

Universidade de Lisboa
Faculdade de Farmácia



Curcumin analogues: novel potential therapeutic agents for the treatment of neurodegenerative diseases

Marta Sofia dos Santos Soares Valente Clariano

Public

Dissertation supervised by Professor Maria de Jesus Perry and co-supervised by Doctor Ana Sofia Falcão.

Master course in Pharmaceutical and Medicinal Chemistry

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Abstract

Neurodegenerative diseases can be related with defects in the autophagy-lysosome pathway. Autophagy is a complex self-degradative process mediated by lysosomes, which is responsible for the bulk degradation of intracellular components, preventing the occurrence of abnormal cellular functions.

Curcumin is a natural polyphenolic compound, derived from the rhizome of turmeric (*Curcuma longa* Linn.), that presents an ample range of biological properties, which we should highlight the improvement of retinal function in retinal degeneration, enhance of autophagy and an antioxidant activity, making it a compound whose application may be effective in the treatment of several diseases, namely cancer, neurodegenerative disorders and ocular diseases. However, the clinical use of curcumin is compromised by pharmacokinetic defects. Therefore, several studies were developed to find novel derivatives and analogues with better pharmacokinetic and pharmacological properties and attention has been focused on monocarbonyl analogues, where the stability, bioavailability and the pharmacokinetic profiles are improved and presents better activities *in vitro* and *in vivo*.

Here the synthesis of a small library of monocarbonyl analogues of curcumin is presented. Also, its structural characterization was performed, and its drug-like properties were evaluated. Lipophilia and stability in physiological conditions, including stability in phosphate buffer and in human plasma were both assessed by HPLC-UV while two different methods were performed to assess the electrophilic character of each compound, monitoring the reaction of the synthesized analogues with cysteamine by UV-spectroscopy and by NMR.

Due to the plethora of biological properties of curcumin, the potential therapeutic effect of the synthesized monocarbonyl analogues of curcumin were evaluated not only in retinal degeneration but also in cancer. The assays were performed in retinal pigmented epithelium (RPE) cells and in colorectal cancer stem cells (CSCs). Dose-response studies in CSCs showed promisor results for compound **1i**. Assays in RPE cells, revealed that both compounds **C1 (1g)** and **C2 (1a)** may have a neuroprotector effect, being promising in age-related macular degeneration treatment.

Keywords: Curcumin; Curcumin monocarbonyl analogues; Age-related macular degeneration

Resumo

As doenças neurodegenerativas podem estar relacionadas com disfunções na autofagia, que consiste num processo complexo de autodegradação, mediado por lisossomas. Estes são organelos celulares cuja principal função é a digestão de componentes intracelulares, com o intuito de prevenir a formação de proteínas tóxicas e a agregação tanto de proteínas como de organelos obsoletos. Uma vez que a autofagia é um processo responsável pela remoção de componentes intracelulares, induzi-la pode constituir um tratamento promissor de doenças neurodegenerativas.

São inúmeras as doenças neurodegenerativas, porém, as mais comuns são a doença de Alzheimer e a doença de Parkinson. Embora estas difiram nos aspetos clínicos, as mesmas partilham entre si características patológicas, que incluem essencialmente o *stress* oxidativo, a inflamação, o processamento defeituoso de proteínas, a disfunção mitocondrial e ainda alterações no processo de autofagia, que, por sua vez, levam à má formação e agregação de proteínas, que se por um lado podem não cumprir todas as suas funções fisiológicas, por outro lado podem tornar-se tóxicas.

A degeneração macular relacionada com a idade constitui atualmente a terceira principal causa de cegueira a nível mundial e, nos países desenvolvidos, é o principal fator de perda de visão em indivíduos com mais de 50 anos. Esta doença degenerativa é caracterizada pela acumulação de aldeídos derivados da vitamina A, que geram a lipofuscina. A lipofuscina é maioritariamente composta por resíduos lipídicos resultantes da digestão lisossomal. Os sintomas da degeneração macular incluem falta de visão ou visão turva ou desfocada e metamorfopsia, que se caracteriza por uma distorção da visão central, na qual as linhas retas aparecem onduladas, observando-se a presença de escotomas centrais. A etiologia desta doença ainda é incerta, porém sabe-se que o *stress* oxidativo é determinante para o desenvolvimento da mesma. Além disso, embora não existam evidências de uma relação causa-efeito direta entre a acumulação de lipofuscina e o desenvolvimento e progressão da doença, é certo que o excesso da mesma é responsável pela perda de células fotorecetoras, assim como das células responsáveis pela sua formação, o epitélio pigmentado da retina.

A curcumina é um composto natural polifenólico, sendo o principal curcuminóide presente no rizoma da curcuma (*Curcuma longa* Linn.). Tem sido atribuída à curcumina uma vasta gama de propriedades biológicas, incluindo atividades antioxidantes, anti-tumorais, anti-inflamatórias e anti-angiogénese. A curcumina apresenta também um efeito neuroprotetor e melhora a função da retina em patologias associadas à degeneração da retina. Estas propriedades que lhe estão associadas juntamente com

a sua baixa toxicidade e a sua capacidade de atravessar a barreira hematoencefálica, tornam a curcumina um composto cuja aplicação pode revelar-se eficaz no tratamento de diversas doenças, abrangendo o cancro, doenças neurodegenerativas e doenças do foro ocular. Contudo, a nível clínico, o uso da curcumina é limitado devido a más propriedades farmacocinéticas e farmacodinâmicas, que incluem a má absorção, baixa biodisponibilidade, rápido metabolismo e má estabilidade química. Como tal, nos últimos anos, o interesse no desenvolvimento de moléculas análogas ou derivadas da curcumina, que apresentem melhores propriedades e que ultrapassem as suas limitações, tem aumentado. Para tal, têm sido exploradas várias tentativas de modificar a estrutura química da curcumina. Dado que a instabilidade e os problemas metabólicos podem estar associados à alta reatividade da porção beta-dicetona, contribuindo para uma rápida degradação e um rápido metabolismo, então a atenção tem sido concentrada em análogos monocarbonilo, isto é, análogos onde este grupo dicetona é removido, ficando apenas um grupo carbonilo. É esperado que para estes análogos, a estabilidade, a biodisponibilidade e os maus perfis farmacocinéticos sejam suplantados. Além disso, estudos anteriores provam que estes análogos apresentam melhores atividades que a curcumina tanto *in vitro* como *in vivo*. Assim sendo, neste trabalho, foram sintetizados vários análogos monocarbonilo da curcumina, em que se fez variar os substituintes e as suas posições nos anéis aromáticos, num total de 12 compostos. As suas propriedades de lipofilia, a estabilidade em condições fisiológicas e o seu carácter eletrofílico foram avaliados.

Os análogos monocarbonilo da curcumina foram obtidos através de condensação aldólica, variando os rendimentos das reações de síntese entre 9.9% e 77.5%. Recorreu-se a técnicas de ressonância magnética nuclear (RMN) mono e bidimensionais, espectrometria de massa, espectrometria infravermelha e ultravioleta e análise elementar para obter uma caracterização estrutural completa de cada análogo.

Os coeficientes de partição e a estabilidade dos compostos sintetizados, em condições fisiológicas, foram avaliados através de HPLC. Os análogos sintetizados cumprem a regra dos cinco de Lipinski e são estáveis tanto em tampão fosfato (pH= 7.4) como em plasma humano, excetuando o composto **1f**, cujo substituinte é um grupo nitro na posição *orto*, que em plasma humano apresentou uma semivida de cerca de 8 horas.

A avaliação do carácter eletrofílico pode ser útil para prever a toxicidade dos compostos, o que é benéfico na otimização e desenvolvimento de fármacos, permitindo eliminar compostos tóxicos ou que constituam falsos positivos, o mais célere possível e consequentemente, reduzindo não só os custos associados, mas também algum sofrimento causado em ensaios biológicos *in vivo*. Os análogos em estudo são compostos carbonílicos α,β -insaturados, sendo que esta classe de compostos é

normalmente evitada no desenvolvimento de fármacos porque pode estar relacionada com danos celulares e citotoxicidade. Contudo, estes podem ser muito úteis devido às suas propriedades eletrofílicas, muitas vezes subjacentes aos mecanismos de ação terapêutica. Neste sentido, dois métodos foram testados para determinar o seu carácter eletrofílico. O primeiro método testado consistia num ensaio cinético de tiol seguido por espectrometria-UV/VIS. No segundo método, a reação dos compostos sintetizados com um nucleófilo é monitorizada por ^1H RMN.

Com estes ensaios, verificou-se que os análogos reagem facilmente com a cisteamina, formando aductos por adição de Michael no carbono β . No método que monitoriza a reação por espectrometria-UV/VIS, para além da cisteamina, testou-se também a reatividade de um dos compostos utilizando um outro nucleófilo, a N-acetilcisteína, porém não se observou qualquer alteração no perfil de UV ao longo do tempo de reação, o que significa que durante os 15 minutos de ensaio, esta reação não ocorre. Contudo, embora estes resultados de carácter eletrofílico dos análogos monocarbonilo da curcumina sejam de elevada importância, é pretensioso transferi-los diretamente para sistemas biológicos, pois a reatividade do grupo tiol pode variar, uma vez que é dependente do ambiente proteico. Por esta razão, a reatividade que utiliza a N-acetilcisteína como nucleófilo não pode ser totalmente descartada.

Para avaliação do potencial terapêutico, os análogos da curcumina sintetizados foram testados em células do epitélio pigmentado da retina e em células estaminais cancerígenas. A viabilidade de células estaminais cancerígenas incubadas com estes análogos ditou que apenas o composto **1i** (grupo trifluorometil na posição *orto*) é considerado um *hit*, enquanto que o composto **1f** é descartado uma vez que pode apresentar toxicidade excessiva. Os resultados dos estudos dose-resposta para o composto **1i** são promissores, prosseguindo para futuros estudos onde será avaliado o seu efeito fenotípico na formação de tumor-esferas.

Os compostos **C1 (1g)** e **C2 (1a)**, cujos substituintes são grupos metoxi, nas posições *orto* e *meta*, respetivamente, foram avaliados em células do epitélio pigmentado da retina, o que revelou que ambos induzem o processo de autofagia, sendo ativadores do fator Nrf2, o que lhes pode, portanto, conferir um efeito neuroprotetor, tornando-os promissores para o tratamento ou para desacelerar ou cessar a progressão de doenças neurodegenerativas. Assim, considerando as suas propriedades “*drug-like*”, isto é, o facto de cumprirem a regra dos cinco de Lipinski e serem estáveis em condições fisiológicas, acrescentando os resultados encorajadores obtidos nos ensaios biológicos em células do epitélio pigmentado da retina para os compostos **C1** e **C2**, estes análogos monocarbonilo da curcumina, revelam ser uma abordagem promissora para o

tratamento de doenças neurodegenerativas, como a degeneração macular relacionada com a idade.

Palavras-chave: Curcumina; Análogos monocarbonilo da curcumina; Degeneração da mácula

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Abbreviations and symbols

α -syn – α -synuclein
A β – Amyloid-beta
Abs – Absorbance
ACN – Acetonitrile
AD – Alzheimer's Disease
AIDS – Acquired immunodeficiency syndrome
Akt – Protein kinase B
ALP – Autophagy-lysosome pathway
AMD – Age-related macular degeneration
APP – Amyloid precursor protein
ARE – Antioxidant response element
ATP – Adenosine triphosphate
BBB – Blood-brain barrier
CAG – Cytosine-adenine-guanine
CFH – Complement factor H
CNS – Central nervous system
CNV – Choroidal neovascularization
COSY – Correlation spectroscopy
COX – Cyclooxygenase
CSCs – Cancer stem cells
Cul3 – Cullin-3
CYP – Cytochrome P450
Cys – Cysteine
DCM - Dichloromethane
DMEM – Dulbecco's modified eagle medium
DMSO – Dimethyl sulfoxide
DNA – Deoxyribonucleic acid
E1cB – Elimination Unimolecular conjugate Base
EMT – Epithelial-mesenchymal transition
ERK – Extracellular signal-regulated kinase
ESI-MS – Electrospray ionization mass spectrometry
EtOH – Ethanol
FBS – Fetal bovin serum
GSH – Glutathione

h – hours
 HBA – Hydrogen bond acceptor
 HBD – Hydrogen bond donor
 HD – Huntington's disease
 HMBC – Heteronuclear multiple bond correlation
 HMQC – Heteronuclear multiple quantum correlation
 HPLC – High Performance Liquid Chromatography
 HPV – Human papilloma virus
 HSAB – Hard and Soft, Acids and Bases
 HTPS – High-throughput screening
HTT – Huntingtin
 IC₅₀ – half maximal inhibitory concentration
 IR – Infrared
 K – Second order rate constant
 Keap1 – Kelch-like ECH-associated protein 1
 K_{obs} – pseudo-first order observed rate constant
 LBs – Lewy bodies
 Log P – Logarithm of the octanol-water partition coefficient
 M – Molar
 MAC – Monocarbonyl analogue of curcumin
 MAPK – Mitogen-activated protein kinase
 MeOH – Methanol
 min – Minutes
 mM – millimolar
 m.p. – Melting point
 m-TOR – Mammalian target of rapamycin
 MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
 MW – Molecular weight
 NAFLD – Non-alcoholic fatty liver disease
 NF_κB – Nuclear factor kappa B
 nm – Nanometre
 NMR – Nuclear magnetic resonance
 Nrf2 – Nuclear factor Erythroid 2-related factor 2
 P – Partition coefficient
 PBS – Phosphate-buffered saline
 PD – Parkinson's disease

PI3K – phosphoinositide 3-kinase
 ps6 – phosphorylated S6 ribosomal protein
 POS – Photoreceptor outer segments
 ppm – Parts per million
 RCCs – Reactive carbonyl compounds
 Rf – Retention factor
 RNA – Ribonucleic acid
 Ro5 – Lipinski's Rule of five
 ROS – Reactive oxygen species
 RPE – Retinal pigmented epithelium
 rpm – Rotations per minute
 Rt – Retention time
 r.t. – Room temperature
 s – Seconds
 SAR – structure-activity relationship
 SN₁- Unimolecular nucleophilic substitution
 SN₂ – Bimolecular nucleophilic substitution
 SN_{Ar} – Aromatic nucleophilic substitution
 STAT3 – signal transducer and activator of transcription 3
 t_{1/2} – Half-life
 TFEB – Transcription factor EB
 TICs – Tumour initiating cells
 TLC – Thin layer chromatography
 UV – Ultraviolet
 UV-Vis – Ultraviolet-visible
 VEGF – Vascular endothelial growth factor

 δ – Chemical shift
 δ⁺ – Partial positive charge
 λ – Wavelength

1 Introduction

1.1 Neurodegenerative diseases

Autophagy is an important and complex self-degradative process mediated by lysosomes that is responsible for the bulk degradation of intracellular components, such as old proteins and organelles, preventing that abnormal cellular functions occurs [1,2]. There are three types of autophagy: microautophagy, macroautophagy and chaperone-mediated autophagy [3]. In the first type, the lysosomal membrane ingests directly small portions of cytoplasm through invaginations of lysosomal membrane. In the chaperone-mediated autophagy process, target proteins are recognized by cytosolic chaperones and transported across the lysosomal membrane. Here, the interest is focused on the macroautophagy, that starts with the encapsulation of cytoplasm and organelles, which are surrounded by double-membraned vesicles, named autophagosomes. The autophagosomes are transported to and fused with lysosomes, forming autolysosomes. In the autolysosomes, the cytoplasmic load and the inner membrane of autophagosome are degraded. The resulting products, including amino acids and fatty acids are transported back to the cytosol by lysosomal permeases and are available for anabolic processes [2,3].

Neuropathological and genetic evidence suggest that neurodegenerative diseases are related with defects in the autophagy-lysosome pathway (ALP) that brings forth toxic protein species, promotes protein aggregation and accumulation of detrimental and obsolete organelles. Therefore, correcting those defects in this process may help treating neuropathies such as Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD) [1,4,5].

Even if neuropathies differ in clinical aspects, the pathobiological processes are largely shared, and includes mostly oxidative stress, inflammation, impaired protein processing, mitochondrial dysfunction and disruptions in the autophagy-lysosomal system, that leads to the presence of misfolded and aggregated proteins that lose their physiological roles and develop neurotoxic properties [6,7]. The compromised pathways include the chaperone-mediated autophagy, the ubiquitin-proteasome system, extracellular clearance by proteases and extrusion into the circulation through the blood brain-barrier (BBB) and glymphatic system [7].

The reason why defects in autophagy may be a mechanism in neurodegenerative diseases is due to the role of mitochondrial damage and consequently deficits in bioenergetics or the excess of reactive oxygen species (ROS) observed in these

diseases. Once autophagy is responsible by the removal of catastrophically damaged mitochondria, dysfunction in the process leads to the accumulation of detrimental mitochondria. It is also important to relate autophagy and aging, since aging is the number one risk factor for these disorders and is associated with down-regulation of autophagy in the brain. Therefore, induction of autophagy may be a possible way to treat the neurodegenerative diseases and it has been reported that the inducement of autophagy may have cytoprotecting or stimulation of apoptosis effects [2,8].

Alzheimer's disease is the most common neurodegenerative disorder and is characterized by a progressive loss of memory and cognitive function due to a widespread and progressive degeneration in the brain, affecting cholinergic neurons, being observed the accumulation of intraneuronal *tau* tangles and extracellular amyloid-beta ($A\beta$) plaques [6,9]. Those $A\beta$ peptides are cleavage products of amyloid precursor protein (APP). Neurons from AD patients shows abnormal accumulation of lysosomes, which is a sign of autophagic deficits, that may be caused by harmed autophagosome formation or faulty lysosomal clearance appear to contribute to the increase of the deposits of $A\beta$ and *tau* and the development of AD features [10].

Following AD, Parkinson's disease is the second most common neurodegenerative disease, being associated with motor and nonmotor symptoms [5]. PD is characterized by gradual degeneration, progressive and selective loss of dopaminergic neurons in the *substantia nigra* pars compacta, associated with the aggregation and accumulation of a α -synuclein (α -syn) cytoplasmic inclusions in neurons on specific brain regions including the brainstem cortical regions, named Lewy bodies (LBs) [5,6,10,11]. Autophagy may be related with PD, once the degradation of α -synuclein is provided by autophagy, endolysosomal pathway and chaperone-mediated autophagy. Defects on those ways promote the accumulation and aggregation of α -syn [12].

Huntington disease is a neurodegenerative, monogenic, autosomal dominant disease, resulting by cytosine-adenine-guanine (CAG) trinucleotide repeated expansions in the first exon of Huntingtin (*HTT*) gene, producing a mutant huntingtin protein [4,6]. Overexpression of transcription factor EB (TFEB) enhances in autophagy-lysosomal pathway, which has proved to have benefits in bettering HD in mammalian cells and mouse models [4].

Lysosomes of retinal pigment epithelial (RPE) cells are crucial in the degradation of phagocytosed photoreceptor outer segments (POS), assuring optimal neuronal activity during the visual cycle [13]. The RPE is part of the blood-ocular barrier and its functions are essential to retinal photoreceptors, having also metabolic and supportive properties. Those functions includes the regeneration of the bleached visual pigment,

converting all *trans* retinol to 11-*cis* retinal, and then allowing it to re-enter the visual cycle (figure 1.1.1), maintain the interphotoreceptor matrix and Bruch's membrane, transport of water, oxygen, ions and nutrients from the choroid vessels to the outer layers of the retina, phagocytic uptake and degradation of apical photoreceptor outer segments [14,15].

