ESI) m/z calcd for $C_{27}H_{40}N_2O_6$ (**46**) $[M+H]^+$: 489.29646; found: 489.29584.



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5. BIOLOGICAL ASSAYS

In vitro studies on the antimalarial activity and on cytotoxicity have been carried out in the laboratory of Prof. Donatella Taramelli, Department of Pharmacological and Biomolecular Sciences of the University of Milan.

5.1 ***Plasmodium falciparum* cultures and drug susceptibility assay**

P. falciparum cultures were carried out according to Trager and Jensen with slight modifications.[83] The CQ-susceptible strains D10 and 3D7 and the CQ-resistant strain W2 were maintained at 5% hematocrit (human type A-positive red blood cells) in RPMI 1640 (EuroClone, Celbio) medium with the addition of 1% AlbuMax (Invitrogen, Milan, Italy), 0.01% hypoxanthine, 20 mM Hepes, and 2 mM glutamine. All the cultures were maintained at 37 °C in a standard gas mixture consisting of 1% O_2, 5% CO_2, and 94% N_2.

Compounds were dissolved in either water or DMSO and then diluted with medium to achieve the required concentrations (final DMSO concentration <1%, which is non toxic to the parasite). Drugs were placed in 96-well flat-bottomed microplates (COSTAR) and serial dilutions made. Asynchronous cultures with parasitaemia of 1-1.5% and 1% final hematocrit were aliquoted into the plates and incubated for 72 h at 37 °C. Parasite growth was determined spectrophotometrically (OD650) by measuring the activity of the parasite lactate dehydrogenase (pLDH), according to a modified version of the method of Makler in control and drug-treated cultures.[84] The antimalarial activity is expressed as 50% inhibitory concentrations (IC_{50}); each IC_{50} value is the mean and standard deviation of at least three separate experiments performed in duplicate.

5.2 ***Promastigote stage of Leishmania spp cultures and antileishmanial activity***

Promastigote stage of *L. infantum* strain MHOM/TN/80/IPT1 (kindly provided by Dr. M. Gramiccia and Dr. T. Di Muccio, ISS, Rome), *L. tropica* (MHOM/IT/2012/ISS3130) and *L. braziliensis* (MHOM/IT/2006/ISS2848) were cultured in RPMI 1640 medium (EuroClone) supplemented with 15% heat-inactivated fetal calf serum (EuroClone), 20 mM Hepes, and 2 mM *L*-glutamine at 22 °C.

To estimate the 50% inhibitory concentration (IC_{50}), the MTT (3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide) method[85,86] was used with modifications. Compounds were dissolved in DMSO and then diluted with medium to achieve the required concentrations. Drugs were placed in 96 wells round-bottom microplates and seven serial dilutions made. Amphotericin B was used as the reference antileishmanial drug. Parasites were diluted in complete medium to 5 x 10^6 parasites/mL and 100 µL of the suspension was seeded into the plates, incubated at 22 °C for 72 hours and then 20 µL of MTT solution (5 mg/mL) was added into each well for 3 hours. The plates were then centrifuged, the supernatants discarded and the resulting pellets dissolved in 100 µL of lysing buffer consisting of 20% (w/v) of a solution of SDS (Sigma), 40% of *N*,*N*-dimethylformamide (Merck) in H_2O. The absorbance was measured spectrophotometrically at a test wavelength of 550 nm and a reference wavelength of 650 nm. The results are expressed as IC_{50} which is the dose of compound necessary to inhibit cell growth by 50%; each IC_{50} value is the mean ± standard deviation of at least three separate experiments performed in duplicate.

5.3 ***Cytotoxicity assay***

The long-term human microvascular endothelial cell line (HMEC-1) immortalized by SV 40 large T antigen[87] was maintained in MCDB 131 medium (Invitrogen, Milan, Italy) supplemented with 10% fetal calf serum (HyClone, Celbio, Milan, Italy), 10 ng/ml of epidermal growth factor (Chemicon), 1 µg/ml of hydrocortisone, 2 mM glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 20 mM Hepes buffer (EuroClone). Unless stated otherwise, all reagents were from Sigma Italia, Milan, Italy. For the cytotoxicity assays, cells were treated with serial dilutions of test compounds for 72 h and cell proliferation evaluated using the MTT assay already described.[88] The results are expressed as IC_{50}, which is the dose of compound necessary to inhibit cell growth by 50%. Each IC_{50} value is the mean and standard deviation of at least three separate experiments performed in duplicate.

