UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

Inducing oxidative stress in parasites: novel hybrid compounds based on peroxides to trap catalytic cysteine residues

Daniela Sofia Ribeiro Coutinho

Dissertação orientada pela Professora Doutora Francisca da Conceição Lopes

E coorientada pela Doutora Rita Sofia Salvador Simões Capela

Mestrado em Química Farmacêutica e Terapêutica

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To my family and my soulmate,

Acknowledgments

First of all, I would like to acknowledge to my supervisor Dr. Francisca Lopes for all the support and for presenting me with this challenging project, which allowed me to learn a lot more in medicinal chemistry.

I would also thank to Professor Rui Moreira, who gave me the opportunity to work in his laboratory and to expand my knowledge.

I am very grateful to Dr. Rita Capela for all the guidance, advices, criticism, patient and encouragement throughout all this year. All the hours in the laboratory would be hardest and certainly less fun.

I would like to thank Dr. Fátima Nogueira for the *in vitro* antimalarial assays, and Dr. Francisco Gamarro for *in vitro* antileishmanial assays.

To all my labmates for all the support, friendship and advices.

I am sure I will forget someone, so I would like to thank to all my friends, specially Miguel, Gonçalo, Hellen, Leo, Sara, and Leonor, whom one way or another helped me along this thesis.

To my childhood friends, Patrícia, Raquel, Clarinha, and Filipa, for cheering me up, for being my refugee, for helping me in the good and bad moments, and for your presence and friendship throughout all these years.

To the friends that Covilhã offered me, my dearests Catarina's, Joana's, Maria and Marta. Thank you for all those amazing years, for always believe in me and being there when I needed the most.

It is not possible to forget the unconditional support from my newest and awesome friends Joana and Andreia, not only in the lab, but also for being my new family in Lisbon. Thank you for all the patient, laughs and friendship.

I would like to express my deep gratitude to my grandmothers Zézinha and Zaia for all the thoughts, teachings, and delicious food. For your unconditional love, for always

believing in my capacities and encourage me to find a better and improved version of myself.

To my four little stars. I know you are always looking up for me.

To all my family, specially my parents, sister, and Martim, for whom a thousand words would not be enough. I will never be able to thank enough for always believing in me throughout all my academic course and for your understanding during my absent.

Last, but not least, I would like to express my endless gratitude to my soulmate and best friend, Daniel Figueiredo, who has always been a constant in my life even now that we are 8000 km apart. Without your patience, encouragement, love and support this would not be possible.

Abbreviations

^{1}H NMR – Proton Nuclear Magnetic Resonance

^{13}C NMR – Carbon Nuclear Magnetic Resonance

ACN – Acetonitrile

AmB – Amphotericin B

APT – Attached proton test

Ar - Aromatic

CL – Cutaneous leishmaniasis

COSY – Proton-proton correlation spectroscopy

Cq – Quaternary carbons

d – Doublet

DCM – Dichloromethane

dd – Double doublet

Equiv – Equivalents

EtOAc – Ethyl acetate

FP – Falcipain

GR – Glutathione reductase

GSH – Glutathione

GSSG – Glutathione disulfide

h – Hour / hours

HMBC – Heteronuclear Multiple Bond Correlation Spectroscopy

HMQC – Heteronuclear Multiple Quantum Correlation Spectroscopy

HPLC – High-performance liquid chromatography

Hz – Hertz

***J* –** Coupling constant

k_{obs} – Rate constant

Log P – Octanol – water partition coefficient

m – Multiplet

m.p. – Melting point

min – Minutes

ML – Mucocutaneous leishmaniasis

NTD – Neglected Tropical Disease

PKDL – Post-kala-azar dermal leishmaniasis

ppm – Parts per million

ROS – Reactive oxygen species

r.t. – Room temperature

R_t – Retention time

s – Singlet

Sb^v – Pentavalent antimonial

$t_{1/2}$ – half-life time

THF – Tetrahydrofuran

TLC – Thin Layer Chromatography

TR – Trypanothione reductase

TS_2 – Trypanothione disulfide

$T(SH)_2$ – Trypanothione

UV/Vis – Ultraviolet-visible spectroscopy

VL – Visceral leishmaniasis

WHO – World Health Organization

δ_C - Carbon nuclear magnetic resonance chemical shift

δ_H – Proton nuclear magnetic resonance chemical shift

η - Yield

Abstract

Infectious diseases caused by viruses, parasites and bacteria are currently the second cause of mortality worldwide. One of these parasites is *Leishamania spp*., which is the protozoa responsible for leishmaniasis. Although, considered the ninth largest infectious disease burden worldwide, is still one of the most neglected tropical disease. Leishmaniasis has three main syndromes, which can range in severity from a relatively benign, self-limiting cutaneous form, to a potentially fatal systemic illness, known as visceral leishmaniasis. While cutaneous leishmaniasis is the most widespread on a global scale, visceral leishmaniasis is the most severe and lethal form of the disease. Another important tropical disease is malaria, caused by *Plasmodium spp*. protozoan parasite and transmitted by a female infected *Anopheles* mosquito. Malaria is regarded to be the world's most devastating tropical disease. The control of both diseases still relies on chemotherapy, but the armamentarium currently used have their use limited due to several adverse effects, elevated cost, lack of efficacy, and appearance of resistant parasites. Additionally, there is no effective vaccine to fight either leishmaniasis or malaria. For these reasons, the search for new therapeutic approaches are urgent. Endoperoxides are known to be reductively activated by iron(II)-heme to form carbon centered radicals and reactive oxygen species which can generate oxidative stress inside parasites. Both, *Leishmania* and *Plasmodium* require high levels of iron at critical steps of their life cycle, and therefore, these compounds could be promising to the design and discovery of new drugs, since this hybrid compounds can act by a dual mechanism and thus can be more effective than single drugs. In this thesis it is proposed the development of endoperoxide-based hybrid compounds which can generate oxidative stress inside the parasite and alkylate biomolecules essential to the parasite survival, and at the same time can deliver an α,β-unsaturated compound able to inhibit essential enzymes to the parasite. The synthetic route occurs in two steps. First, an aldehyde or ketone is treated with formic acid and hydrogen peroxide to give the intermediate *gem*-dihydroperoxide. Then, *trans*-cinnamaldehyde and Re_2O_7 are added to complete conversion into 1,2,4,5-tetraoxane. In this study, seven novel tetraoxanes were synthesized and fully characterized. In addition, the

activation in biomimetic conditions was performed using $FeBr_2$, being the reaction followed by HPLC. Synthesized compounds were evaluated for their antiparasitic activity.

Key words: Leishmaniasis, Malaria, Tetraoxanes, Iron activation.

Resumo

As doenças infeciosas causadas por vírus, parasitas e bactérias são atualmente a segunda maior causa de mortalidade a nível mundial.

Um desses parasitas é a *Leishmania spp*., protozoário responsável pela leishmaniose. Esta doença é atualmente considerada a nona maior doença infeciosa a nível mundial já que afeta cerca de 12 milhões de pessoas em praticamente todos os continentes (Europa, África, América e Ásia). Apesar disso, o interesse por parte da indústria farmacêutica é diminuto sendo classificada como uma doença negligenciada. Encontra-se difundida tanto em áreas tropicais como em áreas subtropicais sendo considerada endémica em 98 países, segundo a Organização Mundial de Saúde (OMS). Esta doença afeta principalmente as populações mais pobres, em países em vias de desenvolvimento e é responsável por cerca de 30 000 mortes por ano, números que têm vindo a aumentar a um ritmo alarmante.

A leishmaniose pode causar um amplo espectro de patologias em humanos, as quais podem variar em severidade desde uma forma relativamente benigna da doença, porém limitante, designada por leishmaniose cutânea, passando por uma variante que consiste num desfiguramento facial, como acontece na leishmaniose mucocutânea, podendo mesmo chegar a uma patologia potencialmente fatal, a leishmaniose visceral. A leishmaniose cutânea é a forma mais comum da doença, sendo que em cada 1,3 milhões de casos totais que surgem anualmente, 1 milhão são referentes à leishmaniose cutânea e apenas 0,3 milhões são relativos à leishmaniose visceral. Esta última, apesar de ser uma forma menos frequente da doença é a mais severa, podendo conduzir à morte se não for devidamente tratada. A leishmaniose mucocutânea representa a forma mais destrutiva da doença.

Existem mais de 20 espécies de *Leishmania* consideradas infeciosas para o ser humano, entre elas estão a *L. donovani*, *L. infantum*, *L. siamensis*, *L. braziliensis* e *L. guyanensis*. A leishmaniose é transmitida através da picada de uma "sandfly" fêmea infetada pelo parasita, que normalmente pertence ao género *Lutzomia* no "Novo Mundo" (América Central e do Sul) e do género *Phlebotomus* no "Velho Mundo" (Europa, África e Ásia).

Uma outra doença tropical é a malária cuja infeção é causada por parasitas do género *Plasmodium spp*., transmitidos aos humanos através da picada de um mosquito fêmea infetado pertencente ao género *Anopheles*.

Existem seis espécies do parasita capazes de infetar humanos, tais como o *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. ovale wallikeri* e *P. knowlesi*. De entre estas espécies o *P. falciparum* é a que origina a forma mais perigosa e fatal da malária e, em conjunto com o *P. vivax* são as espécies mais comuns.

A malária é uma doença tropical que representa um dos problemas públicos mais preocupantes, já que, de acordo com a OMS, existem cerca de 212 milhões de novos casos por ano que conduzem a aproximadamente 430 000 mortes anuais, sendo que a maioria ocorre em África e em crianças com menos de cinco anos de idade.

Para estas duas doenças tropicais a realidade é a mesma, já que não existem vacinas eficientes e o seu controlo encontra-se limitado ao uso de quimioterapia e à prevenção. Infelizmente, a quimioterapia atualmente utilizada para ambas as doenças é a mesma desde há várias décadas, por isso, o seu uso também se encontra limitado devido ao aparecimento de resistências, e ainda devido aos diversos efeitos secundários e ao seu elevado custo. Todas estas razões conduzem a uma procura urgente de novos agentes terapêuticos. É importante que as novas alternativas terapêuticas, para além de eficazes, sejam economicamente acessíveis, uma vez que estas doenças afetam principalmente os países em vias de desenvolvimento.

Neste contexto surgem os endoperóxidos, que são uma família de compostos capazes de serem redutivamente ativados pelo ferro (II), levando à formação de espécies reativas de oxigénio, radicais centrados no carbono e compostos carbonílicos. Assim, os endoperóxidos podem atuar por diferentes mecanismos de ação. Por um lado, estes compostos têm a capacidade de aumentar o *stress* oxidativo dentro do parasita, podem ainda alquilar biomoléculas essenciais para a sobrevivência dos parasitas. Por outro lado, se se libertar o composto carbonílico α,β-insaturado, pode reagir como aceitador de Michael levando à inibição de enzimas necessárias à sobrevivência dos parasitas. Deste modo, estes compostos podem ser utilizados contra qualquer agente infecioso que disponha de elevadas concentrações de ferro em pontos críticos do seu ciclo de vida, tal como acontece com os parasitas dos géneros *Leishmania* e *Plasmodium*.

Seguindo esta linha de raciocínio, nesta tese é reportada a síntese de uma pequena biblioteca de novos compostos 1,2,4,5-tetraoxanos que são capazes de atuar através de um mecanismo de dupla ação, uma vez que, após a ativação pelo ferro (II), irá formar-se uma espécie radicalar, aumentando o *stress* oxidativo. Ao mesmo tempo, liberta-se uma molécula carbonílica α,β-insaturada, neste caso o *trans*-cinamaldeído, capaz de inibir a tripanotiona redutase (TR) no caso da *Leishmania*, e a falcipaína no caso do *P. falciparum*. Estas enzimas desempenham papéis cruciais nos parasitas, a TR participa na defesa antioxidante do parasita *Leishmania*, enquanto que a falcipaína é uma das enzimas presente no vacúolo digestivo do *Plasmodium* responsável pela hidrólise da hemoglobina.

Estes 1,2,4,5-tetraoxanos são preparados com recurso a uma síntese em dois passos. No primeiro passo, os aldeídos ou cetonas reagem com ácido fórmico e com peróxido de hidrogénio dando origem ao intermediário *gem*-dihidroperóxido. O segundo passo usa o óxido de rénio (VII) como catalisador, para formar um complexo com o *trans*-cinamaldeído, que posteriormente é adicionado ao intermediário de modo a permitir a conversão no produto final 1,2,4,5-tetraoxano. Deste modo, o *trans*-cinamaldeído foi escolhido como segundo reagente por ser um composto α,β-insaturado, podendo ser um potencial inibidor das enzimas TR e falcipaína.

Assim, conseguiram-se sintetizar e purificar sete novos compostos híbridos (**21**-**27**) baseados na estrutura tetraoxano com rendimentos moderados a bons. Estes compostos foram ainda caracterizados utilizando-se técnicas de ressonância magnética nuclear, nomeadamente RMN ^{1}H, RMN ^{13}C, COSY, APT, HMQC e HMBC.

De modo a confirmar a ativação pelo ferro (II) e a consequente libertação do composto α,β-insaturado, os compostos foram estudados em condições biomiméticas na presença de brometo de ferro (II), seguindo-se a reação por HPLC. Com estes estudos confirmou-se que o *trans*-cinamaldeído é libertado na presença de brometo ferroso, confirmando-se que o ferro (II) consegue ativar eficientemente esta classe de compostos. Todos os compostos apresentaram tempos de semi-vida curtos, o que indica que são rapidamente ativados pelo ferro (II). Estes estudos foram uma prova de conceito que mostraram que o composto carbonílico α,β-insaturado é rapidamente libertado após a ativação dos tetraoxanos pelo ferro (II), sugerindo que estes endoperóxidos poderão ter comportamentos semelhantes *in vivo*.

A capacidade antiparasitária dos tetraoxanos sintetizados foi avaliada para a malária e para a leishmaniose. Relativamente à malária, os compostos demonstraram ser moderadamente ativos, revelando melhores valores de inibição na gama do sub-μM para a estirpe Dd2 do *Plasmodium falciparum*. A atividade dos compostos foi ainda testada para a forma promastigota de *Leishmania donovani*, porém, devido a problemas de solubilidade, nenhum dos compostos revelou atividade.

Palavras-chave: Leishmaniose, Malaria, Tetraoxanos, Ativação com ferro.

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Chapter 1

1. State of the Art

1.1 Neglected Tropical Diseases

A group of heterogenous infectious diseases are classified as tropical diseases, neglected diseases, diseases of the developing world, and diseases of poverty.[1]

For a better understanding of the topic, Heras, *et al*, explains both terms "tropical" and "neglected". Whereupon, the first term refers to diseases that occur solely, or principally, in tropical areas, such as malaria, leishmaniasis, Chagas disease, African trypanosomiasis and dengue, the second term means that in spite of the high mortality and/or morbidity produced by the disease, little scientific attention and resources have been dedicated to understanding and curing them.[1]

Neglected tropical diseases (NTD) are a group of chronic, disabling and disfiguring conditions that can affect more than two billion people worldwide (about one third of the total population) and nearly two million deaths per year, mostly in the poorest areas of the planet.[1–4]

Common characteristics of NTD include high endemicity in rural and impoverished urban areas of low-income countries, ability to impair childhood growth, and they are both cause and consequence of poverty, profound effect on demography, negative impact in cognitive and educational development as well as adverse effects in work productivity.[1,5]

Chemotherapy plays a key role in treating, helping to control or eliminate diseases in the developing world. Nevertheless, the majority of the drugs used to treat or prevent NTD has remained essentially unchanged since the middle of the 20th century, so many of them have toxicity issues, poor efficacy or have lost efficacy due to emergence and spread drug resistance.[1,5] The fact that, sometimes, the patients do not complete the course of treatment it is because of the cost, availability, invasive route of administration, and long treatment duration, increasing the chances of development of drug resistance. In addition, there are no effective vaccines for most of NTD. So, due to the wide diffusion of these diseases, the small number of effective drugs and the frequent emergence of resistance, the drug discovery in this therapeutic area is a very urgent and challenging task. Unfortunately, big pharmaceutical companies do not invest in drug discovery programs because of low financial returns. However, over the past few years, this gap has started to reduce

considerably as several non-profit organizations have intervened heavily and efficaciously to sustains this research.[2,6]

Two of the most prevalent pathogenic diseases in many countries around the world are, undoubtedly, leishmaniasis and malaria, characterized by a very high morbidity, lethality as well as a widespread diffusion across the planet. [2,5–7]

1.2 Leishmaniasis

Leishmaniasis is a vector-borne disease caused by a heterogeneous group of protozoan parasites of the Kinetoplastidae order, Trypanosomatidae family, and *Leishmania* genus.[1,8–12] There are more than 20 species of *Leishmania* which are infectious to humans, such as *L. donovani*, *L. infantum*, *L. siamensis*, *L. braziliensis*, and *L. guyanensis*. *Leishmania* protozoan species are a hemoflagellate group responsible for human leishmaniasis, which has several other species has reservoir, such as rodents, canines, equines, marsupials and other in the wild animal population. Due to the ability of this infectious agent to circulate among both humans and animals, leishmaniasis can be considered as an important zoonosis.[13–16]

This infectious disease is transmitted by the bite of an infected female sandfly of the genera *Lutzomyia* in the New World (central and south America) and *Phlebotomus* in the Old World (Africa, Asia, Europe).[12,13,15,17] This vectors are smaller than mosquitoes, so the prevention is more difficult, since they can cross standard mosquito nets.[8,18]

Leishmaniasis affects about 12 million people on all continents, being widespread in tropical and subtropical areas and found in 98 countries.[9,19–22] It is regarded as a major public health problem, causing significant morbidity and mortality with near 1.3 million new cases per year, and about 350 million people are globally threatened. Furthermore, the number of cases is increasing globally at an alarming rate.[13,16,22–24] Is reported that although, leishmaniasis represents the ninth largest infectious disease burden worldwide, is still considered one of the most NTD.[7,22,25]

The disease affects largely the poorest of the poor people, mainly in developing countries, because its associated with malnutrition, population displacement, poor housing, weak immune system and lack of resources.[13,24,26] The increase of the incidence of this disease can also be associated to environmental changes, such as deforestation, building of dams, irrigation schemes, global warming, and urban development.[13,16,23]

The main reasons for this blowout in endemic and non-endemic regions, and the primary hurdles in the elimination of leishmaniasis are related to a lack of effective control measures for both parasite and its vector, the poor knowledge about the disease itself, and lack of effective health policies. Furthermore, this infections can last for decades leading to an economic, social, and political consequences.[5,27,28]

The geographic distribution of different *Leishmania* species can induce distinct types of the disease in each region as well as its severity. It is estimated that between 0.2 to 0.4 and 0.7 to 1.3 million people are newly infected every year with visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL), respectively, but only a small fraction of them will develop the disease and about 20 000 to 30 000 will eventually die from leishmaniasis.[22,29]

VL is highly endemic in the Indian subcontinent and in East Africa. According to the World Health Organization (WHO), in 2015, more than 90% of new cases occurred mostly in seven countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan.[11,27] VL emerge essentially in tropical and subtropical areas but, due to global warming the disease is now spreading to temperate regions such as Brazil and Argentina, suggesting that these climates do not restrict the adaption of vectors in these regions.[30]

CL is endemic in more than 70 countries worldwide, and 95% of cases occur in the Americas, the Mediterranean basin, the Middle East and Central Asia (Figure 1.1).[23,31,32] Similar to the previous case, more than two thirds of new CL cases occur mainly in six countries: Afghanistan, Algeria, Brazil, Colombia, Islamic Republic of Iran and Syrian Arab Republic.[11,24,27] Although CL is widespread on a global scale, it is often quite focal in some regions, due to the specific habitat requirements of the sandfly vector and its hosts.[16]

Leishmaniasis causes a wide spectrum of pathologies in humans, which can range in severity from a relatively benign and self-limiting cutaneous form nominated as CL, to facial disfigurements which can occur in mucocutaneous leishmaniasis (ML) or to potentially fatal VL, also known as kala-azar.[6,24,33] Despite the differences between the pathologies of those manifestations, there are some factors that can influence the clinical presentation and prognosis, such as the duration of infection, the host immune response and also depends on the infecting species and, consequently, the endemic region.[8,9,34]

The incubation of this pathology can range from a few days to months, which can lead to misdiagnosis of the skin lesions.[16] When an infected vector bites the host, the infection of CL begins in this precise spot, where a papule appear. It can then enlarge to a nodule and ulcerates with a period of time of 1-3 months.[24,34] Lesions are generally painless unless secondary infection occurs, and the disease is frequently self-cured within months

to years, depending on the *Leishmania* species, leaving behind disfiguring, lifelong cutaneous scars.[16,17,24,35]

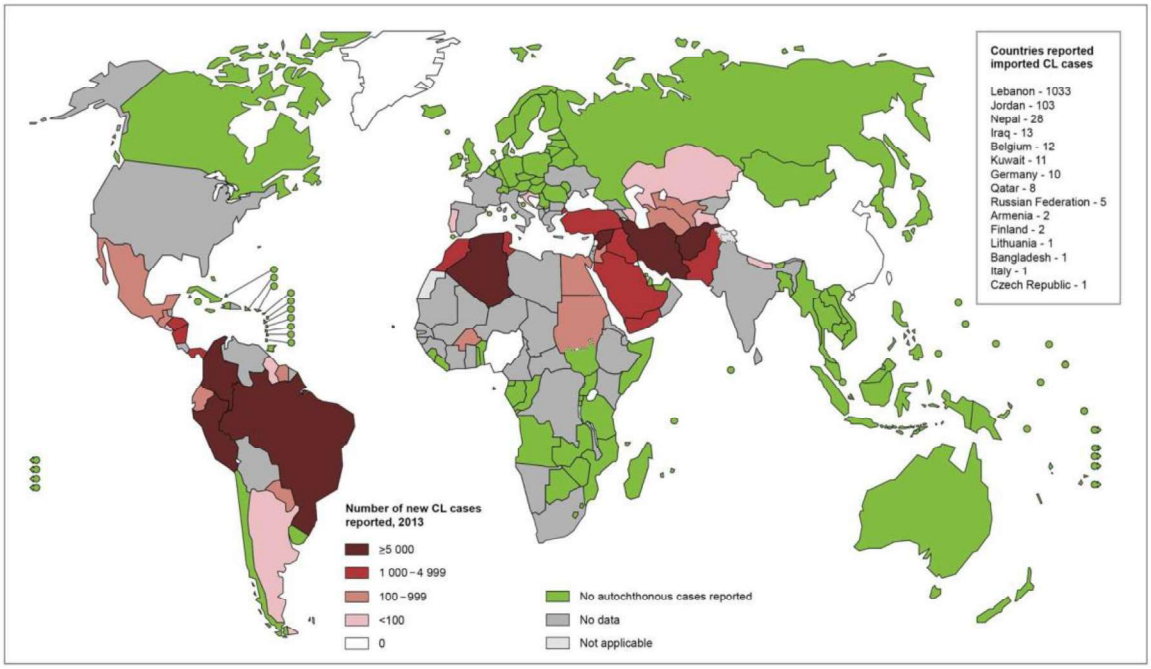


Figure 1.1 – Worldwide status of CL, in 2013. Adapted from WHO 24/06/2017 http://www.who.int/Leishmaniasis/burden/Leishmaniasis_Burden_distribution_VL_CL_2013.pdf

ML, characterized by infection in the mucous membranes of mouth, nose or pharynx, is mainly caused by *L. braziliensis*, *L. panamensis* and *L. guyanensis* and usually appears 1 to 5 years after CL has healed.[16,19,23,36]

The infection starts at the site of inoculation with an erythema and ulcerations at the nasal mucosa, then the disease can spread to the nasal septum causing perforations and even destructive inflammatory lesions. When lesions progress to the pharynx and larynx, it can obstruct theses organs and lead to remarkable disfigurement and sometimes death.[19,23,24,37] In ML, spontaneous self-healing does not occur, and the treatment is difficult. Moreover, it can lead to bacterial coinfections which could be potentially fatal.[23]

VL is the most severe form of leishmaniasis and it can lead to death if untreated, since its lesions affects internal organs like spleen, liver, and lymph nodes. The disease as an incubation period that can range from 2 to 6 months and it can present an acute, subacute or chronic evolution. However, many infected patients can remain asymptomatic.[19,30,34,38,39] The main manifestations of the disease are prolonged fever, cough, weight loss, weakness, diarrhea or dysentery, and abdominal swelling. Some patients can also present progressive

anemia, edema, bleeding episodes, and even splenomegaly, hepatomegaly, and pancytopenia.[19,30,38–40]

In the latent cases, which patients remain undiagnosed, the symptoms can appear several years after infection. In these cases, they become immunocompromised and their skin become darker, defining the origin of the disease synonym kala-azar, which means black fever in the Hindi language.[38] Moreover, patients with no symptoms and no overt signs of VL can function as reservoirs in endemic regions.[30]

Another variant of the main pathologies is post-kala-azar dermal leishmaniasis (PKDL), which is caused by *L. donovani* and can emerge months to years in patients who have been treated against VL.[24,30,38,39] The infection begins around the mouth, nodules can increase in size and become generalized all over the body or even evolve into ulcers, in which PKDL patients become reservoirs of *Leishmania* parasites. In more severe cases, the infection can lead to blindness. [24,30,38]

1.2.1 Life cycle

The life cycle develops in two different organisms, an insect vector (sandfly) and a mammalian host. *Leishmania* parasites have dual-morphological forms: amastigotes, which are intracellular and non-motile, and promastigotes, flagellated motile and extracellular (Figure 1.2). Promastigotes undergoes through several morphological states of differentiation until become amastigote, which is the infectious form of the parasite.[6,8,18]

Besides the fact that iron acquisition has been poorly described, it is known that the low iron environment is a potent trigger for the differentiation of promastigote into infective amastigote.[10,41] Within parasitophorous vacuoles of macrophages, the harsh acidic environment would destroy most microorganisms.[7] However, the intracellular stages of *Leishmania* have developed an adaptive mechanism that allow them to survive. One of the adaptions is the acquisition of essential nutrients, such as iron, which can be obtained as inorganic iron or from heme.[7,10] Promastigotes can activate the expression of ferrous iron transporters, which increase the iron uptake and involves reactive oxygen species (ROS) production.[41] Due to the redox potential of *Leishmania*, iron can be toxic in high amounts, so the acquisition systems must be tightly regulated.[7]

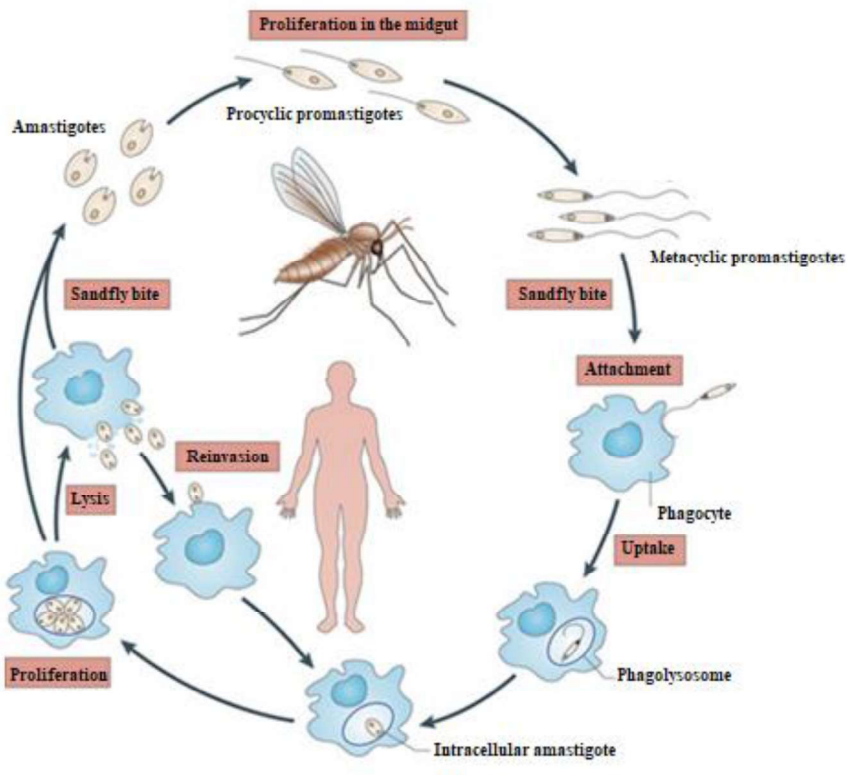


Figure 1.2 – *Leishmania* life cycle. Adapted from[18]

The life cycle starts when a sandfly feeds on a mammalian host, releasing metacyclic promastigotes and several salivary components. Upon inoculation into the vertebrate host, promastigotes are phagocytosed by one of the several cells types, such as macrophage, where they lose the flagella becoming amastigotes.[18,34] This form replicate within the macrophage, which then suffer rupture, releasing the parasites, allowing the reinfection of new cells.[8,18]

The cycle is completed when the amastigote form of *Leishmania* is ingested by another sandfly. Once in the midgut of the vector, the organism is converted into noninfective procyclic promastigotes and then into non-dividing metacyclic promastigotes, which are ready to infect another mammal.[8,18]

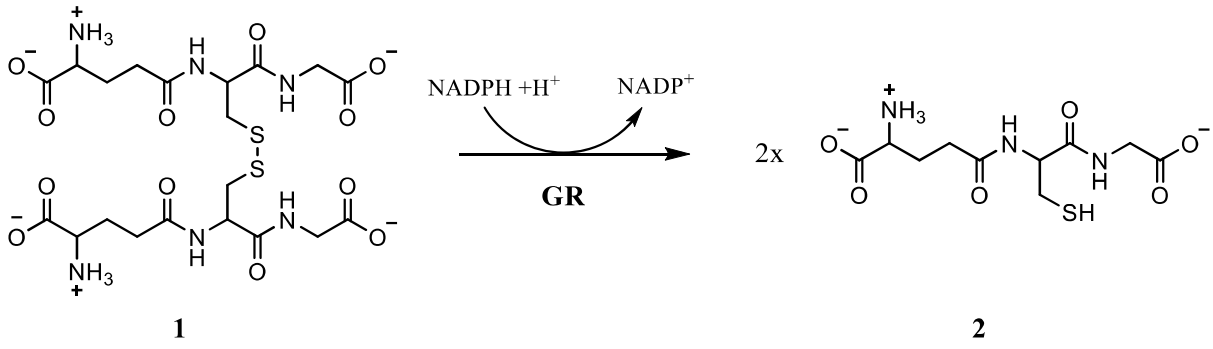
1.2.2 Trypanothione reductase

Trypanothione was discovered, in 1985, as a result of studies on an apparently unusual glutathione reductase (GR) activity in the African trypanosome, *Tryoanosoma brucei brucei*. It is a conjugate of glutathione and the polyamine spermidine, which is a

component of the structure of trypanothione disulfide (TS_2), rationalizing the actions of several antileishmanial drugs.[42,43]

Trypanothione reductase (TR) is a member of the NADPH-dependent flavoprotein oxidoreductase, central to the unique thiol-redox cycling system, which is common to trypanosomiasis and leishmaniasis, and is structurally and mechanistically related to GR of mammals. The glutathione system found in human host, is responsible for the maintenance of an intracellular reducing environment important for the reduction of disulfides, detoxification of peroxides, and the synthesis of DNA precursors. The trypanothione system is thought to protect the parasite against damage by oxidants, xenobiotics and toxic heavy metals.[44,45] The enzymatic regeneration of the thiol pool in *Leishmania* parasites is dependent on TR. This enzyme has a vital physiological role, particularly in highly oxidative environment within the host cells, generated during the antiparasitic defense response.[43,45]

In mammals, a glutathione (GSH)-based system act as a first defense against oxidative stress, leading to glutathione disulfide (GSSG), **1** (Figure 1.3). The regeneration of protective GSH, **2**, from GSSG is catalyzed by GR.



