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# STEROIDS IN A MULTITARGET APPROACH FOR MALARIA ERADICATION. DEVELOPMENT OF HYBRID ANTIMALARIALS

Dissertação de Mestrado em Química Farmacêutica Industrial, orientada pelo Professora Doutora Maria Luísa Campeão Fernandes Sá e Melo e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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Ana Carolina Torres

Para a minha avó

*"The real voyage of discovery consists not in seeking new landscapes, but in having new eyes."*

Marcel Proust

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***ABSTRACT***

Malaria is a public health problem today in more than 100 countries inhabited by some 2,400 million people - 40 percent of the world's population.
While statistics at a first glance might indicate that the global malaria situation has stabilized, an alarming increase in drug resistance and decrease in therapeutic efficacy of available drugs has been observed.

Oxysterols represent a group of lipids, cholesterol derivatives with a growing value due to its relevant bioactivity, that changes according with structural changes in the steroid nucleus. Its evolving in cell biology and pathophysiology systems made this class of oxysterols one of the most extensively studied. Oxysterols have been reported to display some degree of cytotoxicity against cancer cells increasing the sensitivity of tumor cells to other chemotherapeutic agents. 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.

This work aims to exploit simple and profitable reaction for the synthesis of hybrid antimalarials based on a steroid scaffold and a tetraoxane bond.
The first part of this work consists in the synthesis of the oxysterols intermediates, an essential and new moiety of these hybrid drugs.
In the second part of this thesis are performed exploratory procedures for the dihydroperoxide formation and consequent synthesis of the tetraoxane bridge, with the attachment of the third part of the hybrid compound. A spectral characterization of the synthesised hybrid molecules is presented.

The work developed in this thesis, as well as the conclusions achieved are crucial for the understanding and future development of antimalarial hybrid drugs with a steroid scaffold.

***RESUMO***

Atualmente, a malária é um problema de saúde pública em mais de 100 países habitados por cerca de 4,400 milhões de pessoas, 40% da população mundial. Enquanto as estatísticas, numa primeira vista, indicam que a situação global desta doença estabilizou, um aumento alarmante na resistência aos fármacos atualmente usados, assim como uma diminuição na eficácia da terapêutica usada têm sido observados.

Os oxiesteróis representam um grupo de lípidos derivados do colesterol com um valor crescente devido à sua relevante bioatividade, que varia de acordo com mudanças nos grupos funcionais no núcleo esteróide.
Este grupo de compostos está envolvido na biologia da célula e em sistemas patofisiológicos, sendo um dos grupos de compostos mais extensivamente estudados.
No entanto tem sido reportado algum grau de citotoxicidade para com as células tumorais, aumentando a sensibilidade das células neoplásicas a outros fármacos quimioterápicos. Com este pressuposto em mente, os oxiesteróis são excelentes candidatos na síntese de fármacos híbridos, uma vez que eles podem exercer o seu efeito aumentando a sensibilidade das células infectadas a outros fármacos antimaláricos.

Neste trabalho são exploradas reações simples para a síntese de fármacos híbridos antimaláricos baseados num núcleo esteroide e num tetraoxano. Assim, numa primeira parte é discutida a síntese de intermediários oxiesteróis, essenciais na síntese de híbridos antimaláricos,
Numa segunda parte são realizados procedimentos exploratórios para a formação de um dihidroperóxido e consequente síntese do tetraoxano, com a ligação da terceira parte destes fármacos híbridos. A caracterização espectral dos híbridos sintetizados é discutida.

O trabalho desenvolvido com esta tese, assim como as conclusões retiradas são fundamentais no entendimento e futuro desenvolvimento de fármacos híbridos antimaláricos com um núcleo esteroide.

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## *LIST OF ABBREVIATIONS*

**Ac** = Acetyl group

**ACT** = Arthemisin-based combined therapies

**BC** = Before Christ

**HMG-CoA** = 3-hydroxy-3-methyl-glutaryl-Co-enzyme A

**CYP** = Cytochrome

**EMA** = European Medicines Agency

**DDT** = Dichlorodiphenyltrichloroethane

**DEPT** = Distortionless Enhancement by Polarization Transfer

**Eq** = Equivalents

**EtOH** = Ethanol

**FCC** = Flash Column Chromatography

**IR** = Infra Red (Spectroscopy)

***m*CPBA** = *meta*-chloroperoxybenzoic acid

**MMV** = Medicines for Malaria Ventures

**MeOH=** Methanol

**Min** = minute

**MMPP** = Magnesium monoperoxyphthalate hexahydrate

**MP** = Melting Point

**NMR** = Nuclear Magnetic Ressonance

**PABA** = p-aminobenzoic acid

**PCC** = Pyridinium Chlorochromate

**PDC** = Pyridinium Dichromate

**ref** = reflux

**Rf** = retardation factor

**ROS** = Reactive oxygen Species

**rt** = room temperature

**TEA** = Triethylamine

**TLC** = Thin Layer Chromatography

**UV** = Ultra Violet

**WHO** = World Health Organization

## *GENERAL OVERVIEW- THE PROBLEM OF MALARIA*

The high mortality and widespread impact of malaria have resulted in the world's largest parasitic disease, killing more people than any other communicable disease, apart tuberculosis. [1]
Each year, 300-500 million people contract malaria and about 1-2 million die, most of which are children under five years and a significant number of pregnant women in sub-Saharian Africa.
Despite the alarming numbers, malaria is a preventable and treatable disease.

Key interventions to control malaria include: prompt and effective treatment with artemisinin-based combination therapies; use of insecticidal nets by people at risk; and indoor residual spraying with insecticide to control the vector mosquitoes.
The use of insecticides has been inadvisable due to its inherent toxicity both to humans and to the environment. Cases of parasites and vector mosquito's resistance to the usual insecticides have also been reported.
In many parts of the world, the parasites have developed resistance to a higher number of malaria medicines due, to its intensive and self prescribed use, and mostly to poor-quality antimalarial drugs leading in both cases to an inadequate treatment.[2]
Elimination of malaria from high-transmission countries will depend on the success of research to deliver a more robust arsenal of drugs than those available today. However, for such a long term goal, a multifaceted approach is needed.

Malaria vaccines are also an area of intensive research. However, no effective vaccine has been introduced into clinical practice, owing to the complex parasite lifecycle with a wide range of potential targets and to the human immunology system.

In a parallel line of the vaccine development, the research of the influence of the *Plasmodium* parasite in the epigenetics has been emerged.[3]
A proper understanding of epigenetic transcriptional control in the human organism is fundamental once it is known that during its red blood cell stage, the malaria parasite

*Plasmodium falciparum* can switch its variant surface proteins to evade the host immune response, impairing a vaccine achievement.[4]

In spite of vaccines have become available and effective preventive treatments, the endemic malaria areas are among the most rural, isolated and poorest in the whole world, and probably the rates of protection provided will never reach, being their special needs of package, transport, and difficulties in the distribution procedure, important disadvantages of vaccines.

The continuous study and investigation of compounds with proved antimalarial activity is crucial and desirable to a long to medium term.

The association of molecules or the addition to a molecule of a special functional group that mimicry a natural product with antimalarial activity it is presently, the most interesting approach for the design and development of new antimalarial drugs.

## ***OBJECTIVES AND OUTLINE OF THE THESIS***

Taking into account the malaria problem described, the main purpose of this thesis is the synthesis and characterization of hybrid drugs, with antimalarial activity, based on different moieties, a steroidal nucleus, a 1,2,4,5-tetraoxane moiety and the adamantanone or the ethyl 4-oxocyclohexanecarboxylate group.

This dissertation is divided in five chapters.
The first chapter comprehends a general introduction of the malaria disease, an explanation about the life cycle of the parasite and the formation of haemozoin, and the drugs resistance problem. An explanation of what are sterols, oxysterols and, sterols as antimalarials is also present in this chapter. Lastly, a brief clarification of what are hybrid drugs and the importance of the endoperoxide bond in the antimalarial mechanism is explained.

The second chapter is devoted to the synthesis of the intermediates and synthesis of the hybrid compounds, with the oxysterols and the tetraoxane moieties. The synthesis of the intermediates is described, as well as the experimental procedures performed.
Different methodologies for the synthesis of the hybrids are explained and discussed. The methodology chosen is optimized and described in this chapter. The characterization of the synthesized hybrids by NMR and IR is explained in this chapter.

Chapter three states the major findings and conclusions of this work, as well as some future perspectives.

The reagents and solvents, equipment and all the chemical support are presented and described in the fourth chapter.

The last chapter provides the supporting references used in this dissertation.

# CHAPTER I

# INTRODUTION

## *1.1 MALARIA*

*"Children are especially vulnerable to malaria. In Africa,*
*where 80% of malaria cases are treated at home,*
*the disease kills one child in twenty before the age of five."*

Malaria Foundation International (www.malaria.org)

### *1.1.1 A historical overview*

Malaria is a mosquito-borne disease caused by a parasite. This disease has been noticed for more than 4000 years. The first symptoms of what would later be named malaria were described in ancient Chinese medical writings, the *Nei Ching*, the Canon of Medicine.[5]
In the 4$^{th}$ century BC, malaria became responsible for the decline of many of the city-state populations in Greece. A correlation between malaria and mosquitoes was described by a number of roman writers that attributed malaria disease to the swamps.[6]

During the second century BC, in China, the Quinghao plant (*Artemisia annua*) was described in the medical treatise, *52 Remedies*. The discovery of Quinine, a potent antimalarial, happened in the early 17$^{th}$ century with the arrival of Spanish Jesuit missionaries at the "New World" [7].

The discovery of the malaria parasite occurred on 1880, by a French army surgeon, Charles Louis Alphonse Laveran. Laveran was awarded the Nobel Prize in 1907 for his discovery [8].

The naming of human malaria parasites was achieved between 1890 and 1897. The names of *Plasmodium vivax* and *P. Malariae* were introduced in 1890 by two italian investigators. An american scientist review the subject and named the third parasite*: P. Falciparum.* The *P.ovale* and *P.knowlesi* were named in 1922 and 1931 respectively [8].

On 1897, Ronald Ross, a British officer was the first to demonstrate that malaria parasites could be transmitted from infected patients to mosquitoes. Ross also showed that parasites

had a sporogonic cycle, where the parasite developed inside the mosquito. Ross was awarded the Nobel Prize in 1902.

The process of malaria transmission was completely achieved in 1899, with the understanding of the complete sporogonic cycle [9].

In the beginning of the 20th century, malaria was not confined to the tropics (see figure 1).The American Midwest and Scandinavia were two endemic zones of malaria. However, because of the decline in contact between human and vector populations due to the rising prosperity, malaria was in recession in these areas [5].

This recession was aided by measures of vector control which began to be implemented, such as reducing the mosquitoes breeding sites by insecticides applications and controlling water levels in rivers. To purge humans of parasites that might otherwise sustain transmission, quinine was distributed. [10]

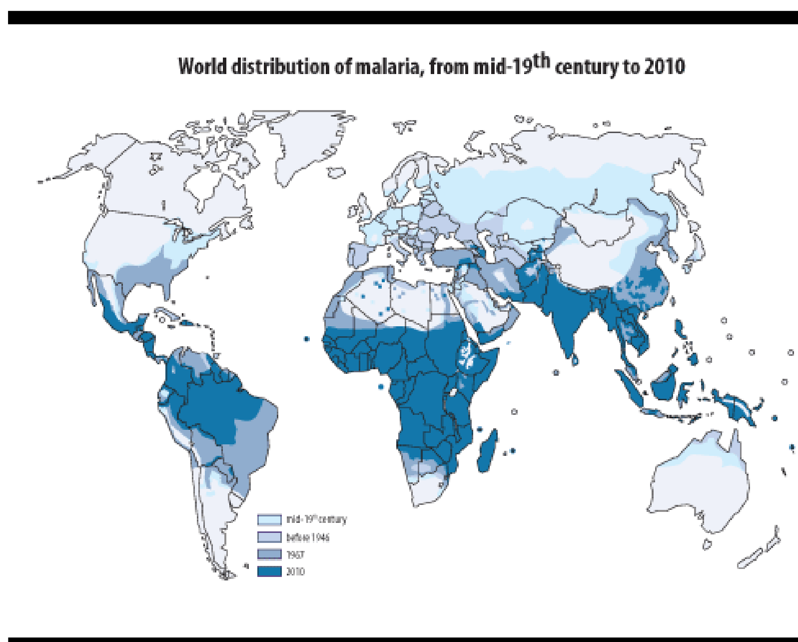



**Figure 1**: An illustration of malaria endemic areas, from the 19th century (light blue) to 2010 (dark blue). Adopted from [11].

The first global strategy for the fight against malaria was adopted in 1955, after World War II. The Global Malaria Eradication Program, a WHO proposal, called for massive and rapid application of DDT (dichlorodiphenyltrichloroethane) to interrupt transmission of the disease in countries around the world. They were the "DDT Golden days". The figure 2 is representative of the massive use of DDT

**Figure 2:** Extensive use DDT at the beach. Adopted from [12].

Eradications efforts began and focused on house spraying with residual insecticides, antimalarial drug treatment, and surveillance. This eradication program was performed in four successive steps: **preparation**, **attack**, **consolidation** and **maintenance**.
Some countries such as India and Sri Lanka had sharp reductions in the number of cases, followed by increases to substantial levels after efforts ceased. Other nations had negligible progress, and others were excluded completely from the eradication campaign (most of sub-Saharan Africa)[13].

Nowadays, the emergence of drug resistance, insecticides widespread resistance, wars and massive population movements, difficulties to obtain sustaining funding from donor countries and lack of community participation made the long-term maintenance of the program unbearable.
Malaria remains one of the most prevalent and deadly infectious diseases across Africa, Asia, and America, in the tropics and subtropic regions [11].
Recent increases in resources, political will and commitment have led again to discussion of the prospects of malaria elimination and, ultimately, eradication.

### *1.1.2 Etiologic Agent – Plasmodium gender*

Malaria is a complex result of the interaction between man, vector and parasite in an environment conductive to the transmission of the pathogen.
The *Plasmodium spp* is the etiological agent of malaria. There are around 155 species but only 6 species are known to cause malaria in man. *P. ovale*, *P. vivax* and *P. falciparum* only occur in man, whilst *P. malariae* is found in man but has also a non-humn primate reservoir. *P. knowlesi*

and *P. cynomolgi cynomolgi* are known to infect occasionally man, once they affect preferentially non-human primates [14].

Malaria control and elimination efforts are primarily directed to the four species of parasites refered previously, *P. vivax*, *P. ovale*, *P. malariae* and *P. falciparum* [15].

*P. vivax* has an extensive distribution, and it is found in most of the endemic malaria regions while *P. ovale* is a rarer parasite and is found mostly in Africa. Both P. *malariae* and *falciparum* are found in tropics.

The pathogenicity is most marked with *P. falciparum* with fatalities rates up to 30%. Most of the fatal cases of *P. falciparum* are hyper-infections with cerebral malaria. [16]

The mosquito *Anopheles* (figure 3) is the biological vector responsible for malaria transmission. Only female anopheline mosquitoes are able to transmit malaria since the male anophelines do not feed on blood. Once the mosquitoes *Anopheles* are night active, the period with higher probability of a bite and consequently transmission is between sunset and sunrise [17].

**Figure 3**: Image of the *Anopheles* mosquito. Adopted from [18].

### *1.1.2.1 Lifecycle of Plasmodium parasites*

*P. falciparum* and *P. vivax*, the parasite species primarily responsible for malaria in humans, have complex lifecycles. Both parasites have multiple developmental stages, each one with its preferred target cell type in humans or mosquitoes, making it a challenge to eliminate infection. [19]

Following the bite, the infected female injects sporozoites of the malaria parasite *Plasmodium* into human bloodstream.

Within 30 to 60 minutes, the sporozoites enter the victim's liver cells. In these cells, they reproduce asexually, forming thousands of merozoites. The merozoites are later released into the blood and invade red blood cells, causing their rupture, releasing more merozoites.
The rupture of infected erythrocytes causes malaria symptoms: fever, chills and progressive anemia. As a result of this invasion, severe anemia, clogging of blood vessels in the brain and in lungs among other organs, may occur, leading to death.
However, the lifecycle doesn't stop here. Some of the merozoites, eventually, develop into male and female gametocytes which can be ingested by an infected mosquito taking a blood meal. At last, sexual reproduction occurs in mosquito's gut, producing an oocyst that releases sporozoites. Once they travel to the mosquito's salivary glands, they are ready to start the cycle all over again [8]. The cycle of life of the parasite is illustrated in the figure 4.

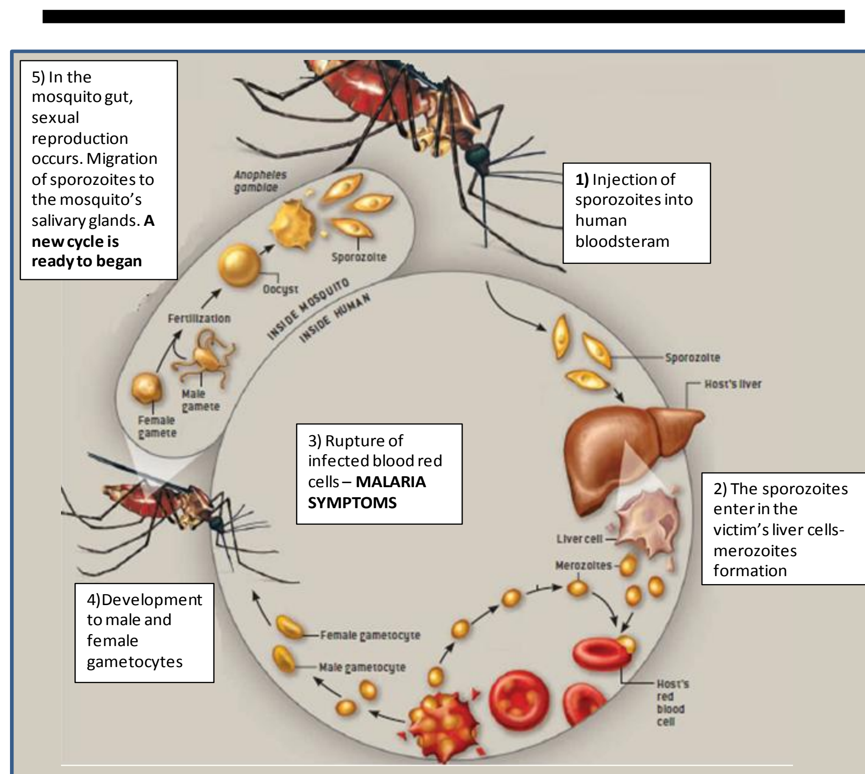



**Figure 4:** Representation of a malaria parasite lifecycle inside a mosquito and inside a human body. Adapted from [10] and [19].

### 1.1.2.2 Haemozoin – A Malaria Pigment

As seen previously, the malaria parasite has a pathogenic blood stage in human. 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 process occurs in an acidic organelle within the parasite called a digestive vacuole (or food vacuole) and is catalyzed by a battery of proteolytic enzymes [21].

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 iron of the heme group is oxidized from Fe (II) to Fe (III) – hematin or also designated Fe(III)PPIX, state by a mechanism that is not yet fully explored, but is presumed to involve $O_2$ as oxidant. Haematin is known to be toxic to microorganisms [22].

By converting haematin to haemozoin, the parasite removes the hematin permanently from the solution and deposits the iron porphyrin in a relatively innocuous crystalline form – the haemozoin (figure 5).

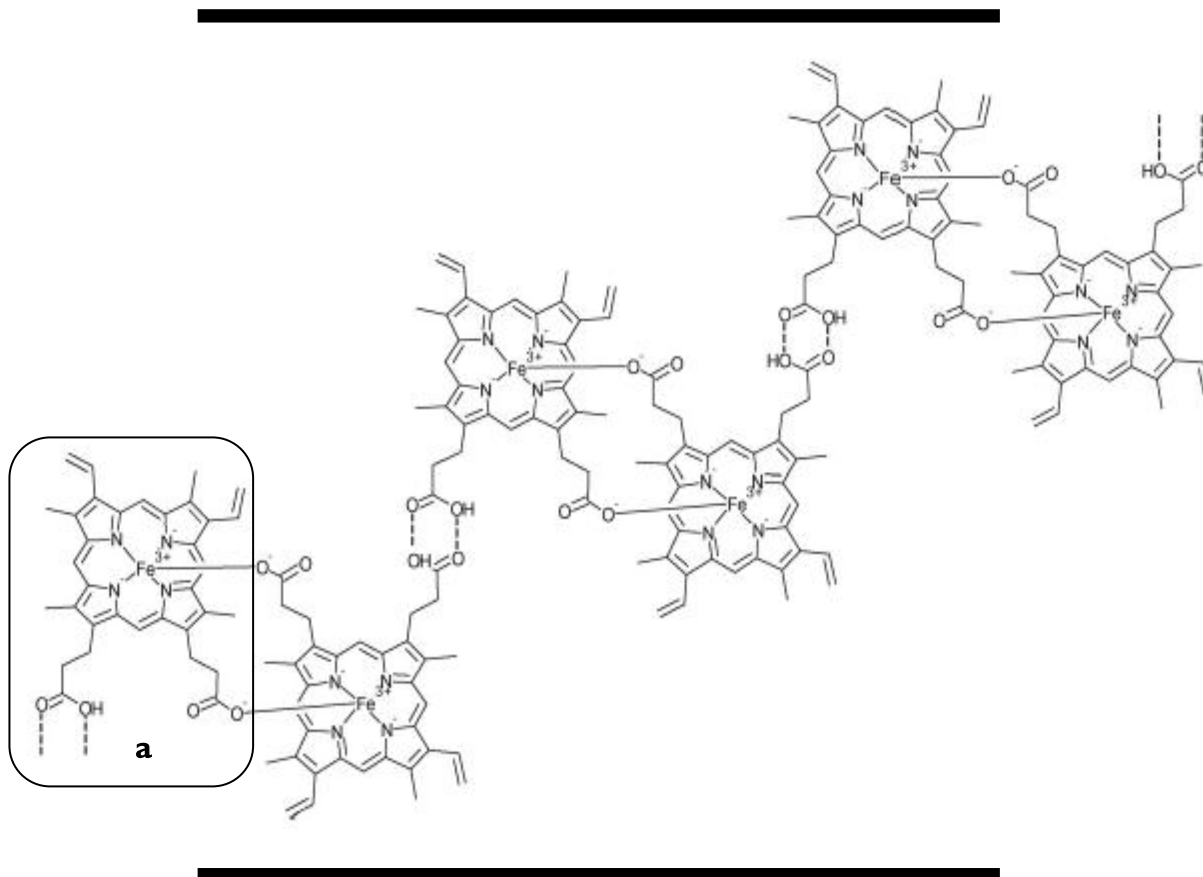



**Figure 5:** Representation of the Haemozoin. A heme group (a) is a prosthetic group that consists of an iron atom in the centre of a heterocyclic porphyrin ring (protophorphyrin IX). Haemozoin consists of dimmers of Fe(III)protoporphyrin IX (hematin), with the deprotonated propionate group of each porphyrin coordinated to the Fe(III) centre of the other. Adapted from [23].

