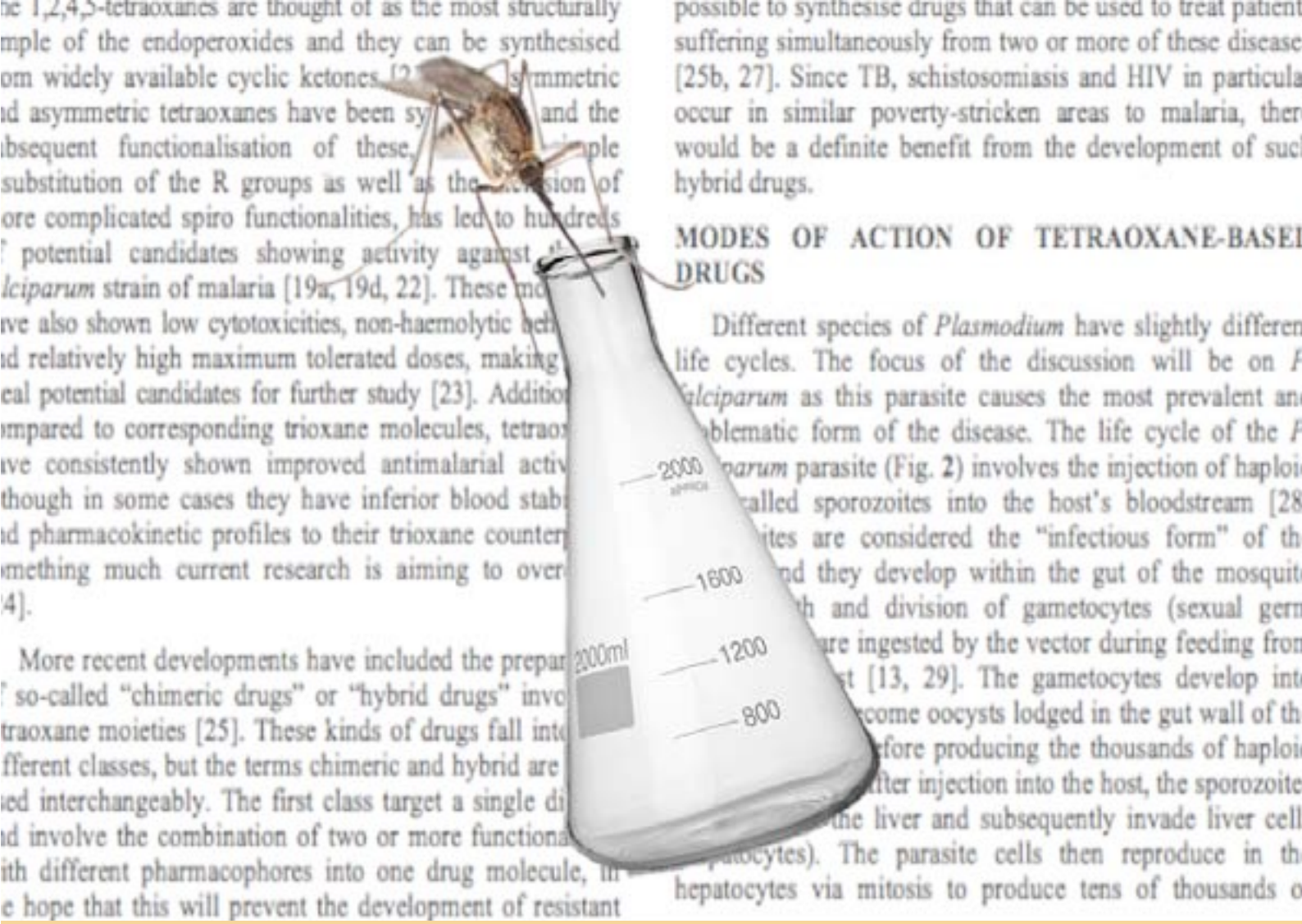


Paula Susana Lopes Rebelo da Costa

Steroidal endoperoxides in the synthesis of novel antimalarial hybrids

Dissertação de Mestrado em Design e Desenvolvimento de Fármacos, orientada pela Professora Doutora Maria Luisa Sá e Melo e Professora Doutora Maria Manuel Cruz Silva e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro 2015

· U C ·

Universidade de Coimbra

Paula Susana Lopes Rebelo da Costa

Steroidal endoperoxides in the synthesis of novel antimalarial hybrids

Dissertação de Mestrado em Design e Desenvolvimento de Fármacos, orientada pela Professora Doutora Maria Luisa Sá e Melo e Professora Doutora Maria Manuel Cruz Silva e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro 2015

Universidade de Coimbra

Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra para obtenção do grau de Mestre em Design e Desenvolvimento de Fármacos, sob orientação científica da Professora Doutora Maria Luisa Sá e Melo e Professora Doutora Maria Manuel Cruz Silva, no Laboratório de Química Farmacêutica da Faculdade de Farmácia da Universidade de Coimbra.

"Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning."
Albert Einstein

AGRADECIMENTOS

Na tentativa de não esquecer ninguém, gostaria de expressar os meus sinceros agradecimentos a todas as pessoas que me apoiaram e incentivaram ao longo deste ano, sem as quais a realização deste trabalho não teria sido possível.

À Professora Doutora Maria Luisa Sá e Melo, agradeço a oportunidade que me proporcionou com um projecto tão aliciante, bem como todos os conhecimentos científicos transmitidos ao longo destes dois anos.

À Professora Doutora Maria Manuel Cruz Silva, pelo incentivo, orientação e toda a dedicação na elaboração deste trabalho. Pelos conhecimentos científicos transmitidos, criticas e sugestões, que contribuíram para a evolução do meu percurso académico.

Ao Pedro Cruz, por partilhar comigo os conhecimentos necessários à análise de espectros e ajuda no esclarecimento e clarificação de algumas dúvidas.

Às colegas de laboratório, Salete Baptista e Sandra Figueiredo, pelo apoio e todos os momentos de boa disposição que deram ânimo aos dias de trabalho.

À Dona Graça Santiago e à Dona Anabela Pinto toda a simpatia e apoio prestado.

À minha família, em especial à Beatriz, pela motivação, compreensão, tolerância e amor, sem os quais a obtenção deste grau não teria sido possível.

Aos meus amigos, o meu muito obrigada pelo constante apoio, motivação, carinho e todos os momentos de amizade partilhados ao longo destes anos.

GENERAL INDEX

LIST OF FIGURES

LIST OF SCHEMES

ABBREVIATION LIST

Ac = Acetyl group

ACT= Arthemisin-based combined therapies

ca. = Circa

$CDCL_3$ = Deuterochloroform

FDA = Food and Drug Administration

eq = Equivalents

FCC = Flash Column Chromatography

IC_{50} = Half minimal inhibitory concentration

MHz =Mega hertz

min = Minute

MMPP = Magnesium Monoperoxyphthalate Hexahydrate

mp = Melting Point

NMR = Nuclear Magnetic Ressonance

ppm = Parts per million

rt = Room temperature

TEA = Triethylamine

TMS = Tetramethylsilane

TLC = Thin Layer Chromatography

WHO = World Health Organization

η = Yields in %

δ = Chemical Shift

ABSTRACT

Malaria is one of the major public health concerns in underdeveloped countries today. The infectious disease infects more than 300 million people a year and kills 1.5 to 2.7 million individuals annually. However, recent reports have indicated that the burden of malaria has significantly declined in some countries and the decline is mainly attributed to intensified control measures such as deployment of artemisinin combination therapy (ACT). ACTs involve the combination of an artemisinin derivative with a partner drug that clear parasites at a slower rate than arteminisin, in order to avoid recrudescence and development of resistant *Plasmodium falciparum* strains.

Hybrid drugs combine two different pharmacophores in a single chemical entity with a dual mode of action. This approach has recently emerged as a strategy to develop new efficient drugs, and in the case of malaria therapy, may represent an attractive alternative to classical ACTs.

Oxygenated derivatives of cholesterol, oxysterols, present a remarkably diverse profile of biological activities. Oxysterols have been implicated in many pathological situations in result of their cytotoxic, oxidative and pro-inflamatory properties. With this assumption in mind, oxysterols are excellent candidates for the synthesis of hybrid drugs once they may increase the sensitivity of the infected cells to other antimalarials drugs.

Recently, Professor Sá e Melo and her co-workers prepared oxysterols with antiplasmodial activity against *P.falciparum* W2 (chloroquine resistance) which present IC_{50} values under 10 micromolar concentrations (unpublished results).

Based on this knowledge, this work explores hybrid compounds based on a steroid scaffold and a tetraoxane bond that interferes in the haemozoin formation, and adamantanone and ethyl 4- oxocyclohexanecarboxylate to complete the hybrid molecule.

So, the main objective of this study was to optimize tetraoxanes synthesis reactions, approaching the one pot technique as an alternative to the method used initially. Another important step was also the comparison between two catalysts used in these reactions.

The work in this thesis and the conclusions reached are important for the future development of antimalarial drugs with a scaffold of steroids.

Keywords: malaria, hybrid compounds, 1,2,4,5-tetraoxanes, oxysterols.

RESUMO

Hoje em dia a Malária é uma das principais preocupações de saúde pública nos países subdesenvolvidos. A doença afecta cerca de 200 a 300 milhões de pessoas por ano e provoca cerca de 1 milhão de mortes anualmente. No entanto, relatórios recentes indicam que o impacto do paludismo diminuiu significativamente em alguns países e o declínio é atribuído principalmente às medidas de controlo intensificadas como a implantação da terapia combinada na administração de artemisininas (ACT).

A ACT tem como fundamento a combinação de um derivado de artemisinina com um outro fármaco antimalárico que tenha maior tempo de semi-vida, com o objectivo de evitar a recrudescência e o desenvolvimento de estirpes resistentes de *Plasmodium falciparum*.

Fármacos híbridos combinam dois farmacóforos diferentes numa única entidade química com duplo modo de acção. Esta abordagem surgiu recentemente como uma estratégia de modo a desenvolver novos fármacos eficazes e pode representar uma alternativa para a terapia de combinação clássica.

Os derivados oxigenados de colesterol, oxiesteróis, apresentam um perfil consideravelmente diverso de actividades biológicas. Os oxiesteróis têm sido implicados em diversas situações patológicas, em consequência das suas propriedades citotóxicas, oxidativas e pro-inflamatórias. Com este pressuposto em mente, os oxiesteróis são excelentes candidatos para a síntese de fármacos híbridos, uma vez que podem aumentar a sensibilidade das células infectadas com outros fármacos antimaláricos.

Recentemente, a Professora Doutora Sá e Melo e seus colaboradores prepararam oxisteróis com actividade antiplasmódica contra *P. falciparum* W2 (resistência à cloroquina) apresentando valores de IC_{50} inferiores a 10 concentrações micromolares (resultados não publicados).

Com base neste conhecimento, este trabalho explora a síntese de antimaláricos híbridos com uma porção de esteróide, uma ligação tetraoxano que interfere na formação da hemozoína, e adamantanona e etil 4-oxociclohexanonacarboxilato para completar a molécula híbrida. Assim, o principal objetivo deste trabalho foi otimizar reações de síntese de tetraoxanos, abordando a técnica *one pot* como uma alternativa ao método utilizado inicialmente. Outro passo igualmente importante foi a comparação entre dois catalisadores usados nestas reacções.

O trabalho nesta tese e as conclusões são importantes para o futuro desenvolvimento de antimaláricos com uma porção de esteróide e um grupo tetraoxano.

Palavras chave: malaria, compostos híbridos, 1,2,4,5-tetraoxanos, oxiesteróis

Chapter 1

INTRODUCTION

1.1 Malaria

Malaria, one of the most dominant human infectious diseases, affects about 200 to 300 million people worldwide and is the cause for about 1 million deaths every year, mostly children under the age of 5 and pregnant women. It is estimated that every 45 seconds a child dies from malaria in Africa. [1,2].

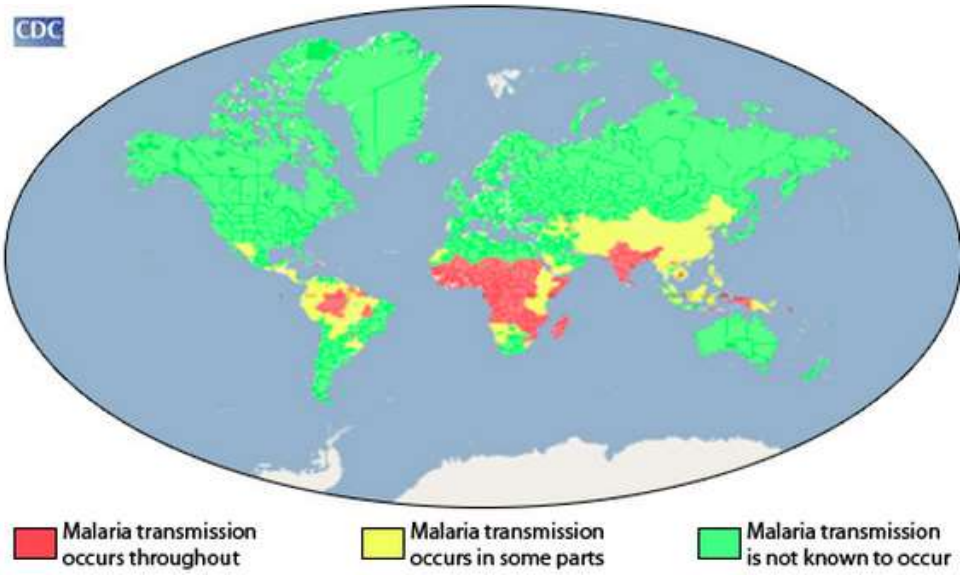


Figure 1: Illustration of the parts of the world where malaria transmission occurs. Adapted from [1] (Accessed in July of 2015).

1.2 Lifecycle of *Plasmodium* parasites

Knowledge of malaria's epidemiology is essential for the design and interpretation of test results with drugs, vaccines or other interventions.

Several species of *Plasmodium* cause malaria in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and the simian *Plasmodium knowlesi*. The most severe species is *P. falciparum* found predominantly in Africa. [3] Furthermore, there is growing evidence that the lethality of *P. vivax* has been underestimated. [4]

The parasite has a complex life cycle (**Figure 2**) and, in order to eradicate the disease, all steps should be considered for treatment. It begins by inoculation of sporozoites into the bloodstream of man, when bite occurs from an infected female mosquito *Anopheles*.

Once inoculated, sporozoites travel through the lymphatic system and blood to the liver where they develop into liver schizonts forms or "exo-erythrocytic" through a process called schizogony, which causes the merozoites. After differentiation of these phenomena, the merozoites are released into the bloodstream where they invade red blood cells of the

host suffering morphological changes to the formation of trophozoites, wich then develop into schizonts. Asexual reproduction by schizogony, the erythrocytic invasion cycles and the release of numerous merozoites, produce a rapid multiplication of parasites that causes infection levels responsible for the disease.

In erythrocytes, some parasites do not suffer schizogony but transform into gametocytes sex-dimorphic (female and male). These gametocytes reach the digestive tract of the mosquito during the blood meal and complete their sexual development until the formation of gametes. The gametes fuse to form a zygote, leading to a mobile ookinete which migrates to the outer wall of the stomach where it forms the oocyst, which undergoes mitotic divisions yielding numerous various sporozoites. When the oocyst ruptures, releases the sporozoites that reach the salivary gland to complete the cycle. [5]

The control of this disease has been severely affected by the emergence of resistant parasites to almost all antimalarial drugs used for prophylaxis and treatment, so the search for new safe and effective anti-malarials, has become a major objective for the control of the disease and high mortality rate. [6]

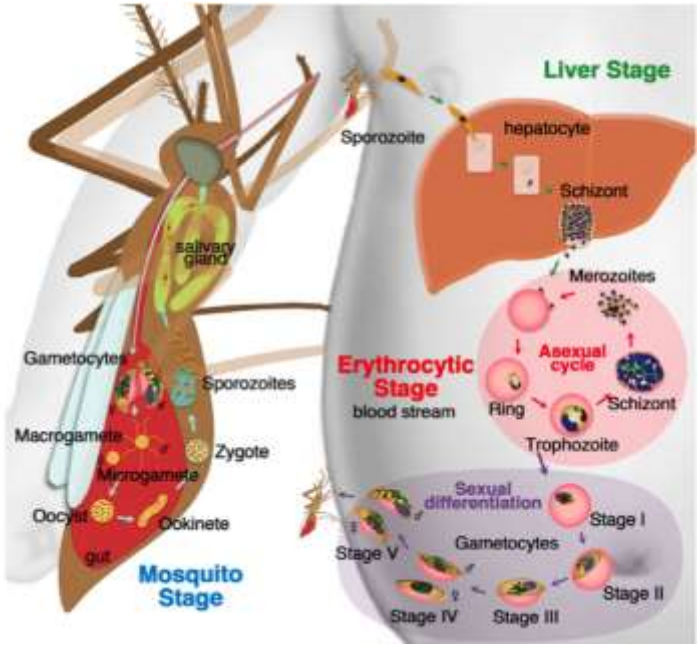


Figure 2: *Plasmodium* life cycle. Adapted from [3]

1.3 Hamaezoin- A Malaria pigment

Haemozoin or malaria pigment has a history in the scientific literature older than the malaria parasite itself, having first been described in the early 18th century by the noted Italian physician Lancisi. Eventually, this pigment played a role in the discovery of the parasite and the elucidation of its life cycle. Its formation occurs in the blood phase of the infectious cycle, resulting from the metabolism of hemoglobin present in erythrocytes. In this phase of the infection, it is known that 60 to 80% of the hemoglobin present in the red blood cell is digested. This metabolic process occurs in the digestive vacuole of the parasite and is catalyzed by a battery of proteolytic enzymes. [7,8] During the process of hemoglobin degradation, all of the heme present in the hemoglobin is released into the digestive vacuole. Free heme is toxic to cells, so the parasites convert it into an insoluble crystalline form.

The possible damage caused by the heme, are minimized by a rapid oxidation of Fe (II) to Fe (III) with formation of hematin (**Figure 3a**). Haematin is known to be toxic to microorganisms [9]. By converting haematin to haemozoin (**Figure 3b**), the parasite removes the hematin permanently from the solution and deposits the iron porphyrin in a relatively innocuous crystalline form – the haemozoin. [10,11,12] Although is not yet completely understood, apparently this is the mechanism used by *Plasmodium* parasites as a way of detoxification of the heme group.

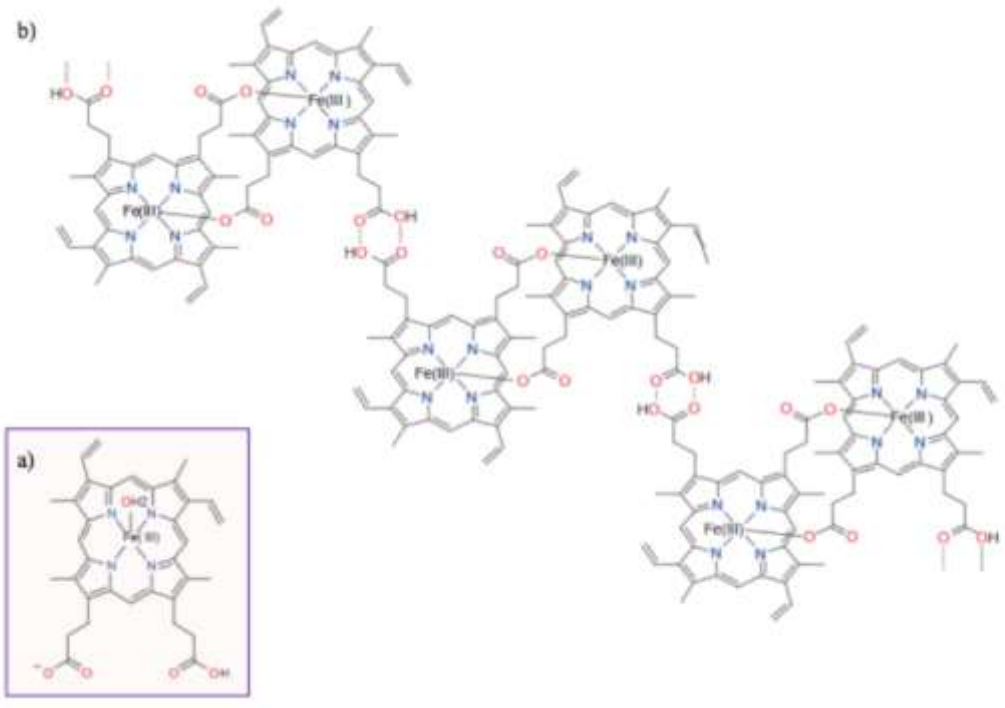


Figure 3: Representation of the Haematin and their chemical interactions with formation of the crystalline structure hemozoin. a) Haematin b)Haemozoin. Adapted from [12]

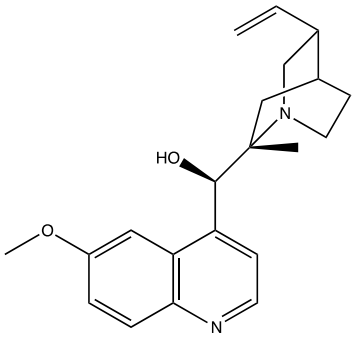
1.4 Development of Antimalarials

The complexity of the parasite's life cycle and its various interactions with the host, man, and the vector, mosquito, suggest several potential targets for pharmacological intervention in the disease and / or transmission. [13]

It is important to mention that the antimalarial drugs can act against more than one form of the parasite and be effective against a species, but totally ineffective against others.

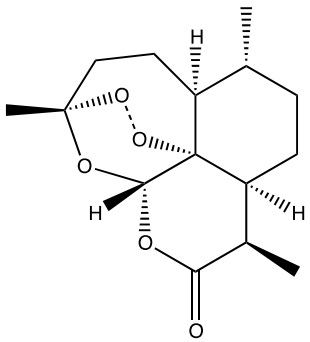
Drugs that target the liver stages are important to prevent the disease from developing (prophylactic treatment) and to provide what is known as a "radical cure" for *P. vivax* and *P. ovale*. On the other hand, drugs that target the blood stages are important to control the symptoms of the disease and associated mortality. Drugs that target the transmission and mosquito stages are important to prevent the infection of other humans, and would benefit an eradication agenda.

The first drug used to treat malaria was quinine, **1.1**, an alkaloid having antipyretic, antimalarial, analgesic and anti-inflammatory properties. It is present in natural trees of Central and South America. [14]



1.1 - Quinine

In the 70s, the crystalline compound artemisinin was isolated, **1.2**, from *Artemisia annua* plant, used in China for over 2000 years due to its antipyretic properties. [6]



1.2 - Artemisinin

Artemisinin is an extremely active antimalarial agent and the only one for which it is not recognized clinical resistance. Structurally, it is a sesquiterpene lactone containing an

endoperoxide linkage, essential for antimalarial activity. This class of antimalarial drugs is effective in drug-resistant strains of *P. falciparum* blood stage having activity at both asexual and sexual forms, which can contribute to a reduction of disease in areas of low transmission. [15]

Artemisinin causes the death of malaria parasites, probably due to the generation of more than one type of cytotoxic intermediates. They are versatile compounds which produce a variety of highly reactive intermediates (carbon and oxygen radicals) and various neutral electrophiles (epoxides, aldehydes and dicarbonyl compounds).This variety of species may explain the difficulty of the malaria parasite to develop resistance. [16] There are several other factors by which the resistance to artemisinin has not yet been developed: [17]

a) The reduced half-lives ($t_{1/2}$) mean that the parasites are exposed to subtherapeutic concentrations of drug over a short period of time;

b) Through their gametocytocidal activity, these derivatives reduces the transmission, reducing the possibility of infection to other mosquitoes;

c) Their antimalarial effect is not exerted in a single biological target, but in several targets simultaneously, with high precision and efficiency. These targets involve enzyme inhibition, lipid peroxidation and effects on heme detoxification;

d) Combination with other antimalarials is quite often.

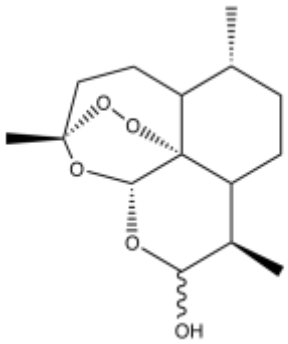
1.5 Semi-synthetics first generation

Although artemisinin has been used clinically in China for the treatment of multidrug resistant *Plasmodium falciparum* malaria, its therapeutic value is limited to a great extent by its low solubility in both oil and water.

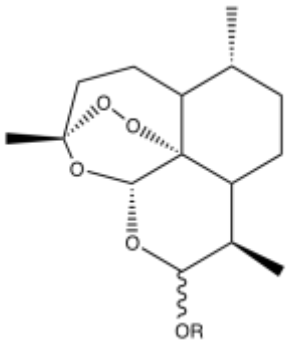
Consequently, in the search for more effective and soluble drugs, Chinese researchers prepared a number of derivatives of the parent drug.