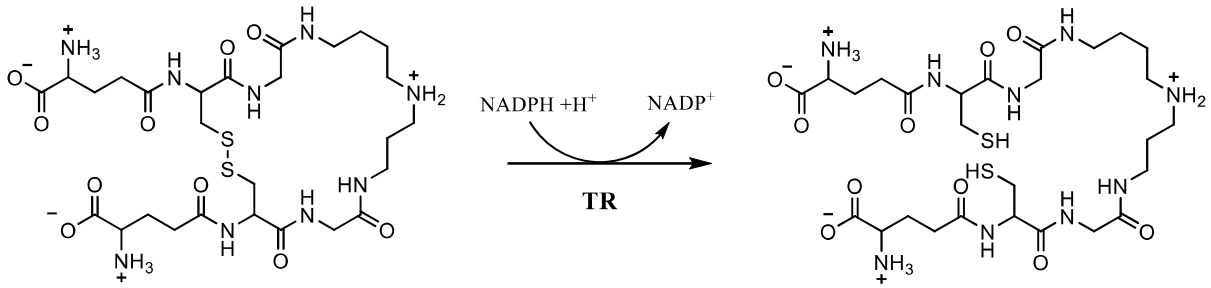
1 **2**

Figure 1.3 – GR-catalyzed reduction of glutathione disulfide **1** to two thiol glutathione **2**. Adapted from[42]

In *Leishmania* parasites occur an analogous system, where dithiol trypanothione ($T(SH)_2$), **4**, differs from GSSG by the presence of a spermidine cross link between the two glycyl carboxyl groups. Additionally, the enzyme TR can reduce TS_2, **3**, to $T(SH)_2$ in a manner analogous to GR (Figure 1.4).[43]

Unlike human hosts, *Leishmania* parasites contain TR instead of analogous enzyme GR to process their cognate substrates trypanothione and glutathione, respectively. Parasite TR does not process GSSG and host does not reduce TS_2. Therefore, the design of selective

inhibitors of TR could be important in the development of drugs against leishmanial infections, due to the mutually exclusive recognition and rejection of cognate substrates between host and parasite.[45]



3 **4**

Figure 1.4 – TR-catalyzed reduction of trypanothione disulfide **3** to dithiol trypanothione **4**. Adapted from[42]

1.2.3 Current Treatments for leishmaniasis

To date, there are no effective vaccines and there is no single effective treatment for all *Leishmania* species and syndromes. The control of leishmaniasis primarily relies on chemotherapy, however current treatment modalities usually do not result in parasitological cure.[39,46] Most of the available therapeutic arsenal for leishmaniasis (Figure 1.5) has several limitations related to adverse effects, elevated cost, need of hospitalization during all treatment, lack of efficacy, and development of drug resistance. Moreover, is important to notice that a response to a drug can vary from an endemic region to another.[39]

Within the armamentarium against leishmaniasis are pentavalent antimonial compounds (Sb^v), which have been the standard first-line treatment recommended for CL, ML and VL, for several decades.[17,38,39] This type of compounds include drugs such as sodium stibogluconate **5** or meglumine antimoniate **6**, however, due to their poor oral absorption, these compounds have to be administered intravenously, intramuscularly or directly into the lesions.[17,38] In addition, these are expensive therapies, adding the fact the need for hospitalization due to cardiac and toxicity problems, increasing the limitation of using this therapy for leishmaniasis.[17,32,47]

It was suggested that Sb^v drugs act with a dual mode of action. When in contact with macrophages, Sb^v can stimulate them to kill the intracellular parasites, and inside parasites

they can undergo with a biological reduction to a more active trivalent antimonite, Sb^{III}, acting as a produg, and kill directly the parasite inside the phagolysosome by inhibiting trypanothione reductase.[35,39,48]

Amphotericin B (AmB) **7**, is another drug used against leishmaniasis. Is a polyene antibiotic isolated from *Streptomyces nodosus*, and have typically been used as the second line of treatment for VL and ML since 1960.[17,38,39]

The mechanism of action is not fully understood, however it is assumed that AmB can interfere with cell membrane of *Leishmania* parasite, changing its permeability and leaking of intracellular components.[39,49] Despite the excellent cure rates, high safety and efficacy, AmB has many adverse effects, which include difficult administration, nephrotoxicity, hypokalemia, and myocarditis, so this treatment also needs hospitalization and close monitoring, thus the cost of this therapy can escalate.[17,38]

Paromomycin **8**, is an aminoglycoside antibiotic isolated from *Streptomyces krestomuceticus*, which is used as a second line drug to circumvent the problems of Sb^{V} resistance.[35,38,39,50] The main adverse effects are fever, reversible ototoxicity, rise in hepatic transaminases, and pain at injection site, which may pose difficulty in its adoption in a control program of a developing country. However this event does not lead to the discontinuation of therapy.[6,38,39] Besides all these disadvantages, this compound continues to be used mainly because of its affordability.[38]

The mechanism of action is through interference with protein synthesis in the ribosome of the target organism.[6,39,50]

Miltefosine **9** belongs to the class of alkylphosphocholine drugs, which are phosphocholine esters of aliphatic long-chain alcohols. Originally developed as an anticancer drug, this compound is the first and only agent used by oral administration for all types of leishmaniasis. This was a major breakthrough, since it does not require hospitalization and it can offer domiciliary treatment, which increases the adhesion for this treatment.[31,39,51–53]

Miltefosine revealed strong possibility of an apoptosis-like mode of cell death in *L. donovani* promastigotes and amastigotes, however the mechanism of action is not fully understood.[33,47,52,54] Moreover, it can produce ROS, inhibition of cytochrome C oxidase

and cell death by necrosis which can be explained through the disturbance on the lipid membranes.[6,39,47,51]

Miltefosine has lower toxicity, however has some limitations, such has teratogenicity effects, therefore the use during the pregnancy is strictly contraindicated. [6,24,39,46,50,51]

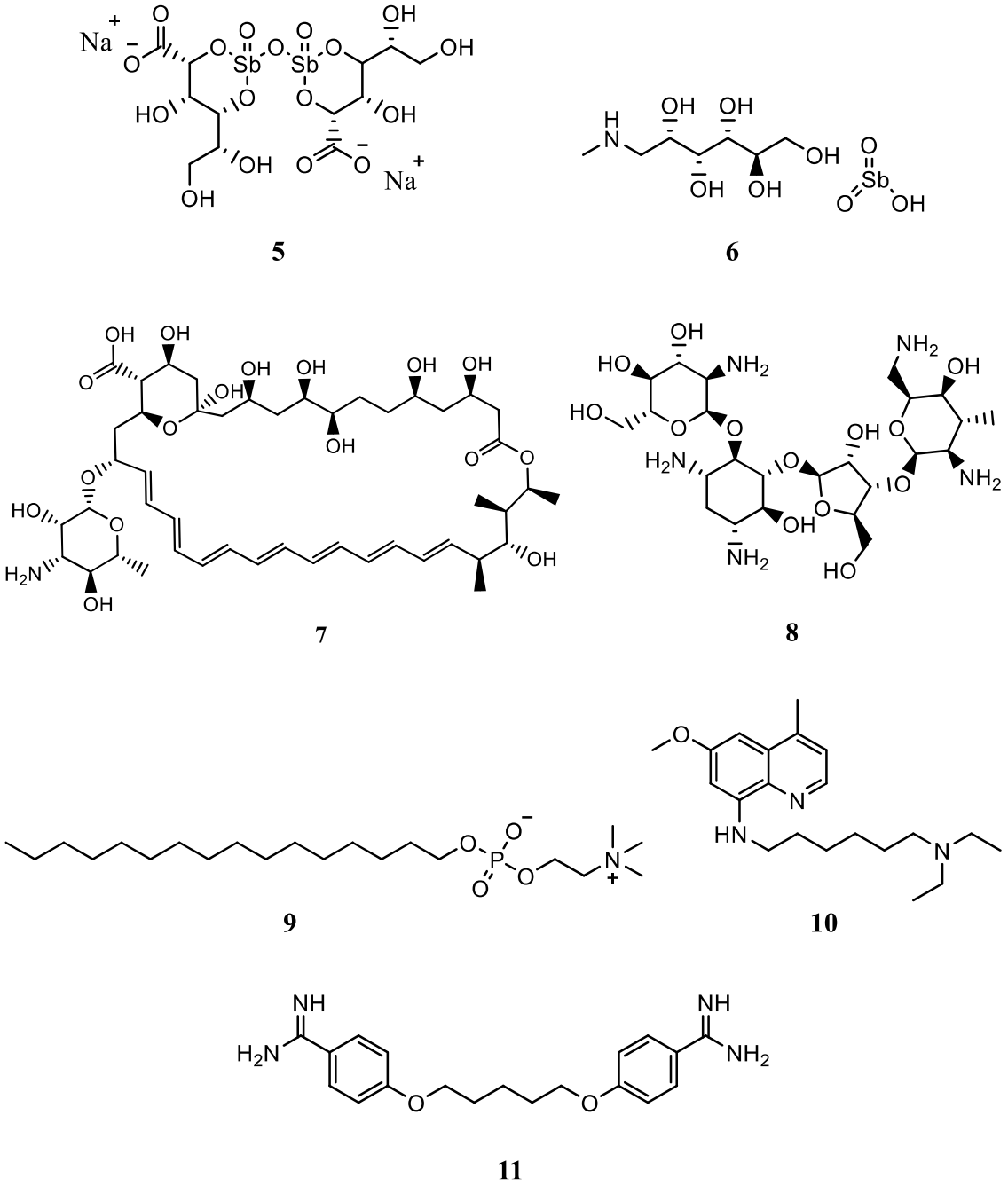


Figure 1.5 – Armamentarium used for the treatment of leishmaniasis

Sitamaquine **10** is an 8-aminoquinoline, and is the second oral drug for leishmaniasis treatment.[50] As a lipophilic weak base, it can be accumulated inside *Leishmania* promastigotes, namely into acidic compartments, allowing their alkalization.[50,55] Sitamaquine causes some adverse effects such as vomiting, abdominal pains, headache, methemoglobinemia, and renal adverse effects.[49,55]

Pentamidine **11** is a synthetic derivative from amidine, and is used in individuals intolerant to antimonial treatment, or in cases of resistance.[17,35,50] It has been used since 1980 for the treatment of VL, CL and ML, however, it is no longer used for VL due to its serious toxicities.[38,49] Other side effects include, pain, nauseas, vomiting, myalgia, headache, hypotension, syncope, transient hyperglycemia and hypoglycemia.[17,39,49] The mechanism of action is the inhibition of active transport system and DNA-mitochondrial complex.[39]

1.3 Malaria

Malaria is caused by infection with parasites of the genus *Plasmodium*, and is transmitted to humans through the bite of infected female mosquitoes of the genus *Anopheles*.[56,57] There are six main strains of human *Plasmodium – falciparum, malariae, vivax*, *ovale, ovale wallikeri* and *knowlesi*-, and among these *P. vivax* and *P. falciparum* are the most common forms, but *P. falciparum* is the most mortal worldwide.[58,59]

Malaria is a tropical disease that represent one of the most important public health problems when it concerns to morbidity and mortality for residents and travelers to endemic regions. According to the WHO about 212 million cases occurred globally in 2015, leading to 429 000 deaths, most of which in children aged under 5 years in Africa. In fact, babies, children under the age of 5 years old, and pregnant women are known to be more susceptible to malaria.[60–64]

The epidemiology of malaria varies geographically, since it depends on the local, intensity of transmission or endemicity class. For instance, while *Plasmodium falciparum* is mostly widespread in tropical Africa, the remaining five strains of *Plasmodium* infections are less common and geographically restricted. On the other hand, most of the vectors

which transmit the parasite has a broad geographic distribution, being more prevalent in Africa.[62,65–67] Within endemic countries, malaria represents a heavy burden on the poorest and most vulnerable communities, leading to these low-income countries to have highest risks associated with malaria, and least access to effective services for prevention, diagnosis and treatment.[68]

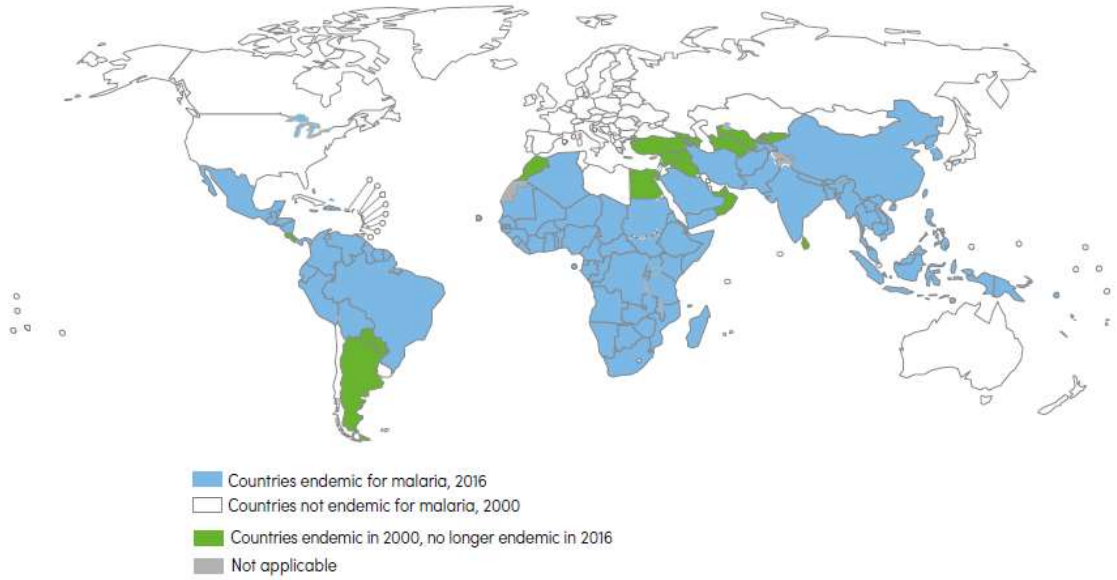


Figure 1.6 – Countries considered endemic for malaria in 2000 and 2016. Adapted from [60]

Between 2000 and 2015, there was a substantial progress in fighting this disease, which can be proved with the latest estimates from WHO, in which malaria incidence was reduced by 41% and malaria mortality rates by 61%. Moreover, at the beginning of 2016 malaria was considered to be endemic in 91 countries and territories, down from 108 in the year 2000, as can be seen in Figure 1.6.[60,69] This decrease in the global burden of malaria can be explained by the scaling up of measures acquired to strengthening prevention, such as the use of insecticide-treated bednets, indoor residual spraying, seasonal malaria chemoprevention, rapid malaria diagnosis, and treatment with artemisinin combination therapy.[62,69]

Despite the remarkable progress towards malaria elimination, the disease continues to have a devastating impact on people's health and livelihoods.[60]

1.3.1 Life cycle

Malaria parasites exhibits a complex life cycle (Figure 1.7) involving two different organisms: a female *Anopheles* mosquito, the vector, and a vertebrate human host.[57,58,62]

The cycle starts with a bite of an infected female mosquito (1), transferring the *Plasmodium* sporozoites to the host during its blood meal (2). These infectious forms of the parasite enter the bloodstream and go to the liver, where they invade hepatocytes and mature into schizonts. During the liver stage (3), parasite reproduces asexually until several thousand of merozoites are produced, which are then released into the bloodstream.[57,58,62,70]

In some strains of *Plasmodium*, such as *P. vivax* and *P. ovale*, some liver parasites remain quiescent in a so-called dormant forms or hypnozoites, which can relapses months or years after the initial infection.[58,62]

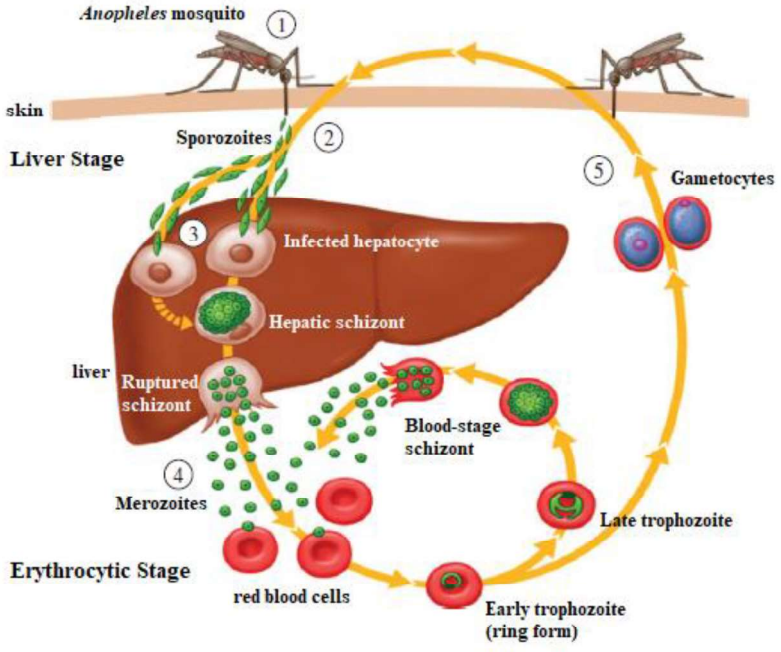


Figure 1.7 – *Plasmodium* life cycle

(1) *Anopheles* mosquito bite (2) Injected sporozoites (3) Liver stage (4) Erythrocytic stage (5) Formation of gametocytes. Adapted from[71]

The erythrocytic stage (4), occurs when the merozoites released from liver invade erythrocytic cells. There, *Plasmodium* parasites undergo through another asexual replication producing mature blood schizonts. Once more, erythrocyte lysis can occur, releasing differentiated merozoites able to invade new red blood cells, restarting the erythrocytic cycle. In this stage, some parasites can differentiate into sexual forms: male and female gametocytes (5).[58,62] When another mosquito takes a blood meal from an infected human, malaria gametocytes are transmitted to the mosquito vector, in which these immature gametes undergo maturation, gamete fusion in the insect midgut, producing a zygote which develops into a ookinete throughout sexual recombination and meiosis. This motile ookinete can transverse the gut wall, and transform into an oocyst, where thousands of new sporozoites are produced.[57,58,62]

When sporozoites are released from oocyst and migrate to the mosquito salivary glands, they are injected in a new human host by the insect bite, restarting the cycle.[58,62]

The pathogenesis is caused by the asexual blood stages, so the first symptoms start 7-10 days after the initial mosquito bite, while the liver phase is asymptomatic.[58,65,70]

If the treatment is ineffective, poor-quality or delayed, the parasite burden continuous to increase and the patient may evolve to severe malaria, which is potentially lethal.[62,72]

1.3.2 Falcipain

In *P. falciparum*, the degradation of the host hemoglobin in a specialized cellular organelle called digestive vacuole, is required for sustaining metabolic needs of rapidly growth. This degradation is due to a group of proteolytic enzymes called falcipains (FP).[73,74]

Falcipains are the best characterized cysteine proteases of the malaria parasite, a family that include four falcipains: facipain-1 (FP1), falcipain-2 (FP2, also known as falcipain-A), falcipain-2' (FP2', also known as falcipain-B), and falcipain-3 (FP3).[73]

All falcipains share a hemoglobinase capacity that appears to be most relevant for FP-2, FP-2', and FP-3. Hemoglobin degradation has proven to be essential for parasite survival, therefore, falcipain inhibitors, in particular FP-2 and FP-3 inhibitors, can block this process leading to parasite's death, thus are logical targets for antimalarial chemotherapy.[75,76]

1.3.3 Current treatment for malaria

There is no effective vaccine for malaria, and there is still no single drug that can act against all *Plasmodium* strains or all the manifestations of the disease. In addition, uncomplicated malaria and severe malaria have distinct treatments. While in uncomplicated malaria the first choice is an oral treatment with high efficacy and a low adverse-effect profile, for severe malaria a rapid treatment is required. Parental administration of an artemisinin derivative is the first choice, because it can rapidly clear the parasites from blood, and is also suitable for patients in coma.[77]

Malaria prevention and treatment currently relies in chemotherapy and vector control. This last one can be achieved by creating a barrier between human host and the mosquitoes, interrupting the life cycle of the parasite. Despite resistance and adverse effects, the use of antimalarial drugs remain a cornerstone for malaria control.[78,79]

1.3.3.1. Quinolines

Quinolines constitute a large group of compounds that are active against malaria parasites (Figure 1.8). Most drugs from this group, namely quinines, 4-aminoqunolines and arylaminoalcohols, are active in the blood stages of parasite's life cycle, but 8-aminoquinolines target the hepatic stages as well.[78]

Quinine **12** is a cinchona alkaloid, extracted from the cinchona tree, which was the first successful chemical compound used to the treatment of an infectious disease, which remains an important antimalarial drug for almost 400 years.[80] It has a rapid schizonticidal action against intra-erythrocytic malaria parasites, gametocytocidal for *Plasmodium vivax* and *Plasmodim malariae*, but not for *Plasmodium falciparum*. It also has analgesic properties, but not antipyretic. Unfortunately, its antimalarial mechanism of action is still unknown.[80]

Quinine present several side effects, such as tinnitus, hypoglycemia, headache, nausea and slight impairment of hearing, which is usually concentration dependent and reversible. More severe manifestations comprise vertigo, vomiting, abdominal pain, diarrhea, marked auditory loss, and visual symptoms that can lead to loss of vision. Less frequent but more severe adverse effects include skin eruptions, asthma, thrombocytopenia, hepatic injury and psychosis. Moreover, intramuscular administration of quinine is painful and may cause sterile abscesses, which lead to difficult acceptation for patients.[80]

Primaquine **13** is an 8-aminoquinoline active against the liver and sexual blood stages of *Plasmodium* and has been widely used since 1950s.[81] Its major advantage is preventing relapses, since it is also active against dormant forms of *P. vivax* (hypnozoites). However, this compound is known for being toxic and poorly tolerated.[82,83]

Chloroquine **14** is a 4-aminoquinoline effective against the asexual forms in blood stage for all types of *Plasmodium* and highly effective, easily administered, well tolerated and inexpensive[84–87] The undesirable side effects are ocular toxicity, dry mouth, fatigue, and loss of appetite and weight. When therapeutic or high doses are administered too rapidly by parental route, it induce acute toxicity, which include several cardiovascular effects.[85,87]

The mechanism of action has not been fully elucidated. However, the most explored and accepted theory is that chloroquine interferes with the detoxification of hemozoin. During the digestion of hemoglobin, the protein moiety is degraded into peptides and heme group, which is toxic to the parasite, so it is transformed in an inert crystalline polymer named hemozoin. Chloroquine can accumulate inside the digestive vacuole, bind to hematin, a heme dimmer, preventing its incorporation into the hemozoin crystal. The detoxification process is compromised and the parasite dies.[84,88]

Amodiaquine **15** is another 4-aminoquinoline, with an aromatic structure instead of the aliphatic side chain of chloroquine. This compound is effective against low-level of chloroquine resistance parasites, but not against highly levels. Biotransformation of this compound leads to the formation of *p*-aminophenol moiety into a quinonimine, which is responsible for severe hepatotoxicity and life-threatening agranulocytosis.[82,89]

Mefloquine **16**, an arylaminoalcohol, was first synthesized in the 1970s. It presents a simplified structure of quinine, but better tolerated and more active against most of the *Plasmodium* strains which are resistant to chloroquine. However, problems of resistance and toxicity, including anxiety, insomnia, depression, hallucinations, acute psychosis, and panic attacks, have limited it use in malaria treatment.[82,90]

This compound is a lipophilic drug, characteristic that can facilitate its delivery inside the parasite or even binding with high affinity to parasite's membranes and uninfected erythrocytes. This feature contribute to the long half-life of mefloquine, as it may provide a reservoir of the drug inside the parasite.[90]

The mechanism of action of arylaminoalcohols interfere with the heme digestion, but it seems to be different than 4-aminoquinolines. However, it is still not known exactly.[82,90]

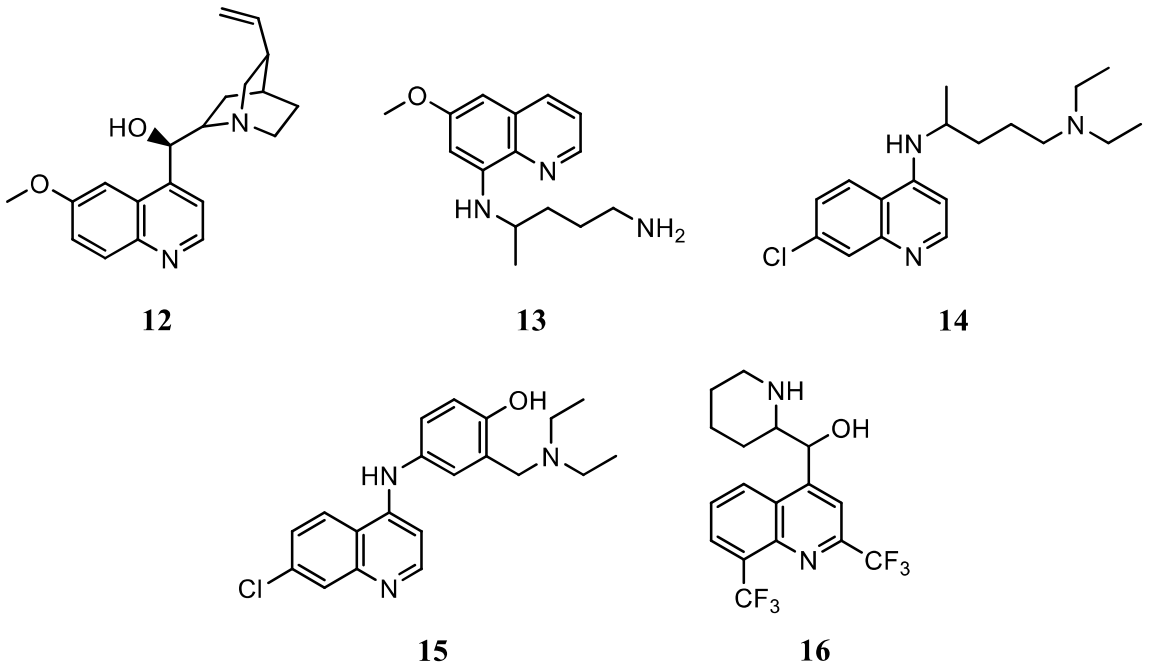
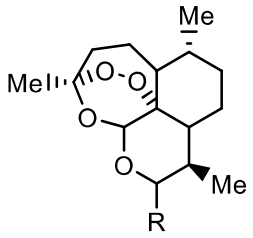


Figure 1.8 – Quinolines used for the treatment of malaria

1.3.3.2 Endoperoxides

Artemisinin **17** is a sesquiterpene lactone isolated from traditional Chinese herb *Artemisia annua L.* in 1972 and its structure was elucidated in 1979 (Figure 1.9). This plant has been used in traditional Chinese medicine as a drug for chills and fevers for more than 2000 years.[91,92]



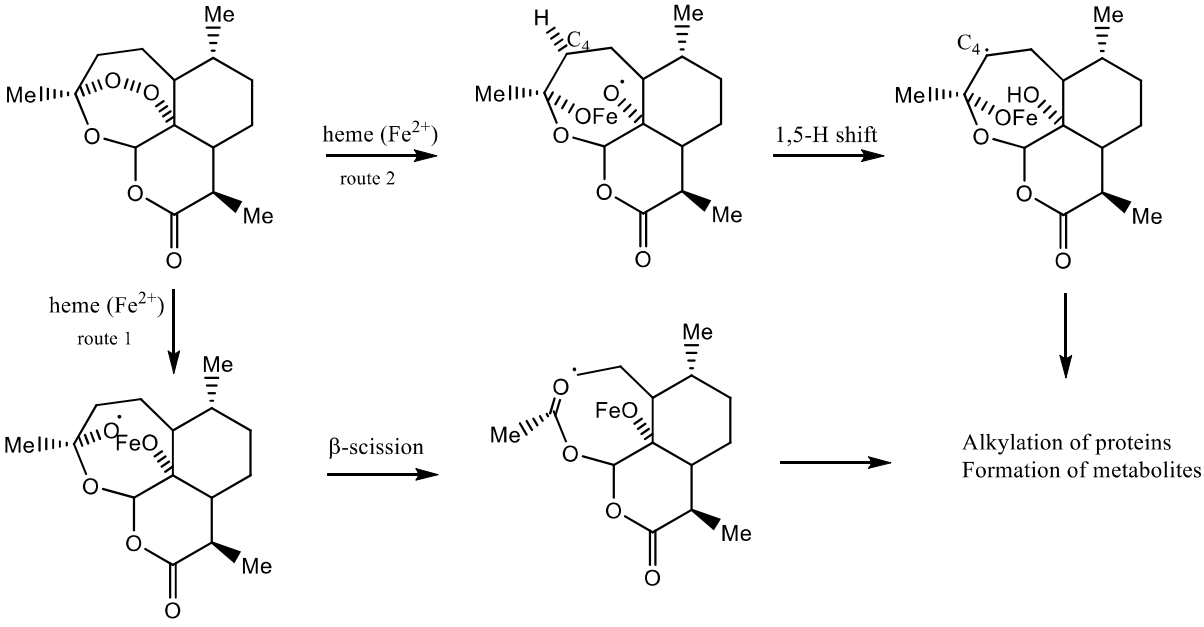
17

Figure 1.9 – Chemical structure of artemisinin and its derivatives

17 R: =O; **17a**: R: OH; **17b** R: OMe; **17c**: R: OEt; **17d**: R: $OC(O)CH_2CO_2Na$

The key pharmacophore of this natural product is the 1,2,4-trioxane group, particularly the endoperoxide bridge, which confers the antimalarial activity. Artemisinin is only active on blood-stage of parasite life cycle.[92,93]

Heme or iron (II), present in high levels inside the parasite, is responsible for the opening of the peroxide bridge of the trioxane in artemisinin (Scheme 1.1), leading to the formation of ROS or carbon centered radicals.[92–94]



Scheme 1.1 – Iron (II)-mediated activation of artemisinin **17**. Adapted from[95]

In the first route, the primary oxygen radical can rearrange by a β-scission process, leading to the formation of carbon centered radicals. While, in the second route, the second radical formed suffers a 1,5-H shift between the oxygen radical and a hydrogen from the C4 atom, leading to the formation of a carbon centered radical, which can react with proteins. These carbon-centered radicals are capable of alkylating parasite biomolecules, which are essential for their survival, leading to parasite's death.[88,92,93,95]

Artemisinin therapeutic effect is limited by its low solubility on both oil and water, consequently, was reduced to give dihydroartemisinin **17a**, which can lead to the preparation of a series of semisynthetic analogues, which include artemether **17b**, arteether **17c**, and artesunate **17d** (Figure 1.9).[82,93]

Both artemether **17b** and arteether **17c** compounds present activity in low single-nanomolar range against chloroquine-resistant *Plasmodium*. Artesunate **17d** has the advantage of being water-soluble becoming possible intravenous administration for the treatment of advanced cases of *P. falciparum*. Dihydroartemisinin **17a** is used for the treatment of uncomplicated malaria. Moreover, all artemisinin derivatives go through an extensive first-pass metabolism and rapid biotransformation to give dihydroartemisinin**17a**, the major active metabolite of artemisinin.[93,94]

Artemisinin derivatives still represent the first-choice treatment for malaria due to its unique endoperoxide structure, however the complex structure makes it difficult and not economically feasible to be chemically synthesized. WHO recommends the use of artemisinin in combination with other antimalarial drugs to delay development of resistance. Unfortunately, this solution may remain a problem once a large population, mostly in Africa, does not have access or financial resources to receive treatment.[96]

18 **19**

Figure 1.10- Chemical structure of **18** - 1,2,4-Trioxolane; **19**- 1,2,4,5-Tetraoxane

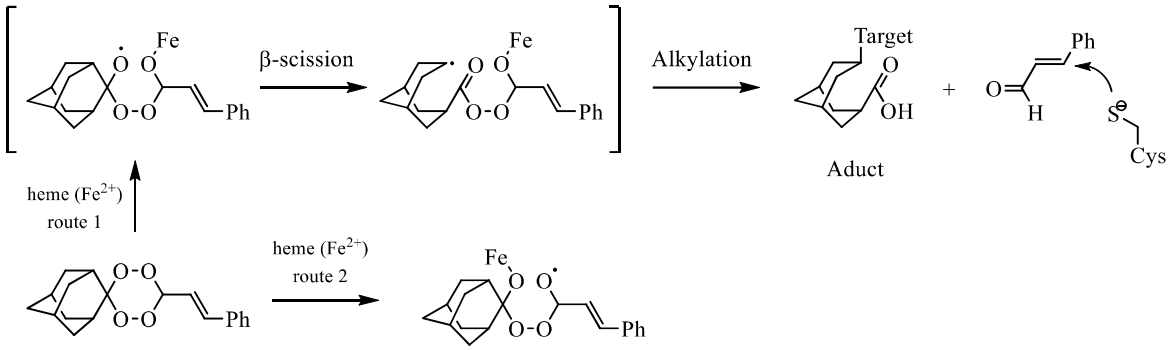
To overcome the problems of the availability of artemisinin, a new class of synthetic peroxides was developed. Among them, trioxolanes **18**, and tetraoxanes **19**, revealed to be potent antimalarial compounds (Figure 1.10).[93,95,97]

Vennerstrom *et al* have described a first class of synthetic endoperoxides, 1,2,4-trioxolanes **18**, however, they had poor water solubility and were chemical unstable.[93,98]

Since endoperoxide is the critical pharmacophoric group for antimalarial activity, the second stage of Vennerstrom research, was to synthesized molecules with two endoperoxide bridges, dispiro 1,2,4,5-tetraoxanes **19**, with antimalarial activity and readily synthesized in one step.[93,95,97,99]

As artemisinin, 1,2,4,5-tetraoxanes are a class of cyclic peroxides, that can be activated by iron or heme inside the parasite (Scheme 1.2), leading to the formation of oxy radicals that rearrange into carbon-centered radicals. This last compound decomposes into two fairly toxic molecules for the parasite, supporting the hypothesis of a dual mechanism of action.[100–102]

One of these toxic molecules is the carbon-centered radical, which not only is highly reactive, leading to alkylation of biomolecules essential for the parasite survival, but also contributes to increase the oxidative stress. The other molecule is an α,β-unsaturated carbonyl, which has an electrophilic character, being susceptible to be attacked by nucleophiles such as proteins containing active cysteine residues. Therefore, this type of compounds are promising inhibitors of both enzymes: TR for *Leishmania* and falcipain for malaria.[100]



Scheme 1.2 – Iron (II)-mediated activation of tetraoxanes derivatives. Adapted from[100]

A key feature of hybrid drugs is the presence of different mechanisms of action, against either a single target or different targets, an interesting method to avoid monotherapies that increase the emergence of new-drug resistance in parasites.[100,103]

Hybrid drugs are defined as chemical entities that combine at least two pharmacophores through covalent bond or through a linker. Thus, this linker should be as simple as possible, once these compounds are bigger than her parent drugs, and often more lipophilic. Recent approaches combines the hybrid and prodrug concepts, in which the selective activation of one pharmacophore triggers the release of the second one at the site of action.[100]

1.4 Work's aim

Leishmaniasis and malaria, which are caused by *Leishmania spp*. and *Plasmodium spp.*, respectively, are two of the most lethal tropical diseases. Besides the fact both diseases entail much concern there are no effective vaccines, so the current armamentarium is limited to chemotherapy. Unfortunately, these chemotherapies have their used limited due to several adverse effects, elevated cost and emergence of resistant strains. For these reasons, the search for new therapeutic approaches is urgent.