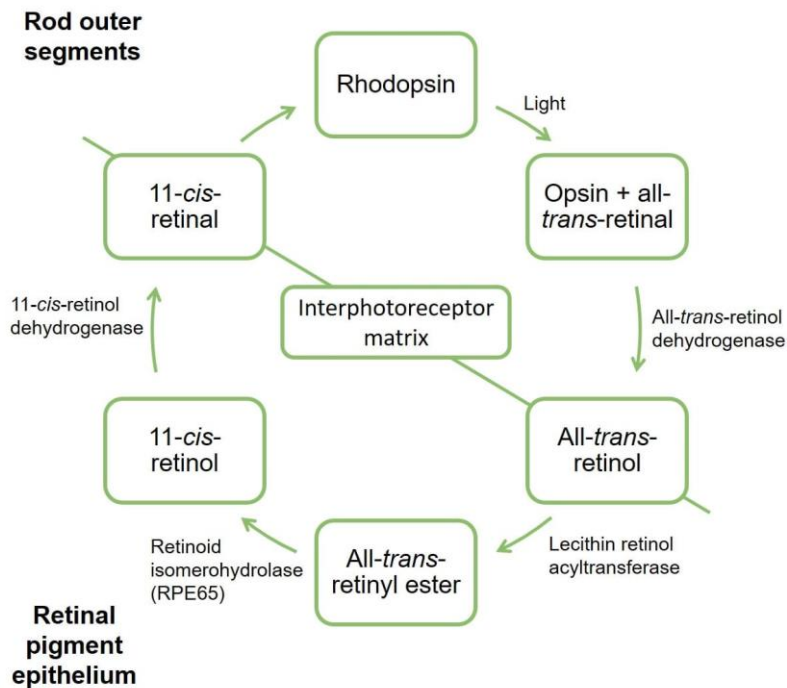


Figure 1.1.1– Visual cycle. Adapted from [16–18].

As the RPE ages, changes occur, including the focal deposition of acellular, polymorphous debris between RPE and Bruch's membrane, called drusen, and the accumulation of lipofuscin. The heavy phagocytic load leads to an oxidative environment in the RPE lysosomes and accumulate lipofuscin, which is composed by autofluorescent undigested aggregates. This process leads to lysosome dysfunction, which affects other cellular functions and ultimately leads to retinal degeneration and blindness [13,15]. Normal aging is associated with the accumulation of vitamin A aldehyde-derived compounds as lipofuscin in RPE cells, which has been linked to some forms of macular degeneration [19].

The aging of RPE cells leads to morphological changes that may contribute to Age-Related Macular Degeneration (AMD), such as the progressive cellular accumulation of lipofuscin, an autofluorescent material that cannot be degraded enzymatically. Probably it occurs due to a decreased phagocytic ability of RPE cells [16,20].

1.2 Age-related macular degeneration

Currently, age-related macular degeneration is the main cause of loss of vision or severe visual impairment in individuals over 50 years old, in developed countries [14,20]. It is the third leading cause of blindness globally [21].

Age-related macular degeneration is a neurodegenerative disorder that affects mainly the macular region of the retina [22]. This is a pathology characterized by accumulation of vitamin A aldehyde-derived compounds, as lipofuscin, which is mainly formed by lipid-containing residues of lysosomal digestion, hyper and hypo-pigmentation of retinal pigment epithelium cells, RPE and photoreceptor apoptosis and choroidal neovascularization (CNV) [14,19].

There are two forms of the disease, the atrophic and the neovascular forms. The atrophic or “dry” form is the most prevalent and consists in a slow progressive dysfunction of the RPE, photoreceptor loss and degeneration of the retina, characterized by the origin of drusen, which are extracellular debris and deposits, between RPE cells and Bruch’s membrane, and is toxic for RPE cells [14,23]. The neovascular or “wet” form is characterized by choroidal neovascularization with intraretinal or subretinal leakage, haemorrhage and RPE detachments. However, the forms can coexist mutually, it means that the atrophic form can develop to CNV and patients with CNV may present some atrophy by the time [23].

AMD is categorized by stages: early, intermediate or advanced, based on the severity of the symptoms, such as the number and size of drusen, hyper and hypopigmentary changes and the presence or non-appearance of choroidal neovascularization [22]. The early stage includes signs as drusen and abnormalities of RPE. The late-stage can be the wet or the dry form and consists in the loss of central visual acuity, resulting in severe and permanent visual impairment and legal blindness [24]. The early stage is asymptomatic [25]. However, it is possible to observe mild central distortion and reduced reading ability with low luminance, being verified scotoma in the central vision. Although, if only one eye is affected, the symptoms may be only revealed when the good eye is occluded. The late stage affects central vision and can progress quickly into the neovascular form and slowly into the atrophic form [24].

People who suffer from this disease may complain symptoms as decreased or blurry vision, metamorphopsia, which consists in distortion of central vision, straight lines appearing wavy and presence of central scotomas [26].

It is estimated that globally, around 30 to 50 million of people suffer from this disease and the tendency is that these number increase over time due to the ageing

population. It is expected that by 2040, in the Europe it will be 14.9 to 21.5 million people with early AMD and 3.9 to 4.8 million in the late stage [27].

The etiology and molecular pathophysiology of AMD are both still uncertain [20]. Although the etiology is unknown, there are strong evidences that the oxidative stress is determinant in the development and progression of the disease [23]. Further, there is no evidences in the direct causal relationship between the accumulation of RPE lipofuscin and the etiology of AMD, but this excess of lipofuscin is surely responsible for the photoreceptor cell loss [28].

The risk factors include age, ethnicity, smoking, genetical, the diet and the sun exposure [26]. The prevalence of this disease increases with age and is higher in non-Hispanic whites than in blacks or Hispanic and Chinese population. Another risk factor is smoking for more than ten years, smokers are twice likely to develop AMD. Although the role of genetic is not already totally understood, it may explain the disease in younger individuals. Also, presence of a polymorphism Tyr402His in the complement factor H (CFH) gene, located on chromosome 1 (1q31), increases the possibility to acquire the disease and presence of Ala69Ser polymorphism on the age-related maculopathy susceptibility 2 gene (LOC387715 or ARMS2), located on chromosome 10 (10q26) has been implicated on the development of the disease, independently of CFH. People with the two variants, Tyr402His and Ala69Ser, have a dramatically higher risk of suffer from the disease. High dietary intakes of antioxidants and zinc decrease the risk in older people. Furthermore, obesity, hypertension and high dietary intake of vegetable fat increases the risk to develop the disease. Higher sun exposure is related to an increased risk of developing the disease [15,26]. Moreover, the presence of ARMS2 Ala69Ser variant together with a history of smoking leads to a synergistically increased risk factor, as compared with those factors alone [15].

The treatment of this disease must include the several factors involved in the development and progression and may imply a combination of therapies after understanding the different phenotypes [29].

AMD can be diagnosed by the clinical examination or assessment of colour fundus photographs [24]. This ophthalmic exam consists in fundus imaging of the retina for observation of symptoms that include drusen deposits, alterations in pigmentary in the RPE and loss and degeneration of neural retinal and exudative changes in the retina [22]. Also, imaging with fluorescein angiography, which allows to visualize the blood vessels, is useful to detect choroidal neovascularization, it is to confirm the presence of CNV, and its location and activity, indicated by the extension of the dye leakage [22,24]. Imaging techniques, including optical coherence tomography angiography, are also useful to confirm the diagnosis. It consists in a non-invasive method that requires no dye

and allows the detection of the choroidal vascular networks present in the choroidal neovascularization. However, it does not detect leakage [22,24].

Currently, there is neither a cure or a treatment to prevent this degenerative disorder [23]. The treatments are limited, and the only treatment proven to be effective in slowing the progression of the wet form of AMD is laser photocoagulation therapy [20,30]. However, only a small portion of patients have shown improvements and the recurrence is high. Further, this treatment is associated with a drop in Snellen visual acuity up to six lines, a consequence that not every patient and ophthalmologists accept, so uptake has been low [30].

Another method used is photodynamic therapy, that was approved in 2000 and consists in injecting intravenously a photosensitizer and then the neovascular lesion is irradiated with a diode laser at 689 nm, for 83 seconds [29,30]. The photosensitizer is excited and generate free radicals that damage vascular endothelium, causing thrombosis within the irradiated vessels [30]. Even though it does not improve vision and it is less efficacious as monotherapy than other treatments, it stops the visual loss in the neovascular form, being safe and with minimal and rare side effects [15]. The photodynamic therapy was the first effective pharmacological treatment for the exudative form of the disease and though it has been less used nowadays, it remains used in patients with systemic or ocular contraindication for intravitreal administration of antiangiogenic drugs. It is also used as an adjuvant, in combination with corticosteroids or antiangiogenic agents and to treat polypoidal choroidal vasculopathy [29]. Transpupillary thermotherapy is also a treatment for AMD, that uses an infrared diode laser, which emission is predominantly collected by the melanin granules of RPE and choroid [30].

Since antioxidants may limit the damage caused by oxidative stress, using antioxidant supplementation, such as vitamins C and E, zinc oxide, cupric oxide and beta carotene suggested to decrease the progression to advanced disease for the dry form in high-risk patients [15,30]. Yet, this treatment may not be appropriate for all patients, once beta-carotene supplementation may be associated with an increase of the risk to develop lung cancer in smokers. Also, a high dose of vitamin E may be related with risk of death and heart failure in diabetic patients or others who also suffer from cardiac disease [15]. Additionally, the inhibition of complement pathway has been identified as an important potential therapeutic intervention for atrophic form of AMD [24]. Moreover, as the risk factors to develop the disease includes environmental factors, a way to manage the disease is to proceed alterations of lifestyle and in the diet, such as quit smoking, maintain healthy weight and blood pressure, decrease the consume of fat and increase the consume food rich in antioxidants [15].

A way to treat neovascular AMD consists in the inhibition of angiogenesis, administering anti-VEGF agents. Those agents inhibit the vascular endothelial growth factor (VEGF), an angiogenic protein produced in the retina and induced by hypoxia, increasing the retinal vascular permeability and promoting neovascularization [24].

1.3 Curcumin interest and properties

Curcumin (diferuloylmethane; 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) (figure 1.3.1) is a natural polyphenolic compound that is bioactive, and it is derived from the rhizome of turmeric (*Curcuma longa* Linn.), which belongs to family *Zingiberaceae* [1,31–34].

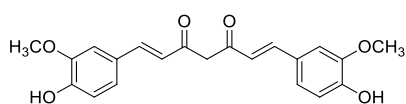


Figure 1.3.1 – Chemical structure of curcumin.

Curcumin is an orange water-insoluble pigment [35,36]. Regarding its chemical structure, curcumin is a symmetric molecule with two aromatic ring systems, *ortho*-methoxy phenolic groups, connected by two α,β -unsaturated carbonyl groups, which are good nucleophilic acceptors of a carbanion or other nucleophiles and the diketones easily form stable enols and are readily deprotonated to form enolates [2,37]. Curcumin occurs in keto and enol tautomeric conformations (figure 1.3.2) and these tautomers also exist in the stereoisomeric forms *cis* and *trans*. In acidic and neutral conditions, keto form is predominant and in alkaline curcumin solutions presents as a stable enol form [38]. In acidic conditions, curcumin is a remarkably potent H-atom donor because in the keto form, the heptadienone linker between the two methoxyphenol groups contains a highly activated carbon atom, and due to the delocalisation of the unpaired electron on the adjacent oxygens, the C-H bonds on this carbon are very weak [36]. The enol form is the most energetically stable not only in solid phase but also in solution, and is able to cross the blood-brain barrier [35]. In this form, curcumin acts as an electron donor, which is a mechanism typical for the scavenging activity of phenolic antioxidants [36]. The *o*-methoxyphenol group and the methylenic hydrogen are responsible for the antioxidant activity. Therefore, this molecule participates in multiple redox reactions as an electron donor, due to the conjugated double bond, stabilizing the structure [2,38].

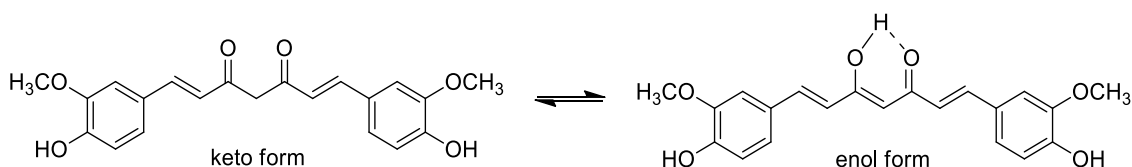


Figure 1.3.2 – Tautomerism of curcumin, that occurs under physiological conditions, where the keto form predominates in acidic and neutral condition and the enol form in basic conditions. Adapted from [36].

This compound was firstly isolated from turmeric in 1815 by Vogel and Pelletier but only in 1870 curcumin was obtained essentially pure [39]. In 1910, Milobedzka and Lampe identified the chemical structure of curcumin as diferuloylmethane, or 1,6-heptadiene-3,5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl)-(1E,6E) [35,40]. Curcumin's chemical structure was determined by Roughley and Whiting in 1973 [35].

The interest of curcumin emerges since the *C. longa* is nontoxic and has a plethora of pharmacologic effects. Turmeric has been used in dietary spice and as a herbal medicine to regulate menstruation, dissolving gallstones, and to treat dyspepsia, wounds, sinusitis, liver and biliary disorders, jaundice, conjunctivitis, flatulence, urinary tract diseases, diabetic ulcers, skin conditions and skin cancer, cold, cough, rheumatoid arthritis and chronic otorrhea, in traditional medicine for a long time due to its anti-inflammatory properties [1,31,32,41,42].

Curcumin has an ample range of biological properties (figure 1.3.3) such as antioxidant [32,43], anti-thrombotic [44], antitumoral [33,45], neuroprotective [46,47] and cognition-enhancing [48,49], anti-infective and antiviral [50], pulmonoprotective [47], anti-inflammatory [50,51], cardioprotective [47], hepatoprotective and lipid-modifying [47], anti-depressive [47], anti-angiogenesis activity [32,52] and pain-relieving and wound healing activities [53]. It has also been reported that curcumin is capable of improve retinal function in retinal degeneration [37,54]. These properties and the fact that this compound is able to cross the BBB, being reported a protective effect in several central nervous system (CNS) disease models, make curcumin a compound whose application may be effective in the treatment of several diseases, namely cancer, neurodegenerative disorders and ocular diseases [55–57].

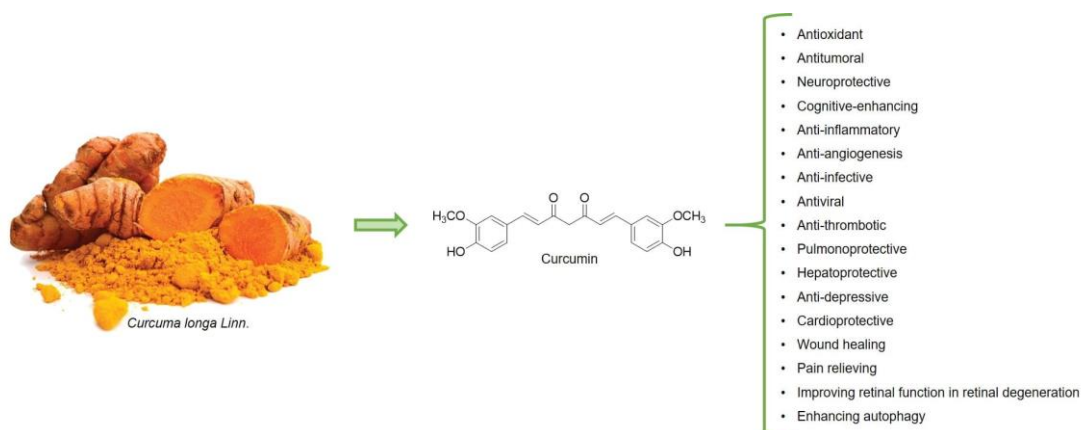


Figure 1.3.3 – Curcumin's biological activities.

Curcumin is capable of inhibit cell growth and induce apoptosis, by modulating the activities of several transcription factors, growth regulators, adhesion molecules, apoptotic genes and cellular signalling pathways. Therefore, curcumin has antitumoral proprieties, inhibits the cell proliferation in several tumours and has anti-metastatic activity possibly due to its ability to inhibit the activation of prosurvival transcription factor nuclear factor kappa B (NF κ B), leading to apoptosis [32,33]. Once it has anti-cancer activity and presents extremely low toxicity, this compound is an ideal candidate for treating cancer [33].

Curcumin inhibits vascular endothelial growth factor (VEGF)-mediated angiogenesis [52]. The anti-angiogenesis activity is attributed to the fact that curcumin irreversibly inhibits the CD13/aminopeptidase N, that is important to angiogenesis process. The anti-inflammatory activity of curcumin is possibly related to its ability to inhibit up-regulation of cyclooxygenase-2 (COX-2) [32,52]. Once inhibitors of VEGF are used as a treatment for AMD [29], curcumin having this property may have interest as an agent to treat this disease.

It is unclear if the potent antioxidant activity is the basis for most of other biological activities of this compound. Also, it has been suggested that this activity depends the presence of phenolic functional groups [32] and that the central methylene hydrogens are also important for anti-oxidant activity [43], so it is the phenolic and the methoxy group on the aromatic ring and the 1,3-diketone moiety that contribute to this activity. It was verified that the antioxidant activity increases when there is a methoxy group in *ortho* position, because it can form an intramolecular hydrogen bond (figure 1.3.4) with the phenolic hydrogen, abstracting easily the H-atom from the *ortho*-methoxyphenols [58,59]. Additionally, it has already been demonstrated that the central methylene and the phenolic hydrogens may be involved in the mechanism of formation of phenoxy radical, depending of reaction conditions [32]. Curcumin scavenges directly or indirectly

reactive oxygen species (ROS) [60]. It is suggested that the donation of H-atom from β -diketone moiety to lipid alkyl or lipid peroxy radical contributes antioxidant activity [34].

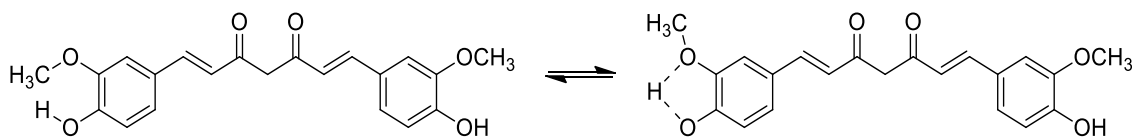


Figure 1.3.4 – Intramolecular hydrogen bond between methoxy and phenyl groups.

Curcumin is able to induce the expression of nuclear factor Erythroid 2-related factor 2 (Nrf2)-dependent phase II and anti-oxidant enzymes [61]. The transcription factor Nrf2, which is found throughout the CNS, regulates phase II detoxification and antioxidant genes and anti-inflammatory and neuroprotective genes, being also an important regulator of brain inflammation. This factor is responsible for coordination of expression of genes needed for free radical scavenging, detoxification of xenobiotics and to maintain the redox potential [61,62]. In normal conditions, Nrf2 in the cytosol complexes with Kelch-like ECH associated protein (Keap1) and Cullin-3 (Cul3), which is an adaptor to link Nrf2 to E3 ubiquitin ligase complex. The freshly synthesized Nrf2 in the cells is degraded by ubiquitination and proteasomal degradation, limiting the concentration of Nrf2 in the cytosol. In situations of oxidative and electrophilic stresses, the cysteine residues of Keap1 are modified, promoting changes in the interaction with Nrf2 and unlocking Keap1 from the Nrf2 complex, which allows the accumulation and translocation of Nrf2 to the nucleus, where it interacts with small Maf proteins and binds to promoters with anti-oxidant response element (ARE) sequences [61]. Curcumin interacts with the Keap1-Nrf2-ARE antioxidant system (figure 1.3.5) through its electrophilic properties and promotes the dissociation of Keap1 unleashing the Nrf2, which then migrates to the nucleus, where it binds to antioxidant response elements. The binding of Nrf2 with ARE not only induces the expression of antioxidant enzymes but also increases detoxification enzymes production, being responsible for diminishing the oxidative stress and apoptosis [34,56]. This activation of Nrf2/ARE signal pathway is one of the potential mechanisms that explains the neuroprotector effect of curcumin, as this compound activates the Nrf2, resulting in the upregulation of antioxidative and detoxifying enzymes [56].