6. RESULTS AND DISCUSSION

6.1 *4-Aminoquinoline derivatives*

The synthesized compounds **1-3** have been tested *in vitro* against D10 (CQ-S) strain and W2 (CQ-R) strain of *P.falciparum*. Their antiplasmodial activity was quantified as inhibition of parasite growth, expressed as IC_{50} (nM), and it is reported in Table 5. The activities of the related compound (-)-AM-1[35] and of chloroquine as reference drug have been also reported. The ratios between the IC_{50} of each compound against the CQ-R and CQ-S strains of *P. falciparum* are also indicated. This value (resistance index, R.I.) is suggestive of cross resistance between the compound and chloroquine. Cytotoxicity on the human endothelial cell line HMEC-1 or on the murine cell line WEHI Clone 13 was assayed using the MTT test and the selectivity index (S.I.; IC_{50} HMEC-1 or WEHI 13/IC_{50}W2 strain) was calculated (Table 5).

Table 5. Antimalarial activity of 4-aminoquinoline derivatives and (-)-AM-1 on D10 and W2 strains of *P. falciparum* and their cytotoxicity on the human cell line HMEC-1 or on the murine cell line WEHI Clone 13.

Compound	D10 (CQ-S) IC_{50} (nM)[a]	W2 (CQ-R) IC_{50} (nM)[a]	R.I.[b]	HMEC-1 IC_{50} (nM)[c]	WEHI 13 IC_{50} (nM)[c]	S.I.[d]
1	22.5±9.6	475.3±145.7	21.2	>26939	n.t.	>56.7
2	27.1±7.9	194.3±74.6	7.2	9211.2±938.3	n.t.	47.4
3	8.9±3.7	25.9±3.7	2.8	5494.8±697.5	n.t.	212
(-)-AM-1[35]	26.4±15.3	41.9±19.9	1.6	n.t.	9206	220
CQ	19.8±4.6	320.3±50.9	16.2	>38000	n.t.	>118

n.t.: not tested.
[a]Results are expressed as IC_{50} ± SD of at least three different experiments, each performed in duplicate.
[b]Ratio between IC_{50} (W2) and IC_{50} (D10) calculated for each compound.
[c]The cytotoxicity evaluation has been performed using the MTT assay.
[d]Ratio between IC_{50} (HMEC-1 or WEHI 13) and IC_{50} (W2) calculated for each compound.

All compounds showed IC_{50} comparable to that of chloroquine against the D10 (CQ-S) strain of *P. falciparum*, whereas the activity against the W2 (CQ-R) strain greatly improved with the elongation of the alkyl chain (n= 8; IC_{50}= 26 nM, 12-fold more active than chloroquine and 1.6-fold more

active than (-)-AM-1), with very low resistance index. Moreover, results clearly showed that there was a linear improvement of antiplasmodial activity with the gradual increase of the number of terms of the alkyl chain. The most interesting compound (**3**), in addition to its excellent antiplasmodial activity, showed also a good resistance index and a remarkable safety profile, with a selectivity index similar to that of (-)-AM-1 and to that of chloroquine.

6.2 ***Hybrids of 4-aminoquinoline***

The synthesized compounds **4-9** have been tested *in vitro* against D10 (CQ-S) strain and W2 (CQ-R) strain of *P. falciparum*. Their antiplasmodial activity, expressed as IC_{50}±SD (nM), and their selectivity index are reported in Table 6. Even the reference drug (SAHA) and some intermediates (**65**, **67**, **68** and **74**) were tested as terms for comparison, in order to better evaluate the importance of the hydroxamic acid group for the antiplasmodial activity. Cytotoxicity on the human endothelial cell line HMEC-1 of almost all compounds is also indicated.

Table 6. Antimalarial activity of hybrids of 4-aminoquinoline on D10 and W2 strains of *P. falciparum* and their cytotoxicity on the human cell line HMEC-1.