Formation of haemozoin appears to be a detoxification mechanism which packages the potentially harmful Fe(III)PPIX in a highly insoluble and compact crystalline form, essential to the survival of these parasites.

Several antimalarial drugs like chloroquine and mefloquine act by interfering with the haemozoin formation by inhibition of the biocrystallization [24].

Artemisin and its derivatives are another class of drugs thought to exert its effect by interfering with the plasmodial hemoglobin catabolic pathway and inhibition of Haemozoin formation [24,25].

### *1.1.3 Antimalarials drugs and the resistance problem*

As seen previously, the malaria parasite has a complex life cycle and in order to eradicate the disease, every stage should be considered for treatment.

A mosquito control, anti-*Plasmodium* treatments and a general improvement of hygiene and sanitation are ideal strategies to take into account.

The exclusive use of antimosquito measures, such as insecticides (DDT) helped in the eradication of malaria around 1979s. However, besides its toxicity, the insecticides are efficient indoors and most of the bites happen outdoors. Resistance of anophelines to insecticidal and larvicidal agents like the chlorinated hydrocarbon has been notified, leading to a loose of interest in this kind of "weapon".

Many efforts to avoid the first contact between mosquitoes and humans are beginning to win some space in the mosquito stage. A recent notice the development of an application for cell phones that send a mosquito repellent sound [27], is an example of such efforts.

The oldest treatment against malaria parasite is quinine (figure 6). Quinine is a natural white crystalline alkaloid, occurs naturally in the bark of the cinchona tree and has antipyretic, analgesic, anti-inflammatory and antimalarial properties [28], [29]. Despite the mechanism of action is not totally understood, it is thought that quinine is a schizonticide, active only against malaria pigment producing stages [30], [31]. Being alkaline, the drug reaches in high concentrations within the food vacuoles of the parasite and raises its pH, inhibiting the biocristalization of the heamozoin.

Because of its severe side effects like cardiotoxicity, ototoxicity and ocular toxicity, quinine is no longer a first line treatment for uncomplicated malaria. The use of Qualaquin® (quinine sulphate) remains a FDA approved emergence treatment for uncomplicated malaria caused by the *P. falciparum* for infected patients that arrive to the USA [29-31].

Since early, chloroquine (figure 6) proved to be one of the most successful and important drugs ever deployed against an infectious disease [35]. Chloroquine is a 4-aminoquinoline, Being alkaline, and similarly to quinine, the drug reaches high concentration within the food vacuoles of the parasite and raises its pH, inhibiting the biocristalization of the heamozoin.
 Chloroquine also inhibits the parasitic enzyme heme polymerase that converts the toxic heme into non-toxic hemazoin, thereby resulting in the accumulation of toxic heme within the parasite. However, due to its massive use, the development of resistances did not take long and nowadays, chloroquine, as a single drug, it isn't a first line therapy [33,34]. Resochina® is the commercial name of chloroquine.

Mefloquine (figure 6) is a synthetic derivative of quinine used in malaria treatment. It is the only registered drug effective in a single dose [38] and the commercial name is Mephaquin®. This drug is another example of other blood schizontocidal antimalarial drug, inhibitor of the heme detoxification. The serious neuropsychiatric side effects and notifications of resistance, diminishes the use of this drug [39].
 The combination of pyrimethamine (inhibitor of folic acid redutase) and sulfadoxine (antagonist of the PABA- enhancer of the antimalarial activity of other drugs) is a blood stage drug, effective as a single dose is ®, although the problem of resistance is documented [40], [41].

Other association with resistance documented cases is Malarone® (atovaquone and proguanil). Atovaquone inhibits mitochondrial electron transport at the level of the cytochrome $bc_1$ complex and collapses the electropotential across the mitochondrial inner membrane[42]. Proguanil is an acid folic reductase inhibitor and despite their action in multi stages of the parasite cycle of life, the use of proguanil is preferential in associations once the parasite develops resistance in a short period of time[43].

The liver stage is one of the most relevant steps to consider in the disease development. Once the parasite reaches the liver, it starts to replicate there. *P. vivax* and *P. ovale* can remain dormant in the liver once they form hypnozoines [44] (not shown in lifecycle scheme). Primaquine, an 8-aminoquinoline (figure 6) is the only drug approved to eliminate hypnozoites. The principal biotransformation process leads to an oxidant metabolite that will destroy the parasite in the liver stage, where the hypnozoites are.

Another important class of drugs are the artemisin-based combined therapies (ACT). These therapies are the current standard of malaria treatment [37]. Artemisin (figure 6) is an endoperoxide sesquiterpene lactone produced by a Chinese herb and is poorly soluble in water. Two derivatives, artemether and dihydroartemisin were synthesized in order to solve the solubility problem.

Artemisin and its derivatives have a fast onset of action. However, due to its fast clearance, artemisin derivatives are combined with slow-clearing drugs to kill residual parasites [45]. Two combinations are known, Coartem® contains artemether and lumefantrine, a antimalarial drug with the same mechanism of action of chloroquine and mefloquine. Eurartesim® is another combination therapy of dihydroartemisin and piperaquine, a bisquinolone with antimalarial properties thought to act blocking the metabolism of the parasite, approved in 2011 by EMA (figure 6).

Based in the mechanism of action of the endoperoxide bond of the artemisin, some synthetic compound are a fundamental *via* in the development of antimalarial drugs.

OZ439 (figure 6).is a second-generation peroxide with promising antimalarial results. The trioxane moiety in this compound mimicries the artemisinin endoperoxide. This molecule has significant prophylactic activity and is now in clinical trials, phase II studies [45] [46].

RKA182 (figure 6) is also a synthetic drug candidate, with a tetraoxane function, a more stable endoperoxide than trioxane. [47]

Another compound in clinical trials, phase II is NITD609 (figure 6). NITD 609 belongs to a compound collection identified as spiroindolones. It is a potent inhibitor of gametocytogenesis and block transmission to mosquitoes. The Medicines for Malaria Ventures (MMV) selected the spiroindolone project as the Project of the Year 2009 [42,43]

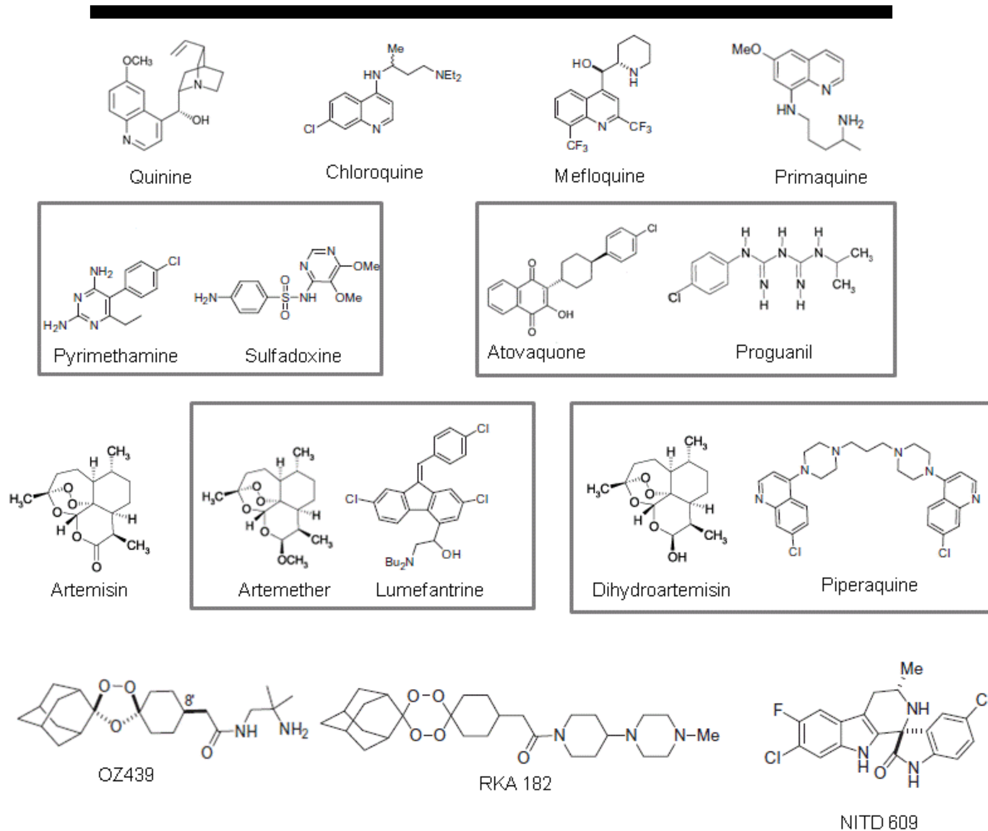



**Figure 6**: Representation of antimalarial drugs structures. The combination drugs are inside squares.

## *1.2 BIOACTIVE STEROLS*

*"Steroids constitute a family of products with great therapeutic value. In this way, beyond the effort to design and synthesize new compounds, there is a great interesting in the isolation and identification of natural steroids with undisclosed bioactivities."*

Carvalho, J.F.S. Bioactive Sterols: Structure, Antitumoral and SAR studies, 2010

### *1.2.1. Natural Products - 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 [44,45].

Steroids are a group of complex organic compounds, more specifically a group of lipids, which chemical structures are characteristics of the living world. They comprise a wide collection of structurally related natural compounds with important functions *in vivo*. In fact, they may act as physiological regulators, regulating for example the properties of cell membranes in mammalian cells [46,47], as hormones like testosterone and estrogen ,[48,49] and even as provitamins [56].

Structurally, the cyclopentaneperhydrophenantrene hydrocarbon framework of steroids possess a tetracyclic carbon skeleton. This skeleton has seventeen atoms of carbon, bounded to form a molecule, composed of three fused cyclohexane rings (A, B and C) and a cyclopentane ring (D) (figure 7) [57].

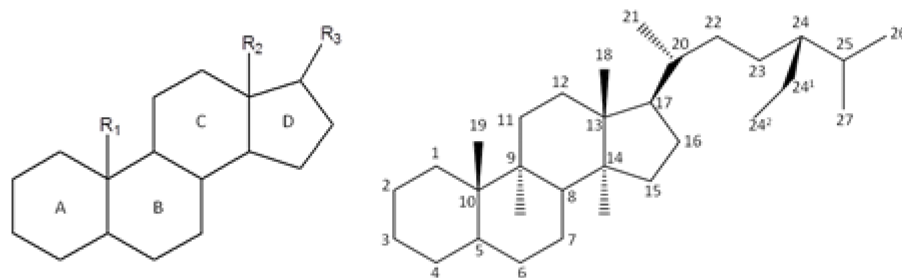



**Figure 7:** Representation of the steroid skeleton. Numbering of the steroid carbons and side chain.

Usually, the $R_1$ and $R_2$ are methyl groups while $R_3$ is a more or less extended carbon chain. As observed in **Table 1**, the combination of the substituents $R_1$, $R_2$ and $R_3$ defines the steroid class.

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

| | *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 |

The variations of $R_1$, $R_2$ and $R_3$ as well as nuclear substitutions with different functional groups, number of unsaturations and severals nucleus configurations are responsible for the

natural diversity of steroids. Not only the chemical reactivity but also the biological activity is affected by all these structural features.

Due to the ring fusions, steroids are rigid molecules and can assume different three dimensional structures according to the fusion type, along with different chiral centers.
Most of the biologically active steroids present an unsaturation at C4 (testosterone, progesterone and aldosterone) and C5-6 (cholesterol and pregnenolone).

Due to their great value in therapeutics for several pathologies like cancer or neurologic diseases, [52-55], 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.

### *1.2.2 Sterols*

Sterols, a group of steroids compounds possess a 3β-hydroxyl group and an aliphatic chain at C17 in the tetracyclic carbon skeleton (figure 7).
Because of the refered characteristics, sterols are usually amphiphilic molecules, 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 [62].

#### *1.2.2.1 Cholesterol and its function in cells*

One of the most important and studied sterol is cholesterol. Cholesterol is present in plants and in some marine red algae, and it represents more than 99% of the sterols in mammals.[57-59]
Cholesterol is a 27 carbon polycyclic alcohol, with a β-hydroxyl group at C3, an unsaturation at C5-6 and an eight-carbon side-chain in the C17 position. Because of the double bond at B-ring, cholesterol is prone to oxidation processes.

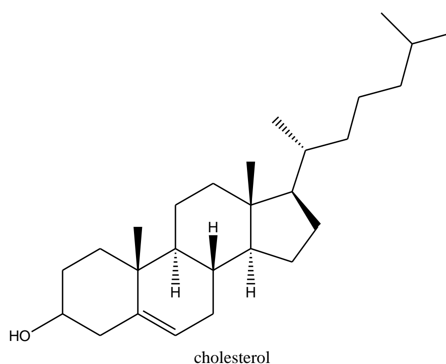



**Figure 8:** Representation of the cholesterol chemical structure.

Cholesterol 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.

In eukaryotic cells, 40-90% of the free cholesterol, upon the cell type, resides in the plasma membrane, while only a residual amount of cholesterol esters (5-10%) is stored in the cytoplasm as lipid droplets [66].

The percentage of free cholesterol present in plasma membranes plays a principal role as modulator of fluidity and phase transitions in the plasma membrane. It is also fundamental for the formation of plasma membrane rafts or caveolae, which are microdomain sites depending on cholesterol, where signaling molecules are concentrated and consequently involved in the regulation of many biological functions [67].

### *1.2.3 Oxysterols*

The oxidation of cholesterol leads to a group of oxygenated derivatives of cholesterol named oxysterols. These derivatives usually contain one or more additional oxygen atoms in the steroid skeleton or in the lateral chain. However, according to the oxygen positions, differences in the biophysiological properties and on the transfer ability through membranes, can be observed [68].

The isolation of the first oxysterol, 7β-hydroxycholesterol from bovine liver was in 1939 [69]. On the 70s, after almost thirty years of its isolation, the biologic effects started to be known, specially its proliferative effects [68].

Their selective synthesis won its heyday in the last years, due to the relevant bioactivity and the wide range of biological phenomena in which they are involved. Apparently, their activity covers fundamental and manifold processes as the cholesterol homeostasis, inflammatory disease, cellular differentiation and atherosclerosis [70].

More recently, antiviral and antifungal properties have been described for this complex group of compounds [65-67].

*In vivo,* oxysterols can be the result of the oxidation of cholesterol, involving oxidative enzymes or non enzymatic oxidations (figure 9).

The oxysterols formed due to enzymatic oxidation can be used as endogenous markers once the balance between synthesis, degradations and cholesterol conversion by the enzymatic via is a CYP 450 process [74] [75].

The non enzymatic *via* to form oxysterols consists auto oxidations of the cholesterol molecule. These oxidations may occur inside the cell membranes or inside lipoproteins. The ROS are determinant for this *via* of cholesterol oxidation (figure 9).

These non enzymatic oxidations occur mostly in ring B unsaturation (5,6-double bond) and in the 7-position, vulnerable to the attack of free radicals or transition metals radicals. The auto oxidation may also occur in the tertiary carbons of the side chain; however this oxidation is not so common [69,70].

The allylic position in C4 also represents a putative oxidation position although ,due to the 3-hydroxyl group and the trisubstituted C5 this oxidation rarely occurs [77].

One of the major functions of oxysterols is their role in the cholesterol metabolism. Their extra oxygen atom and the amphiphilic nature allows them to cross cell membranes more easily than cholesterol, helping the cholesterol output from the organism[78].

A hypothesis of the oxidized form of cholesterol could function as an *in vivo* antioxidant was been discussed [79] but more studies are required.

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 [73,74].

The mediation of cholesterol oxidative derivatives in osteoporosis is also a point of interest in the this disease, However, this mediation is not completely clear and contradictory information exists [75-77].

The oxysterols have been reported to have some level of cytotoxic selectivity, though, some anticancer properties [52,62,64]. One of the cytotoxic mechanisms is the increase of the sensitivity of tumor cells to irradiation and to other antineoplasic agents.

Based on this hypothesis, the use of oxysterols as part of a hybrid drug can present a major advantage.

### *1.2.4 Steroids as antimalarials*

The association of malaria and steroids isn't recent. 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 [85], [86]

Furthermore, the skeleton nucleus of cholic acid derivatives have been used as carriers for 1,2,4,5,-tetraoxacycloalkenes as antimalarials and start to be investigated more deeply in 2000 [87]. After 2000 more studies were performed using cholic acid and endoperoxide bond [88], [89], however the evaluation of the steroidal moiety to antimalarial activity was not evaluated.

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

### *1.3 HYBRID DRUGS – A NEW STRATEGY*

*"At the border between bio-inspired design and rational design,*
*one can imagine preparation of hybrid molecules with*
*a dual mode of action to create efficient new drugs."*

Meunier, B, Hybrid Molecules with a Dual Mode of Action: Dream or Reality?, 2007

The drug discovery process is highly challenging for scientific reasons, time needed and money consuming. These factors represent tremendous costs for the R&D companies, costs that are very difficult or almost impossible to afford.

The synthesis of hybrid drugs represents a viable alternative to these costs. However, this is not the main reason of what is expected to be "The Golden Age of Hybrids"

A major driving force and motivation in the hybrid drug development is to overcome one of the worst things that can happen to a drug: the development of resistance in its target population.

So, a hybrid molecule is a chemical entity with two or more structural domains, pharmacophores, having different biological functions and dual activity.

Hybrid molecules can be classified as:

A) <u>Conjugates</u> - 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;

B) <u>Cleavage conjugates</u> – have a linker designed to be metabolized to release the two drugs that interact independently with each target;

C) <u>Fused hybrid</u> – the two farmacophores are connected, the size of the linker decreased such that the framework of the pharmacophores is essentially touching;

D) <u>Merged hybrid</u> – have their frameworks merged by taking advantage of commonalities in the structures of the starting compounds. Usually are smaller and simplest molecules.

One of the major advantage of the research area of the hybrid compounds is the synergic effect that only one molecule can have in a specific disease, instead of the need of using different molecules for that disease. Another advantage is that the lower risk of drug-drug adverse interactions compared to multicomponent drugs.

In the development of a hybrid molecule, some of the physical and chemical properties of the moieties used will be changed, such as pKa, liposolubility, among others. One of the critical points of the hybrid drug research is the improvement of some of those characteristics, for example, the solubility of one molecule can be improved adding a more hydrophilic group present in the second moiety of the hybrid. However, due to the changing molecules together, it is imperative to ensure the toxicity levels of the hybrid drug, as well as define the therapeutic range [90]. Once the hybrid drug is synthesized, this molecule has to be considered as a new chemical entity.

### *1.3.1 Hybrid drugs in the treatment of malaria- the importance of the endoperoxide bond*

Recently, through rational drug design approach, single hybrid molecules with dual functionality and/or targets and none or minimum toxicity has been developed as novel antimalarial drugs. So far none of these hybrid antimalarials have reached clinical applications [91]

Most of the hybrid drugs developed newly for the treatment of malaria present a singular characteristic, an endoperoxide ring [22], [37], [85-87]

This fact is easly explained once the endoperoxide ring is an essential farmacophore for the antimalarial activity of the model, the artemisin.

The peroxide moiety in artemisinin reacts in the presence of the flat achiral iron (II)-heme. The reductive activation of the peroxide function generates a short-lived alkoxy radical, which quickly rearranges to a C-centered primary radical.

This radical alkylates heme via an intramolecular process to produce covalent heme-drug adducts. A consequence of heme alkylation leads to the increase of non-polymerizable redox-active heme derivatives, thought to be toxic for the parasite.

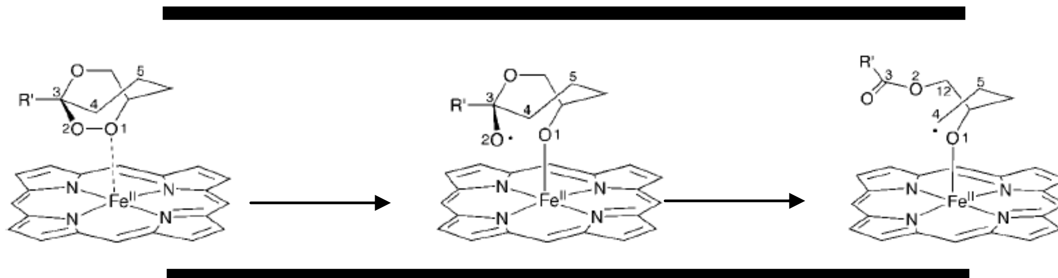


**Figure 9:** Schematic representation of the interaction of the trioxane and the heme group. The tetraoxane has similar mechanism.

Based on this mechanism of action, new hybrid molecules with an endoperoxide bond and dual mode of action have been designed.
Synthetic trioxanes as well as trioxolanes and tetraoxanes alkylate the heme group (figure 9).

The first molecules synthesized with an endoperoxide ring were trioxaquines, the covalently linkage of a trioxane moiety with a quinoline entity in a single molecule [95]. As the trioxane or trioxolane moiety is a potential alkylating agent after reductive activation by heme, and the 4-aminoquioline entity easly penetrates into infected erythrocytes and then interacts with heme, such modular molecules are expected to combine the dual activity of both fragments [96].