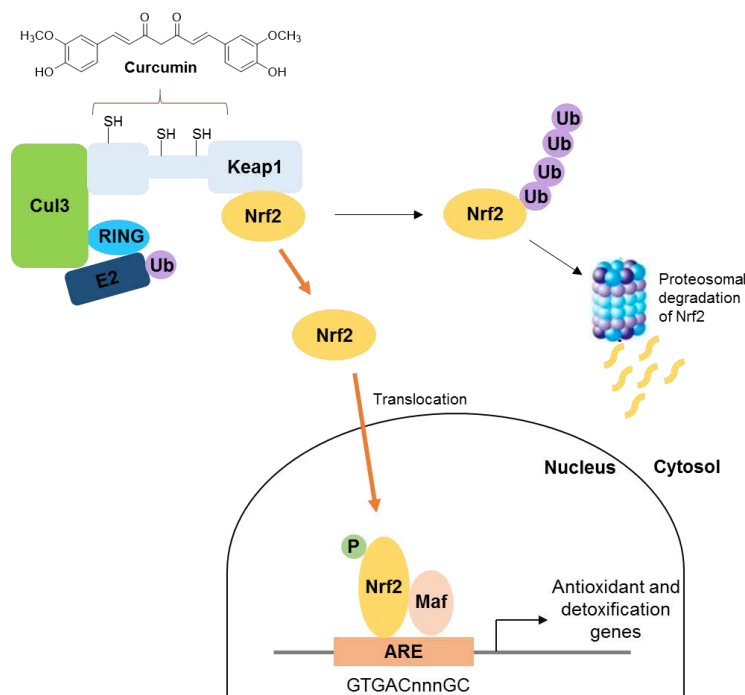


Figure 1.3.5 – Interaction of curcumin with Keap1-Nrf2-ARE antioxidant system. Adapted from [63].

Also, curcumin has potential to target cancer stem cells (CSCs), regulating CSCs self-renewal pathways, including Wnt/ β -catenin, Notch and sonic hedgehog, and the specific microRNAs involved in acquisition of epithelial-mesenchymal transition (EMT). Curcumin may be a promising adjunct to traditional cancer treatments [64]. Further, curcumin inhibits the Signal transducer and activator of transcription 3 (STAT3) and Nuclear factor kappa B signalling pathways, both having key-roles in the development and progression of cancer [51]. The ability of curcumin to inhibit the activation of NF κ B is responsible for induction of the apoptosis, which makes curcumin able to stop the proliferation of tumour cells and it confers its anti-metastatic activity [32].

It has been reported that autophagy induction may have cytoprotective or apoptotic effects and there are several molecular mechanisms for curcumin to induce autophagy [2]. For instance, curcumin increases autophagy and lysosomal activation, once it inhibits the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K-Akt-mTOR) signalling pathway, being a direct inhibitor of mTOR [1,65]. Additionally, it was evaluated the effect of curcumin in apoptosis and it was verified that the cell treatment with curcumin in combination with piperine results in the activation of autophagy mediated protein degradation and induction of anti-apoptotic events [66].

Transcription factor EB (TFEB) is a master regulator of autophagy and lysosomal biogenesis and has been gaining interest once it induces the expression of several lysosomal genes and consequently may contribute to the clearance of pathogenic proteins [67]. Under normal conditions, TFEB is phosphorylated and located in the cytoplasm. In situations of stress, as starvation, lysosomal dysfunction or inhibition of

mechanistic target of rapamycin (serine/threonine kinase) complex 1 (mTORC1), TFEB is dephosphorylated and translocate to the nucleus, where it binds to coordinated lysosomal expression and regulation elements and activates genes involved in autophagy and lysosomal biogenesis [1,4,68]. TFEB leads to an increase of lysosomal exocytosis and promotes the degradation of autophagic substrates and clearance of lipids and mitochondria [67]. By promoting the expression of TFEB, it is stimulated the clearance of lysosomal substrates and toxic protein aggregates, curcumin have a neuroprotective effect [1]. Therefore, molecules that increase the expression or promotes the nuclear translocation of this transcription factor, could be a promising way to stop the progression of neurodegeneration, functioning as a disease-modifying therapy [1]. Also, it was already reported that curcumin is able to mark the anti-amyloidogenic activity, which is interesting to mind in Alzheimer's disease [42].

Curcumin has been investigated in several clinical trials, minding its biological properties [42,69,70]. Focusing on its anti-inflammatory efficacy, trials were performed on patients of osteoarthritis and rheumatoid arthritis, confirming its anti-arthritic effect and showing that curcumin is useful to manage and in the therapy of these diseases, being a promising and safe complementary therapy [42,70]. Curcumin proved to be effective in skin diseases, as psoriasis, pruritis, vitiligo and radiation-induced dermatitis [42,70].

Clinical trials on ophthalmological disorders proved that curcumin is highly effective, not only by oral intake but also when applied locally or systemically [42]. The efficacy of curcumin in Chronic Anterior Uveitis were assessed and compared with the only available treatment, corticosteroid therapy, and the main advantage was the lack of adverse effects [71].

This compound was also studied in central nervous system diseases. The first clinical trial in Alzheimer's disease was discouraging, there was no significant difference observed between curcumin and placebo group. However, another following study demonstrated that curcumin can disaggregate A β -deposits. Also, a novel curcumin formulation (Longvida®), with higher bioavailability, have shown good results from the first dose, where it meliorates the attention and working memory tasks and when the administration is four weeks, it improves memory, mood, alertness and contentedness [42].

Clinical trials on depression and anxiety proved that curcumin improved depressive symptoms, being the modulation of plasma endothelin-1 and leptin, a possible mechanism of action of this compound. Only in one trial, it was verified that curcumin reduced anxiety but not depression, but it was justified with the fact that the

time of administration were only four weeks contrasting with other studies that were between five and eight weeks [42,70].

Curcumin was also object of study in clinical trials of respiratory and cardiovascular diseases, proving its cardioprotective effect. It was also proved to be effective in gastrointestinal, genitourinary tract and liver diseases, confirming its hepatoprotective effect [42]. Curcumin was evaluated in inflammatory bowel diseases, including ulcerative colitis and Chron's disease, concluding that curcumin is safe and useful in treating inflammatory bowel diseases [70].

This compound was also evaluated in metabolic disorders, including diabetes, obesity, β -thalassemia and acquired immunodeficiency syndrome (AIDS) and only on AIDS it didn't showed improvements [42,69]. It was reported that curcumin may be advantageous on the management of diabetic complications, glycaemic and lipid control [70]. Also, curcumin were tested to treat non-alcoholic fatty liver disease (NAFLD) and proved to have a positive effect, resulting in a significant diminishing of the liver fat contend, body mass index, serum levels of total cholesterol, triglycerides, aspartate and alanine aminotransferases, glucose and glycated haemoglobin [70,72].

The ability to neutralize toxins were also object of clinical studies and it was observed that curcumin has potential in reduction of alcohol intoxication [42].

Some studies also evaluate the effect of curcumin in premenstrual syndrome symptoms and in postmenopausal health. It was reported that curcumin can reduce premenstrual syndrome symptoms, possible through the modulation of neurotransmitters and the anti-inflammatory activity of this compound. Regarding its effect on postmenopausal health, it was verified that combining the ingestion of curcumin with exercise have positive effects [70].

Due to curcumin's anti-microbial activity, it was shown that it is capable to reduce human papilloma virus (HPV) that causes cervical cancer [69]. Furthermore, several clinical trials evaluate the effects of curcumin in cancer treatment, leading to improvement of symptoms and other markers and parameters of tumour and meliorating the quality of life [42]. Curcumin proved to be efficient in treatment of colorectal, breast, prostate, pancreatic and biliary cancer and also showed to slow the progression of monoclonal gammopathy of undefined significance, a condition that can develop into multiple myeloma [69,70].

Despite the plethora of properties of curcumin and low toxicity, its clinical use is restricted. What limits the clinical use of curcumin, is that this compound has pharmacokinetic defects, that includes poor absorption, low bioavailability, rapid metabolism and poor chemical stability [1,31,45]. However, the low bioavailability of curcumin may be a consequence of its poor absorption, fast metabolism and rapid

systemic elimination [38]. Therefore, several studies have been developed to find novel derivatives with improved pharmacokinetic and pharmacological properties [1,31].

1.4 Analogues of curcumin

In order to achieve better pharmacological and pharmacokinetic profiles, there have been attempted chemical modifications of curcumin [1,31]. There are several sites where the modifications can be done to achieve analogues of curcumin with properties improved (figure 1.4.1). Such modifications can occur in the aryl side chain, in the double bond, in the diketo moiety and in the active methylene functionality [73].

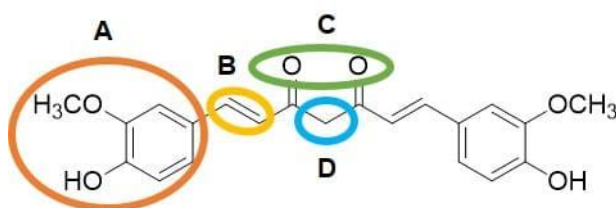


Figure 1.4.1 – Possible sites to perform modifications on curcumin's structure: (A) aryl side chain; (B) double bond; (C) diketo moiety; (D) central methylene. Adapted from [73].

Weber *et al.* reported the antioxidant activities of curcumin and enone analogues, including compounds with 7-carbon, 5-carbon and 3-carbon spacers [32].

The instability and metabolic defects may be derived from the high reactivity of the beta-diketone moiety, that contributes to the fast degradation and metabolism. Therefore, the attention has been focused on monocarbonyl analogues of curcumin (MAC), where this moiety has been removed, so the stability, bioavailability and the pharmacokinetic profiles are improved and presents better activities *in vitro* and *in vivo* [1,31,33,45].

Several monocarbonyl analogues of curcumin have been reported. Lee *et al.* reported three series of MAC. The first set has a carbonyl group, the second a cyclopentenone and the last a cyclohexanone in the centre of the spacer (figure 1.4.2) [74].

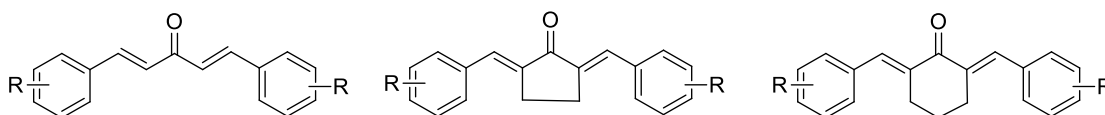


Figure 1.4.2 – Structures of analogues reported by Lee *et al.* [74].

Beyond the analogues referred by Lee *et al.*, Leong *et al.* also focus on another two general structures of MAC and in analogues preserving the β -diketone moiety (figure 1.4.3), that hopefully constitute additional hydrogen-bond acceptors or donors and

consequently better bioactivity. These compounds were evaluated as anti-inflammatory agents [75].

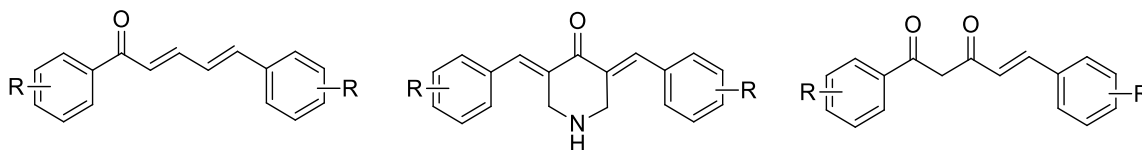


Figure 1.4.3 – General structures of the series of MAC reported by Leong *et al.* [75].

Yin *et al.* focused on MAC (figure 1.4.4) and on its potential anticancer activity, verifying that these compounds overcome the problem of poor stability of curcumin and some of them presented better activity than curcumin [45].

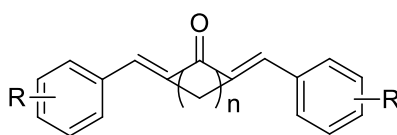


Figure 1.4.4 – Structure of MAC reported by Yin *et al.* [45].

Zhang *et al.* synthesized asymmetrical monocarbonyl analogues (figure 1.4.5) and evaluated them also as anti-inflammatory agents, concluding that some of them have a higher anti-inflammatory activity than curcumin [31].

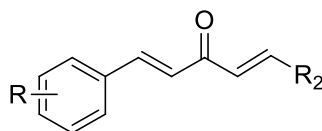


Figure 1.4.5 – General structure of the asymmetrical monocarbonyl analogues reported by Zhang *et al.* [31].

The monocarbonyl analogues of curcumin have demonstrated superior efficacy than curcumin relatively to antitumoral and anti-inflammatory activity [76,77].

Regarding the cytotoxicity, it has already been reported that the position of the substituents on the aromatic ring is critical and independently of what substituent, either it is electro withdrawing or electro donating, being in position *ortho* to the five carbons spacer raise the cytotoxicity against adenocarcinomic human alveolar basal epithelial cells (A549 cells), which is called the *ortho* effect. Also, it is described that to increase the activity is better use electron withdrawing groups [77].

Baiwra *et al.* review the synthesis and structure-activity relationship (SAR) of several distinct analogues of curcumin from 1993 to mid-2013, including substitutions on the aromatic ring, symmetrical and asymmetrical monocarbonyl analogues, β -diketone substitution and heterocyclic analogues, being the structures summarized in figure 1.4.6. In the same review is reported that antioxidant activity is better with the increase of

phenolic groups on the aryl rings. Also, analogues with cyclohexanone moiety presented an enhanced anti-inflammatory and cytotoxic activities than analogues with ketone and cyclopentanone moieties. Some of the heterocyclic analogues have shown promising cyclooxygenase (COX) inhibitory activities and pyrazole analogues presented better antioxidant, COX-2 inhibitory, antitumor and antimalarial activities compared with curcumin. Also comparing with curcumin, hydrogenated analogues presents better antioxidant and similar cytotoxic and anti-inflammatory activities [78].

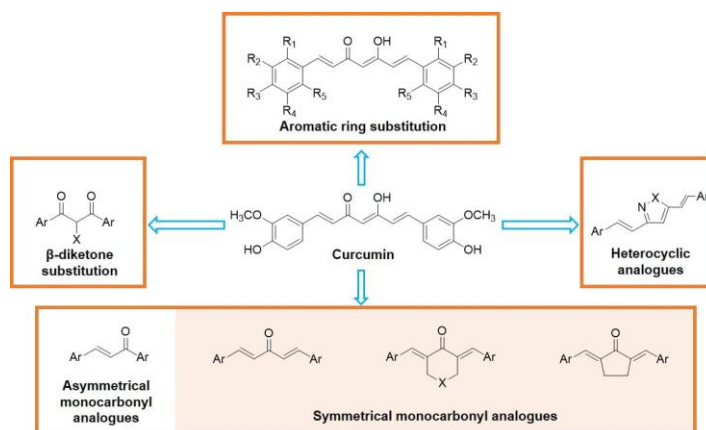


Figure 1.4.6 – General structure of analogues of curcumin reviewed by Baiwra *et al.*. Adapted from [78].

In this work, several symmetrical monocarbonyl analogues (figure 1.4.7) will be synthesized by aldol condensation of modified benzaldehyde and ketone, in presence of Sodium Hydroxide (NaOH), some of them already presented positive feedback.

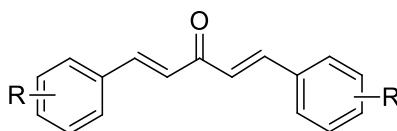


Figure 1.4.7 – General chemical structure of monocarbonyl analogues of curcumin.

Among those already investigated, 1,5-Bis(2-methoxyphenyl)-1,4-pentadien-3-one (**C1**) (figure 1.4.8), a MAC which has a methoxy group in *ortho* position, is the one that is most promising to date, being already identified to be a potent TFEB activator, without inhibiting mTOR activity, which suggests a potential efficacy to treat neurodegenerative diseases once it promotes autophagy [79]. Contrarily to curcumin that improves autophagy by inhibiting the PI3K-Akt-mTOR signalling pathway, **C1** binds directly to TFEB and promoting its entrance to the nucleus, without affecting TFEB phosphorylation or inhibiting the activities of mTOR and mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) pathways, MAPK1/ERK2 and MAPK3/ERK1 [1,79]. Also, **C1** presents a good structural stability and blood barrier permeability, being orally effective in improving autophagy and lysosome biogenesis in

the brain, which makes it a good drug candidate although further studies in animal models of neurodegenerative diseases are required [1].

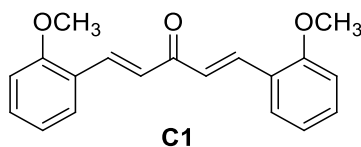


Figure 1.4.8 – Chemical structure of monocarbonyl analogue of curcumin **C1**.

Song *et al.* reported that **C1** potently induces nuclear translocation of endogenous TFEB in Neuro-2a cells and that **C1** doesn't inhibit serine phosphorylation of TFEB or MAPK1/3 activity. Same study refers that the binding site of **C1** localizes within the N-terminal Gly and Ala-rich domain of TFEB. A possible mechanism of action of **C1** on TFEB could be that its binding with TFEB at the N terminus changes TFEB conformation to expose the nuclear localization signal as TFEB dephosphorylation in Ser211 does alternatively or in addition, **C1** binding to TFEB may block TFEB-YWHA interaction and therefore, assisting its nuclear translocation [1].

In several cell lines, **C1** upregulated some autophagy-lysosomal genes and proteins controlled by TFEB and its ability to increase autophagy was blocked by knocking down TFEB and its target gene Becn1, which supports that **C1** promotes TFEB-mediated autophagy and lysosome biogenesis [1].

Curcumin and its analogue, difluorinated-curcumin (figure 1.4.9) have been shown promising results in the area of cancer. Both have potential to inhibit CSCs in several types of cancer, such as glioma, breast, colorectal, pancreatic, brain and oesophageal cancers [64].

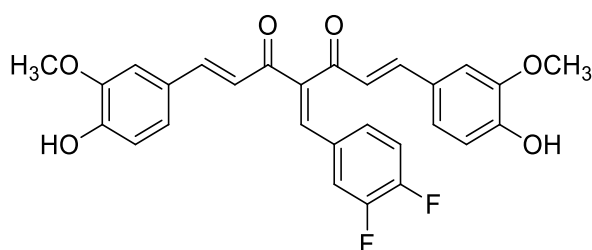


Figure 1.4.9 - Chemical structure difluorinated-curcumin.

Jankun *et al.* reviewed the information available regarding the SAR of curcumin analogues (figure 1.4.10), reporting that the antitumoral activity depends the presence of OH groups in the phenolic ring (*para* position), that are electron donor to free radicals, and if solubilizing groups were attached to these OH groups, the analogue turns cytotoxic. The antioxidant activity is attributed to the methoxy group at *meta* position but if one of these methoxy groups is eliminated it is reported anti-tuberculosis activity while

converting the methoxy groups in hydroxyl increases anti-HIV activity. Substitutions at *ortho* position increases all activities comparing with unsubstituted analogues. Additionally, the cyclization in the central moiety of the compound and the introduction of heteroatoms, such as oxygen and nitrogen, lead to compounds with better antitumoral and anti-angiogenic activities [80].