Compound	D10 (CQ-S) IC_{50} (nM)[a]	W2 (CQ-R) IC_{50} (nM)[a]	R.I.[b]	HMEC-1 IC_{50} (nM)[c]	S.I.[d]
4	105.9±25.0	2037.5±212.4	19.2	13929±2672	7
5	262.9±74.3	2775.4±159.9	10.5	32597±3306	12
6	664.2±131.0	846.2±174.1	1.3	23435±931	28
7	675.8±93.1	506.7±178.3	0.7	82107±17378	162
8	362.4±75.5	267.6±102.6	0.7	13722±3129	51
9	359.5±162.3	384.1±109.2	1.1	9820±2499	26
65	524.7±172.3	994.5±295.5	1.9	>108460	>109
67	40.2±6.2	76.0±10.9	1.9	n.t.	-
68	1133.4±323.2	>9328	>8.2	>93278	>10
74	1796.9±388.7	853.0±306.0	0.5	80163±22607	94
SAHA	138.6±4.5	177.1±14.6	1.3	908	5
CQ	20.1±6.1	305.8±44.2	15.3	>38000	>124

n.t.: not tested.
[a]Results are expressed as IC_{50} ± SD of at least three different experiments, each performed in duplicate.
[b]Ratio between IC_{50} (W2) and IC_{50} (D10) calculated for each compound.
[c]The cytotoxicity evaluation has been performed using the MTT assay.
[d]Ratio between IC_{50} (HMEC-1) and IC_{50} (W2) calculated for each compound.

All the tested hybrid compounds were able to inhibit the *in vitro* growth of the considered strains of *P. falciparum*, with IC_{50} in the nanomolar or low micromolar range (IC_{50} against CQ-S= 105.9-675.8 nM and IC_{50} against CQ-R= 267.6-2775.4 nM). Results showed that the presence of a dithiolthione moiety (compounds **4** and **5**) was not positive for the activity against the W2 (CQ-R) strain of *P. falciparum*, as it was much lower than the activity against the D10 (CQ-S) strain, suggesting that this modification could favor the outbreak of resistance mechanisms. Moreover, their selectivity indexes were quite low, even if better than that of SAHA. Concerning the other compounds (**6**-**9**), despite the loss of potency compared to chloroquine and SAHA, the presence of

an hydroxamic acid group seemed useful to contrast resistance onset, leading to very low resistance indexes (0.7-1.1). It is well-known that hydroxamic acid groups possess an antiproliferative and not specific activity, but in this case the presence of the quinoline moiety decreased the cytotoxicity of the hybrids (S.I.= 26-162), compared to the reference drug SAHA (S.I.= 5). In particular, compound **7** exhibited a very high S.I., suggesting that, quite unexpectedly, the cinnamoyl moiety could be useful to obtain more selective compounds.

The importance of the hydroxamic acid group as Zn^{2+}-chelating moiety for the antiplasmodial activity was confirmed by comparing compound **7** to the corresponding carboxylic acid (**68**) and comparing compound **8** to the corresponding methyl ester (**74**). A particular and unexpected case is that of the ethyl cinnamate derivative (**67**), which showed a great antiplasmodial activity on both CQ-S and CQ-R strains of *P. falciparum*, much higher than the activity of the corresponding hydroxamic acid (compound **7**). This could be explained with the fact that probably, as the ester is more lipophilic, it could easily pass the membranes and, once inside the *Plasmodium*, it could exert its antiplasmodial activity. In this case, the mechanism of action could be ascribed to the quinoline moiety or also to the cinnamoyl moiety, that could react with different substrates as a Michael reaction acceptor. It would be interesting also to evaluate the real mechanism of action of these hybrids, first of all evaluating their ability to inhibit HDAC enzimes of *P.falciparum* and also performing the BHIA (β-hematin inhibitory activity) assay,[89] that underlines the ability to inhibit β-hematin formation (chloroquine's mechanism of action).

6.3 ***Indeno[2,1-c]quinolines***

All compounds were tested *in vitro* against D10 and/or 3D7 (CQ-S) and W2 (CQ-R) strains of *P. falciparum*. Their antiplasmodial activity was quantified as inhibition of parasite growth, expressed as IC_{50} (μM), and it is reported in Table 7. Resistance index (R.I.), cytotoxicity on the human endothelial cell line HMEC-1 and the selectivity index (S.I.) on W2 strain were also indicated (Table 7).