The reduction of the lactone in artemisinin led to the formation of dihydroartemisinin, **1.3**. Later on the derivatives of dihydroartemisinin ether, artemether, **1.4**, and arteether, **1.5**, were prepared, both characterized by an acetal group. [18] **1.4** and **1.5** derivatives suffer oxidative dealkylation resulting in the dihydroartemisinin, suggesting that this may be the active metabolite. [19]

Both of these compounds, artemether and arteether, are more potent than artemisinin. However, they present short plasma half-lives and produce fatal central nervous system (CNS) toxicity in chronically dosed rats and dogs. [20]



1.3 - Dihydroartemisinin



1.4, R= CH_3 – Artemether
1.5, R=C_2H_5 – Arteether
1.6, R=$C(O)CH_2CH_2CO_2Na$ – Sodium artesunate
1.7, R=$CH_2C_6H_4$-4CO_2Na – Sodium artelinato

For the treatment of advanced cases of *P. falciparum* malaria, a water-soluble derivative of artemisinin is desired.

Other semisynthetic artemisinin derivative is sodium artesunate **1.6**, that due to the carboxylate group, is water soluble and can be administered intravenously, which is very important in cases of cerebral malaria. [21]

The artesunate is rapidly hydrolyzed, releasing dihydroartemisinin and is eliminated with a $t_{1/2}$ less than 10 min, while its metabolite, dihydroartemisinin, is eliminated with a $t_{1/2}$ 40-60 minutes, similar to that obtained for the artemether. [22] Sodium artelinato, **1.7**, an other water-soluble artemisinin derivative, has an acetal group metabolically more stable and a carboxylate group like artesunate, and has $t_{1/2}$ 1.5-3 hours. [23]

The neurotoxicity is the major concern with all artemisinin derivatives because they originate dihydroartemisinin by biotransformation, wich is a potential neurotoxic agent. The neurotoxicity observed in studies with animal models, has not been observed in humans, despite the widespread use of artemisinin in China for over 30 years. [24]

Artemisinin and its derivatives are toxic to the malaria parasites in the order of nM concentrations, but for mammalian cells concentrations in the range of μM are required. Regarding malaria prophylaxis, the use of artemisinin and its derivatives is prohibited because of possible risk of neurotoxicity and to minimize the emergence of resistance. [25]

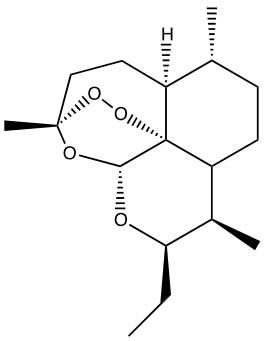
1.6 Semi-synthetics of second generation

In order to overcome the problems presented by the first generation derivatives of artemisinin (reduced $t_{1/2}$ and dihydroartemisinin formation), semisynthetic derivatives of second generation were developed. [18,23,26]

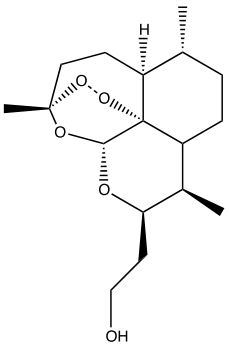
To reduce the sensitivity of the metabolic acetal group, most variations were made in the C_{10} position of artemisinin, where the exocyclic oxygen atom was substituted by carbon substituents. Various compounds were developed containing alkyl (**1.8**, **1.9**, **1.10**), aryl (**1.11**) and heteroaryl residues (**1.12 ,1.13**).

Synthetic derivatives containing variations in the C_{16} position, such as compound **1.14**, and dimers, **1.15** were also prepared. [27,28] The compound **1.10** does not give rise to the metabolite dihydroartemisinin and contains a side chain that originates a water soluble salt, having values of *in vitro* activity higher than artemether and artesunate. [29]

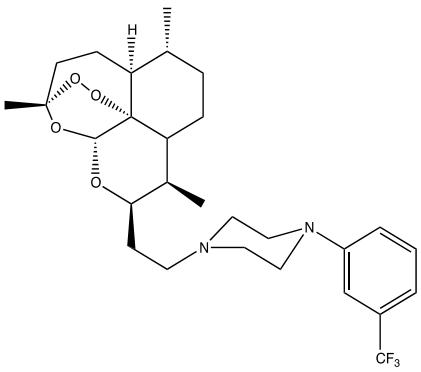
In general, compounds were synthesized with antimalarial activity *in vitro* superior to the activities of the first generation derivatives, with sometimes promising results *in vivo* but none of the derivative has reached clinical phases.



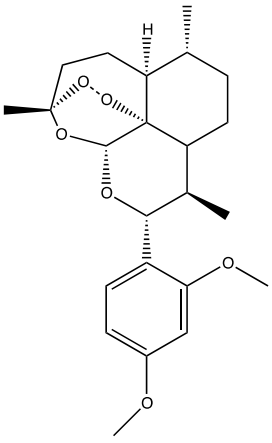
1.8



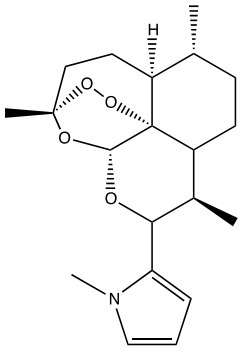
1.9



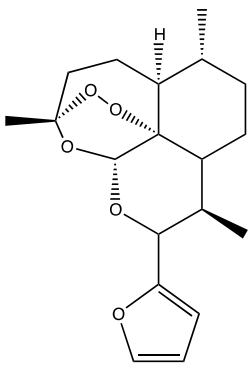
1.10



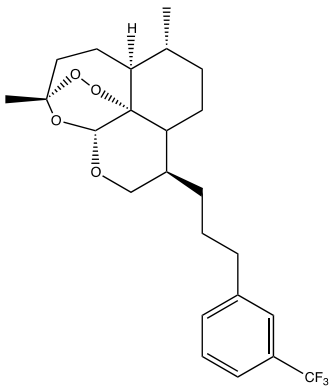
1.11



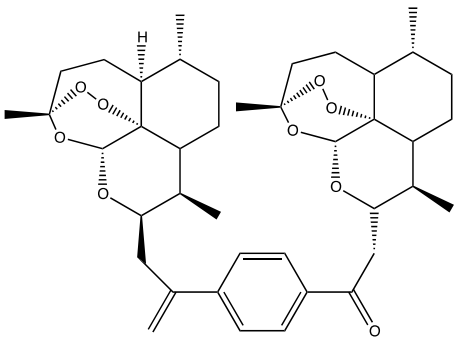
1.12



1.13



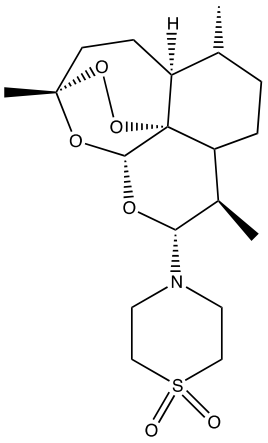
1.14



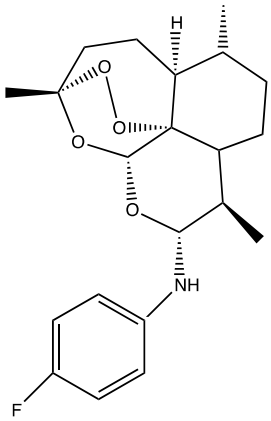
1.15

Other second-generation derivatives have also been developed, comprising compounds **1.16** to **1.19**, which contain nitrogen substituents at C_{10}, yielding to N, O-acetal derivatives of artemisinin. [18,23,26]

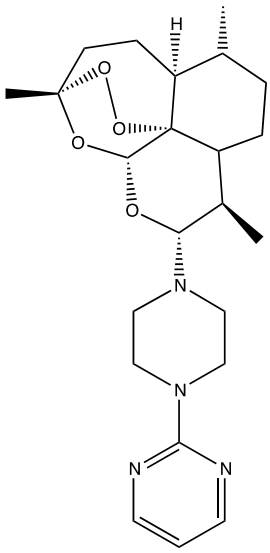
The derivative **1.16** artemisona, despite not being the most active compound, was the most promising candidate, showning to be 2-5 times more active *in vivo* that artesunate, deprived of neurotoxicity or cytotoxicity. Therefore, this derivative has been in clinical trials phase II, although studies are currenty suspended. [30,31]



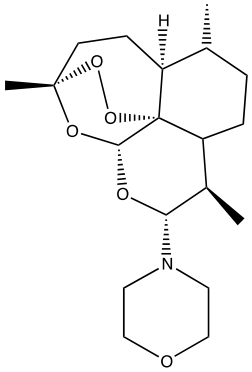
1.16



1.17



1.18



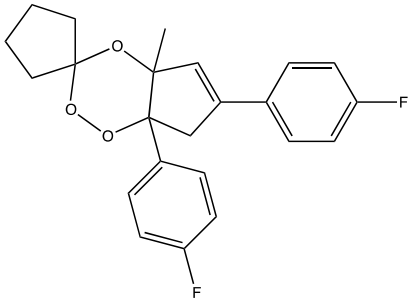
1.19

1.7 Synthetic peroxides

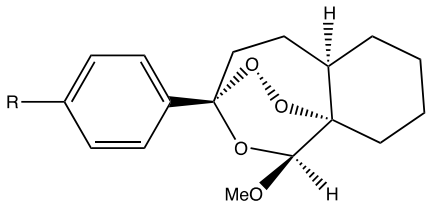
The disadvantage of all the semisynthetic artemisinin derivatives is the fact that they are from a natural origin of limited access.

In order to avoid this problem and knoing that the endoperoxide bond in artemisinin and its derivatives has been identified as the key pharmacophore in the antimalarial activities of these molecules, the development of a range of peroxide-containing molecules, like 1,2,4-trioxanes, **1.20** to **1.22**, 1,2,4-trioxolanes **1.23** to **1.25**, tetraoxanes, **1.26** to **1.28**, and other endoperoxide structures **1.29** to **1.30** has been pursued.

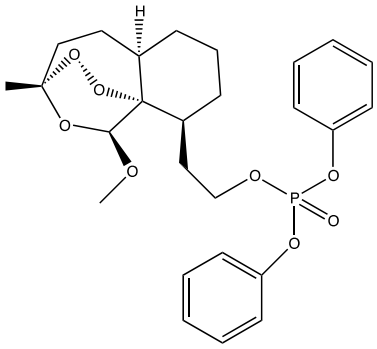
These derivatives, such as artemisinin, are activated by Fe II and exert their mode of action through the formation of radical species. [18,23,26]



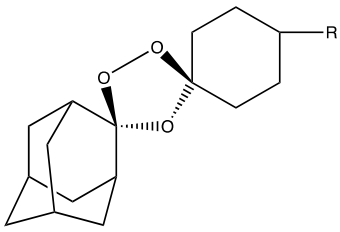
1.20



1.21

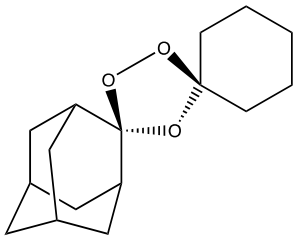


1.22

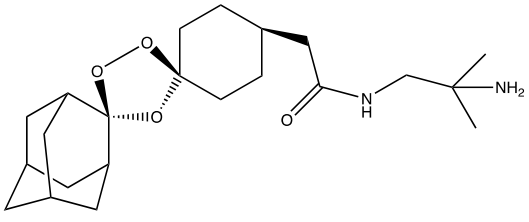


R=OH ,OR, NHR
where R=aryl , alkyl

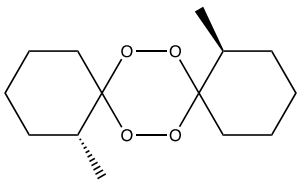
1.23



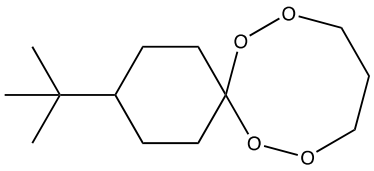
1.24



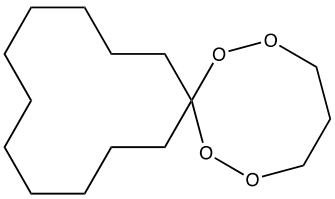
1.25



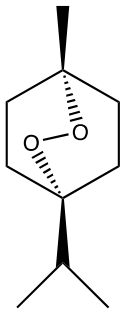
1.26



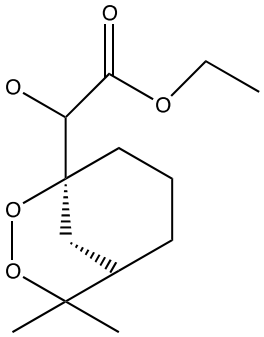
1.27



1.28



1.29



1.30

It was found that, generally, this kind of analogue exhibits activity's values superior to the values of artemisinin and, when administered orally in mice, they are very effective and don't have relevant toxic effects. [18,23,26]

The 1,2,4,5-tetraoxanes are thought of as the most structurally simple of the endoperoxides and they can be synthesised from widely available cyclic ketones. [33] Both symmetric and asymmetric tetraoxanes have been synthesised, and the subsequent functionalisation of these, both by simple disubstitution of the R groups as well as the inclusion of more complicated spiro functionalities, has led to hundreds of potential candidates showing activity against the *P. falciparum* strain of malaria. [34,35,36] These molecules have also shown low cytotoxicities, non-haemolytic behaviour and relatively high maximum tolerated doses, making them ideal potential candidates for further study. [37] Additionally, compared to corresponding trioxane molecules, tetraoxanes have consistently shown improved antimalarial activities, although in some cases they have inferior blood stabilities and pharmacokinetic profiles as compared to their trioxane counterparts, something that current research is aiming to overcome. [38]

1.8 Combating resistance

The malaria parasite has developed resistance to most anti-malarial drugs used in clinic, for example, chloroquine, pyrimethamine, sulfadoxine, amodiaquine and mefloquine. In some cases resistance appeared in the year it was introduced, as in the case of sulfadoxine pyrimethamine. [39]

Although until the moment there is no relevant clinical evidence of *plasmodium* resistance to artemisinin, a decreased susceptibility of parasites to treatment with artemisinin on the border between Thailand and Cambodia was observed, demonstrating the possible development of the drug resistant parasites.

The three main reasons for the appearance of the artemisinin resistance phenomenon is probably due to the use of monotherapy; to subtherapeutic doses administered and falsifications of this product. [40]

This emerging situation of possible resistance, led the WHO to recommend the use of combination therapy in the management of artemisinin (ACT - Artemisinin-based Combination Therapy) as first-line treatment of malaria since 2001. [41]

1.9 Artemisin-based Combination Therapy

The concept of combination therapy is based on the synergistic or additive potential of two or more drugs, to improve therapeutic efficacy and to delay the development of resistance to individual components of the combination.

The principle of combining different drugs in order to improve its pharmaceutical properties is not new and has been applied in the treatment of infections such as tuberculosis, HIV and in the chemotherapy of various types of cancer. [42,43,44]

In monotherapy, artemisinin acts rapidly against the parasites and has faster clearance of the parasites from the blood than any other antimalarial drugs, resulting in faster relief of clinical symptoms. [45,46,47] However, patients must take the drug for at least 7 days to maximize cure rates due to its very short half-life, otherwise some parasites could escape from the action of the drug during treatment. Thus, the failure to complete the whole drug treatment timeline, due to misleading rapid improvement in clinical symptoms, can lead to high levels of treatment failure. [48]

An approach to circumvent this problem is to use combination therapy comprising artemisinin derivatives plus another antimalarial drug with longer half-life and a different mode of action, known as artemisinin-based combination therapy or ACT. [49] These ACTs

can be taken for shorter durations (<3 days) than artemisinin monotherapy, and importantly, can increase patient compliance thus reducing the risk of resistant parasites arising during therapy.

By the end of 2013, ACTs had been adopted as national policy for first-line treatment in 79 of 88 countries where *Plasmodium falciparum* is endemic. Chloroquine was being used in 10 Central American and Caribbean countries where it remains efficacious. The number of ACT courses procured from manufacturers – for both the public and private sectors – rose from 11 million in 2005 to 392 million in 2013. This increase has been largely driven by procurements for the public sector. [1]

1.10 Hybrids drugs

Malaria remains a devastating disease in the tropics and sub-tropics despite the availability of a large number of antimalarial drugs. Part of this problem is due to the disadvantages of the drugs in use, which include (depending on the drug) side effects, low efficacy due to resistance, and high cost.

Traditional and innovative approaches to the discovery and development of new antimalarial drugs are needed to provide new drugs to better control of malaria. The innovative approach of hybrid drugs has been pursued to address these problems.

A hybrid drug can be defined as an entity composed by two or more drugs with different complimentary effects, linked covalently, in order to create a more effective molecule compared to their individual components.

It can be said that hybrid drugs act by two or more different pharmacophores, each with different molecular mechanisms of action. The drugs can be linked directly or via a linker susceptible to cleavage. [50]

According to Morphy and Rankovic [51] the hybrid molecules can be classified into:

1. **Conjugates**, the molecular frameworks that contain the pharmacophores for each target are separated by a distinct linker group metabolically stable that is not found in either the individual drugs;
2. ***Cleavage conjugates*** have a linker designed to be metabolized to release the two drugs that interact independently with each target;
3. ***Fused hybrid*** the two farmacophores are connected, the size of the linker decreased such that the framework of the pharmacophores is essentially touching;

4. ***Merged hybrids*** have their frameworks merged by taking advantage of commonalities in the structures of the starting compounds, which give rise to smaller and simpler molecules.

Drugs are usually linked via labile linkers with ester function, amide, carbamate and others, which can be cleaved chemically and enzymatically to release the active drug molecules at the site of action in the organism.

Several diseases are treated by a combination of therapeutic agents administered in separate dosages.However, there are potential advantages to administering drugs in a single chemical entity compared to the separate mixing agents such as:

a) to improve the distribution and pharmacokinetic properties easing the distribution of drugs in specific sites of action, through appropriate design of linker (s) hydrolysable;

b) to improve the physical and chemical properties (such as solubility and polarity), compared to the molecules shown separately;

c) to improve the chemical stability of the formulation and the metabolic stability;

d) to achieve greater synergy between the two drugs, due to its proximity;

e) to minimize side effects. [52,53]

The downside, however, is that it is more difficult to adjust the ratio of activities at the different targets.

There are several hybrid agents acting in various therapeutic areas. For instance, hybrids of aspirin [54] and ibuprofen [55] were synthesized for the treatment of inflammation. There are also hybrids with antihistaminic action [56] and anti-depression. [57]

Despite the many advantages of this type of drugs, they present as main drawback the high molecular weight often greater than 500 Da, violating a Rule of Five of Lipinsky [58] and, as such molecules are not favorable for the development of an oral, or topical drug, neither to act on the central nervous system.

However, it is noted that there are drugs whose five Lipinsky criteria are not fully satisfied. An ideal hybrid drug should be stable enough to support the development of the formulation, but can not be too stable, compromising the release of the drug *in vivo*.

Another interesting aspect in the design of hybrid drugs is its meaning for toxicological. As new chemical entities considered, irrespective of its constituents, the drugs have to be treated as new xenobiotic by the Food and Drug Administration (FDA), since they are new drugs that have never been previously administered to humans. For this reason, the toxicity tests should be carried out. [59]

Most developing hybrids contain at least one of two pharmacophores: quinoline nucleus essential in inhibiting the formation of hemozoin, leading to the death of the parasite and the endoperoxide bridge for the reasons described above. [60,61]

1.11 Hybrids with endoperoxide

Recently, in a deliberate rational design of antimalarials acting specifically on multiple targets, several hybrid molecules have been developed in what has been termed "covalent biotherapy."

One of the first reports of a hybrid drug for malaria treatment came in 2000 from Meunier *et al.,* wherein a series of chimeric molecules were produced by the attachment of a trioxane to an aminoquinoline moiety, forming "trioxaquines" (**Figure 4**), a concept referred to as "covalent biotherapy", and thus possess a dual mode of action, namely heme alkylation with the trioxane entity heme stacking with the aminoquinoline moeity and inhibition of haemozoin formation. It was proved that the trioxaquines had improved antimalarial activity than their individual fragments, indicating a potential additive/synergistic effect of the hybrids. [62]

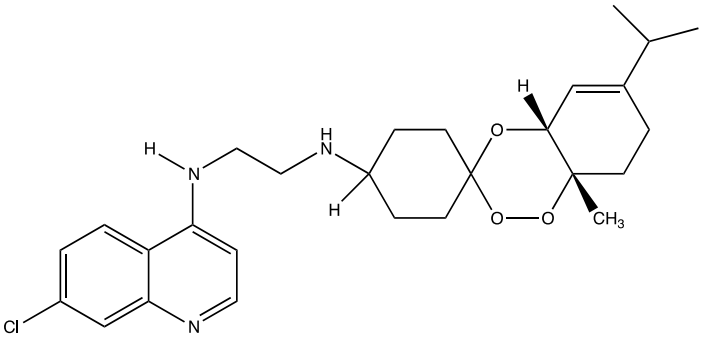


Figure 4: Chemical structure of a tetraoxaquine

Trioxolaquines (**Figure 5**) are hybrid molecules similar to trioxaquines except that they contain a trioxolane motif, namely an ozonide, instead of a trioxane entity. The use of tetraoxanes covalently conjugated with quinolone systems yielded to another hybrid class called tetraoxaquines. The tetraoxanes are characterized by antimalarial activity in the order of magnitude of nM and for not having toxicity *in vitro* and *in vivo.* [63]

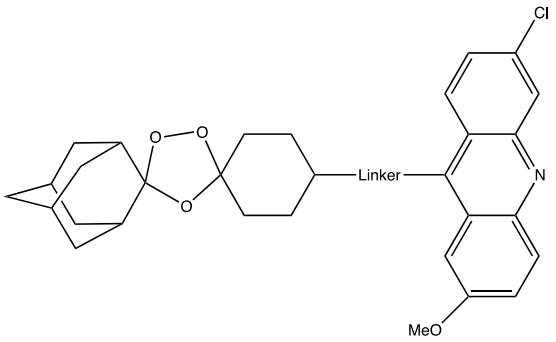


Figure 5: Chemical structure of a trioxolaquine

More recent studies on endoperoxide stability have shown that 1,2,4,5-tetraoxanes have significantly higher stability than the 1,2,4-trioxanes. [64]

The only drug-development candidate with a tetraoxane moiety is so far the RKA 182 (**Figure 6**). Concerted efforts geared towards development of fully synthetic alternatives, which retain the peroxide pharmacophore have been applied for almost decades.

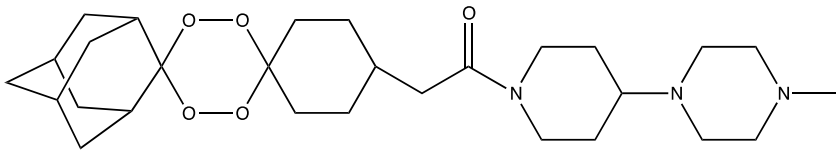


Figure 6: Chemical structure of the RKA 182

1.12 Mechanism of action

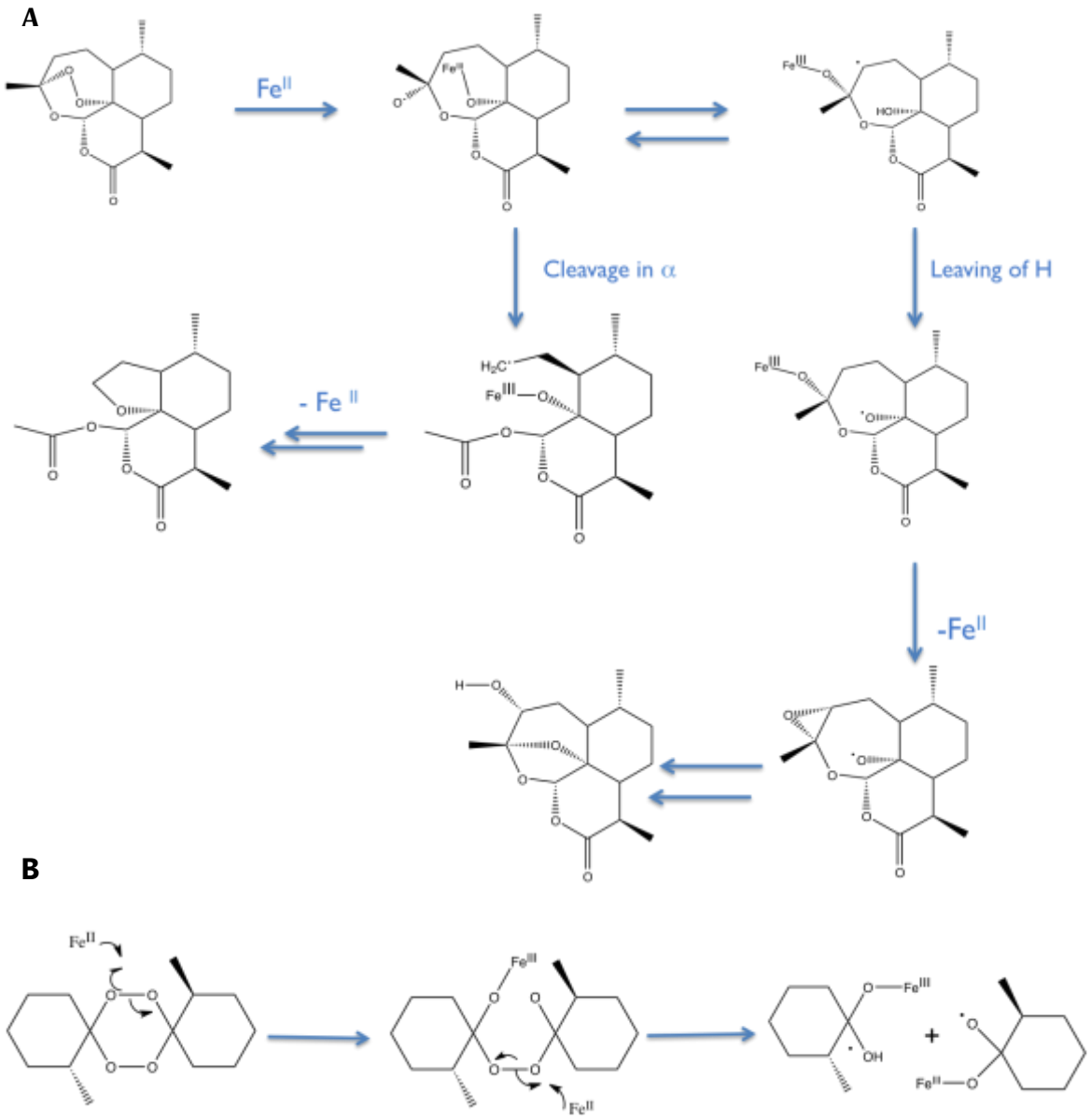
Although artemisinin is an antimalarial agent used in clinic therapeutic, its mechanism and site of action continue to be debated in the scientific community. There are several theories about the mode of action of artemisinin and its derivatives, but they all point to the need of the presence of Fe^{2+}. [65]

1.12.1 Interaccion with the heme

The first sign that allowed proposing a mechanism of action arose from evidence that peroxidic bridge, characteristic of the chemical structure of these compounds, was fundamental for its antimalarial activity. [66]

Meshnick et al proposed in 1991 that the Fe^{2+} atom present in the hemoglobin heme triggered the reduction of the peroxidic bridge and consequently breakage and formation of oxygen-centered radicals atom.