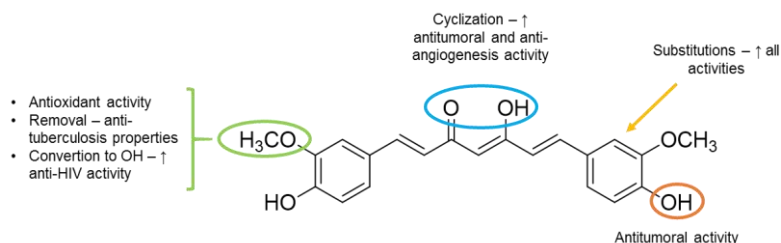


Figure 1.4.10 – Structure-activity relationship of analogues of curcumin. Adapted from [80].

1.5 Goal of the thesis

The ample range of actions of curcumin makes it a compound whose application may be effective in the treatment of several diseases, namely cancer, neurodegenerative disorders and ocular diseases. Still, the clinical use of curcumin is compromised by pharmacokinetic defects. Therefore, the main goal of this research project is to find novel derivatives with improved pharmacokinetic and pharmacological properties, focusing on monocarbonyl analogues (figure 1.5.1) where the stability, bioavailability and the pharmacokinetic profiles are improved and has been reported to have better activities *in vitro* and *in vivo*.

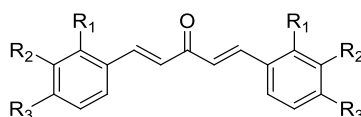


Figure 1.5.1 – General structure of curcumin monocarbonyl analogues.

This work consists in:

- Synthesis of the monocarbonyl analogues of curcumin and structural characterization – the compounds were synthesized through an aldol condensation reaction and then were characterized by NMR spectroscopy (^1H , ^{13}C), Mass Spectroscopy, Elemental analysis, IR and UV spectrometry;
- Evaluation of the stability in physiological conditions – each compound was evaluated in physiological conditions, which include stability studies in phosphate buffer (PBS) and in human plasma;
- Assessment of the “drug-likeness” properties – it was determined the lipophilicity of the compounds using the octanol-water model;

- Electrophilicity assessment by kinetic assay and by NMR assay – this part was important to identify the potential electrophilic character of the compounds and cytotoxic mechanisms;
- Biological evaluation – for the last, two of the monocarbonyl analogues of curcumin were evaluated in human RPE cells where the lysosomal dysfunction was induced. The synthesized monocarbonyl analogues were also tested in cancer stem cells.

2 Synthesis of monocarbonyl analogues of curcumin

This chapter reports on the synthesis of the monocarbonyl analogues of curcumin **1a-l**. The reaction methodologies and mechanisms involved, and the structural characterization of the synthesized compounds carried out by nuclear magnetic resonance spectroscopy (^1H -NMR, ^{13}C -NMR, COSY, HMBC, HMQC), electrospray ionization mass spectrometry (ESI-MS), elemental analysis, infrared (IR) spectroscopy and ultraviolet-visible (UV-Vis) spectroscopy are thus presented.

By varying functional groups in aromatic ring, a small library of compounds was obtained. Mainly, the substituent on the *ortho* position is varied. The idea of switching the groups in this position comes to the fact that the compound **1g (C1)**, which has a methoxy group in *ortho* position, was shown to be promising improving autophagy, once it was already identified as a good activator of TFEB [1,79]. These different compounds correspond to different modified benzaldehydes in the synthesis, which general reaction is presented in figure 2.1. This small library of compounds was later screened, and a structural-activity relationship study is proposed.

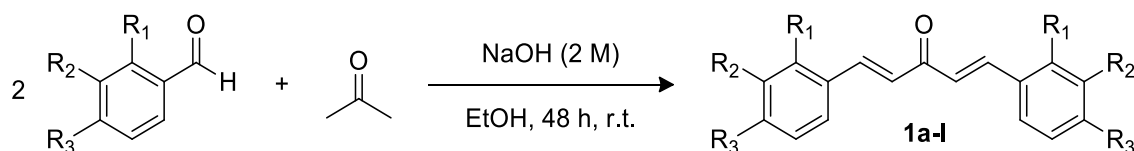


Figure 2.1 – General reaction for the synthesis of monocarbonyl analogues of curcumin **1a-l**.

The different substituents on the aromatic ring (R_1 , R_2 and R_3 groups) for each monocarbonyl analogue of curcumin **1a-l** are presented in table 2.1.

Table 2.1 – Substituents for each monocarbonyl analogue of curcumin **1a-l**.

Compound	Substituents		
	R ₁	R ₂	R ₃
1a (C2)	H	OCH ₃	H
1b	OCH ₃	OCH ₃	OCH ₃
1c	H	H	OCH ₃
1d	Br	H	H
1e	H	H	H
1f	NO ₂	H	H
1g (C1)	OCH ₃	H	H
1h	OCH ₂ CH ₃	H	H
1i	CF ₃	H	H
1j	CH ₃	H	H
1k	F	H	H
1l	OCF ₃	H	H

Synthesis of monocarbonyl analogues of curcumin occurs by an aldol condensation reaction which consists in a condensation reaction that happens with carbonyl compounds that behave simultaneously as nucleophiles and electrophiles.

In aldol condensation, aldehydes or ketones having an acidic proton in α -position relatively to carbonyl group, in alkaline medium, may form enolate ions, which are nucleophilic species. The enolate ions may, in the first step, add to carbonyl groups (electrophilic centres), forming products named “aldol” because of being bifunctional, it means they have a carbonyl group and a hydroxyl group in β -position, constituting hydroxy carbonyl compounds. To cross aldol reaction work, it is important to have only one reagent capable of enolization, in our specific case the ketone, and the other reagent (modified benzaldehyde) must be incapable to form enolate ion and be more electrophilic than the first. The last step of the aldol condensation consists in an elimination reaction where occurs a dehydration. In basic solution, it is not possible to just eliminate water from an alcohol, since the hydroxide (HO⁻) group is a bad leaving group. In this case, it is the carbonyl group that allows the elimination to occur, consisting in an elimination E1cB reaction, where a second enolization allows the loss of the HO⁻ group. E1cB reactions is a unimolecular elimination, and because of this, it is some kind of E1 reaction. However, the leaving group is not lost from the starting molecule but from the conjugate base, therefore cB stands for conjugate base. In this kind of reactions, is important to remind that negative charges are stabilized by conjugation with carbonyl groups and that the proton adjacent to the carbonyl group is acid (pK_a about 20). This proton is removed by the base without the simultaneous depart of the leaving group, forming an anion that is stable enough because it can be delocalized on the carbonyl group, but still prefers to lose a leaving group and become an alkene, which is the rate-

-determining step of the elimination. The dehydration occurs at room temperature, since the final product is stabilized by resonance, being more stable than the intermediate. The formation of *trans* isomer is favoured, because the *trans* form is lower in energy than the *cis* isomer, due to steric factors: *trans* isomer has less steric hinderance [81]. The *trans* geometry of our analogues was later confirmed in the NMR spectrum.

The mechanism of the aldol condensation that origin the monocarbonyl analogues of curcumin **1a-l** is presented in the figure 2.2.

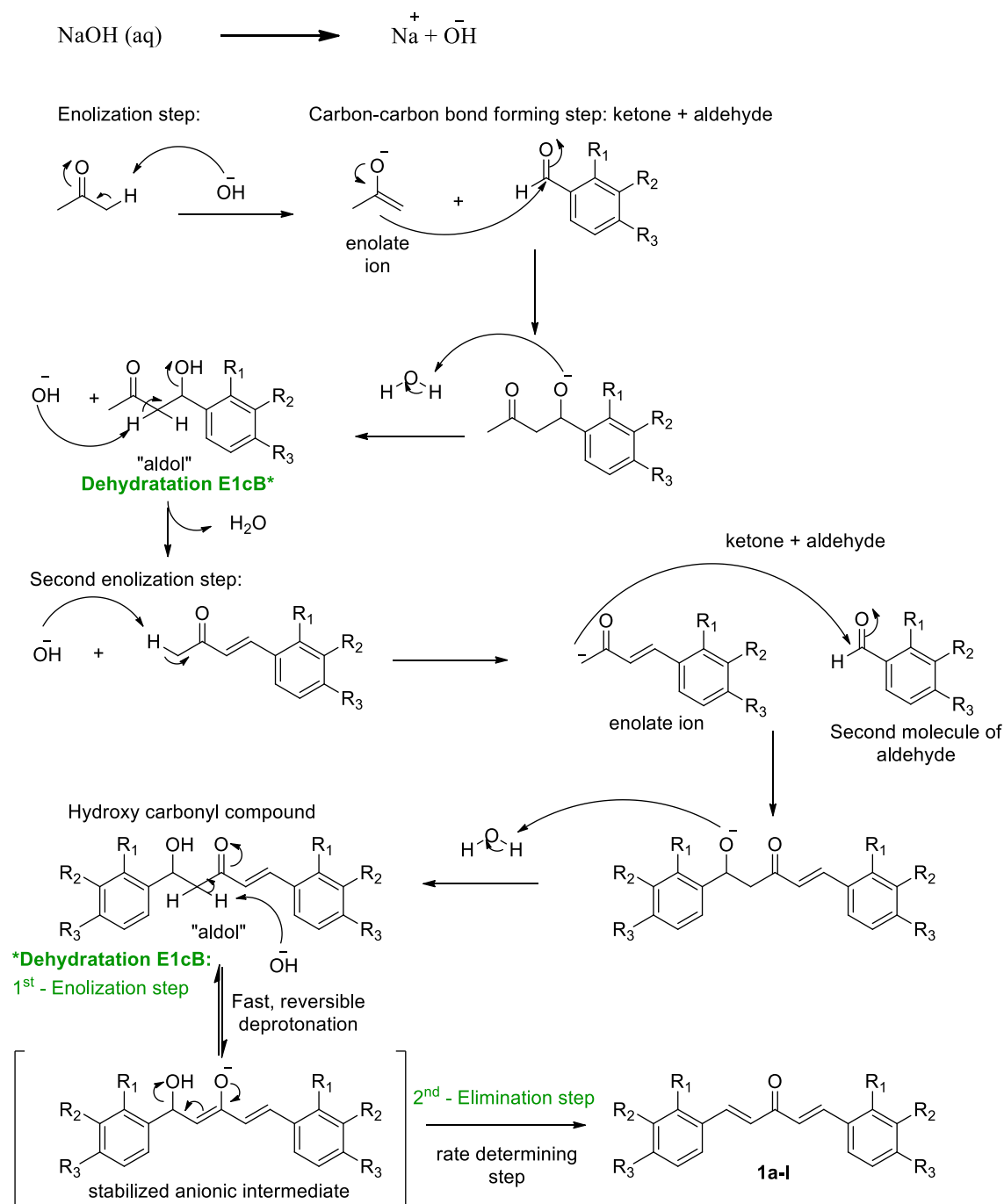


Figure 2.2 – Mechanism of the general aldol reaction to synthesize the monocarbonyl analogues of curcumin **1a-l**.

The yield of the synthesis of each compound was calculated and the melting point of the synthesized compounds was measured. Both are presented in table 2.2.

Table 2.2 – Yield and melting point of synthesized monocarbonyl analogues **1a-l**.

Compound	Substituents			Yield (%)	Melting point (°C)
	R ₁	R ₂	R ₃		
1a (C2)	H	OCH ₃	H	21.6	(¹)
1b	OCH ₃	OCH ₃	OCH ₃	46.8	(¹)
1c	H	H	OCH ₃	59.6	124-125
1d	Br	H	H	20.7	64-66
1e	H	H	H	10.5	58-59
1f	NO ₂	H	H	9.9	158-159
1g (C1)	OCH ₃	H	H	77.5	123-124
1h	OCH ₂ CH ₃	H	H	35.3	83-84
1i	CF ₃	H	H	49.7	131-132
1j	CH ₃	H	H	70.1	90-91
1k	F	H	H	82.4*	(¹)
1l	OCF ₃	H	H	11.8*	(¹)

*compounds not pure (¹) oil

The yield of the reaction reflects the reactivity of the reagents and all the procedures performed to obtain the final pure product. The yields of the synthesis of the monocarbonyl analogues of curcumin **1a-j** vary from 9.9 to 77.5%.

Observing the obtained yield values and considering what is reported in literature [82–84] about the reactivity of substituted benzaldehydes is possible to relate the yields with the functional groups present on the aromatic ring.

The aldol condensation reaction velocity may be influenced by several factors, such as inductive effect of the aryl substituents, resonance effects and steric or proximity effects. The resonance effect may occur with the ring or with the functional groups. A resonance polar effect contributes to electron availability or deficit at the different positions on the ring and can be subdivided in: (i) direct influence exerted from the *ortho* and *para* positions; (ii) resonance inductive effect exerted by the *meta* position due to the charges induced *ortho* to functional group. The steric effects can alter either the interaction of the substituent with the ring or affect the coupling of the carbonyl group with the aromatic system [83]. Also, a hydrogen bonding may occur between the formyl hydrogen and electron-rich substituents in *ortho* position. Such hydrogen bonding would be expected to withdraw positive charge from the carbonyl group and stabilize the starting material and, therefore, it would decrease the susceptibility to nucleophilic attack, and consequently, the reactivity. Although this possibility is not much considered

because it doesn't explain the observed, for instance, Wolfenden *et al.* detected an increased rate for reaction with a methyl in *ortho* position, what cannot be explained by such hydrogen bonding since methyl group has no available free electron pair [84].

Our yields suggest the reactivity of the substituted benzaldehydes in *ortho* position would be: $\text{OCH}_3 > \text{CH}_3 > \text{CF}_3 > \text{OCH}_2\text{CH}_3 > \text{Br} > \text{H} > \text{NO}_2$. These results are consistent with what was reported by Wolfenden *et al.* for the reactivity of substituted benzaldehydes for a reaction similar to our aldol condensation, that also have two steps: an addition of a nucleophile to the carbonyl followed by dehydration. For the reaction for benzaldehydes substituted in *ortho* position, velocity constant is higher for methoxy followed by methyl group and then the non-substituted benzaldehyde [84]. Additionally, Das *et al.* reported that the reactivity for *ortho* substituted benzaldehydes is: $\text{OCH}_3 > \text{Br} > \text{F} > \text{CH}_3 > \text{CF}_3 > \text{NO}_2$ [82]. Our compound with fluorine group were not obtained essentially pure, so the yield can't be considered to compare with the others. Considering our yields, only the one obtained for compound with the bromo in *ortho* position is not in agreement with this reactivity. However, as it was previously referred, the yields don't reflect only the reactivity of the reaction, so it is possible that the compound react very well but in the followed procedures to get the compound pure may contribute to the diminishing of the yield.

Comparing the yields of compounds **1a**, **1c** and **1g**, it is verified that the presence of methoxy group in the *ortho* position leads to an higher yield (77.5%), followed by the group in *para* position (59.6%) and for the last, the lower yield (21.6%) occurs to the compound with the methoxy group in *meta* position. These yields are consistent on what is reported for the reactivity of the methoxy-benzaldehydes: the *ortho* substituted is reported to be more reactive than the *meta* and *para* substituted benzaldehydes [82–84].

Steric effects may justify the yield of compound **1h** being lower than compound **1g**. Ethoxy groups are larger than methoxy groups, therefore, it is possible that the ethoxy group causes a hinderance, diminishing the access to carbonyl group, making it difficult the enolate nucleophilic attack over the electrophilic carbonyl group and, consequently, lowering the formation of the final product.

The lowest yield (9.9%) is observed for the compound that has a nitro group in *ortho* position. For the benzaldehydes with the nitro group in this position, the information available in literature is diverse. Heitler *et al.* and for Wolfenden *et al.* the *ortho*-nitro-benzaldehydes are the more reactive ones while for Das *et al.* these *ortho*-substituted benzaldehydes are the less reactive. For nitro substituted benzaldehyde occurs a movement for electrons towards the nitro group (figure 2.3) that leads to a positive charge on the carbon bond to carbonyl group, that consequently diminishes the partial positive charge (δ^+) on the carbonyl and therefore difficult the nucleophilic attack,

reducing its reactivity. Another hypothesis is that the steric effect reduces the reactivity of the compound, i.e., the size of the nitro group causes hinderance, making it difficult the reaction with the carbonyl group [82–84].

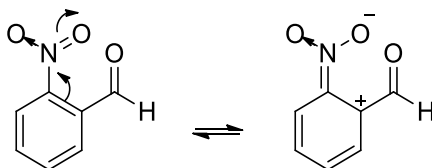


Figure 2.3 – Resonance structure of *ortho*-nitro-benzaldehyde.

Compounds **1k** and **1l** were not obtained in an essentially pure state. Therefore, the presence of impurities on compounds **1k** contribute to an increase of mass obtained and, consequently, to a high yield. Contrarily, compound **1l**, presented a low yield, which may be due to the attempts to purify this compound. Firstly, it was performed a column chromatography and then a preparative thin layer chromatography, which lead to an inevitable loss of product.

The higher melting point occurs in the compound with a nitro group in the *ortho* position while the lower is reported to the compound without substituents.

2.1 Structural characterization

The full structural characterization of the synthesized compounds was carried out by nuclear magnetic resonance spectroscopy (^1H -NMR, ^{13}C -NMR, COSY, HMBC, HMQC), electrospray ionization mass spectrometry, infrared spectroscopy, elemental analysis and a determination of maxima absorbance in UV-Vis of each compound. In this chapter, a resume of the main characteristics of each compound for each analysis is reported. The spectra recorded are presented in Appendices section.

2.1.1 NMR Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is the main technique that allows us to identify the compounds. For each monocarbonyl analogue of curcumin (figure 2.1.1.1), ^1H -NMR, ^{13}C -NMR, COSY, HMBC, HMQC were recorded.

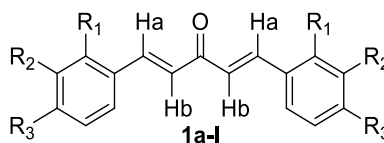


Figure 2.1.1.1 – General structure for monocarbonyl analogues of curcumin **1a-j**.

In tables 2.1.1.1 and 2.1.1.2 are reported special features of ^1H and ^{13}C NMR characterization of the synthesized compounds.

Monocarbonyl analogues of curcumin **1a-j** have a symmetrical molecular structure, therefore, the spectra look simplified. In the ^1H NMR spectra, the compounds are mainly identified by the presence of the vinylic hydrogens and by the signals of the substituents on the aromatic ring.

In most spectra, vinyl hydrogens produce doublets that integrate for two hydrogens with a coupling constant of 16 Hz, that corresponds to geometry *trans*, which region varies between 7.69 and 8.16 ppm for H_a and 6.97 and 7.23 ppm for H_b . These variations regarding the chemical shifts are the result of the influence of the substituents present on the aromatic ring. Curiously, it is verified that the higher value for H_a corresponds to the lowest value for H_b and that both occur for compound **1f**, which has a nitro group in the *ortho* position. Also, it is important to highlight that for compound **1i**, the vinylic hydrogen H_a , produces a double doublet, contrary to all the other compounds that exhibit a doublet. This multiplicity may be explained by the influence of CF_3 group, once the presence of atoms of fluorine in a molecule results in the existence of multiplicity in both spectra of ^1H and ^{13}C NMR, due to ^{19}F - ^1H and ^{19}F - ^{13}C coupling [85].