Table 7. *In vitro* antimalarial activities and cytotoxicity on the human cell line HMEC-1 of the prepared indeno[2,1-c]quinolines.

Comp.	D10 (CQ-S) IC_{50} (μM)[a]	R.I. (W2/D10)[b]	3D7 (CQ-S) IC_{50} (μM)[a]	R.I. (W2/3D7)[b]	W2 (CQ-R) IC_{50} (μM)[a]	HMEC-1 IC_{50} (μM)[c]	S.I.[d]
10	n.t.	-	1.713±0.992	0.4	0.626±0.191	n.t.	-
11	n.t.	-	2.339±0.351	0.5	1.172±0.216	n.t.	-
12	3.757±0.421	0.7	6.775±1.117	0.4	2.554±0.671	n.t.	-
13	0.703±0.050	0.8	1.031±0.242	0.5	0.568±0.231	1.937±0.715	3.4
14	0.468±0.186	1.1	n.t.	-	0.534±0.186	3.125±0.642	5.9
15	1.636±0.181	0.7	n.t.	-	1.186±0.262	4.034±1.220	3.4
16	6.077±0.686	1.1	n.t.	-	6.936±2.525	n.t.	-
17	n.t.[e]	-	n.t.[e]	-	n.t.[e]	n.t.[e]	-
18	0.850±0.181	0.3	0.843±0.096	0.3	0.255±0.119	3.122±0.491	12.0
19	n.t.	-	0.530±0.193	0.5	0.244±0.125	2.342±0.058	9.6
20	2.839±0.745	1.0	5.390±1.357	0.5	2.728±0.753	n.t.	-
21	0.667±0.050	1.0	0.818±0.175	0.8	0.699±0.187	2.585±0.719	3.7
22	0.676±0.064	1.6	n.t.	-	1.055±0.328	2.938±0.233	2.8
23	6.612±2.427	1.0	n.t.	-	6.807±2.628	>38.000	>5.6
24	4.046±1.019	1.0	n.t.	-	4.160±0.540	>35000	>8.4
25	>5.000	-	n.t.	-	>5000	n.t.	-
26	0.360±0.020	1.2	n.t.	-	0.432±0.105	1.723±0.128	4.0
27	4.578±1.236	0.7	n.t.	-	3.157±0.623	5.279±0.454	1.7
CQ	0.020±0.005	16	0.012±0.003	26	0.316±0.051	>38.000	>120

n.t.: not tested

[a]The results are expressed as IC_{50} ± SD of at least three different experiments each performed in duplicate or triplicate.

[b]Ratio between the IC_{50} values of each compound against the two strains of *P. falciparum*.

[c]The cytotoxic activity was assayed *in vitro* using the MTT assay.

[d]IC_{50} HMEC/IC_{50} W2 strain of *P. falciparum*.

[e]Compound **17** could not be tested for solubility problems.

Table 8. Inhibition of β-hematin formation (BHIA method).

Compound	IC_{50} Drug: haemin molar ratio[a]
18	>4[b]
CQ	1.71± 0.6

[a]The IC_{50} represent the molar equivalents of test compounds, relative to haemin, required to inhibit β-hematin by50%. (Results represent the mean of three different experiment, each performed in duplicate.
[b]Higher concentration could not be used for solubility problems.

All the tested compounds, with the exception of **17** and **25**, were able to inhibit the *in vitro* growth of the considered CQ-S (IC_{50}= 0.36-6.61 μM) and CQ-R (IC_{50}= 0.24-6.94 μM) strains of *P. falciparum.* Among them, six compounds (**13**, **14**, **18**, **19**, **21** and **26**) exhibited IC_{50}< 1 μM on all the tested strains, suggesting that the presence of the indeno[2,1-c]quinoline scaffold is suitable for the antimalarial activity, which can be modulated through the modifications of the substituents.

Interestingly, resistance index was always very low (range 0.3-1.6); this is indicative that this kind of compounds do not share the same resistance mechanisms of CQ. Moreover, the W2 (CQ-R) strain is often more sensitive to our compounds than the CQ-S strains and this unusual result confers interest to this class of compounds, despite their moderate potency.