More recent studies on endoperoxide stability have shown that 1,2,4,5-tetraoxanes have significantly higher stability than the 1,2,4-trioxanes [89,90]. 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.

# CHAPTER II

# SYNTHESIS AND CHARACTERIZATION OF NEW HYBRID ANTIMALARIALS

***Synthesis and Characterization of New Hybrid Antimalarials***

*"In the coming years, the world will need all*
*the malaria weapons it can muster. After all, malaria not only kills,*
*it holds back human and economic development. ´*
*Tackling is now an international imperative."*

*Dunavan, C.P. Tackling Malaria, 2005*

The importance of an endoperoxide moiety as an inhibitor of the haemozoin formation appears to be crucial in the development of antimalarial hybrid drugs. Based on this mechanism, antimalarial hybrid drugs with a tetraoxane and a steroidal moiety are synthesized in this work.
Thus, this thesis consists in two parts; the first one describes the synthesis of the oxysterols intermediates while the second part describes the exploratory work to the synthesis of oxysterol tetraoxanes hybrid compounds.

Recently, Sá e Melo and her co-workers prepared oxysterols with surprisingly antimalarial properties, such as compounds (**4**), (**7**) and (**9**). Their antiplasmodial activity against *P.falciparum* W2 (chloroquine resistance) present $IC_{50}$ values under 10 micromolar concentrations (unpublished results).
Based on this knowledge, the synthesis of three oxysterols was planned, the 5α-hydroxy-6β-methoxycholestan-3-one (**5**), the cholest-5-ene-3,7-dione (**8**) and the 5α-hydroxy-3-oxocholestan-6β-yil, 6-hemiphthalate (**11**). The difference between the compounds (**4**), (**7**) and (**9**) is related to a ketone group at C3, essential to the formation of a dihydroperoxide and sequentially the tetraoxane moiety.

A simpler molecule, the 5α-cholest-3-one (**20**) without functional groups in the steroid backbone was also used as starting material for the reactions under research.

The strategy to build a 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 its proved antimalarial activity and chemical stability, when compared with artemisin [99].

Moreover, it can be used as a substitute for artemisin, when resistance becomes to emerge. The therapeutic target of these endoperoxides is the blood stage of malaria disease, allowing alleviating symptoms in the patients.

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 may contribute to balance the physical and chemical properties of a steroid scaffold while the steroid nucleous may improve the transport of the tetraoxane for inside cells.

The attachment of chemical diverse cycloalkanones can be important to modulate physiological characteristics of the whole hybrid. Moreover, this type of scaffold can be design to allow the attachment of liver stage active molecules, as 8-amino quinolines.

The syntheses of these oxysterol tetraoxane hybrids were performed taking into account the processes global costs, the reagents toxicity and the reuse of solvents. The use of a lipase in a synthetic step was another synthetic methodology considered.

The second part of the experimental work, describing the development of a method for the synthesis of hybrid molecules and the characterization of these compounds, was started in the faculty of pharmacy of the University of Lisbon and has been developed in the laboratory of pharmaceutical chemistry in the faculty of pharmacy of the University of Coimbra.

### *2.1. Synthesis of Oxysterols Intermediates*

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

For the synthesis of the 5α-hydroxy-6β-methoxycholestan-3-one (**5**), the cholesterol acetate (**1**) was the starting material chosen and four steps were necessary to achieve the intermediate (**5**). The first three procedures have been extensively described [100].

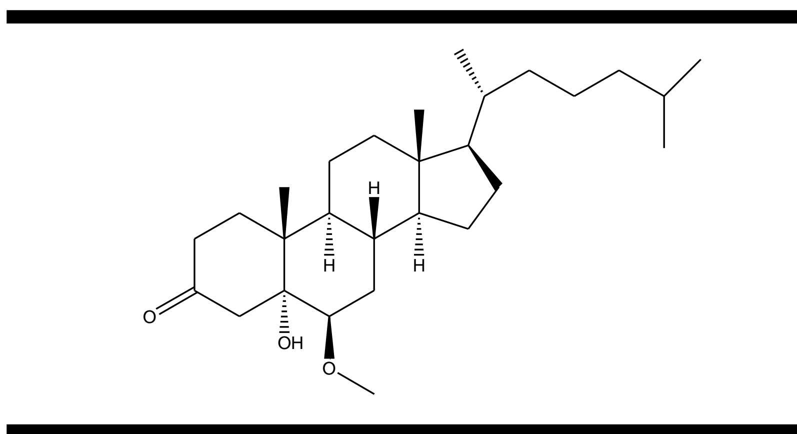



**Figure 10:** Chemical structure of the 5α-hydroxy-6β-methoxycholestan-3-one (**5**).

The first intermediate was the 5,6-epoxycholestan-3-yl acetate (**2**) as epoxidation of cholesterol acetate (**1**) using a magnesium salt, the magnesium bis(monoperoxyphthalate) hexahydrate (MMPP) commercially available and inexpensive[101]. It emerged as an alternative to the expensive and potentially explosive oxidant reagent, *meta*-chloroperoxybenzoic acid, mCPBA [102].
As the result of the epoxidation reaction, a mixture of two compounds was obtained, the 5α,6α- and 5β,6β-epoxysteroids in a good yield (93,6%) with a diastereoselectivity of 76:24 (α/β), according to the literature [101].

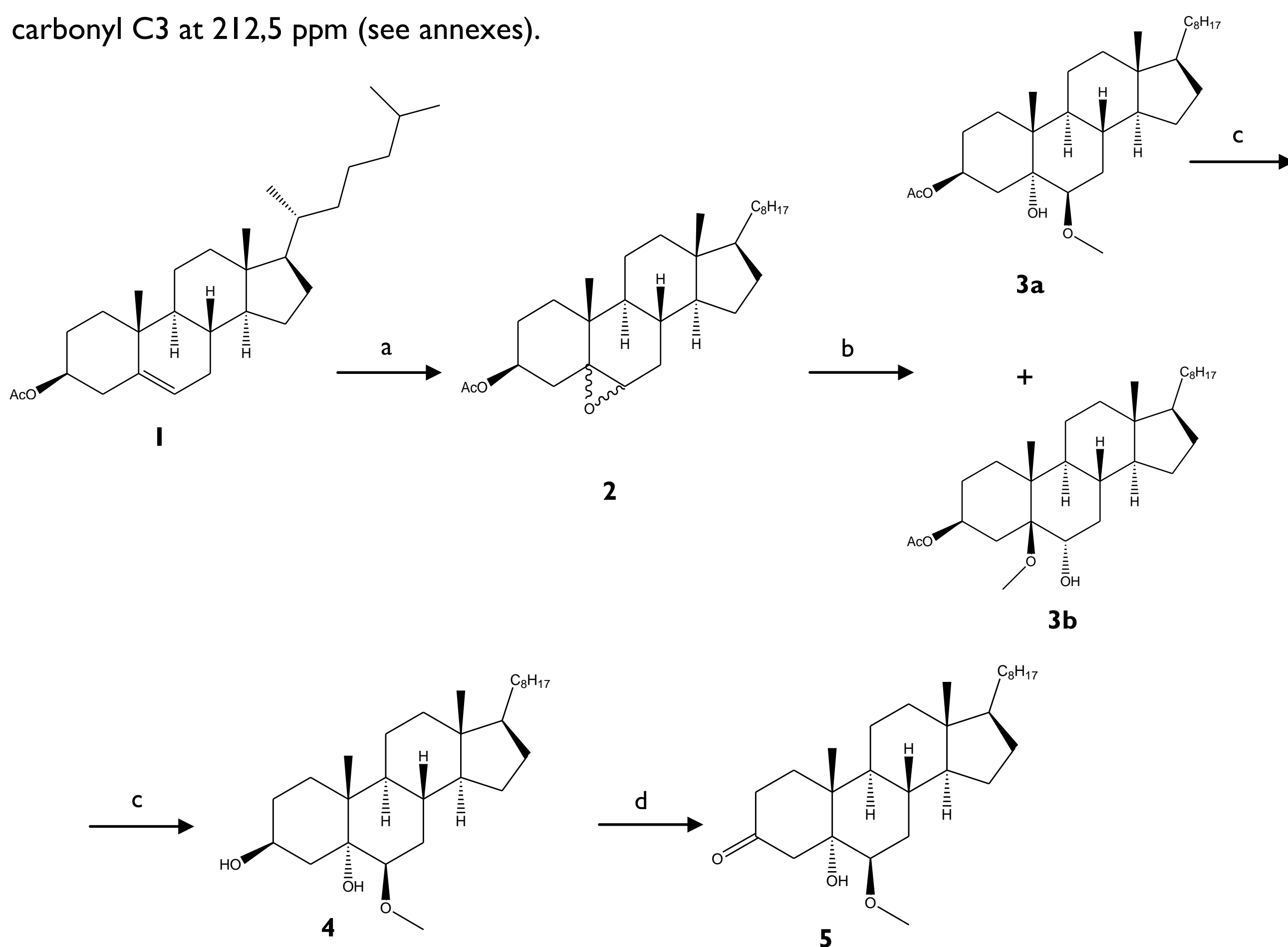
The next step was the epoxidation opening. This reaction was dependent of the catalytic properties of $Bi(OTf)_{3.}$
Due to the formation of isomers in the synthesis of the intermediate (**2**), the result of this reaction has been a mixture of two derivative compounds, (**3a**) and (**3b**) which have been separated by Flash Column Chromatography (FCC) with silica in a diastereoselectivity of 74:21, which reflect the ration in the mixture (**2**).

The reaction to afford the intermediate 6β-methoxycholestan-3β,5α-diol (**4**) from the corresponding 3β-acetate (**3a**) was an alkaline hydrolysis in EtOH. The advantages of an

alkaline hydrolysis, using the NaOH are the irreversibility of the reaction and an easy separation of the product [103]. The purpose of this hydrolysis was to remove the acetate group to form a primary alcohol.

Due to the need of a ketone group C3 to form the hybrid compound, the oxidation of the 3β-hydroxyl group was achieved via a Jones oxidation. This reaction is very rapid and exothermic so it is done in ice, and the yield was typically high, as expected [104].

The first intermediate to prepare an antimalarial hybrid was achieved. The compound (**5**) was confirmed by NMR techniques, comparing with the compound (**4**), characterized in [105]. A signal at 4.08 ppm (m, 1H, J=10.5; 5.2 Hz) corresponding to the 3α-H of the compound (**4**), disappear in the compound (**5**) $^{1}$H NMR spectrum, proving the oxidation of the 3-OH (see annexes). Observing the $^{13}$C NMR spectrum, it is possible to identify the carbonyl C3 at 212,5 ppm (see annexes).




**Scheme 1:** Synthesis of the 5α-hydroxy-6β-methoxycholestan-3-one (**5**). a) MMPP, $CH_3CN$, under reflux temperature, 20 min; b) $Bi(TOf)_3$, dry methanol, rt, 2h30; c) NaOH 10%, EtOH, $CH_2Cl_2$, rt, 2h; d) Jones reagent, dry acetone, ice.

*2.1.2 Synthesis of the cholest-5-ene-3,7-dione (**8**)*

The chosen strategy for the synthesis of another oxysterols moiety, the cholest-5-ene-3,7-dione (**8**), was again the cholesterol acetate (**1**) as starting material although the synthesis of this intermediate was shorter. The allylic oxidation to form a ketone group at C7 was performed in a single reaction. The acetate group was removed by an alkaline hydrolysis and a Jones oxidation to form a ketone group at C3, essential for the hybrid formation was performed.

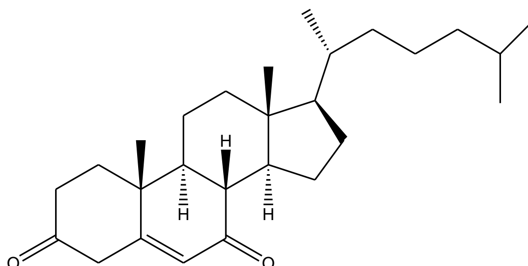
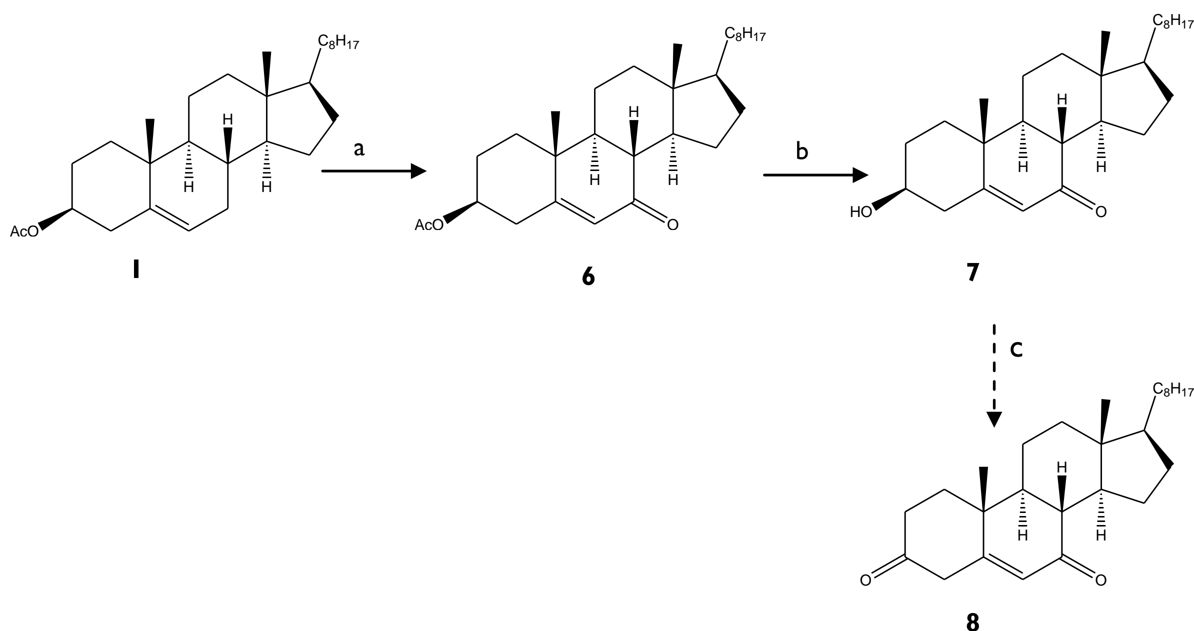



**Figure 11:** Chemical structure of the cholest-5-ene-3,7-dione (**8**).

An allylic oxidation at the cholesterol acetate using the *t*-BHP, *tert*-Butyl hydroperoxide as oxidant agent and copper iodide as a catalyst was performed to prepare the 7-oxocholest-5-en-3yl acetate (**6**) [106].
An allylic oxidation is defined as a reaction where a new C-O connection (in ketones, aldehydes, esthers and ethers) is formed in an allylic position [107].

An alkaline hydrolysis in EtOH was performed to remove the acetate group in C3 of the 7-oxo-cholest-5-en-3β-yl acetate (**6**) and form the tertiary alcohol (**7**). The conditions used were the same as used in the reaction described to achieve the intermediate **4**.

The final step to the synthesis of the diketone from the 3β-hydroxycholest-5-en-7-one (**7**) was an oxidation in C3 to form the ketone group, essential for the preparation of tetraoxane. To prepare the 3,7-diketone (**8**), a Jones oxidation was performed in the same conditions used to obtain the compound (**5**).




**Scheme 2:** Synthesis of the cholest-5-ene-3,7-dione (**8**). a) CuI, CH3CN, t-BHP, reflux temperature; b) NaOH 10%, EtOH, CH2Cl2, rt, 2h; c) Jones reagent, dry acetone, ice.

Despite the simple synthesis, the characterization of this product was not clear by $^1$H NMR. The IR spectroscopy was used as a complementary technique for this identification, although a FCC with silica was used as an attempt to purify the compound (**8**) was not successful. Further investigation to discover a more suitable oxidation method is needed as well as a more efficient method to achieve the pure compound (**8**).

### *2.1.3 Synthesis of the 5α-hydroxy-3-oxocholestan-6β-yl, 6-hemiphthalate (11)*

Phthalic acid is an aromatic dicarboxylic acid, with molecular formula $C_6H_4(CO_2H)_2$. It has a broad application in chemistry, especially in the synthesis of condutive polymers[98,99]. It is also known that phthalic acid acts as starting material to the development of insecticides. [100,101]. Its insectide properties may contribute to the treatment of malaria.

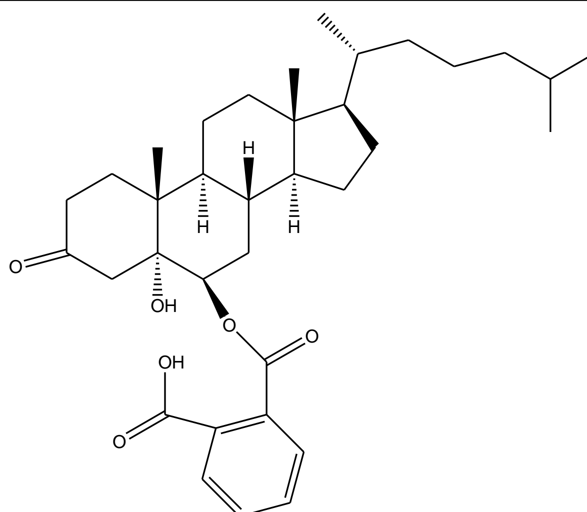



**Figure 12:** Chemical structure of the 5α-hydroxy-3-oxocholestan-6β-yil, 6-hemiphthalate (**11**).

*a) 1st attempt to the synthesis of the 5α-hydroxy-3-oxocholestan-6β-yil, 6-hemiphthalate (**11**)*

The starting material for the formation of the hemiphthalate (**9**) was the cholesterol acetate (**1**). This reaction was simple and the reagents were the same used in the epoxidation to form the compound (**2)**. However, the time for the complete consumption of the starting material (**1**), is much longer. The explanation for the time variation is related with the acidity in the reaction media, which in the 20 min of reaction is not enough to promote the epoxide opening.

Therefore, it is hypothesized that, the phthalic acid generated *in situ,* when the reaction is under reflux could act as nucleophile and attack the epoxide affording the hemiphthalate derivatives [100].

The reaction was checked by TLC and a product with very low Rf was observed, according to the literature [100] should be compared with (**9**) The product was purified by a careful FCC and the sample analyzed by NMR techniques. For instance, the protons corresponding to the aromatic ring are present at 7.3 ppm (1H, t, J=7.1Hz), 7.6 ppm (1H, t, J=7.0 Hz), 7.7 ppm (1H, d, J=7.1 Hz) and 7.8 ppm (1H, d, J=7.0 Hz) and the proton corresponding to the 6α-H at 4.36 ppm are similar to the signals present in literature [100].

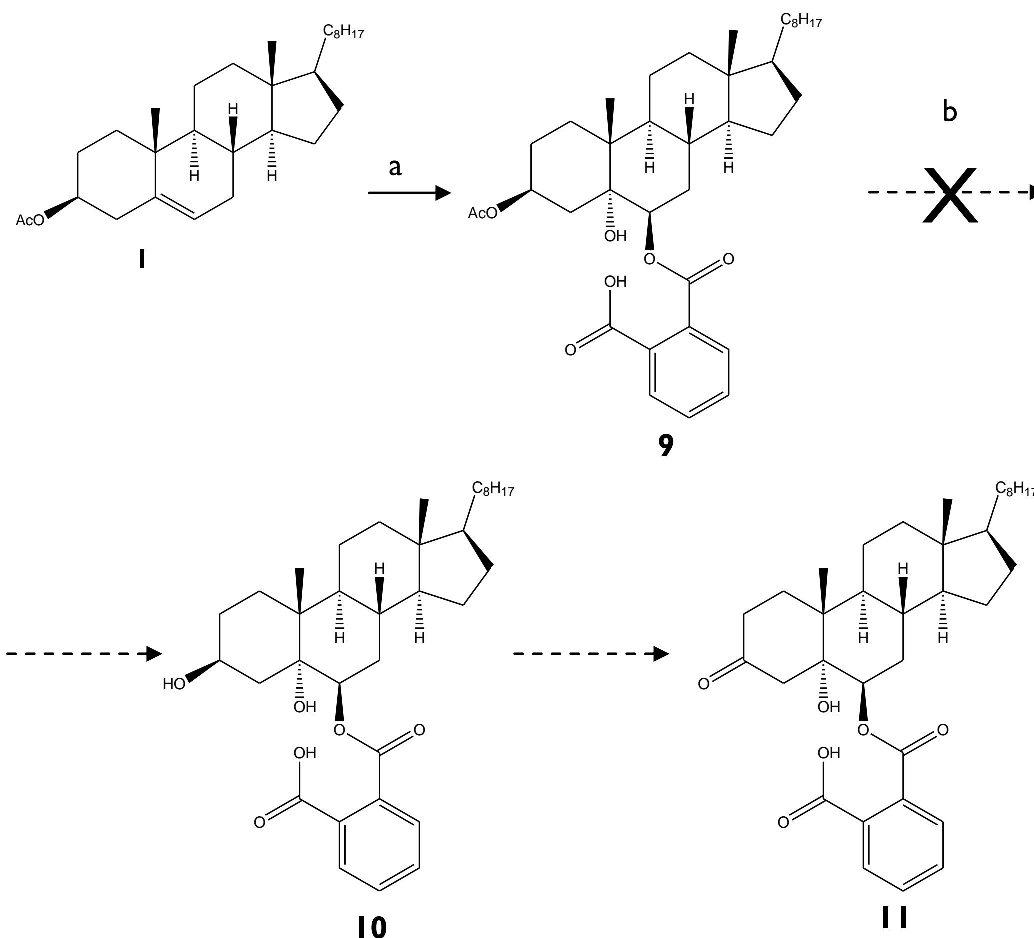
Since an alkaline hydrolysis would remove not only the Ac group, but also the hemiphthalate, mild hydrolytic conditions are needed.

Using an isolated enzyme, a triacylglycerol lipase (EC 3.1.1.3) catalyzes the hydrolysis of lipids and contributes to the lipid and energy metabolism of different tissues and can be used in this case [112]. Indeed this enzyme was already explored as a deacetylating agent for 3β-acetoxyepoxysterols [100].