Regarding the signals correspondent to the substituents on the aromatic ring, compounds **1a**, **1c** and **1g**, all have a methoxy group, respectively, in *meta*, *para* and *ortho* position, it is observed that the singlet is present at 3.82, 3.85 and 3.91 ppm, respectively. These different values result from the different resonance that can occur with the three structures. In the compounds **1g** and **1c**, the hydrogens of the methoxy group are more deshielded, which result from the conjugated and extended resonance with the substituents, the double bond and the carbonyl group.

Table 2.1.1.1 – Special features of ¹H NMR characterization of synthesized monocarbonyl analogues **1a-j**.

Compound	Substituent			δ (ppm)						
	R ₁	R ₂	R ₃	H _a	H _b	R ₁	R ₂	R ₃	R ₄ (H)	R ₅ (H)
1a (C2)	H	OCH ₃	H	7.69 (2H, d, J=16 Hz)	7.85 (2H, d, J=16 Hz)	7.12 (2H, t, J=2 Hz)	3.82 (6H, s)	6.94 (2H, dq J=8 Hz)	7.31 (2H, t, J=8 Hz)	7.19 (2H, d, J=8 Hz)
1b	OCH ₃	OCH ₃	OCH ₃	7.71 (2H, d, J=16 Hz)	7.00 (2H, d, J=16 Hz)	3.80 (6H, s)	3.79 (6H, s)	3.86 (6H, s)	6.62 (2H, d, J=9 Hz)	7.27 (2H, d, J=9 Hz)
1c	H	H	OCH ₃	8.09 (2H, d, J=16 Hz)	7.03 (2H, d, J=16 Hz)	–	7.71 (2H, dd, J=2; 8 Hz)	7.57 (4H, d J=9 Hz)	7.36 (2H, t J=7 Hz)	7.64 (2H, d, J=8 Hz)
1d	Br	H	H	7.75 (2H, d, J=16 Hz)	7.10 (2H, d, J=16 Hz)	–	7.62 (4H, m)	3.85 (6H, s)	6.94 (4H, d J=9 Hz)	–
1e	H	H	H	8.16 (2H, d, J=16 Hz)	6.97 (2H, d, J=16 Hz)	–	8.08 (2H, d, J=8 Hz)	R ₁ = R ₅	7.41 (6H, m)	7.58 (2H, td, J=2; 8 Hz)
1f	NO ₂	H	H	8.07 (2H, d, J=16 Hz)	7.18 (2H, d, J=16 Hz)	–	7.72 (4H, m)	R ₂ = R ₄	7.62 (2H, dd, J=2, 8 Hz)	–
1g (C1)	OCH ₃	H	H	8.05 (2H, d, J=16 Hz)	7.23 (2H, d, J=16 Hz)	–	6.94 (2H, d, J=8 Hz)	R ₃	6.99 (2H, t, J=8 Hz)	–
1h	OCH ₂ CH ₃	H	H	8.09 (2H, dd, J= 16, 2 Hz)	7.01 (2H, d, J=16 Hz)	4.14 (4H, q, J=7 Hz) 1.51 (6H, t, J=7 Hz)	7.37 (2H, td, J=2, 8 Hz)	7.60 (2H, dd, J=2, 8 Hz)	6.98 (2H, td, J=1, 8 Hz)	7.34 (2H, ddd, J=1, 2, 8 Hz)
1i	CF ₃	H	H	8.09 (2H, dd, J= 16, 2 Hz)	7.01 (2H, d, J=16 Hz)	–	7.74 (2H, d, J=8 Hz)	7.51 (2H, t, J=8 Hz)	7.61 (2H, t, J=7 Hz)	7.79 (2H, d, J=8 Hz)
1j	CH ₃	H	H	8.05 (2H, d, J=16 Hz)	7.00 (2H, d, J=16 Hz)	2.49 (6H, s)	7.22-7.34 (6H, m)	7.66 (2H, d, J=8 Hz)	–	–

Table 2.1.1.2 – Special features of ¹³C NMR characterization of synthesized monocarbonyl analogues **1a-j**.

	1j	1i	1h	1g (C1)	1f	1e	1d	1c	1b	1a (C2)	Compound			
											Carbonyl	Vinyl		R ₁
												a	b	
	189.06	188.44	190.32	190.13	188.46	189.04	188.82	188.99	189.43	188.76				
	141.04	139.39	138.65	138.33	139.57	143.44	142.14	142.81	137.71	143.17				
	126.85	129.02	126.56	126.40	129.36	125.47	127.87	123.66	124.83	125.63				
	138.34 (CCH ₃)	128.65 (CCF ₃) 124.12 (CF ₃)	158.19 (COCH ₂ CH ₃) 64.13 (COCH ₂ CH ₃) 14.98 (COCH ₂ CH ₃)	158.71 (COCH ₃) 55.65 (COCH ₃)	148.67 (CNO ₂)		126.01 (CBr)	R ₁ = R ₅ 130.21 (CH)	155.56 (COCH ₃) 55.97 (COCH ₃)	113.30 (CH)				
	126.50 (CH)	126.45 (q, CH)	112.23 (CH)	111.33 (CH)	125.23 (CH)	R ₁ = R ₅ 128.50 (CH)	128.00 (CH)	R ₃ 161.70 (COCH ₃) 55.53	142.26 (COCH ₃) 60.81 (COCH ₃)	159.93 (COCH ₃) 55.25 (COCH ₃)				
	R ₃ = R ₄ 126.56 (CH)	129.96 (CH)	129.41 (CH)	131.68 (CH)	133.82 (CH)	R ₂ = R ₄ 130.62 (CH)	131.55 (CH)	(COCH ₃) R ₂ = R ₄ 114.56 (CH)	153.46 (COCH ₃) 61.39 (COCH ₃)	116.30 (CH)				
	R ₅ 131.03 (CH)	132.33 (CH)	120.76 (CH)	120.87 (CH)			127.92 (CH)		107.59 (CH)	129.91 (CH)				
		128.76 (CH)	131.58 (CH)	128.84 (CH)	130.69 (CH)		133.70 (CH)		123.32 (CH)	121.06 (CH)				

In the ^{13}C NMR, the compounds are mainly identified by the presence of the signals for the mono-carbonyl group and by the signals of the substituents on the aromatic ring. It is observed that signals for carbonyl have an insignificant variation between compounds, all of them are between 188 and 190 ppm, typical value for carbonyl compounds.

Regarding the vinylic carbons, it is possible to observe that the values for carbon bond to H_a vary between 138 and 143 ppm and for carbon bond to H_b , vary between 124 and 129 ppm, characteristic values for these carbons.

Comparing the values obtained for carbons on *ortho* positions, they vary between 113 and 158 ppm, due to the influence of substituents on these carbons. The lower values are observed for compounds which present a hydrogen on this position. The higher values, above 155 ppm results from the influence of substituents with an oxygen (methoxy and ethoxy groups) which turn the carbons more deshielded. The same is verified for the *meta* and *para* positions, which means that the lower values (111 ppm for *meta* position and 116 for *para* position) result from the absence of substituents on these positions. Also, the higher values (160 ppm for *meta* position and 162 ppm for *para* position) are due to the presence of methoxy groups, being these values higher than the presented for *ortho* position due to the resonance existent with the double bond and the carbonyl group.

As already referred, the existence of fluorine atoms in a molecule results in the presence of multiplicity on ^{13}C NMR, due to ^{19}F - ^{13}C coupling [85]. Minding compound **1i**, it is interesting to mention the presence of a quartet, as a result of the splitting observed, which was induced by the presence of a trifluoromethyl substituent.

2.1.2 IR Spectroscopy

The solid compounds were analysed by Infrared spectroscopy, using KBr tablets. IR spectroscopy helps us to confirm the presence of specific groups of the molecule, such as the carbonyl group and some of the substituents on the aromatic ring. These relevant absorption bands are resumed in table 2.1.2.1.

Table 2.1.2.1 – Main characteristic bands on IR spectra of each monocarbonyl analogue of curcumin analysed.

Compound	IR absorption band (cm ⁻¹)			
	Substituents			Carbonyl
	R ₁	R ₂	R ₃	
1a (C2)*	H –	OCH ₃ –	H –	–
1b*	OCH ₃ –	OCH ₃ –	OCH ₃ –	–
1c	H	H	OCH ₃ 1240 and 1176	1597
1d	Br 1192	H	H	1647
1e	H	H	H	1647
1f	NO ₂ 1508 and 1340	H	H	1624
1g (C1)	OCH ₃ 1332 and 1252	H	H	1614
1h	OCH ₂ CH ₃ 1333, 1250 and 556	H	H	1670
1i	CF ₃ 1155 – 1198	H	H	1591
1j	CH ₃ 2945 – 2924	H	H	1612

* IR spectroscopy was not performed for oils

The tabled shifts for aldehydes and ketones presume that the carbonyl group presents absorption bands at the region 1690-1750 cm⁻¹. All the compounds present the absorption band derived from the carbonyl is between 1591-1670 cm⁻¹, being observed a lower value than the bibliographic values and this may be explained by the highly conjugation that occurs between the substituted aromatic ring and the carbonyl group [86].

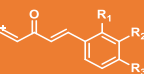
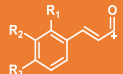
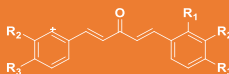
For compound **1f**, it is observed two N-O stretching bands at 1508 and 1340 cm⁻¹. These values are accordingly to expected, once nitro groups attached to aromatic rings present stretching bands at regions 1550-1475 cm⁻¹ and 1360-1290 cm⁻¹ [87].

Comparing the shifts for methoxy groups in compounds **1c** and **1g**, it is verified that they are lower for the compound which has the methoxy group in *para* position, which was expected once the conjugation is higher in that compound. The same occurs for the shift of the carbonyl, the compound **1c** presents the carbonyl band at lower frequency.

2.1.3 ESI mass spectroscopy

In order to confirm the structure of the synthesized monocarbonyl analogues, electrospray ionization mass spectrometry was performed, which allowed to identify the molecular ion and some fragments of the compounds (table 2.1.3.1).

Table 2.1.3.1 – m/z values of the fragments of the analogues **1a-j**, identified by ESI⁺-MS.

Compound	1a (C2)	1b	1c	1d	1e	1f	1g (C1)	1h	1i	1j	1k	1l
[M+H] ⁺	295 (100)	415 (100)	295 (100)	393 (25)	235 (100)	325 (10)	295 (100)	323 (100)	371 (70)	263 (100)	271 (100)	403 (45)
M+Na		437	317				317 (15)	345 (25)				
2M+H		829	589	785			589				805 (10)	
2M+Na	611	852						667 (10)	763			
2M+K					507 (10)							
3M+H					703							
								135 (43)		105 (23)		
							187 (20)	210 (30)				
	162 (11)		162 (2)		131 (10)		162 (6)	173 (50)		145 (6)	149 (55)	215 (60)
						280 (12)				247 (6)		

Observing the table 2.1.3.1, it is possible to observe that although the compounds have the same general structure, not all of them fragment the same way. Four types of fragments were detected. One of the types of the fragments occurs to eight of the twelve compounds, so the monocarbonyl analogues of curcumin mostly fragment on the carbonyl group.

The presence of $[M+H]^+$ peak was important to confirm the molecular weight of the synthesized monocarbonyl analogues of curcumin. Also, to some of the compounds (**1a-c**, **1e** and **1g-i**) it was possible to identify adducts with sodium and potassium. For compounds **1b**, **1c**, **1g** and **1h** peaks for $M+Na$ for compounds were detected while for compounds **1a**, **1b**, **1h** and **1i** peaks for $2M+Na$ for compounds were identified. Only for compound **1e**, an adduct with potassium corresponding to $2M+K$ was observed.

Regarding compound **1d**, which has bromine group as an *ortho* substituent, and an exact mass of 389.93, it was detected the $[M+H]^+$ at 392.8. This may be explained by the simultaneous presence of ^{79}Br and ^{81}Br , in the two aromatic rings.

Although it wasn't possible to get compounds **1k** and **1l** totally purified, the electrospray ionization mass spectrometry proved their presence in the final product.

2.1.4 Elemental Analysis

Elemental analysis was performed to confirm the purity of the solid synthesized monocarbonyl analogues. The theoretical and the practical determination is reported in table 2.1.4.1.

Table 2.1.4.1 – Elemental analysis of synthesized monocarbonyl analogues **1c-j**.

Compound	Theoretical (%)				Experimental (%)							
	C	N	H	O	C		N		H		O	
					Average	Deviation	Average	Deviation	Average	Deviation	Average	Deviation
1a (C2)*	–	–	–	–	–	–	–	–	–	–	–	–
1b*	–	–	–	–	–	–	–	–	–	–	–	–
1c	77.53	0	6.16	16.31	77.19	0.34	–	–	6.18	0.02	16.55	0.24
1d	52.08	0	3.08	4.08	53.25	1.17	–	–	3.88	0.80	5.38	1.30
1e	87.15	0	6.02	6.83	85.02	2.13	–	–	6.71	0.69	7.83	1.00
1f	62.96	8.64	3.73	24.67	63.06	0.10	8.52	0.12	4.05	0.32	25.25	0.2
1g (C1)	77.53	0	6.16	16.31	77.44	0.09	–	–	6.30	0.14	16.50	0.19
1h	78.23	0	6.88	14.89	77.97	0.26	–	–	6.98	0.10	15.07	0.18
1i	61.63	0	3.27	4.32	61.52	0.11	–	–	3.47	0.20	6.99	2.67
1j	86.99	0	6.92	6.10	86.68	0.31	–	–	7.06	0.14	6.21	0.11

* Elemental analysis was not performed for oils

In general, the structural analysis of the synthesized monocarbonyl analogues of curcumin showed deviations from theoretical percentages below 0.4% (control criteria), except for compounds **1d**, **1e** and **1i**. For compounds **1d** and **1i**, the deviations might be explained by the presence of substituents on the aromatic ring with halogens (Br and CF₃, respectively). Regarding the deviation between duplicates, only compound **1i** showed a deviation above 0.4%, probably due to the presence of fluorine.

2.1.5 UV spectroscopy

For each synthesized monocarbonyl analogue of curcumin **1a-l**, a scan was performed between 400 and 200 nm, in a UV-spectrophotometer in order to determine the maximum of absorbance (abs). The compounds were dissolved in a solution of acetonitrile (ACN), which concentration is in the order of 10^{-5} M. The values of the absorption maximum of each spectrum are presented in table 2.1.5.1.

Table 2.1.5.1 – UV maximum of absorbance of each compound.

Compound	Abs	λ (nm)
1a (C2)	1.613	316.40
	0.860	242.40
	2.626	211.00
1b	0.292	340.80
	0.285	333.20
	1.073	203.80
1c	1.608	356.40
	0.887	238.00
1d	0.124	295.60
	0.124	292.80
1e	0.679	303.20
	0.526	224.00
1f	0.788	273.60
1g (C1)	0.597	350.60
	0.867	280.20
1h	0.621	358.80
	0.393	307.60
	0.287	239.60
	0.832	210.20
1i	1.197	301.60
	0.927	224.60
1j	0.849	329.60
	0.375	232.20
	1.104	203.80
1k	0.836	284.60
1l	0.884	284.60

The compounds have its maximum of absorbance between 273 and 358 nm and these values are fundamental to the identification and quantification of compounds in High Performance Liquid Chromatography with UV-Vis detectors (HPLC-UV/Vis) experiments.

3 Assessment of “drug-likeness” properties

“Drug-likeness” consists in the sum of several characteristic properties of drugs, being a concept related and that can be explained by Lipinski’s Rule of Five (Ro5) [88]. These properties may help medicinal chemists in the design and discover of hits with good pharmacokinetic and pharmacodynamic profiles. Usually, the “drug-likeness” properties comprise lipophilicity, polarity, size and solubility and several biological properties, including none or low cytochromes P450 (CYP) inhibition, metabolic stability and no potential toxicity [89].

Predict the toxicity is crucial in order to reduce costs and consequences of drug evaluations in cellular, animal and clinical tests. The risks of clinical trials reveals the importance of detect the toxicity of compounds as early as possible, preventing toxic drugs to reach those trials [90]. There are several reasons to perform the assessment of “drug-likeness” properties, for instance, to determinate the electrophilicity of the compounds. These assays are important to identify false positives in drug discovery [91]. Additionally, our synthesized compounds may act as Michael acceptors, which might be related with toxicity. Thus, these assays are important to evaluate the interactions of our compounds with endogenous molecules. However, the assessment is limited, even if the assays mimics the physiological conditions, there are always differences, once the reactivity of cysteine depends on the protein environment [91]. In fact, understanding the reactivity and characterize the electrophilic character of the β -position of α,β -unsaturated carbonyl compounds allows the rational design of potential drugs [92].

This chapter reports on drug-like properties of the synthesized monocarbonyl analogues of curcumin that were evaluated, including the lipophilicity, stability in physiological conditions and the electrophilic character of each compound, as a measure of the potential toxicity.

3.1 Partition coefficients determination

Lipophilicity is an extremely important physicochemical feature in drug discovery and development, once it affects the drug activity in its pharmaceutical, pharmacokinetic and pharmacodynamic action. This molecular property gives information about drug behaviour, such as its ability to cross cell membranes, its absorption and distribution, and, therefore, its interaction with the targets [93]. The lipophilicity is intrinsically

associated with the biological activity, once the affinity for a lipophilic environment facilitates the passive diffusion through membranes and the formation of complexes between compounds and the receptor binding site [93]. Contrarily, a low lipophilicity corresponds to a high solubility in aqueous phase, which is important for drug-likeness but when it is extremely low, it could compromise the ability of the drug to achieve the target. Since it possesses poor permeability through cell membranes, its absorption or the transport across the blood-brain barrier is limited. So, an efficient solubility and permeability is associated with a low range of lipophilicity. Also, high lipophilicity is associated with high clearance and low metabolic stability [94].

The lipophilicity of a compound can be quantified by its partition coefficient (P), which is defined as the ratio of the concentrations of a compound in two immiscible solvents, constituting an organic and an aqueous phases, generally water and *n*-octanol, respectively, at a thermodynamic equilibrium [93,94].

The experimental determination of lipophilicity was done using the shaking flask method, which permits to calculate the partition coefficient using a biphasic system water-octanol, being a good approximation of the partitioning between the cytosol and lipidic membranes of living systems [93]. The procedure consists in an extraction in *n*-octanol and phosphate phase. It is usually expressed in a logarithmic scale, log P, as shown in equation (1).

$$\text{Log } P = \log_{10} \left(\frac{[\text{compound}]_{\text{octanol}}}{[\text{compound}]_{\text{phosphate}}} \right) \text{ (Eq. 1)}$$

Christopher Lipinski, a Medicinal Chemist from Pfizer, published in 1997 a set of rules for properties of well absorbed drugs, which is known by Lipinski's Rule of Five (Ro5). This rules predicts that a good absorption or permeation is more likely to occurs when there has no more than one violation of the following criteria: no more than 5 hydrogen bond donors (HBD), no more than 10 hydrogen bond acceptors (HBA), the molecular weight (MW) lower than 500 Da and the quantified log P below 5. Although, this set of rules only applies to compounds that are not substrates for active transporters [95,96].

The log P values obtained experimentally were compared with those calculated by the computational programs ALOGPS 2.1, Chemdraw and Molinspiration software and are depicted in table 3.1.1.

Table 3.1.1 – Calculated and experimental log P values of monocarbonyl analogues of curcumin **1a-j**.