In addition, the ability of a representative compound (**18**) to inhibit β-hematin formation was evaluated using the BHIA (β-hematin inhibitory activity) assay.[89] Results reported in Table 8 shows that compound **18** has no, or very low ability to interfere with the process of heme crystallization, compared to CQ. Therefore the mechanism of the antiplasmodial activity of compound **18** and, likely, of this class of compounds, should be different from that of CQ.

Unfortunately, the most potent compounds tested on a human endothelial cell line exhibited a fairly high toxicity, which however is in line with what observed with the indenoquinoline derivatives described by Upadhayaya *et al.*[48] This result, could be related to the chemical similarity of these compounds to the known antiproliferative agent TAS-103.

Concerning the structure-activity relationships (SAR), we can observe the following:

a) the presence of the chlorine in position 2 or 3 of the indenoquinoline nucleus is generally irrelevant, except for the two couples of compounds **10**, **11** and **21**, **22** where the 3-chloro-substituted compound is clearly less active on W-2 (CQ-R) strain of *P. falciparum*.

b) When R is a piperidinoethylamino moiety, 7-carbonyl derivatives are less active than corresponding oximes, whereas the opposite happens for compounds **14** and **22**; the other compounds didn't present significant differences in their antimalarial potency.

c) Differently from what reported for the analogous bromo-derivatives,[47] the esterification of the oxime group with propionic acid improved only modestly the activity (**25** vs **23**).

d) Among the basic heads inserted in position 6 of the indenoquinoline nucleus, the piperidinoethylamino and methylpiperazinoethylamino moieties seem associated to the better activity, whereas the more polar morpholino- and 2-pyridinyl-4-piperazinyl derivatives are clearly less potent.

e) The shift of the basic side chain to position 7 showed contrasting results, with the aminoguanidone derivative **26** exhibiting a good activity, whereas the product of condensation of 2-chloro-6-methoxyindeno[2,1-c]quinolin-7-one with 1-amino-4-methylpiperazine (**27**) was scarcely active. Compound **26** could represent a starting point to prepare new analogs, bearing different substituents in position 6.

6.4 ***Riminophenazines***

Riminophenazines were tested *in vitro* against D10 (CQ-S) and W2 (CQ-R) strains of *P. falciparum*. Their antiplasmodial activity was quantified as inhibition of parasite growth, expressed as IC_{50} (μM), and it is reported in Table 9. Resistance index (R.I.), cytotoxicity on the human endothelial cell line HMEC-1 and the selectivity index (S.I.) were also indicated (Table 9).

Table 9. *In vitro* data on antiplasmodial activity against D10 and W2 strains of *P. falciparum* and cytotoxicity on the human endothelial cell line (HMEC-1).

Compound	D10 (CQ-S) IC_{50} (μM)[a]	W2 (CQ-R) IC_{50} (μM)[a]	R.I.[b]	HMEC-1 IC_{50} (μM)[c]	S.I.[d]
28	0.34±0.02	0.29 ±0.03	0.9	1.90±0.37	6.5
29	0.19 ±0.04	0.45 ±0.10	2.4	0.98±0.10	2.2
30	0.21±0.04	0.40±0.11	1.9	1.57±0.34	4.0
31	0.21±0.03	0.27±0.03	1.3	1.23±0.03	4.5
32	0.17±0.05	0.21±0.07	1.2	1.24±0.12	6.0
33	0.24±0.02	0.39±0.05	1.6	1.96±0.25	5.0
34	0.28±0.08	0.34±0.07	1.2	1.87±0.10	5.5
35	0.31±0.06	0.57±0.03	1.9	1.35±0.17	2.4
36	0.19±0.03	0.27±0.03	1.4	11.06±3.70	41
37	0.28±0.05	0.21±0.06	0.8	3.89±0.73	18
38	0.81±0.25	0.35±0.12	0.4	6.32±1.64	18
39	0.22±0.03	0.18±0.05	0.8	2.33±1.03	13
40	0.40±0.03	0.28±0.04	0.7	4.66±1.61	16
Clofazimine	4.82 ±0.82	7.02±1.10	1.5	18.59[a]	2.6
CQ	0.02±0.005[e]	0.36±0.06[e]	16	>38	>119

n.t.: not tested.
[a]Results are expressed as IC_{50} ± SD of at least three different experiments, each performed in duplicate.
[b]Ratio between IC_{50} (W2) and IC_{50} (D10) calculated for each compound.
[c]The cytotoxicity evaluation has been performed using the MTT assay.
[d]Ratio between IC_{50} (HMEC-1 or WEHI 13) and IC_{50} (W2) calculated for each compound.
[e]Mean value from many different experiments.