Endoperoxides are known to be reductively activated by iron(II)-heme to form carbon-centered radicals, reactive oxygen species and carbonyl species which can generate oxidative stress in the parasite. Both, *Leishmania* and *Plasmodium* are infectious agents that require high levels of iron at critical steps of their life cycle, therefore this kind of compounds could be promising to the design and discovery of new drugs.

1,2,4,5-Tetraoxanes, which have the key factor of possessing an endoperoxide bridge, have shown higher stability and potent antimalarial activity.

Following this concept, the aim of this thesis is to report the synthesis of a small library of new compounds containing a 1,2,4,5-tetraoxanes scaffold (Figure 1.11) to selectively deliver an electrophile capable of reacting with essential enzymes of both parasites, trypanothione reductase for leishmaniasis and falcipain for malaria.

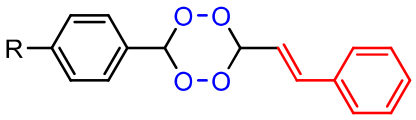


Figure 1.11 – Tetraoxanes specific designed for the aim of this work.

These hybrid compounds can be activated by iron inside parasites, acting through two distinct mechanisms of action, decomposing the tetraoxane into two molecules with different but supportive roles: one with the formation of radicals from the endoperoxide structure represented in blue in the Figure 1.11, which will contribute to increase the oxidative stress. On the other hand, an α,β-unsaturated aldehyde, represented in red in the Figure 1.11, which can react with nucleophilic biomolecules essential for the parasite, acting as inhibitor of TR or falcipain enzymes. Pursuing this idea, cinnamaldehyde was chosen as our starting reagent, since it is an α,β-unsaturated aldehyde that can act as TR or falcipain inhibitor. The benzaldehyde has the major advantage of being easily

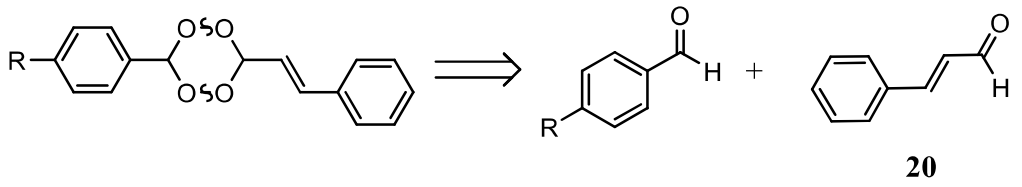
functionalized, allowing the opportunity to have structural diversity for the synthesis of several compounds for our library. Moreover, all the substituents were in the *para* position of the phenyl group, once it has already shown good antimalarial activities.[104]

In addition, other objective of this work is to prove that *trans*-cinnamaldehyde is released, using biomimetic conditions, by activation in the presence of iron (II) bromide. Finally, the biological activity of hybrid compounds will be assessed.

Chapter 2

2. Synthesis of 1,2,4,5-tetraoxanes derived from *trans*-cinnamaldehyde

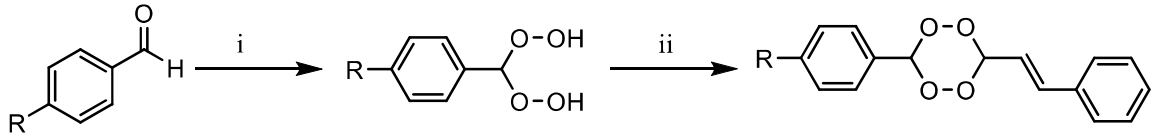
As already mentioned, the tetraoxane moiety is a critical pharmacophoric group for antimalarial activity. This class of compounds can eventually also be used against other parasites such as *Leishmania*. In this chapter, we describe the design and synthesis of new hybrid molecules containing a 1,2,4,5-tetraoxanes. Scheme 2.1 allowed the observation of the retrosynthetic analysis, were *trans*-cinnamaldehyde, an α,β-unsaturated carbonyl compound, and the other starting reagents, an aldehyde or ketone, were selected in order to obtain a small library with a range of stability, lipophilicity, and more importantly antiparasitic activity.



20

Scheme 2.1 – Retrosynthetic analysis for the synthesis of 1,2,4,5-tetraoxanes derived from *trans*-cinnamaldehyde

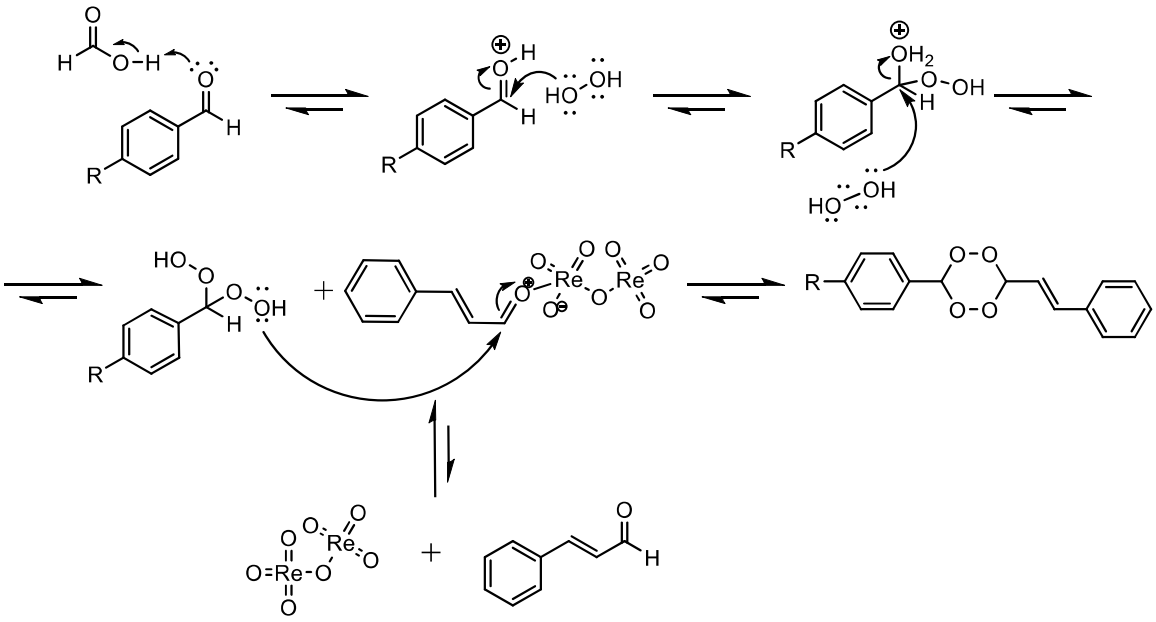
Several methodologies have already been described for the synthesis of this type of compounds, however, due to simplicity, efficiency, and relatively low price of reagents, we used the synthesis of tetraoxanes according to the methodology developed by O'Neill *et al* (Scheme 2.2).[101,105]



Scheme 2.2 – Methodology for the synthesis of tetraoxanes

i) HCO_2H, CH_3CN, H_2O_2 50%, r.t.; ii) Re_2O_7, *trans*-cinnamaldehyde, CH_2Cl_2, 0ºC

1,2,4,5-Tetraoxanes were prepared using a two-step synthesis depicted in Scheme 2.2. In the first step, appropriate aldehyde or ketone after acid-catalysis condensation with formic acid, react with hydrogen peroxide 50% to give the corresponding *gem*-dihydroperoxide, as it can be seen in Scheme 2.3.



Scheme 2.3 – Proposed reaction mechanism for the synthesis of 1,2,4,5-tetraoxanes

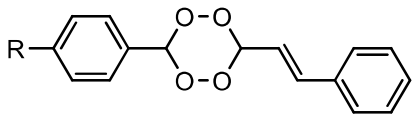
In the final step, the *gem*-dihydroperoxide intermediate reacts with the complex previously formed between the catalyst rhenium (VII) oxide and the *trans*-cinnamaldehyde, allowing the complete conversion to the 1,2,4,5-tetraoxane.

2.1 Synthesis of tetraoxanes hybrids derived from *p*-benzaldehyde

In this thesis, a small library of tetraoxanes-based hybrid compounds was synthesized, using the technique described above, by varying benzaldehyde moiety. All compounds were purified by column chromatography with moderate yields (Table 2.1), and were characterized by uni- and bi-dimensional NMR techniques, such as ^{1}H NMR,

COSY (Correlation Spectroscopy), ^{13}C NMR, APT (Attached Proton Test), HMQC (Heteronuclear Multiple Quantum Correlation) and melting point determination.

Table 2.1 – Synthesized tetraoxane hybrids derived from *p*-benzaldehyde, theoretical Log P, yields and melting point determination



Compound	R	Log P[a]	Yield (%)	m.p. (ºC)
21	H	3.96	44	134-136
22	Cl	4.63	39	171-173
23	Br	4.76	15	196-198
24	F	4.12	47	158-160
25	CH_3	4.40	20	166-168
26	CF_3	4.85	39	163-165
27	OCF_3	4.92	33	155-157

[a] *calculated by software http://www.molinspiration.com/cgi-bin/properties*

As example, the assignment for compound **22** will be presented in this chapter. The full characterization of all the other compounds is available in chapter 5 and 7.

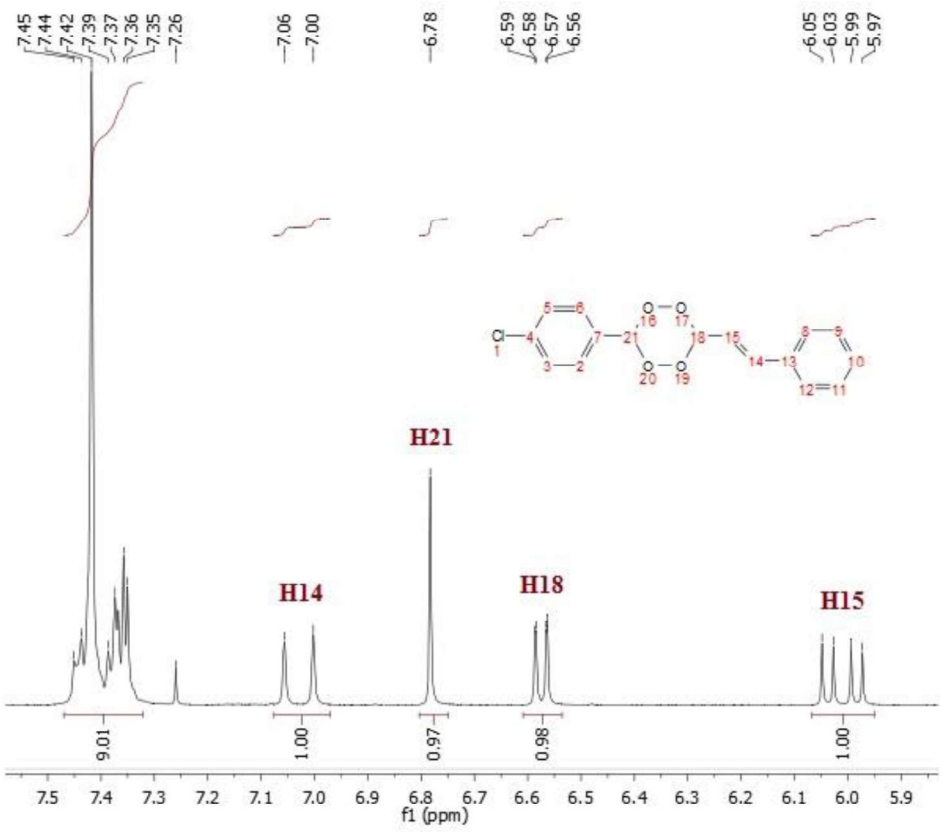


Figure 2.1 – Chlorobenzaldehyde-tetraoxane **22** ^{1}H NMR spectra ($CDCl_3$)

Regarding ^{1}H NMR spectra (Figure 2.1), aromatic signals from phenyl rings at a downfield shift, corresponding to 9 protons, appeared as a multiplet between a ^{1}H chemical shift (δ_H) value of 7.45 to 7.35 ppm. The assignment of the chemical shifts on the aliphatic region, reveals two vinylic protons attributed to H14 and H15, with δ_H values of 7.03 ppm and 6.01 ppm, as a doublet (*d*) and a double-doublet (*dd*), respectively. As it can be seen in 2D NMR COSY spectra (Figure 2.2), proton H15 interacts with proton H14 and H18 with a vicinal coupling constant of 16.3 Hz and 6.4 Hz, respectively, while proton H14 only interacts with H15, exhibiting the coupling constant of 16.3 Hz. This is characteristic of the *trans* configuration. The signal with δ_H value of 6.58 ppm correspond to proton H18, once the COSY spectra reveals its interaction with H15 with a vicinal coupling constant of 6.4 Hz. The last signal, integrating for 1 proton, correspond to proton H21, which appeared as a singlet with δ_H value of 6.78 ppm. Additionally, ^{1}H NMR signals are in agreement with the chemical structure of compound **22** and have adequate integrals to fit the 13 protons.

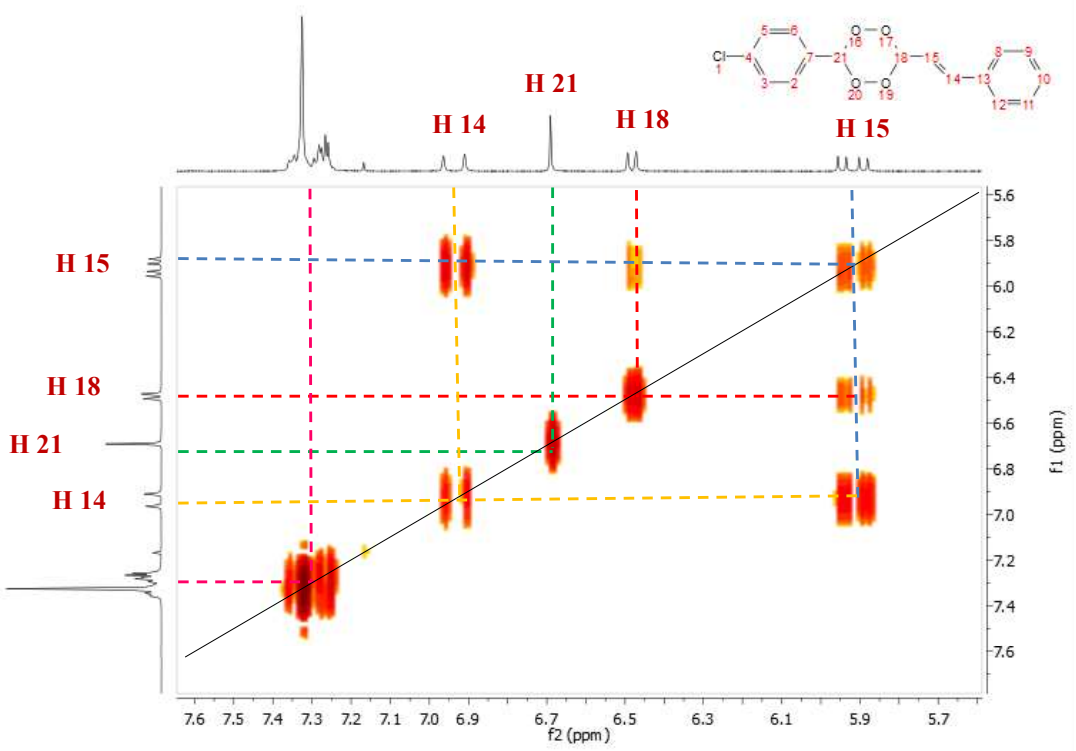


Figure 2.2 - Chlorobenzaldehyde-tetraoxane **22** COSY spectra ($CDCl_3$)

Compound **22** has a total of 16 carbons, but it only was expected 12 signals in ^{13}C NMR spectra, since the pairs C2-C6, C3-C5, C8-C12, and C9-C11 are equivalent and belonging to both aromatic rings. However, the ^{13}C NMR (Figure 2.3) only shows 11 signals, which means that two of them are overlapped. The aromatic carbons correspond to ^{13}C chemical shifts (δ_C) 129.66, 129.14, 128.83, and 127.32 ppm.

To identification of quaternary carbons (Cq) it was required an APT spectrum (Figure 2.3), which shows the signals of primary and tertiary carbons as positive peaks (pointed upward), and the signals of secondary and quaternary carbons as negative peaks (pointed downwards). Once tetraoxane **22** only has quaternary and tertiary carbons, the identification of the chemical shifts to the three quaternary carbons correspond to δ_C of 137.47, 134.67, and 129.43 ppm.

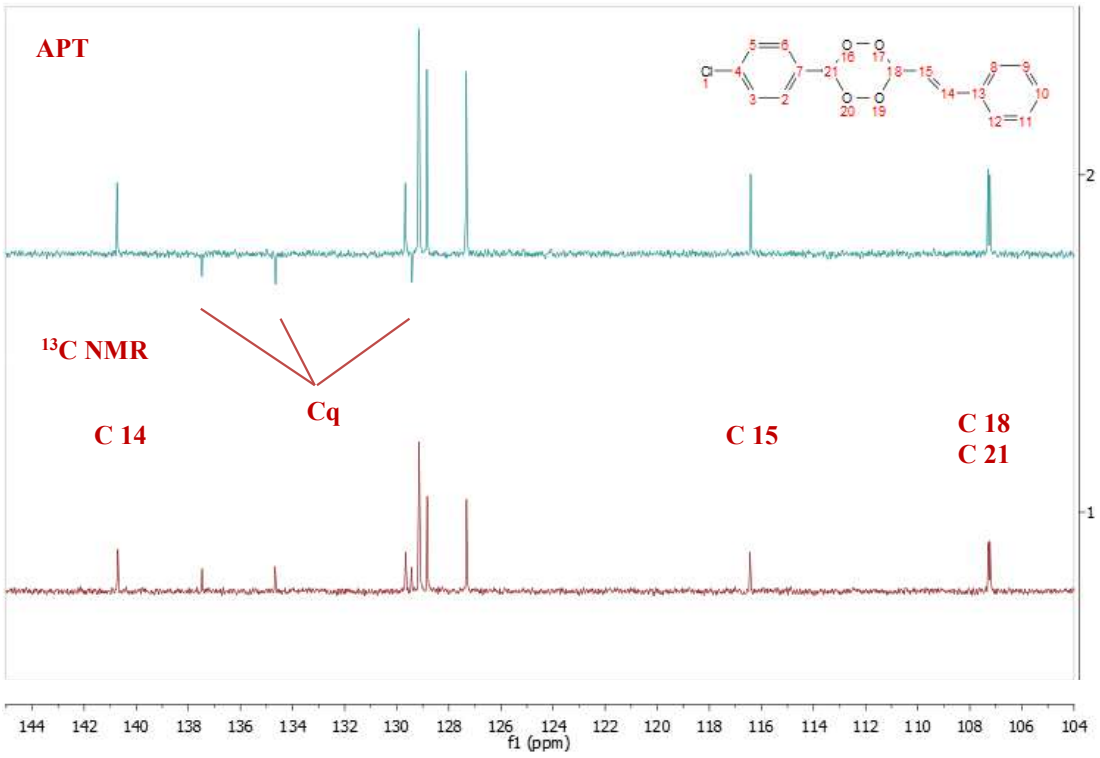


Figure 2.3 - Chlorobenzaldehyde-tetraoxane **22** APT and ^{13}C NMR spectrum ($CDCl_3$)

For complete assignment of carbon signals, it was performed ^{1}H-^{13}C heterocorrelation experiments (HMQC – Heteronuclear Multiple Quantum Correlation, Figure 2.4). Regarding to C14 and C15, HMQC shows clearly that H15 interacts with δ_C of 116.44 ppm, while H14 interacts with δ_C of 140.71 ppm. Finally, the tetraoxane core can be identified through two characteristic signals of C18 and C21 with δ_C of 107.22 ppm, and 107.29 ppm, respectively.

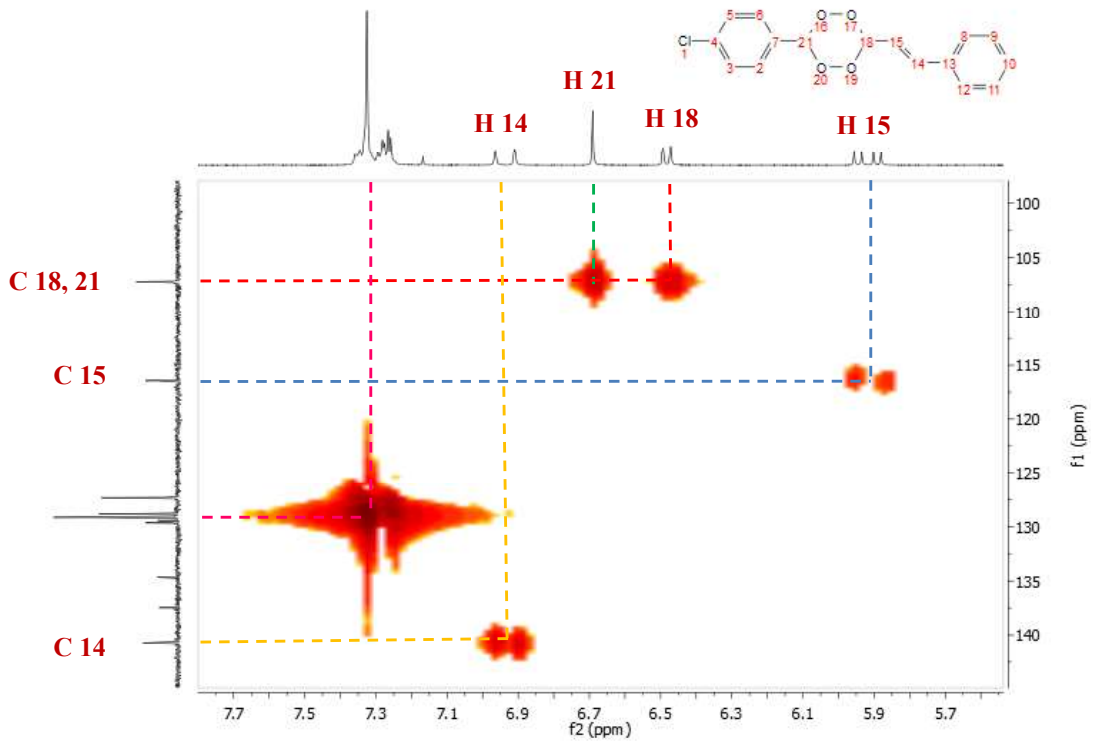


Figure 2.4 - Chlorobenzaldehyde-tetraoxane **22** HMQC spectra ($CDCl_3$)

Using Heteronuclear Multiple Bond Correlation Spectroscopy (HMBC, Figure 2.5), it was possible to conclude that the Cq with δ_C of 137.47 ppm correspond to C4, once it is coupled at distance only with a phenyl group. The Cq with δ_C of 134.67 ppm only interact at distance with H15, which means that it could only correspond to C13. Finally, as it can be seen in this spectrum, the Cq with δ_C of 129.43 ppm can couple at distance with both aromatic ring and H21, corresponding to C7.

In addition to the NMR spectroscopy, all the compounds were characterized through melting point determination (Table 2.1). For all of them, the melting point determination range was lower than 2ºC, revealing the pure degree of the synthesized compounds.

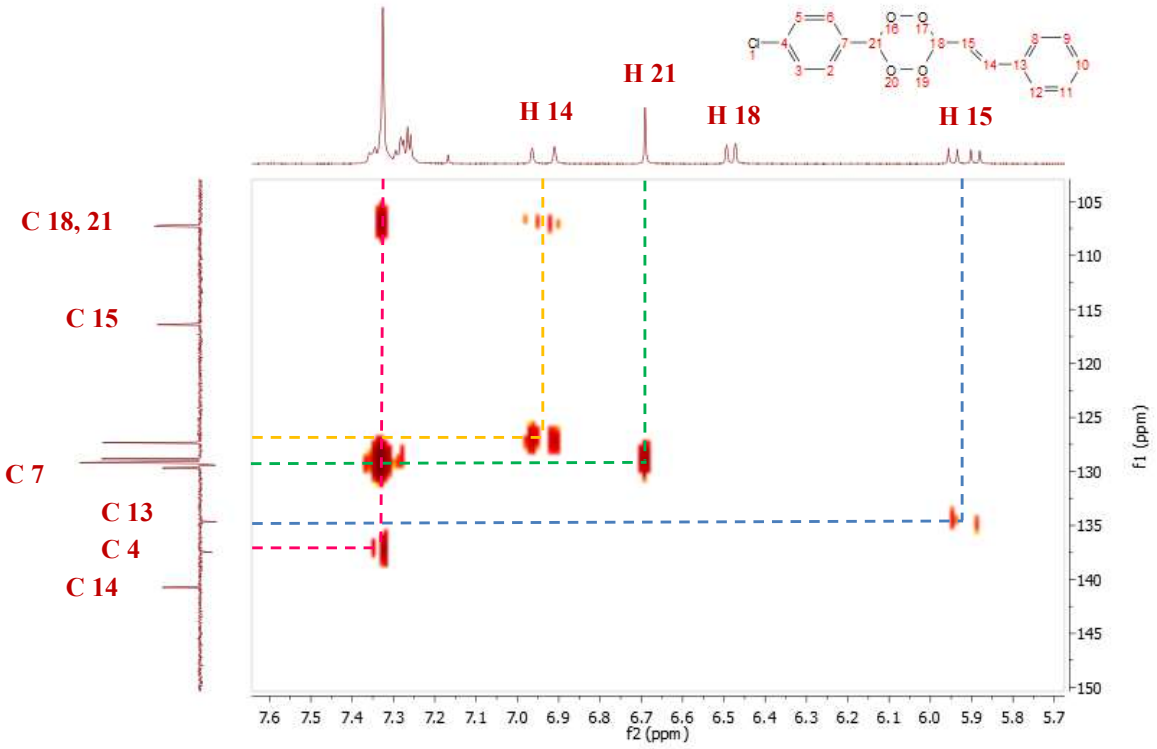


Figure 2.5 - Chlorobenzaldehyde-tetraoxane **22** HMBC spectra ($CDCl_3$)

In Table 2.2 is presented a summary of δ_H for all tetraoxanes hybrid compounds synthesized.

Table 2.2 – ^{1}H chemical shift of synthesized hybrid tetraoxanes derived from *p*-benzaldehyde

	δ (ppm); multiplicity					
Compound	**CH Ar**	**CH=CH-Ar**	**HCOO**	**OOCH**	**CH=CH-Ar**	**CH_3**
21	7.53-7.3; *m*	7.05; *d*	6.84; *s*	6.62; *dd*	6.05; *dd*	-
22	7.47-7.33; *m*	7.03; *d*	6.78; s	6.57; *dd*	6.01; *dd*	-
23	7.53-7.23; *m*	6.94; *d*	6.69; *s*	6.49; *dd*	5.92; *dd*	-
24	7.51-7.35; *m* 7.12; *t*	7.03; *d*	6.79; *s*	6.58; *dd*	6.02; *dd*	-
25	7.35-7.12; *m*	6.92; *d*	6.67; *s*	6.48; *dd*	5.92; *dd*	2.28; *s*
26	8.05-7.69; *m*	7.51; *d*	7.29; *s*	7.06; *dd*	6.48; *dd*	-
27	7.57-7.28; *m*	7.05; *d*	6.83; *s*	6.60; *dd*	6.03; *dd*	-

During the synthesis of compound **21** it was observed the formation of 1,2,4,5,7,8-hexaoxonane as a side product (Figure 2.6).

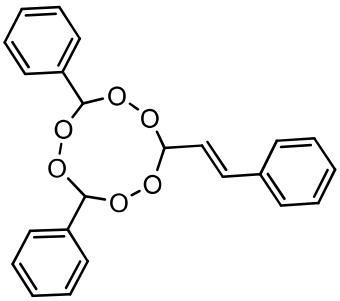


Figure 2.6 – Chemical structure of 1,2,4,5,7,8-hexaoxonane

After several modifications in the amount of H_2O_2 throughout the first step of the synthesis, it was observed that an excess of H_2O_2 leads to the formation of this trimer. This observation has also been reported by other groups.[106]

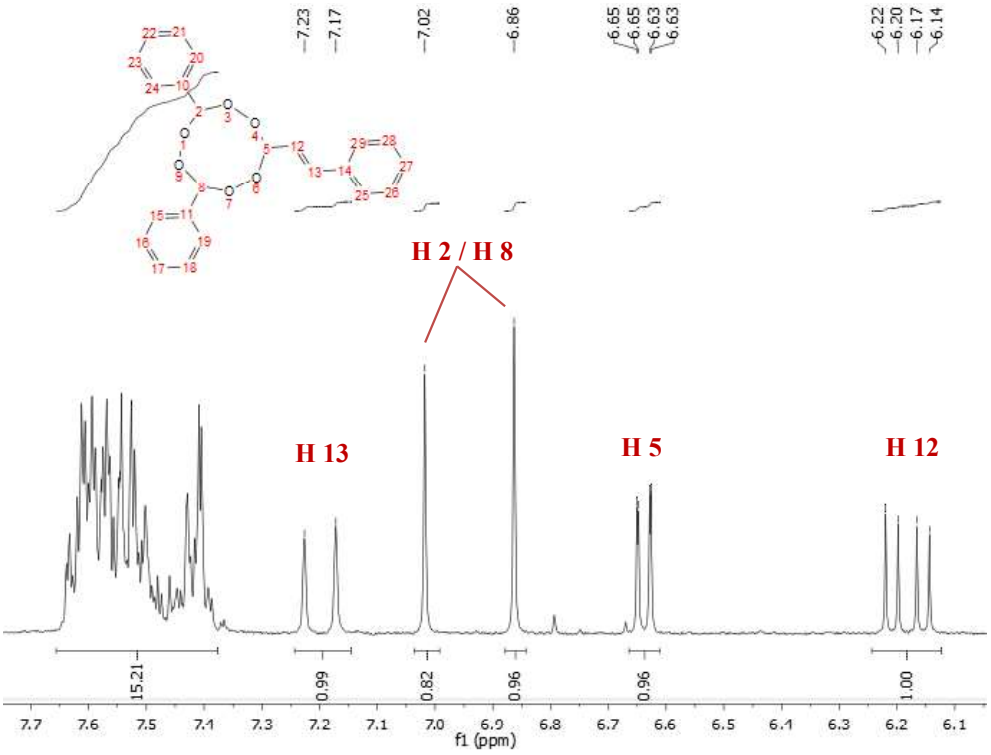
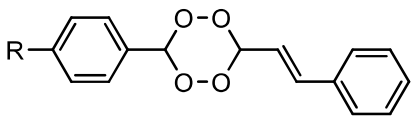


Figure 2.7 – 1,2,4,5,7,8-Hexaoxonane ^{1}H NMR spectra ($CDCl_3$)

In the ^{1}H NMR spectra (Figure 2.7) it is possible to observe that instead of a multiplet that integrate to 10 protons regarding to the two aromatic rings, a signal appeared as a downfield shift multiplet integrating to 15 protons, what opens the hypothesis of three aromatic rings, instead of the two predicted.