Compound	Substituents			Log P						
	R ₁	R ₂	R ₃	Calculated			Experimental	Difference		
				(1)	(2)	(3)		(1)	(2)	(3)
1a (C2)	H	OCH ₃	H	4.23	3.77	4.25	2.83	1.40	0.94	1.42
1b	OCH ₃	OCH ₃	OCH ₃	4.28	3.26	3.48	ND ^a	–	–	–
1c	H	H	OCH ₃	4.24	3.77	4.3	3.81	0.43	-0.04	0.49
1d	Br	H	H	5.38	5.68	5.35	3.58	1.80	2.10	1.77
1e	H	H	H	3.88	4.02	4.18	3.25	0.63	0.77	0.93
1f	NO ₂	H	H	3.86	4.01	3.65	2.15	1.71	1.86	1.50
1g (C1)	OCH ₃	H	H	4.18	3.77	3.84	ND ^a	–	–	–
1h	OCH ₂ CH ₃	H	H	5.06	4.44	4.59	3.36	1.70	1.08	1.23
1i	CF ₃	H	H	5.12	5.86	5.52	3.88	1.24	1.98	1.64
1j	CH ₃	H	H	4.71	4.99	4.63	4.08	0.63	0.91	0.55

Predicted by ⁽¹⁾ ALOGPs 2.1 ⁽²⁾ ChemDraw ⁽³⁾ Molinspiration; ^a Not determinated

Results from table 3.1.1 shows that in general, the synthesized monocarbonyl analogues of curcumin present an experimental log P value lower than the calculated. Analysis of the results obtained let us verify that the compounds have very close values of log P, except compounds **1a**, **1f** which has lower values of 2.83 and 2.15, respectively and **1j** presenting a higher value of 4.08, all the others vary between 3.25 and 3.88, so all synthesized monocarbonyl analogues are lipophilic but still presenting log P values under 5, contrary to calculated for compounds **1d**, **1h** and **1i**.

The compound with the lowest log P value is compound **1f**. This analogue has a nitro group in *ortho* position, which is an electron withdrawing group and consists in a charged group, leading to a decrease the lipophilicity. It is known that electron-withdrawing groups diminishes lipophilicity [97], which may explain this log P value obtained for compound **1f**. On the other hand, alkyl groups and halogens are responsible for increasing the lipophilicity [98], what explains the fact that compound **1j**, which has a methyl group in *ortho* position, presented the higher experimental log P value.

The difference in the log P values obtained experimentally for the mono substituted methoxy analogues **1a**, **1c** and **1g** makes it possible to note the influence of the position of the substituent in the aromatic ring over the lipophilicity. For compound **1g**, which has a methoxy group in *ortho* position it was not possible to determine the log P value because the compound is too lipophilic, as it can't be quantified in the phosphate phase. Compound **1c**, which has a methoxy group in *para* position, has a higher log P

value than **1a**, *meta* substituted. It occurs once the methoxy groups interact strongly as an electron donating by resonance with the aromatic ring and the conjugated system and having also an inductive effect, being those interactions mostly marked when the methoxy group occupies the *ortho* and *para* positions [99]. Further, when in *ortho* position, it may occur a through-space level of interactions, such as (i) intramolecular H-bonds, which partially or completely reduce the H-bonds with the environment, being responsible for an increase of lipophilicity of the compounds and/or (ii) it may occur a steric hindrance with out-of-plane rotation, promoting a decrease in lipophilicity [99,100].

Furthermore, the other parameters referred on Lipinski rule of 5 were also considered for all the compounds (table 3.1.2).

Table 3.1.2 – “Lipinski’s rule of 5” parameters of monocarbonyl analogues of curcumin **1a-j**.

Compound	Substituents			HBA	HBD	MW
	R ₁	R ₂	R ₃			
1a (C2)	H	OCH ₃	H	3	0	294.34
1b	OCH ₃	OCH ₃	OCH ₃	7	0	414.45
1c	H	H	OCH ₃	3	0	294.34
1d	Br	H	H	3	0	392.08
1e	H	H	H	1	0	234.29
1f	NO ₂	H	H	3	0	324.29
1g (C1)	OCH ₃	H	H	3	0	294.34
1h	OCH ₂ CH ₃	H	H	3	0	322.40
1i	CF ₃	H	H	3	0	370.29
1j	CH ₃	H	H	1	0	262.35

All the compounds fulfil the parameters described in rule of five. Considering the molecular weight, all monocarbonyl analogues presented MW lower than 500 Da, varying between 234 and 414 Da, so all of them, theoretically, can permeate across biological membranes.

3.2 Stability in physiological conditions

It is important to evaluate how the curcumin monocarbonyl analogues behave in conditions that mimics the ones they would find in the human body, evaluating its chemical and enzymatic stability. Stability studies under physiological conditions were performed in PBS with 20% of acetonitrile and in human plasma diluted to 80% (v/v) with PBS (0.01 M, pH 7.4), at 37 °C, for 48 hours. The assays were monitored by HPLC-UV.

3.2.1 Stability in Phosphate buffered saline

Phosphate-buffered saline (PBS) consists in a buffer solution, which is widely used in research, composed by a water-based salt solution with sodium phosphate (Na_2HPO_4), sodium chloride (NaCl), and in some formulations, potassium chloride (KCl) and potassium phosphate (KH_2PO_4) [101,102]. This buffer is commonly used in biological studies due to its non-toxicity and because it is an isotonic buffer that mimics the pH, osmolarity and ion concentrations of the human body [103].

Stability of the synthesized monocarbonyl analogues **1a-j** were evaluated at physiological pH in PBS (0.01 M, pH = 7.4) with 20% ACN at 37 °C. These assays were monitored by HPLC-UV, following the diminishing of the concentration of each compound along the time, which means loss of the compounds.

The pseudo first-order rate constants (k_{obs}) were obtained from the slope of the straight line from the plot of natural logarithm of the concentration *versus* time, as give in equation (2). Half-life ($t_{1/2}$) values, corresponding to the time that it takes for a substance to decrease one-half of its initial value, were calculated using equation (3).

$$\ln(A) = \ln(A_0) - K_{\text{obs}} \times t \quad (\text{Eq. 2})$$

A = concentration of compound at time t; A_0 = concentration of compound at time zero; t = time

$$t_{1/2} = \frac{\ln 2}{K_{\text{obs}}} \quad (\text{Eq. 3})$$

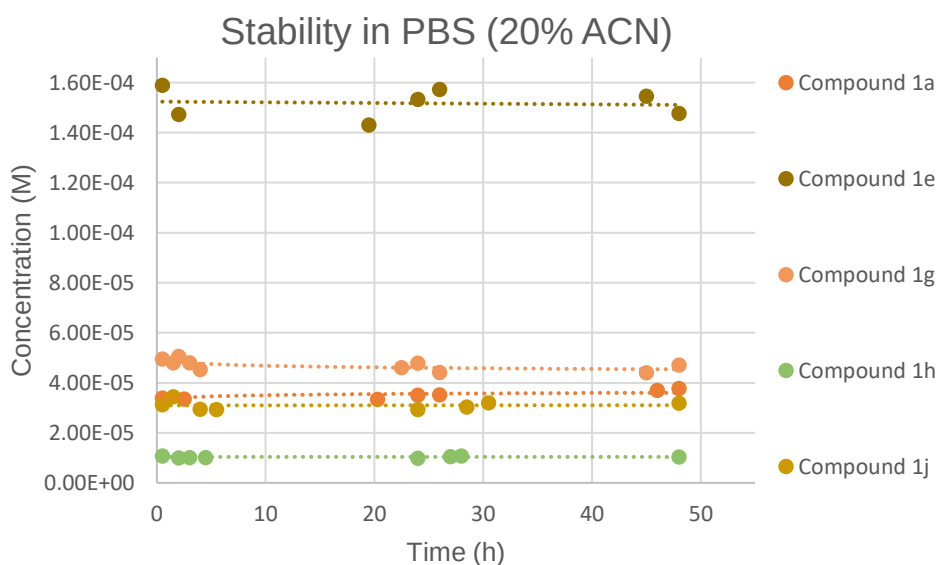


Figure 3.2.1.1 Stability in PBS of compounds **1a**, **1e**, **1g**, **1h** and **1j**.

Observing the graphic in the figure 3.2.1.1 is possible to note that during the 48 hours of assay, compounds **1a**, **1e**, **1g**, **1h** and **1j** maintain the concentration presented at the beginning of the assay. It means, the compounds are stable in PBS and therefore it was not possible to determine neither the first-order rate constant and the half-life values.

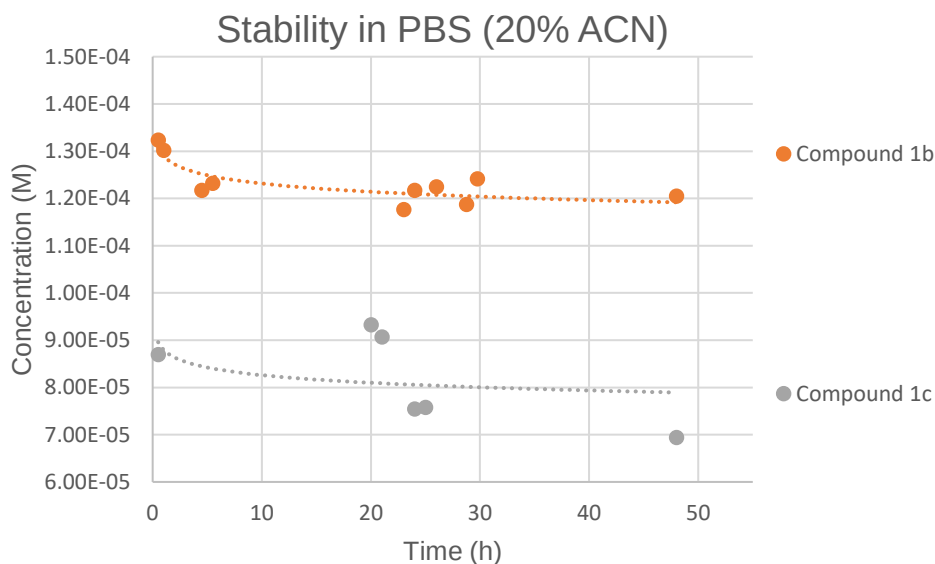


Figure 3.2.1.2 – Stability in PBS of compounds **1b** and **1c**.

However, for compounds **1b** and **1c**, the figure 3.2.1.2 allows us to see a decrease on the concentration of those compounds, along the 48 hours. Table 3.2.1.1 present the values for the percentage of degradation of the compounds **1a-j** after 48 h in physiologic conditions.

Table 3.2.1.1– Percentage of degradation for compounds **1a-j** after 48 h of incubation in PBS.

Compound	Degradation (%)
1a (C2)	0
1b	9.09
1c	18.66
1d	0
1e	1.5
1f	0
1g (C1)	1.29
1h	0
1i	0
1j	0

Analysing table 3.2.1.1, it is observed that compounds **1b**, **1c**, **1e** and **1g** presented a little degradation, being the higher degradation for compound **1c** (around 19%).

Results for compounds **1d**, **1f** and **1i** were different (figure 3.2.1.3).

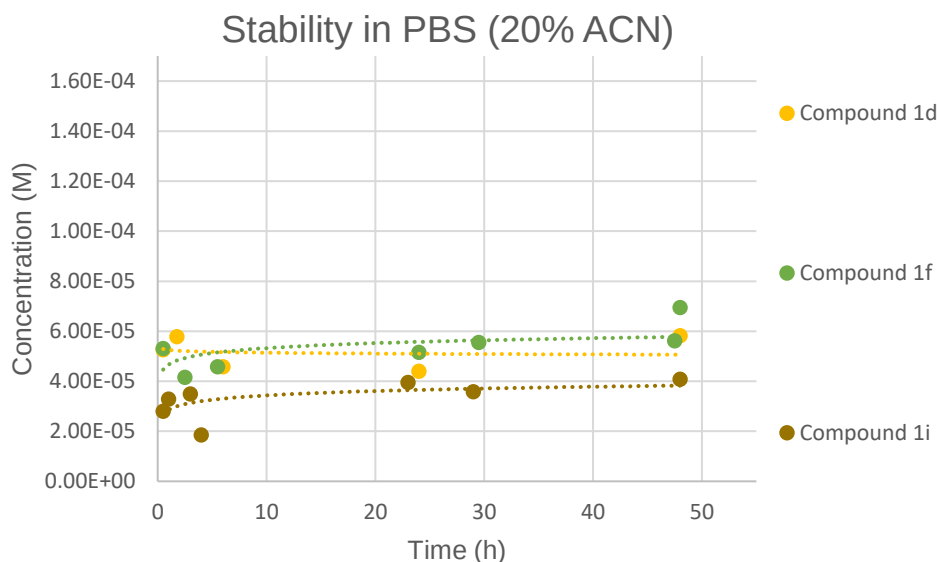


Figure 3.2.1.3 – Stability of compounds **1d**, **1f** and **1i** in PBS with 20% of ACN.

Compounds **1d**, **1f** and **1i**, presented the typical behaviour of compounds which solubility is poor, which is seen by the increase of concentration along the time.

Briefly, all the synthesized monocarbonyl analogues of curcumin demonstrate to be very stable in PBS with 20% ACN, at 37 °C, for 48 hours. Our results are consistent with what was reported by Dai *et al.*, where the evaluated monocarbonyl analogues of curcumin showed a significantly slow decay, it is, they proved to be stable in PBS [77].

3.2.2 Stability in human plasma

Human plasma is composed by water, proteins such as serum albumins, globulins and fibrinogen, hormones, nutrients and metabolites, electrolytes including Na^+ , Ca^+ , Mg^{2+} , HPO_4^{2-} / H_2PO_4 , Cl^- and HCO_3^- , glucose, clotting factors, oxygen and carbon dioxide [104].

Plasma is rich in hydrolytic enzymes, including aldolase, cholinesterase, lipase, alkaline and acid phosphatases and dehydropeptidases that can be responsible for the degradation of the compounds [105]. Once the hydrolysis in plasma occurs, the efficacy of the drug may be compromised because the concentrations may not be achievable *in vivo* or the compound is unable to achieve the target. Therefore, it is important to evaluate the stability of the monocarbonyl analogues of curcumin in human plasma. To

achieve that, the assay was performed at 37 °C in human plasma diluted to 80%(v/v) with PBS (0.01 M, pH 7.4).

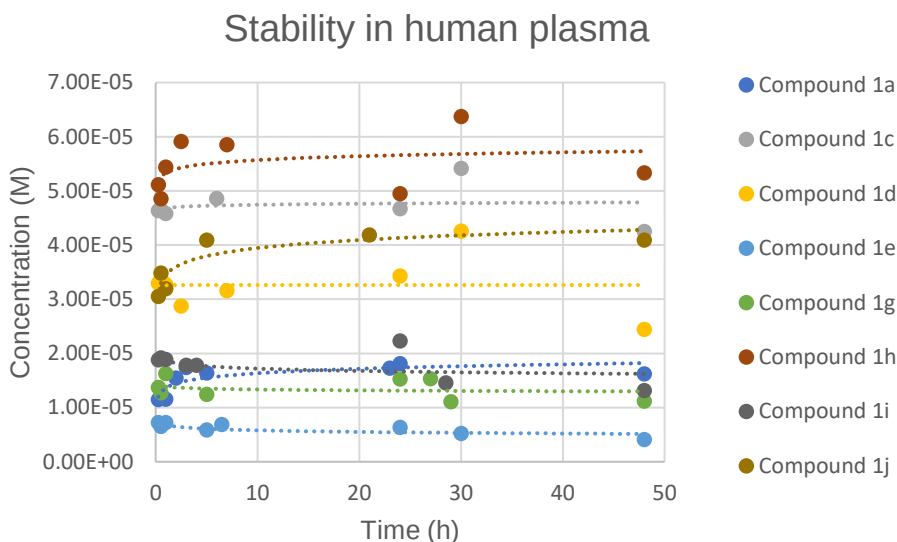


Figure 3.2.2.1 – Stability of compounds **1a**, **1c-1e** and **1g-1j** in human plasma (80%).

As it can be seen by the graphic (figure 3.2.2.1), compounds are stable in human plasma. The exceptions (figure 3.2.2.2) are compounds **1b** and **1f**.

Most compounds presented the typical behaviour of compounds which solubility is poor, which is verified by the variations of concentration (decreasing and then increasing) along the time.

Liang *et al.* reported the stability of compound **1d** in plasma of healthy rats, having a $t_{1/2}$ of about 3 hours [106]. Here we observe that compound **1d** is stable in human plasma. This difference may be explained because the plasma stability can vary among the species and typically compounds are less stable in rodents plasma than in human plasma [105].

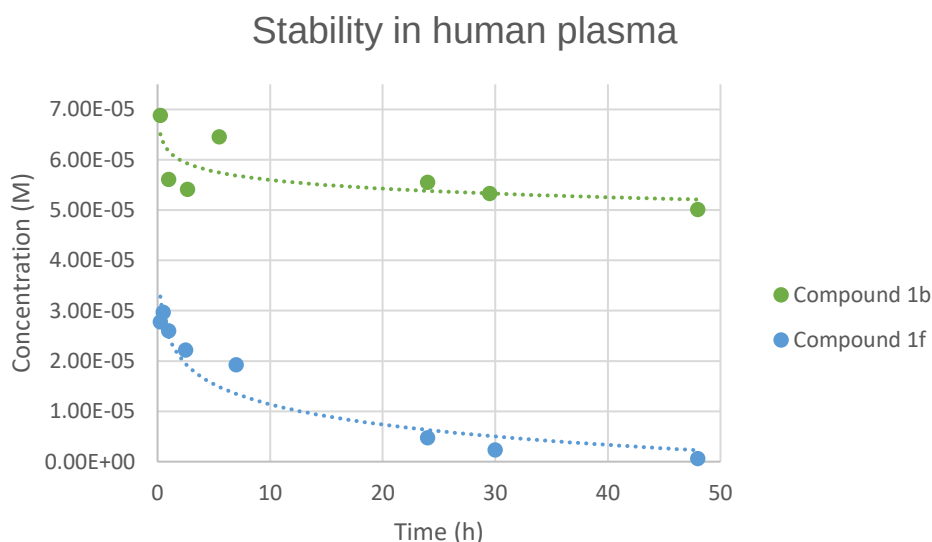


Figure 3.2.2.2 – Stability of compounds **1b** and **1f** in human plasma.

For the compound with the nitro group in *ortho* position, the assay was repeated in triplicate. The kinetic constants (K_{obs}) and half-lives values ($t_{1/2}$) are presented in table 3.2.2.1.

Table 3.2.2.1 – kinetic constants of compounds **1b** and **1f**.

Compound	K_{obs} (h^{-1})	$t_{1/2}$ (h)
1b	0.007	97.63
1f	0.082 ± 0.008	8.04 ± 0.71

Compound **1f** is the only one with short half-life (≈ 8 hours) contrasting with the other synthesized compounds.

Also, it is interesting to note that the compound **1f** is the one who presented a lower lipophilicity. That means that this analogue would have more difficulties to reach the target than the others, once it has less facility to cross membranes and because it is rapidly degraded by enzymes present in plasma.

3.3 Electrophilicity assessment

Several biological effects, including toxicological events, initiate by the interaction of the compounds with endogenous macromolecules [107,108]. It is important analyse these interactions regarding the organic chemical mechanisms involved because it might predict their potential toxicity, especially when the interaction between the drug molecule and the endogenous macromolecules consist in a covalent binding [107]. There are two mechanisms that explain how covalent binding might produce toxicity: (i) with the interaction of the compound with the macromolecules, the normal function of this ones can't be maintained; (ii) formation of toxic adducts between the compound and the

macromolecule [109]. Normally, in these potential interactions, the drug molecule act as an electrophile and the endogenous macromolecules as nucleophiles. The electrophilic centres include carbonyl groups, unsaturated bonds and halogenated carbons while the nucleophilic centre consist in sulphur, oxygen and nitrogen atoms [108].