Once formed, these radicals may react with intracellular targets of parasitic cells, such as proteins and/or lipids, or undergo rearrangement with the formation of carbon-centered radicals. (**Scheme 1**) [67]



Scheme 1: Mechanism postulated of interaction of iron present in the digestive vacuole with artemisinin (A) and 1,2,4,5 tetraoxanes derivatives (B). (Adapted from [68a,68b])

1.13 Bioactive Sterols

1.13.1 Steroids

Lipids are starting to gain an interest in research mostly due to a clear knowledge of its important role in cellular membranes and in the regulation of some intracellular processes as cell signaling and receptor function among others. [69,70]

One major class of lipids is the steroids, which have structures totally different from the other classes of lipids. Steroids may be recognized by their tetracyclic skeleton, consisting of three fused six-membered and one five-membered ring. The four rings are designated A, B, C & D and the ring carbon atoms are conventionally assigned (**Figure 7**). The substituents designated by R are often alkyl groups, but may also other functionalities. The R group at the A:B ring fusion is most commonly methyl or hydrogen, whereas the C:D fusion is usually methyl. The substituent at C-17 varies considerably. The most common locations of functional groups are C-3, C-4, C-7, C-11, C-12 & C-17. Ring A is sometimes aromatic. [71]

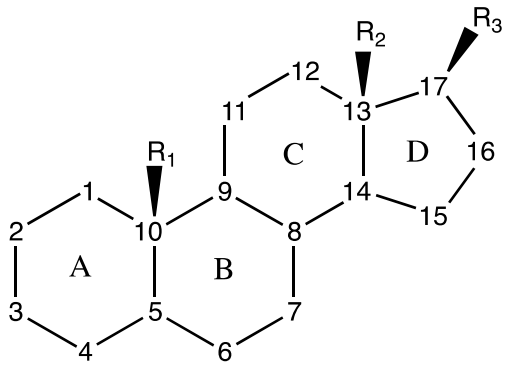


Figure 7: Representation of the steroid skeleton

As observed in **Table I** the combination of the substituents R_1, R_2 and R_3 defines the steroid class.

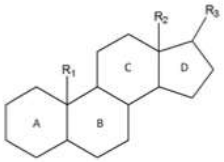
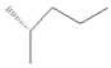
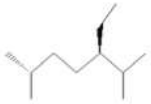
	Substituent			Number of C	Steroid Class
	R_1	R_2	R_3		
	H	H	H	17	Gonanes
	H	CH_3	H	18	Estranes
	CH_3	CH_3	H	19	Androstane
	CH_3	CH_3	CH_2CH_3	21	Pregnanes
	CH_3	CH_3		24	Cholane
	CH_3	CH_3		27	Cholestane
	CH_3	CH_3		29	Stigmastane

Table 1: Classification of the steroids classes according to the substituents and the whole number of carbons.

Due to their great value in therapeutics for several pathologies like cancer or neurologic diseases, [72,73,74] the interest for steroids has increased exponentially in the last decades. Hundreds of steroids were extracted and isolated from natural sources, while others were modified based on the natural ones by hemi-synthesis, or by total synthesis. The wide range of possibilities offered by steroids in Medicinal Chemistry is far from being completely deciphered.

1.13.2 Sterols

Sterols, also known as steroid alcohols, are a subgroup of steroids with a hydroxyl group at the 3-position of the A-ring. They are amphipathic lipids, possessing both hydrophilic and lipophilic properties, essential for their incorporation into the lipid bilayer of the cytoplasmatic membrane of the eukaryotic organisms as well as by many procaryotic ones, regardless of their status as plants, animals, or Protista. [75]

1.13.3 Cholesterol

Cholesterol, the best known and most abundant steroid in the body. It is a lipid with a unique structure consisting of four linked hydrocarbon rings forming the bulky steroid structure. [71,76,77] There is a hydrocarbon tail linked to one end of the steroid and a hydroxyl group linked to the other end (**Figure 8**). The hydroxyl group is able to form hydrogen bonds with nearby carbonyl oxygen of phospholipid and sphingolipid head groups. Cholesterol is known as a "sterol" because it is a steroid alchohol.

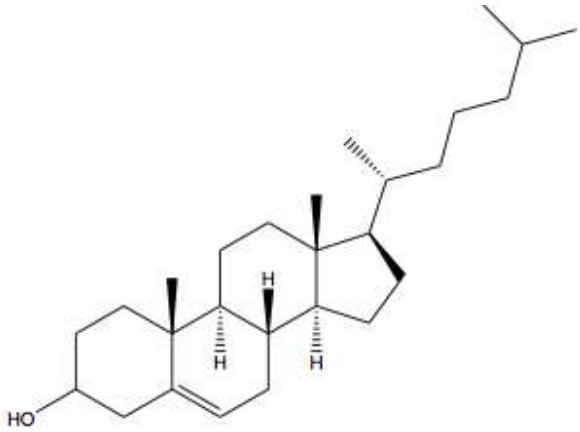


Figure 8: Cholesterol chemical structure

This steroid is the preferential starting material for the steroid biochemistry. Several steroidal hormones, as progesterone, testosterone and estradiol represent a glaring example of how the steroid skeleton is used to form molecules biologically essential.

Cholesterol is required to build and maintain membranes; it modulates membrane fluidity over the range of physiological temperatures. Within the cell membrane, cholesterol also functions in intracellular transport, cell signaling and nerve conduction. [78]

1.13.4 Oxysterols

Oxysterols are usually defined as oxygenated derivatives of cholesterol that are intermediates or even end products in cholesterol excretion pathways. With few exceptions, introduction of an oxygen function in the cholesterol molecule drastically reduces the half-life of the molecule and directs it to excretion or to further oxidation to water-soluble bile acids. [79]

The rapid degradation and excretion of oxysterols are facilitated by their physical properties, allowing them to pass lipophilic membranes and to be redistributed in the cell at a much faster rate than cholesterol itself. [80]

The oxysterols are a controversial group of molecules once they are involved in the pathogeneses of some diseases but recent studies have proved their therapeutic effects in other pathologies.

Oxysterols formed by non-specific oxidative mechanisms have been associated with atherosclerosis and more recently, age related macular degeneration, and cataracts have been suggested to be mediated by this group of sterols. [81,82]

The potential antitumor activity of oxysterols is known from long ago. One of the

cytotoxic mechanisms is the increase of the sensitivity of tumor cells to irradiation and to other antineoplasic agents. [72,75,83] Based on this hypothesis, the use of oxysterols as part of a hybrid drug can present a major advantage.

1.14 Steroids as Antimalarials

The use of steroids for the treatment of malaria is known for a long time ago. Several studies refer the use of testosterone and dexamethasone in the treatment of malaria symptoms, and the association of these compounds for the immune response of the human organism to malaria. [84,85]

The use of oxysterols as antimalarial moiety, from the best of our knowledge, has not been explored.

1.15 Thesis Scope and Goals

The eradication of malaria requires new prophylactic and therapeutic approaches that act simultaneously at different stages in the parasite's life cycle.

The WHO recommendation on the use of artemisinin and its derivatives in combination therapy was the major starting point for the synthesis of the compounds described in this thesis.

The hybrid compounds, as mentioned above, are chemical entities with two or more structural domains, pharmacophores, having different biological functions and dual activity. Some of the hybrid molecules already developed in antimalarial therapy, where two antimalarials were combined via a linker, showed promising results, and effective action against the respective targets in the parasite.

Taking into account the malaria problem described and the recommendation of the WHO, the main purpose of this thesis is a) the optimization of the reaction conditions for the synthesis of steroidal tetraoxanes. b) the synthesis and characterization of hybrid compounds, with antimalarial activity, based on different moieties, a steroidal nucleus, a 1,2,4,5-tetraoxane moiety and the adamantanone group.

Chapter II

SYNTHESIS AND CHARACTERIZATION OF NEW HYBRID ANTIMALARIALS

Synthesis and Characterization of New Hybrid Antimalarials

The endoperoxide linkage in artemisinin and its derivatives has been identified as the key pharmacophore in the antimalarial activities of these molecules, and this has led to subsequent interest in the development of a range of peroxide-containing molecules, particularly 1,2,4,5-tetraoxanes. Based on this knowledge, antimalarial hybrid drugs with a tetraoxane and a steroidal moiety are synthesized in this work.

Our strategy to build an hybrid drug with a 1,2,4,5 tetraoxane moiety involves an oxysterol and a cycloalkanone as chemical entities. The relevance of this synthetic endoperoxide is based on the proved antimalarial activity and chemical stability of tetraoxanes, when compared with artemisin. [86]

Unpublished results from our group have disclosed the antimalarial activity of synthetic oxysterols.

The combination of an oxysterol scaffold and an artemisin-like compound has never been tested and the contribution of each pharmacophore is not completely understood. The endoperoxide moiety is the key pharmacophore in the antimalarial activities and may contribute to balance the physical and chemical properties of a steroid scaffold while the steroid nucleous may improve the transport of the tetraoxane to the cells.

An oxysterol intermediate was synthesized, the *5α-hydroxy-6β-methoxycholestan-3-one* and the synthesis of oxysterol tetraoxanes hybrid compounds has been pursued.

A simpler steroid, the 5α-cholest-3-one without additional functional groups in the steroid backbone was used as starting material to study the reactions of the synthetic pathway.

The hybrid compounds synthesized were characterized by ^{1}H and ^{13}C NMR.

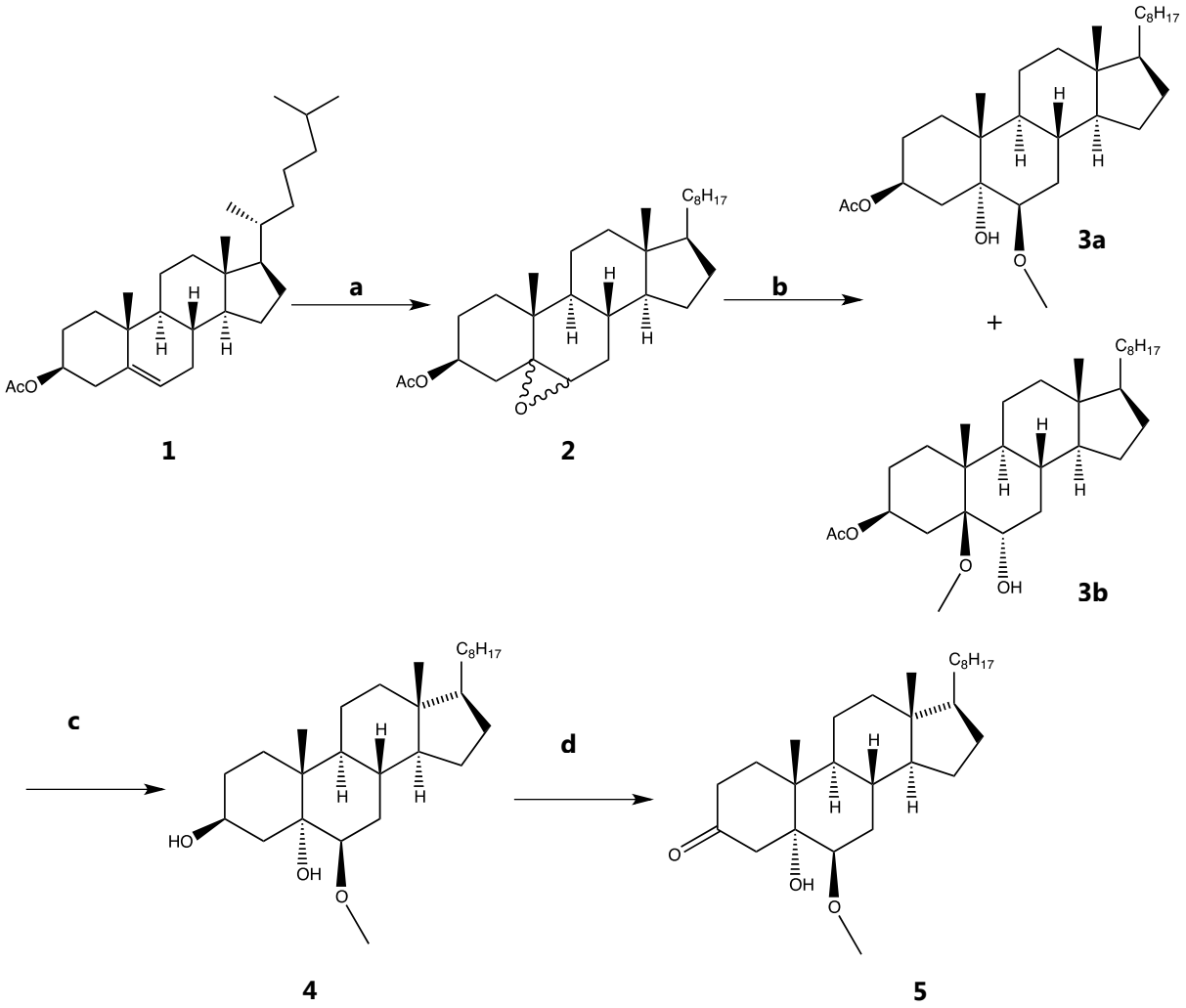
2.1 Synthesis of the 5α-hydroxy-6β-methoxycholestan-3-one (**5**)

The synthesis of 5α-hydroxy-6β-methoxycholestan-3-one (**5**) involves four steps and the starting material is the cholesterol acetate (**1**). [87]

The first step in the synthesis corresponds to the cholesterol acetate epoxidation (**1**), leading to the formation of an epimeric mixture of compounds, the 5α,6α- and 5β,6β-epoxysteroids, in a good yield (80%) with a diastereoselectivity of 76:24 (α / β), which is according to the literature. [88]

Then, the epoxide opening was undertaken in methanol aiming to obtain the 6β-methoxycholestan-3β,5α-diol (**4**). Due to the formation of isomers in the synthesis of the intermediate (**2**), the result of this reaction has been a mixture of two derivative compounds, **3a** and **3b** which have been separated by Flash Column Chromatography (FCC) in a diastereoselectivity of 74:21, which reflect the ratio in the epoxide (**2**). The next step is an alkaline hydrolysis of the 3β-acetate (**3a**) in EtOH/NaOH to afford the intermediate 6β-methoxycholestan-3β,5α-diol (**4**) which was easily isolated in good yield and was easily separated. [89] The aim of this hydrolysis was to remove the acetate group to form a secondary alcohol at C3. To form the tetraoxane derivative it is necessary to have a ketone at C3, which was obtained via Jones oxidation of the 3β-hydroxyl group. This reaction is very fast, is done in ice due to the presence of acid in the Jones reagent and the yield is high, as expected. [90]

After synthesis, the compound was analyzed by NMR. A peak at 4.08 ppm (m, 1H, J=10.5; 5.2 Hz) corresponding to the 3α-H of the compound (**3**) disappear in the compound (**4**) ^{1}H NMR spectrum, proving the oxidation of the 3-OH (see annexes). [91] Observing the ^{13}C NMR spectrum, it is possible to identify the carbonyl C3 at 212,5 ppm (see annexes).



Scheme 2: Synthesis of the 5α-hydroxy-6β-methoxycholestan-3-one (5) (a) MMPP, CH_3CN, under reflux temperature, 20 min; (b) $Bi(OTf)_3$, dry methanol, rt, 2h30min; (c) NaOH 10%, EtOH, CH_2Cl_2, rt, 2h; (d) Jones reagent, dry acetone, ice. η = 92%

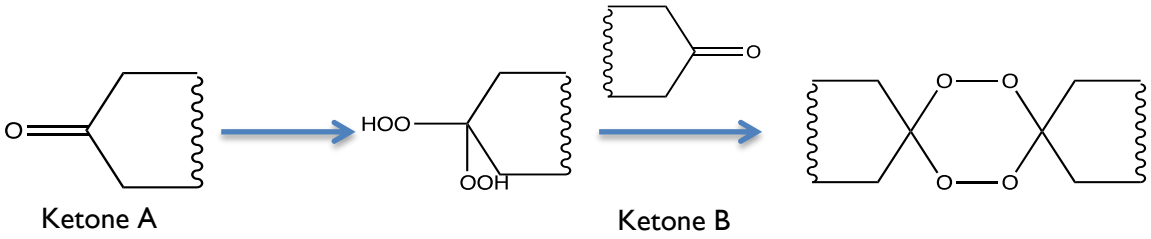
2.2 Synthesis of 1,2,4,5 - tetraoxane hybrid compounds

The endoperoxide linkage in artemisinin and its derivatives has been identified as the key pharmacophore in the antimalarial activities of these molecules, and this has led to subsequent interest in the development of a range of peroxide-containing molecules. These compounds might potentially circumvent the high cost and bioavailability issues associated with artemisinin and its derivatives

Studies of endoperoxide stability have shown that 1,2,4,5-tetraoxanes have significantly higher stability than their 1,2,4-trioxolane or 1,2,4-trioxane counterparts. The

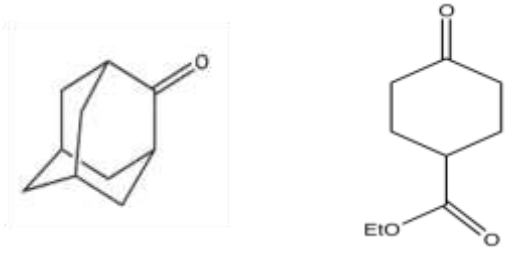
tetraoxanes appear to lack cytotoxic effects *in vitro* and to be safe *in vivo.* [92]

The general scheme of an endeperoxide formation involves two steps: a ketone is converted into the dihydroperoxide, which is then coupled with another ketone.



Scheme 3: General sequence of the formation of the endoperoxide bond.

For the synthesis of the hybrids, adamantanone (**6**) and ethyl 4-oxo cyclohexanecarboxylate (**7**) were chosen to bind to the dihydroperoxide function.



6 **7**

Figure 9: Chemical structure of the adamantanone (**6**) and ethyl 4-oxo cyclohexanecarboxylate (**7**).

The choice of these compounds was motivated by the stabilizing effect of the cycloalkane scaffold on the endoperoxide bridge.

According to the literature, [86,93] for good levels of antimalarial activity in the ozonide series, 1,2,4,5-tretraoxane ring system to an adamantane core provide the best biological activity.

The ethyl 4-oxo cyclohexanecarboxylate is also a cycloalkane structure, although due to the more polar water-solubilizing group, it can be important to counterbalance the very low polarity of the cholestane structure. Moreover, this molecule is more versatile, since it contains a potential reactive group, which allowing further functional group interconvertions, can be used to prepare molecules with improved pharmacokinetic or pharmacodynamic

properties.

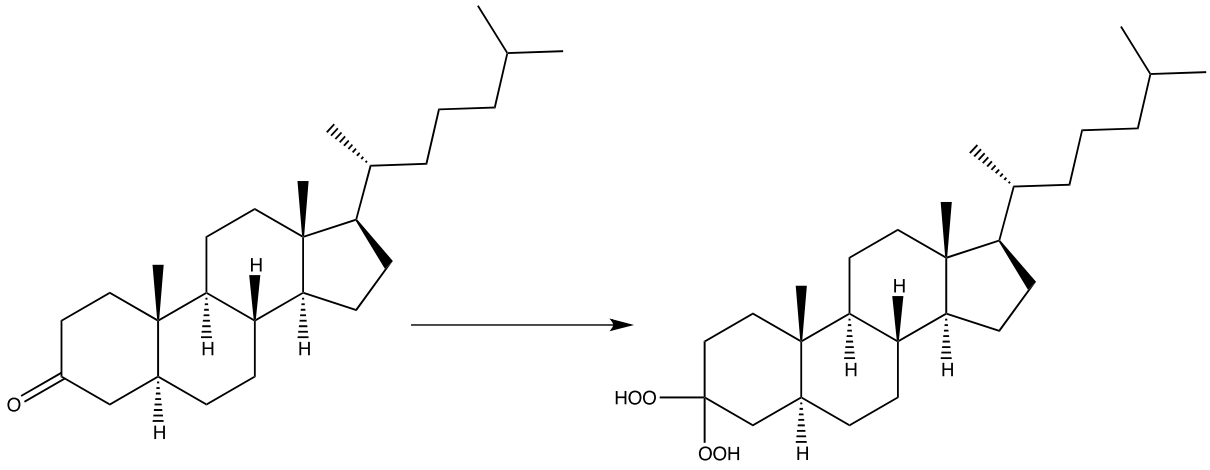
Several procedures were exploited in order to optimize the synthesis of the hybrid compounds planned. The methodologies will be presented and discussed throughout this thesis.

2.2.1 Optimization of dihydroperoxide intermediates

The synthesis of dihydroperoxide was carried out under various reaction conditions and in different ketones.

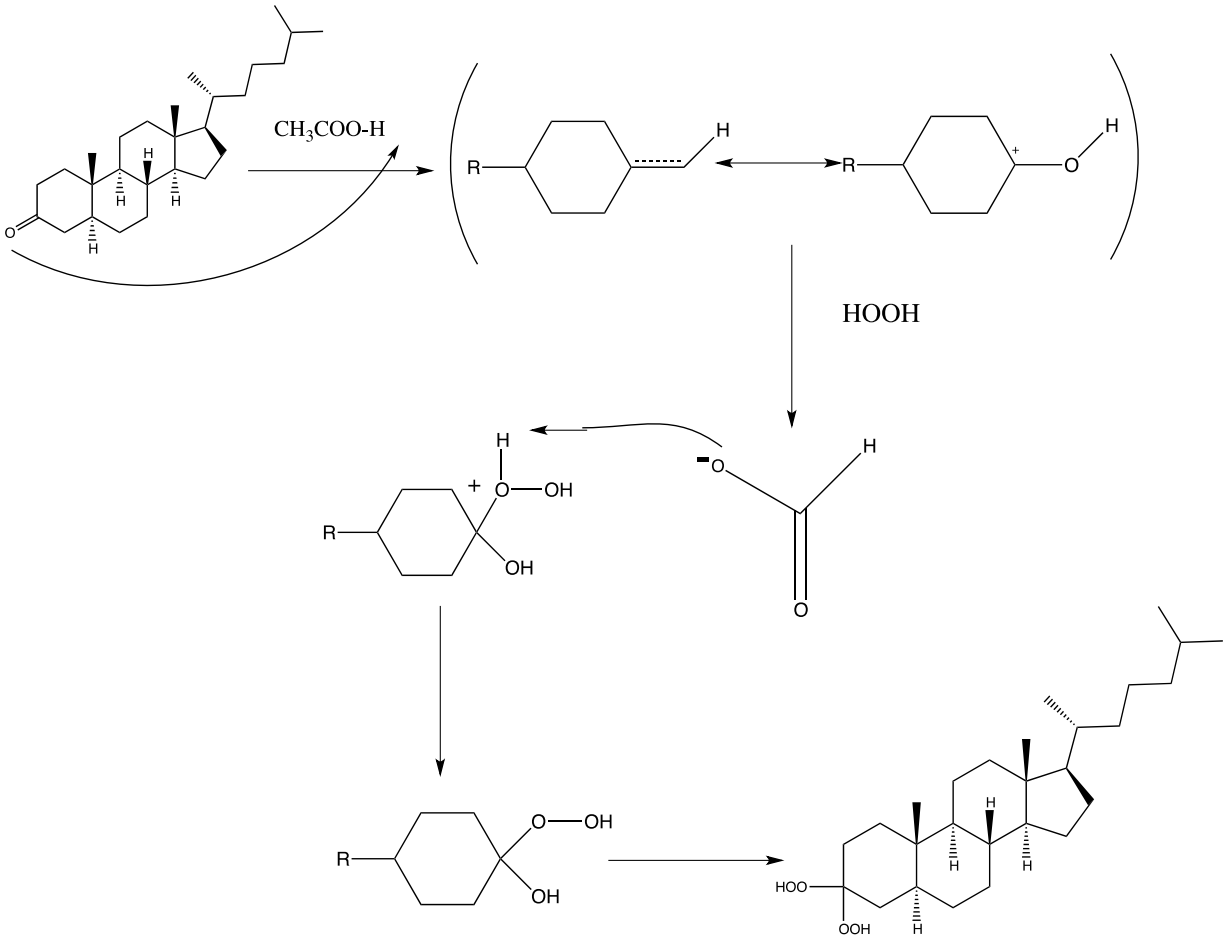
The first reaction was with 5α-cholest-3-one (**8**) as starting material.