The compound (**9**) was dissolved in saturated di-iso-propyl ether and after that, the enzyme was added to the suspension. The mixture was incubated at 45°C, 200 rpm. However, after one month of shaking at 45°C, no products were formed.

Probably, owing to the presence of a large group as the phthalic acid, the enzymatic hydrolysis didn't work as expected.

Further solutions will be required to overcome these difficulties in the preparation of the desired intermediate (**10**).




**Scheme 3:** 1st attempt to the synthesis of the 5α-hydroxy-3-oxocholestan-6β-yil, 6-hemiphthalate (**11**). a) MMPP, $CH_3CN$, reflux temperature, 48h; b) lipase. EC 3.1.1.3., di-iso-propyl, 45°C, 200 rpm, 31 days.

*b) 2nd attempt to the synthesis of the 5α–hydroxy-3-oxocholestan-6β-yil, 6-hemiphthalate (**11**)*

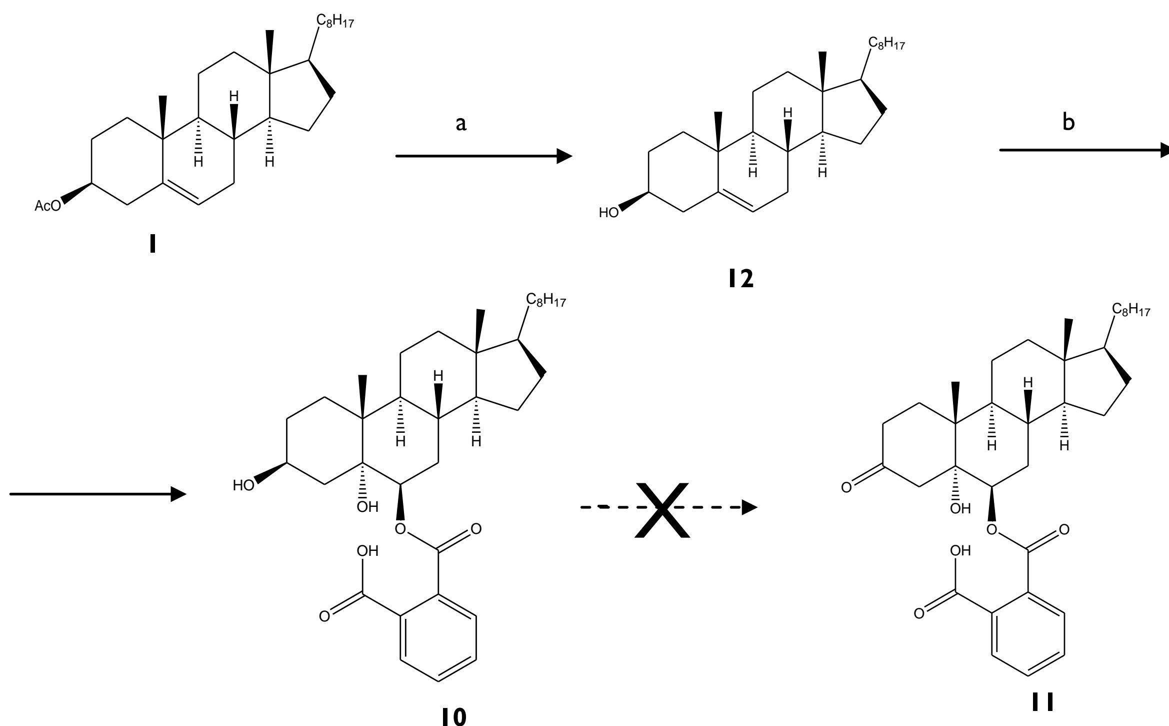
In order to solve the hydrolysis of the acetate group without removal of the hemiphthalate group, an alternative approach was investigate once the enzymatic method didn't work even after 1 month of incubation.

To overcome the hydrolytic step tried in the previous attempt, the starting material for this reaction was cholesterol (**12**), result of the cholesterol acetate hydrolysis.

Because of the acidic media and drastic conditions used of the Jones oxidation, the hemiphthalic group was removed as seen in the $^1$H NMR spectrum (the signals corresponding to the aromatic ring disappear). As an alternative reaction pyridinium dichromate (PDC) was used as oxidant agent. Pyridinium salt of dichromate is obtained by addition of pyridine to a solution of chromium trioxide in water.[113]

PDC is less acidic than pyridinium chlorochromate (PCC), a similar salt, and can be more suitable for the oxidation of acid-sensitive substrates.[106,107]

However, the $^1$H NMR analysis revealed that the aromatic group wasn't present, thought the hemiphthalate group was removed.




**Scheme 4**: 2ndattempt to the synthesis of the 5α-hydroxy-3-oxocholestan-6β-yil, 6-hemiphthalate (**11**). a) NaOH 10%, EtOH, CH2Cl2, rt, 2h; b) MMPP,$CH_3CN$, reflux temperature, 24h.

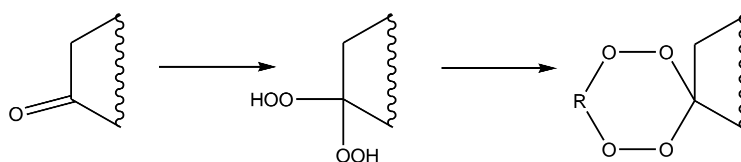
For a future investigation, we plan to use oxone as an oxidant agent since it has emerged as an alternative to traditional metal –mediated oxidations [116].

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

Efforts to build a tetraoxane ring using adamantanone or ethyl 4-oxocyclohexanecarboxylate group and an oxysterols moiety will be described.

An endoperoxide-based therapy appeared as an alternative for the first generation analogues of artemisinin, which have a limited availability, high cost and poor bioavailability.
The endoperoxide bridge is the crucial structure functionality within artemisinin and synthetic 1,2,4-trioxanes. However, studies of endoperoxide stability have shown that 1,2,4,5-tetraoxanes have significantly higher stability. The tetraoxanes appear to lack cytotoxic effects *in vitro* and to be safe *in vivo* [117].

The general scheme of an endoperoxide formation involves two steps: the first part comprehends the preparation of a dihydroperoxide from a ketone, while the second part consists in the coupling with a third moiety.




**Scheme 5**: General sequence of the formation of the endoperoxide bond.

Several procedures were executed in order to optimize the formation of the hybrid compounds. The methodologies will be presented and discussed.

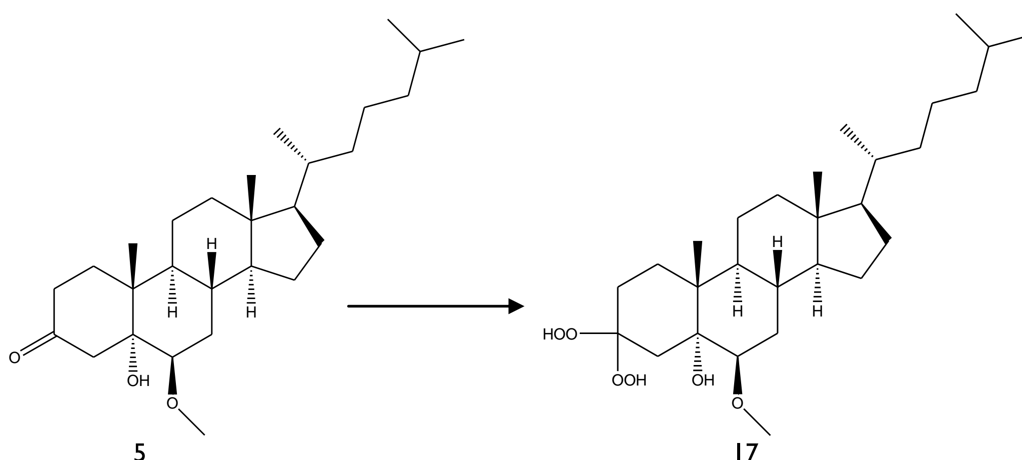
### *2.2.1 Optimization of dihydroperoxide intermediates*

The synthesis of the dihydroperoxides was attempted with the reaction of the ketone in C3 of an oxysterols intermediate (**5**). Based on the conditions used by Opsenica et al [89] and [97], the oxysterol (**5**) was dissolved, at 0°C in a mixture of $CH_2Cl_2$/ $CH_3CN$ (1: 2). Two drops of HCl 5% were added to the solution, and sequentially, 0.1 eq of 30 % $H_2O_2$ were also added. A TLC was immediately done and a unique more polar product was seen although some starting material still remained. After 1h of stirring, several spots were visualized, probably due to the formation of secondary products. The reaction was left

overnight once the TLC showed any substantial difference from the previous one. The extraction was performed to remove the $H_2O_2$ and the $H_2O$. Anhydrous conditions should be ensured to perform the next step for the hybrid synthesis.

In order to study the influence of the solvents in the reaction, the $CH_3OH$ was used to dissolve the oxysterol (**5**). After complete dissolution at room temperature, the reaction was performed in the same manner. The outcome of the reaction was about the same.

Trying to overcome the formation of secondary products in these reactions, glacial acetic acid was used to replace HCl, as suggested by O'Neill [99].
Immediately after the addition of all reagents, a TLC revealed only the presence of the starting material (**5**). Since after 1h a tenuous more polar spot appeared, the reaction was left overnight. Although the reaction was not complete, the secondary products were vestigious.




**Scheme 6**: Synthesis of the dihydroperoxide, using the oxysterols (**5**) as starting material. $CH_2Cl_2$/ $CH_3CN$ (1: 2), acetic acid, $H_2O_2$.

Another attempt for the optimization of the dihydroperoxide synthesis was the use of adamantanone as starting material, instead the oxysterol (**5**).

The adamantanone was dissolved in $CH_3CN$, the 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. The reaction was quenched with $H_2O$, $CH_3CN$ and $H_2O_2$ and worked-up in the usual manner. A TLC control revealed an extensive reversibility of the reaction, with the major formation of adamantanone.

From the studies performed, the best conditions found for the formation of a dihydroperoxide moiety were the use of a steroidal dihydroperoxide, $CH_2Cl_2$ and $CH_3CN$ (1:2) as solvent and glacial acetic acid as catalyst.

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

The synthesis of an antimalarial hybrid compound comprehends the formation of an endoperoxide moiety with the attachment of cycloalkanones to the dihydroperoxide function. The adamantanone and ethyl 4-oxo cyclohexanecarboxylate were chosen for this purpose.

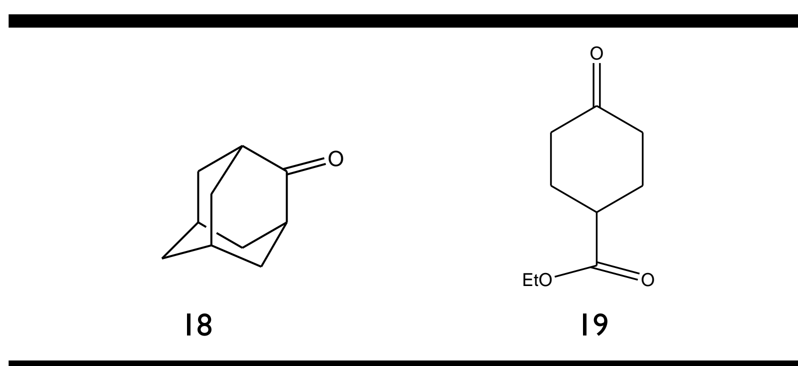



**Figure 13:** Chemical structure of the adamantanone (**18**) and ethyl 4-oxo cyclohexanecarboxylate (**19**).

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

According to the literature, [99] and [118], the fusion of the 1,2,4,5-tretraoxane system to the adamantane core was found to be essential for good levels of antimalarial 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 non 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.

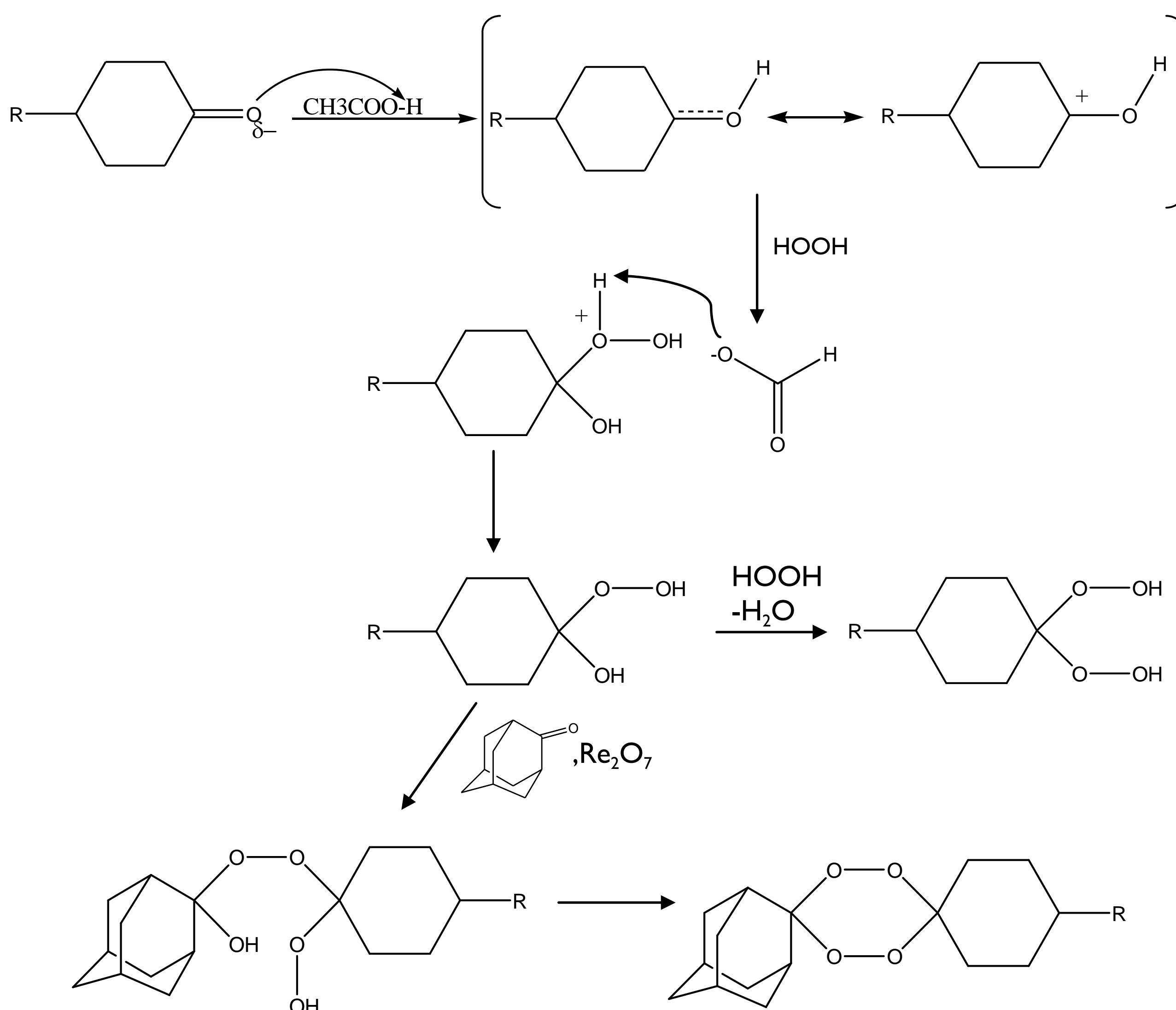
The coupling of a cycloalkanone to a steroidal dihydroperoxide can follow two different methodologies. The first methodology used is based on the work of Opsenica with deoxycholic acid [88].

The dihydroperoxide (**17**) and the adamantanone were dissolved in toluene, at 0°C. A drop of an ice solution of $H_2SO_4$: $CH_3CN$ (1:10) was added. The dihydroperoxide was consumed

instantaneously, as seen on TLC, although no new main product was formed. Part of the dihydroperoxide (**17**) probably yielded the intermediate (**5**) with formation of by-products.

A second methodology using rhenium (VII) oxide ($Re_2O_7$) followed by O'Neill [99] has been tried for our dihydroperoxide as an alternative to the previous method tried.
To a solution of adamantanone 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 (**17**) were added. The reaction was left stirring at room temperature for 2h. The consumption of (**17**) and formation of a major product were seen on TLC.

The mechanism for the 1,2,4,5- tetraoxane hybrid compound is illustrated in scheme 7.



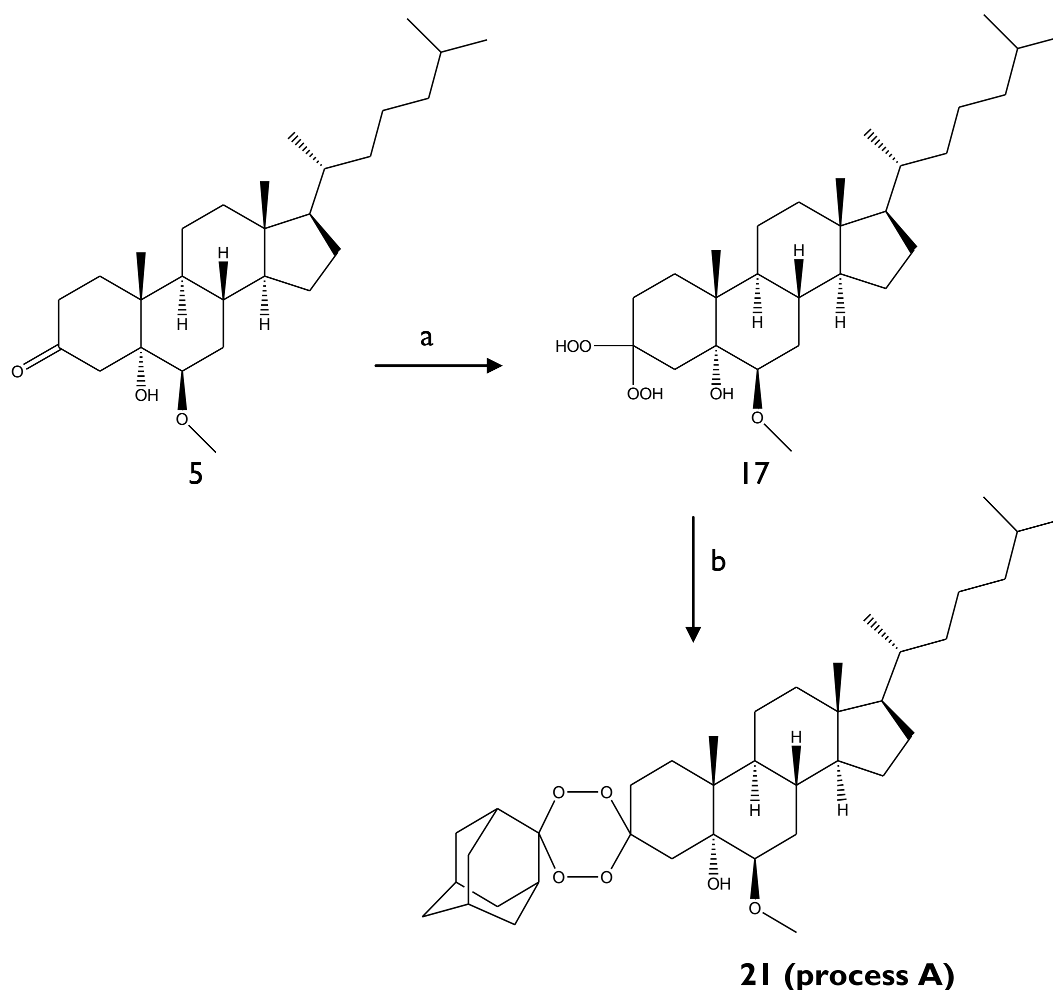

**Scheme 7**: Proposed mechanism for the synthesis of the hybrid drug, with the formation of an 1,2,4,5-tetraoxane.

### *2.3 Synthesis of mixed steroidal 1,2,4,5 – tetraoxanes*

#### *2.3.1 Synthesis of a 5,6-oxygenated cholestane - 1,2,4,5 – tetraoxaadamantane (**21**)*

***Process A – starting material: oxysterol (5)***

Using the best conditions studied, 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 0ºC, and thereafter, $H_2O_2$ .To form an excess of the dihydroperoxide (**17**), the reaction was maintained under stirring for at least one week, with additions of $H_2O_2$ every day. After the usual work-up, the (**17**) was added to a solution of adamantanone and $Re_2O_7$ in dry $CH_2Cl_2$ under an inert atmosphere. The reaction was instantaneous, and the oxysterol (**5**) did not interfered with the hybrid formation. The product formed was purified by FCC with petroleum ether and $CH_3CN$ to afford the hybrid **21 (process A)**.




**Scheme 8:** Synthesis of the hybrid molecule (**21 (process A)**) using the oxysterol (**5**) as starting material. a) $CH_2Cl_2$/ $CH_3CN$ (1: 2), acetic acid, $H_2O_2$, b) adamantanone, $Re_2O_7$ ,dry $CH_2Cl_2$.

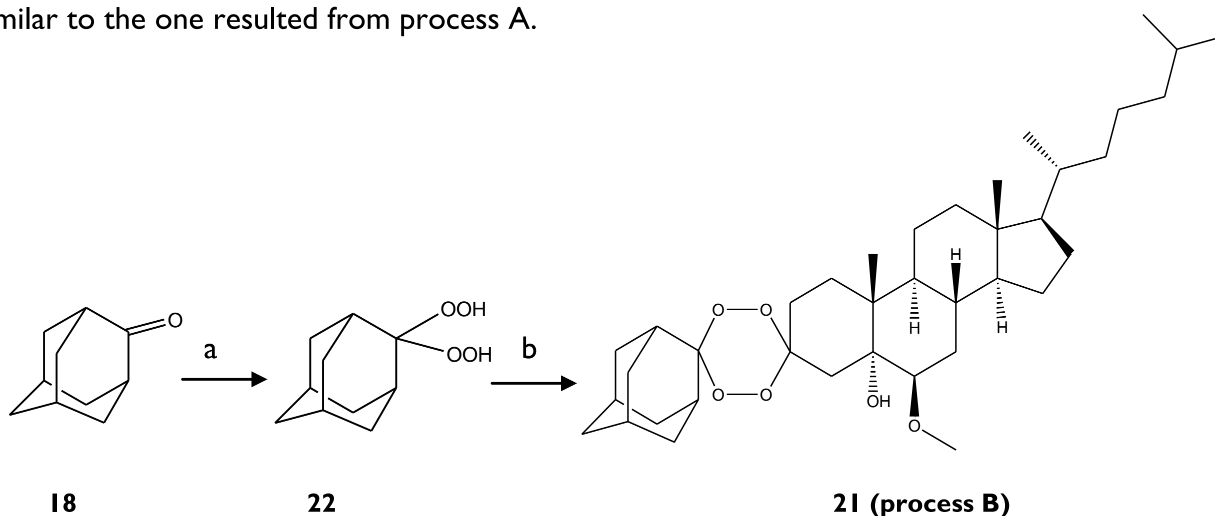
After drying in vacuum, the product (**21 (process A)**) was analyzed by NMR techniques.