There are several reactions that can occur due to the reactivity of α,β -unsaturated carbonyl compounds (figure 3.3.1), including Michael addition, radical scavenging, oxidation and double bond isomerization, being the nature of the acyl substituent groups (figure 3.3.1 – R_1 and R_2) essential for the reactivity observed. For instance, hydroxyl groups are oxidized to quinones and are the key to antioxidant activity. This activity is important, once it consists in the prevention or delay of the oxidation of other molecules, being also referred as scavenging activity, since the radicals are reduced or “trapped”, as nonradical species. This radical scavenging activity consists in trapping and stabilizing free radicals and occurs in α,β -unsaturated carbonyl compounds, when conjugated π -electron systems are present [92].

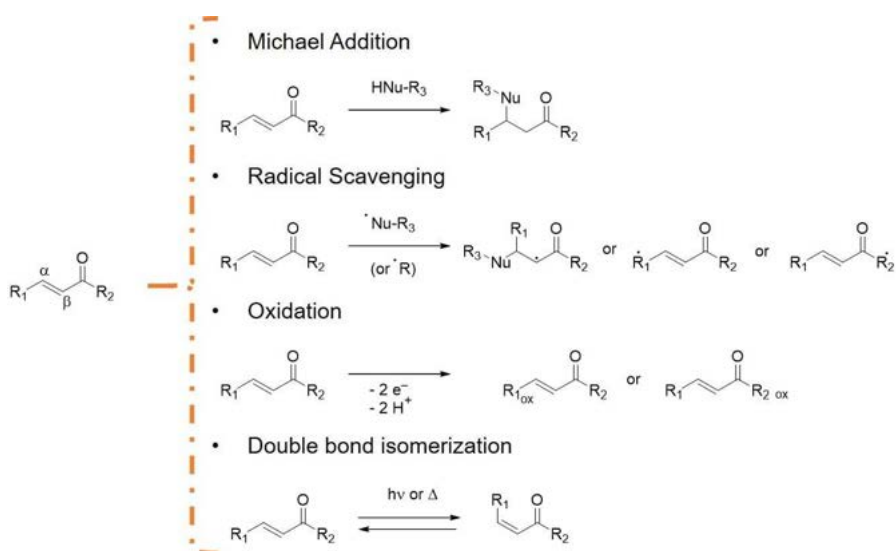


Figure 3.3.1 – Possible reactivities of α,β -unsaturated carbonyl compounds. Adapted from [92].

The electrophilic reactivity assessment is an important topic in the prediction of toxicity and also for recognize Pan-assay interference compounds (PAINS), which is very relevant in the case of Michael acceptors. Reaction with biological molecules includes conjugation, substitution and addition reactions. Predict the toxicological effects of compounds based on their structure and their physicochemical properties is extremely important, once it allows to optimize the product development process, eliminating toxic compounds early, and therefore, reduce costs and animal suffering (*in vivo* experiments) in risks evaluation process [110,111]. The initiating steps for many mechanisms of toxicological action comprise the reactive, covalent binding between an electrophile, the xenobiotic molecule, and an endogenous nucleophile [110].

Our monocarbonyl analogues of curcumin have potential Michael acceptor activity. This activity of α,β -unsaturated carbonyl compounds may be responsible for adverse effects, caused by covalent attack at DNA and proteins, including cell damage, cytotoxicity, mutagenesis, skin sensation and tumorigenicity [92,112].

The experimental reactivity of the synthesized compounds is important to determine the electrophilic nature of compounds, if they can react covalently with a biologically important nucleophile and/or identify which nucleophile. Therefore, the measurement of the reactivity of the compound with a reference nucleophile is required, being the choice of the reference nucleophile dependent on the expected mechanism and the site of action. Thiol groups of cysteinyl residues and glutathione (GSH) are considered soft endogenous nucleophiles. Also, GSH is reactive toward soft electrophiles as the α,β -unsaturated compounds, which act predominantly as Michael acceptors [110].

Hard and Soft, Acids and Bases (HSAB) general principle suggests that electrophiles will preferentially react (and more rapidly) forming covalent adducts with nucleophiles with the same softness or hardness. Additionally, even if there are occasional exceptions to HSAB theory, it is widely applicable and therefore is important to mention that relative reactivities determined with one model nucleophile may be useful to estimate reactivities toward unknown biological nucleophiles, being the thiol or amino-based compounds just as good as a model nucleophile as cysteine, lysine, glutathione or other amino acids and peptides with reactive side chains [110,111]. Thus, the electrophilicity assessment is fundamental to identify the potential electrophilic character of monocarbonyl analogues of curcumin and cytotoxic mechanisms. To accomplish these studies, two methods were tested: a kinetic thiol assay in UV-spectroscopy and an NMR assay.

Cysteamine (figure 3.3.2) also named as β -mercaptoethylamine, 2-aminoethanethiol, 2-mercaptoethylamine, decarboxycysteine, thioethanolamine and mercaptamine, consists in an amino thiol, that is endogenously derived from coenzyme A degradation. However, plasma concentrations of cysteamine are low [113]. Since cysteamine has a pK_a of 8.3, having the same reactivity as several surface thiols of proteins, the reaction with cysteamine is a good way to predict the reactivity of compounds with those thiol groups. Joining the fact that cysteamine is an aliphatic thiol that doesn't absorb in the UV range of interest, turns cysteamine a perfect thiol model for reactivity screening by UV spectroscopy [91,114].

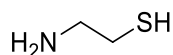


Figure 3.3.2 – Molecular structure of cysteamine.

Another method used to evaluate electrophilic character of the drugs is to perform the reaction with the nucleophile N-acetylcysteine (figure 3.3.3), also owning a nucleophilic thiol group, which can form stable covalent adducts with electrophilic compounds [115].

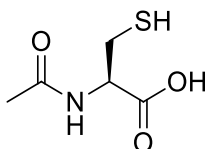


Figure 3.3.3 – Molecular structure of N-Acetylcysteine.

Cysteamine hydrochloride and N-acetylcysteine are both mono-thiol antioxidants that contain a sulphur-hydrogen functional group [116]. N-acetylcysteine has a highly reactive soft thiol group, being used for the comparison of reactivity with and without α -amine side reactions. Cysteamine is also a soft nucleophile. Both, modelling cysteine (cys) reactivity, being cysteamine able to model also GSH reactivity [110]. Also, N-acetylcysteine is a powerful nucleophile responsible for scavenging of free radicals [116].

Monocarbonyl analogues of curcumin act as reactive carbonyl compounds (RCCs) that react and form covalent bonds with biological substances, including proteins, phospholipids and DNA [112,117]. Chemicals with reactive electrophilic centres form adducts with proteins, but the mechanistic pathways differ from compound to compound and may be determinant in the type and strength of the adduct that is formed. There are five most common mechanistic domains, such as Michael acceptors, acylating agents, Schiff base formers, S_NAr electrophiles and S_N1/S_N2 electrophiles [108,118]. The monocarbonyl analogues of curcumin **1a-j** are α,β -unsaturated carbonyl compounds, having a double bond conjugated with a carbonyl group, which makes them ambident electrophiles. It means, the nucleophiles may react not only with the carbonyl group but also on the beta carbon, and either mechanism can predominate, being the most reactive mechanism dependent on the substituents at the beta carbon [110,119]. Therefore, cysteamine may react with the carbonyl group [117,120]. The expected mechanism involved in the reaction of cysteamine with the carbonyl group of the synthesized analogues leading to adduct **a1** is presented in figure 3.3.4.

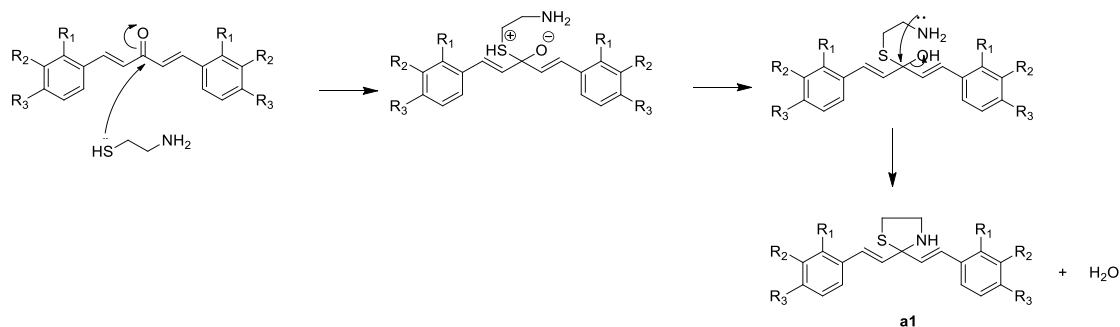


Figure 3.3.4 – Mechanism of the addition of cysteamine to carbonyl group of the synthesized analogues **1a-j**.

However, the majority of the electrophiles have unsaturated π -bonds polarized by neighbouring substituents and that's the case of synthesized monocarbonyl analogues of curcumin. The soft nucleophilic addition of the thiol group occurs to the beta carbon. This 1,4- or Michael addition is thought to be a two-step process, in which forms a covalent adduct at a soft electrophilic centre without a leaving group in the molecule. Esters, aldehydes and ketones where the carbonyl is conjugated to the polarized double bond of an olefin are important electrophiles regarding toxicological response. A molecular rearrangement turns out in a relatively diffuse and polarizable electron density of the π -bond, making these molecules the softest electrophiles. It means, when the electron density at beta carbon is lower, more likely to undergo nucleophilic attack, especially by soft thiol groups [109,110]. So, another possible reaction for the curcumin monocarbonyl analogues with cysteamine is that the thiol group of cysteamine reacts with the beta carbon, originating adduct **a2**. The mechanism involved is presented in figure 3.3.5.

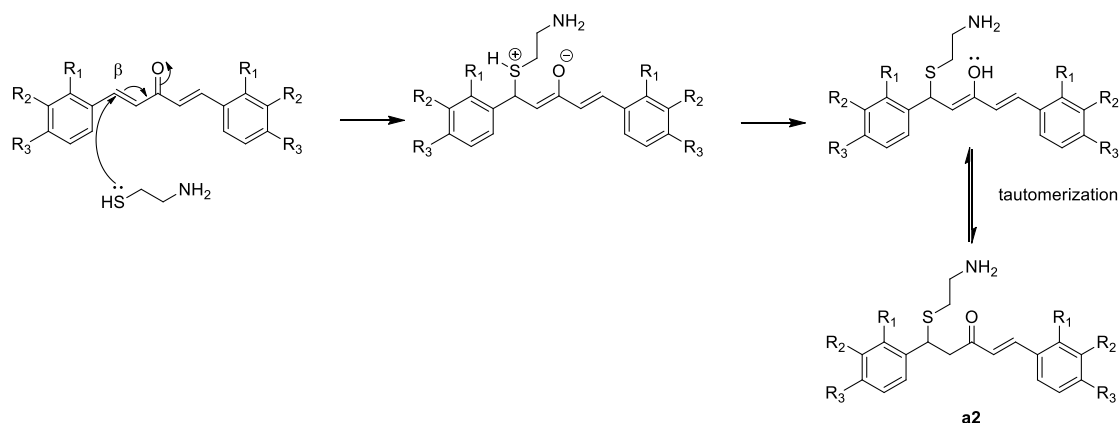


Figure 3.3.5 – Mechanism of 1,4 addition of cysteamine to monocarbonyl analogues of curcumin **1a-j**.

The experiments performed proved that our compounds **1a (C2)** and **1g (C1)** react with cysteamine, forming the adduct **a2**.

3.3.1 Electrophilicity assessment by a kinetic thiol assay

In the first assay, it is evaluated the electrophilicity by a kinetic thiol assay. The reaction is followed in UV-Vis spectrophotometer. Performing a scan from 400 to 200 nm, during 15 min, it is possible to note the changes in the UV profile of each compound.

An example is presented for the reaction of compound **1g** with the lower concentration of cysteamine (8.34×10^{-4} M), in figure 3.3.1.1.

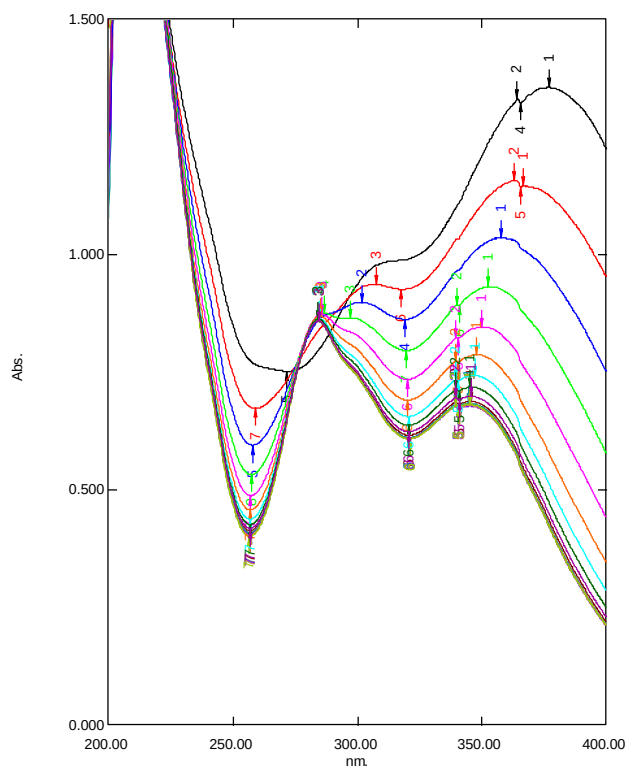


Figure 3.3.1.1 – UV-Vis spectrum of the reaction of cysteamine (8.35×10^{-4} M) and compound **1g** (10^{-5} M), with repetition interval of 1 min, during 15 min.

Observing the spectrum in the figure 3.3.1.1, it is possible to note that the profile of compound **1g** is suffering changes by the time of reaction, verifying a great decrease in the absorbance at 356 nm, being this wavelength that is used to follow the reaction. Furthermore, an isosbestic point is observed at about 275 nm.

In order to identify the structure of the adduct formed, the resulting product of the reaction of compound **1a** with cysteamine was sent to ESI⁺-MS analysis. The exact mass of the expected adduct is 353.14 g/mol. The mass spectrum is presented in figure 3.3.1.2.

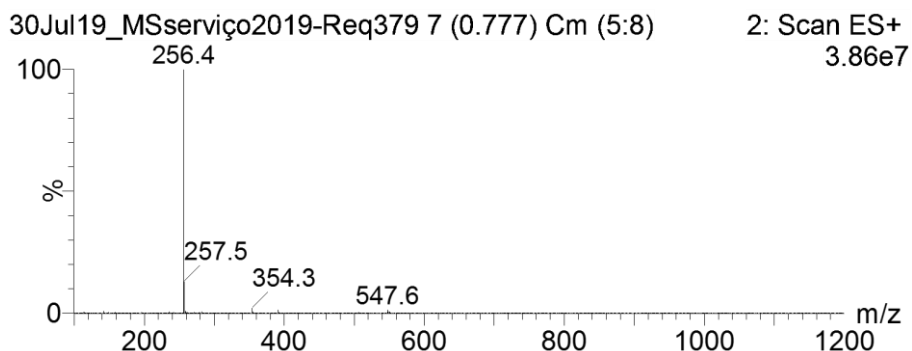


Figure 3.3.1.2 – Mass spectrum of the adduct of the reaction of analogue **1a** with cysteamine (8.35×10^{-3} M).

Observing the spectrum, we can confirm the presence of the adduct **a2** there, which is confirmed by $[M+H^+]$ peak. However, it was done an expansion (figure 3.3.1.3).

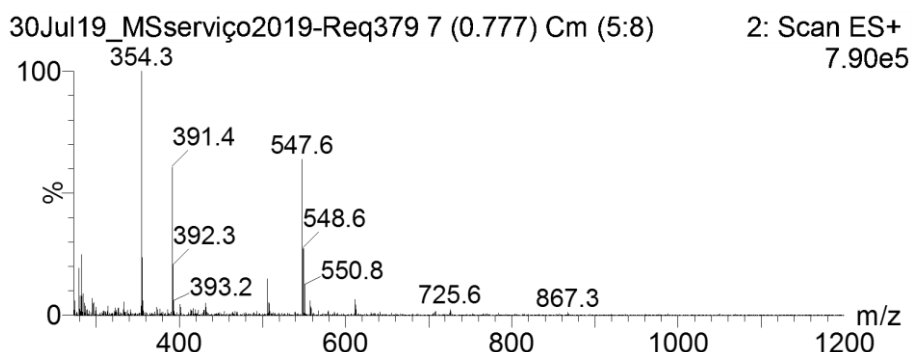


Figure 3.3.1.3 – Expansion of the mass spectrum of the adduct of the reaction of analogue **1a** with cysteamine (8.35×10^{-3} M).

By the expansion, it is verified the base peak, $[M+H^+]$, 354.3 m/z, with a percentage of relative abundance of 100%. Also, it is observed an adduct with potassium (392.3 m/z).

The reactivity rate constants (k), which are a measure of the velocity at an electrophile binds to a nucleophile [118] were calculated for each concentration of cysteamine and for each compound. Each compound reacted with cysteamine in different concentrations. Firstly, for each concentration it was obtained the pseudo 1st order observed constant (K_{obs}) by the slope of the graph $\ln(A-A_0)$ by time. Then, it was constructed a graph of the K_{obs} vs concentration of cysteamine, that allows us to calculate K , the 2nd order rate constant for the catalytic effect of the cysteamine. An example of the determination of the K , catalytic constant is presented in figure 3.3.1.4.

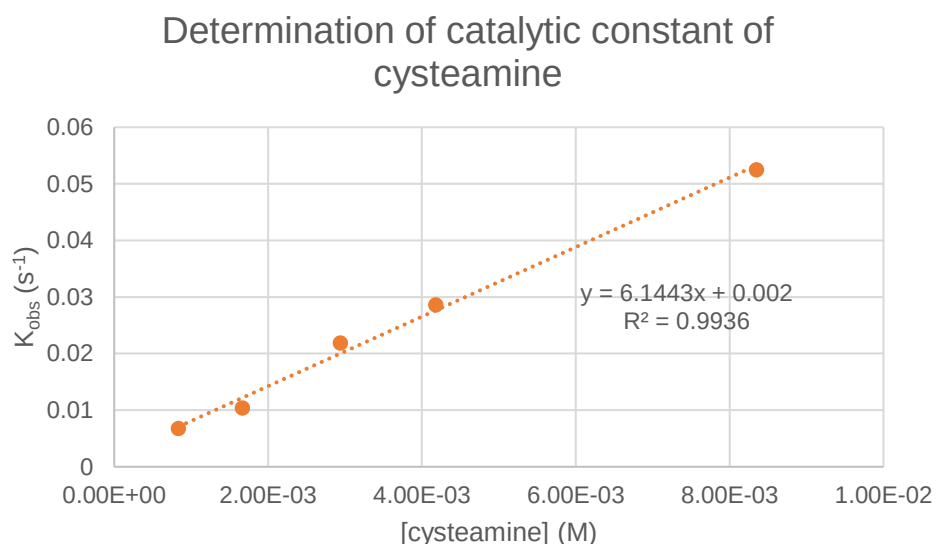


Figure 3.3.1.4 – Determination of the catalytic constant of cysteamine over the reaction with compound **1a**.

By the slope we obtain the K value for each compound **1a-j**, that is presented in table 3.3.1.1.