The dihydroperoxide, a more polar product, was the only one observed on TLC. (**Scheme 4**), and the reaction sequence proceeded to the tetraoxane synthesis. The desired product was obtained in 11% yield. The molecular mechanism describing the dihydroperoxide synthesis is shown in **Scheme 5**.



8 **9**

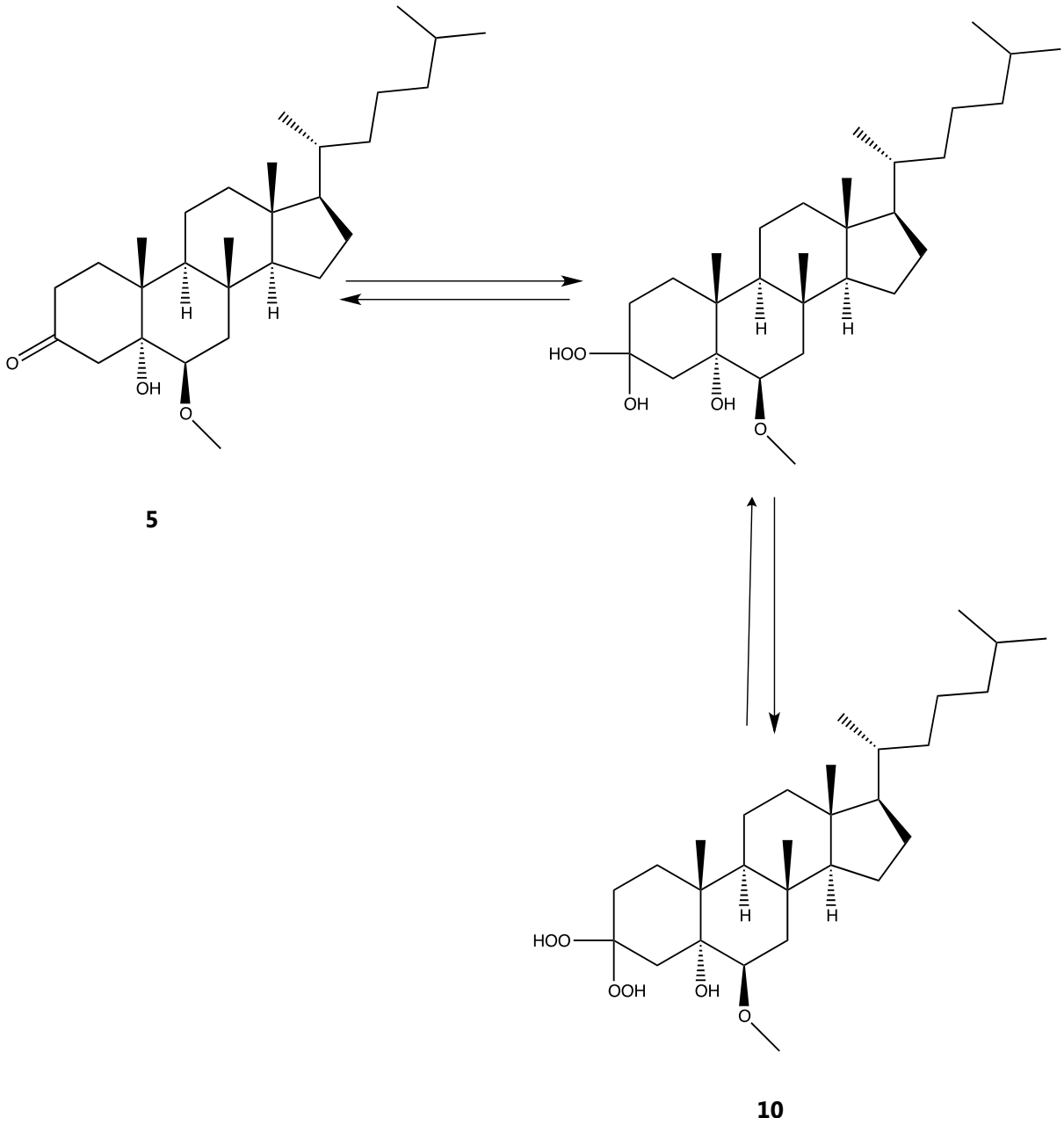
Scheme 4: Synthesis of the dihydroperoxide, using the 5α-cholest-3-one (**8**) as starting material.



Scheme 5: Proposed mechanism for the synthesis of dihydroperoxide **9.**

The dihydroperoxide synthesis from the oxysterol intermediate (**5**) and adamantanone (**6**) was more difficult (**Scheme 6**). According to the procedure described by O'Neill, [87] the oxysterol (**5**) was dissolved in a mixture of CH_2Cl_2/ CH_3CN (1: 2), acetic acid was added at 0°C followed by 0.1 eq of 30% H_2O_2. A TLC was immediately done and a unique more polar product was seen although some starting material still remained.

To allow the full oxysterol conversion, the reaction was maintained stirring, at rt, for at least one week, with additions of H_2O_2 every day. Although the reaction was not complete, the secondary products were vestigious. However, the final product was not the desired one and was not further characterized.

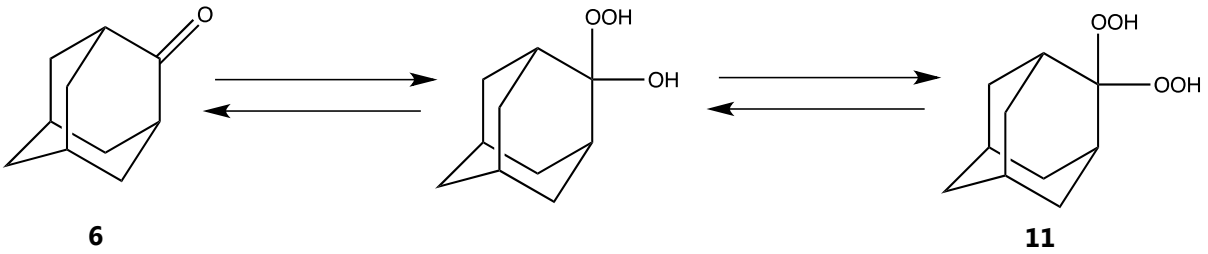


5

10

Scheme 6: Proposed mechanism for the formation of the dihydroperoxide steroidal (**10**).

Another attempt for the optimization of the dihydroperoxide synthesis (**Scheme 7**) was in the use of adamantanone (**6**) as starting material. The adamantanone (**6**) was dissolved in CH_3CN, acetic acid was added at 0°C followed by H_2O_2. After stirring for c.a 3h, the reaction was completed, as seen in TLC. A TLC control revealed extensive reversibility of the reaction, with conversion of the dihydroperoxide back to adamantanone.



6 **11**

Scheme 7: Proposed mechanism for the formation of the steroid in the adamantanone.

Given these problems of reversibility and instability of the dihydroperoxide and after some research on this type of reactions, the one-pot approach was exploited.

It should be noted that several attempts were made to isolate the dihydroperoxide to be characterized, but because of its instability it was not possible to conclude anything.

2.2.2 Optimization of 1,2,4,5-tetraoxanes

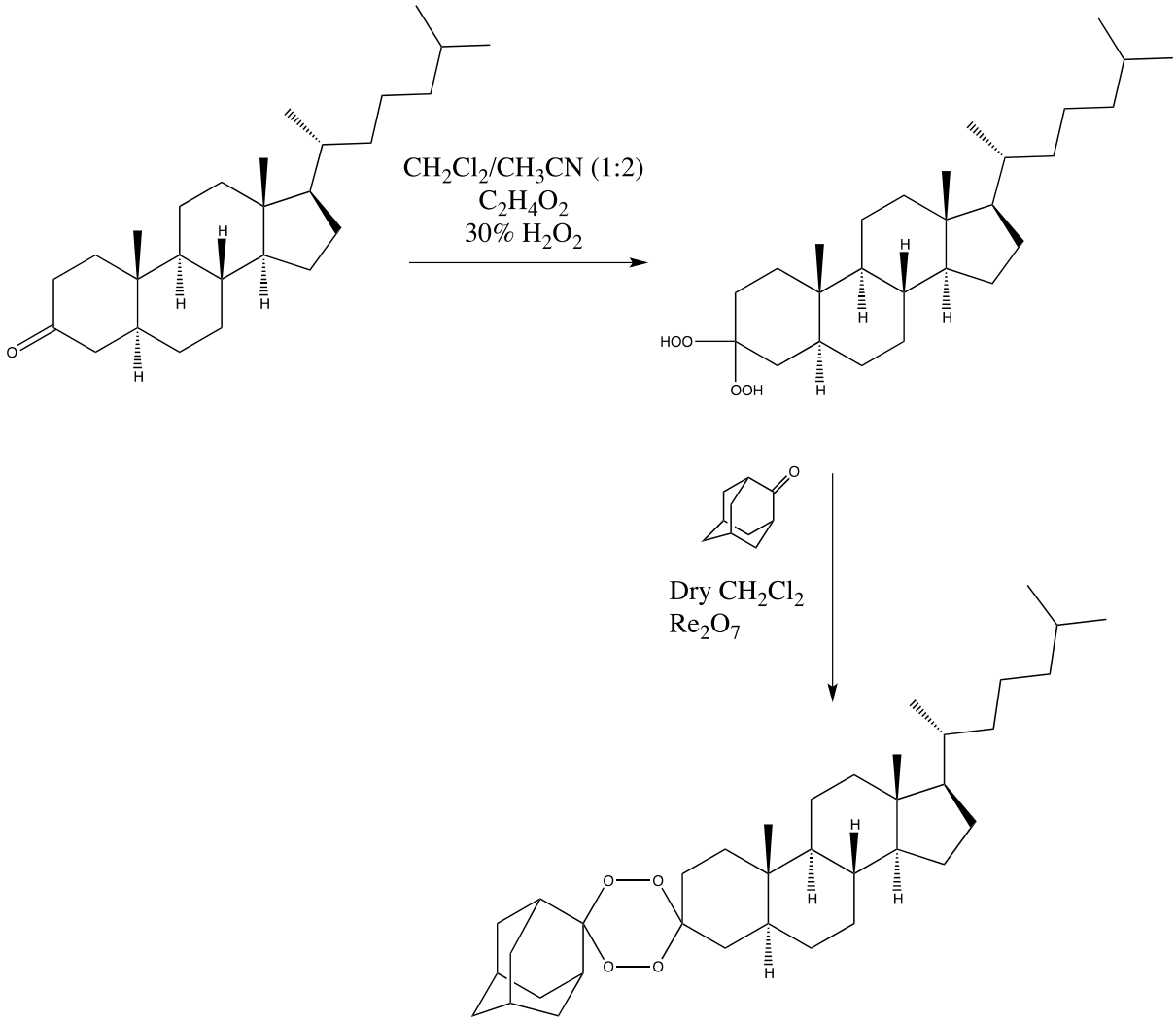
The most widely used approaches to the synthesis of 1,2,4,5-tetraoxanes are based on the reaction of ketones and aldehydes with hydrogen peroxide or gem-bishydroperoxides in the presence of protic or aprotic acids. Moreover, Re_2O_7, I_2, PMA (Phosphomolybdic acid), and CAN (Ceric Ammonium Nitrate) were also used as catalysts for these transformations. [94] However, most of these methods suffer from noteworthy limitations, such as use of high concentration of H_2O_2, the need for excess acid, long reaction times and use of toxic catalysts. So, another methodology using $Bi(OTf)_3$ as catalyst has been tried as an alternative to the method used with Re_2O_7. [95] $Bi(OTf)_3$ is and environmentally friendly catalyst, readily available and easy to handle.

In short, this work exploited three different methodologies.

Process A

The first methodology using rhenium (VII) oxide (Re_2O_7) followed by O'Neill [86] has been tried for our dihydroperoxide (**9**), from the 5α-cholest-3-one (**8**) (**Scheme 8**). To a solution of adamantanone (**6**) dissolved in dry CH_2Cl_2 at 0 °C and at an inert atmosphere, a small amount of Re_2O_7 and the crude product (**9**) were added.

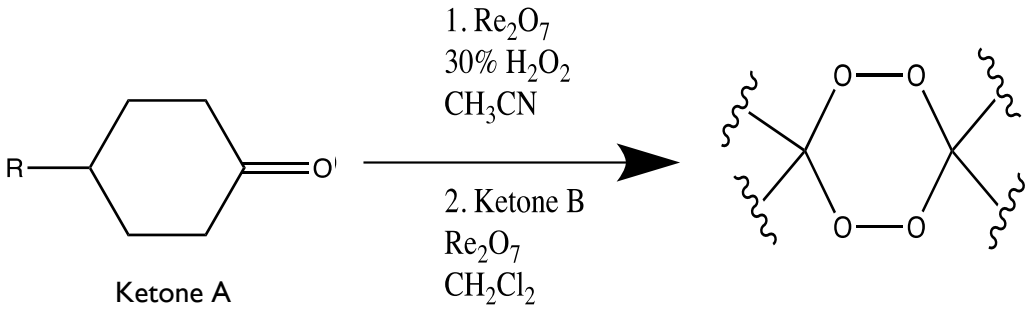
The reaction was left stirring at room temperature for 1h. The consumption of (**9**) and formation of a major product were seen on TLC.



Scheme 8: Proposed mechanism for the **Process A** in 5α-cholest-3-one (**8**).

Process B

The second methodology is the one-pot synthesis, two solvent approach [96] using rhenium (VII) oxide (Re_2O_7) as catalyst (**Scheme 9**). Thus after the addition of 0.05 equiv of Re_2O_7 are added to the solution of the ketone (1.0 equiv) and 30% H_2O_2 (4 equiv) in CH_3CN. Although the total consumption of the substrate was not observed, the reaction was stopped after 1h stiring at r.t., following the procedure described. Then the reaction was partially concentrated at reduced pressure due to the presence to remove CH_3CN, and the second ketone was added as a solution in CH_2Cl_2.



Scheme 9: General scheme of the approach one-pot by synthesis of tetraoxanes from ketones.

Process C

The third methodology is also the one-pot approach but using $Bi(OTf)_3$ as catalyst instead of Re_2O_7 [90].

Therefore, in general, the reaction occurs as follows. Addition of 4 equiv of 30% aq. H_2O_2 to a solution of ketone (1.0 equiv) and $Bi(OTf)_3$ (0.05 equiv) in CH_3CN/CH_2Cl_2 (1:1) led to partial comsuption of the ketone. Then the reaction mixture was partially concentrated under reduced pressure, followed by addition of second ketone to this reaction mixture.

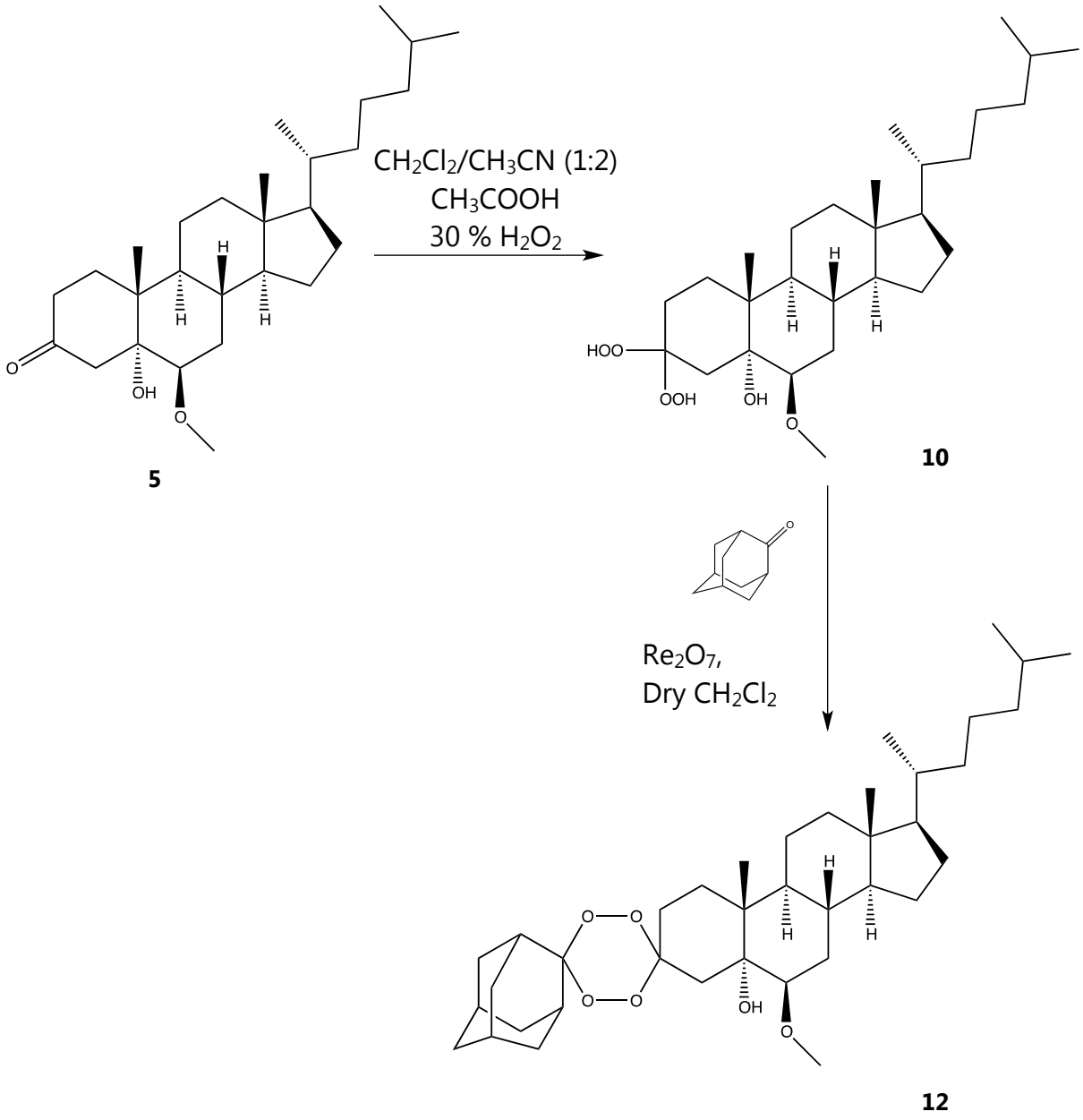
2.3 Synthesis of Hybrid Compounds

2.3.1 Synthesis of a 5,6-oxygenated cholestane-1,2,4,5-tetraoxaadamantane (**12**)

a) Synthesis of a *5,6-oxygenated cholestane-1,2,4,5–tetraoxaadamantane (**12**)* – ***Process A***

The oxysterols intermediate (**5**) was dissolved in CH_2Cl_2 and CH_3CN (1:2) at rt. After complete dissolution, glacial acetic acid was added at 0°C, and thereafter, H_2O_2 . The reaction was maintained under stirring for at least one week, with additions of H_2O_2 every day. After the usual work-up, dihydroperoxide (**10**) was added to a solution of adamantanone (**6**) and Re_2O_7 in dry CH_2Cl_2 under an inert atmosphere. Ten minutes after TLC, four products were detected by TLC, all less polar that the dihydroperoxide. The products formed were purified by FCC with petroleum ether and CH_3CN. After drying in vacuo, the four products were analyzed by NMR and none of them was the desired product.

In view the whole process of synthesis (**Scheme 10**), the most likely to have happened is that at the time of formation of dihydroperoxide there was a reversion of this since this step is sequential and the dihydroperoxide is not formed at once.

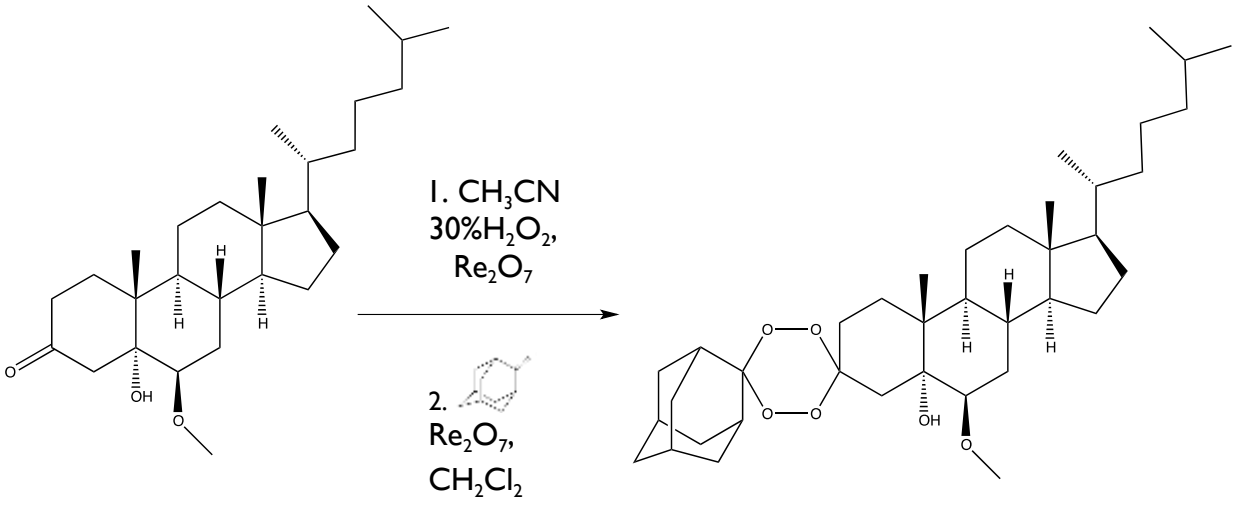


Scheme 10: Synthesis of the hybrid molecule (**12 (Process A)**) using the oxysterol (**5**) as starting material.

b) Synthesis of a *5,6-oxygenated cholestane -1,2,4,5-tetraoxaadamantane (**12**) – **Process B***

The oxysterol (**5**) was dissolved in CH_3CN. The 30% H_2O_2 (4 equiv) and the Re_2O_7 (0.05 equiv) were added and after one hour, the TLC revealed a new more polar product was formed (**10**). Then the reaction was partially concentrated at reduced pressure and the adamantanone (**6**) added as solution in CH_2Cl_2. After 15 minutes, two products were detected by TLC, all more non polar than the dihydroperoxid .The reaction time was about 1 hour and 15 minutes. (**Scheme 11**)

The products formed were purified by FCC with petroleum ether and CH_3CN to afford the hybrid **12**.