Moreover, ^{1}H NMR spectra shows an extra singlet integrating to 1 proton. These two singlets correspond to H2 and H8 with δ_H of 7.02 and 6.86 ppm. This observation confirms the hypothesis of the trimer formation, with two molecules of benzaldehyde and one of cinnamaldehyde.

Table 2.3 – Conditions tried for the synthesis of tetraoxane hybrids **28-32**



Compound	R	n(mmol) H_2O_2	Time (h) t1	t2
28	OH	23	3	3
		11	3	5
		45	7	7
29	$N(CH_3)_2$	23	1.5	3
30	NO_2	23	3	3
		45	3	3
		45	7	7
31	OCH_2Ph	23	3	3
		45	3	3
		45	7	7
32	$OCH_2CH{=}CH_2$	23	3	3
		45	3	3

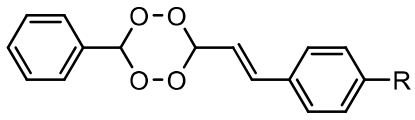
t1 and **t2** – reaction times for the first and second step of the synthesis respectively

Besides the tetraoxanes **21**-**27** synthesized with yields between 15 and 47%, other compounds were tried to be synthesized but without success. From these attempts it can be rationalized that the intermediate *gem*-dihydroperoxide in the substituted benzaldehyde is formed, based on thin-layer chromatography (TLC). However, it was not possible to quantify the intermediate formed, since it cannot be isolated, due to the instability of the *gem*-dihydroperoxide.[107] Nevertheless, the problem seems to appear in the second step of the synthesis, since there was no evidence of the tetraoxane formation neither by TLC nor in NMR experiences.

With the aim to synthesize the tetraoxanes **28**-**32**, several attempts were made changing the amount of H_2O_2 and the reaction time (Table 2.3).

Higher temperature and reactions overnight were avoided due to the instability of tetraoxanes. Moreover, it has already been reported that the reaction of hydroperoxidation of aromatic aldehydes is long and quite complicated. Adding the fact that symmetric tetraoxanes are more easily formed than asymmetric ones.[108] Also, powerful electron-withdrawing substituents on aromatic aldehydes, such as 4-nitrobenzaldehyde, are unfavorable as very long reaction times are demand to convert to *gem*-dihydroperoxide, leading to its decomposition.[107,109] All of that can explain why we were not able to obtain this set of compounds.

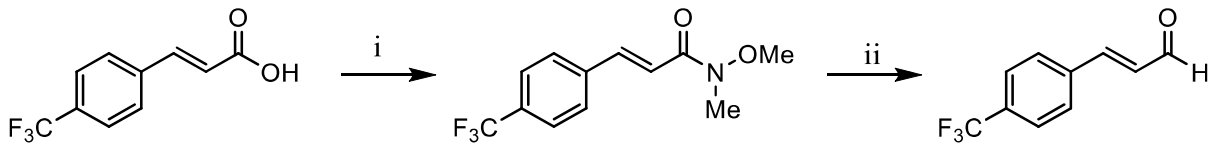
A second group of compounds were tried to be synthesize, using *para*-substitute cinnamaldehyde, as it can be seen in Figure 2.8.



R = CF_3 **33** R = OCH_3 **34**

Figure 2.8 – Tetraoxane designed with *p*-substituents in cinnamaldehyde

The same synthetic route as described above (Scheme 2.2) was followed for the synthesis of these two compounds. Compound *trans*-4-(trifluoromethyl)cinnamaldehyde was not commercially available, so the reduction of the available *trans*-cinnamic acid to the correspondent aldehyde was performed. Briefly, *trans*-4-(trifluoromethyl)cinnamic acid reacted with TBTU and TEA, followed by the reaction with N,O-dimethylhydroxylamine to give the intermediate hydroxamate, which was confirmed by ^{1}H NMR. In the final step, the intermediate was reduced with $LiAlH_4$ to give *trans*-4-(trifluoromethyl)cinnamaldehyde (Scheme 2.4).



Scheme 2.4 – Synthetic methodology for the synthesis of *trans*-4-(trifluoromethyl)cinnamaldehyde.

i) TBTU, TEA, N,O-dimethylhydroxylamine, CH_2Cl_2, r.t.; ii) $LiAlH_4$, THF, 0°C, N_2.

The synthesis of compound **33** was accomplished (Table 2.4), however after purification with flash column chromatography, the compound was not isolated.

Table 2.4 – Conditions tried for the synthesis of tetraoxane hybrids derived from substituted *p*-cinnamaldehyde

Compound	**R**	**Time (h)**	
		t1	**t2**
33	CF_3	3	3
34	OCH_3	3	3
		3	4
		3	5
		7	6

t1 and **t2** – reaction times for the first and second step of the synthesis, respectively

For compound **34** were made some changes in reactions times in the first and second step of the synthesis, as described in Table 2.4. The first attempt to synthesize compound **34** was similar to the synthesis previously described, using 3 hours reaction for both steps. Nevertheless, the synthesis of this compound was not possible. To overcome the problem, the time of the second step of the reaction was increased, since TLC of the first step showed the formation of the intermediate *gem*-dihydroperoxide. However, this change did not lead to the formation of the final tetraoxane.

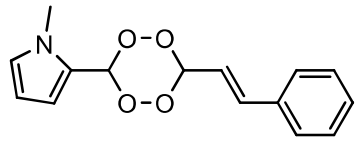
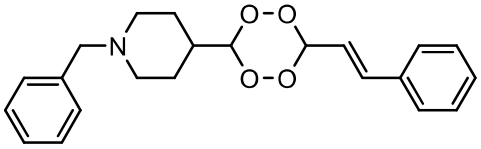
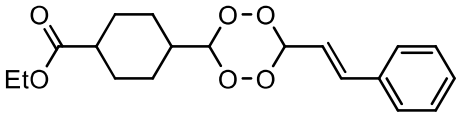
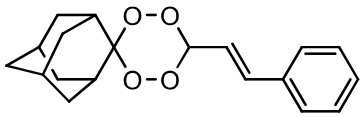
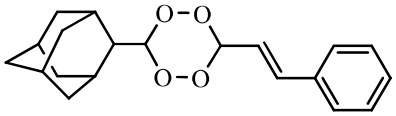
A new and successful attempt was made increasing both reaction times, in which t1 and t2 were 7 and 6 hours, respectively. In this case, the first step was longer, comparing with the first attempt of only 3h. However, after purification using flash chromatography no pure compound **34** was isolated.

Since the *gem*-dihydroperoxide was not quantified for reasons already explained above, probably the synthesized amount of this intermediate in the first attempts was not enough to proceed the second step with good yields.

2.2 Synthesis of tetraoxanes hybrids with improved solubility

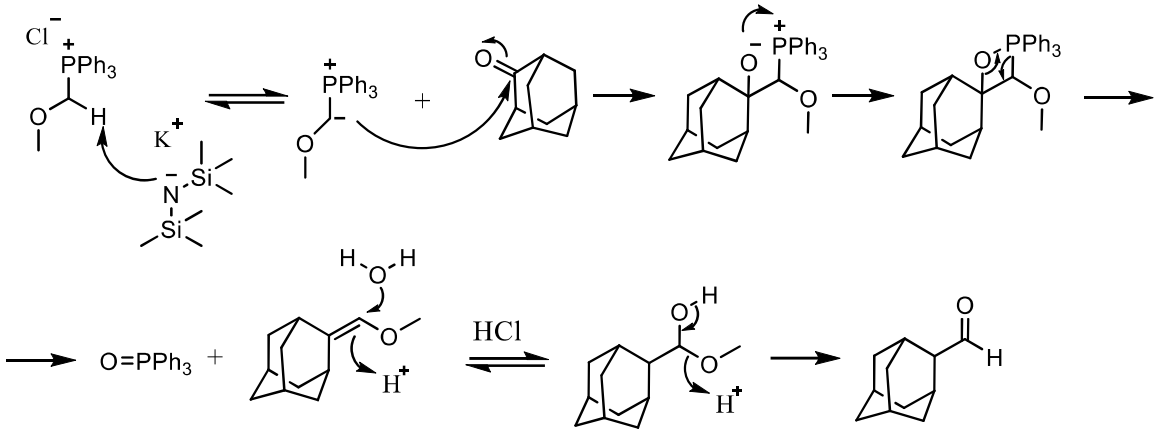
In order to improve water solubility, a new set of compounds were synthesized. The rationale was to incorporate ionizable groups or groups with a higher sp3 content (Table 2.5).

Table 2.5 – Possible hybrid tetraoxanes to be synthesized and its theoretical Log P

Compounds	R	Log P[a]
35		3.18
36		4.26
37		3.98
38		5.21
39		5.51

[a] calculated by software *http://www.molinspiration.com/cgi-bin/properties*

In order to obtain adamantanone-2-carbaldehyde from 2-adamantanone it was necessary a Wittig-Horner reaction followed by hydrolysis in acidic conditions, as it can be seen in Scheme 2.5.[110]



Scheme 2.5 – Synthetic methodology for the synthesis of adamantanone-2-carbaldehyde

Reaction conditions were optimized in order to obtain compounds **35-39**. In particular, the number of molar equivalents of H_2O_2, the reaction time and temperature of each step of the tetraoxane synthesis, and the solvent used in the second step, as presented in Table 2.6. Once more, high temperatures and long reactions overnight were avoided due to the reactivity of peroxides.

Compound **36** was designed as a precursor of a secondary amine (piperidine) by removing the benzyl group via hydrogenation. Such piperidine derivative would have a log P value of 2.27 which is significantly more polar than the remaining tetraoxanes. This could also improve water solubility, and thus oral absorption when compared to more lipophilic tetraoxanes. The pyrrole derivative **35** was also selected for its polarity. Unfortunately, none of the methods afforded compounds **35** and **36**. Since the corresponding *gem*-dihydroperoxide are more polar when compared from the previous series, we hypothesized that dichloromethane (DCM), used as solvent in the second step of tetraoxane synthesis, could be replaced by the more polar solvents ethyl acetate or trifluoroethanol. However, none of the modifications afforded the expected target compound.

Table 2.6 – Conditions tried for the synthesis of other hybrid tetraoxanes

Compound	n(mmol) H_2O_2	Time (h) t1	t2	Temp (ºC) T2	Solvent S2
35	23	1	3	0	DCM
	23	1.5	3	r.t.	
36	23	3	3	0	DCM
	23	3	3	0	THF
	4	3	120	r.t	CF_3CH_2OH
37	23	3	3	r.t.	DCM
	4	3	3		CF_3CH_2OH
	23	3	4	0	CF_3CH_2OH
	45	3	3		CF_3CH_2OH
38	23	3	3	0	DCM
	23	6	3		
	23	3	3	r.t.	
39	23	2	3	r.t.	DCM

t1- reaction time for the first step of the synthesis; **t2**- reaction time for the second step of the synthesis; **T2**- temperature during the second step of the synthesis; **S2**- solvent used during the second step of the synthesis

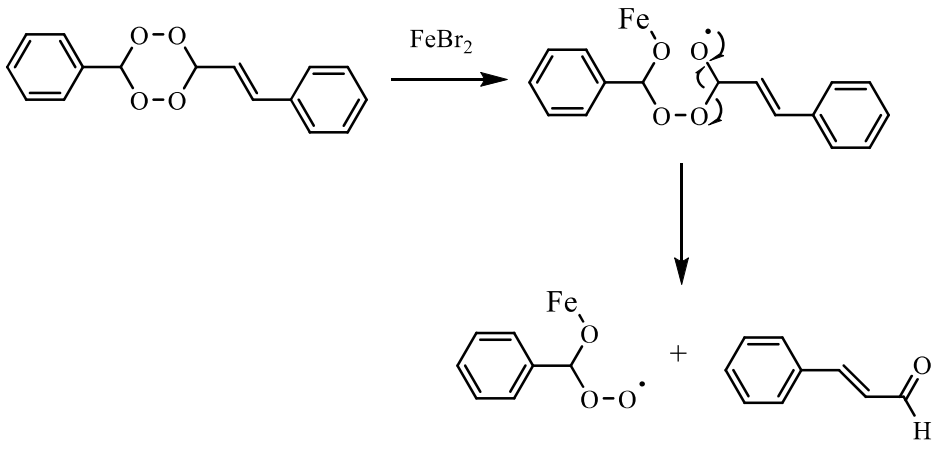
For compounds **37**-**39**, the reaction mixtures were complex, and we were not able to obtain the desired compounds with adequate purity.

Chapter 3

3. Biomimetic activation and antiparasitic activity

3.1 Activation of tetraoxanes with iron (II)

The hybrid compounds were designed to act by two different mechanisms of action. The endoperoxide bond can be activated by iron (II) into radicals leading to the increase of the oxidative stress. In addition, this activation lead to the formation of an α,β-unsaturated compound, *trans*-cinnamaldehyde, (Scheme 3.1) that can react with nucleophiles causing the possible inhibition of TR or falcipain, in leishmaniasis and malaria, respectively. To evaluate this hypothesis, we monitored by HPLC the activation of the compounds by incubate them in the presence of iron (II) bromide.



Scheme 3.1 – Activation of tetraoxanes with $FeBr_2$

For the HPLC studies of the tetraoxanes **21**-**27**, a column RP-18e (5μm) was used, isocratic gradient with MeOH/H_2O (95:5) as eluent with a flow rate of 1.0 mL/min, and wavelength of 264 nm.

Table 3.1 shows the retention times (R_t) obtained for the aldehydes used as starting reagents and hybrid tetraoxanes **21**-**27**.

Table 3.1 – Retention times for tetraoxanes synthesized and respective aldehydes

Compounds	Retention time (min)	Aldehydes	Retention time (min)
		20	2.48
21	3.54	**Benzaldehyde**	2.46
22	3.59	**4-Chlorobenzaldehyde**	2.53
23	3.99	**4-Bromobenzaldehyde**	2.64
24	3.25	**4-Fluorobenzaldehyde**	2.43
25	3.55	***p*-Tolualdehyde**	2.64
26	3.52	**4-(Trifluoromethyl)benzaldehyde**	2.42
27	3.30	**4-(Trifluorometoxy)benzaldehyde**	2.49

Fresh solutions containing tetraoxane, iron (II) bromide, and ACN/H_2O were kept in a thermostated bath at 25ºC. Activation assays were done in triplicate and calibration curves were also obtained for all compounds. Figure 3.1 shows the calibration curve of compound **22**, in which is possible to see a direct proportionality between concentration and area.

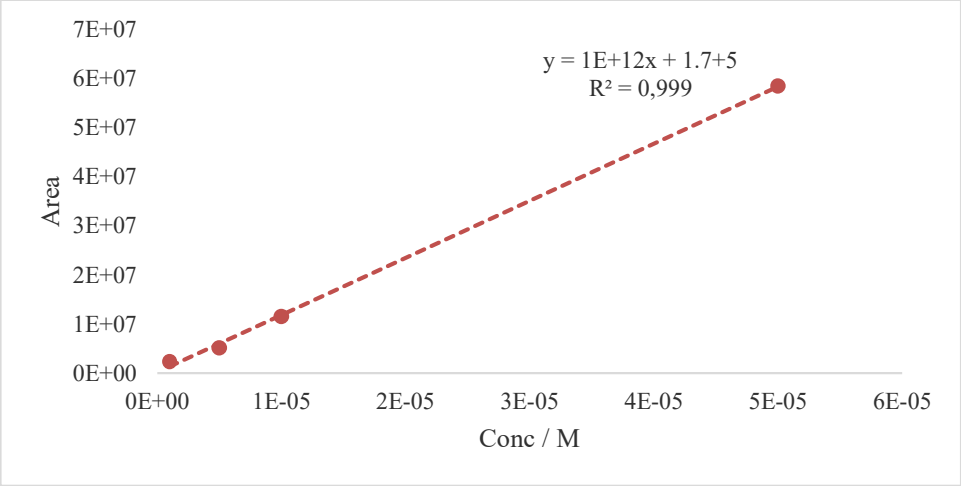


Figure 3.1 – Calibration curve of tetraoxane **22**

The reaction of activation was monitored for 2 h by HPLC, in which is possible to observe the disappearance of the hybrid tetraoxane and the appearance of a new compound with a lower R_t, coincident to both cinnamaldehyde and parental aldehydes. The results for all the compounds were identical, so the interpretation of the results was only done for compound **22** (Figure 3.2).

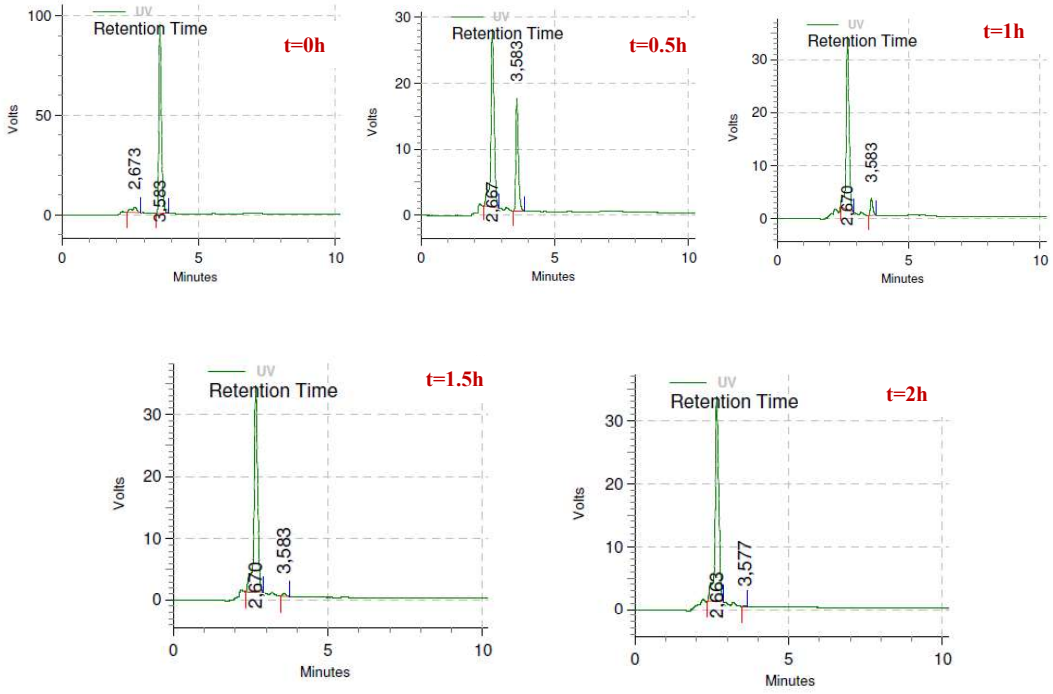


Figure 3.2 – Activation of tetraoxane **22** with iron (II) bromide

The graph in Figure 3.3 summarizes the results obtained, in which is possible to see the disappearance of compound **22** in blue and the appearance of a new compound in red.

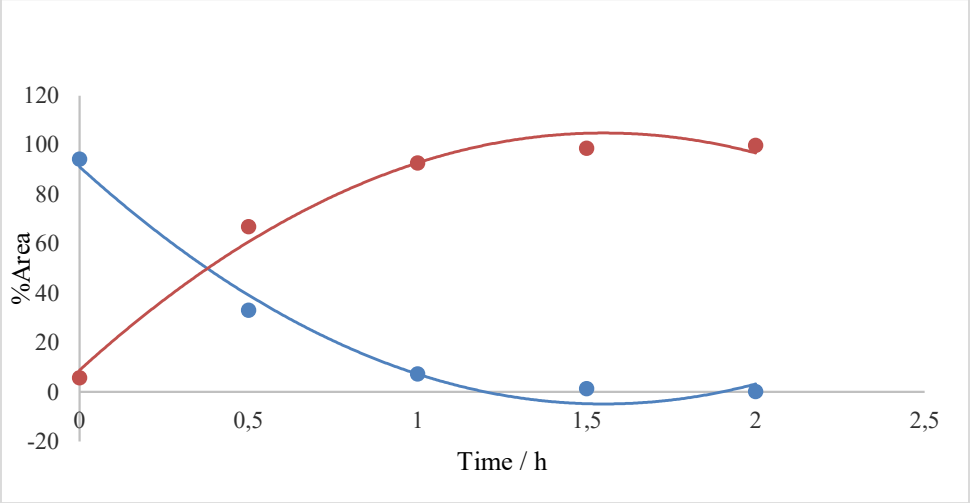


Figure 3.3 – Time-dependence profile for the activation of tetraoxane **22**

The rate constant (k_{obs}) for the decreasing of compound **22** is given by the slope of a linear regression of Ln(Area) vs time. (Figure 3.4).

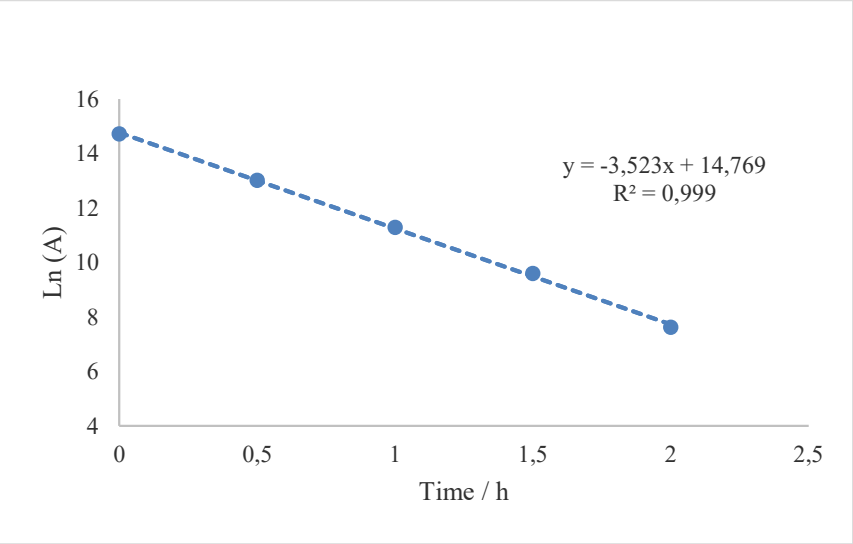


Figure 3.4 – Kinetic of the disappearance of tetraoxane **22** throughout time

This HPLC studies were made in pseudo first-order conditions, once the concentration of iron (II) bromide was much higher than the concentration of the substrate (tetraoxanes **21**-**27**).

Rate constant and half-life time ($t_{1/2}$) of the compounds were determined through Equation 1 and 2, respectively. Table 3.2 has a summary of all the calculated half-lives times for tetraoxanes **21**-**27**.

$$v = k_{obs}[A], \quad \text{in which} \quad k_{obs} = k_B[B] \qquad \text{Equation 1}$$

$$t_{1/2} = \frac{\ln 2}{k_{obs}} \qquad \text{Equation 2}$$

Table 3.2 – Half-lives times and rate constant of the hybrid compounds **21**-**27**

Compound	k_{obs} (h^{-1}) [a]	$t_{1/2}$ (min) [a]
21	2.76	15.6
22	3.35	13.2
23	3.23	13.2
24	2.40	17.4
25	3.27	13.2
26	2.35	18
27	2.06	21

[a] calculated through the average of the three assays

The kinetic of activation revealed a complete consumption of all the hybrid compounds and appearance of *trans*-cinnamaldehyde and their respective aldehydes. However, the quantification of these products was not accomplished since both compound have similar R_t. *Trans*-cinnamaldehyde, as an α,β-unsaturated compound, can act as inhibitor of TR or falcipain, which are enzymes that play a key role in leishmaniasis and malaria, respectively. Moreover, these compounds have demonstrated small half-lives times which indicates that they are rapidly activated by iron (II), so it is expected that they have similar behavior when it comes to *in vivo* studies.

3.2 Antiparasitic activity

The tetraoxanes synthesized were evaluated against *Plasmodium falciparum* Dd2 and 3D7 which is a strain resistant to both chloroquine and mefloquine, and a strain sensitive to both chloroquine and mefloquine, respectively. These studies were carried out in the Institute of Hygiene and Tropical Medicine (Universidade Nova de Lisboa), following a reported protocol and briefly described in chapter 5.[111]

The results of their inhibitory activity are summarized in Table 3.3 for *P. falciparum* Dd2 and 3D7 strains, and are expressed in IC_{50}, which means the concentration of the drug that inhibits 50% of the parasite growing.

Table 3.3 – IC_{50} values for tetraoxanes **21**-**27** in *P. falciparum* Dd2 and 3D7 strains

	IC_{50} (μM)	
Compound	Dd2	3D7
21	1.34	1.67
22	0.68	2.06
23	0.42	1.36
24	1.65	1.20
25	0.74	0.59
26	0.63	1.02
27	1.64	3.07
Chloroquine	0.36	0.02

Compounds **22**, **23**, **25** and **26** revealed the best inhibition action in *P. falciparum* Dd2 strains, with IC_{50} values ranging between 0.42 and 0.74 μM. Compounds **21**, **24** and **27** revealed to inhibit with less efficacy *P. falciparum* Dd2 strains, what can be seen by their IC_{50} values ranging between 1.34 and 1.65 μM.

Compound **25** revealed the best inhibition in *P. falciparum* 3D7 strains, with IC_{50} value of 0.59 μM. The remaining compounds revealed to inhibit with less efficacy *P. falciparum* 3D7 strains, what can be seen by their IC_{50} values ranging between 1.02 and 3.07 μM.

Hybrid compounds have demonstrated to be moderately active, showing better inhibition results against *Plasmodium falciparum* Dd2 strain, in which several compounds revealed sub-μM values.

As mentioned previously, TR is a cysteine dependent oxy-reductase crucial to maintain the redox balance in *Leishmania spp*.. To evaluate the potential as antileishmanial agents, the compounds were screened at Francisco Gamarro Lab, Instituto de Parasitologia y Biomedicina "Lopez-Neyra", Navarra.

The compounds were tested against the promastigote form of *Leishmania donovani*, in which under the conditions used some compounds have shown solubility issues. All compounds tested did not shown activity, even at the higher concentration tested of 1 mM. These results can indicate that: i) the compounds do not have activity against the promastigote form of *L. donovani*; ii) the compounds are unable to cross the plasmatic membrane of parasites; iii) the compounds are not well dissolved in the media used and, consequently, precipitated.

Based in the results obtained, less lipophilic compounds should be synthesized and tested against promastigote and amastigote forms of *L. donovani*.

Chapter 4

4. Conclusions and future work

4.1 Conclusions

The main objective of this thesis was to prepare a small library of hybrid compounds with 1,2,4,5-tetraoxane scaffold that, upon activation by intracellular iron(II), can selectively deliver an electrophile capable of reacting with essential enzymes of the parasites causing leishmaniasis and malaria. The electrophile is an α,β-unsaturated aldehyde that has the potential to react via Michael addition with the catalytic cysteine residues of trypanothione reductase for leishmaniasis and falcipain for malaria.

Seven 1,2,4,5-tetraoxanes were synthesized in moderate yields by first reacting the appropriate benzaldehyde with hydrogen peroxide, and then converting the resulting *gem*-dihydroperoxide into the final product by reaction with *trans*-cinnamaldehyde in the presence of rhenium (VII) oxide. The chemical structure of tetraoxanes was confirmed through unidimensional - ^{1}H NMR, ^{13}C NMR – and bidimensional – COSY, HMQC, and HMBC – NMR spectroscopy. Since the initial compounds displays high lipophilicity and low solubility, a second set of tetraoxanes were designed to incorporate solubility-conferring structural features such as amines and 5-membered heterocyclic moieties. Unfortunately, all attempts to synthesize these compounds failed, suggesting that the synthetic method has stringent structural requirements that limit its usefulness.

The activation of tetraoxanes was studied by monitoring the reaction of compounds with iron(II) by HPLC. Incubation of tetraoxanes with $FeBr_2$ in ACN/H_2O led to complete consumption of the starting material and the formation of the corresponding benzaldehyde and cinnamaldehyde, thus confirming that iron (II) efficiently activates this class of endoperoxides. The half-lives times for activation ranged from 13 to 21 minutes, indicating that the reaction is not significantly affected by the substituents on the benzaldehyde moiety. This is consistent with a radical based mechanism, in which the initial oxygen-centered radicals are poorly stabilized by the substituents. Overall, these studies were a proof-of-concept showing that a α,β-unsaturated carbonyl compound is rapidly released upon activation of tetraoxane by iron (II), suggesting that these endoperoxides would have similar behavior *in vivo*.

Tetraoxanes were screened against the chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum* strains, 3D7 and Dd2 respectively, showing IC_{50} values in the sub-micromolar range for Dd2 strain. The results are consistent with efficient activation of tetraoxanes in the infected erythrocytes to generate toxic radical species and the release of cinnamaldehyde. Unfortunately, the tetraoxanes were not soluble in the conditions used for the screening against *Leishmania donovani*, thus precluding evaluation as antileishmanial agents.

4.2 Future Work

The work reported in this thesis is a novel approach, in the context of selective antiparasitic agents. As *Leishmania* and *Plasmodium* parasite mobilize relatively high amounts of iron during critical steps of their life-cycle, it is expected that tetraoxanes can be activated in similar ways in both parasites.

The major challenge that needs to be addressed in the future is the poor solubility and high lipophilicity displayed by tetraoxanes. Different approaches can be envisaged to overcome the problems faced in preparing more polar derivatives. First, a different set of substituents could be used in the benzaldehyde or cinnamaldehyde starting materials, such as carboxylic acid (or an ester that can be subsequently hydrolyzed) or an amide. Secondly, optimization of the synthetic method could be performed in order to extend its scope. Thirdly, encapsulation in nanoparticles could be performed as a strategy to improve solubility and selective delivery to macrophages. This is particularly attractive for leishmaniasis, because *Leishmania* parasites tend to accumulate in these cells.

Future work should also involve a more detailed kinetic study of tetraoxane activation in infected cells in order to evaluate if rates of delivery of the electrophiles are identical to those determined in the biomimetic experiments. Including, more substituents in both sides of the tetraoxane scaffold would also improve our understanding of the structural requirements for rapid and efficient activation.

Chapter 5

5. Materials and Methods

5.1 Equipment

NMR spectra were recorded in a Bruker 300 Ultra Shield. Chemical shifts values of δ_H and δ_C are reported in parts per million (ppm), using d_3-chloroform as internal reference. Coupling constants (*J*) are reported in hertz (Hz).

Melting points were determined on a Kofler Bock Monoscop M. camera and are uncorrected.

UV-visible spectra were record in 1 cm pathlength quartz cuvettes at 25 ºC using a Shimadzu UV-1603 UV-visible spectrophotometer.

HPLC data were obtained on a Hitachi Elite Lachrom with a L-2130 pump, a L-2300 column oven, a Rheodyne injector of 20 μL, and a L-2400 UV-detector. The column used was a LichroCart ® RP-18E 250-4 purospher ®STAR with a guard column RP-18E.

5.2 Chromatography

Thin-layer chromatography (TLC) was performed using coated silica gel plates from Merk Kieselgel 60 F_{254}, 200 μm thickness. Visualization was carried out by a CAMAG UV lamp at a wavelength of 254 nm and 366 nm, and revealed with iodine, *p*-anisaldehyde and ninhydrin dip.

Purification of compounds by column chromatography was performed using silica gel 60 M, 0.040-0.063 mm from Merk.

Purification of compounds by preparative thin-layer chromatography were performed in coated silica-gel plates, silica gel 60 GF_{254}, 0.040-0.063 mm, from Merk.

5.3 Reagents and Solvents

5.3.1 Reagents

All the reagents used during the experimental synthesis were of analytical grade and were purchased from Sigma-Aldrich, Alfa Aesar, and Merk and used without further purification.

5.3.2 Solvents

Solvents used were of analytical grade. Dichloromethane (DCM) and acetonitrile (ACN), were dried from calcium chloride and from phosphorous pentoxide, respectively, and distilled at atmospheric pressure. Tetrahydrofuran was distilled from sodium-benzophenone system. All the solvents used in purification were distilled under reduced pressure.

NMR analysis were performed using chloroform deuterated ($CDCl_3$) with a degree of purity higher than 95%.