All compounds were also tested *in vitro* against different species of *Leishmania* promastigotes and the results, expressed as IC_{50}±SD (μM), are reported in Table 10 together with their corresponding selectivity indexes (S.I.).

Table 10. *In vitro* data on antileishmanial activity against different species of *Leishmania* promastigotes and cytotoxicity on the human endothelial cell line (HMEC-1).

Compound	*L.infantum* IC_{50}(μM)[a]	S.I.[b]	*L.tropica* IC_{50}(μM)[a]	S.I.[b]	*L.braziliensis* IC_{50}(μM)[a]	S.I.[b]	HMEC-1 IC_{50}(μM)[c]
28	0.91±0.26	2.1	1.10±0.33	1.7	n.t.	-	1.90±0.37
29	0.30±0.03	3.3	0.54±0.30	1.8	n.t.	-	0.98±0.10
30	0.46±0.20	3.4	0.47±0.23	3.3	n.t.	-	1.57±0.34
31	0.37±0.11	3.3	0.22±0.08	6	0.17±0.08	7.2	1.23±0.03
32	0.23±0.05	5	0.12±0.03	10	0.14±0.03	8.9	1.24±0.12
33	0.48±0.26	4	0.84±0.53	2.3	n.t.	-	1.96±0.25
34	0.43±0.17	4	0.33±0.07	5.5	n.t.	-	1.87±0.10
35	0.35±0.08	4	0.24±0.07	5.5	n.t.	-	1.35±0.17
36	2.67±1.53	4	1.09±0.34	10	n.t.	-	11.06±3.70
37	6.02±2.70	0.6	2.01±0.51	1.9	n.t.	-	3.89±0.73
38	4.44±0.21	1.4	2.32±0.24	2.7	n.t.	-	6.32±1.64
39	0.34±0.07	7	0.34±0.11	7	n.t.	-	2.33±1.03
40	0.41±0.21	11	0.77±0.31	6	n.t.	-	4.66±1.61
Clofazimine	4.48±1.06	4.2	2.96±1.25	6.3	n.t.	-	18.59[a]
Amph B	0.08±0.02	321	0.09±0.04	286	0.09±0.02	286	25.70±1.90

[a]The results are expressed as IC_{50}±SD of at least three different experiments each performed in duplicate or triplicate.
[b]Selectivity Index: IC_{50} HMEC/IC_{50} different species of *Leishmania.*
[c]The cytotoxic activity was assayed *in vitro* using the MTT assay.
n.t.= not tested.

All the tested compounds inhibited the growth of different species of *Leishmania* promastigotes as well as CQ-S and CQ-R strains of *P. falciparum*. Most of them exhibited IC_{50} in the nanomolar range, with a clear improvement of potency compared to clofazimine, which was, generally, from one to two orders of magnitude less potent. These results confirm the importance of the introduction of a basic head on the imino nitrogen in position 3 on the phenazine.

Concerning the antiplasmodial activity, the tested compounds exhibited similar activity against CQ-S and CQ-R strains of *P. falciparum*, with very low R.I. (0.4-2.4), thus demonstrating that this kind of compounds do not share the same resistance mechanisms of CQ.

The most active compound on both *P. falciparum* strains was compound **32**, which inhibited the D10 (CQ-S) and W2 (CQ-R) strains with IC_{50}= 0.17 and 0.21 μM, respectively, thus showing a R.I.= 1.2.

In the series of symmetrical quinolizidinylalkyl derivatives (**31**-**39**), the different length of the alkyl chain (4 or 6 CH_2) didn't influence the antiplasmodial activity.