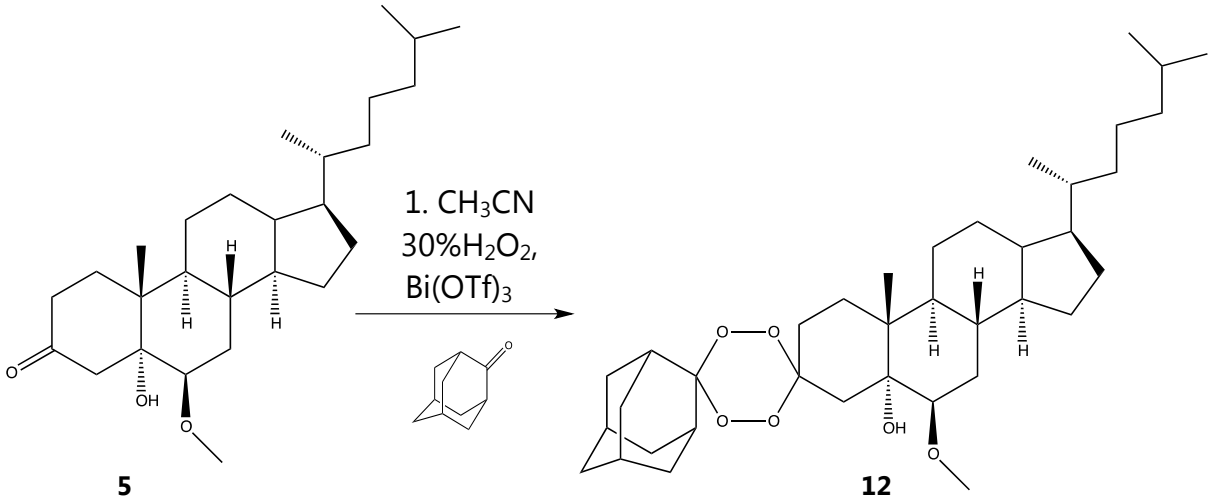
Scheme 11: Synthesis of the hybrid compound (**12** (**Process B**)) using the oxysterol (**5**) as starting material.

c) Synthesis of a *5,6-oxygenated cholestane -1,2,4,5 tetraoxaadamantane (12) – **Process C***

This method is very similar to the one described above and the main difference is the catalyst used. In this case, the $Bi(OTf)_3$ replaced Re_2O_7.

The profile of the secondary products in this process was similar to the one resulted from the Process B and the reaction time was slightly lower, instead of 1 hour and 15 minutes was about 50 minutes.

The (**12 (Process C)**) compound was purified by FCC. After drying in vacuum, this product was characterized by NMR techniques.



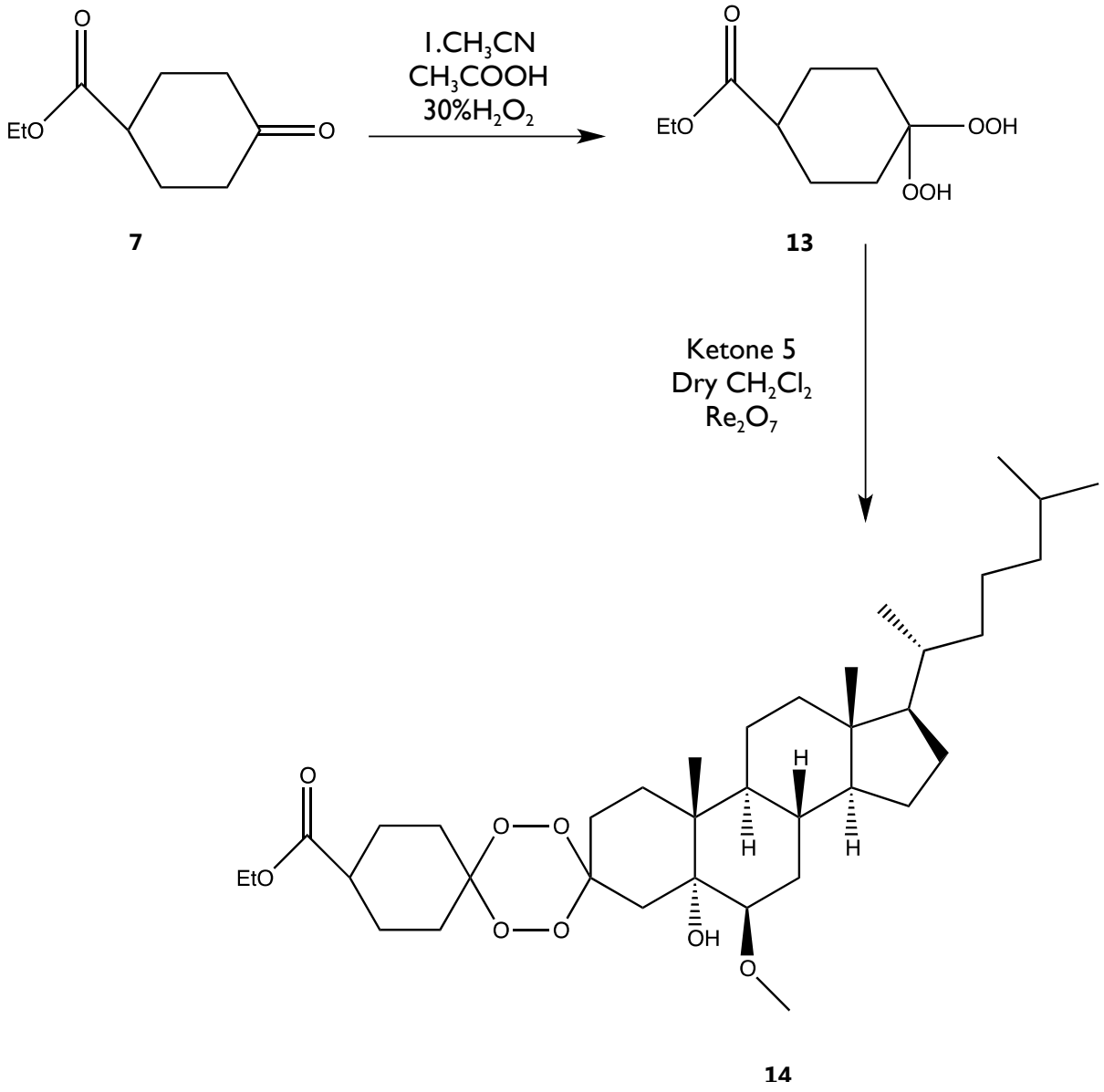
Scheme 12: Synthesis of the hybrid molecule (**12** (**Process C**)) using the oxysterol (**5**) as starting material.

The results of these three methods will be discussed in the chapter conclusions.

2.3.2 Synthesis of a 5,6-oxygenated cholestane 1,2,4,5-tetraoxacyclohexane derivative (**14**)

The sarting material is the ethyl 4-oxocyclohexanecarboxylate (**7**) which is dissolved in CH_3CN in an ice cold bath. After complete dissolution, the acetic acid and H_2O_2 were added. The reaction was maintaining stirring for 6h, with additions of H_2O_2 every hour. Then the reaction was stopped adding H_2O_2, CH_3CN to maintain the reaction conditions longer.

Meanwhile, the oxysterol (**5**) was dissolved in dry CH_2Cl_2, and Re_2O_7 was added in an inert atmosphere. The dihydroperoxide (**13**) was then added to this solution. A prevalent product was formed immediately.



7 **13** **14**

Scheme 13: Synthesis of the hybrid molecule (**14**) using the intermediate (**7**) as starting material.

After drying in vacuum, the hybrid (**14**) couldn't be characterized once the crude yield was very low which hampered its purification.

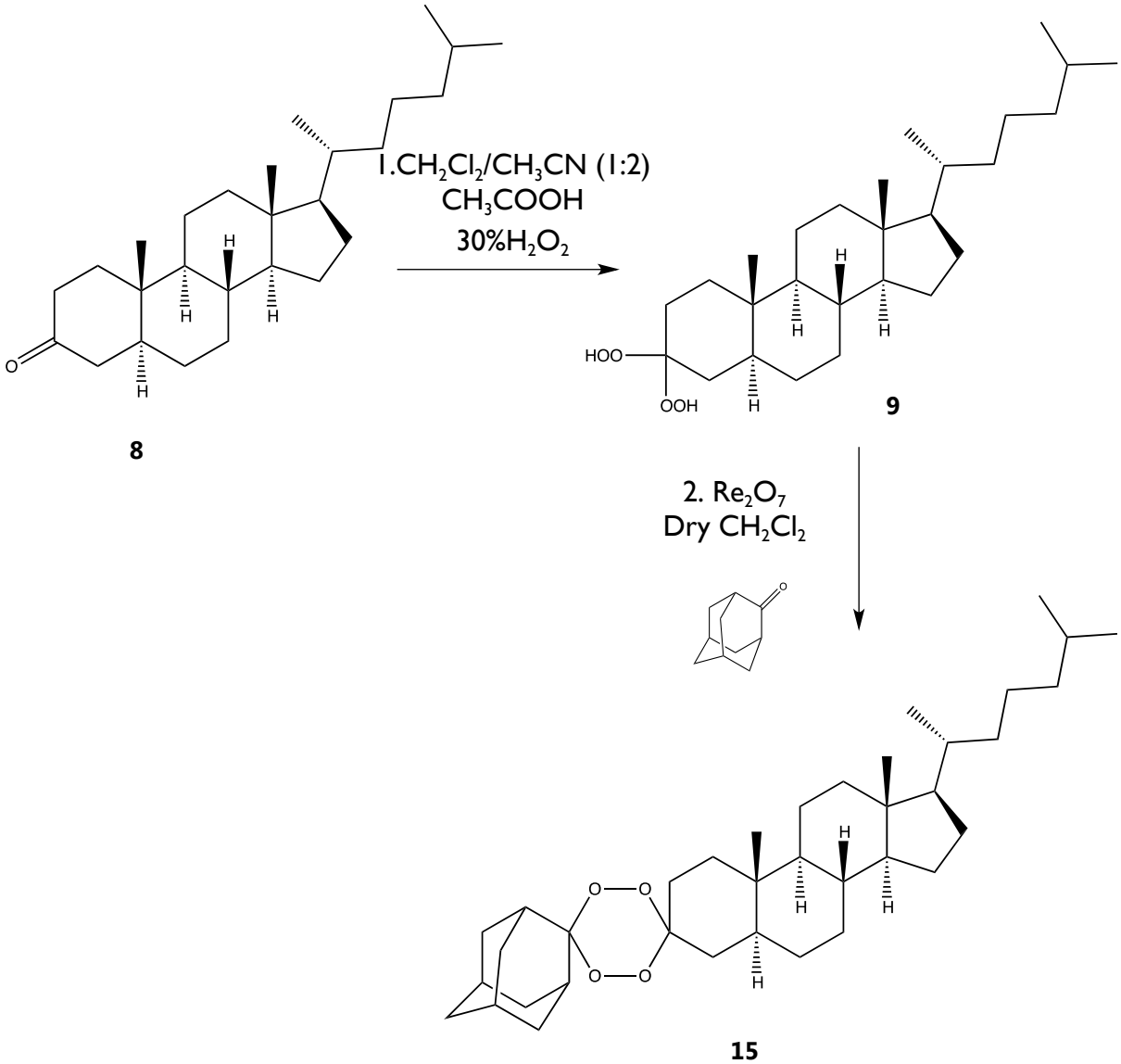
2.3.3 Synthesis of a cholestane-1,2,4,5-tetraoxaadamantane (**15**)

The synthesis of the hybrids (**15**) and (**16**) were performed with 5α-cholestan- 3-one (**8**) as starting material. The choice of this compound (**8**) was based on the absence of other functional groups, apart of a ketone at C3 which reduces the possibility of formation of undesirable products.

a) Synthesis of a *cholestane -1,2,4,5-tetraoxaadamantane* – ***Process A***

The 5α-cholestan-3-one (**8**) was dissolved in CH_2CL_2 and CH_3CN (1:2), following the previously procedure. The reaction was maintained stirring for 48h and the formation of an insoluble white product was noticed. The work-up was performed as usual.

To a solution of adamantanone (**6**) in dry CH_2Cl_2 in ice bath under inert atmosphere, Re_2O_7 was added and then the intermediate (**9**). The reaction was instantaneous, and the presence of cholestanone (**8**) did not interfered with the hybrid formation. The presence of secondary products, more nonpolar than the dihydroperoxide, was noticed on TLC. (**Scheme 14**)



8 **9** **15**

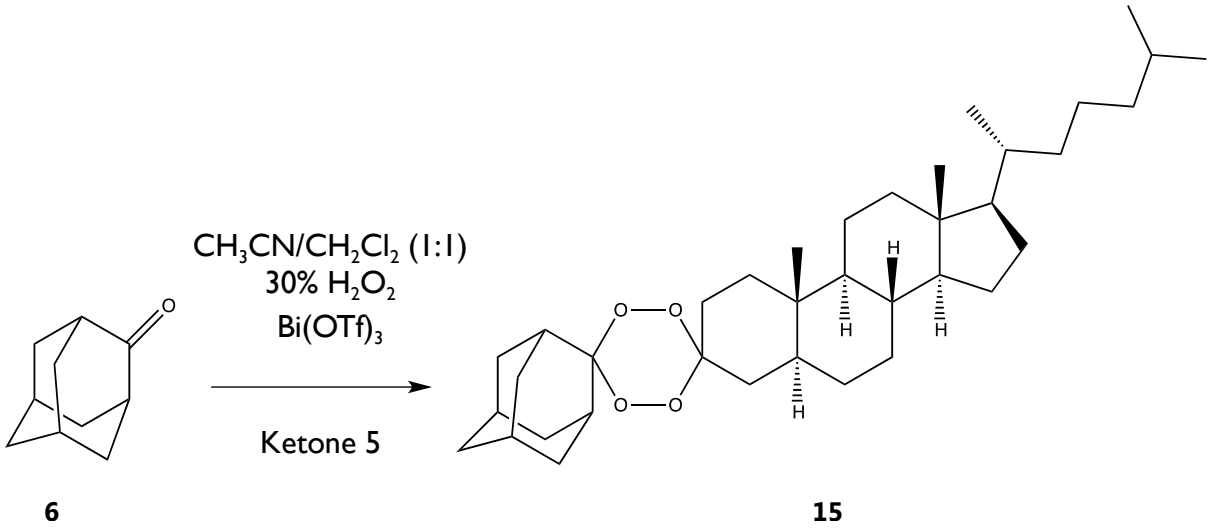
Scheme 14: Synthesis of the hybrid molecule (**15** (**Process A**)) using the intermediate (**8**) as starting material.

The compound was purified by FCC. After drying in vacuum, this product (**15**) was characterized by NMR techniques.

b) Synthesis of a *cholestane -1,2,4,5-tetraoxaadamantane* – ***Process B***

Another methodology starting with adamantanone (**6**) and using $Bi(OTf)_3$ as catalyst has been tried for the synthesis of a cholestane -1,2,4,5-tetraoxaadamantane.

So, adamantanone (**6**) was dissolved in CH_3CN/CH_2Cl_2 (1:1). Bi(OTf)3 and the H_2O_2 were added and after 1 hour the TLC revealed that a new more polar product was formed. Although the reaction was not complete, the intermediate (**5**) was added to this reaction mixture and the reaction took about 50 minutes. The presence of secondary products was noticed on TLC and the adamantone did not interfere with hybrid formation. The product formed was purified by FCC with petroleum ether and CH_3CN to afford the hybrid **15**. (**Scheme 15**)



6 **15**

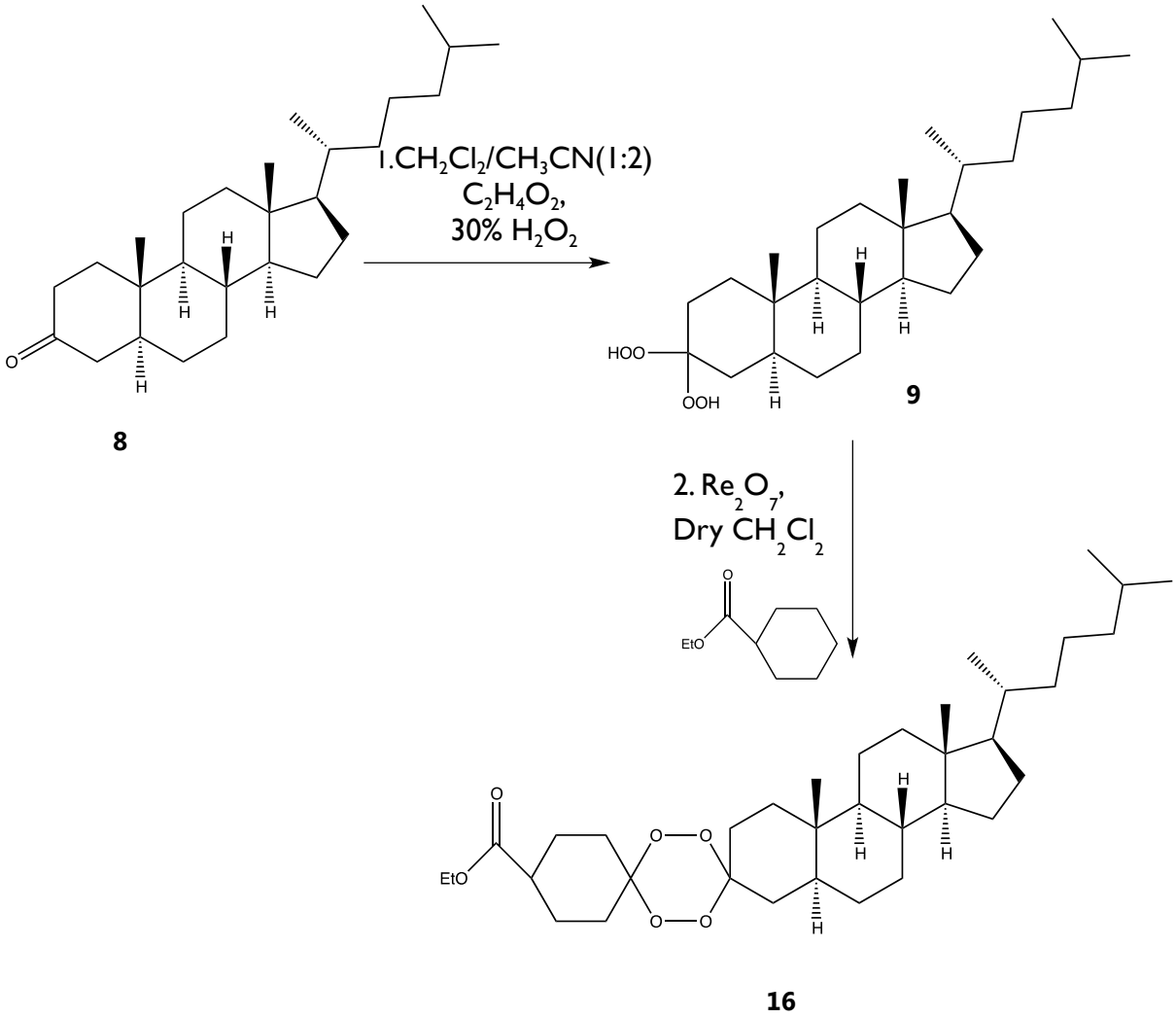
Scheme 15: Synthesis of the hybrid molecule (**15** (**Process B**)) using the adamantanone (**6**) as starting material.

2.3.4 Synthesis of a cholestane-1,2,4,5-tetraoxacyclohexane derivative (**16**)

The first part of the reaction occurs as described above for the synthesis of (**9 (Process A)**). The second part of the synthesis was also performed in the same way as the process A, but instead of adding adamantanone (**6**), the ethyl 4-oxocyclohexanecarboxylate (**7**) was added to form tetraoxane. Again, the reaction was instantaneous although the

presence of secondary products was also observed. (**Scheme 16**)

The crude was purified by FCC and analyzed by NMR techniques.

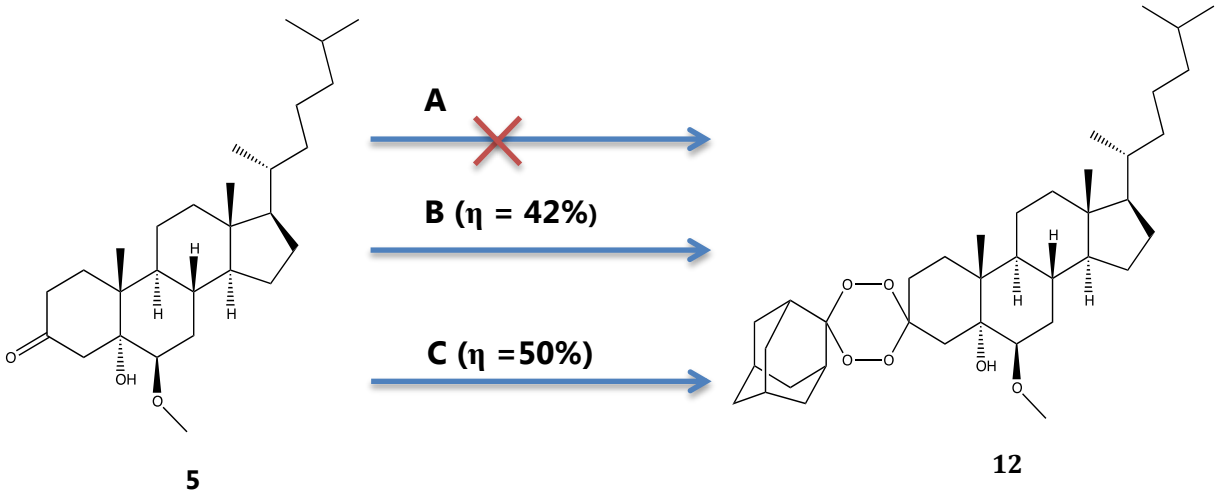


8 **9** **16**

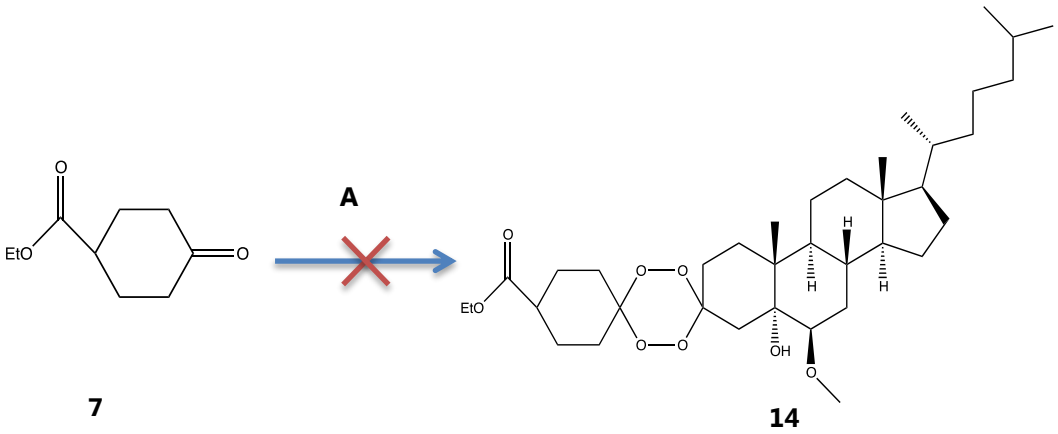
Scheme 16: Synthesis of the hybrid molecule (**16**) using the intermediate (**8**) as starting material.

In **Scheme 17** features all hybrid synthesis reactions with their respective processes and yields.

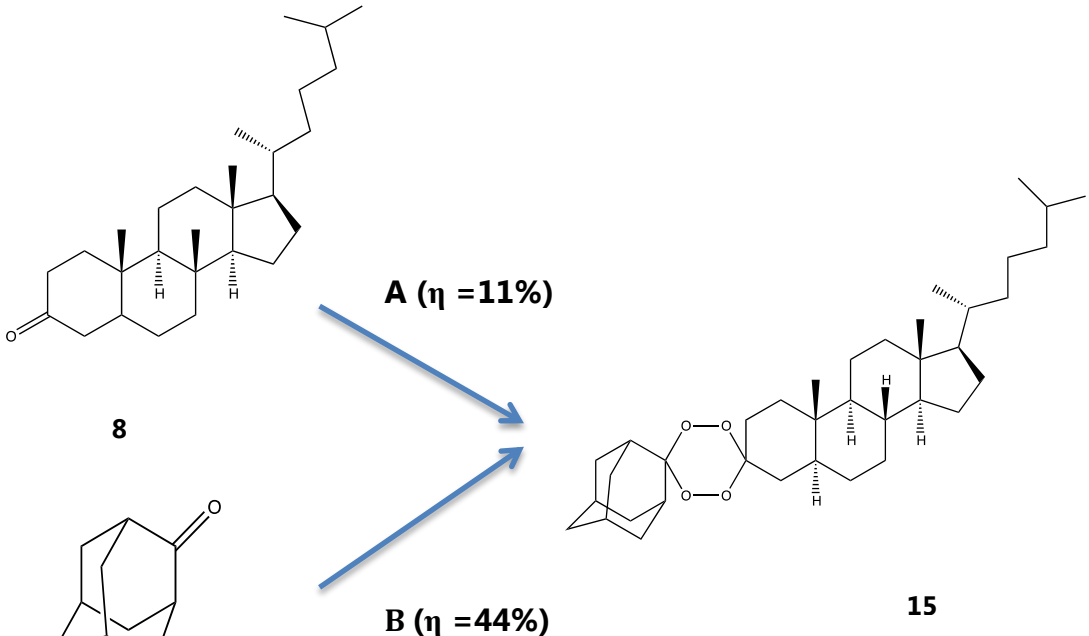
Starting Process Hybrid



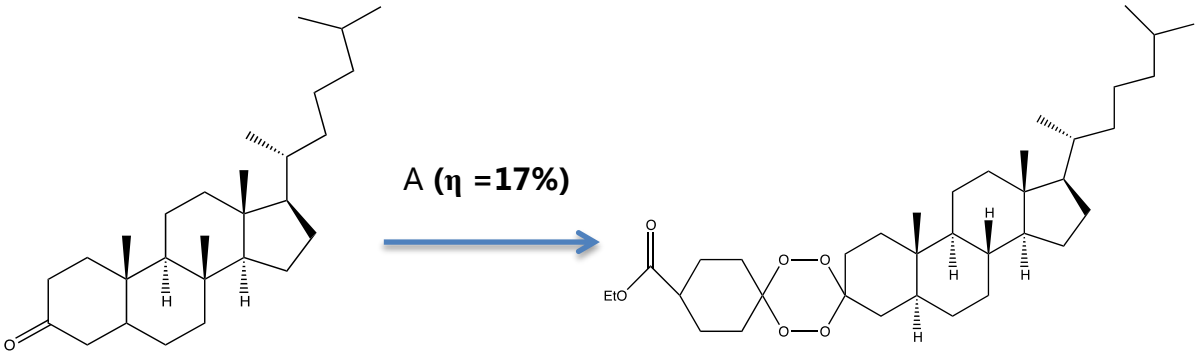
5 **12**



7 **14**



8 **6** **15**



8 **16**

Scheme 17: Hybrid synthesis reactions with their respective processes and yields.