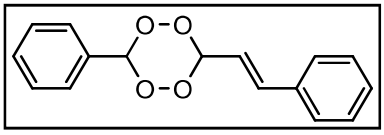
5.4 Synthesis of 1,2,4,5-tetraoxanes derivatives and it structural characterization

General procedure for the synthesis of **21**-**27**:

To a stirring solution of the appropriate benzaldehyde (0.9 mmol; 1 equiv.) in dry acetonitrile (1.3 mL) was added formic acid (34 equiv.) at 0°C under nitrogen atmosphere. Hydrogen peroxide 50% (24 equiv.) was added slowly and then the solution was allowed to warm to room temperature and stirred for 2-3 h under nitrogen atmosphere. After 1h of reaction, hydrogen peroxide 50% (24 equiv.) was added in the same conditions. The solution was diluted with brine solution (10 mL) and extracted with DCM (3 × 15 mL). The combined organic layers, containing the *gem*-dihydroperoxide, were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. At 0°C and under nitrogen atmosphere the *gem*-dihydroperoxide intermediate was added to a stirring solution of *trans*-cinnamaldehyde (1.6 equiv.) and rhenium (VII) oxide (0.05 mmol%) in dry DCM (1.6 mL). The reaction was followed by TLC, and after 2-3 h at 0°C and under nitrogen atmosphere, the mixture was filtered with a plug of silica and concentrated under reduced pressure.

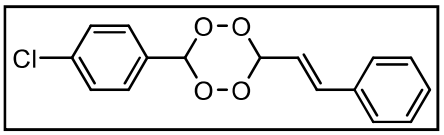
The purification by flash column chromatography on silica gel using DCM:n-Hexane (1:1) as eluent gave the pure compound.

Compound 21 - *(E)-3-phenyl-6-styryl-1,2,4,5-tetraoxane*



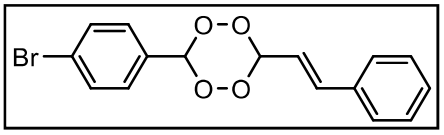
Prepared according to the general procedure to give **21** as a white solid with a yield of 44%, **m.p** 133-136°C; **^{1}H NMR (300 MHz, $CDCl_3$):** δ_H (ppm) 7.53 – 7.37 (*m*, 10H, Ar(C<u>H</u>)), 7.05 (*d*, *J* = 16.3 Hz, 1H, CH=C<u>H</u>-Ar), 6.84 (*s*, 1H, <u>H</u>COO), 6.62 (*dd*, *J* = 6.3, 0.6 Hz, 1H, OOC<u>H</u>), 6.05 (*dd*, *J* = 16.3, 6.3 Hz, 1H, C<u>H</u>=CH-Ar). **^{13}C NMR (75 MHz, $CDCl_3$):** δ_C (ppm) 140.47 (CH=<u>C</u>H-Ar), 134.78 (<u>Cq</u>), 131.32 (Ar(<u>C</u>H)), 131.06 (<u>Cq</u>), 129.57 (Ar(<u>C</u>H)), 128.81 (Ar(<u>C</u>H)),127.80 (Ar(<u>C</u>H)), 127.31 (Ar(<u>C</u>H)), 116.70 (<u>C</u>H=CH-Ar), 108.08 (H<u>C</u>OO), 107.20 (OO<u>C</u>H). **% purity by HPLC:** 99%

Compound 22 - *(E)-3-(4-chlorophenyl)-6-styryl-1,2,4,5-tetraoxane*



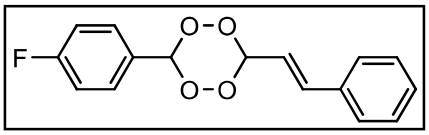
Prepared according to the general procedure to give **22** as a white solid with a yield of 39%, **m.p** 171-173°C; **^{1}H NMR (300 MHz, $CDCl_3$):** δ_H (ppm) 7.47 – 7.33 (*m*, 9H, Ar(C<u>H</u>)), 7.03 (*d*, *J* = 16.3 Hz, 1H, CH=C<u>H</u>-Ar), 6.78 (*s*, 1H, <u>H</u>COO), 6.58 (*dd*, *J* = 6.4, 0.7 Hz, 1H, OOC<u>H</u>), 6.01 (*dd*, *J* = 16.3, 6.3 Hz, 1H, C<u>H</u>=CH-Ar). **^{13}C NMR (75 MHz, $CDCl_3$):** δ_C (ppm) 140.71 (CH=<u>C</u>H-Ar), 137.47 (<u>Cq</u>), 134.67 (<u>Cq</u>), 129.66 (Ar(<u>C</u>H)), 129.43 (<u>Cq</u>), 129.14 (Ar(<u>C</u>H)), 128.83 (Ar(<u>C</u>H)), 127.32 (Ar(<u>C</u>H)), 116.44 (<u>C</u>H=CH-Ar), 107.29 (H<u>C</u>OO), 107.22 (OO<u>C</u>H). **% purity by HPLC:** 99%

Compound 23 - *(E)-3-(4-bromophenyl)-6-styryl-1,2,4,5-tetraoxane*



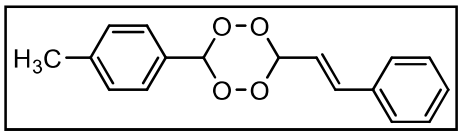
Prepared according to the general procedure to give **23** as a white solid with a yield of 15%, **m.p** 196-198°C; **^{1}H NMR (300 MHz, $CDCl_3$):** δ_H (ppm) 7.53 – 7.23 (*m*, 9H, Ar(C<u>H</u>)), 6.94 (*d*, *J* = 16.2 Hz, 1H, CH=C<u>H</u>-Ar), 6.69 (*s*, 1H, <u>H</u>COO), 6.49 (*dd*, *J* = 6.4, 0.8 Hz, 1H, OOC<u>H</u>), 5.92 (*dd*, *J* = 16.3, 6.4 Hz, 1H, C<u>H</u>=CH-Ar). **^{13}C NMR (75 MHz, $CDCl_3$):** δ_C (ppm) 140.74 (CH=<u>C</u>H-Ar), 132.10 (Ar(<u>C</u>H)), 134.66 (<u>Cq</u>), 129.90 (<u>Cq</u>), 129.67 (Ar(<u>C</u>H)), 129.35 (Ar(<u>C</u>H)), 128.84 (Ar(<u>C</u>H)), 127.33 (Ar(<u>C</u>H)), 125.27 (<u>Cq</u>), 116.41 (<u>C</u>H=CH-Ar), 107.31 (H<u>C</u>OO), 107.18 (OO<u>C</u>H). **% purity by HPLC:** 98%

Compound 24 - *(E)-3-(4-fluorophenyl)-6-styryl-1,2,4,5-tetraoxane*



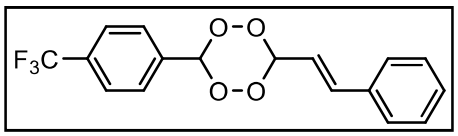
Prepared according to the general procedure to give **24** as a white solid with a yield of 47%, **m.p** 158-160°C; **^{1}H NMR (300 MHz, $CDCl_3$):** δ_H (ppm) 7.51 – 7.35 (*m*, 7H, Ar(C<u>H</u>)), 7.12 (*t*, *J* = 8.7 Hz, 2H, Ar), 7.03 (*dd*, *J* = 16.3 Hz, 1H, CH=C<u>H</u>-Ar), 6.79 (*s*, 1H, <u>H</u>COO), 6.58 (*dd*, *J* = 6.3, 0.6 Hz, 1H, OOC<u>H</u>), 6.02 (*dd*, *J* = 16.3, 6.3 Hz, 1H, C<u>H</u>=CH-Ar). **^{13}C NMR (75 MHz, $CDCl_3$):** δ_C (ppm) 166.07 (<u>Cq</u>), 162.74 (<u>Cq</u>), 140.68 (CH=<u>C</u>H-Ar), 134.68 (<u>Cq</u>), 130.08 (Ar(<u>C</u>H)), 129.96 (Ar(<u>C</u>H)), 129.66 (Ar(<u>C</u>H)), 128.84 (Ar(<u>C</u>H)), 127.33 (Ar(<u>C</u>H)), 116.17 (Ar(<u>C</u>H)), 115.88 (Ar(<u>C</u>H)), 116.46 (<u>C</u>H=CH-Ar), 107.28 (H<u>C</u>OO), 107.23 (OO<u>C</u>H). **% purity by HPLC:** 99%

Compound 25 - *(E)-3-styryl-6-(p-tolyl)-1,2,4,5-tetraoxane*



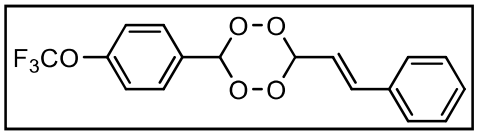
Prepared according to the general procedure to give **25** as a white solid with a yield of 20%, **m.p** 166-168°C; **^{1}H NMR (300 MHz, $CDCl_3$):** δ_H (ppm) 7.35 – 7.12 (*m*, 9H, Ar(C<u>H</u>)), 6.92 (*d*, *J* = 16.3 Hz, 1H, CH=C<u>H</u>-Ar), 6.67 (*s*, 1H, <u>H</u>COO), 6.48 (*dd*, *J* = 6.3, 0.7 Hz, 1H, OOC<u>H</u>), 5.92 (*dd*, *J* = 16.3, 6.3 Hz, 1H, C<u>H</u>=CH-Ar), 2.28 (*s*, 3H, C<u>H</u>$_3$). **^{13}C NMR (75 MHz, $CDCl_3$):** δ_C (ppm) 141.65 (<u>Cq</u>), 140.38 (CH=<u>C</u>H-Ar), 134.80 (<u>Cq</u>), 129.54 (Ar(<u>C</u>H)), 129.46 (Ar(<u>C</u>H)), 128.80 (Ar(<u>C</u>H)), 128.20 (<u>Cq</u>), 127,76 (Ar(<u>C</u>H)), 127.29 (Ar(<u>C</u>H)), 116.77 (<u>C</u>H=CH-Ar),108.10 (H<u>C</u>OO), 107.08 (OO<u>C</u>H), 21.50 (<u>C</u>H$_3$). **% purity by HPLC:** 98%

Compound 26 - *(E)-3-styryl-6-(4-(trifluoromethyl)phenyl)-1,2,4,5-tetraoxane*



Prepared according to the general procedure to give **26** as a white solid with a yield of 39%, **m.p** 163-165°C; **^{1}H NMR (300 MHz, $CDCl_3$):** δ_H (ppm) 8.05 – 7.69 (*m*, 9H, Ar(C<u>H</u>)), 7.51 (*d*, *J* = 16.3 Hz, 1H, CH=C<u>H</u>-Ar), 7.29 (*s*, 1H, <u>H</u>COO), 7.06 (*dd*, *J* = 6.4, 0.7 Hz, 1H, OOC<u>H</u>), 6.48 (*dd*, *J* = 16.3, 6.4 Hz, 1H, C<u>H</u>=CH-Ar). **^{13}C NMR (75 MHz, $CDCl_3$):** δ_C (ppm) 151.20 (<u>C</u>F$_3$), 140.78 (CH=<u>C</u>H-Ar), 134.64 (<u>Cq</u>), 129.68 (Ar(<u>C</u>H)), 129.56 (Ar(<u>C</u>H)), 129.48 (<u>Cq</u>), 128.84 (Ar(<u>C</u>H)), 127.33 (Ar(<u>C</u>H)), 122.03 (<u>Cq</u>), 121.05 (Ar(<u>C</u>H)), 116.36 (<u>C</u>H=CH-Ar), 107.33 (OO<u>C</u>H) and 107.01 (H<u>C</u>OO). **% purity by HPLC:** 98%

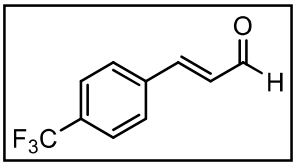
Compound 27 - *(E)-3-styryl-6-(4-(trifluoromethoxy)phenyl)-1,2,4,5-tetraoxane*



Prepared according to the general procedure to give **27** as a white solid with a yield of 33%, **m.p.** 155-157°C; **^{1}H NMR (300 MHz, $CDCl_3$):** δ_H (ppm) 7.57 – 7.28 (*m*, 9H, Ar(C<u>H</u>)), 7.05 (*d*, *J* = 16.3 Hz, 1H, CH=C<u>H</u>-Ar), 6.83 (*s*, 1H, <u>H</u>COO), 6.60 (*dd*, *J* = 6.4, 0.6 Hz, 1H, OOC<u>H</u>), 6.03 (*dd*, *J* = 16.3, 6.4 Hz, 1H,

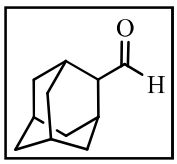
C<u>H</u>=CH-Ar). **^{13}C NMR (75 MHz, CDCl$_3$):** δ_C (ppm) 151.18 (O<u>C</u>F$_3$), 140.78 (CH=<u>C</u>H-Ar), 134.64 (<u>Cq</u>), 129.68 (Ar(<u>C</u>H)), 129.55 (Ar(<u>C</u>H)), 129.48 (<u>Cq</u>), 128.84 (Ar(<u>C</u>H)), 127.32 (Ar(<u>C</u>H)), 122.03 (<u>Cq</u>), 121.05 (Ar(<u>C</u>H)), 116.35 (<u>C</u>H=CH-Ar), 107.33 (OO<u>C</u>H), 107.00 (H<u>C</u>OO). **% purity by HPLC:** 99%

5.5 Synthesis of *trans*-4-(trifluoromethyl)-cinnamaldehyde



To a stirring solution of *trans*-4-(trifluoromethyl)cinnamic acid (0.84 mmol) in DCM (3 mL) was added TBTU (1 equiv.) and TEA (1 equiv.) at room temperature. After 30 minutes of reaction, N,O-dimethylhydroxylamine (1.1 equiv.) and TEA (1 equiv.) were added. The stirring continued overnight at room temperature, then the mixture was diluted with DCM (20 mL) and washed with $NaHCO_3$ (2 × 20 mL) and brine solution (2 × 20 mL). The combined organic layers, were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure to give the Weinreb amide intermediate. The compound was purified by flash column chromatography on silica gel using EtOAc:n-Hexane (1:1). In the second step, to the stirring solution of the intermediate in dry THF (8 mL) was slowly added $LiAlH_4$ (1 equiv.) at 0°C under nitrogen atmosphere. The reaction was followed by TLC and after completion a solution of potassium hydrogenosulfate (2 equiv.) in water (2 mL) was added. The mixture was diluted with water (40 mL) and extracted with diethylic ether (3 × 30 mL). The combined organic layers were washed with $NaHCO_3$ (3 × 30 mL) and brine solution (3 × 30 mL) and dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure, to give an orange oil.

5.6 Synthesis of adamantane-2-carbaldehyde



Methoxymethyltriphenylphosphonium (5.8 mmol; 1.4 equiv.) and potassium bis(trimethylsilyl)amide (0.5M in hexane, 11.66 mL) were added in dry THF (7.5 mL) at 0 ºC under nitrogen atmosphere. After 15 minutes, 2-adamantanone (1 equiv) in dry THF (2.5 mL) was added. The stirring continued for 2h at 0 ºC, then the mixture was diluted with water (30 mL) and extracted with diethyl ether

(3 × 30 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude was dissolved into ACN (25 mL) and HCl (1 M; 6.3 mL) was added. After 21 h at room temperature the solution was diluted with brine solution (30 mL) and extracted with diethyl ether (3 × 30 mL) and the combined organic layers dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure, to give pure compound. White solid, 35% yield, **m.p** 130-132°C; **^{1}H NMR (300 MHz, $CDCl_3$):** δ_H (ppm) 10.11 (*s*, 1H, CO<u>H</u>), 2.78 (*s*, 3H), 2.34-2.01 (*m*, 12H).

5.7 Activation of tetraoxanes with iron bromide

Stock solutions of compounds **21**-**27** (10 mM in ACN), iron (II) bromide (100 mM in H_2O) were freshly prepared. For the activation assays, in an eppendorf kept in a water bath at 25 ºC and containing 200 μL of compounds **21**-**27** (1 mM) and 1.44 mL of ACN/H_2O (1:1) were added 360 μL of iron (II) bromide (18 mM). The activation begins when iron (II) bromide was added (t=0) and several aliquots were injected in the HPLC system during 2h.[103]

Chromatographic separation was achieved using a RP-18e (5μm) column and isocratic gradient with MeOH/H_2O (95:5) at a 1.0 mL/min flow rate. The activation was monitored at 264 nm wavelength obtained from the maximum absorption from the UV-visible spectra of compounds **21**-**27**.

The activation assays were done in triplicate and calibration curves were also obtained for all compounds.

5.8 In vitro antimalarial assays

Dd2 and 3D7 strains were cultured using human erythrocytes suspension with a 5 % hematocrit and maximal parasitemia of 5 %. The cultures were set up in microplates and synchronized by double sorbitol treatment prior to the assays. To determine the correspondent IC_{50}, Dd2 and 3D7 strains were cultivated for 48 h in the presence of

different concentrations of tetraoxanes, then a solution of SYBR Green was added to each well and incubated for 60 minutes. Fluorescence is collected in a fluorimeter plate reader. The activity was determined through parasitemia counting using optical microscopy.[111]

5.9 In vitro antileishmanial assays

L. donovani promastigotes with the luciferase gene integrated into the parasite genome (Luc line) were grown at 28°C in an RPMI 1640-modified medium (Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum (hiFBS, Invitrogen). The antileishmaial activity of compounds was determined measuring the luciferase activity in parasites.[112]

Chapter 6

6. References

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Chapter 7

7. Annexes

7.1 Compound 21 – (*E*)-3-phenyl-6styryl-1,2,4,5-tetraoxane

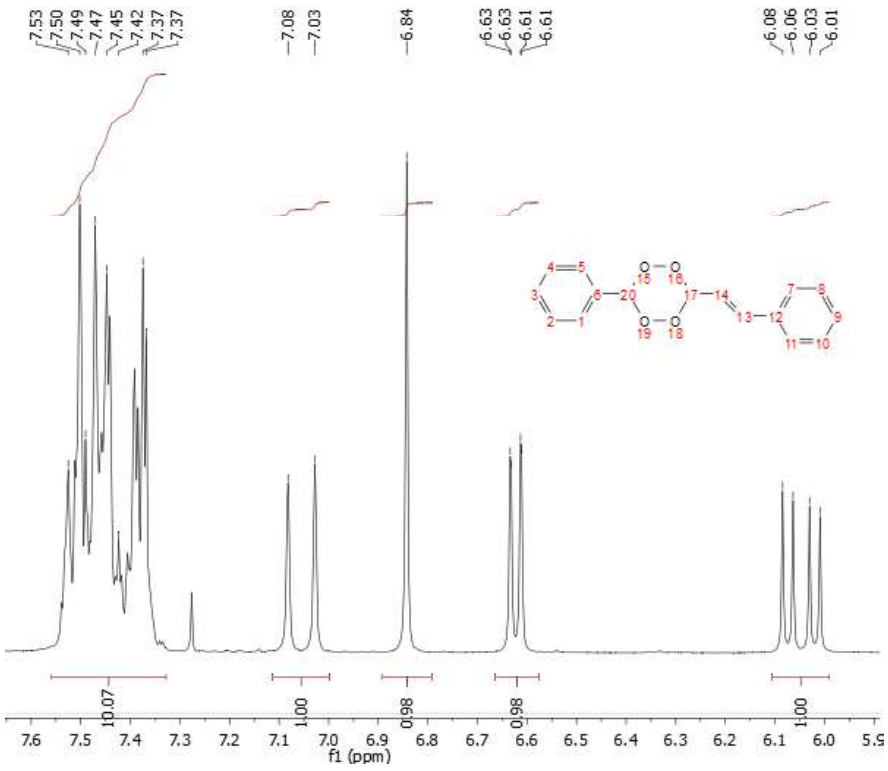

Figure 7.1 – Benzaldehyde-tetraoxane **21** ^{1}H NMR spectra ($CDCl_3$)

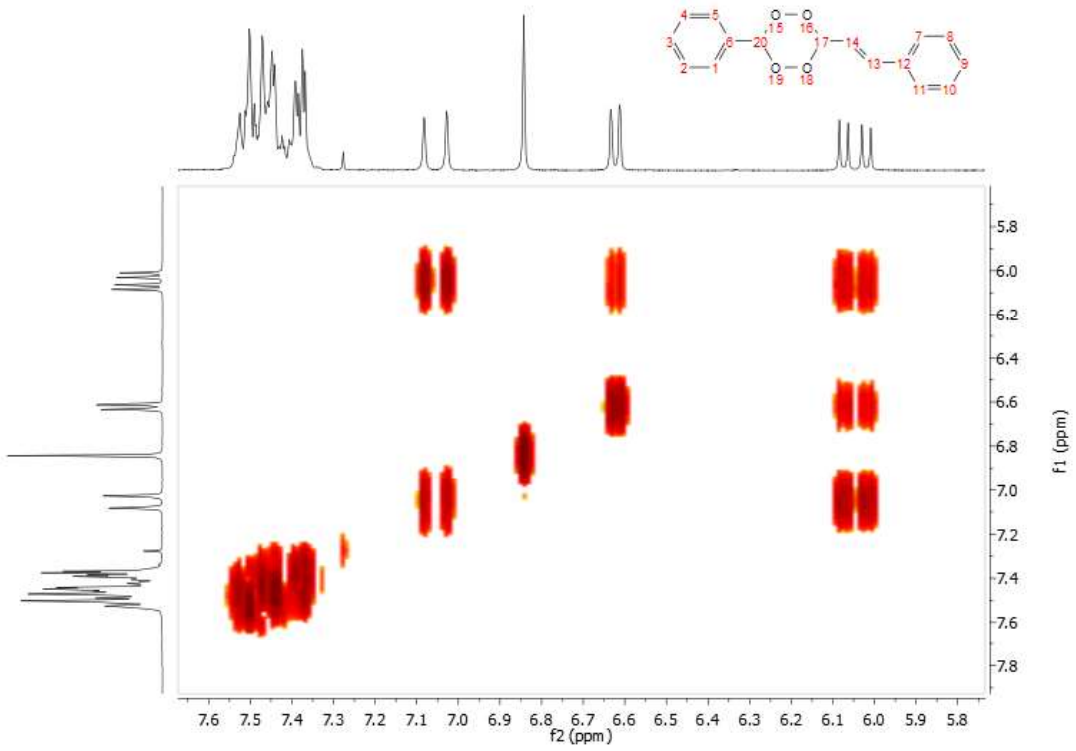

Figure 7.2 – Benzaldehyde-tetraoxane **21** COSY spectra ($CDCl_3$)

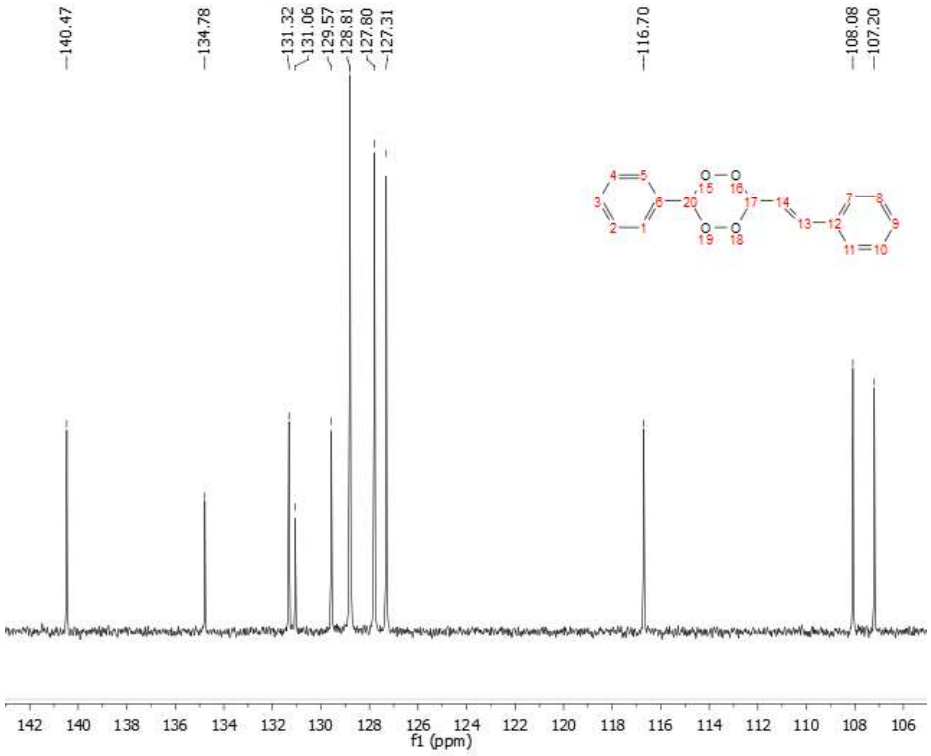

Figure 7.3 – Benzaldehyde-tetraoxane **21** ^{13}C NMR spectra ($CDCl_3$)

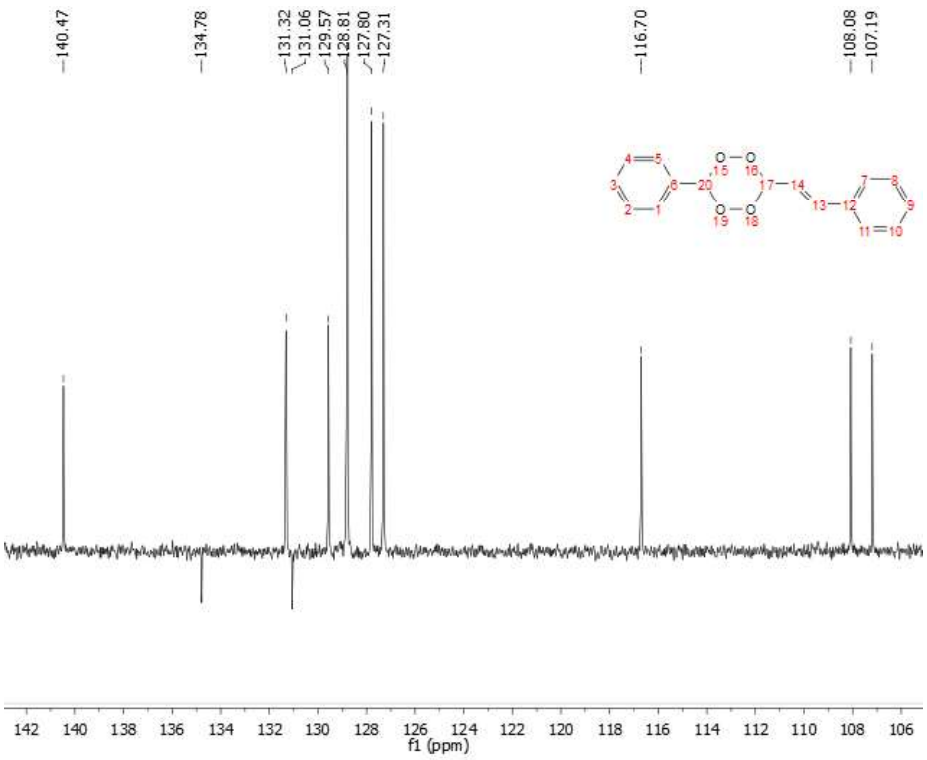

Figure 7.4 – Benzaldehyde-tetraoxane **21** APT spectra ($CDCl_3$)

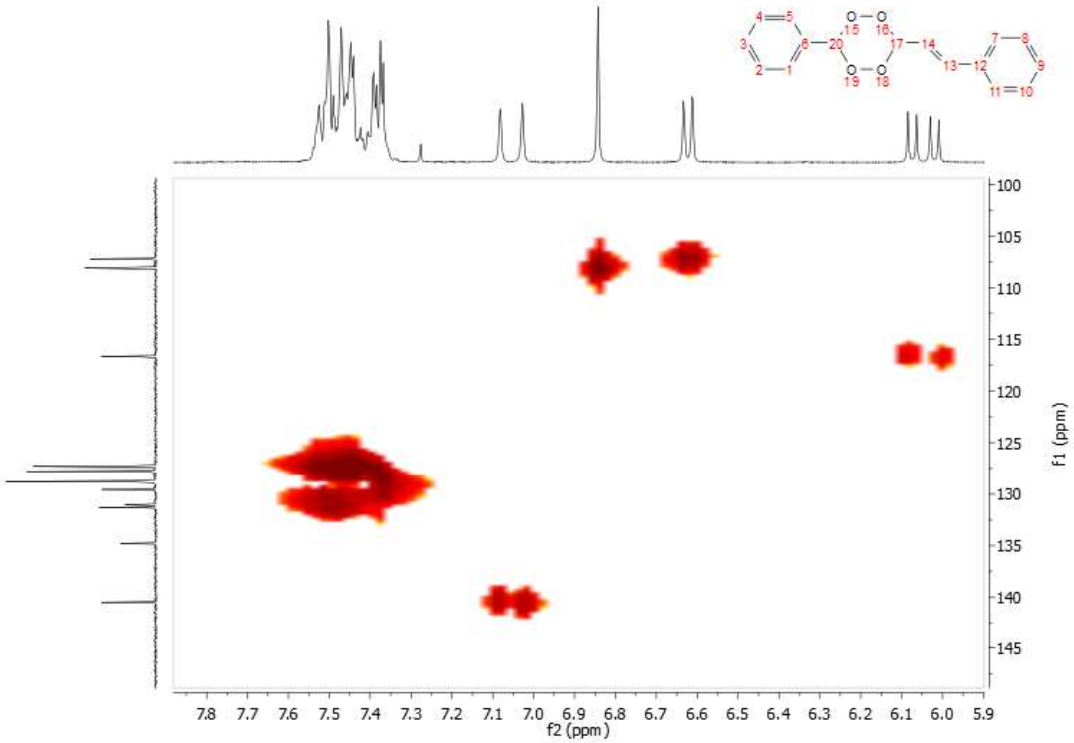

Figure 7.5 – Benzaldehyde-tetraoxane **21** HMQC spectra ($CDCl_3$)

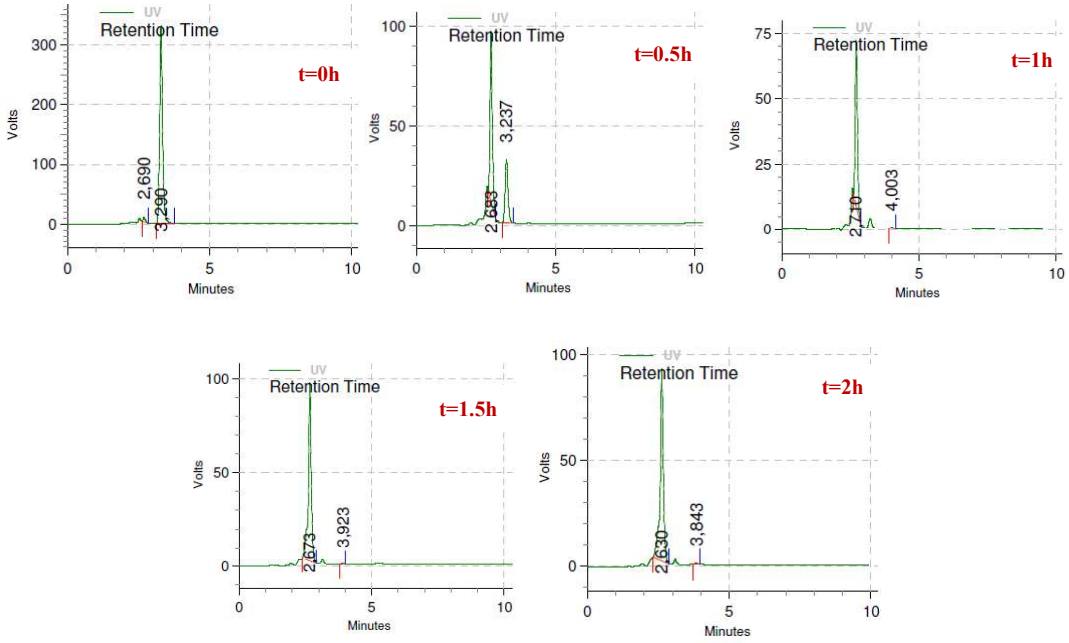

Figure 7.6 – Activation of tetraoxane **21** with $FeBr_2$

7.2 Compound 22 – (*E*)-3-(4-chlorophenyl)-6-styryl-1,2,4,5-tetraoxane

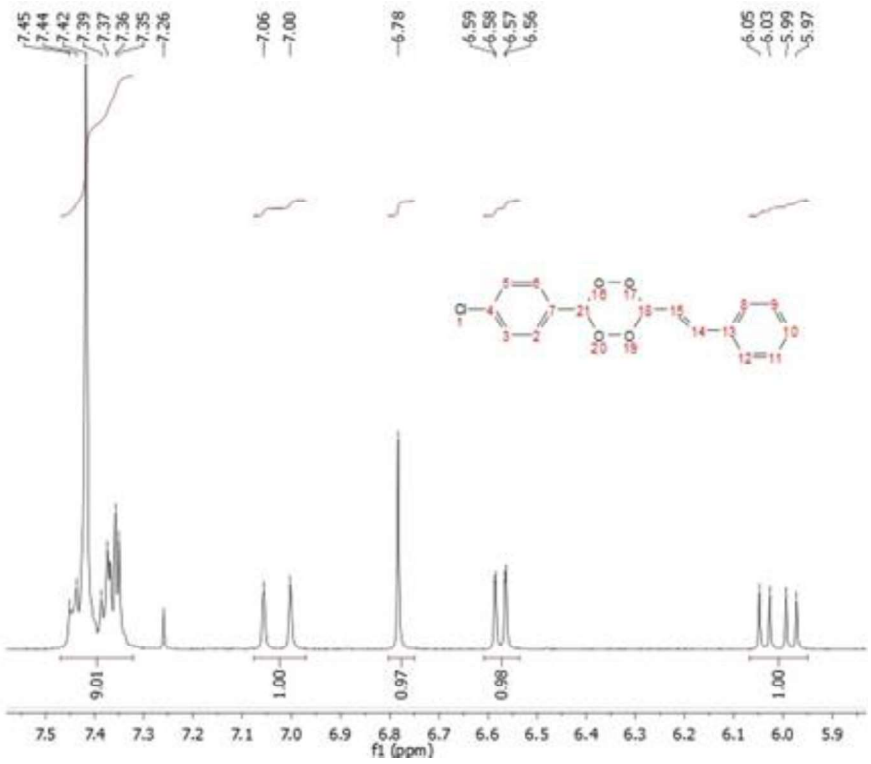

Figure 7.7 – Chlorobenzaldehyde-tetraoxane **22** ^1H NMR spectra ($CDCl_3$)