***Process B – starting material: adamantanone (18)***

The adamantanone (**18**) was dissolved in $CH_3CN$ in a cold ice bath. The acetic acid and the $H_2O_2$ were added and after 4h, the TLC revealed that a new more polar product was formed (**22**).The work-up was performed as usual.

The intermediate (**5**) was dissolved in dry $CH_2Cl_2$, in an inert atmosphere, and the $Re_2O_7$ was added followed by the dihydroperoxide **22**.

The reaction was instantaneous. The profile of the secondary products in this process was similar to the one resulted from process A.




**Scheme 9:** Synthesis of the hybrid molecule (**21 (process B)**) using the intermediate (**18**) as starting material. a) $CH_2Cl_2$/ $CH_3CN$ (1: 2), acetic acid, $H_2O_2$; b) oxysterols (**5**), $Re_2O_7$, dry $CH_2Cl_2$.

After drying in vacuum, the hybrid (**21 (process B)**) couldn't be characterized once the crude yield was very low which hampered its purification.

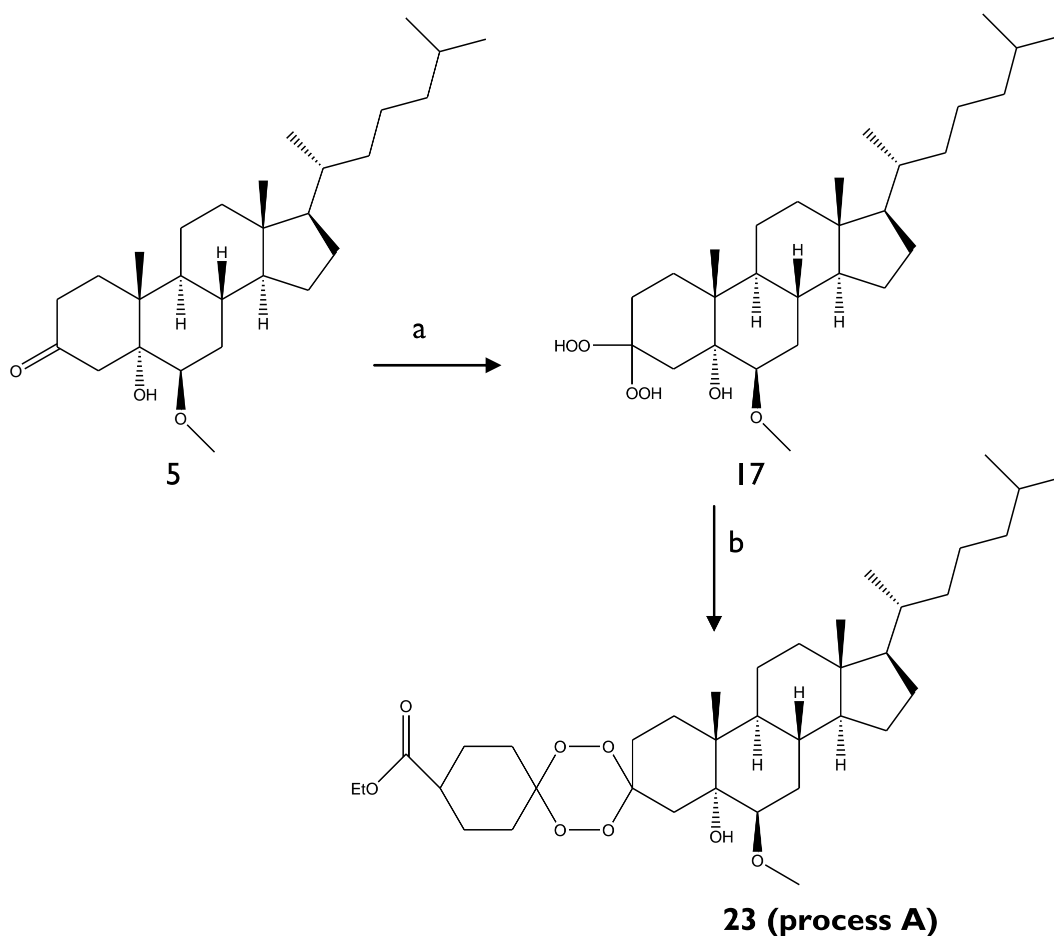
*2.3.2 Synthesis of a 5,6-oxygenated cholestane 1,2,4,5 – tetraoxacyclohexane derivative (**23**)*

***Process A – starting material: oxysterol (5)***

The first part of the reaction occurs as described above for the synthesis of (**17**). After one week of stirring, the reaction was stopped, the extraction was performed and the compound (**17**) was used for the synthesis of the (**23 (process A)**).

The ethyl 4-oxocyclohexanecarboxylate (**19**) was dissolved in dry $CH_2Cl_2$, and $Re_2O_7$ at 0°C, in an inert atmosphere. The reaction was instantaneous.

The work-up was performed as mention for the hybrid **21 (process A).**



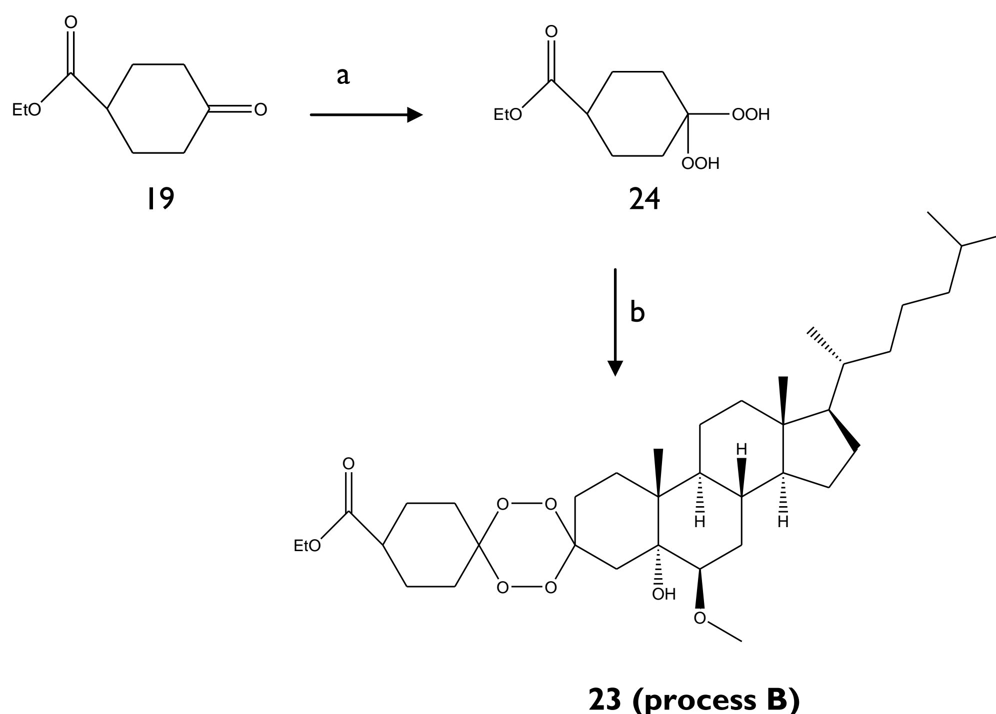

**Scheme 10:** Synthesis of the hybrid molecule (**23 (process A)**) using oxysterol (**5**) as starting material. a) $CH_2Cl_2$/ $CH_3CN$ (1: 2), acetic acid, $H_2O_2$; b) ethyl 4-oxocyclohexanecarboxylate, $Re_2O_7$, dry $CH_2Cl_2$.

The (**23 (process A)**) compound was purified by FCC. After drying in vacuum, this product was characterized by NMR techniques. However, these techniques weren't conclusive and clear to identify correctly the synthesised compound.

***Process B – starting material: ethyl 4-oxocyclohexanecarboxylate (19)***

The (**19**) was dissolved in $CH_3CN$ in an ice cold bath. Glacial acetic acid and $H_2O_2$ were added. After 4h the reaction was complete and stopped adding $H_2O$, $CH_3CN$ and $H_2O_2$ in the usual way.

Meanwhile, the oxysterol (**5**) was dissolved in dry $CH_2Cl_2$, and $Re_2O_7$ was added in an inert atmosphere. The dihydroperoxide (**24**) was then added to this solution. A prevalent product was formed immediately.




**Scheme 11:** Synthesis of the hybrid molecule (**23 (process B)**) using the intermediate (**19**) as starting material. a) $CH_2Cl_2$/ $CH_3CN$ (1: 2), acetic acid, $H_2O_2$; b) oxysterols (**5**), $Re_2O_7$ ,dry $CH_2Cl_2$.

The crude was purified by FCC and crystallization to yield (**23** (process B)) and analyzed by NMR techniques and IR spectroscopy.

### *2.3.3 Synthesis of a cholestane -1,2,4,5 – tetraoxaadamantane (**26**)*

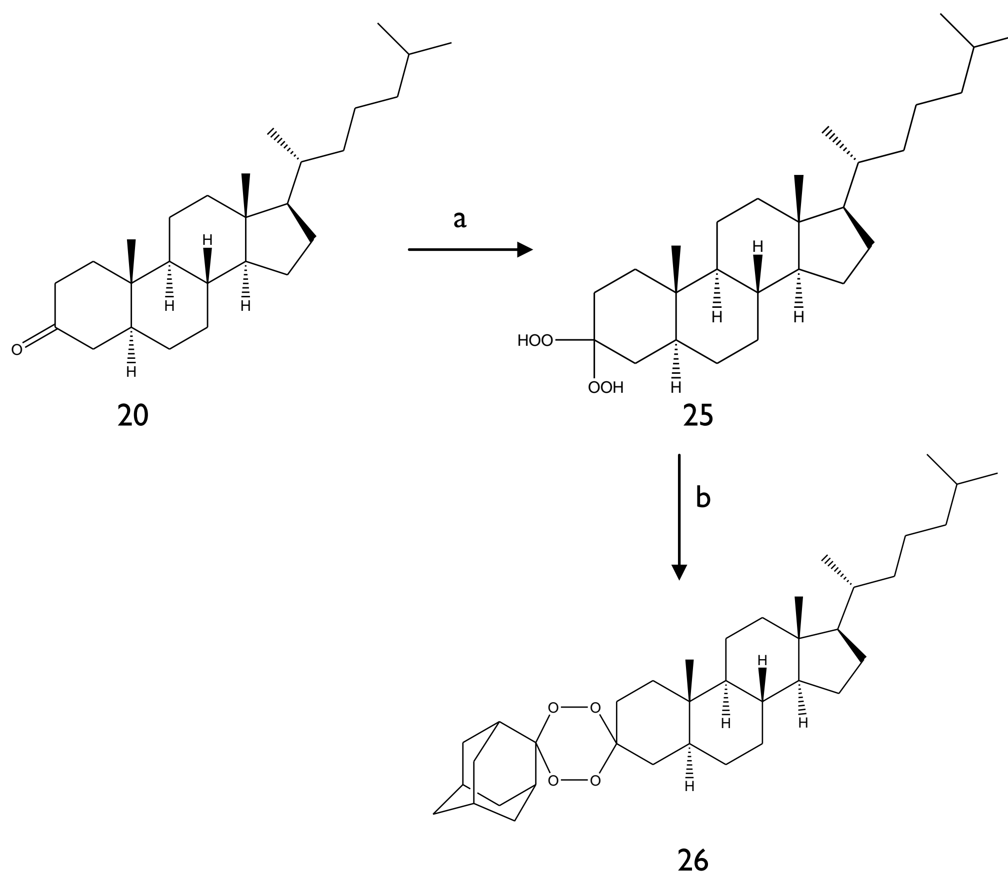
The synthesis of the hybrids nominated by (**26**) and (**27**) were performed with 5α-cholestan-3-one (**20**) as starting material. The choice of this compound (**20**) was based on the absence of other functional groups, apart of a ketone at C3.

The cholestanone (**20**) was dissolved in $CH_2Cl_2$ and $CH_3CN$ (1:2), following the previously procedure. The reaction was left over night and the formation of an insoluble white product was noticed.

The reaction was stopped and the work-up was performed as before.

To a solution of adamantanone (**18**) in dry $CH_2Cl_2$ in ice bath under an inert atmosphere, the $Re_2O_7$ was added and then the intermediate (**25**).

The reaction was instantaneous but the presence of secondary products was noticed on TLC.

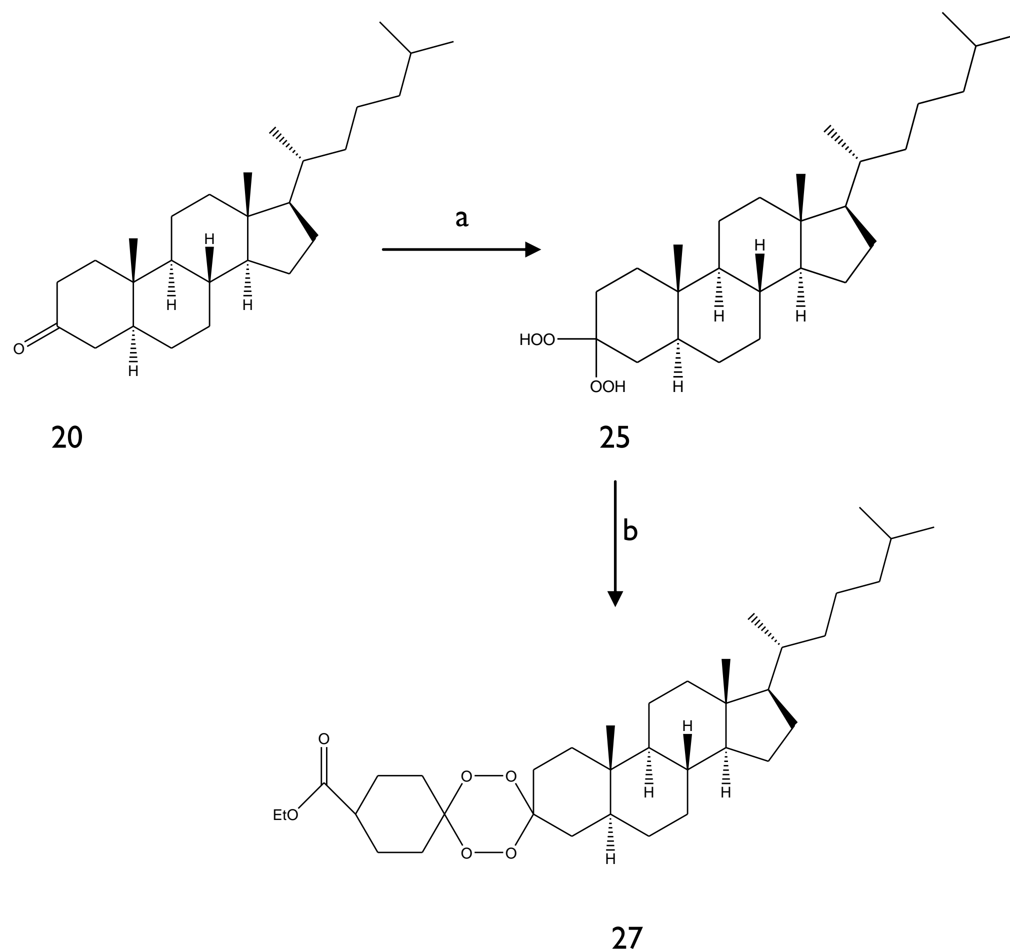



**Scheme 12:** Synthesis of the hybrid molecule (**26**) using the intermediate (**20**) as starting material. a) $CH_2Cl_2$/ $CH_3CN$ (1: 2), acetic acid, $H_2O_2$; b) adamantanone, $Re_2O_7$, dry $CH_2Cl_2$.

The crude product was purified by FCC and the yielded the hybrid (**26**) which was analysed by NMR techniques.

*2.3.4 Synthesis of a cholestane - 1,2,4,5 – tetraoxacyclohexane derivative (**27**)*

The dihydroperoxide (**25**) was prepared as described previously. The second part of the synthesis was also performed in the same way as usual. Again, the reaction was instantaneous although the presence of secondary products was also observed.




**Scheme 13:** Synthesis of the hybrid molecule (**27**) using intermediate (**20**) as starting material. a) $CH_2Cl_2$/ $CH_3CN$ (1: 2), acetic acid, $H_2O_2$; b) ethyl 4-oxocyclohexanecarboxylate, $Re_2O_7$ ,dry $CH_2Cl_2$.

The crude was purified by FCC and yields the hybrid (**27**). Analyses by NMR techniques were performed.

***2.4 Spectral characterization of the hybrid compounds***

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

*2.4.1 5,6-oxygenated cholestane - 1,2,4,5 – tetraoxaadamantane (**21**)*

The hybrid (**21**) characterized was prepared by process A. In the $^{1}H$ NMR spectra, a constant pattern was observed. The methyl groups of the steroid moiety were easily identified by the proton chemical shifts 0.66 ppm (s,18-$CH_3$), 0.85ppm (d, 26 -$CH_3$), 0.87

ppm (d,27-$CH_3$), 0.87 ppm (d,21-$CH_3$) and 1.05 ppm (19-$CH_3$), characteristic of a cholestan. The signal at 3.28 ppm can be assigned for the 6β-O$\underline{CH_3}$.

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

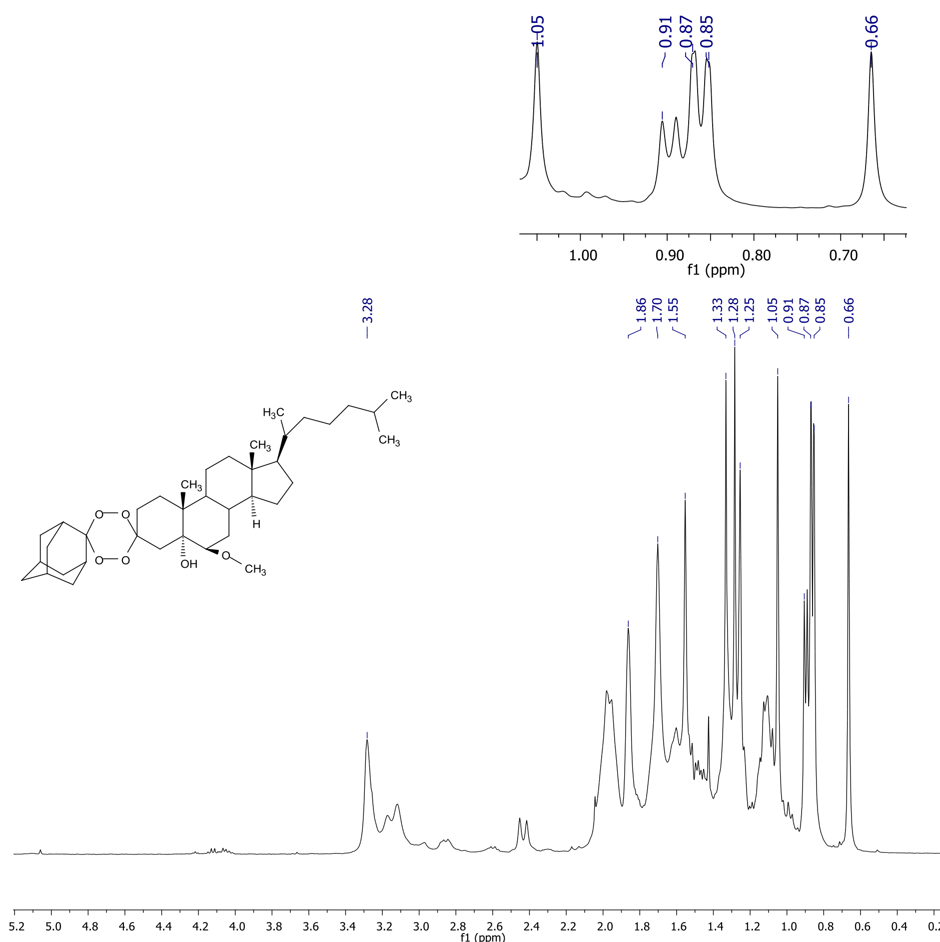
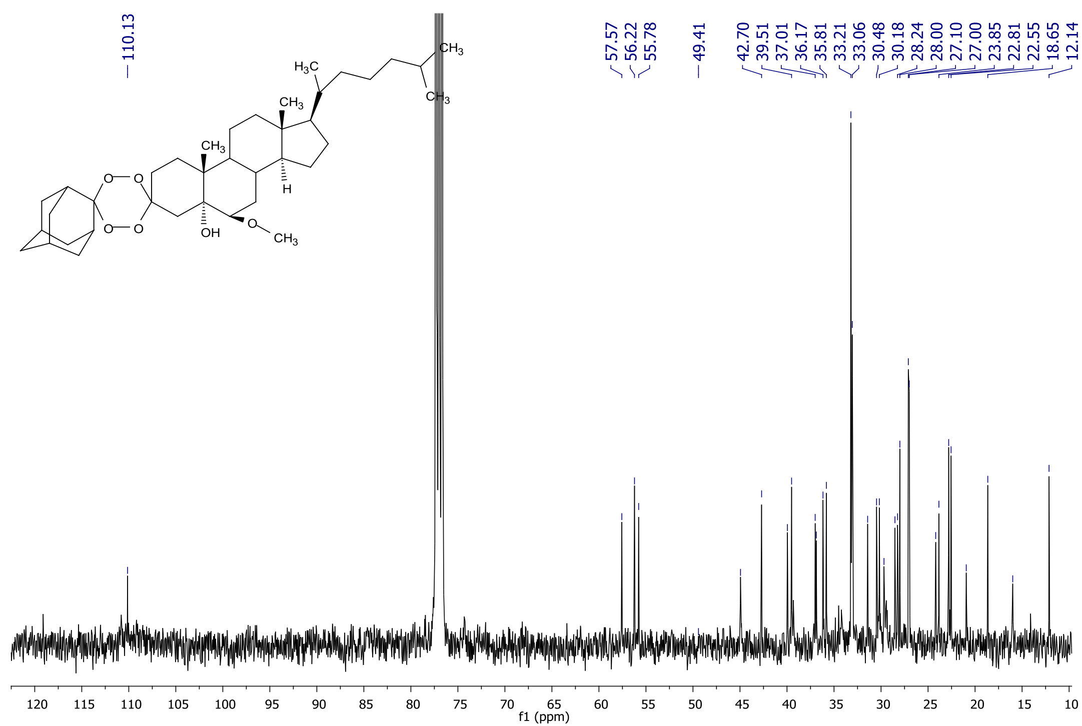



**Figure 14:** $^{1}H$ NMR spectrum of compound (**21**)

The $^{13}C$ NMR and DEPT 135 were important to clarify the synthesized molecule.