2.4 Spectral characterization of the hybrid compounds

The structural elucidation of the synthesized hybrids was performed by ^{1}H and ^{13}C NMR.

2.4.1 5,6-oxygenated cholestane-1,2,4,5-tetraoxaadamantane (**12**)

The hybrid (**12**) characterized was prepared by process C.

There was a pattern in the ^{1}H in all hybrids. The methyl groups of the steroid moiety are easily identified by the signals 0.66 ppm (3H, s, 18-CH_3), 0.85ppm (3H, 2d, 26-CH_3), 0.87ppm (3H, 2d, 27-CH3), 0.89ppm (d, 21-CH_3) and 1.05ppm(3H, s, 19-CH3), all these signs are characteristic of cholestan.

The signal at 3.28 ppm can be assigned for the 6β-OCH_3, finding himself more deshielding.

The signals from 1.25 to 1.33 prove the presence of adamantanone.

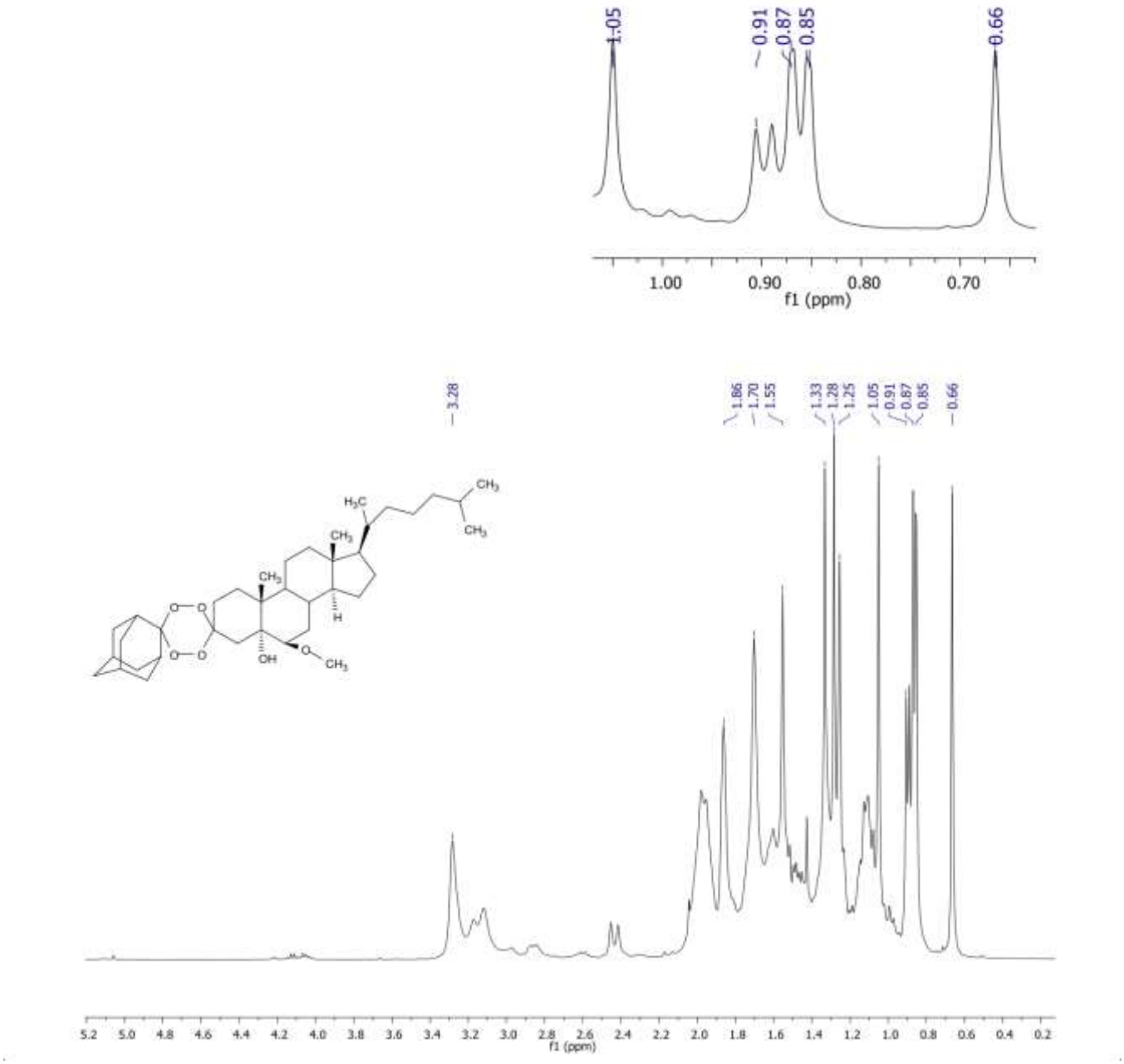


Figure 10: ^{1}H NMR spectrum of compound **12**.

The ^{13}C was very important to check the total number of carbon.

The signal at 110.13 ppm corresponds to a carbon in the OO-C-OO moiety.

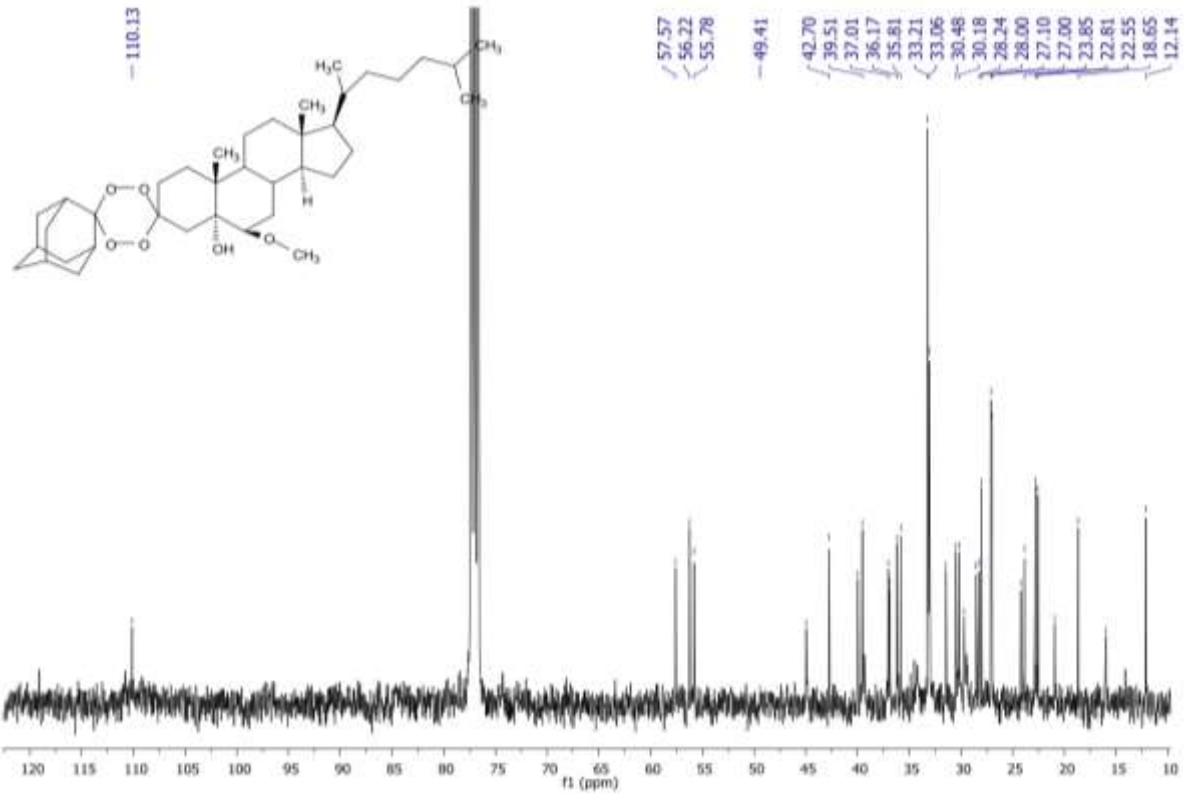


Figure 11: ^{13}C NMR spectrum of compound **12**.

2.4.2 Cholestane-1,2,4,5-tetraoxaadamantane (**15**)

The structural elucidation was performed in compound (**15**) prepared by process A. The hybrid compound is similar to the compound (**12**) in respect to the steroidal moiety, but without functional groups. The steroid moiety showed the same pattern as observed for the previous hybrid compound, apart the position of the singlet 19-CH_3 which moved from 1.00ppm in the 5α-cholestan-3- one (**8**) to 0.81 ppm in the hybrid (**15**).

The presence of adamantanone is proved mainly by the signals from 1.25 to 1.33.

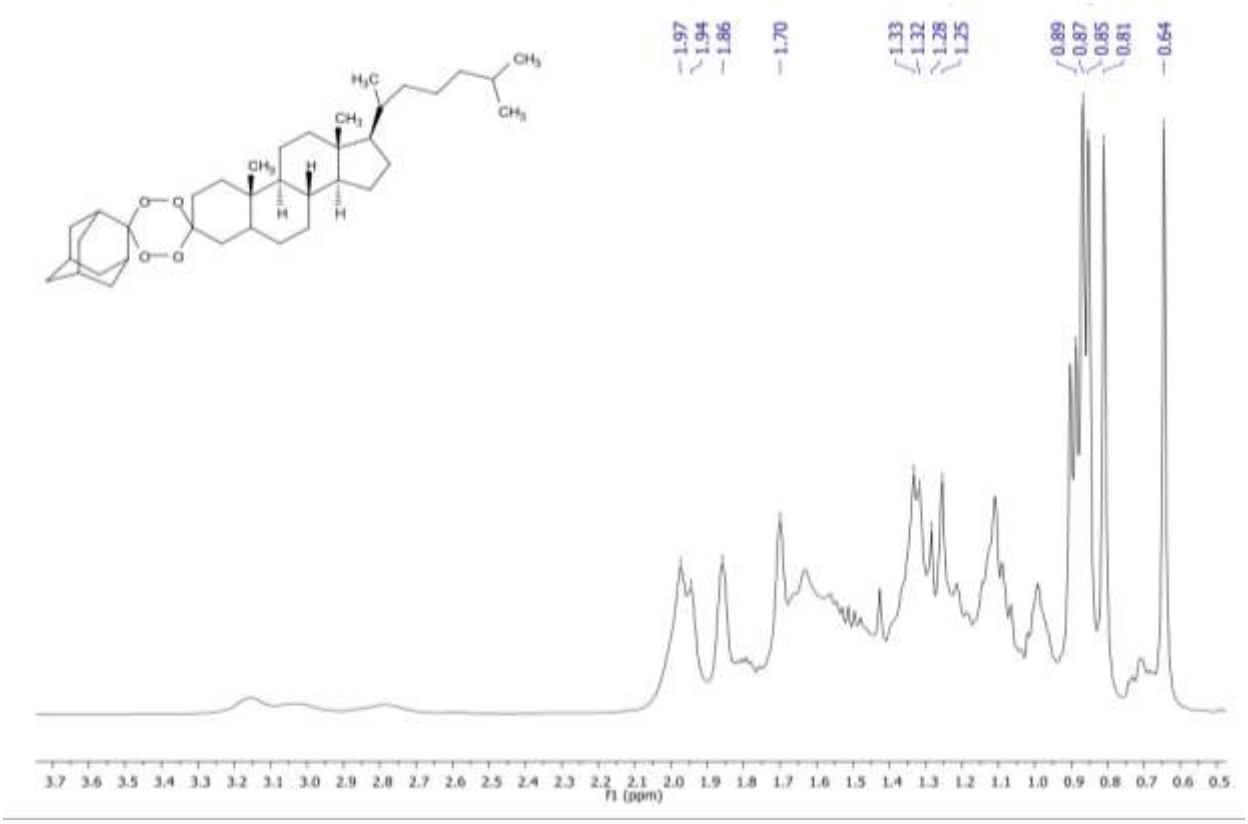


Figure 12: ^{1}H NMR spectrum of compound **15**.

The ^{13}C NMR was important to clarify the synthesized molecule.

The C19 is slightly shifted compared to the same carbon in hybrid **12**, in this case corresponds to the signal 11.4 instead of 12.14 (**12**).

It was possible to identify the quaternary carbons corresponding to the OO-C3-OO moiety at 1088.4 ppm and OO-C1'-OO at 110.3 ppm which confirms the tetraoxane bridge.

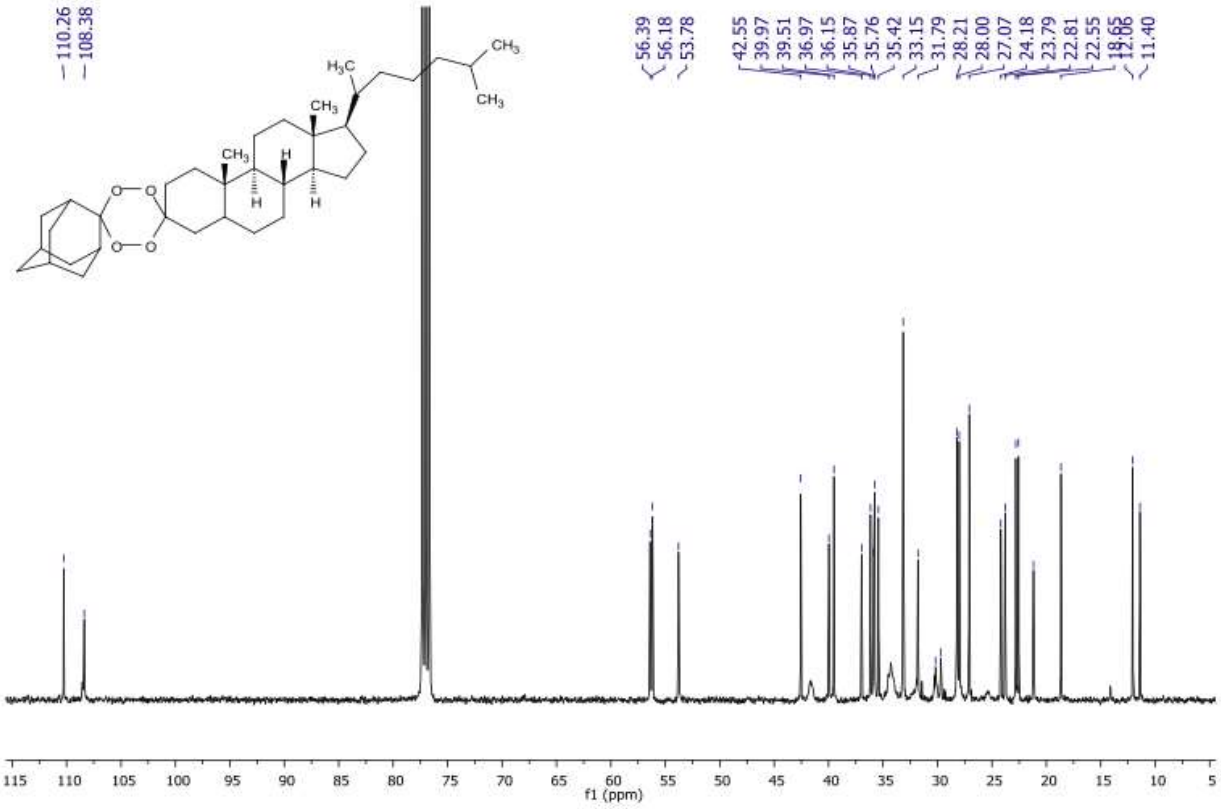


Figure 13: ^{13}C NMR spectrum of compound **15**.

2.4.3 Cholestane-1,2,4,5–tetraoxacyclohexane derivative (**16**)

The ^{1}H spectrum of the hybrid compound reveals a steroid scaffold like the *cholestane -1,2,4,5 - tetraoxaadamantane (**15**)*. The same typical signals already observed for compound (**15**) such as 19-CH_3 from 1.00 ppm to 0.81 ppm due to the attachment of the tetraoxane are noted too. The triplet at 1.24 ppm correspond to the CH_3CH_2 group and the quartet at 4.12 ppm correspond to the OCO - group.

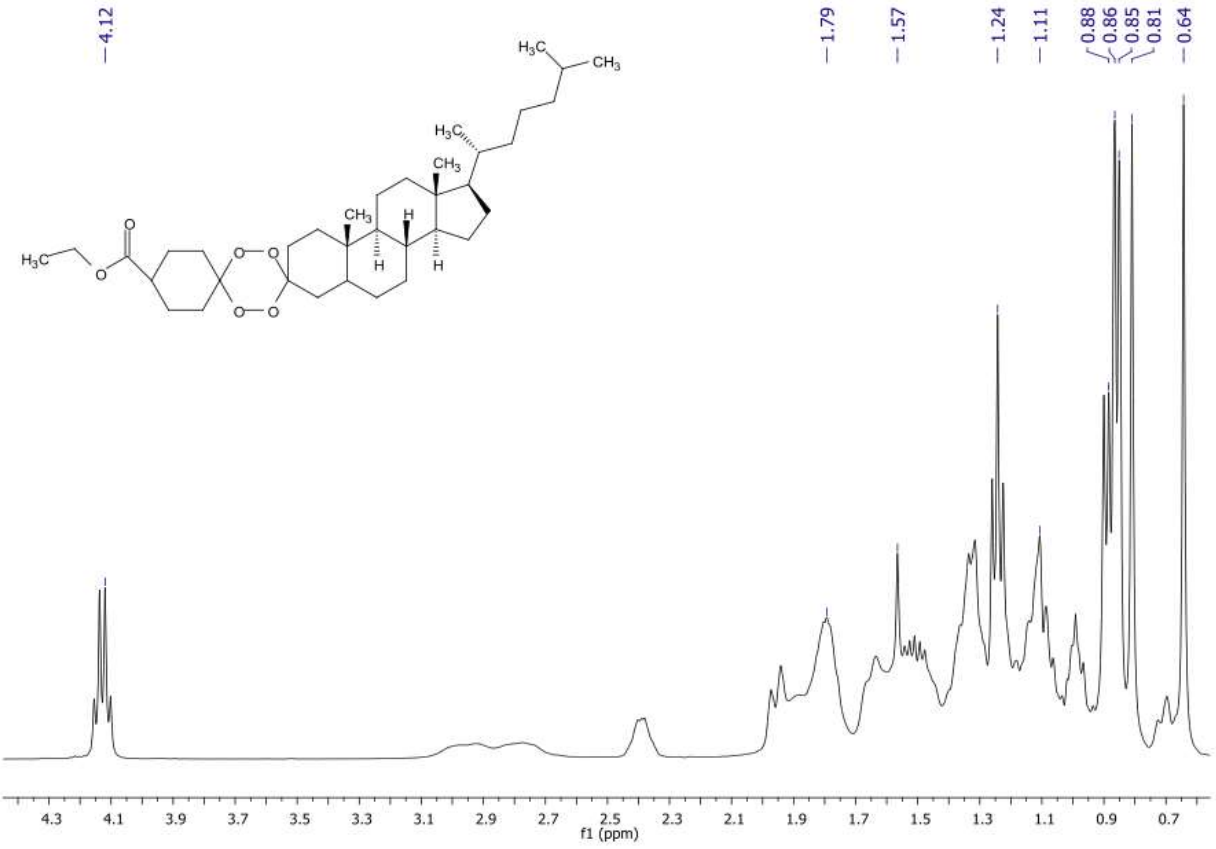


Figure 14: ^{1}H NMR spectrum of compound **16**.

The ^{13}C spectrum show the carbons in the tetraoxane bridge and the one from OCO-group The signal for the C19 was again deviated to upfield like in compound **15**. The signal at 174.6 ppm corresponds to the C=O of the hybrid.

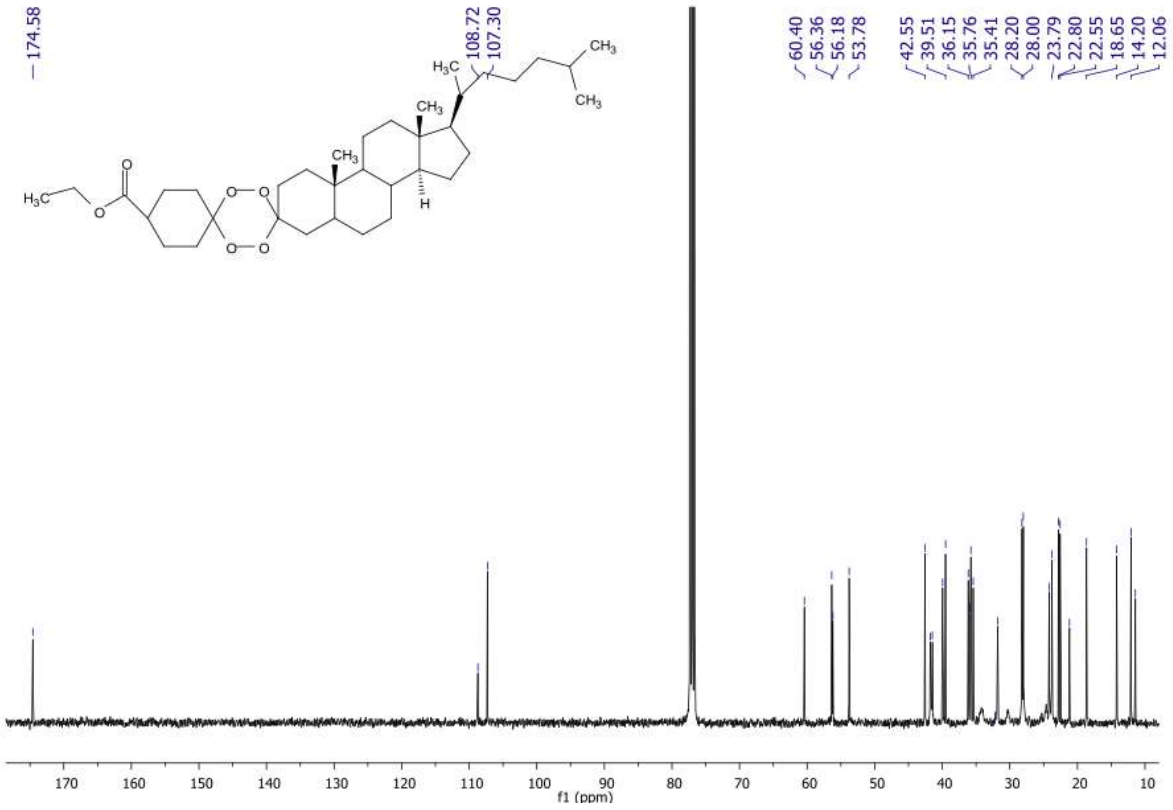


Figure 15: ^{13}C NMR spectrum of compound **16**.

Chapter III

CONCLUSIONS

Conclusions

Efforts to discover and to develop new medicines for malaria disease have increased dramatically in recent years, both as a result of the recognition of the global importance of the fight against malaria and as a result of the public-private partnership strategy dedicated to discovering, developing and provide new drugs.

The antimalarial therapy involves the use of combinations of drugs as a way to improve the efficiency of treatment and reduce the development of resistance. The emphasis in therapeutic combination of antimalarial drugs logically led to the development of hybrid molecules.

This work aims to exploit simple and profitable reactions for the synthesis of hybrid antimalarials based on a steroid scaffold and a tetraoxane bond.

The steroid part was tested with the compound 5α-hydroxy-6β-methoxycholestan-3-one (**5**), which was synthesized in the laboratory, and with the compound cholestan- 5α-3-one (**8**), commercially obtained.

Three different methodologies for the 1,2,4,5-tetraoxane synthesis, based on the literature, were tested. The first methodology, was followed by O'Neill, [87] using rhenium (VII) oxide Re_2O_7 as catalyst. The second one is the one-pot synthesis, two solvent approach [91] using Re_2O_7 as catalyst too, and the third methodology is also the one-pot approach but using bismuth (III) trifluoromethanesulfonate $Bi(OTf)_3$ as catalyst instead of Re_2O_7.[90] Comparing the two catalysts used, $Bi(OTf)_3$ is less toxic than Re_2O_7, which makes it an ecofriendly catalyst, fulfilling one of the principles of Green Chemistry.