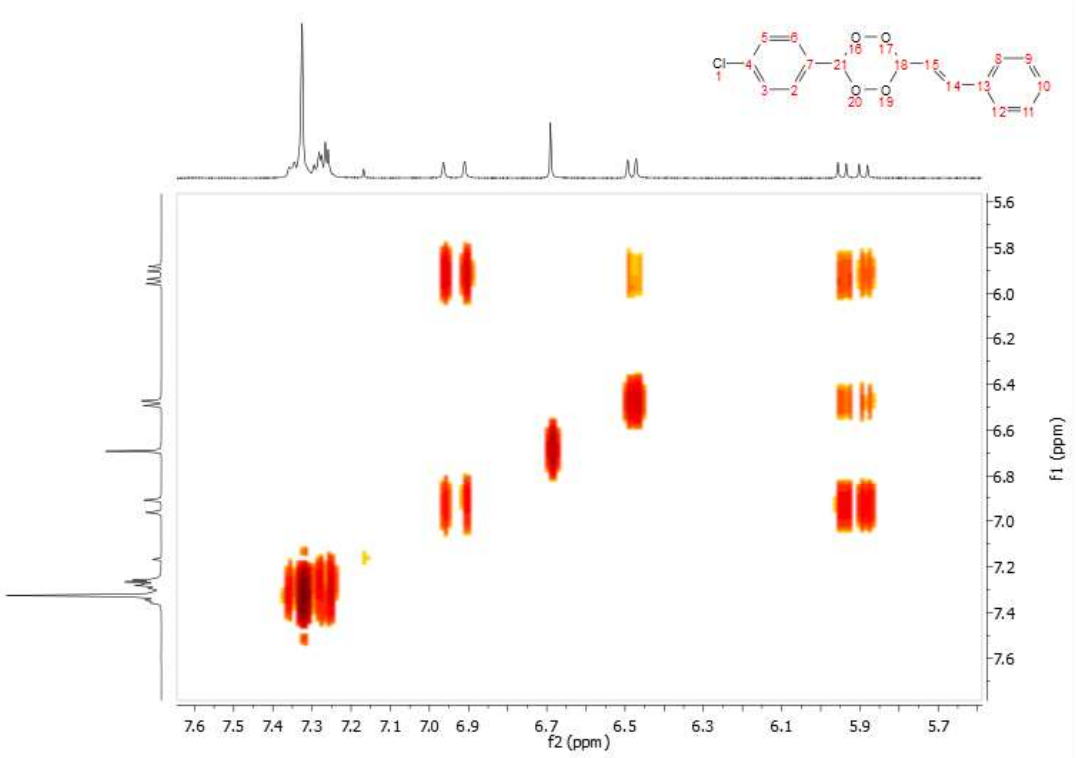

Figure 7.8 – Chlorobenzaldehyde-tetraoxane **22** COSY spectra ($CDCl_3$)

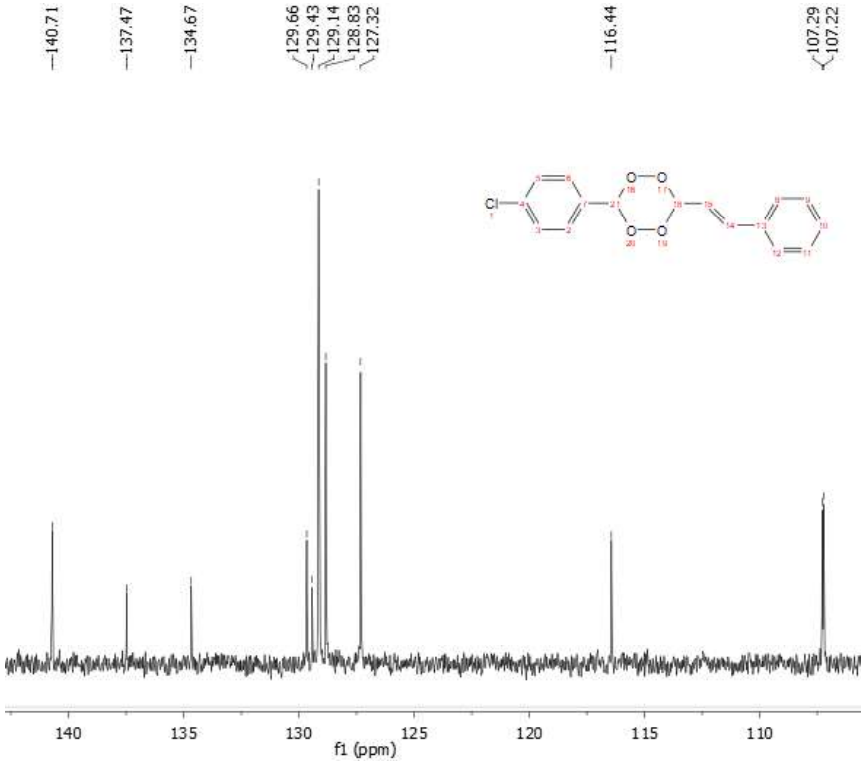

Figure 7.9 – Chlorobenzaldehyde-tetraoxane **22** ^{13}C NMR spectra ($CDCl_3$)

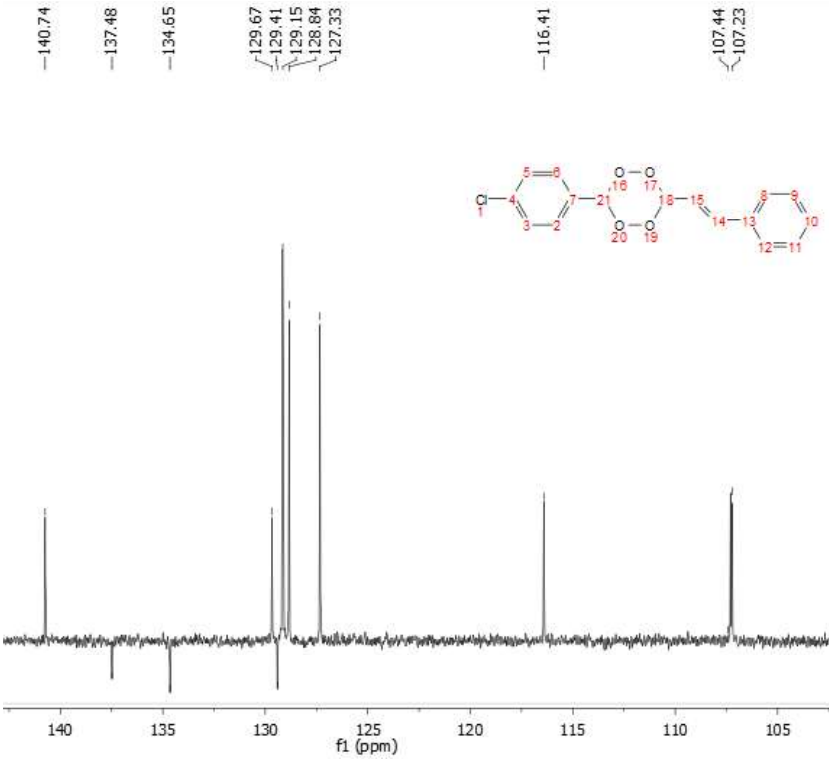

Figure 7.10 – Chlorobenzaldehyde-tetraoxane **22** APT spectra ($CDCl_3$)

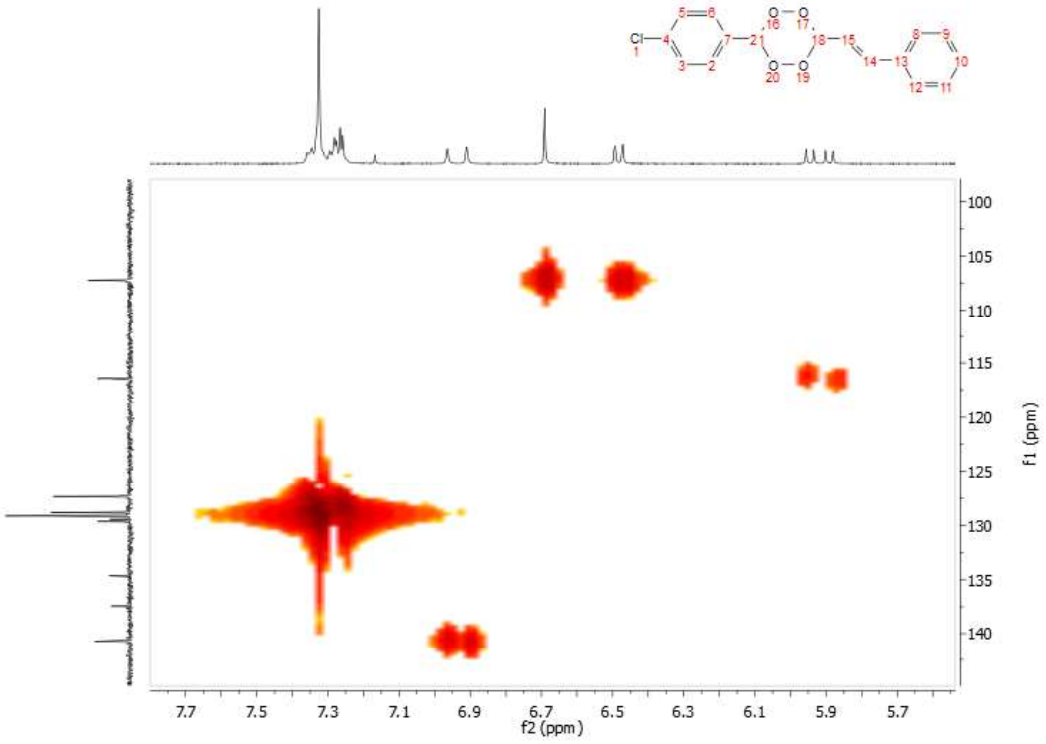

Figure 7.11 – Chlorobenzaldehyde-tetraoxane **22** HMQC spectra ($CDCl_3$)

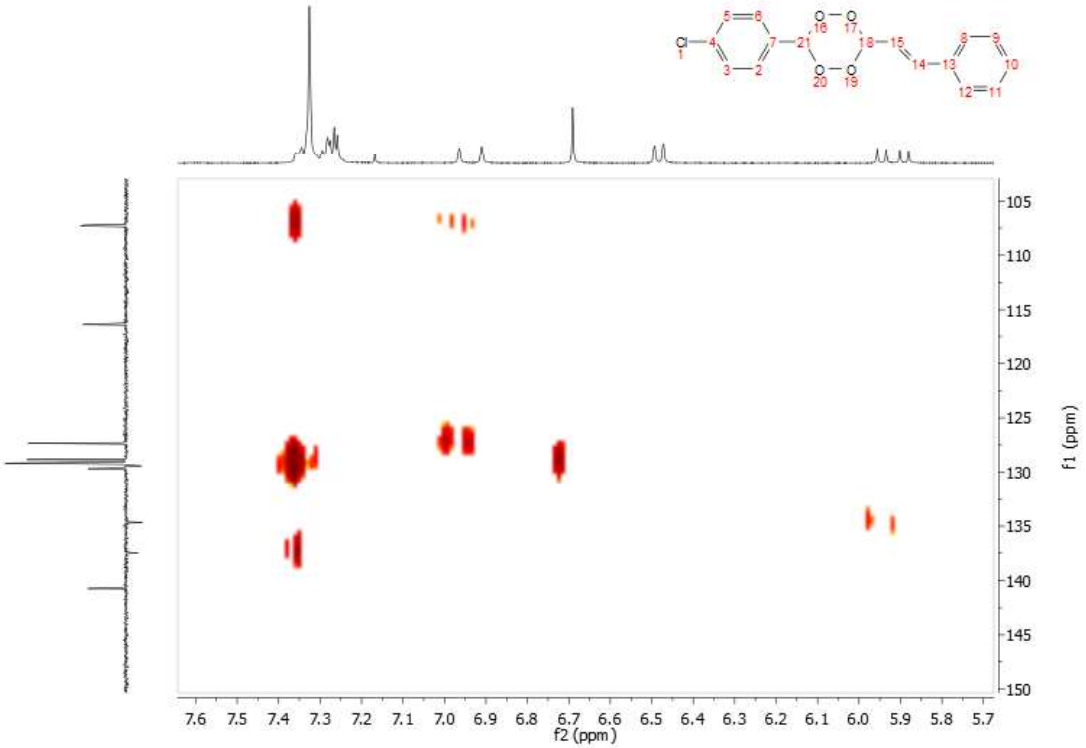

Figure 7.12 – Chlorobenzaldehyde-tetraoxane **22** HMBC spectra ($CDCl_3$)

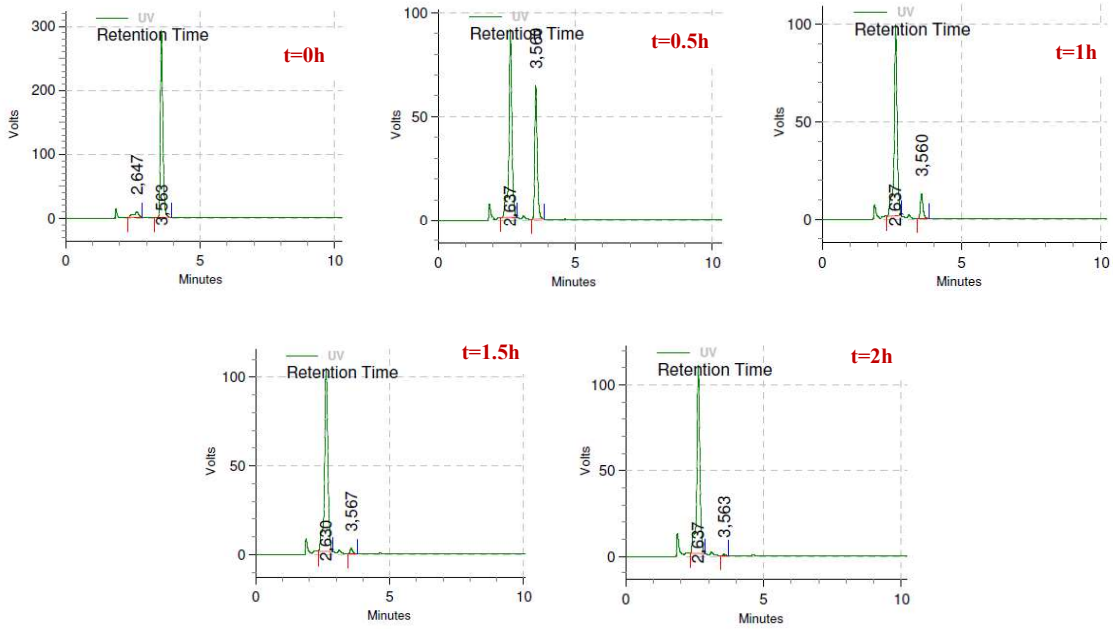

Figure 7.13 – Activation of tetraoxane **22** with $FeBr_2$

7.3 Compound 23 – (*E*)-3-(4-bromophenyl)-6-styryl-1,2,4,5-tetraoxane

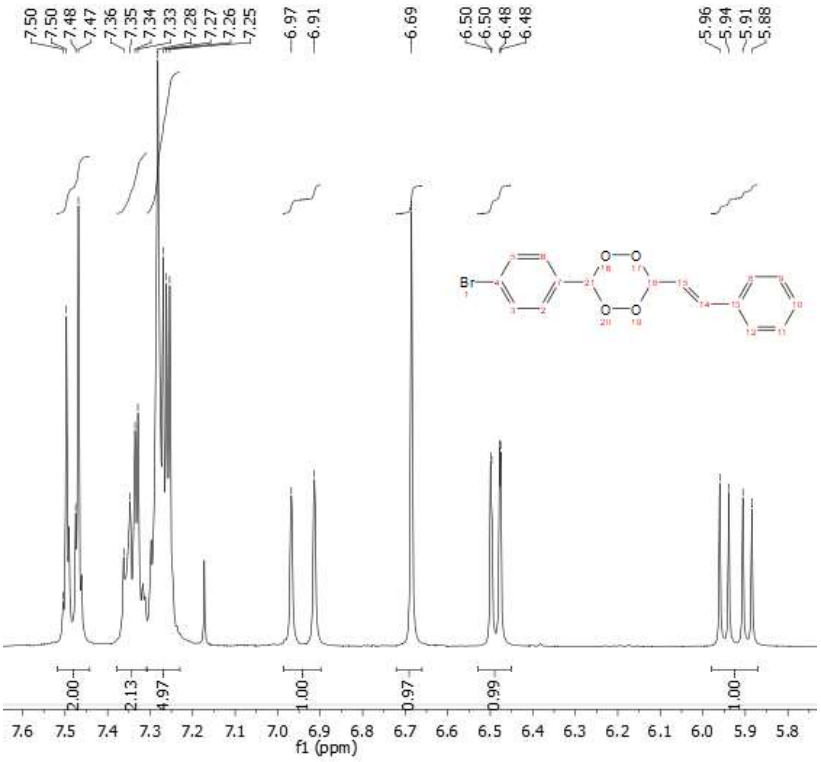

Figure 7.14 – Bromobenzaldehyde-tetraoxane **23** ^{1}H NMR spectra ($CDCl_3$)

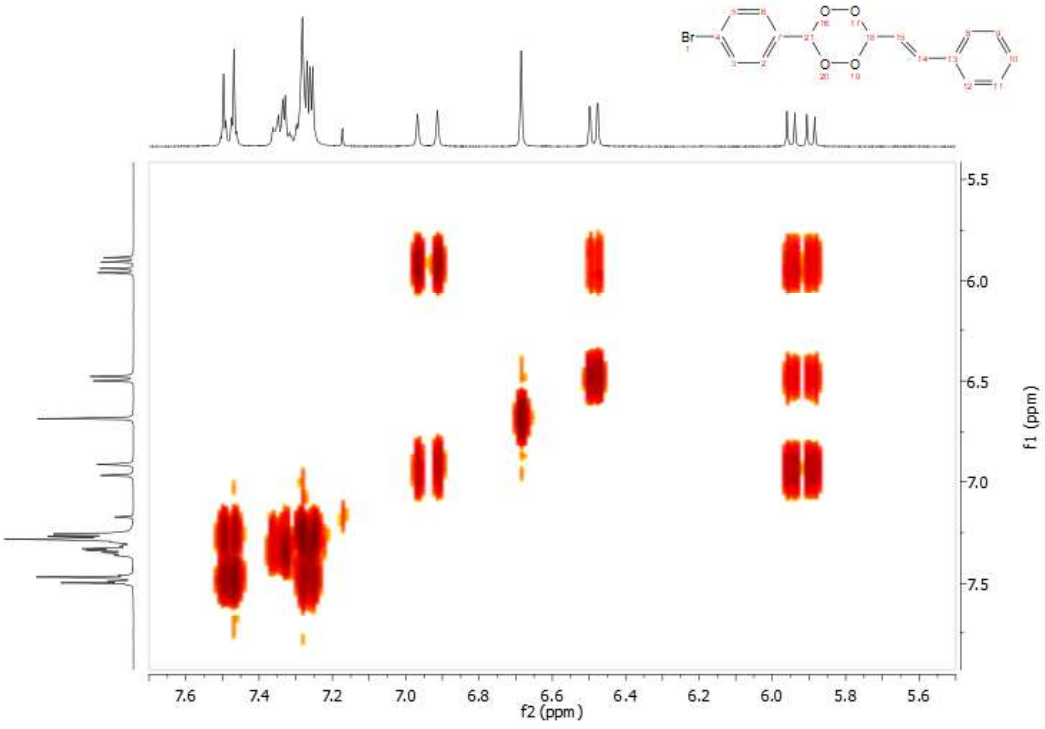

Figure 7.15 – Bromobenzaldehyde-tetraoxane **23** COSY spectra ($CDCl_3$)

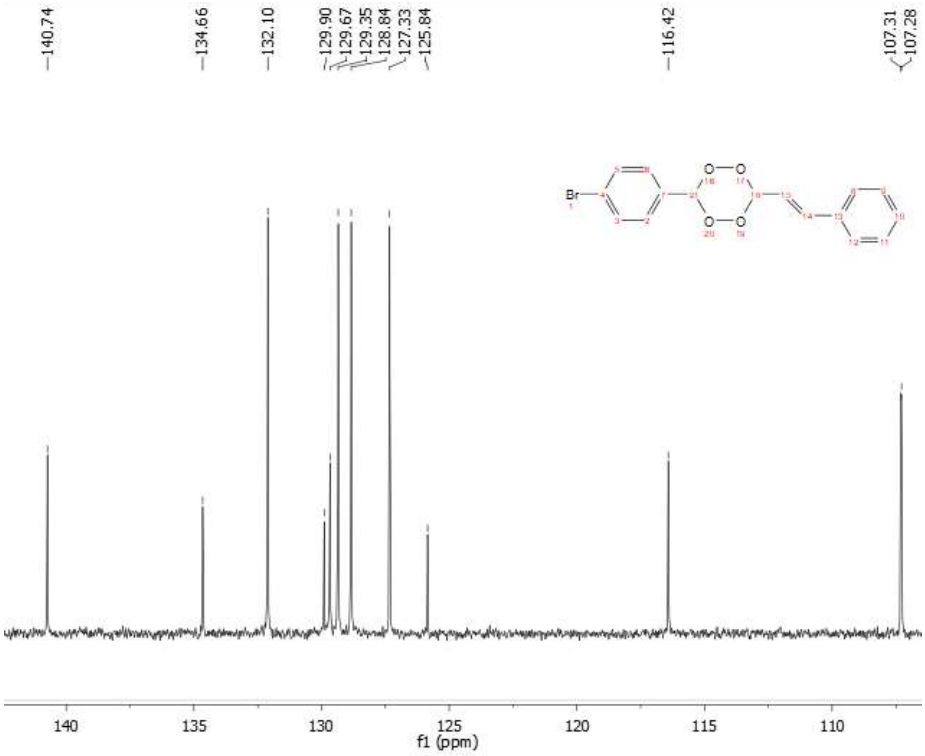

Figure 7.16 – Bromobenzaldehyde-tetraoxane **23** ^{13}C NMR spectra ($CDCl_3$)

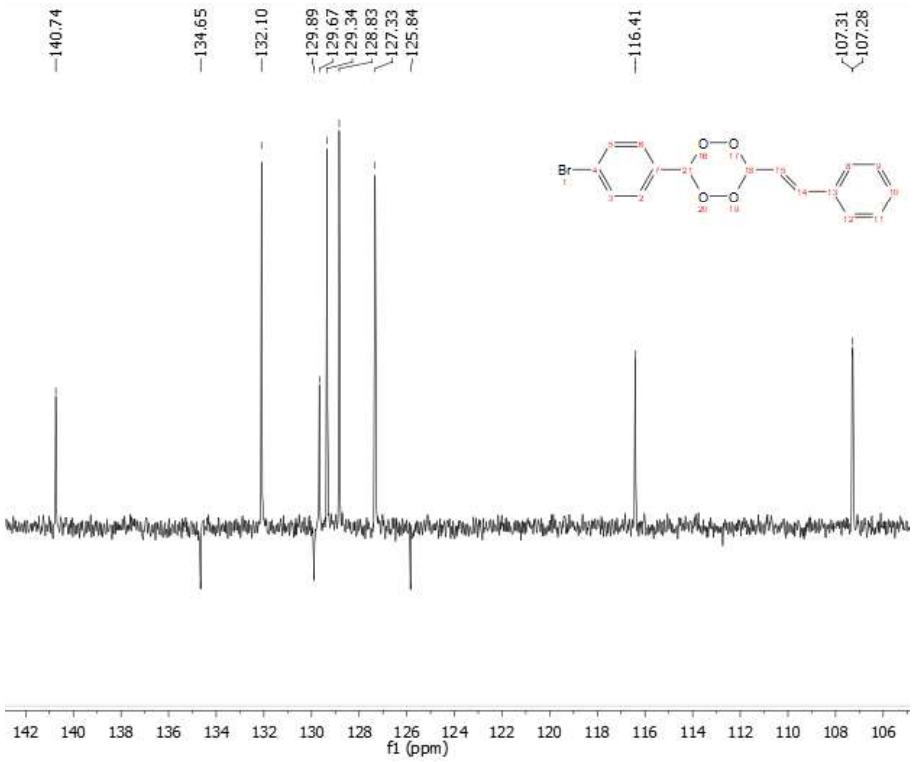

Figure 7.17 – Bromobenzaldehyde-tetraoxane **23** APT spectra ($CDCl_3$)

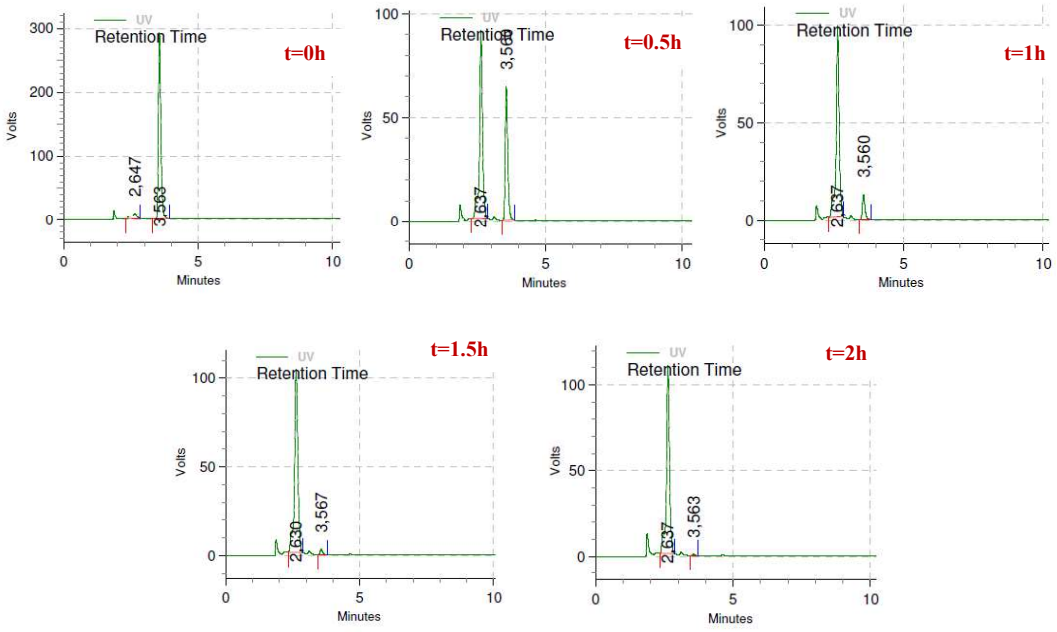

Figure 7.18 – Activation of tetraoxane **23** with $FeBr_2$

7.4 Compound 24 – (*E*)-3-(4-fluorophenyl)-6-styryl-1,2,4,5-tetraoxane

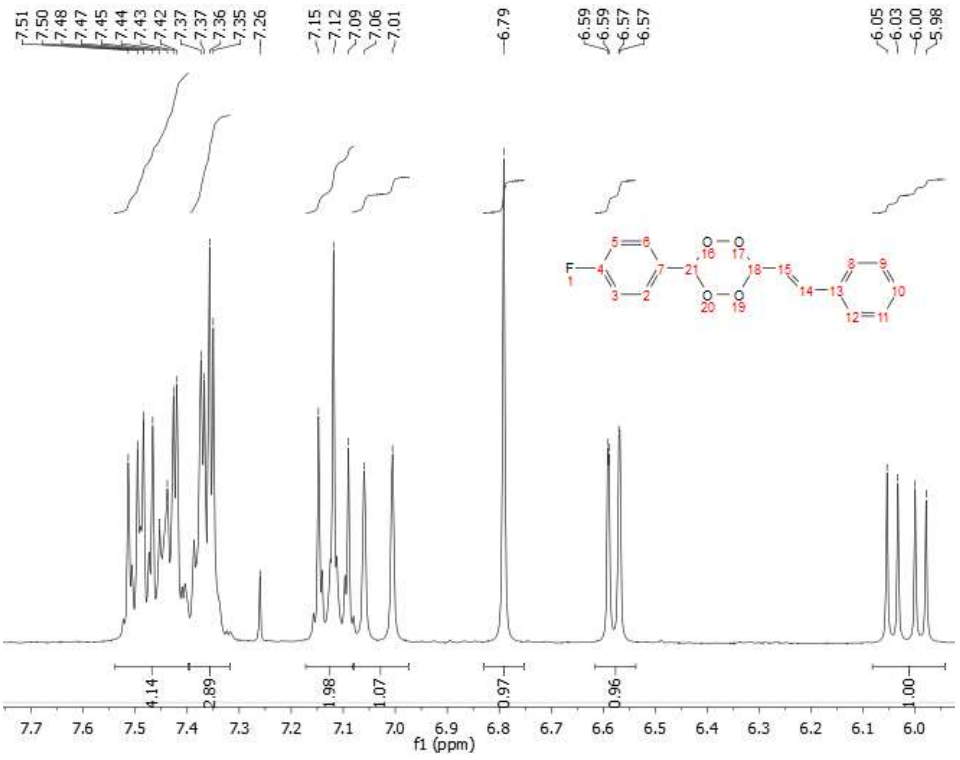

Figure 7.19 – Fluorobenzaldehyde-tetraoxane **24** ^1H NMR spectra ($CDCl_3$)

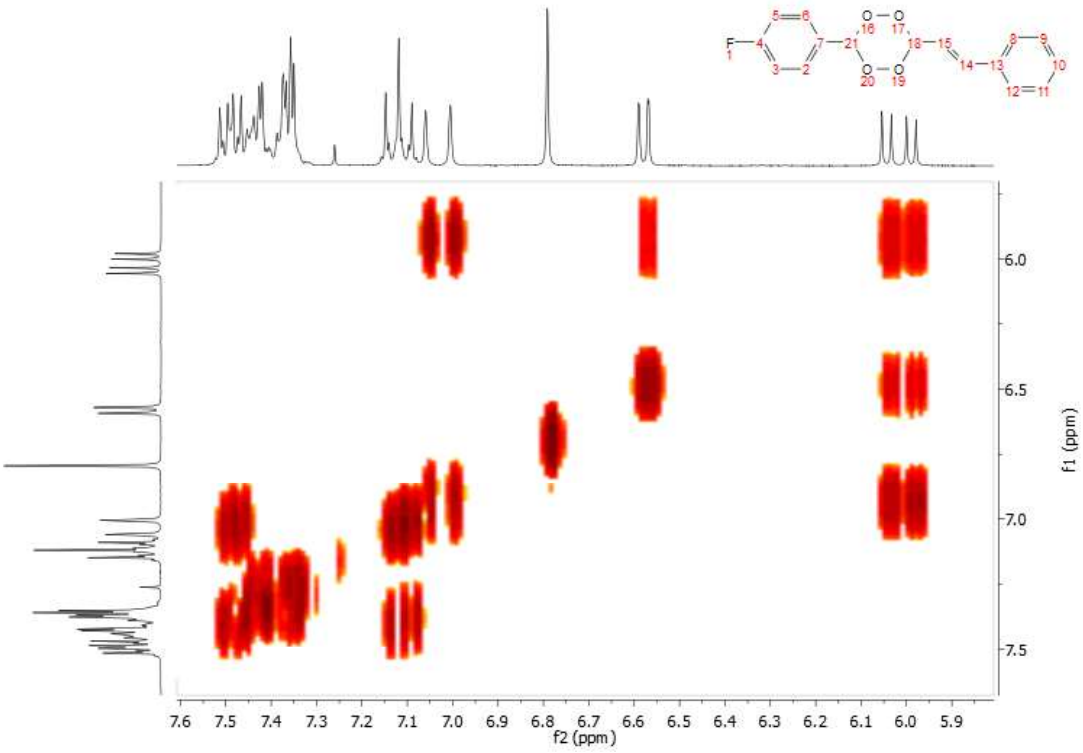

Figure 7.20 – Fluorobenzaldehyde-tetraoxane **24** COSY spectra ($CDCl_3$)