Through the $^{13}C$ NMR and DEPTs it was possible to calculate the total number of carbon. The signal at 57.57 ppm was assigned for the $CH_3$ of the methoxy group while the signal at 77.21 ppm, only seen in the DEPT 135, corresponds to the C6.

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



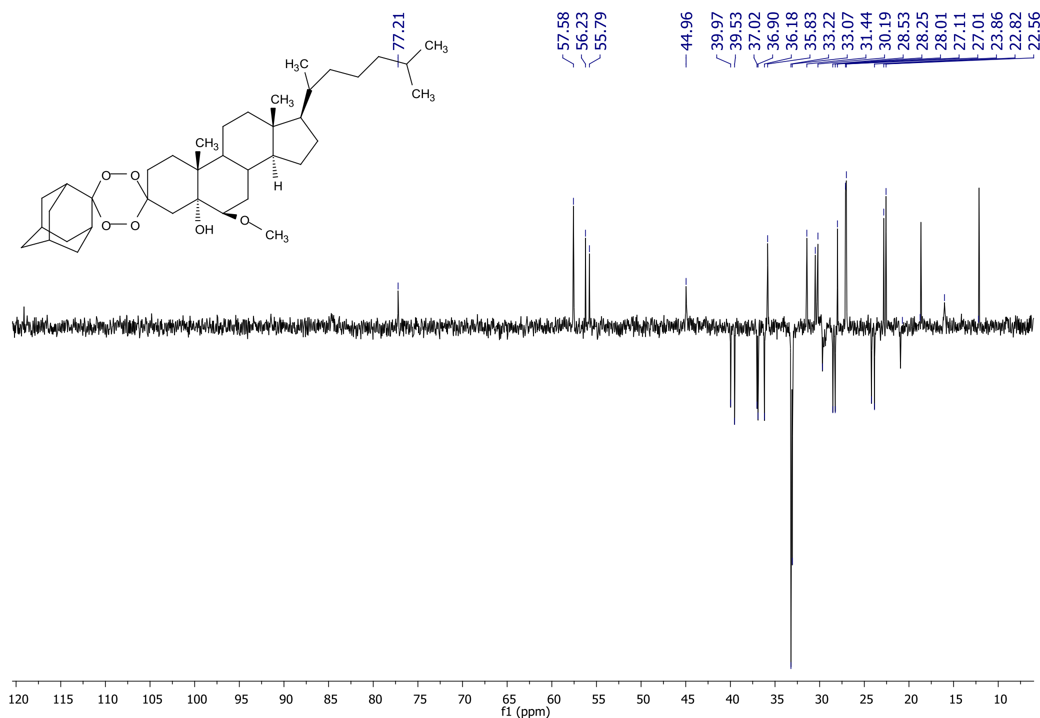


**Figure 15:** $^{13}C$ and DEPT NMR spectra of compound (**21**).

### *2.4.2 5,6-oxygenated cholestane 1,2,4,5 – tetraoxacyclohexane derivative (**23**)*

The structural elucidation was performed in compound (**23**) prepared by process B.

When the ^{1}H NMR spectra is observed, the hybrid compound was similar to the compound (**21**) in respect to the steroidal moiety. On the upfield spectrum, the methyl groups were easily identified, being the proton chemical shifts identical to the observed in the[1]H NMR for hybrid (**21**).

The singlet at 3.29 ppm integrates 3 protons and was assigned for the 6β-O$\underline{CH_3}$,

The triplet at 1.25 ppm (J=6,6 Hz) corresponds to the $CH_3$ of the ester group of the hybrid (**23**) and the quadruplet at 4.14 ppm (J=6.6) to the $CH_2$ of the same group.

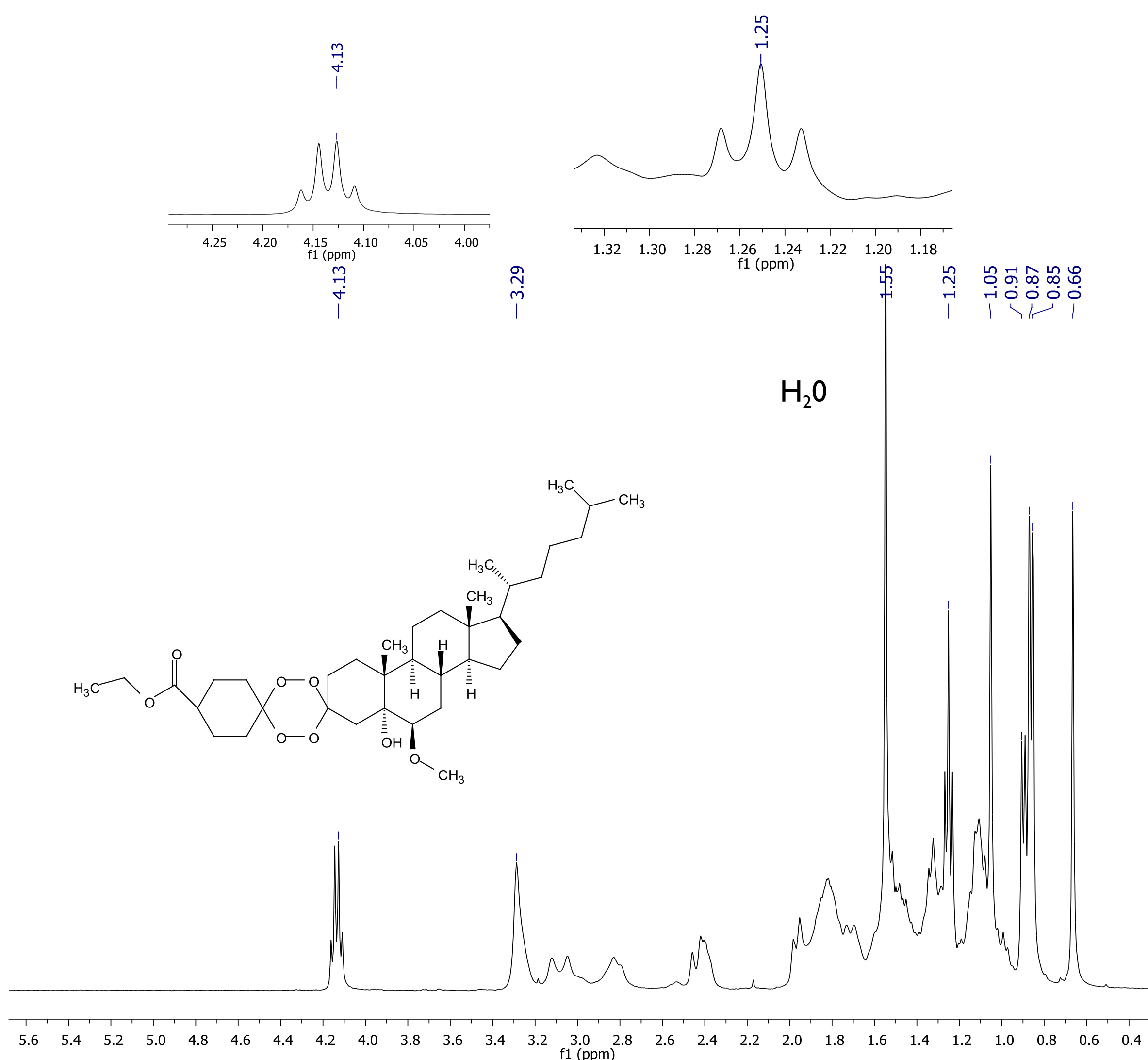



**Figure 16:** ^{1}H NMR spectrum of compound (**23**).

The ^{13}C NMR and DEPT 135 and 90 were performed in order to clarify the carbon signals.

From these spectra, it was possible to identify the quaternary carbons corresponding to the OO-C3-OO moiety at 107.8 ppm and OO-C1'-OO at 109.5 ppm which confirms the tetraoxane bridge.

A signal at 57.5 ppm reveals the presence of the $OCH_3$ and the signal at 174.5 ppm corresponds to the C=O of the hybrid.

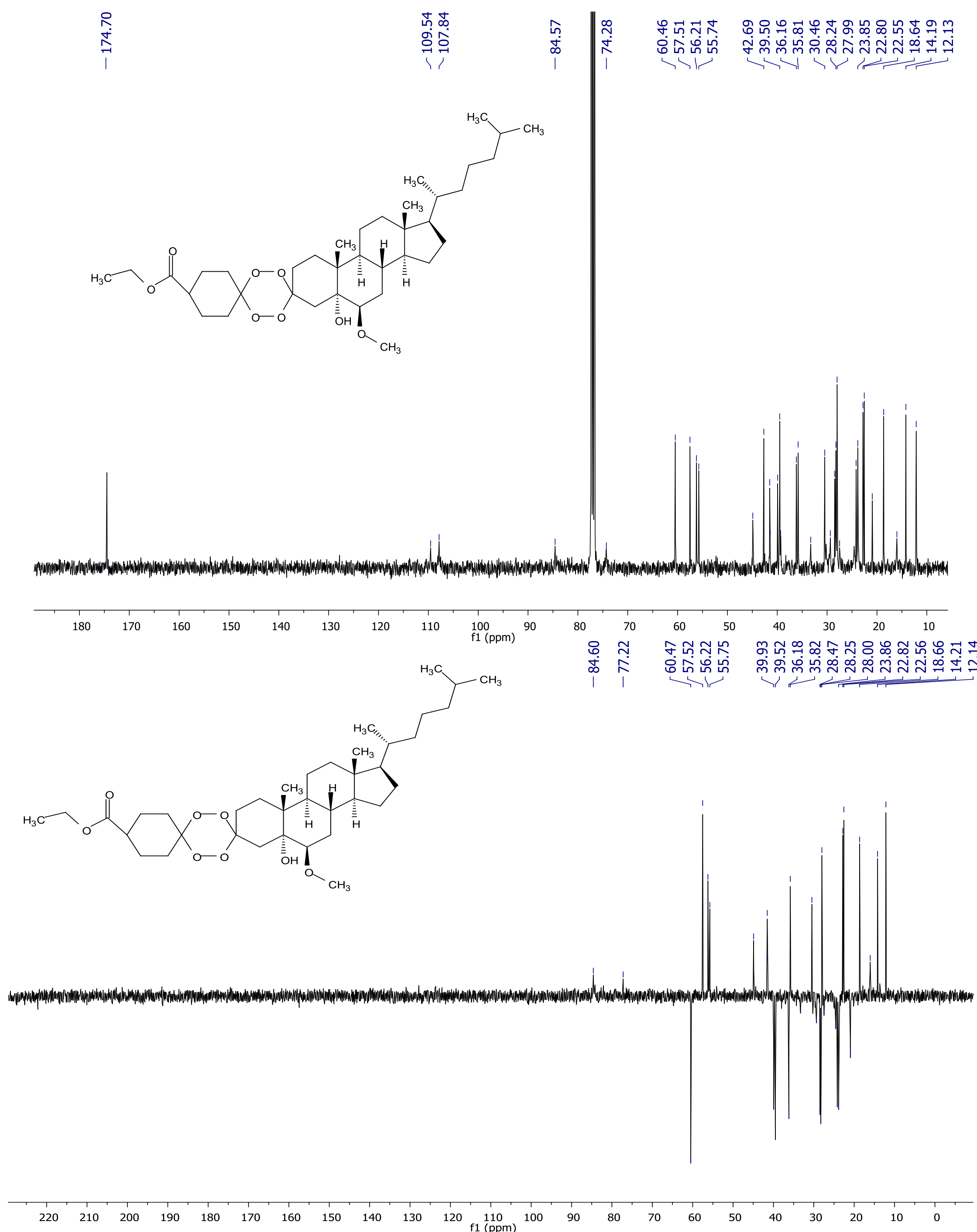


**Figure 17:** $^{13}$C NMR and DEPT 135 spectra of compound (**23**).

From the IR spectrum of the compound (**23**) it is possible to identify some functional groups presents in the molecule, according with [119]. We have identified an O-H stretch at 3620 $cm^{-1}$.

A strong band at 2932 $cm^{-1}$ corresponds to the methyl groups present in the molecule.

A strong band at 1722 $cm^{-1}$ corresponds to the saturated carbonyl of the ester group of the hybrid.

The C-O stretch bands, corresponding to ether, ester groups, tertiary alcohol and C-O-O at can be assigned between 1000 and 1200 $cm^{-1}$. However, a more detailed study is necessary to achieve the specific band of each C-O stretch.

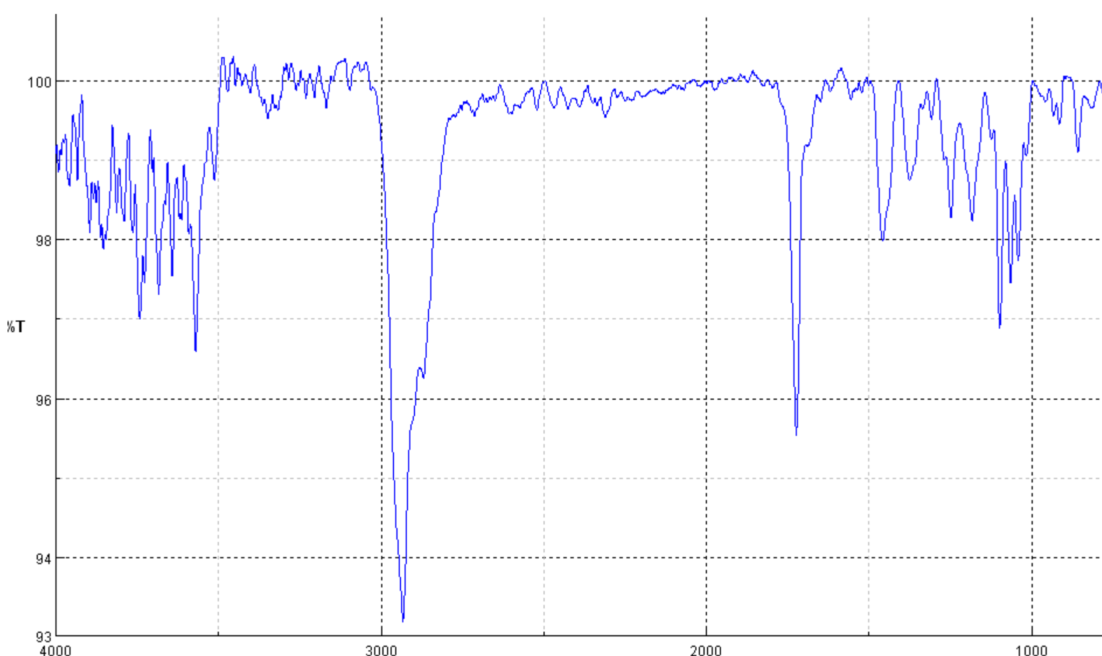



**Figure 18:** IR spectrum of the compound (**23**).

*2.4.3 Cholestane -1,2,4,5 - tetraoxaadamantane (**26**)*

The $^{1}$H NMR spectrum of the (**26**) reflects a steroid scaffold, without functional groups. The steroid moiety showed the same pattern as observed for the previous hybrid compounds, apart the position of the singlet 19-$CH_3$ which moved from 1.00ppm in the 5α-cholestan-3-one (**20**) to 0.81 ppm in the hybrid (**26**) (see annexes).

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

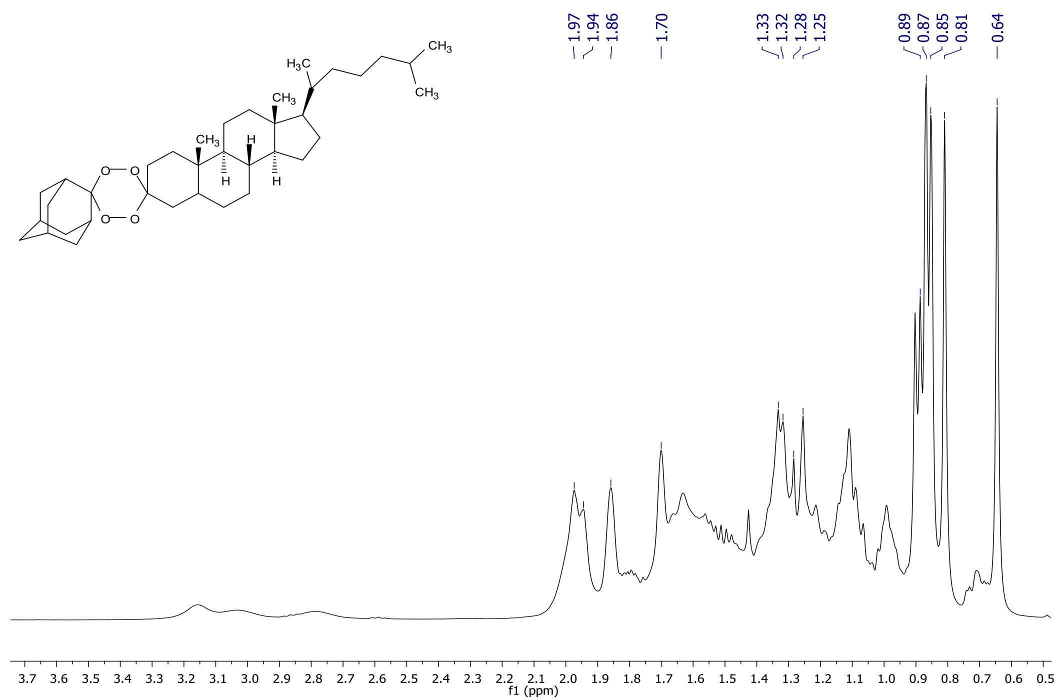


**Figure 19:** $^1$H NMR spectrum of compound (**26**).

The $^{13}$C NMR and DEPT 135 were also performed in order to clarify the number of carbons of th hybrid compound synthesised.

At 11.4 ppm is the signal corresponding to the C19, which suffers a deviation upfield comparing with the spectra of the (**21**) and (**23**) hybrids.

From these spectra, it was possible to identify the quaternary carbons corresponding to the OO-C3-OO moiety at 108.4 ppm and OO-C1'-OO at 110.3 ppm which confirms the tetraoxane bridge.