Interestingly, the quaternarization of the quinolizidine nitrogen with a methyl group (compound **36**) increased significantly the selectivity index on *Plasmodia*, lowering the toxicity on the tested human cell line, while leaving unchanged the antiplasmodial activity. Thus the effect of the quaternarization of the basic head is surely worth of a more extensive investigation.

The introduction on the phenyl rings of a substituent like Cl, F or CH_3 didn't affect significantly the antiplasmodial activity or toxicity on the human cell line.

The replacement of the aniline moiety in position 2 with a differently substituted aminopyridine (compounds of series B) slightly increased the selectivity indexes on *Plasmodia* and the resistance indexes (R.I.=0.4-0.8). Indeed compounds of this series were always more active on W2 (CQ-R) strain of *P. falciparum* than on D10 (CQ-S) strain.

Finally, the nature and the size of the basic head didn't influence significantly the antiplasmodial activity.

Concerning the antileishmanial activity, all the tested compounds inhibited the growth of *L. infantum* and *L. tropica* promastigotes with IC_{50} in the range 0.12-6.02 μM and seven of them showed $IC_{50} \leq 0.5$ μM.

9-Quinolizidinyl derivatives (**31-39**) were the most active and in this group the different length of the chain spacer, as well as the substitution with alogen atoms on the phenyl rings, didn't affect significantly the antileishmanial activity whereas, when the aniline moiety in position 2 was replaced by an aminopyridine (compounds of series B), the presence of a chlorine atom on the phenyl ring was clearly favourable (compare compound **37** with **39**).

Differently from antiplasmodial activity, the quaternarization of the quinolizidine ring with a methyl group was detrimental for the antileishmanial activity (compound **36** vs compound **31**).

The most potent compound **32** showed activity against different *Leishmania* promastigotes comparable with that of the reference drug Amphotericin B and activity against the W2 (CQ-R) strain of *P. falciparum* better than that of chloroquine and a selectivity index (S.I.) more favorable than that of clofazimine.

In series B, the replacement of the bulky bicyclic basic head (quinolizidine) of compound **37** with the small dimethylamino group (compound **40**) led to a compound with somewhat reduced activity against both the *Leishmania* promastigotes and the *P. falciparum* strains, but also with reduced toxicity on the endothelial cell line HMEC-1.

6.5 Aphidicolin derivatives

The synthesized aphidicolin derivatives (**41-44)** and their parent compounds were tested *in vitro* against different species of *Leishmania* promastigotes and the results, expressed as IC_{50}±SD (μM), are reported in Table 11, together with their cytotoxicity on the human endothelial cell line HMEC-1.

Table 11. *In vitro* data on antileishmanial activity against different species of *Leishmania* promastigotes and cytotoxicity on the human endothelial cell line (HMEC-1).

Compound	*L.infantum* IC_{50}(μM)[a]	*L.tropica* IC_{50}(μM)[a]	*L.braziliensis* IC_{50}(μM)[a]	% of inhibition at 5 μg/ml (*L.tropica*)[b]	HMEC-1 IC_{50}(μM)[c]
41	24.39±12.91	25.06±10.04	n.t.	-	2.21±0.47
42	n.t.	>16	>16	37.23	>79
44	n.t.	>21	17.51±6.75	33.34	29.27±6.85
MF29	10.00±3.50	12.80±5.80	n.t.	-	4.72±1.38
Eflornithine	n.t.	>46	>46	-	>229
Aphidicolin	6.29±2.01	6.61±7.17	n.t.	-	8.61±2.29
Amph B	0.08±0.02	0.09±0.04	0.09±0.02	-	25.70±1.90

[a]The results are expressed as IC_{50}±SD of at least three different experiments each performed in duplicate or triplicate.
[b]Inhibition (%) of *L.tropica* promastigotes at the higher tested concentration.
[c]The cytotoxic activity was assayed *in vitro* using the MTT assay.
n.t.= not tested.
n.c.= not calculable.

Even though they exhibited the ability to inhibit the growth of *Leishmania* in the micromolar range, all synthesized hybrids didn't increase the antileishmanial activity of their parent compounds. Compared to aphidicolin, hybrids exhibited a lower antileishmanial activity maybe due to their inappropriate solubility properties or because they could be partially hydrolyzed by some esterases present in the culture medium before entering into the promastigotes and, in this case, only the resulting free aphidicolin could pass the membranes and carry out its antileishmanial activity.