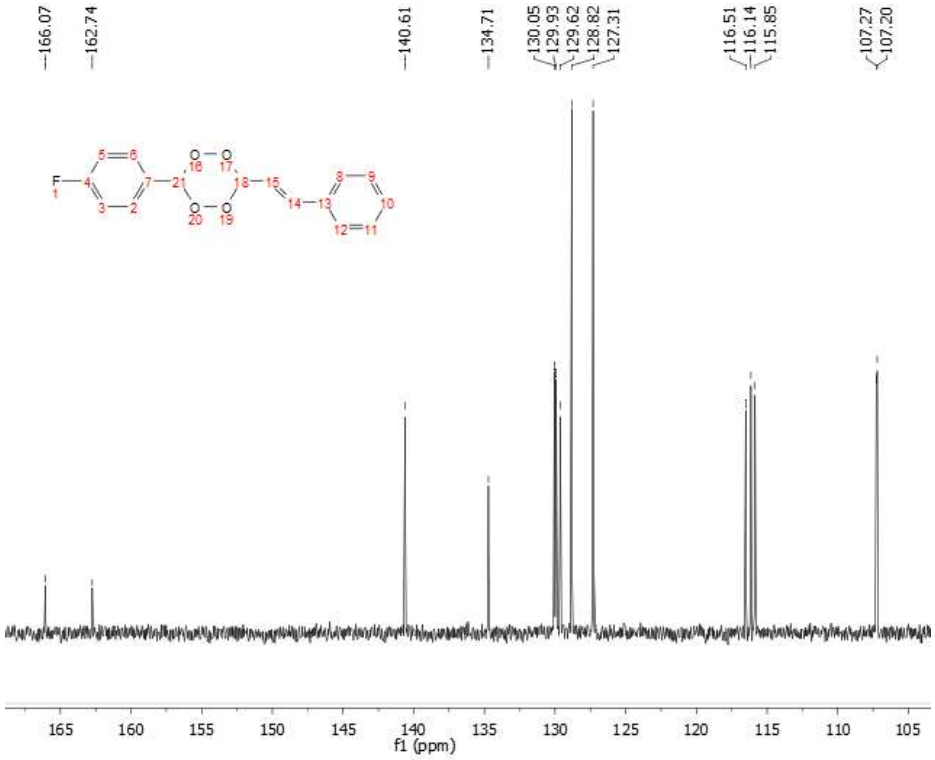

Figure 7.21 – Fluorobenzaldehyde-tetraoxane **24** ^{13}C NMR spectra ($CDCl_3$)

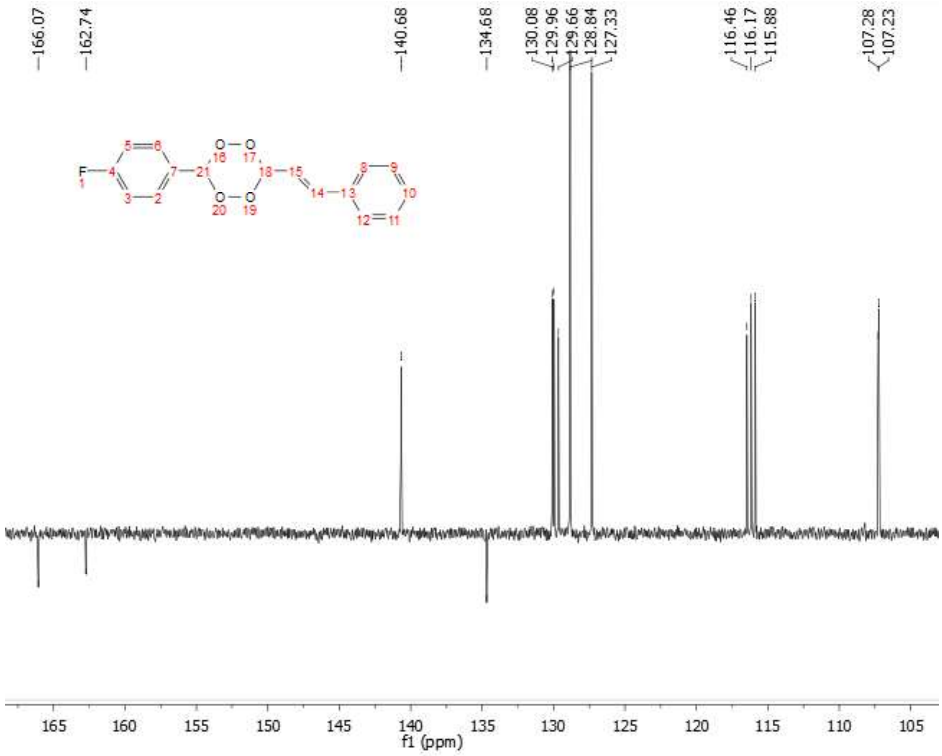

Figure 7.22 – Fluorobenzaldehyde-tetraoxane **24** APT spectra ($CDCl_3$)

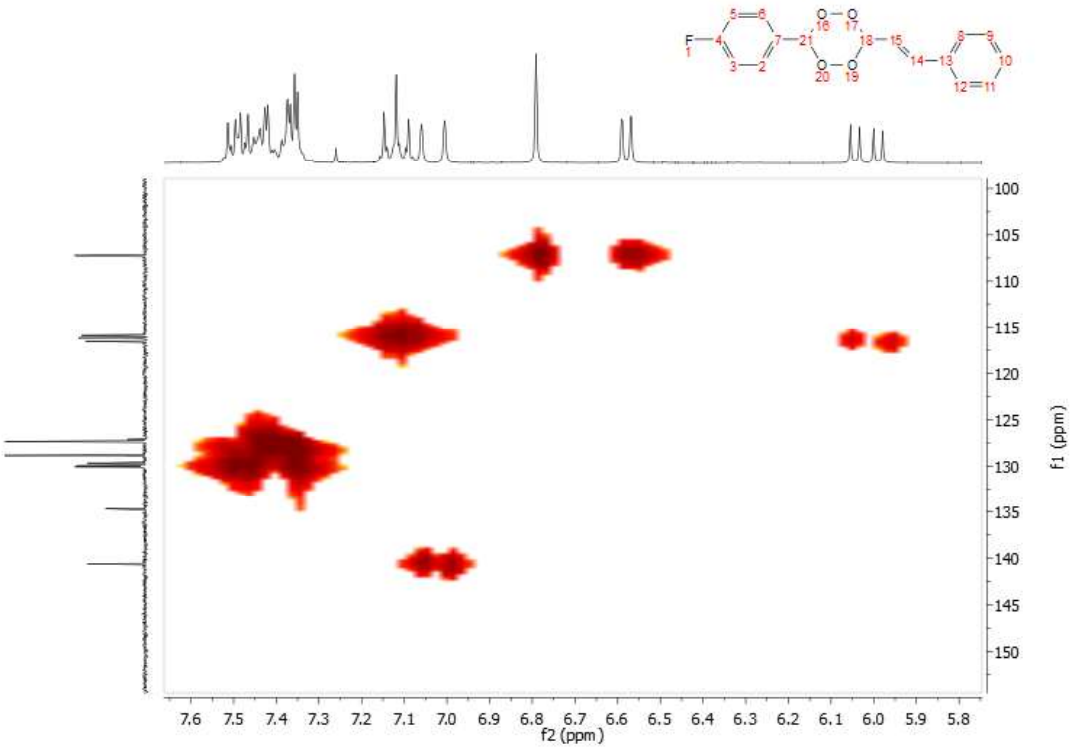

Figure 7.23 – Fluorobenzaldehyde-tetraoxane **24** HMQC spectra ($CDCl_3$)

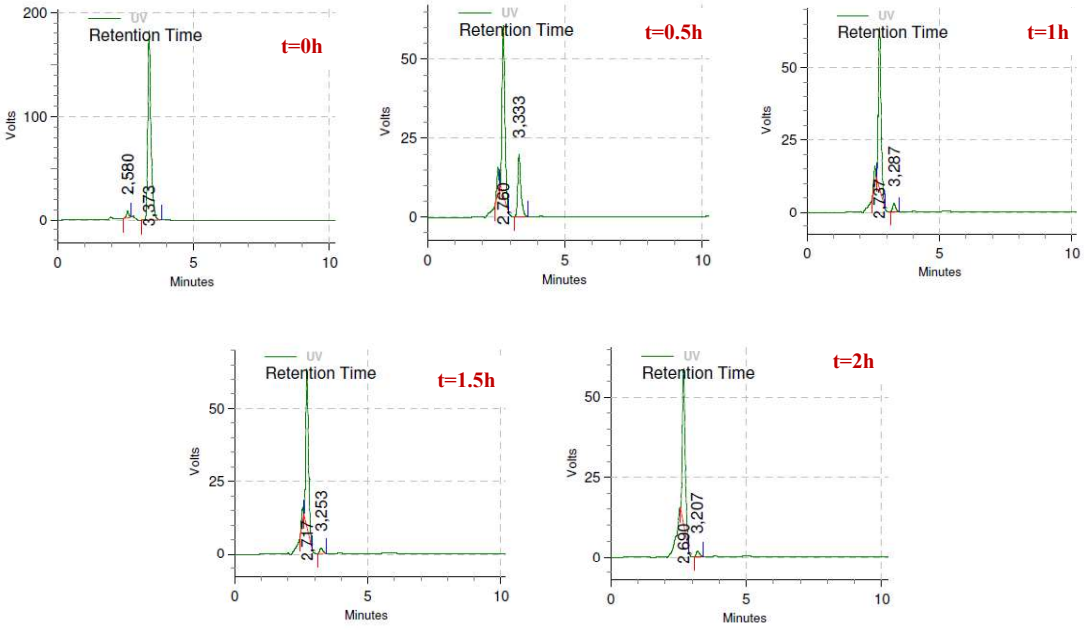

Figure 7.24 – Activation of tetraoxane **24** with $FeBr_2$

7.5 Compound 25 – (*E*)-3-styryl-6-(*p*-tolyl)-1,2,4,5-tetraoxane

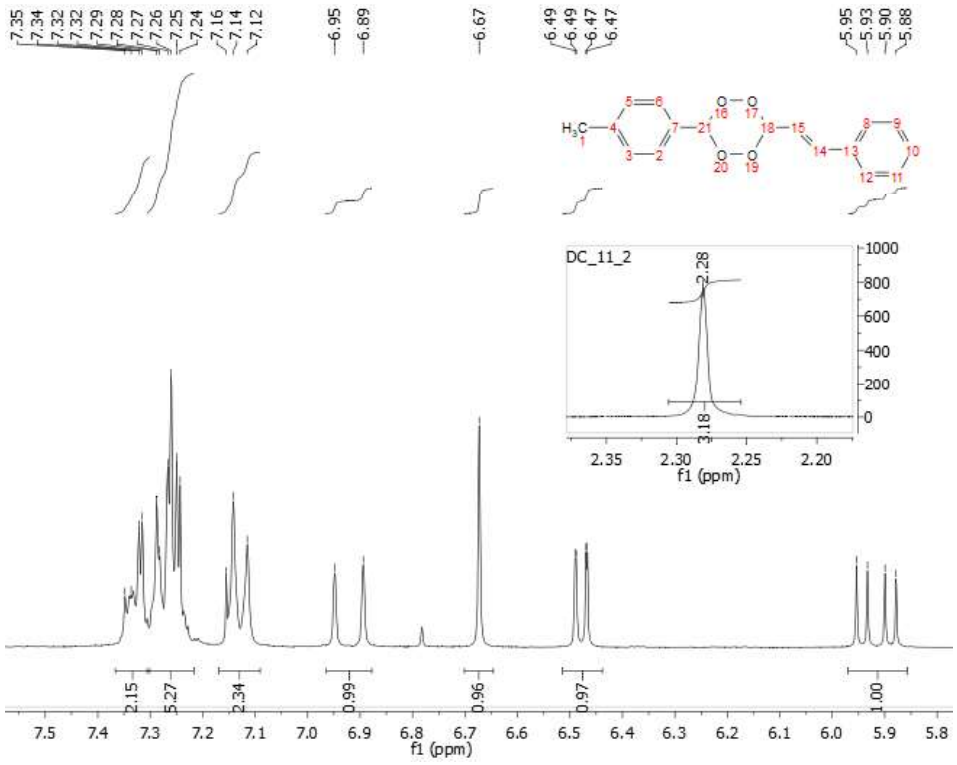

Figure 7.25 – *p*-Tolylbenzaldehyde-tetraoxane **25** ^1H NMR spectra ($CDCl_3$)

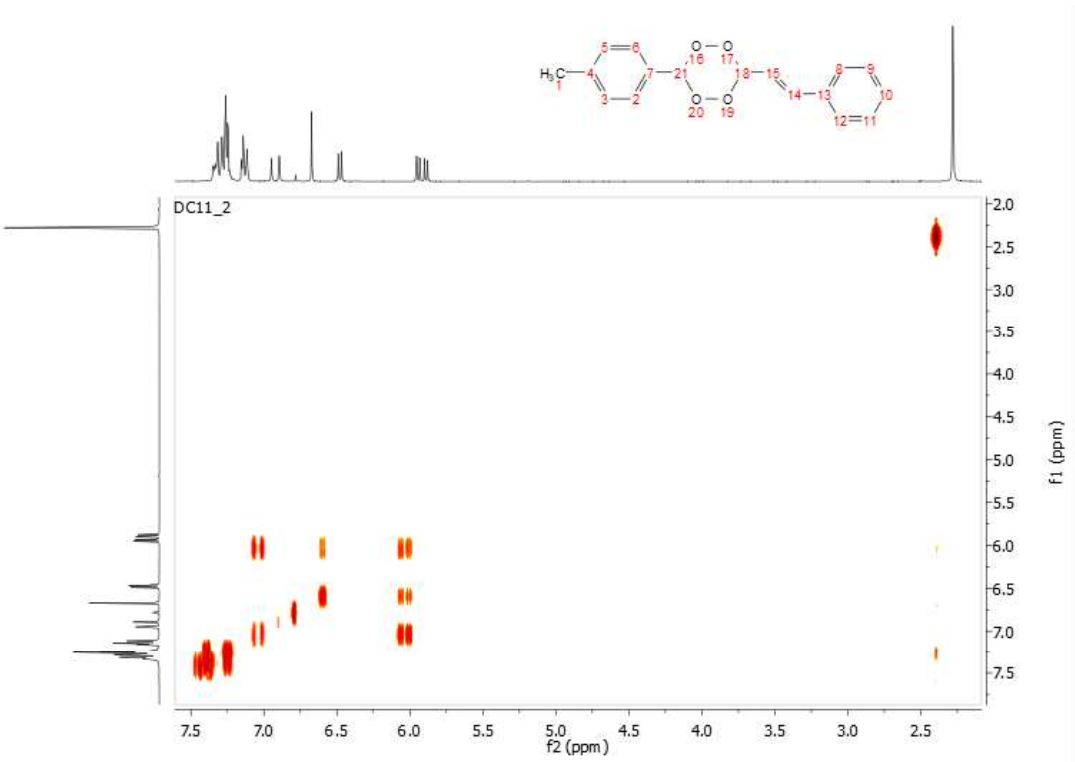

Figure 7.26 – *p*-Tolylbenzaldehyde-tetraoxane **25** COSY spectra ($CDCl_3$)

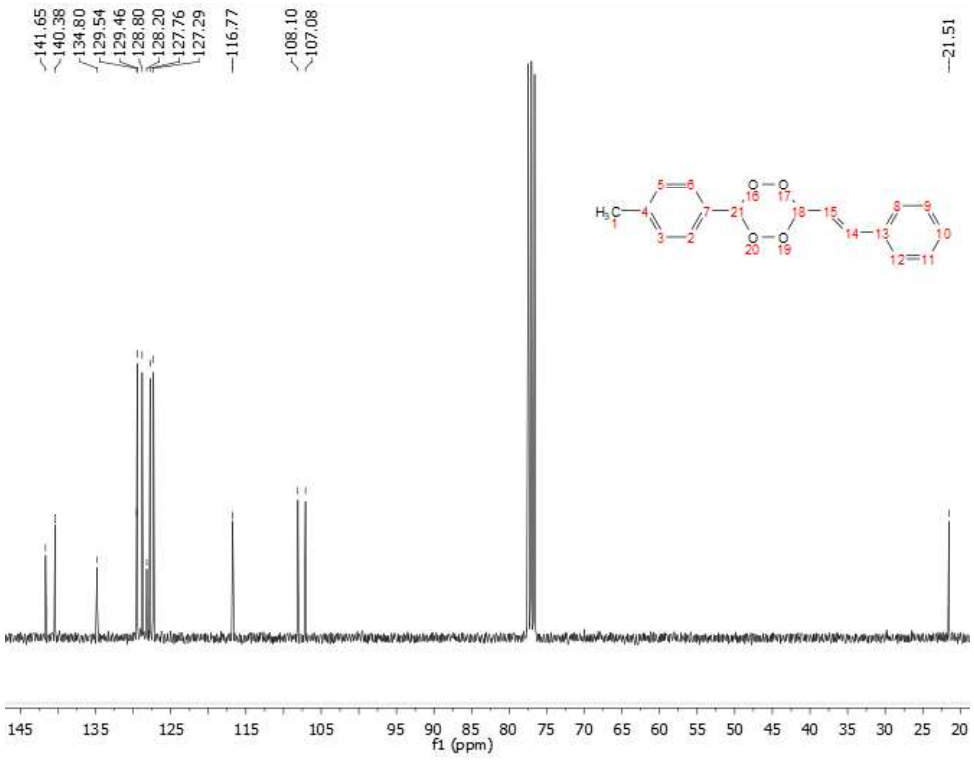

Figure 7.27 – *p*-Tolylbenzaldehyde-tetraoxane **25** ^{13}C NMR spectra ($CDCl_3$)

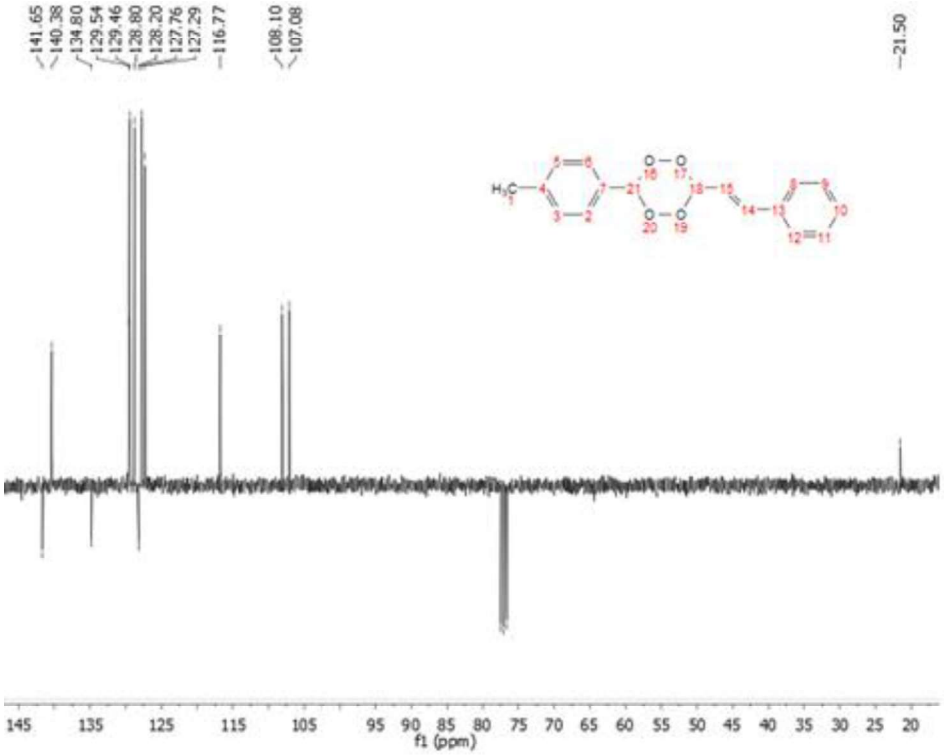

Figure 7.28 – *p*-Tolylbenzaldehyde-tetraoxane **25** APT spectra ($CDCl_3$)

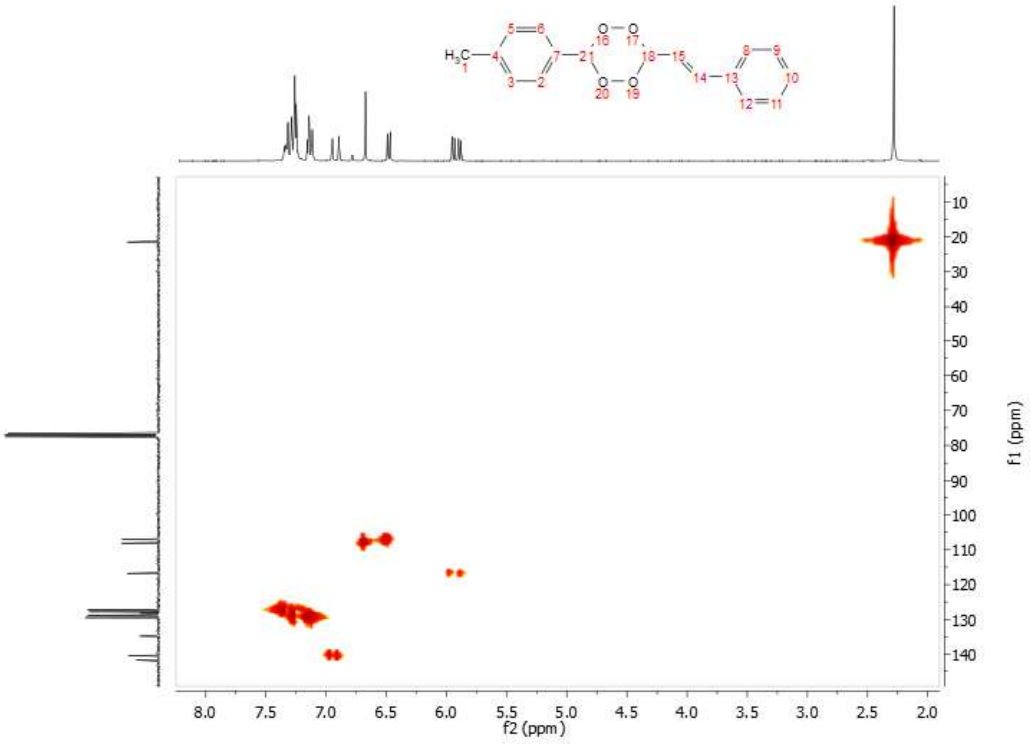

Figure 7.29 – *p*-Tolylbenzaldehyde-tetraoxane **25** HMQC spectra ($CDCl_3$)

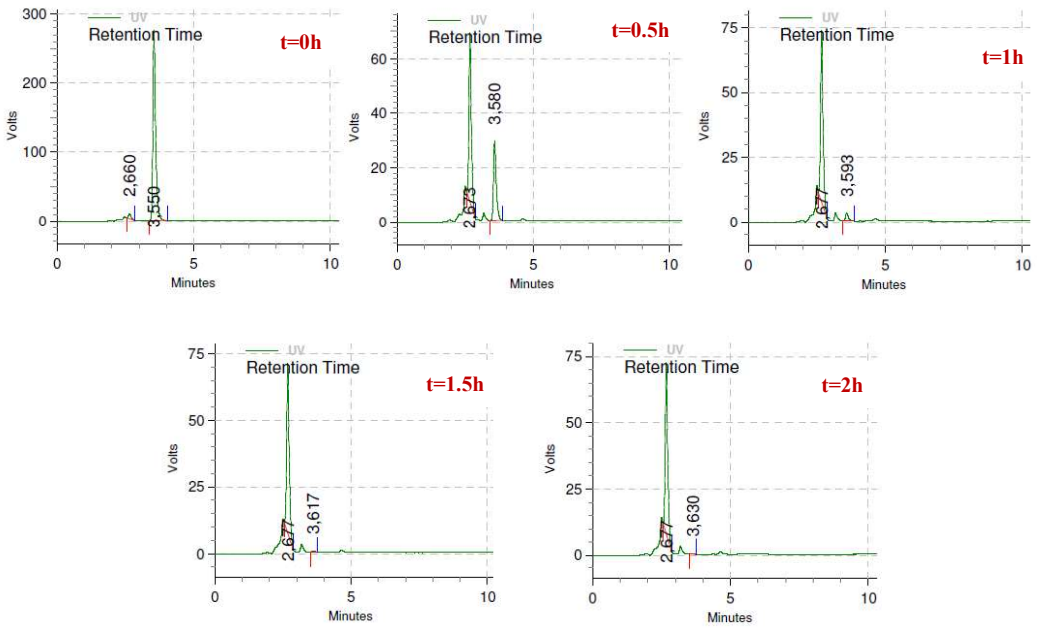

Figure 7.30 – Activation of tetraoxane **25** with $FeBr_2$

7.6 Compound 26 – (*E*)-3-styryl-6-(4-(trifluoromethyl)phenyl)-1,2,4,5-tetraoxane

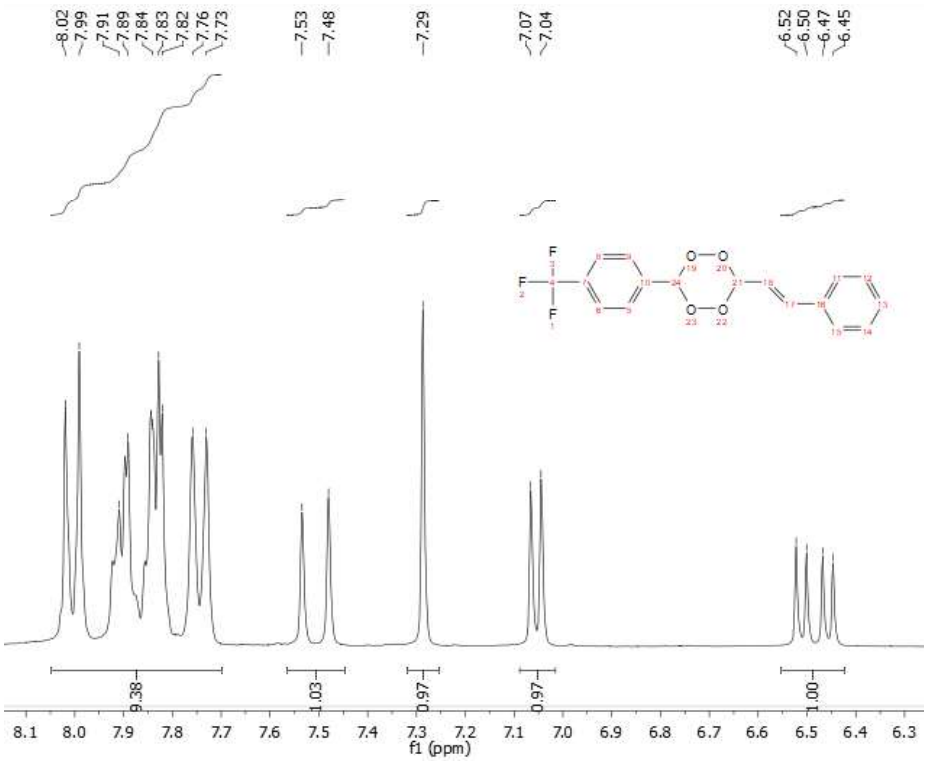

Figure 7.31 – Trifluoromethylbenzaldehyde-tetraoxane **26** ^1H NMR spectra ($CDCl_3$)

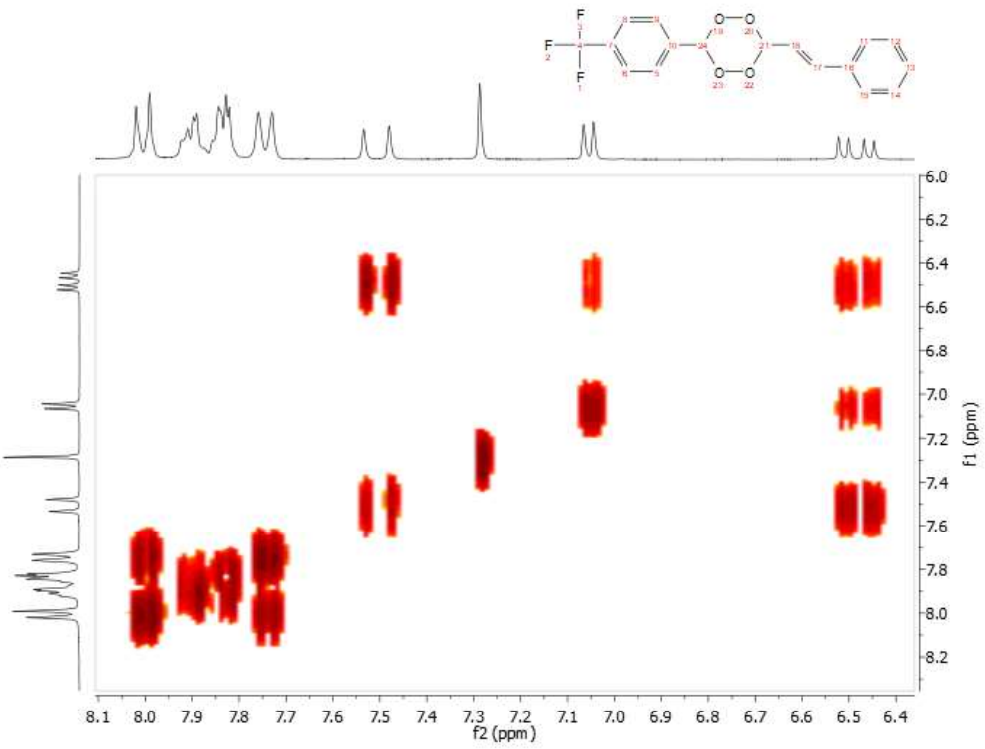

Figure 7.32 – Trifluoromethylbenzaldehyde-tetraoxane **26** COSY spectra ($CDCl_3$)

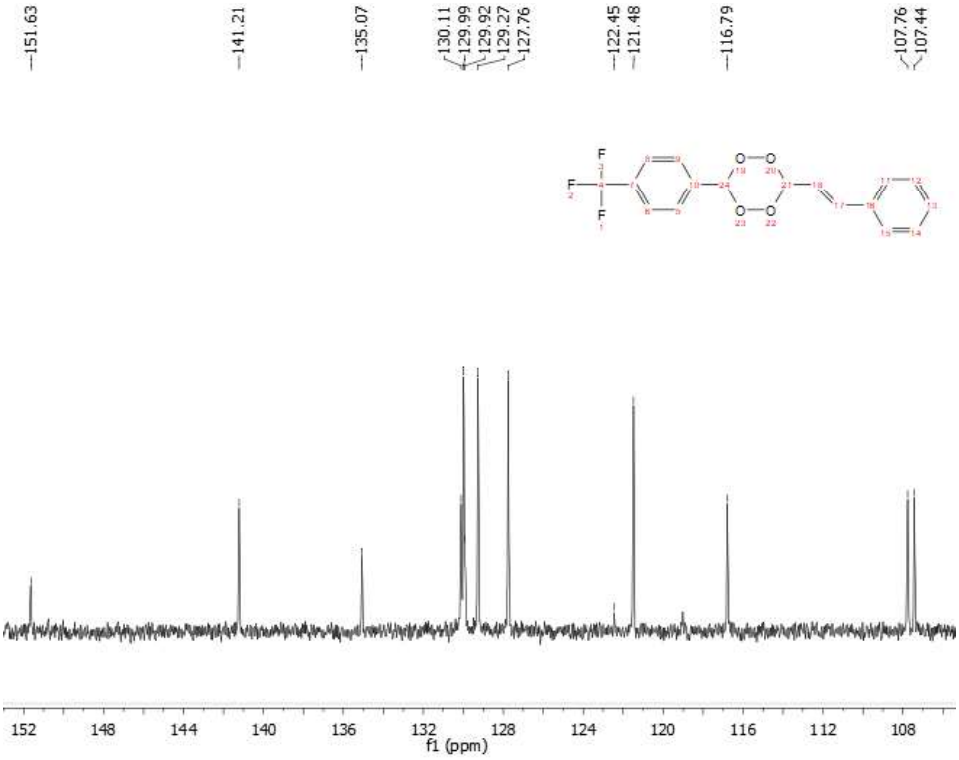

Figure 7.33 – Trifluoromethylbenzaldehyde-tetraoxane **26** ^{13}C NMR spectra ($CDCl_3$)