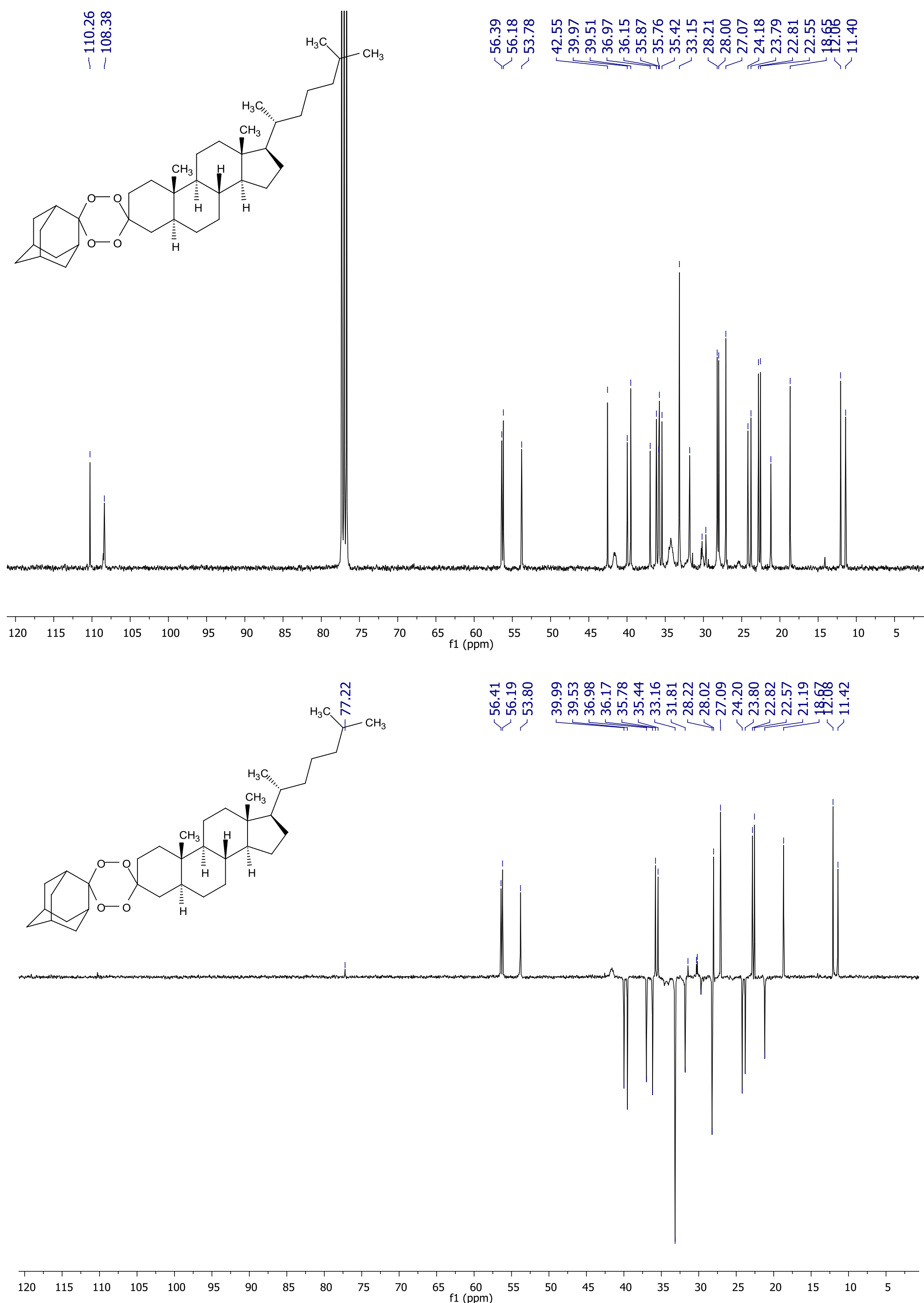



**Figure 20:** $^{13}$C NMR and DEPT 135 spectra of compound (**26**).

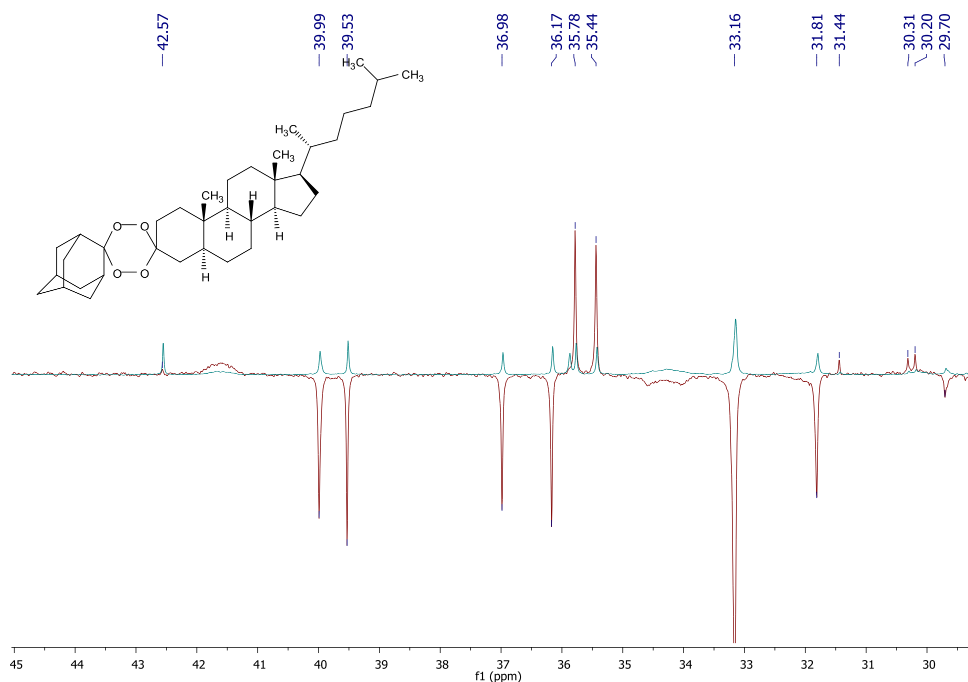



**Figure 21:** Detail of the overlap of the $^{13}C$ NMR spectrum with the DEPT 135 of the compound (**26**).

*2.4.4. Cholestane - 1,2,4,5 – tetraoxacyclohexane derivative (**27**)*

In the $^{1}H$ NMR spectrum of the (**27**), the same typical signals already observed for compound (**26**) such as 19-$CH_3$ from 1.00 ppm to 0.81 ppm due to the attachment of the tetraoxane. The triplet at 1.24 ppm and the quartet at 4.12 ppm correspond to the $CH_3CH_2$ OCO - group.

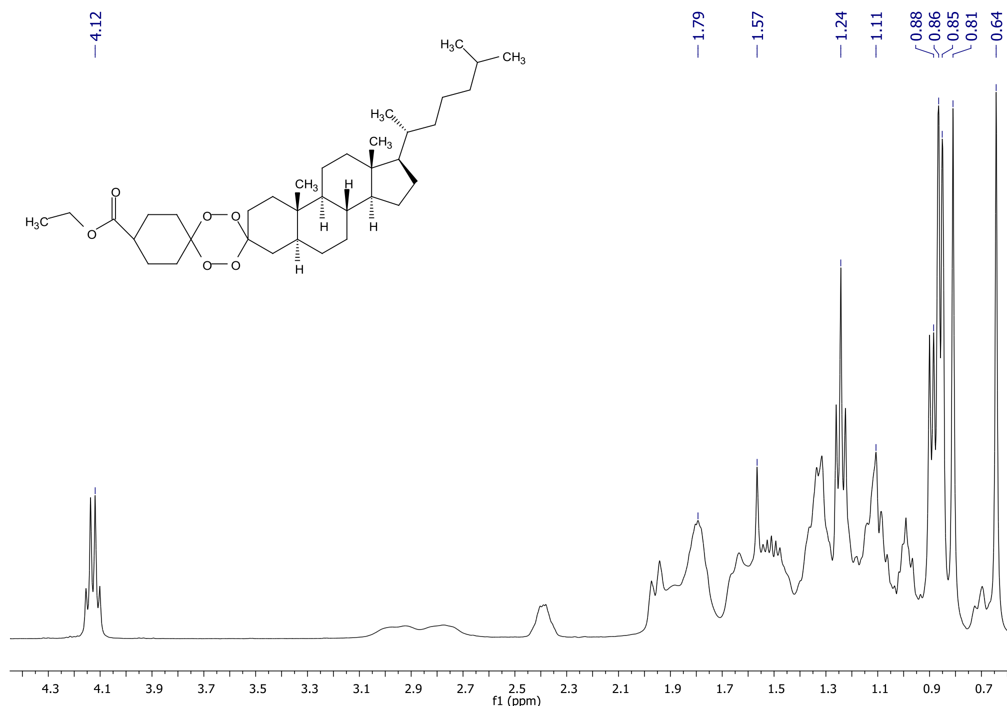



**Figure 22:** $^{1}$H NMR spectrum of compound (**27**).

In the $^{13}$C spectrum of the compound (**27**), the carbons in the tetraoxane bridge were also seen, as well as the signal for the C=O. The signal for the C19 was again deviated to upfield like in compound (**26**)**.** The signal at 174.6 ppm corresponds to the C=O of the hybrid.

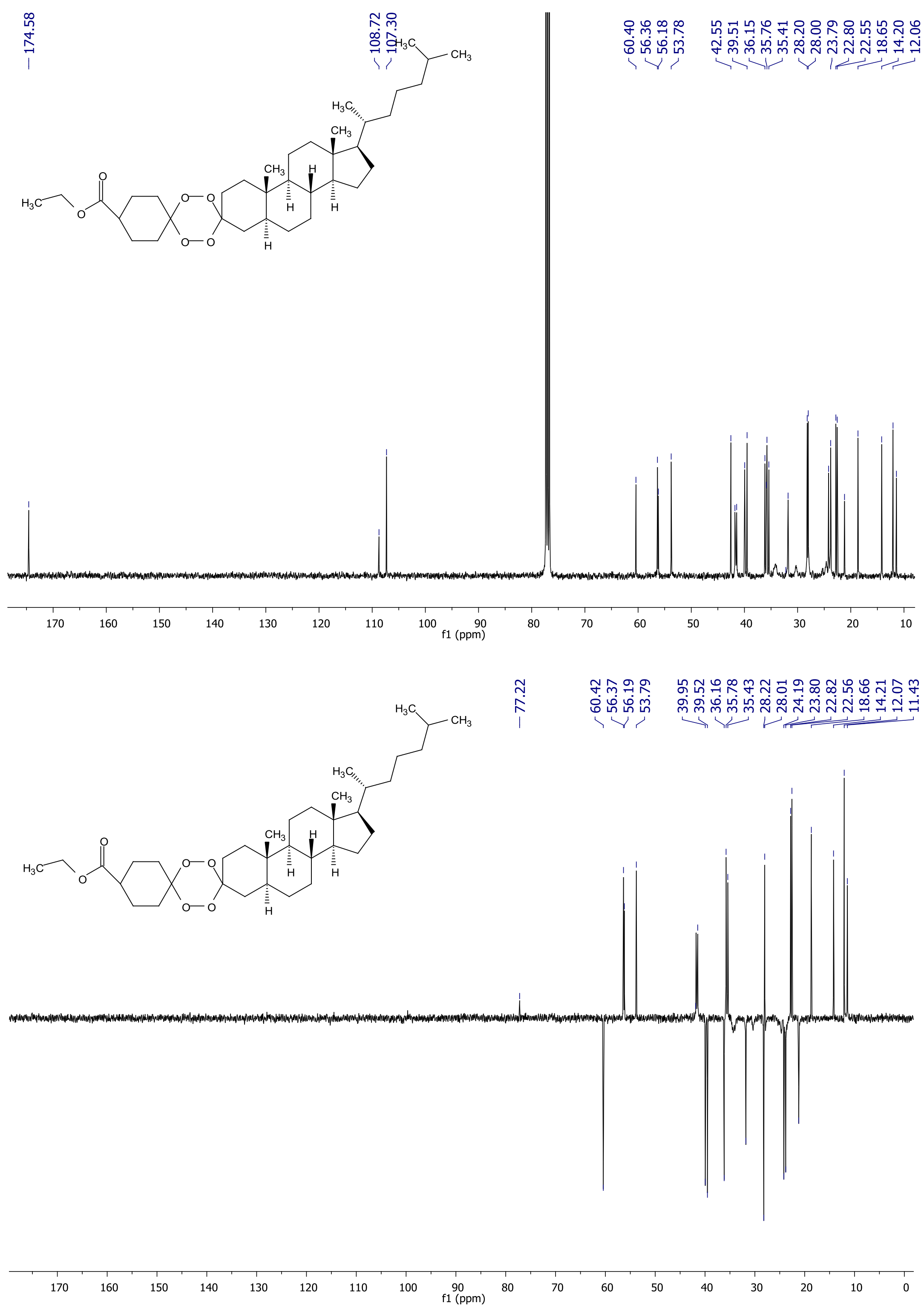



**Figure 23:** $^{13}$C NMR and DEPT spectra of the compound (**27**).

# CHAPTER III

# CONCLUSIONS

***3.1 Conclusions and Future Perspectives***

The synthesis of hybrid drugs shows up as a fashionable alternative to the synthesis of new molecules either due to the reduction of funds in the I&D units, either due to different mechanisms inherent to the scaffolds, in different stages of the same disease.
The aim of this thesis was the synthesis of hybrid antimalarials with a steroid scaffold, a tetraoxane bond that interferes in the haemozoin formation, and adamantanone or ethyl 4-oxocyclohexanecarboxylate to complete the hybrid molecule.
The steroid part was only tested with the compounds (**5**) and (**20**).
The compound (**11**) couldn't be use due to the impossibility to form a ketone group at C3, despite the attempt with a soft oxidant. More oxidative reagents are needed to investigate.

The compound (**8**) was synthesized, however due to the difficulties in its purification this compound couldn't be use as starting material for the synthesis of hybrid compounds.

In a near future, the synthesis of more oxysteroids scaffolds are needed in order to study the influence of ring A, B and C substituents.

Two different methodologies for the dihydroperoxide formation and 1,2,4,5-tetraoxane synthesis, based on the literature, were tested. However, none of the methodologies described was fully applicable and the protocol chosen was a mixture of the two methodologies, adapted to the starting material.
The procedure to form a dihydroperoxide and a 1,2,4,5-tetraoxone was optimized. After this optimization and once the reaction time was extended, an alternative formation of the dihydroperoxide was tested. The opening of the ketone group of the ethyl 4-oxo cyclohexanecarboxylate (**19**) to form the dihydroperoxide was tested and this methodology presents clear advantages in the reaction time, being one of the preferential procedures for the synthesis of this kind of hybrid molecules.

Due to the complexity of the molecules, the techniques of NMR and IR were complementary but all of them present specific handicaps. However, a proper pattern can be identified for future studies, like the signals of OO-C3-OO and OO-C1'-OO in the $^{13}$C NMR.

A more green procedure and high yield method is something that needs to be refined in a short period of time.

A complete investigation to determine the function of the steroid moiety in the hybrid drug is mandatory, once the steroid may act synergistically as an antimalarial scaffold and as a carrier for the other antimalarial moieties.

The design of these hybrid molecules with a steroid moiety is now starting their new "golden era". The simplicity of their synthesis, allied to their abundance in the nature, make the steroids the perfect scaffold for the design of new hybrid molecules.

# CHAPTER IV

# EXPERIMENTAL SECTION

## *4.1 Experimental Section*

### *4.1.1 Reagents and Solvents*

All the reagents commercially available were used as supplied by the manufactures. Cholesterol acetate, 5α-cholestan-3one, ethyl 4-oxo cyclohexanecarboxylate, adamantanone, rhenium (VII) oxide ($Re_2O_7$), copper iodide (CuI), bismuth triflate ($Bi(OTf_3)$), magnesium monoperoxyphthalate hexahydrate (MMPP) and the lipase (E.C. 3.1.1.3) were acquired to Sigma-Aldrich. The tert-butyl hydroperoxide (*t*-BHP) was purchased to Fluka.

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

The acetonitrile, $CH_3CN$, dichloromethane $CH_2Cl_2$, sodium hydroxide (NaOH), celite® 545, silica gel 60 (column chromatography) and TLC silica gel 60 $F_{254}$ were obtained from MERK Co.

The solution of 30% of hydrogen peroxide ($H_2O_2$), as well as the Jones reagent (25g of sodium dichromate $Cr(VI)O_3$ diluted in 25 mL/75 mL of sulphuric acid/$H_2O$) were 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 solution used in thin layer chromatography was a mixture of ethanol, sulphuric acid, acetic acid and p-anisaldehyde purchased at MERK Co.

### *4.1.2 Equipments*

The $^1H$ NMR $^{13}C$ NMR and DEPT spectra were performed in a 400 MHz Bruker Avance III spectrometer.

The IR spectra were obtained in a Jasco FT/IR-420 spectrometer. The MS spectra were obtained in a mass spectrometer quadrupolo/ion trap (QIT-EM), LCQ Advantage MAX from Thermo Finnigan. The melting points were determined in a Büchi-540 and they are not corrected.

### *4.1.3 Chemistry*

#### *4.1.3.1 Synthesis of the intermediates*

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

To a solution of cholesterol acetate (**1**, 800 mg, 1,887 mmol) in acetonitrile (28mL), MMPP (1.1 eq) was added as a single portion. The suspension was stirred for 20 min at reflux temperature (83°C). After the substrate consumption, 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 (785 mg, 93.6%).

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

To a solution of **2**, (785 mg, 1.765 mmol) in dry methanol (40 mL), 0,05 eq of $Bi(OTf)_3$ were added. The solution was stirred at room temperature until substrate consumption (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** (622 mg, 74%).

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

The resulting **3a** (622mg, 1.306 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. When the consumption of the substrate was complete, 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 (523 mg, 93%). The spectral analysis was accordingly to the literature [100].

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

After the addition of 523 mg of **4** (1.202 mmol) to 22 ml 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.

After evaporation, 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 (480 mg, 93%).

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

**$^1$H NMR** (400 MHz, *CDCL$_3$*) δ ppm: 0.69 (3H, s, 18-$CH_3$), 0.86 e 0.87 (each 3H, 2d, *J*= 6.7 Hz, 26-$CH_3$ e 27-$CH_3$), 0.89 (3H, t, J=7.5 Hz, 21-$CH_3$), 1.25 (3H, s, 19-$CH_3$), 2.95 (1H, s, 6α-H), 3.27 (3H, s, 6β-$OCH_3$).

**$^{13}$C NMR** (100 MHz, *CDCL$_3$*) 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).

*Synthesis of the 7-oxocholest-5-en-3β-yl acetate (**6**)*

To a solution of 1950mg, 4.55mmol of acetate cholesterol (**1**) and CuI (520 mg, 2,729 mmol) in $CH_3CN$ (30 mL) at reflux temperature, was added 1.171 mL of *t*-BHP (70%, 45,49 mmol) each 45 min, 5 times. After the last addition, the reaction was remained stirring for 1h, and stopped under vacuum.

The work-up was performed using diethyl ether to dissolve the residue. The resulting solution was filtered in a celite column to extract the inorganic CuI. The filtered solution was put into stirring over night with $Na_2SO_3$ (10%) and $H_2O$. After drying with anhydrous $Na_2SO_4$the solution was filtered an evaporated. A yellow product was achieved.

The 7-oxocholest-5-en-3-yl acetate (**6**) was isolated by FCC using as eluent a mixture of petroleum ether and AcOEt in the proportion 10:1, resulting in a pure white product (2.035 g, 46%).

*Synthesis of the 3β-hydroxycholest-5-en-7-one (**7**)*

The resulting **6** (890mg, 2.101mmol) was dissolved in 17,5 mL of EtOH and 9mL of $CH_2Cl_2$, and 4 eq of NaOH (10%) were added. The reaction was stirred during 2h, at rt. When the consumption of the substrate was complete, the reaction was 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 (850mg, 96%).

*Synthesis of the cholest-5-ene-3,7-dione (**8**)*

After the addition of 850 mg, 2.125 mmol of the compound **7** to 40 ml of dry acetone, at 0°C, the Jones reagent was added dropwise. The procedure was the same as described for compound **5**.
After filtration and evaporation, a golden product (800mg, 94.2%) was obtained.

*Synthesis of the 5α-hydroxycholestane-3β,6β- diyl 3-acetate, 6-hemiphthalate (**9**)*

To a solution of cholesterol acetate (**I**, 600 mg, 1,400 mmol) in acetonitrile (42mL), MMPP (1.1 eq) was added as a single portion. The suspension was stirred at reflux temperature (83°C) for 24h. After the substrate consumption, the reaction mixture was stopped by evaporation under vacuum. The resulting white solid residue was dissolved in diethyl ether and washed with water, filtered and evaporated again to dryness. Careful FCC of the crude material, starting with chloroform as eluent and then in slow gradient (chloroform: ethanol 50:1 to 5:1) afforded the pure white solid **9** (290 mg, 34%).

*Synthesis of the 5α-hydroxy-3-oxocholestan-6β-yl, 6-hemiphthalate (**10**)*

To a solution of cholesterol (**I3**, 655.5 mg, 1,72 mmol) in acetonitrile (52 mL), MMPP (1.1 eq, 1.90 mmol) was added as a single portion. The procedure was the same as described for compound **9**.
Careful FCC of the crude material, starting with chloroform as eluent and then in slow gradient (chloroform: ethanol 50:1 to 1:1) afforded the pure white solid **10** (301 mg, 31%).

**Formula:** $C_{37}H_{54}O_7$

**$^1$H NMR** (400 MHz, *$CDCL_3$*) δ ppm: 0.66 (3H, s, 18-$CH_3$), 0.85 e 0.87 (each 3H, 2d, *J*= 6.7 Hz, 26-$CH_3$ e 27-$CH_3$), 0.88 (3H, t, J=7.5 Hz, 21-$CH_3$), 1.17 (3H, s, 19-$CH_3$), 4.02 (1H, m, 3α-H) 4.36 (1H, m, 6α-H), 7.34 ppm (1H, t, J=7.1Hz, Ph), 7.6 ppm (1H, t, J=7.0 Hz, Ph), 7.7 ppm (1H, d, J=7.1 Hz, Ph), 7.8 ppm (1H, d, J=7.0 Hz, Ph).

*4.1.3.2 Synthesis of the hybrid compounds*

*Synthesis of 5,6-oxygenated cholestane - 1,2,4,5 – tetraoxaadamantane (**21**) – Process A*

152,7 mg, 0.34mmol of the intermediate (**5**) was dissolved in 4mL of $CH_2Cl_2$ and 8mL of $CH_3CN$, at rt. After complete dissolution, 1.45 mL of acetic acid was added at 0°C, and thereafter, 500 μL of

$H_2O_2$ was also added. The reaction was maintained stirring, at rt, for at least one week, with additions of $H_2O_2$ every day. The reaction was stopped adding $CH_2Cl_2$, $H_2O_2$ and $H_2O$. After the extraction, the solution was dried with anhydrous $Na_2SO_4$, filtered and the excess of $CH_2Cl_2$ evaporated.

The resulting dihydroperoxide (**17**) was added to a solution of 1.6 eq of adamantanone (**18**) (76.03 mg), $Re_2O_7$ (5mg) and 5 mL of dry $CH_2Cl_2$, in an inert atmosphere. The reaction is instantaneous. 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 is purified by FCC starting with petroleum ether and adding carefully $CH_3CN$ to achieve the gradient 98:2, afforded the hybrid (**21**) (34.5 mg, 0.0561mmol, 16.5%).

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

**$^1$H NMR** (400 MHz, *CDCL₃*) δ ppm: 0.66 (3H, s, 18-$CH_3$), 0.85 e 0.87 (each 3H, 2d, *J*= 6.7 Hz, 26-$CH_3$ e 27-$CH_3$), 0.91 (3H, d, *J*=6.6 Hz, 21-$CH_3$), 1.05 (3H, s, 19-$CH_3$), 3.12 (1H, s, 6α-H), 3.28 (3H, s, 6β-$OCH_3$).

**$^{13}$C NMR** (100 MHz, *$CDCL_3$*) δ ppm: 12.1 ($CH_3$), 16.0 ($CH_3$), 18.6 ($CH_3$), 20.9, 22.5 ($CH_3$), 22.8 ($CH_3$), 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 ($CH_3$), 77.2, 110.1 (C).