Alternatively, hybrids could enter into the promastigotes but they couldn't be hydrolyzed and therefore the single parts could not exert their own activity. Actually, none synergistic effect was observed and other structural modifications should be done to obtain more potent compounds and to evaluate the real potentiality of these scaffolds as antileishmanial agents. It would be interesting to test the activity of these compounds against amastigotes, which are the replicative forms of the parasite and are much more susceptible to drugs than promastigotes. A limitation on the development of this kind of hybrids is that their activity was not specific and they were quite toxic on the human cells. In particular, compound **41** was more active against the human cells than against *Leishmania* promastigotes. The introduction of a more hydrophilic moiety, as in compound **42**, led to a decrease of the cytotoxicity; however further investigations should be carried out to improve this aspect.

6.6 Rosmaricine and derivative

Rosmaricine and its derivative (**46**) were tested *in vitro* against D10 (CQ-S) and W2 (CQ-R) strains of *P. falciparum* and also against *L. tropica* promastigotes. Their antiprotozoal activities were expressed as IC_{50} (μM), and it is reported in Table 12. Resistance index (R.I.), cytotoxicity on the human endothelial cell line HMEC-1 and the selectivity index (S.I.) were also indicated (Table 12).

Table 12. *In vitro* data on antimalarial activity against D10 and W2 strains of *P. falciparum* and antileishmanial activity against *L. tropica* promastigotes and cytotoxicity on the human endothelial cell line (HMEC-1).

Compound	D10 (CQ-S) IC_{50} (nM)[a]	W2 (CQ-R) IC_{50} (nM)[a]	R.I.[b]	HMEC-1 IC_{50} (nM)[c]	S.I.[d]	*L. tropica* IC_{50} (nM)[e]
Rosmaricine	>14000	11901±2658	<0.85	19502±1639	1.6	>57000
46	5621±777	6541±1559	1.2	34999±9647	5.4	>43000
CQ	31.3±4.1	712.5±105.8	23	>38000	53	-
Amph B	-	-	-	n.t.	120±30	0.12±0.03

[a]The results are expressed as IC_{50} ± SD of at least three different experiments each performed in duplicate or triplicate.
[b]Ratio between the IC_{50} values of each compound against the two strains of *P. falciparum*.
[c]The cytotoxic activity was assayed *in vitro* using the MTT assay.
[d]IC_{50} HMEC/IC_{50} W2 strain of *P. falciparum*.
[e]Results are expressed as IC_{50} and are preliminary.
n.t. = not tested.

Results suggested that both rosmaricine and compound **46** were able to inhibit the growth of the tested strains of *P. falciparum*, despite a quite poor antiplasmodial activity. On the other hand, they were clearly characterized by an insignificant antileishmanial activity (IC_{50} >57000 and >43000 respectively). However, compound **46** was more active than rosmaricine on both the CQ-S and CQ-R strains of *P. falciparum*. This aspect confirmed the hypothesis that the introduction of a side chain on the nitrogen of rosmaricine could increase the antiplasmodial activity. For this reason, further studies should be carried out and other rosmaricine derivatives, mainly with a basic side chain, as initially projected, should be prepared in order to assess this issue and to obtain more active compounds.

7. CONCLUSIONS

In conclusion, different sets of novel antiprotozoal compounds were designed and synthesized and some of them exhibited very interesting antiplasmodial activities, in the nanomolar range. These molecules, in particular 4-aminoquinoline derivatives, indeno[2,1-c]quinolines and riminophenazines, could represent potential lead compounds to be optimized in order to discover more potent antiplasmodial and antileishmanial agents, following the encouraging strategies already used for the development of the presented compounds. They are new tiles in the complicated and alarming mosaic of the antiprotozoal drugs discovery, which is always threatens by the worldwide spread of drug resistant species of parasites.

In this context, also compounds derived from natural products, such as aphidicolin and rosmaricine derivatives, that we have only started to explore, demonstrated to be an alternative that should be more investigated and improved to fight against protozoan diseases.

8. REFERENCES

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