In the synthesis of 5,6-cholestane oxygenated-1,2,4,5-tetraoxaadamantane (**12**), the three methodologies were exploited. In spite of several attempts, the desired product was not obtained using the first method, while using one pot approach, it was possible to synthesize the desired hybrid. However, there are differences in yields and reaction times between the second and third methodology. It was found that by using $Bi(OTf)_3$ as catalyst, the yield was higher (50% vs. 42%), and the reaction time is shorter when using the same catalyst (50 min vs 1h15min).

For the synthesis of cholestane-1,2,4,5-tetraoxaadamantane (**16**), the first and the third methodologies were compared. We could observe that that when using one pot approach the yield is higher (44% vs. 11%) and the reaction time is shorter when using the same approach.

In the case of synthesis of cholestane-1,2,4,5-tetraoxacyclohexane derivative (**17**), the first methodology was the only one used and low yield has been verified (16%).

The structure of the compounds developed was confirmed by ^{1}HNMR, ^{13}CNMR, which allowed us to identify the characteristic signals of the steroidal moiety and the signals of OO-C3-OO and OO-C1'-OO in the ^{13}CNMR which confirms the tetraoxane bridge.

Finally, the use of one pot approach is beneficial since it affords higher yields, shorter reaction times and reduced use of solvents.

In the future, oxysterols with different structures should be synthesized in order to study influence of ring A, B and C substituents. Furthermore, the reaction conditions for the synthesis of steroid tetraoxanes should be optimized and a high yielded method is something that needs to be improved.

Chapter IV

EXPERIMENTAL SECTION

4.1 Equipments

Nuclear Resonance Spectroscopy

Nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR) were performed in a 400 MHz Bruker Avance III spectrometer. The solvent used was deuterochloroform ($CDCl_3$). The values of chemical shifts are reported in ppm relative to internal standard tetramethylsilane (TMS) and values of coupling constants (J) are expressed in Hz.

Melting Point

The melting points were determined in a Büchi-540.

Thin Layer Chromatography (TLC)

In the reactions whose evolution was monitored by thin layer chromatography, silica 60 F_{254} plates in aluminum support were used and they were provided by Merck.

The samples were concentrated, evaporated and dried in R-215 Rotavapor® from Buchi.

4.2 Reagents and Solvents

Cholesterol acetate, 5α-cholestan-3one, ethyl 4-oxo cyclohexanecarboxylate, adamantanone, rhenium (VII) oxide (Re_2O_7) bismuth triflate ($Bi(OTf_3)$), magnesium monoperoxyphthalate hexahydrate (MMPP), hydrogen peroxide solution 30% , deuterated chloroform ($CDCL_3$) and triethylamine (TEA) were purchased to Sigma-Aldrich.

Methanol (MeOH), acetone ($(CH_3)_2CO$)), ethanol (EtOH) and diethyl ether were obtained from VWR Co.

The acetonitrile, CH_3CN, dichloromethane CH_2Cl_2, sodium hydroxide (NaOH), celite® 545 were obtained from MERK Co.

Glacial acetic acid (CH_3COOH) and ethyl acetate were purchased from Fisher Chemical.

Aqueous solutions of Na_2SO_3 (10%), $NaHCO_3$ (sat), HCl (5%), NaOH (10%), NaCl (10%) were prepared in the laboratory in accordance with certificate protocols.

Dry dichloromethane was prepared in the laboratory. The CH_2Cl_2 was refluxed in the presence of calcium chloride for 3 hours and then distilled .

The Jones reagent (25g of sodium dichromate $Cr(VI)O_3$ diluted in 25 mL/75 mL of sulphuric acid/H_2O) was prepared in the laboratory according to certificate protocols. The solvents used in the work up and chromatography columns were from analytical grade suitable for chemistry research according to the specifications of the suppliers and were obtained from Fisher Scientific.

The revelation solutions used in thin layer chromatography were also prepared in the laboratory. The p-anisaldehyde solution (2.5 mL p-anisaldehyde, 93 ml of ethanol, 3.5 ml of sulfuric acid H_2SO_4 and 1 ml of acetic acid (CH_3COOH) held on ice with stirring) and steroid revelation solution (95 ml ethanol and 5 ml of sulfuric acid) were prepared according certified protocols.

4.3 Chemistry

4.3.1 Synthesis of the intermediate 5α-hydroxy-6β-methoxycholestan-3-one

*Synthesis of the 5,6-epoxycholestan-3-yl acetate (**2**)*

A solution of cholesterol acetate (**1**) (1000mg, 2.33 mmol) was dissolved in acetonitrile (34.95mL) and then the MMPP (1.1 eq) was added as a single portion. The suspension was stirred for 20 min at reflux temperature (83^{0}C). After the substrate consumption (monitored by TLC), the reaction mixture was cooled, filtered and concentrated under vacuum. The white solid residue was dissolved in diethyl ether and washed with Na_2SO_3 (10% aq.sol.), $NaHCO_3$ (sat. aq. sol.) and water. Then was dried with anhydrous Na_2SO_4, filtered and evaporated to yield a white foam crude product (839mg).

$\underline{\eta}$ =80%

*Synthesis of the 5α-hydroxy-6β-methoxycholestan-3β-yl acetate (**3a**)*

To a solution of **2**, (839mg, 1.886mmol) in dry methanol (42.75mL), 0,05 eq of $Bi(OTf)_3$ were added. The reaction mixture was stirred at room temperature. After completion of reaction mixture (ca. 2h30), the reaction was stopped with silica and few drops of triethylamine.

After evaporation under vacuum, the resulting white product was purified with FCC (petroleum ether/ ethyl acetate 1:15), afforded the pure 5α-hydroxy-6β-methoxycholestan-3β-yl acetate **3a** (739mg).

η =82%

*Synthesis of the 6β-methoxycholestan-3β,5α-diol (**4**)*

The resulting **3a** (739mg, 1.55 mmol) was divided in two equal portions and dissolved in EtOH and CH_2Cl_2. 4 eq of NaOH (10%) were added to each solution. The reactions were stirred during 2h, at room temperature. After completion of reaction, the reactions were stopped under evaporation. The residue was dissolved in CH_3CN and washed with HCl (5% aq.sol.), $NaHCO_3$ (sat. aq. sol.) and water, dried with anhydrous Na_2SO_4 filtered and evaporated to yield a white crude product (568mg). The spectral analysis was accordingly to the literature. [87]

η =84%

*Synthesis of the 5α-hydroxy-6β-methoxycholestan-3-one (**5**)*

To a solution of **4** (568mg, 1.31mmol) in 23.89mL of dry acetone, at 0°C, the Jones reagent was added dropwise. In the beginning, the solution turns green which means that all chromium was reduced. To ensure the oxidation of the entire substrate, a few more drops were added until the solution turned yellow-brown again. To reduce again the excess of the chromium, methanol was added until the solution turns dark green. The reaction mixture was concentrated under reduced pressure the crude material was dissolved in saturated diethyl ether, washed with water and NaCl 10% and dried with anhydrous Na_2SO_4. After filtration and evaporation, a white product was achieved (523mg).

Formula: $C_{28}H_{48}O_3$

White solid

η= 92%

mp: (MeOH) = 174.7°C -176.4°C.

RMN ^1H (400 MHz, *CDCL3*) δ ppm: 0.69 (3H, s, 18-CH3), 0.86 e 0.87 (each 3H, 2d, *J*= 6.7 Hz, 26- CH3 e 27-CH3), 0.89 (3H, t, J=7.5 Hz, 21-CH3), 1.25 (3H, s, 19-CH3), 2.95 (1H, s, 6α-H), 3.27 (3H, s, 6β-OCH3).

RMN ^{13}C (100 MHz, *CDCL3*) δ ppm: 12.1 (CH_3), 15.9 (CH_3), 18.6 (CH_3), 21.2, 22.5 (CH_3), 22.8 (CH_3), 22.8, 24.2, 28.0, 28.9, 30.3, 33.8, 35.8, 36.1, 37.8, 38.9 (C), 39.5, 39.8, 42.7 (C), 45.9, 49.6, 55.7, 56.2, 57.6, 78.3 (C), 85.1, 212.5 (C).

4.3.2 Synthesis of the hybrid compounds

*Synthesis of the final product 5,6-oxygenated cholestane-1,2,4,5 tetraoxaadamantane (**12- Process B)***

The 5α-hydroxy-6β-methoxycholestan-3-one (**5**) (96mg, 0.22 mmol) was dissolved in 10ml of CH_3CN. 30% aq H_2O_2 (100 µL) and Re_2O_7 (6mg) were added, and then the reaction mixture was stirred at rt for 1 hour. Then the reaction was partially concentrated under reduced pressure and the adamantanone (**6**) (52.87mg, 0,352 mmol) added as solution in 10mL of CH_2Cl_2. The suspension was stirred for 1hour and 15 min at room temperature. Then the reaction mixture was concentrated under vacuum, and extracted with CH_2Cl_2.The organic layer was dried over anhydrous Na_2SO_4 , and solvent removed under reduced pressure, further purified by column chromatography (with petroleum ether and CH_3CN) (98:3), to provide compound **12** (58mg, 0,09 mmol).

Formula: $C_{38}H_{62}O_6$

White solid

*Synthesis of the final product 5,6-oxygenated cholestane-1,2,4,5tetraoxaadamantane (**12- Process C)***

101mg, 0.23mmol of 5α-hydroxy-6β-methoxycholestan-3-one (**5**) was dissolved in 10 ml of CH_3CN/CH_2Cl_2 (1:1), at rt. After complete dissolution, 30% aq H_2O_2 (100 µL) was added and thereafter, $Bi(OTf)_3$ (6mg) was also added. The reaction was maintained stirring, at rt, for 1hour, and then was partially concentrated under reduced pressure, followed by addition of adamantanone (**6**) (55.28mg, 0.368mmol) to this reaction mixture. The reaction was stopped after 50min. After evaporation under vacuum, the extraction was made with CH_2Cl_2 and thereafter, the solution was dried with anhydrous Na_2SO_3, filtered and the excess of CH_2Cl_2 evaporated. The product formed is purified by FCC starting with petroleum ether and adding carefully CH_3CN to achieve the gradient 99:1 to 98:3 afforded the hybrid **12**

(72mg, 0,117mmol).

Formula: $C_{38}H_{62}O_6$

White solid

η (Process B) = 42%

η (Process C)= 50%

mp (Process B): 159.5°C -162.3°C.

mp (Process C): 158.9°C -161.1°C.

RMN ^1H (400 MHz, *CDCL3*) δ ppm: 0.66 (3H, s, 18-CH3), 0.85 e 0.87 (each 3H, 2d, *J*= 6.7 Hz, 26- CH3 e 27-CH3), 0.91 (3H, d, *J*=6.6 Hz, 21-CH3), 1.05 (3H, s, 19-CH3), 3.12 (1H, s, 6α-H), 3.28 (3H, s, 6β-OCH3).

RMN ^{13}C (100 MHz, *CDCL3*) δ ppm: 12.1 (CH3), 16.0 (CH3), 18.6 (CH3), 20.9, 22.5 (CH3), 22.8 (CH3), 23.8, 24.2, 27.0, 27.1, 28.0, 28.2, 28.5, 29.7, 30.2, 30.5, 31.4, 33.1, 33.2, 35.8 (C), 36.2, 36.9, 37.0, 39.5, 39.9, 42.7 (C), 44.9 , 55.8, 56.2, 57.6 (CH3), 77.2, 110.1 (C).

*Synthesis of the final product cholestane-1,2,4,5-tetraoxaadamantane (**15-Process A**)*

500mg, of 5α-cholestan-3-one (**8**) was dissolved in 7.4mL of CH_2Cl_2 and 14.7mL of CH_3CN, at rt. After complete dissolution, 4.7mL of acetic acid was added at 0^0C, and thereafter, 1.47mL of H_2O_2 was also added. The reaction was maintained stirring, at rt, for 48h, with additions of H_2O_2. The reaction was stopped adding CH_2Cl_2 and H_2O_2 . Then the reaction mixture was concentrated under vacuum, and extracted with CH_2Cl_2 .The organic layer was dried over anhydrous Na_2SO_4, and solvent removed under reduced pressure.

The resulting dihydroperoxide (**9**) was added to a solution of 1.6 eq of the adamantanone (**6**) (275mg, 1.83mmol), Re_2O_7 (6mg) and 17.65mL of dry CH_2Cl_2 , in an inhert atmosphere. The mixture was left stirring at rt for 15min. When the reaction was completed, the mixture was diluted with CH_2Cl_2 and vacuum filtered through a silica plug, to remove the catalyst. After dried with anhydrous Na_2SO_4 the solution was filtered. The product formed was

purified by FCC starting with petroleum ether and adding carefully CH_3CN to achieve the gradient 99:1 afforded the hybrid **15** (83mg, 0,14mmol).

Formula: $C_{37}H_{66}O_4$

White solid

*Synthesis of the final product a cholestane-1,2,4,5-tetraoxaadamantane (**15-Process B**)*

66.01mg of adamantanone (**6**) was dissolved in 5mL of CH_3CN and 5mL of CH_2Cl_2 , at rt. After complete dissolution, 550 µL of H_2O_2 was added, and thereafter, 6mg of $Bi(OTf)_3$ was also added. The reaction was maintained stirring, at rt, for 1hour.

Then, the 5α-hydroxy-6β-methoxycholestan-3-one (**5**) (170mg, 0.39mmol) was added to the reaction mixture and the reaction was maintained stirring, at rt, for 50 min. When the reaction was completed, the reaction mixture was concentrated under vacuum, and extracted with CH_2Cl_2 .The solution was dried with anhydrous Na_2SO_3, filtered and the excess of CH_2Cl_2 evaporated.

The product formed is purified by FCC starting with petroleum ether and adding carefully CH_3CN to achieve the gradient 99:1 to 90:10 afforded the hybrid **15** (98mg, 015mmol).

Formula: $C_{37}H_{66}O_4$

White solid

η (Process A)= 11%

η (Process B)= 44%

mp (Process A): 157.4°C -159.3°C.

mp (Process B): 158.5°C -160.1°C.

RMN ^1H (400 MHz, *CDCL3*) δ ppm: 0.64 (3H, s, 18-CH_3), 0.81 (3H, s, 19-CH_3), 0.85 e 0.87 (each 3H,2d, *J*= 6.7 Hz, 26-CH_3 e 27-CH_3), 0.89 (3H, d, *J*=6.6 Hz, 21-CH_3).

RMN ^{13}C (100 MHz, *CDCL3*) δ ppm: 11.4 (CH_3), 12.1 (CH_3), 18.7 (CH_3), 21.2, 22.6 (CH_3),

22.8 (CH_3), 23.8, 24.2, 27.1, 28.0, 28.2, 29.7, 30.2, 30.3, 31.4, 31.8, 33.2, 35.4, 35.8, 35.9 (C), 36.2, 36.9, 39.5 , 40.0, 42.8 (C) , 53.8, 56.2, 56.4, 60.4, 77.2, 108.4 (C), 110.3 (C).

*Synthesis of the final product a cholestane-1,2,4,5-tetraoxacyclohexane derivative (**16-Process A**)*

500mg of 5α-cholestan- 3-one was dissolved in 7.4mL of CH_2Cl_2 and 14.7mL of CH_3CN, at rt. After complete dissolution, 4.7mL of acetic acid was added at 0^0C, and thereafter, 1.47mL of H_2O_2 was also added. The reaction was maintained stirring, at rt, for 48h, with additions of H_2O_2. The reaction was stopped adding CH_2Cl_2 and H_2O_2 . Then the reaction mixture was concentrated under vacuum, and extracted with CH_2Cl_2 .

The organic layer was dried over anhydrous Na_2SO_4, and solvent removed under reduced pressure. The resulting dihydroperoxide (**9**) was added to a solution of 1.6 eq of the ethyl 4-oxo cyclohexanecarboxylate (0.185mL), Re_2O_7 (6mg) and 17.65mL of dry CH_2Cl_2, in an inhert atmosphere.

The mixture was left stirring at rt for 15min. When the reaction was completed, the mixture was diluted with CH_2Cl_2 and vacuum filtered through a silica plug, to remove the catalyst. After dried with anhydrous Na_2SO_4 the solution was filtered. The product formed was purified by FCC starting with petroleum ether and adding carefully CH_3CN to achieve the gradient 99:1 afforded the hybrid **16** (92mg, 0.15mmol).

Formula: $C_{36}H_{60}O_6$

White solid

η = 16%

mp (MeOH): 159.3°-161.4°C

RMN ^{1}H (400 MHz, *CDCL$_3$*) δ ppm: 0.64 (3H, s, 18-CH_3), 0.81 (3H, s, 19-CH_3), 0.85 e 0.87 (each 3H, 2d, *J*= 6.7 Hz, 26-CH_3 e 27-CH_3), 0.88 (3H, d, *J*=6.6 Hz, 21-CH_3), 1.24 (2H, t, J= 6.8 Hz, OCH_2CH_3), 4.12 (3H, q, J= 6.8Hz, OCH_2CH_3)

RMN ^{13}C (100 MHz, *CDCL$_3$*) δ ppm: 11.4 (CH_3), 12.1 (CH_3), 14.2 (CH_3), 18.6 (CH_3), 21.2 (CH_2), 22.6 (CH_3), 22.8 (CH_3), 23.7, 24.2, 28.0, 28.2, 31.8, 35.4, 35.8, 36.2, 38.9 (C), 39.5, 39.9 , 41.5, 41.8, 42.6(C) , 53.8, 56.2, 56.4, 60.4, 107.3 (C), 108.7 (C), 174.6 (C).

Chapter V

REFERENCES

1 http://www.cdc.gov

2 WHO, World Malaria Report 2010.

3 Marco A. Biamonte , Jutta Wanner , Karine G. Le Roch , Recent advances in malaria drug discovery , Bioorganic & Medicinal Chemistry Letters, 23 ,2013, 2829-2843.

4 Battle, K. E.; Gething, P. W.; Elyazar, I. R.; Moyes, C. L.; Sinka, M. E.; Howes, R. E.; Guerra, C. A.; Price, R. N.; Baird, K. J.; Hay, S. I. Adv. Parasitol. 2012, 80, 1.

5 Ramya, T. N. C., Surolia, N., Surolia, A., Survival strategies of the malarial parasite *Plasmodium falciparum*, Curr. Sci., 2002, 83, 818-825.

6 O´Neill, P. M., Posner, G. H., A Medicinal Chemistry Perspective on Artemisinin and Related Endoperoxides, J. Med. Chem., 2004, 47, 2945-2964.

7 Shio, M. T., Kassa, F. A., Bellemare, M-J. and Olivier, M.. Innate inflammatory response to the malarial pigment hemozoin. Microbes Infect. 2010. 12:889-99.

8 Stiebler, R., Correa Soares, J. B. R., Timm, B. L., Silva, J. R., Mury, F. B., Dansa-Petretski, M. and Oliveira, M. F.. On the mechanisms involved in biological heme crystallization. J Bioenerg Biomembr. 2011. 43:93-99.

9 Francis, S. E., Sullivan, D. J. and Goldberg, D. E.. Hemoglobin metabolism in the malaria parasite Plasmodium Falciparum. Annu. Rev. Microbiol. 1997. 51:97-123.

10 Trager, W. - The formation of haemozoin – further intrigue. Trends in Parasitology 19 (2003) 388.

11 Egan, T. J.. Haemozoin (malaria pigment): a unique crystalline drug target. Targets. 2003. 2:115-124.

12 Phillips, R. S., Current Status of Malaria and Potencial for Control, Clin. Microbiol. Rev., 2001, 14, 208-226.

13 Wells, T. N. C. In Treatment and Prevention of Malaria; Staines, H. M., Krishna, S., Eds.; Springer: Basel, 2012; p 227.

14 Jane Achan, Ambrose O Talisuna , Annette Erhart , Adoke Yeka , James K Tibenderana, Frederick N Baliraine , Philip J Rosenthal and Umberto D'Alessandro, Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria, Malaria Journal. Rev 2011, 10:144

15 Krishna, S., Uhlemann, A., Haynes, R. K., Artemisinins: mechanisms of action and potential for resistance, Drug Resist. Updat., 2004, 7, 233-244.

16 Olliaro, P. L., Haynes, R. K., Meunier, B., Yuthavong, Y., Possible modes of action of the artemisinin-type compounds, Trends Parasitol., 2001, 17, 122-126.

17 Meshnick, S. R., Artemisinin: mechanisms of action, resistance and toxicity, Int.

J.Parasitol., 2002, 32, 1655-1660.

18 Tang, Y., Dong, Y., Vennerstrom, J. L., Synthetic Peroxides as Antimalarials, Med. Res. Rev., 2004, 24, 425-448.

19 Schlitzer, M., Antimalarial Drugs-What is in Use and What is in the Pipeline, Arch.Pharm. Chem. Life. Sci., 2008, 341, 149-163.

20 Brewer, T.G. ; Grate,S.J. ; Peggins, J. O. ; Weina, P.J. ; Petras, J.M. ; et al. Fatal Neurotoxicity of Arteether and Artemether. Am. J.Trop.Med.HYG.1994,51,251-259]

21 Meshnick, S. R., Taylor, T. E., Kamchonwongpaisan, S., Artemisinin and the Antimalarial Endoperoxides: from Herbal Remedy to Targeted Chemotherapy, Microbiol. Rev., 1996, 60, 301-315.

22 Posner, G. H., O´Neill, P. M., Knowledge of the Proposed Chemical Mechanism of Action and Cytochrome P450 Metabolism of Antimalarial Trioxanes Like Artemisinin Allows Rational Design of New Antimalarial Peroxides, Acc. Chem. Res., 2004, 37, 397-404.

23 Schlitzer, M., Malaria Chemotherapeutics Part I: History of Antimalarial Drug Development, Currently Used Therapeutics, and Drugs in Clinical Development,ChemMedChem, 2007, 2, 944-986.

24 Schmuck, G., Roehrdanz, E., Haynes, R. K., Kahl, R., Neurotoxic Mode of Action of Artemisinin, Antimicrob. Agents Chemother., 2002, 46, 821-827.

25 Agtmael, M. A., Eggelte, T. A., Boxtel, C. J., Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication, Trends Pharmacol. Sci., 1999, 20.

26 Ploypradith, P., Development of artemisinin and its structurally simplified trioxane derivatives as antimalarial drugs, Acta Trop., 2004, 89, 329-342.

27 Chaturvedi, D., Goswami, A., Saikia, P. P., Barua, N. C., Rao, P. G., Artemisinin and its derivatives: a novel class of anti-malarial and anti-cancer agents, Chem. Soc. Rev.,2010, 39, 435-454.

28 Posner, G. H., Northrop, J., Paik, I., Borstnik, K., Dolan, P., Kensler, T. W., Xie, S., Shapiro, T., New Chemical and Biological Aspects of Artemisinin-Derived Trioxane Dimers, Bioorg. Med. Chem., 2002, 10, 227-232.

29 Biagini, G. A., O´Neill, P. M., Nzila, A., Ward, S. A., Bray, P. G., Antimalarial chemotherapy: young guns or back to the future?, Trends parasitol., 2003, 19, 479-487.

30 Steyn, J. D., Wiesner, L., Plessis, L. H., Grobler, A. F, Smith, P. J., Chan, W. C., Haynes, R. K., Kotzé, A. F., Absorption of the novel artemisinin derivatives artemisone and artemiside: Potencial application of PheroidTM technology, Int. J. Pharm., 2011, 414, 260-266.

31 - Dunay, I. R., Chan, W. C., Haynes, R. K., Sibley, L. D., Artemisone and Artemiside Control Acute and Reactivated Toxoplasmosis in a Murine Model, Antimicrob. Agents Chemother., 2009, 53, 4450-4456.

32 Robert, A., Benoit-Vical, F., Meunier, B., The key role of heme to trigger the antimalarial activity of trioxanes, Coord. Chem. Rev., 2005, 249, 1927-1936.

33 J. L. Vennerstrom, H.-N. Fu, W. Y. Ellis, A. L. A. Jr., J. K. Wood, S. L. Andersen, L. Gerena, W. K. Milhous; Dispiro-1,2,4,5- tetraoxanes: A New Class of Antimalarial Peroxides. *J. Med. Chem.,* 1992, *35*(16).

34 F. Marti, J. Chadwick, R. K. Amewu, H. Burrell-Saward, A. Srivastava, S. A. Ward, R. Sharma, N. Berry, P. M. O'Neill; Second generation analogues of RKA182: synthetic tetraoxanes with outstanding *in vitro* and *in vivo* antimalarial activities. *MedChemComm,* 2011, *2*(7), 661-665.

35 H.-S. Kim, Y. Shibata, Y. Wataya, K. Tsuchiya, A. Masuyama, M. Nojima; Synthesis and Antimalarial Activity of Cyclic Peroxides, 1,2,4,5,7-Pentoxocanes and 1,2,4,5-Tetraoxanes. *J. Med. Chem.,* 1999, *42*, 2604-2609.