*Synthesis of 5,6-oxygenated cholestane 1,2,4,5 – tetraoxacyclohexane derivative (**23**) – Process B*

28.68 μL, 0.18 mmol of the compound (**19**) was dissolved in 400 μL of $CH_3CN$, at rt. After complete dissolution, 135 μL of acetic acid was added at 0ºC, and thereafter, 69 μL of $H_2O_2$ was also added. The reaction was maintained stirring, at rt, for 6h, with additions of $H_2O_2$ every hour. The reaction was stopped adding $CH_2Cl_2$, $H_2O_2$ and $H_2O$. After the extraction, the solution was dried with anhydrous $Na_2SO_4$, filtered and the excess of $CH_2Cl_2$ evaporated.

The resulting dihydroperoxide (**17**) was added to a solution of 1.6 eq of the intermediate **5** (117 mg, 0.27 mmol), $Re_2O_7$ (5mg) and 1.5 mL of dry $CH_2Cl_2$, in an inert atmosphere. The reaction is instantaneous. 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 to 98:2 afforded the hybrid **23** (75.7 mg, 0.119 mmol, 66.2%).

**Formula:** $C_{37}H_{62}O_8$

**MP.**: 157.8 - 159.5 °C.

**IR** (film): 3610, 2932, 1722, 1457, 1373, 1243, 1184, 1096, 1063, 1038, 856 $cm^{-1}$.

$^{1}$**H NMR** (400 MHz, *CDCL₃*) δ ppm: 0.66 (3H, s, 18-$CH_3$), 0.85 e 0.87 (each 3H, 2d, *J*= 6.7 Hz, 26-$CH_3$ e 27-$CH_3$), 0.91 (3H, d, *J*=6.6 Hz, 21-$CH_3$), 1.05 (3H, s, 19-$CH_3$), 1.25 (3H, t, J= 6.6 Hz, $OCH_2C\mathbf{H_3}$), 3.28 (3H, s, 6β-$OCH_3$), 4.14 (2H, q, J= 6.6Hz, $OC\mathbf{H_2}CH_3$)

$^{13}$**C NMR** (100 MHz, *CDCL₃*) δ ppm: 12.1 ($CH_3$), 14.2 ($CH_3$), 16.0 ($CH_3$), 18.6 ($CH_3$), 20.9, 22.5 ($CH_3$), 22.8 ($CH_3$), 23.8, 24.2, 27.5, 28.0, 28.2, 28.5, 29.4, 30.5, 33.3, 35.8, 36.1, 39.5, 39.9, 41.4 (C), 41.5, 42.7(C), 44.9, 55.7, 56.2, 57.5 ($CH_3$), 60.5, 74.33 (C), 77.2, 84.6, 107.8 (C), 109.5 (C), 174.5 (C).

*Synthesis of the* cholestane -1,2,4,5 – tetraoxaadamantane (**26**)

170 mg, 0.439 mmol of the compound (**20**) was dissolved in 2.5mL of $CH_2Cl_2$ and 5mL of $CH_3CN$, at rt. After complete dissolution, 1.6 mL of acetic acid was added at 0°C, and thereafter, 550 µL 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 $CH_2Cl_2$, $H_2O_2$ and $H_2O$. After the extraction, the solution was dried with anhydrous $Na_2SO_4$, filtered and the excess of $CH_2Cl_2$ evaporated.
The resulting dihydroperoxide (**25**) was added to a solution of 1.6 eq of the adamantanone (93.5 mg, 0.622 mmol), $Re_2O_7$ (6mg) and 6 mL of dry $CH_2Cl_2$, in an inert atmosphere. The mixture was left stirring at rt for 15 min. 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 (**26**) (50 mg, 0.087 mmol, 19.8%).

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

$^{1}$**H NMR** (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-$CH_3$).

**$^{13}$C NMR** (100 MHz, *CDCL$_3$*) 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 cholestane - 1,2,4,5 – tetraoxacyclohexane derivative (**27**)*

170 mg, 0.439 mmol of the compound (**20**) was dissolved in 2.5mL of $CH_2Cl_2$ and 5mL of $CH_3CN$, at rt. After complete dissolution, 1.6 mL of acetic acid was added at 0ºC, and thereafter, 550 µL 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 $CH_2Cl_2$, $H_2O_2$ and $H_2O$. After the extraction, the solution was dried with anhydrous $Na_2SO_4$, filtered and the excess of $CH_2Cl_2$ evaporated.
The resulting dihydroperoxide (**25**) was added to a solution of 1.6 eq of the ethyl 4-oxocyclohexanecarboxylate (62.9 µL, 0.395 mmol), $Re_2O_7$ (6 mg) and 6 mL of dry $CH_2Cl_2$, in an inert atmosphere. The mixture was left stirring at rt for 15 min. 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 **27** (55 mg, 0.068 mmol, 21.28%).

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

**$^1$H NMR** (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= 6.6Hz, $OCH_2CH_3$)

**$^{13}$C NMR** (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

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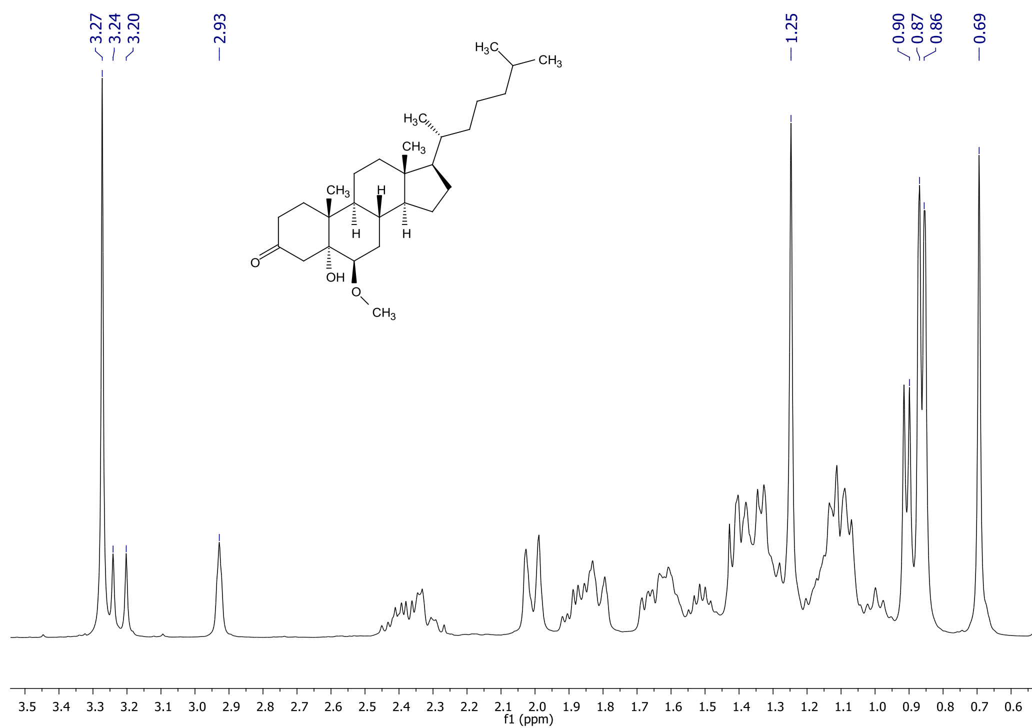
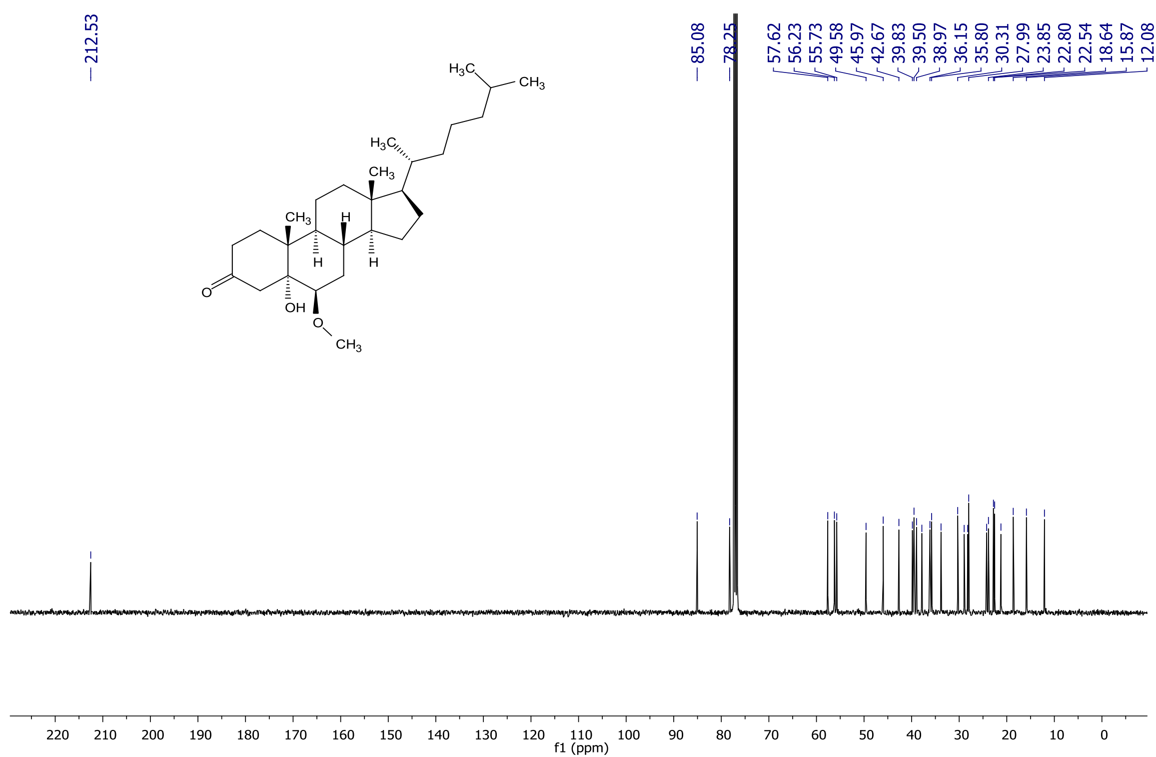
# CHAPTER VI

# ANNEXES

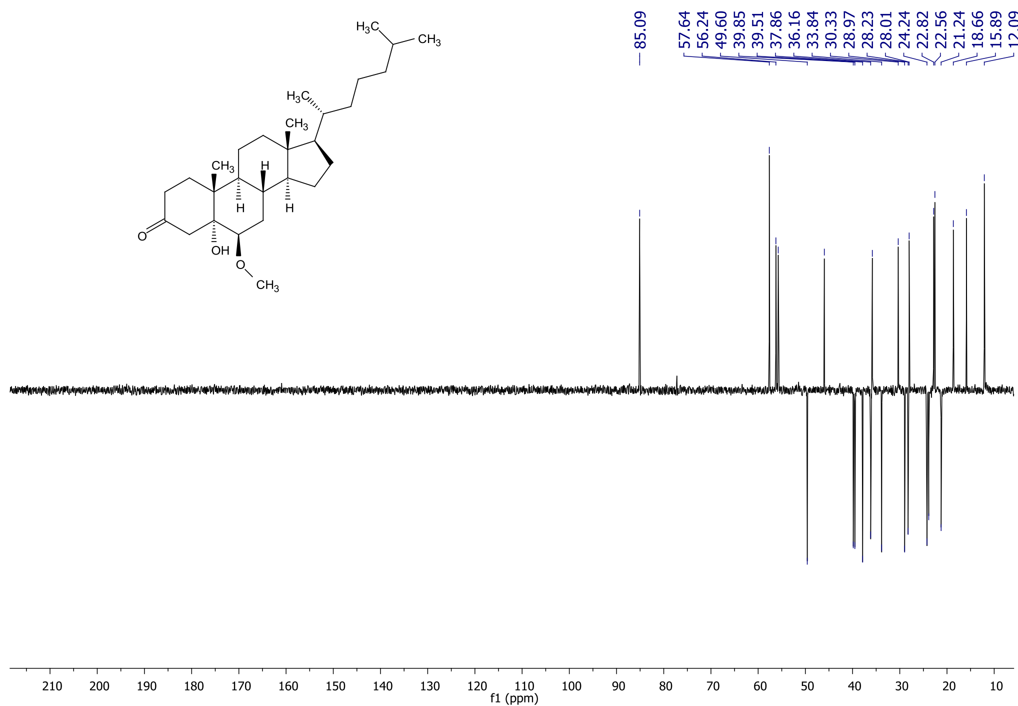
## $^{1}$H NMR spectrum of the intermediate **5**



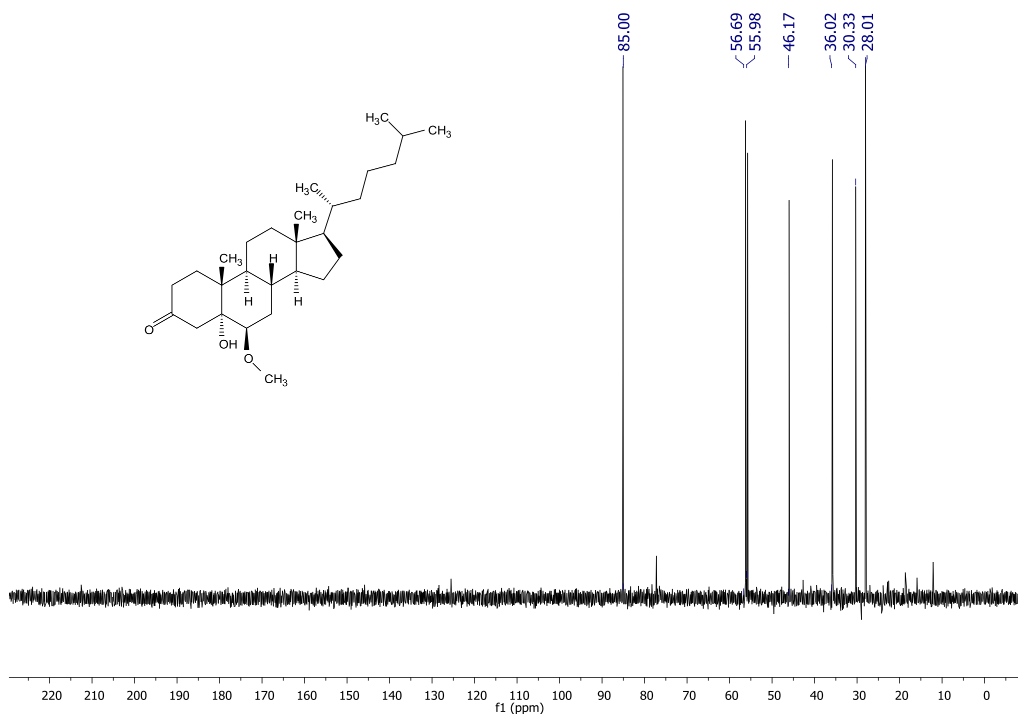

## $^{13}$C NMR spectrum of the intermediate **5**



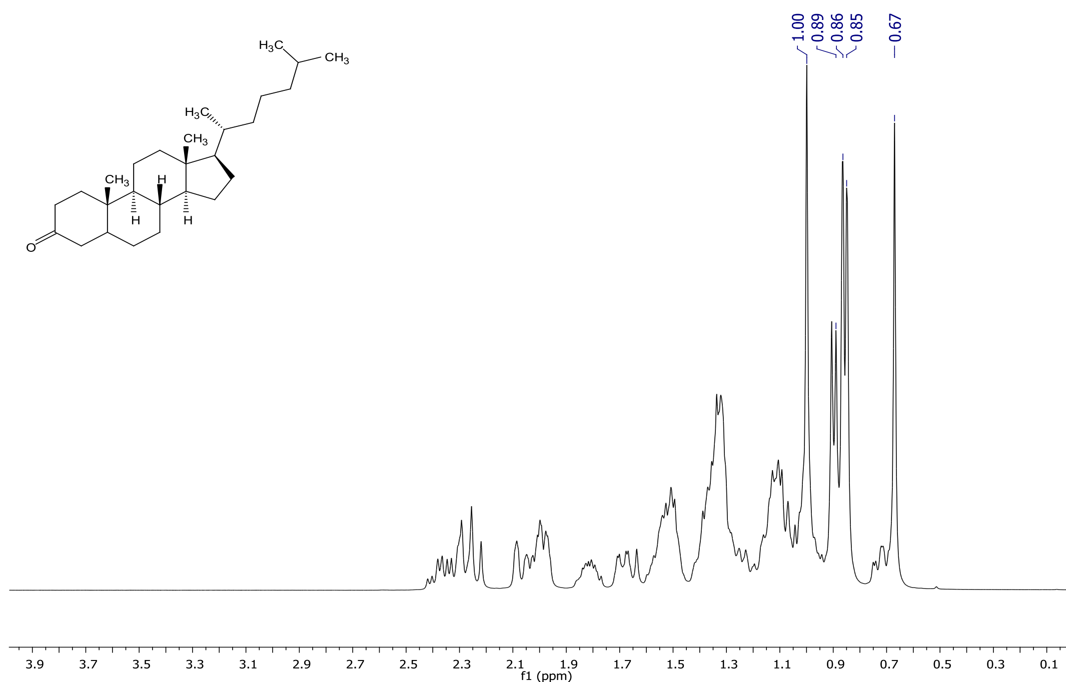
DEPT 135 spectrum of the intermediate **5**



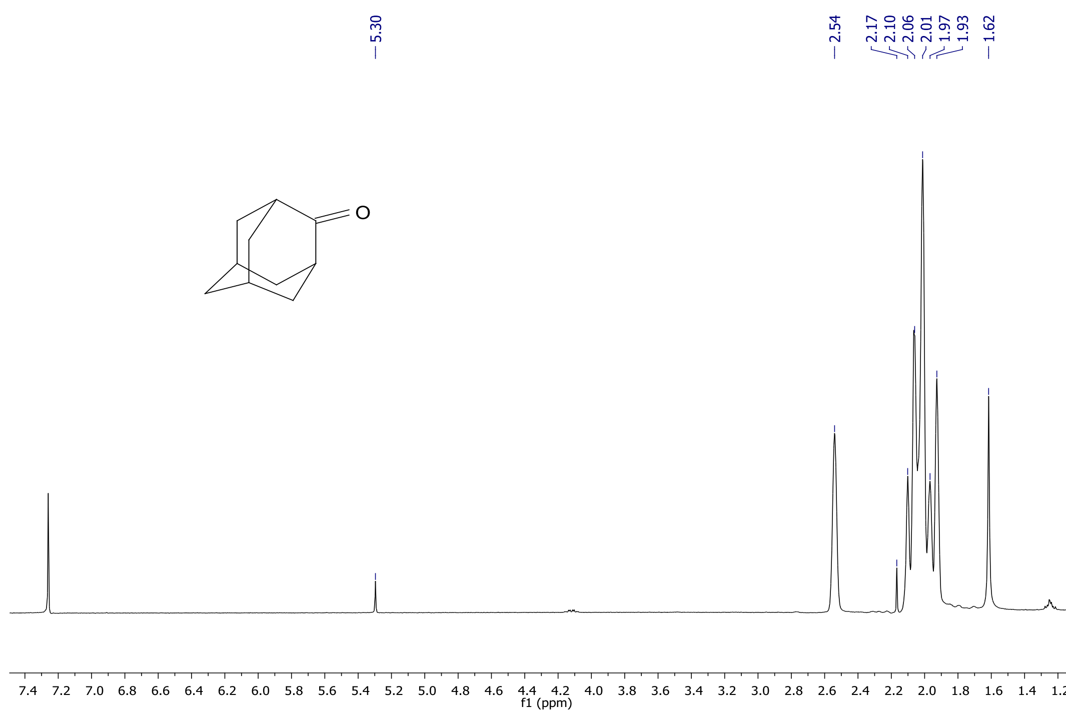

DEPT 90 spectrum of the intermediate **5**



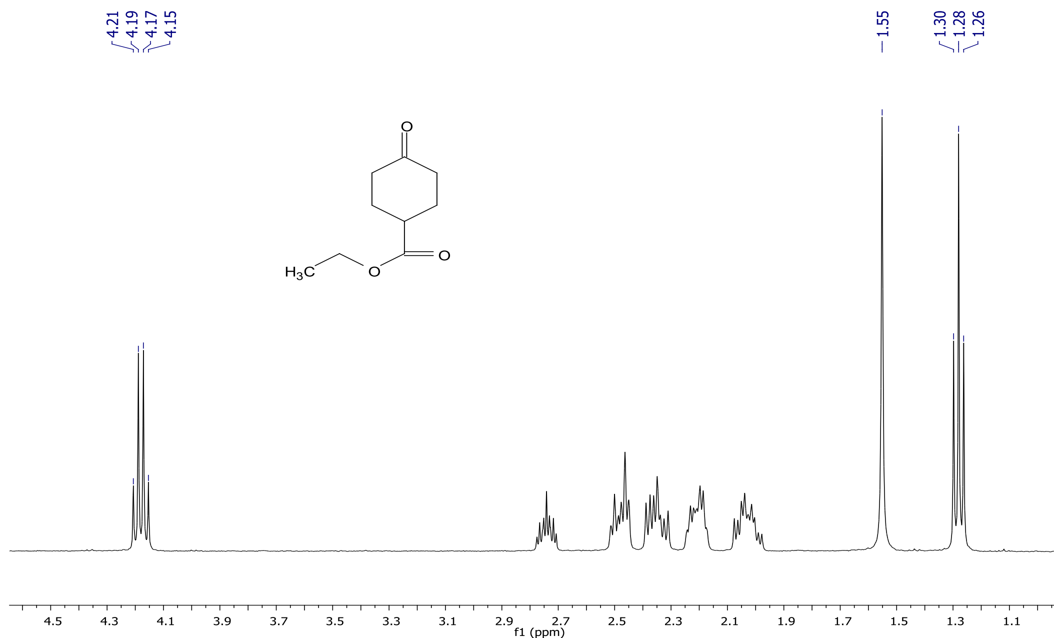
^{1}H NMR spectrum of the intermediate **20**- 5α-cholestan-3-one




^{1}H NMR spectrum of the intermediate **18**- adamantanone



$^{1}$H NMR spectrum of the intermediate **19**- ethyl 4-oxo cyclohexanecarboxylate




$^{13}$C NMR spectrum of the intermediate **19**- ethyl 4-oxocyclohexanecarboxylate