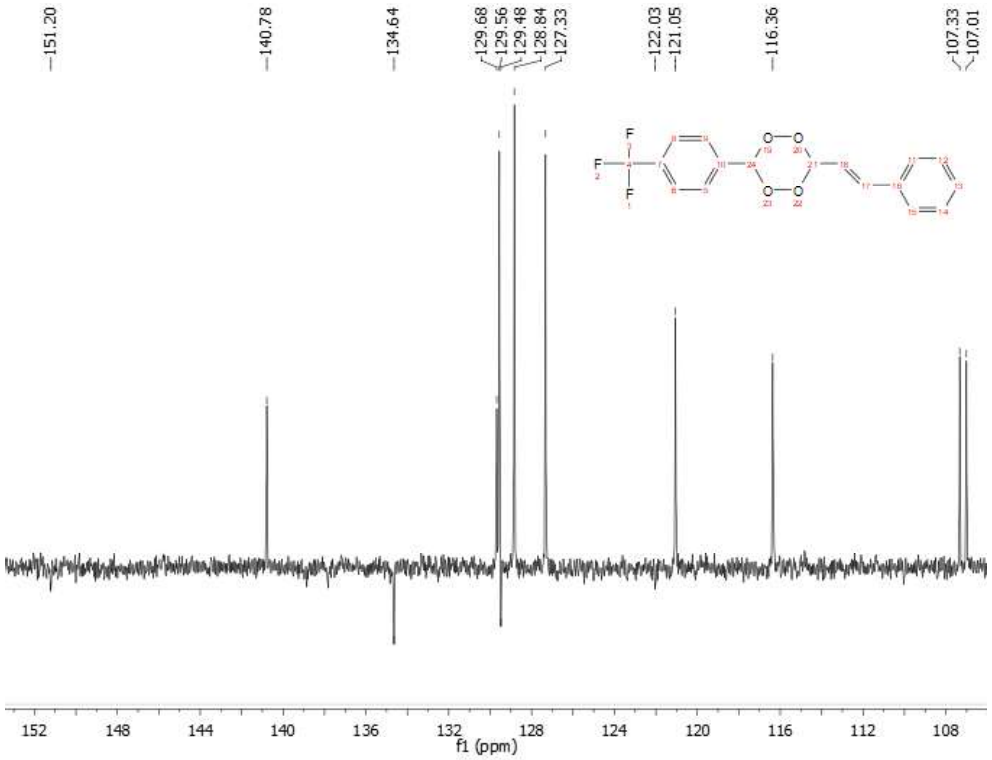

Figure 7.34 – Trifluoromethylbenzaldehyde-tetraoxane **26** APT spectra ($CDCl_3$)

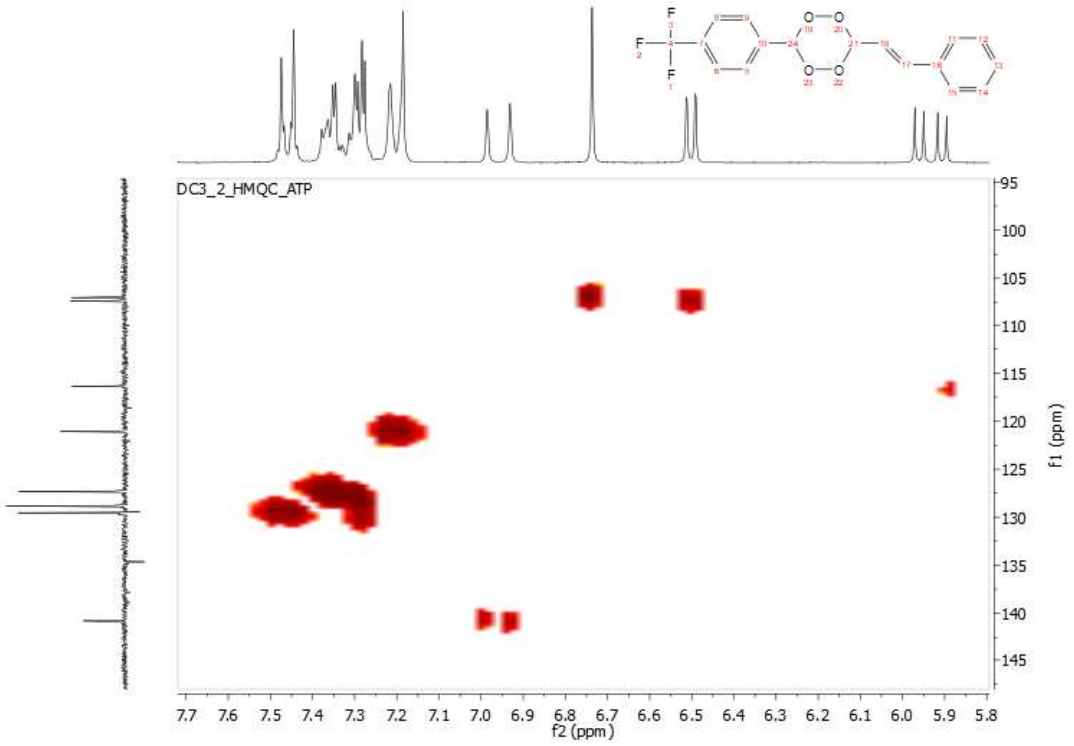

Figure 7.35 – Trifluoromethylbenzaldehyde-tetraoxane **26** HMQC spectra ($CDCl_3$)

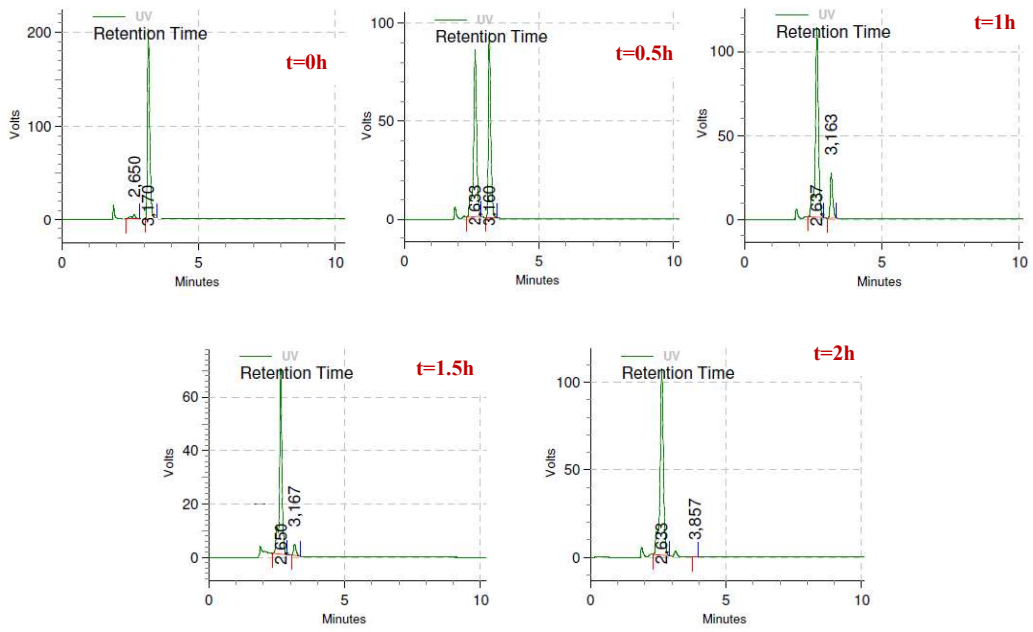

Figure 7.36 – Activation of tetraoxane **26** with $FeBr_2$

7.7 Compound 27 – (*E*)-3-styryl-6-(4-trifluoromethoxy)phenyl)-1,2,4,5-tetraoxane

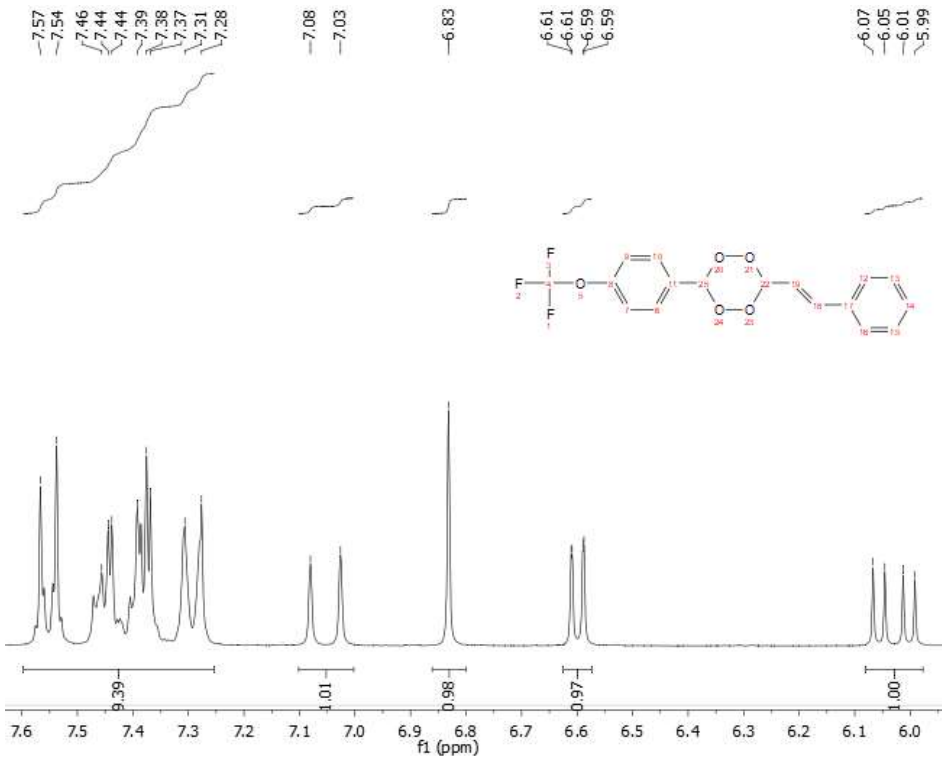

Figure 7.37 – Trifluoromethoxybenzaldehyde-tetraoxane **27** ^1H NMR spectra ($CDCl_3$)

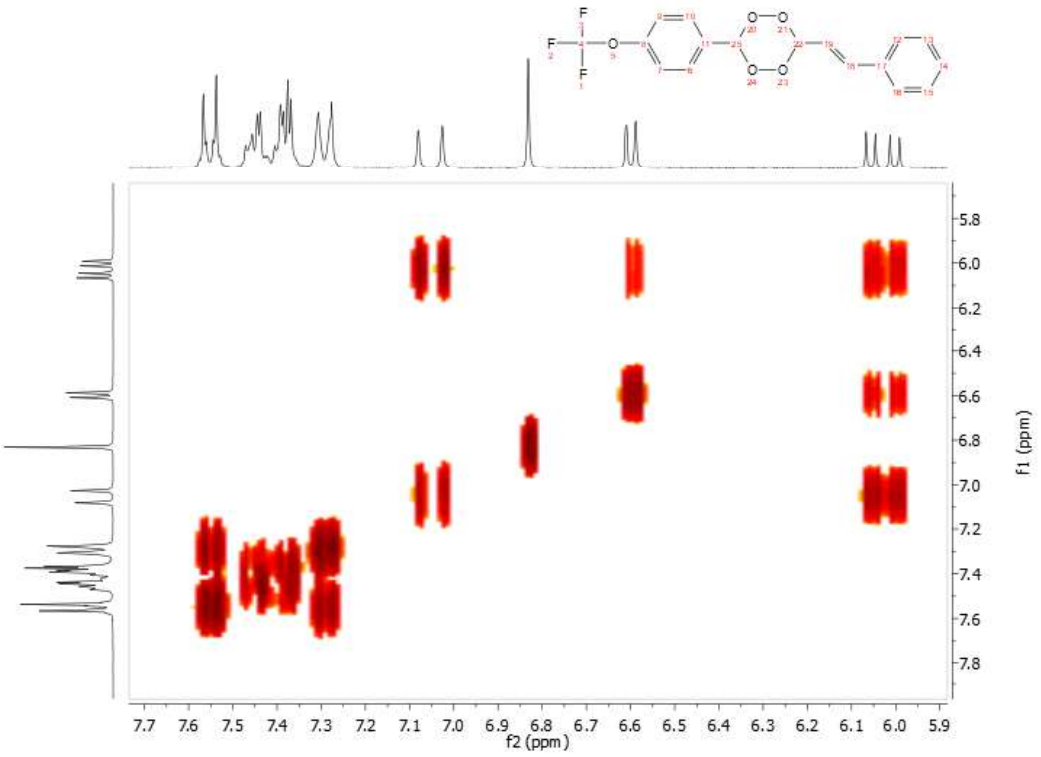

Figure 7.38 – Trifluoromethoxybenzaldehyde-tetraoxane **27** COSY NMR spectra ($CDCl_3$)

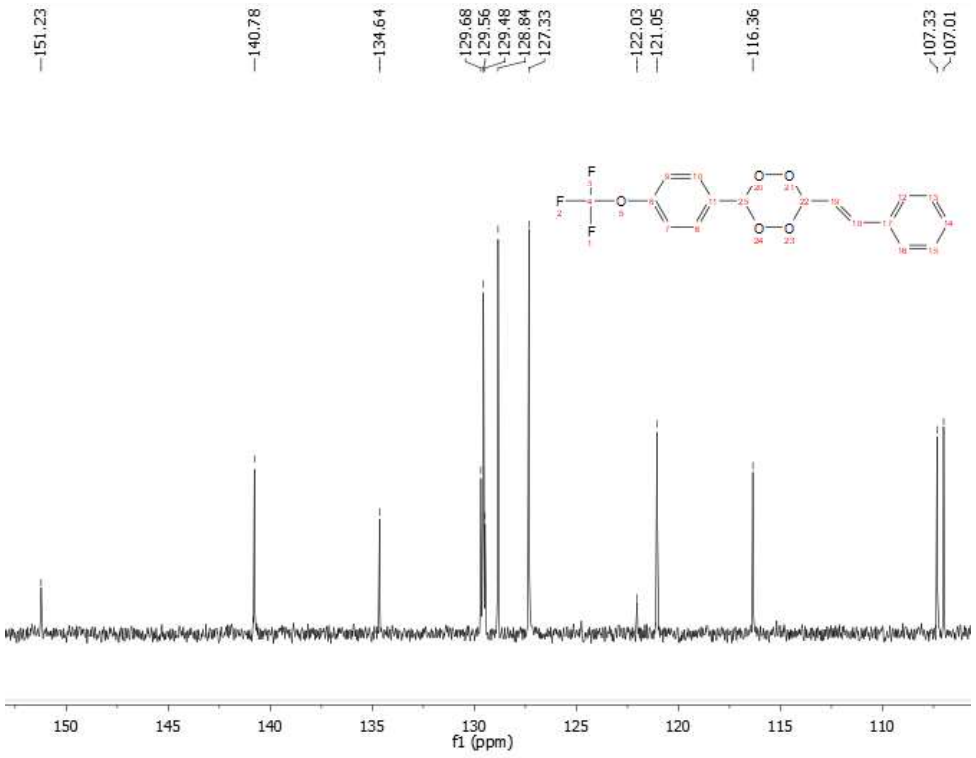

Figure 7.39 – Trifluoromethoxybenzaldehyde-tetraoxane **27** ^{13}C NMR spectra ($CDCl_3$)

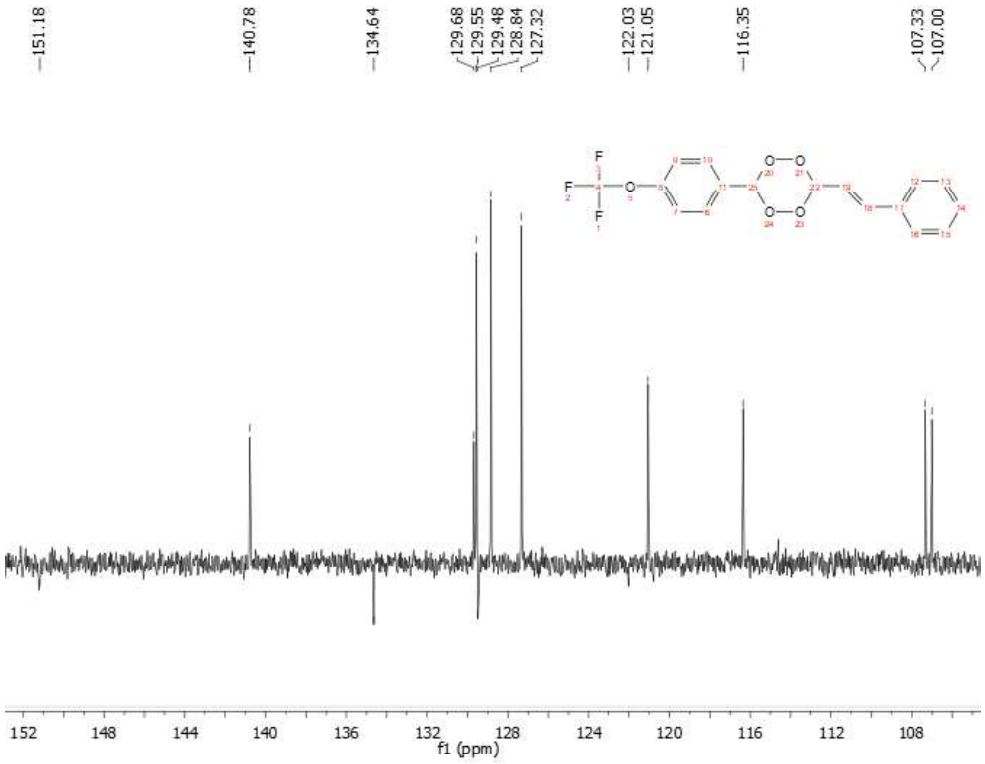

Figure 7.40 – Trifluoromethoxybenzaldehyde-tetraoxane **27** APT NMR spectra ($CDCl_3$)

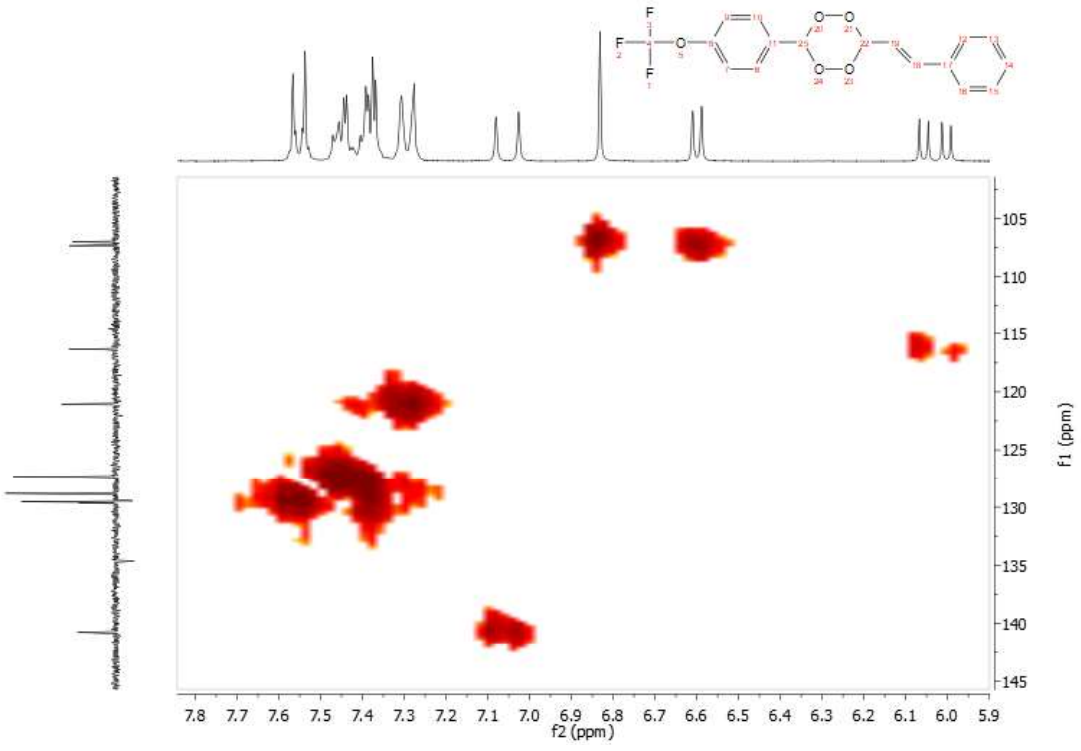

Figure 7.41 – Trifluoromethoxybenzaldehyde-tetraoxane **27** HMQC NMR spectra ($CDCl_3$)

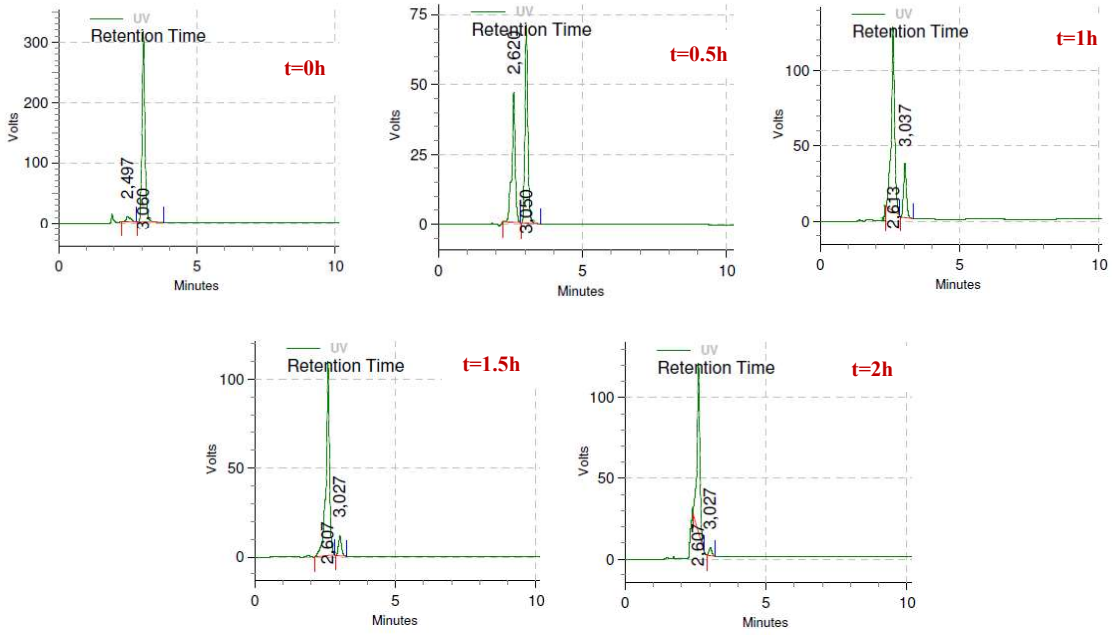

Figure 7.42 – Activation of tetraoxane **27** with $FeBr_2$

7.8 (*E*)-*N*-methoxy-*N*-methyl-3-(4-(trifluoromethyl)phenyl)acrylamide

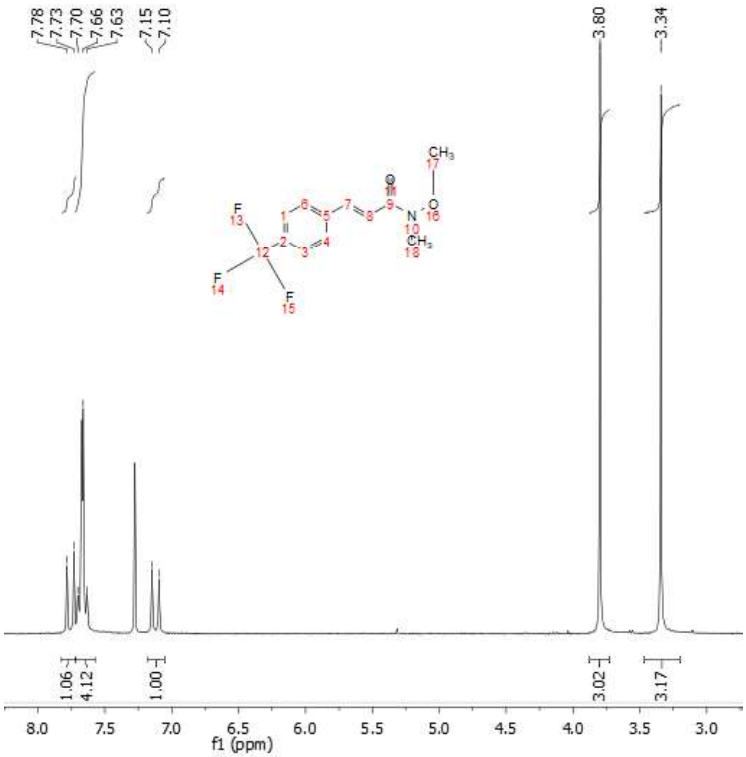

Figure 7.43 – (*E*)-*N*-methoxy-*N*-methyl-3-(4-(trifluoromethyl)phenyl)acrylamide ^{1}H NMR spectra ($CDCl_3$)

7.9 Compound 34 – (*E*)-3-(4-methoxystyryl)-6-phenyl-1,2,4,5-tetraoxane

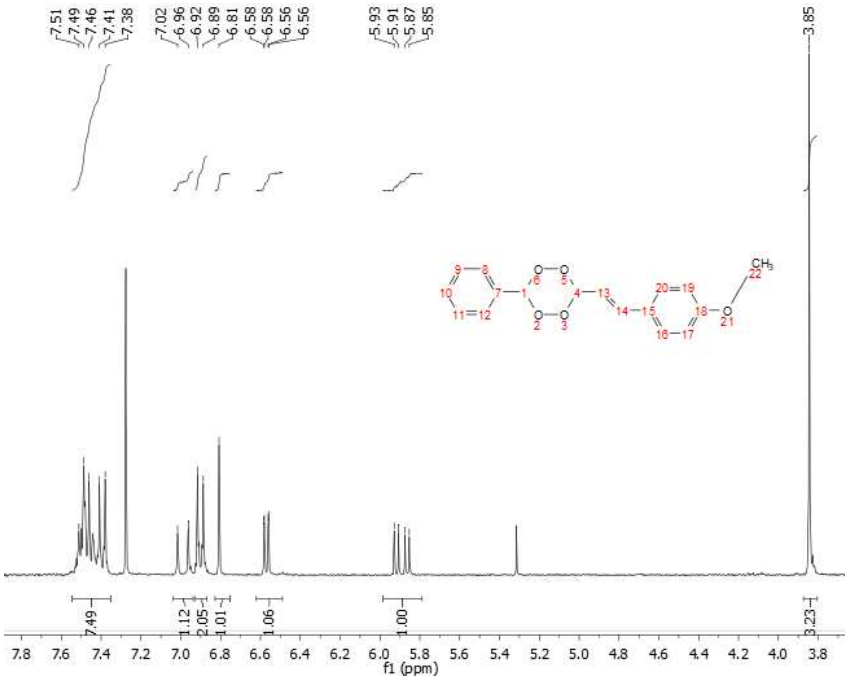

Figure 7.44 – (*E*)-3-(4-methoxystyryl)-6-phenyl-1,2,4,5-tetraoxane **34** ^{1}H NMR spectra ($CDCl_3$)

7.10 Compound 38 – (1*r*, 3*r*, 5*r*, 7*r*)- 6'-((*E*)-styryl)spiro[adamantane-2, 3'-[1,2,4,5]tetraoxane]

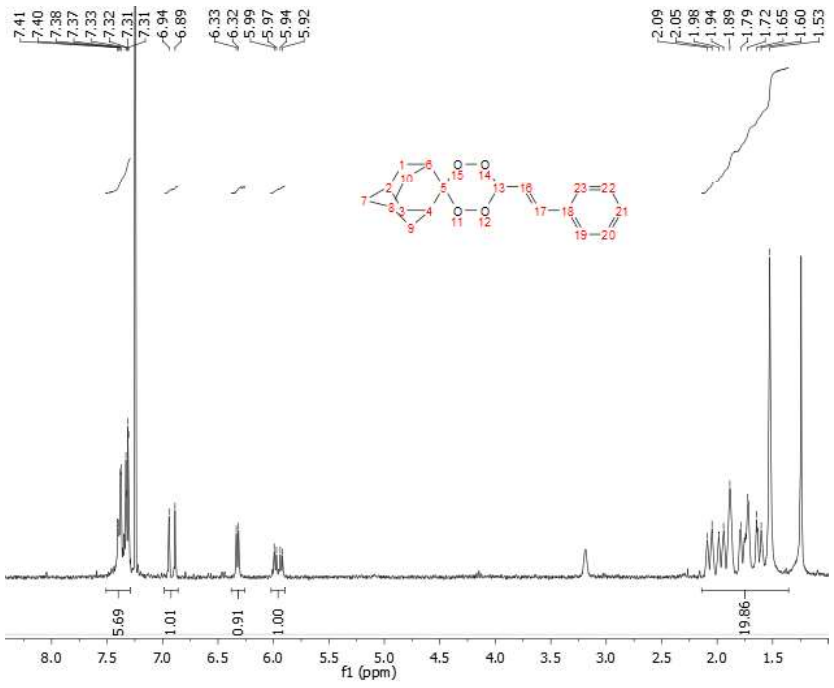

Figure 7.45 - (1*r*, 3*r*, 5*r*, 7*r*)-6'-((*E*)-styryl)spiro[adamantane-2,3'-[1,2,4,5]tetraoxane] **38** ^{1}H NMR spectra ($CDCl_3$)

7.11 Compound 39 - 3-((1*r*, 3*r*, 5*r*, 7*r*)-adamantan-2-yl)-6-((*E*)-styryl)-1,2,4,5-tetraoxane

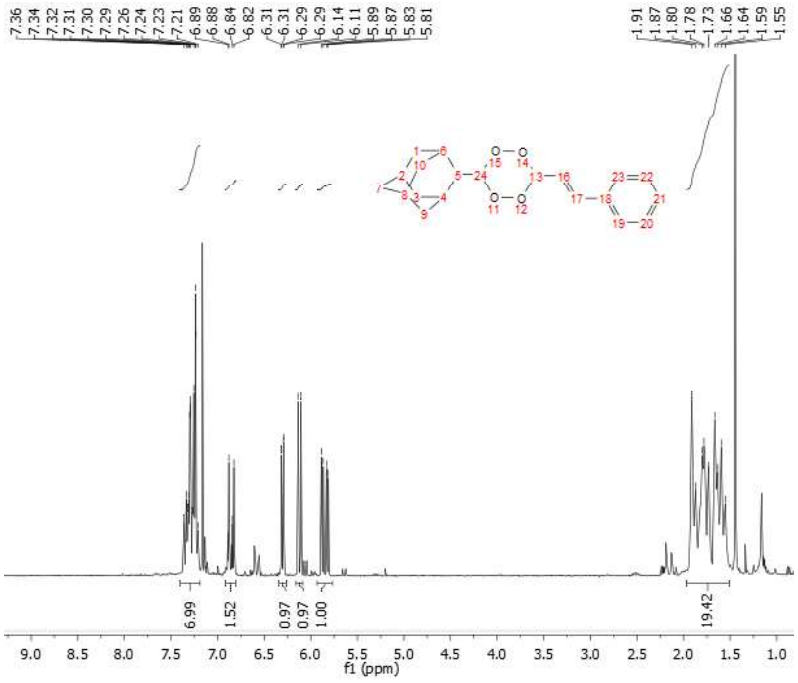

Figure 7.46 - 3-((1*r*, 3*r*, 5*r*, 7*r*)-adamantan-2-yl)-6-((*E*)-styryl)-1,2,4,5-tetraoxane **39** ^{1}H NMR spectra ($CDCl_3$)