36 H. Atheaya, S. I. Khan, R. Mamgain, D. S. Rawat; Synthesis, thermal stability, antimalarial activity of symmetrically and asymmetrically substituted tetraoxanes. *Bioorg. Med. Chem. Lett.,* 2008, *18*(4), 1446-1449.

37 N. Terzié, D. Opsenica, D. Milié, B. Tinant, K. S. Smith, W. K. Milhous, B. A. Solaja; Deoxycholic Acid-Derived Tetraoxane Antimalarials and Antiproliferatives. *J. Med. Chem.,* 2007, *2007*(50), 5118-5127.

38 X. Wang, Y. Dong, S. Wittlin, S. A. Charman, F. C. Chiu, J. Chollet, K. Katneni, J. Mannila, J. Morizzi, E. Ryan, C. Scheurer, J. Steuten, J. Santo Tomas, C. Snyder, J. L. Vennerstrom; Comparative antimalarial activities and ADME profiles of ozonides (1,2,4-trioxolanes) OZ277, OZ439, and their 1,2-dioxolane, 1,2,4- trioxane, and 1,2,4,5-tetraoxane isosteres. *J. Med. Chem.,* 2013, *56*(6), 2547-2555.

39 http://www.mmv.org/, accessed in 11-06-2015.

40 Krungkai, J., Imprasittichai, W., Otjungreed, S., Pongsabut, S., Krungkai, S. R., Artemisinin resistance or tolerance in human malaria patients, Asian Pac. J. Trop. Med., 2010, 748-753.

41 World Health Organization. Antimalarial drug combination therapy: report of WHO technical consultation. Geneva: WHO, 2001.

42 Schmidt, L. H., Alexander, S., Allen, L., Rasco, J., Comparison of the Curative Antimalarial Activities and Toxicities of Primaquine and Its d and l Isomers, Antimicrob. Agents. Chemother., 1977, 12, 1, 51-60.

43 Krungkai, J., Imprasittichai, W., Otjungreed, S., Pongsabut, S., Krungkai, S. R., Artemisinin resistance or tolerance in human malaria patients, Asian Pac. J. Trop. Med., 2010, 748-753.

44 Ridley, R. G., Medical need, scientific opportunity and the drive for antimalarial drugs, Nature, 2002, 415, 686-692.

45 NJ White Qinghaosu (artemisinin): the price of success Science, 320 (2008), pp. 330–334.

46 SR Krungkrai, Y Yuthavong The antimalarial action on *Plasmodium falciparum* of qinghaosu and artesunate in combination with agents which modulate oxidant stress Trans R Soc Trop Med Hyg, 81 (1987), pp. 710-714.

47 S Meshnick, TE Taylor, S Kamchonwongpaisan Artemisinin and he antimalarial endoperoxides: from herbal remedy to targeted chemotherapy Microbiol Rev, 60 (1996), pp. 301-315.

48 W Li, W Mo, L Sun, J Wang, S Lu, JM Gitschier, *et al.* Yeast model uncovers dual roles of mitochondria in action of artemisinin Plos Genet, 1 (2005), p. e36.

49 J Wang, L Huang, J Li, Q Fan, Y Long, Y Li, *et al.* Artemisinin directly targets malarial mitochondria through its specific mitochondrial activation Plos One, 5 (2010), p. e9582.

50 Silverman, R. B., The organic chemistry of drug design and drug action, 2004, 146-147, 2nd Edition, Elsevier, Oxford.

51 Morphy, R., Rankovic, Z., Designed Multiple Ligands. An emerging Drug Discovery Paradigm, J. Med. Chem., 2005, 48, 6523-6543.

52 Walsh, J. J., Bell, A., Hybrid drugs for malaria, Curr. Pharm. Des., 2009, 15, 2970–2985.

53 Howard, M., Al-Ghananeem, A., Crooks, P. A., A novel chemical delivery system comprising an ocular sustained release formulation of a 3α, 17α, 21-trihydroxy-5β-pregnan-20-one-BIS-5-Flouroucil codrug, Drug. Dev. Ind. Pharm., 2007, 33, 677-682.

54 Cena, C., Lolli, M. L., Lazzarato, L., Guaita, E., Morini, G., Coruzzi, G., McElroy, S. P., Megson, I. L., Fruttero, R., Gasco, A., Antiinflammatory, Gastrosparing, and

Antiplatelet Properties of New NO-Donor Esters of Aspirin, J. Med. Chem., 2003, 46, 747-754.

55 Lolli, M. L., Cena, C., Medana, C., Lazzarato, L., Morini, G., Coruzzi, G., Manarini, S., Fruttero, R., Gasco, A., A New Class of Ibuprofen Derivatives with Reduced Gastrotoxicity, J. Med. Chem., 2001, 44, 3463-3468.

56 Aslanian, R., Mutahi, M., Shih, N.-Y., Piwinski, J. P., West, R., Williams, S. M., She, S., Wu, R.-L., Hey, J. A., Identification of a Dual Histamine H1/H3 Receptor Ligand Based on the H1 Antagonist Chlorpheniramine, Bioorg. Med. Chem. Lett., 2003, 13, 1959-1961.

57 Perez, M., Pauwels, P. J., Pallard-Sigogneau, I., Fourrier, C., Chopin, P., Palmier, C., Colovray, V., Halazy, S., Design and Synthesis of New Potent, Silent 5-HT1A Antagonists by Covalent Coupling of Aminopropanol Derivatives With Selective Serotonin Reuptake Inhibitors, Bioorg. Med. Chem. Lett., 1998, 8, 3423-3428.

58 Lipinski C. A., Lombardo, F., Dominy, B. W., Feeney, P. J., Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Adv. Drug Deliver Rev., 2001, 46, 3-26.

59 Hamad, M. O., Kiptoo, P. K., Stinchcomb, A. L., Crooks, P. A., Synthesis and hydrolytic behavior of two novel tripartate codrugs of naltrexone and 6β-naltrexol with hydroxybupropion as potencial alcohol abuse and smoking cessation agents, Bioorg. Med. Chem., 2006, 14, 7051-7061.

60 Egan, T. J., Structure-Function Relationships in Chloroquine and Related 4-Aminoquinolines Antimalarials, Mini Reviews Med. Chem., 2001, 1, 113-123.

61 O´Neill, P. M., Bray, P. G., Hawley, S. R., Ward, S. A., Park, B. K., 4-Aminoquinolines- Past, Present, and Future: A Chemical Perspective, Pharmacol. Ther., 1998, 77, 29-58.

62 Araújo, N. C. P., Barton, V., Jones, M., Stocks, P. A., Ward, S. A., Davies, J., Bray, P. G., Shone, A. E., Cristiano, M. L. S., O'Neill, P. M., Semi-synthetic and synthetic 1,2,4-trioxaquines and 1,2,4-trioxolaquines: synthesis, preliminary SAR and comparison with acridine endoperoxide conjugates, Bioorg. Med. Chem. Lett., 2009, 19, 2038-2043.

63 Meunier, B. [et al] - Hybrid molecules with a dual mode of action: dream or reality? Acc. Chem. Res. 41 (2008) 69-77.

64 Solaja, B. A. [et al] - Mixed Steroidal 1,2,4,5-tetraoxanes: Antimalarial and Antimycobacterial Activity. J. Med. Chem. 45 (2002) 3331-3336.

65 O´Neill, P. M., Barton, V. E., Ward, S. A., The Molecular Mechanism of Action of Artemisinin - The Debate Continues, Molecules, 2010, 15, 1705-1721.

66 Shio, M. T., Kassa, F. A., Bellemare, M-J. and Olivier, M.. Innate inflammatory response to the malarial pigment hemozoin. Microbes Infect. 2010. 12:889-99.

67 Meunier, B. and Robert, A.. Heme as trigger and target for trioxane-containing antimalarial drugs. Acc Chem Res. 2010. 43:1444-1451.

68 a) Haynes, R. K. and Krishna, S.. Artemisinins: activities and actions. Microbes and infection. 2004. 6:1339-46; b) Opsenica, I., Terzić, N., Opsenica, D., Angelovski, G., Lehnig, M., Eilbracht, P., Tinant, B., Juranić, Z., Smith, K. S., Yang, Y. S., Diaz, D. S., Smith, P.L., Milhous, W. K., Doković, D. and Solaja, B. A.. Tetraoxane antimalarials and their reaction with Fe(II). J Med Chem. 2006. 49:3790-9.

69 Markus, M. B. - Malaria: origin of the term 'hypnozoite'. J. Hist. Biol 44 (2011) 781–786.

70 Biamonte, M. A., Wanner, J. and Le Roch, K. G. - Recent advances in malaria drug discovery. Bioorg. Med. Chem. Lett. 23 (2013) 2829-2843.

71 Moss, G. P. [et al] - International Union of Pure Joint Commission on Biochemical Nomenclature. 61 (1989) 1783-1822.

72 Schroepfer, G. J. - Oxysterols: modulators of cholesterol metabolism and other processes. Physiol. Rev. 80 (2000) 361-554.

73 Doisneau-Sixou, S. F. [et al] - Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. Endocrine-related cancer, 10 (2003) 179-186.

74 Simons, K. and Ikonen, E. - How cells handle cholesterol. Science 290 (2000) 1721-1726.

75 Alberts, B. [et al] - Molecular Biology of the Cell. 4th Ed. New York: Garland Science; 2002. The Lipid Bilayer. Available from: http://www.ncbi.nlm.nih.gov/books/NBK26871/

76 Carvalho, J. F. S. [et al] - Sterols as anticancer agents: synthesis of ring-B oxygenated steroids, cytotoxic profile, and comprehensive SAR analysis. J. Med. Chem. 53 (2010) 7632-7638.

77 Salvador, J. A. R. [et al] - Anticancer steroids: linking natural and semi-synthetic compounds. Nat. Prod. Rep. 30 (2013) 324-374.

78 Yeagle, P. L. - Cholesterol and the cell membrane. Biochim. Biophys. Acta, Rev. Biomembr. 822 (1985) 267-287.

79 Carvalho, J. F. S. [et al] - Selective cytotoxicity of oxysterols through structural

modulation on rings A and B. Synthesis, in vitro evaluation, and SAR. J. Med. Chem. 54 (2011) 6375-6393.

80 Lund, E. [et al] - Importance of a Novel Oxidative Mechanism for Elimination of Intracellular Cholesterol in Humans. Arterioscl. Throm. Vas. 16 (1996) 208-212.

81 Blanc, M. [et al] -The transcription factor STAT-1 couples macrophage synthesis of 25-hydroxycholesterol to the interferon antiviral response. Immunity 38 (2013) 106-118.

82 Shinkyo, R. [et al] - Conversion of 7-dehydrocholesterol to 7-ketocholesterol is catalyzed by human cytochrome P450 7A1 and occurs by direct oxidation without an epoxide intermediate. J. Biol. Chem. 286 (2011) 33021-33028.

83 Diener, A. C. - Sterol Methyltransferase 1 Controls the Level of Cholesterol in Plants. The Plant Cell. 12 (2000) 853-870.

84 Neill AL, Hunt NH.,Effects of Endotoxin and Dexamethasone on Cerebral Malaria in Mice. , Parasitology. 1995 Nov; 111 (Pt 4):443-54.

85 DHKIL, M. A. [et al] - Testosterone-induced persistent susceptibility to Plasmodium chabaudi malaria: Long-term changes of lincRNA and mRNA expression in the spleen. Steroids 78 (2013) 220-227.

86 O'Neill, P. [et al] - Identification of a 1,2,4,5-tetraoxane antimalarial drug-development candidate (RKA 182) with superior properties to the semisynthetic artemisinins. Angew. Chem. Int. Ed. 49 (2010) 5693-5697.

87 Carvalho, J.F.S. Bioactive Sterols: Synthesis, Antitumoral Evaluation and Structure-Activity Studies. University of Coimbra, (2010).

88 Carvalho, F. S., Silva, M. M. C. and Sa E Melo, M. L. - Highly efficient epoxidation of unsaturated steroids using magnesium bis (monoperoxyphthalate) hexahydrate. Tetrahedron 65 (2009) 2773-2781.

89 Bruice, T.C. and FIFE, T.H. - The Nature of Neighboring Hydroxyl Group Assistance in the Alkaline Hydrolysis of the Ester Bond. JACS 10 (1962) 1973-1979.

90 Bowden, K. [et al] -The preparation of acetylenic ketones by oxidation of acetylenic carbinols and glycols. J. Chem. Soc. 39 (1946).

91 Carvalho, J. F. S. [et al] - Efficient chemoenzymatic synthesis, cytotoxic evaluation, and SAR of epoxysterols. J. Med. Chem. 52 (2009) 4007-4019.

92 Opsenica, I. [et al] - New chimeric antimalarials with 4-aminoquinoline moiety linked to a tetraoxane skeleton. J. Med. Chem. 51 (2008) 6216-6219.

93 Vennerstrom, J. L. [et al] - Identification of an antimalarial synthetic trioxolane drug

development candidate. Nature 430, (2004) 900-904.

94 Alexander O. Terent'ev, Dmitry A. Borisov , Vera A. Vil' and Valery M. Dembitsky, Synthesis of five- and six-membered cyclic organic peroxides: Key transformations into peroxide ring-retaining products, Beilstein J. Org. Chem. (2014), 10, 34-114.

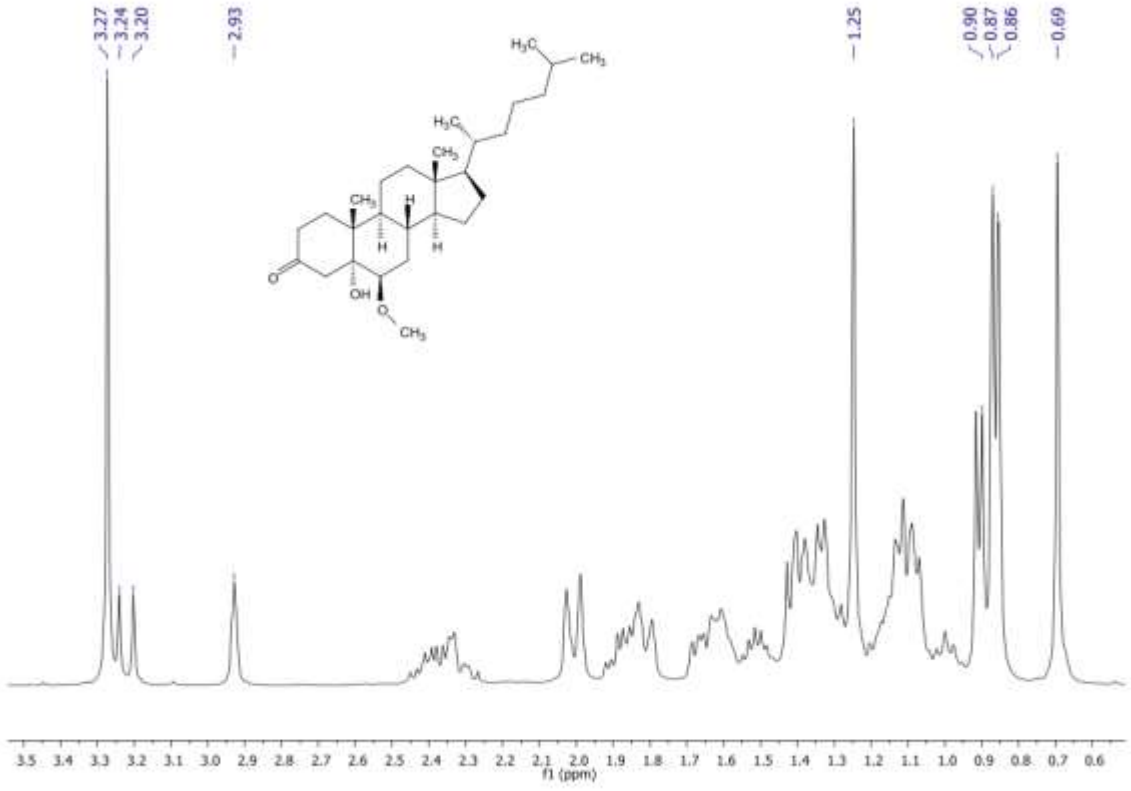
95 Koneni V. Sashidhara, Srinivasa Rao Avula, L.Ravithej Sing, Gopala Reddy Palnati, A facile and efficient Bi(III) catalyzed synthesis of 1,1-dihydroperoxide and 1,2,4,5-tetraoxanes, Tetrahedron Letters 53, (2012), 4880-488.

96 Prasanta Ghorai , Patrick H. Dussault, Broadly Applicable Synthesis of 1,2,4,5-Tetraoxanes, Organic Letters, 11 (2009), 213-216.

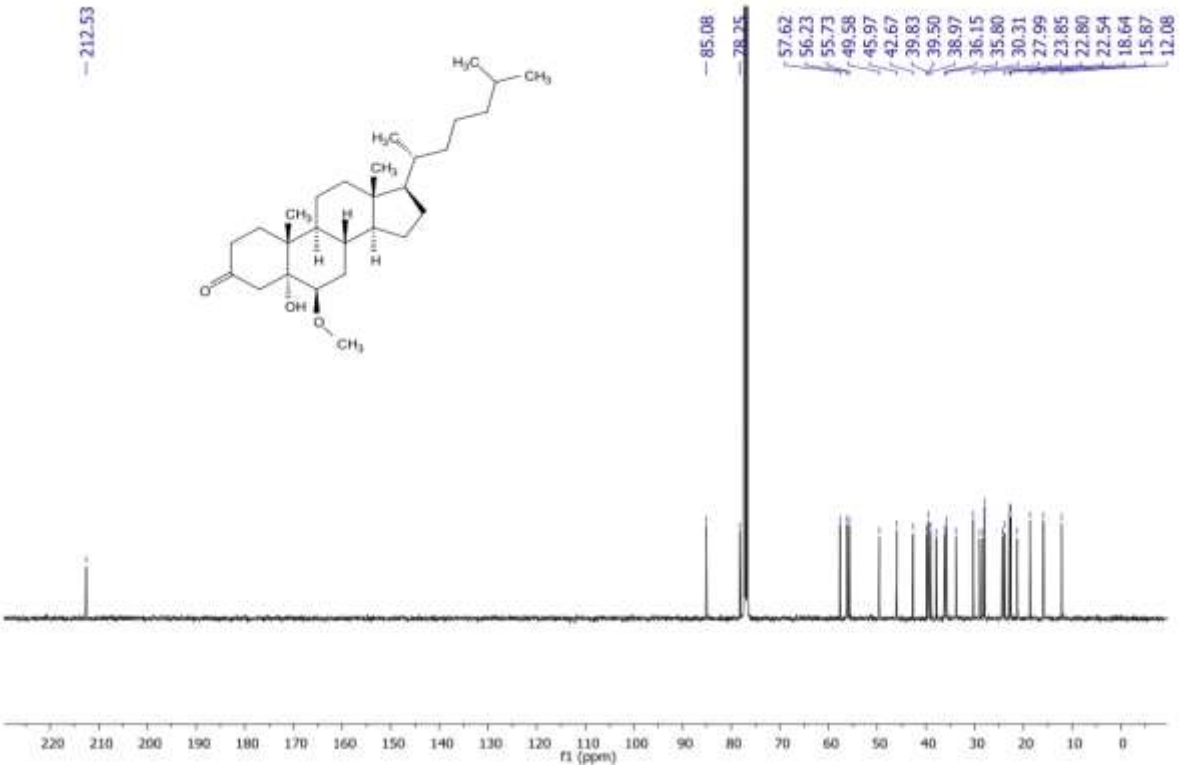
Chapter VI

ANNEXES

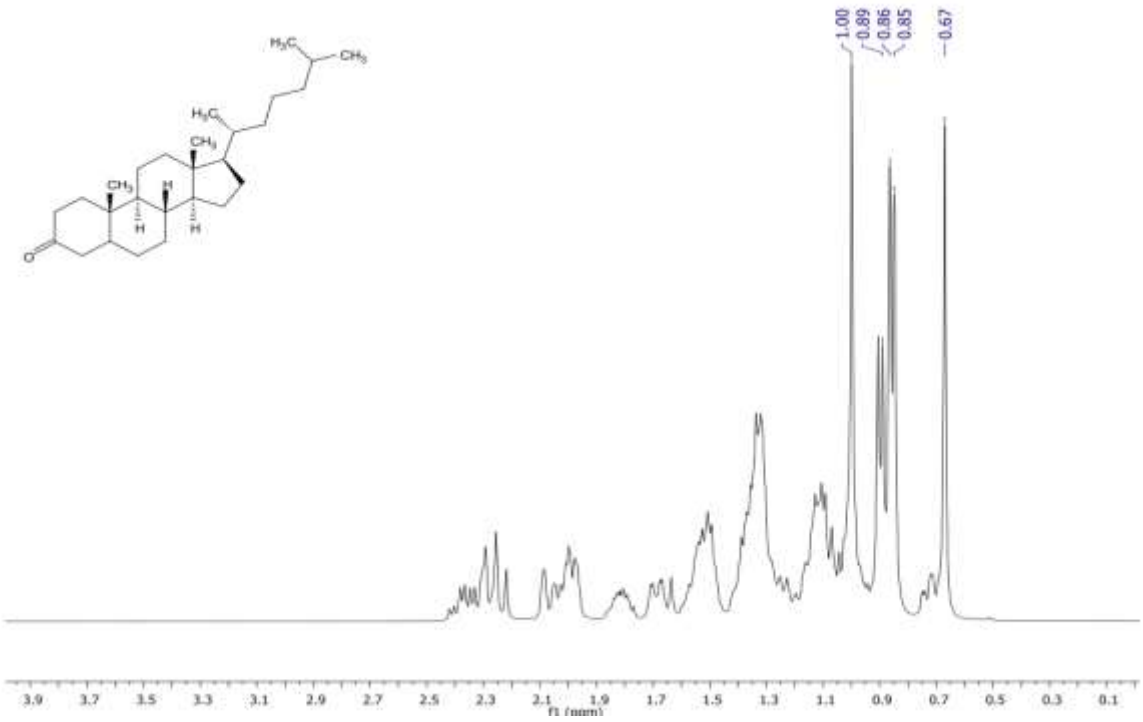
^{1}H NMR Spectrum of the intermediate 5



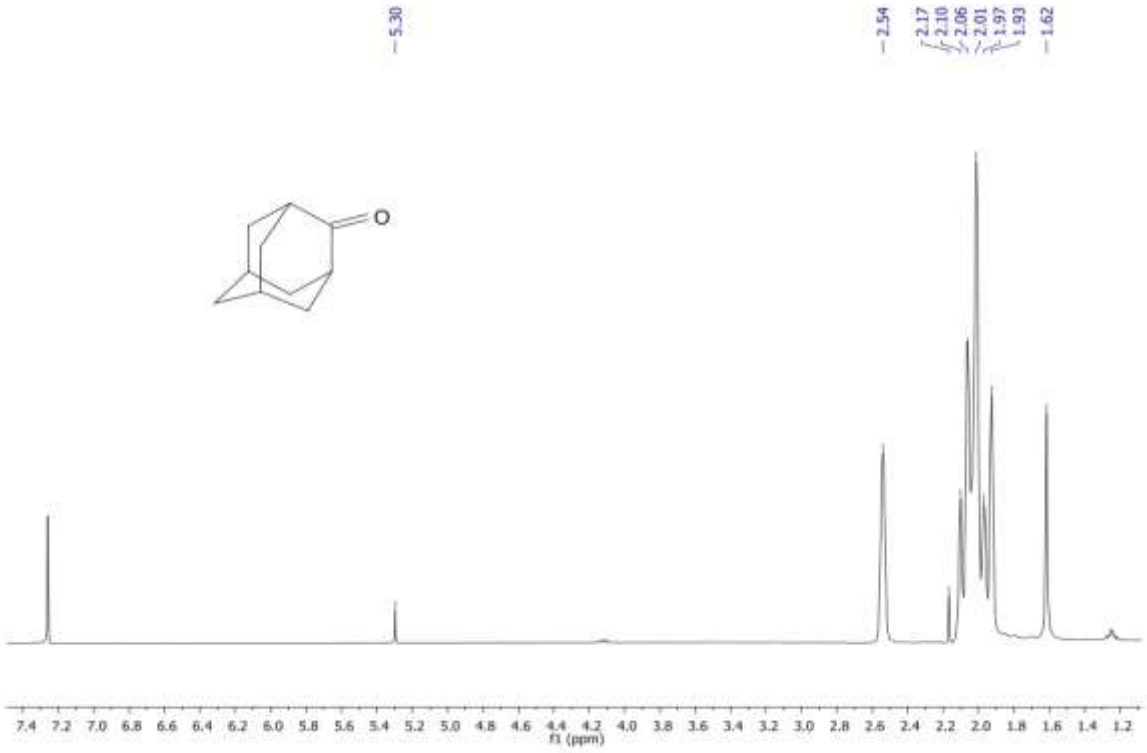
^{13}C NMR Spectrum of the intermediate 5



^{1}H NMR Spectrum of the intermediate 8 - 5α-cholest-3-one



^{1}H NMR Spectrum of the intermediate 6 - adamantanone



^{1}H NMR Spectrum of the intermediate 7 - ethyl 4-oxo cyclohexanecarboxylate