Table 3.3.1.1 – Values of K for monocarbonyl analogues **1a-j**.

Compound	Substituents			K ($M^{-1}s^{-1}$)
	R ₁	R ₂	R ₃	
1a (C2)	H	OCH ₃	H	6.144
1b	OCH ₃	OCH ₃	OCH ₃	2.021
1c	H	H	OCH ₃	1.461
1d	Br	H	H	1.683
1e	H	H	H	11.773
1f	NO ₂	H	H	0.452
1g (C1)	OCH ₃	H	H	1.895
1h	OCH ₂ CH ₃	H	H	0.504
1i	CF ₃	H	H	3.459
1j	CH ₃	H	H	6.140

Analysing the table 3.3.1.1, it is assumed that the electrophilic character of each compound varies, which might be explained by the fact that adduct formation is dependent on the substituents on the aromatic ring. Also, differences on the reactivity could be explained by steric factors and electronic properties that lead to alterations on the conjugation of the π -system. Briefly, the effect of the substituents occurs in function of three effects: steric, inductive and electronic effects. The steric effect is a result of the steric hinderance caused by the substituent in *ortho* position, the position closer to the β -carbon. The inductive effect regards the electron withdrawing or electron donating

character of the substituent, that depends on the position on the aromatic ring (*ortho*, *meta* and *para*), and decreases with the distance. The electronic effect is a result of the conjugated resonance between the aromatic ring and substituents [91,114]. However, since the majority of the synthesized compounds are *ortho*-substituted, it is difficult to evaluate the contribution of the electronic effect or the inductive effect over the electrophilic character of the β -carbon.

Looking for a dependence of the aryl substituents, our K values experimentally obtained order is: **1e** (no substituents) > **1a** (*m*-OCH₃) $\approx$ **1j** (*o*-CH₃) > **1i** (*o*-CF₃) > **1b** (*o,m,p*-OCH₃) > **1g** (*o*-OCH₃) > **1d** (*o*-Br) > **1c** (*p*-OCH₃) > **1h** (*o*-CH₂CH₃) > **1f** (*o*-NO₂). Therefore, it is possible to deduce that the *ortho* substituents determines less reactivity (less Michael addition behaviour), probably due to the steric hindrance, since the *ortho* position is the nearest to the β -carbon (electrophilic centre). This steric hindrance of the *ortho* substituents is confirmed when we compare **1g** with **1h**, since the expansion with a CH₂ group gives a compound four times less reactive to cysteamine Michael Addition.

Comparing **1c** (*p*-OCH₃), which is an electron donating by resonance, with **1a** (*m*-OCH₃), which is an electron withdrawing by inductive effect, we can infer a more electrophilic β -carbon for analogues *m*-electron withdrawing substituents.

In order to visualize better the difference in the electrophilic character of each synthesized MAC, the behaviour of all of them in the presence of cysteamine (8.35X10⁻³ M), were compared by the graph of ln(A-A₀) by time (figure 3.3.1.5).

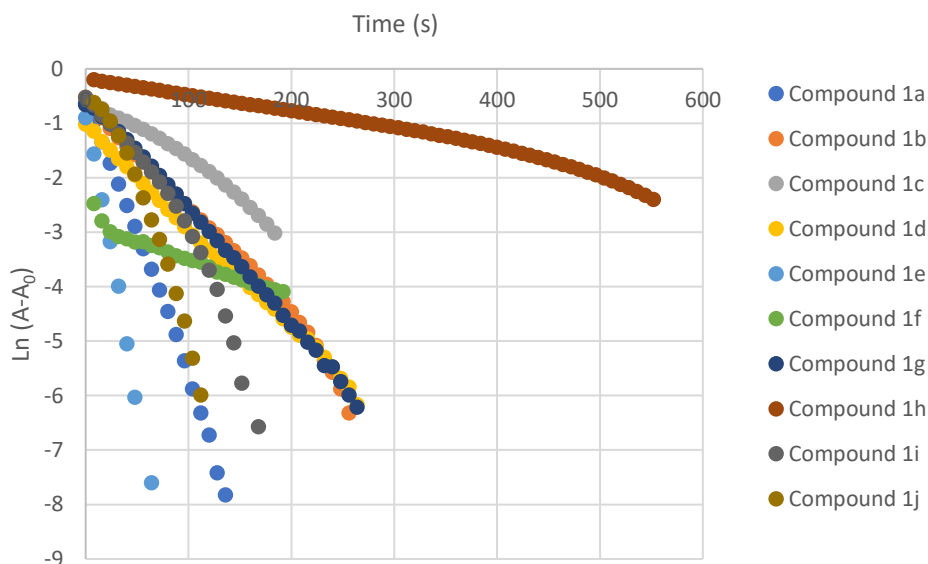


Figure 3.3.1.5 – ln(A-A₀) in function of time (s) for each analogue **1a-j** in the presence of cysteamine (8.35X10⁻³ M).

Analysing the figure 3.3.1.5, it is concluded that compound **1e** is the most electrophilic one while compound **1h** is the least electrophilic one, once it takes more

time to react with the nucleophile. Table 3.3.1.2 resumes K_{obs} (s^{-1}) values for each analogue in the reaction of cysteamine ($8.35 \times 10^{-3} \text{ M}$).

Table 3.3.1.2 – K_{obs} (s^{-1}) values for the monocarbonyl analogues of curcumin **1a-j** in the presence of cysteamine ($8.35 \times 10^{-3} \text{ M}$).

Compound	Substituents			K_{obs} (s^{-1})	$t_{1/2}$ (s)
	R ₁	R ₂	R ₃		
1a (C2)	H	OCH ₃	H	0.052	13.203
1b	OCH ₃	OCH ₃	OCH ₃	0.020	33.812
1c	H	H	OCH ₃	0.015	46.416
1d	Br	H	H	0.018	37.467
1e	H	H	H	0.107	6.466
1f	NO ₂	H	H	0.008	86.643
1g (C1)	OCH ₃	H	H	0.021	32.850
1h	OCH ₂ CH ₃	H	H	0.005	150.684
1i	CF ₃	H	H	0.033	20.815
1j	CH ₃	H	H	0.052	13.433

Analysing the K_{obs} values ranking: **1e** (no substituents) > **1a** (*m*-OCH₃) $\approx$ **1j** (*o*-CH₃) > **1i** (*o*-CF₃) > **1g** (*o*-OCH₃) > **1b** (*o,m,p*-OCH₃) > **1d** (*o*-Br) > **1c** (*p*-OCH₃) > **1h** (*o*-CH₂CH₃) > **1f** (*o*-NO₂), we can find the same relation for the reaction constants with the aromatic substituents that we previously discussed. It is confirmed that compound **1e** is the most electrophilic one while compound **1h** is the least electrophilic.

We also promote the reaction of compound **1c** with N-acetylcysteine, performing overlaid UV scans along the time (figure 3.3.1.6).

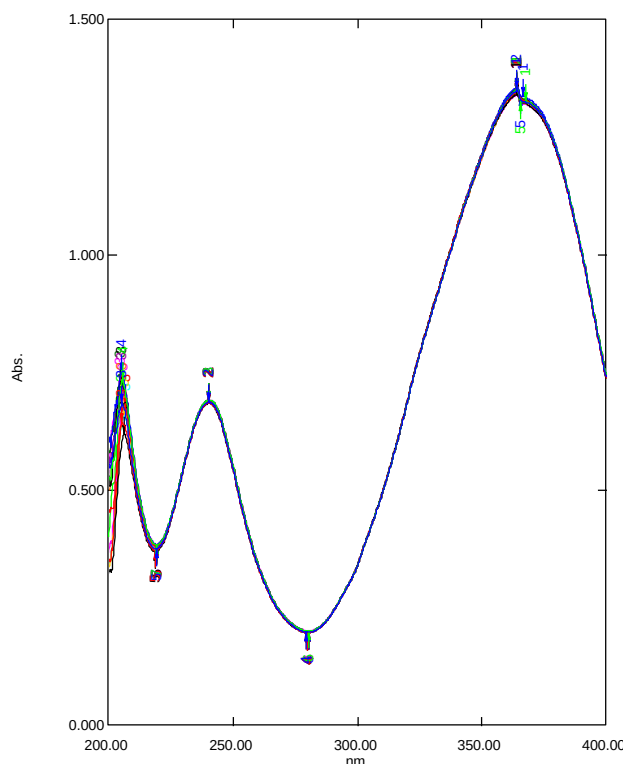


Figure 3.3.1.6 – UV-Vis spectrum of the reaction of N-acetylcysteine (8.35×10^{-4} M) and compound **1c** (10^{-5} M), with repetition interval of 1 min, during 15 min.

In this experiment, we observed no alterations in the UV spectrum, so there is no formation of any adducts between N-acetylcysteine and the monocarbonyl curcumin analogues **1a-j**, during the 15 minutes of the assay. This was not surprising since a previous study performed by Vidal *et al.* tested the reaction of α,β -unsaturated aldehydes, verifying that glutathione and N-acetylcysteine didn't show strong reactivity while cysteamine showed high efficiency in reacting with the evaluated electrophiles [121]. Additionally, Arsovska *et al.* refers an assay performed by Mendgen, where were tested compounds which have already presented activity against one or more enzymes using glutathione as a biological nucleophile. In spite of that, when the NMR assay was realized, the addition of glutathione was not detected. As such, it is not possible to assert that the reaction of our compounds **1a-j** with N-acetylcysteine doesn't happen, i.e., the reactivity of these compounds on their target proteins can't be completely excluded and we should bear in mind that reactivity of cysteine sulphur depends on the protein environment [91].

Since there are several electrophilic reaction mechanisms and also different rates of reactivity within mechanisms of action, it is understandable that not all electrophiles react with all potential biological nucleophiles [110]. Therefore, is important to consider the selectivity of a compound toward a specific molecular site of action, which may be explained by the classification of electrophiles and nucleophiles according to their

polarizability, it is, the chemical “hardness” and “softness” of the electrophilic or nucleophilic centres. A hard electrophile has low polarizability and has an electron deficiency, i.e., a localized electrostatic positive charge while in a soft electrophile the electron deficiency is spread over a molecular region, being highly polarized [109,110]. Application of the HSAB principle imply that soft electrophiles of several chemical classes selectively origin covalent Michael-type adducts with soft, highly reactive cysteine thiolate nucleophile, it consists in a simplification “like-reacts-with-like” [109–111].

The unionized thiol group of N-acetylcysteine and cysteamine act as general acid but the protonated amino group of cysteamine also act as general acid catalyst [122]. Furthermore, as reported for other compounds [122], where the high nucleophilic rate constant for cysteamine is attributed to its ability of acting as an intramolecular general-acid catalyst, may explain why the reaction with cysteamine occurs but the monocarbonyl analogues of curcumin don't form adducts with N-acetylcysteine. Is important to mind that N-acetylcysteine at high pH exists predominantly in a *trans* thiolate-carboxylate rotamer form, where protonation promotes the occurrence of intramolecular hydrogen bond-forming isomers (figure 3.3.1.7). Also, this nucleophile has a pK_a of the thiol group of 9.6 and pK_a of carboxylic acid moiety of 3.3, which means that N-acetylcysteine is monoanionic at pH range between 3.3 and 9.6 and dianionic pH higher 9.6 [111,123]. Consequently at physiological pH, the thiol group doesn't exist in the reactive state, which may justify why the reaction with this nucleophile doesn't occur [111].

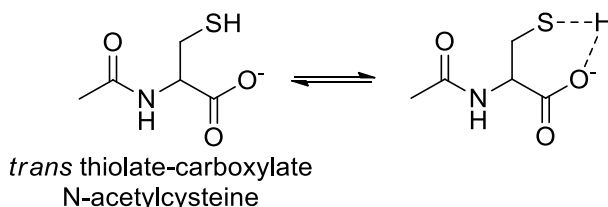


Figure 3.3.1.7 – Hydrogen bond for N-acetylcysteine in alkaline medium.

3.3.2 Electrophilicity assessment by NMR assay

Another method to study the electrophilicity was applied and consists in following the reaction through ^1H NMR, comparing the spectra and verifying the formation of an adduct, accordingly to a procedure adapted from Avonto *et al.* [124] and Autelitano *et al.* [125]. Three solvents were tested during the assay, methanol- d_4 , acetone- d_6 and DMSO- d_6 and the reversibility of the reaction was also evaluated.

The electrophilicity assessment by NMR assay was performed reacting monocarbonyl analogue of curcumin **1g** (figure 3.3.2.1) with 10 molar equivalents of cysteamine hydrochloride and it was verified the formation of adduct **a2**, as expected.

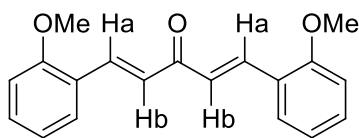


Figure 3.3.2.1 – Chemical structure of compound **1g**.

When evaluating the electrophilicity of compound **1g** using methanol as solvent, it was observed that the monocarbonyl analogue of curcumin **1g** has low solubility in this solvent, which did not allow the assay to be performed in methanol. However, the two spectra, one spectrum of the compound and another after addition of cysteamine, were performed and are presented in figure 3.3.2.2.

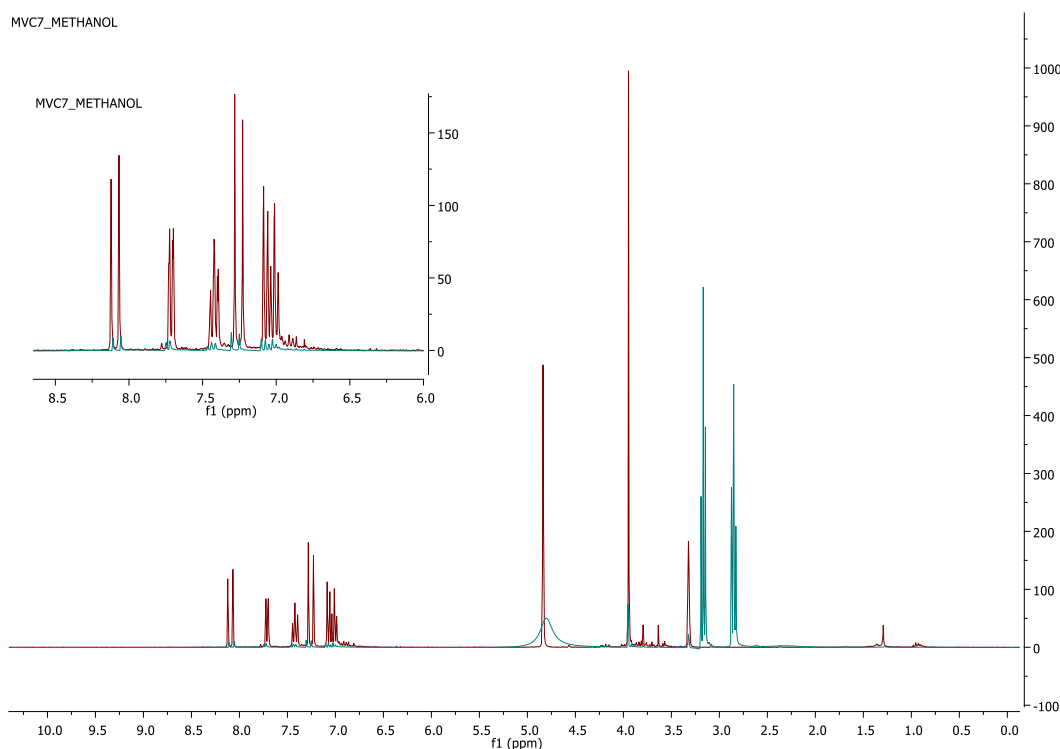


Figure 3.3.2.2 – ^1H NMR spectra for thiol assay in methanol. In red, the spectrum of compound **1g** and in blue the spectrum of compound **1g** after the addition of cysteamine.

Observing the spectrum presented in figure 3.3.2.2, is verified that the signals of compound **1g** maintain, as it is emphasized by the expansion of the aromatic signals. Additionally, occurs the appearing of the signals of cysteamine but not observing any alteration that would correspond to the occurrence of the reaction. Therefore, for the assay in methanol, the low solubility of the compound **1g** limited the assay.

For the assessment of the electrophilicity of compound **1g** using acetone as solvent, it was observed that cysteamine has low solubility in acetone, which did not allow the use of this solvent to perform the assay. The ^1H NMR spectra obtained for the assay in acetone are presented in figure 3.3.2.3.

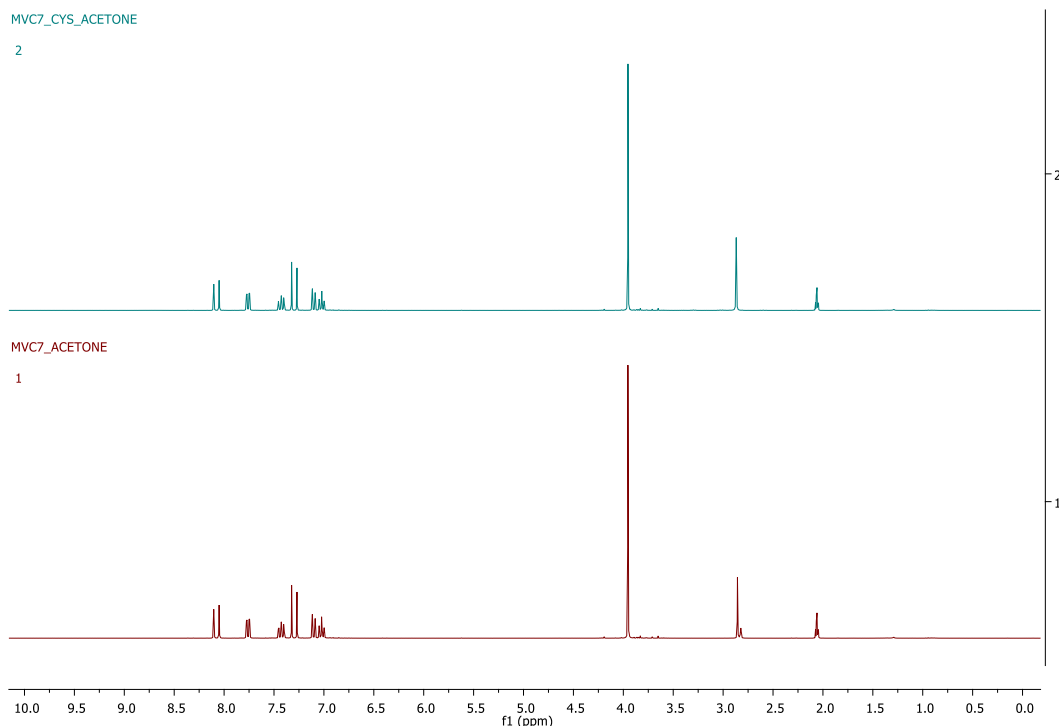


Figure 3.3.2.3 – ^1H NMR spectra for thiol assay in acetone. In red, the spectrum of compound **1g** and in blue the spectrum of compound **1g** after the addition of cysteamine.

Cysteamine is not soluble in acetone, which explain why the spectra before and after the addition of cysteamine present the same signals (figure 3.3.2.3), not being observed the signals correspondent to cysteamine. Therefore, the reaction of compound **1g** with cysteamine doesn't occur in acetone, once the low solubility of cysteamine in acetone limited the use of this solvent in the assay.

Since methanol and acetone aren't suitable to proceed the assay, the assessment of the electrophilicity was only performed in DMSO. Therefore, firstly it was performed an ^1H NMR spectrum of monocarbonyl analogue of curcumin **1g** in DMSO, which is presented in figure 3.3.2.4.

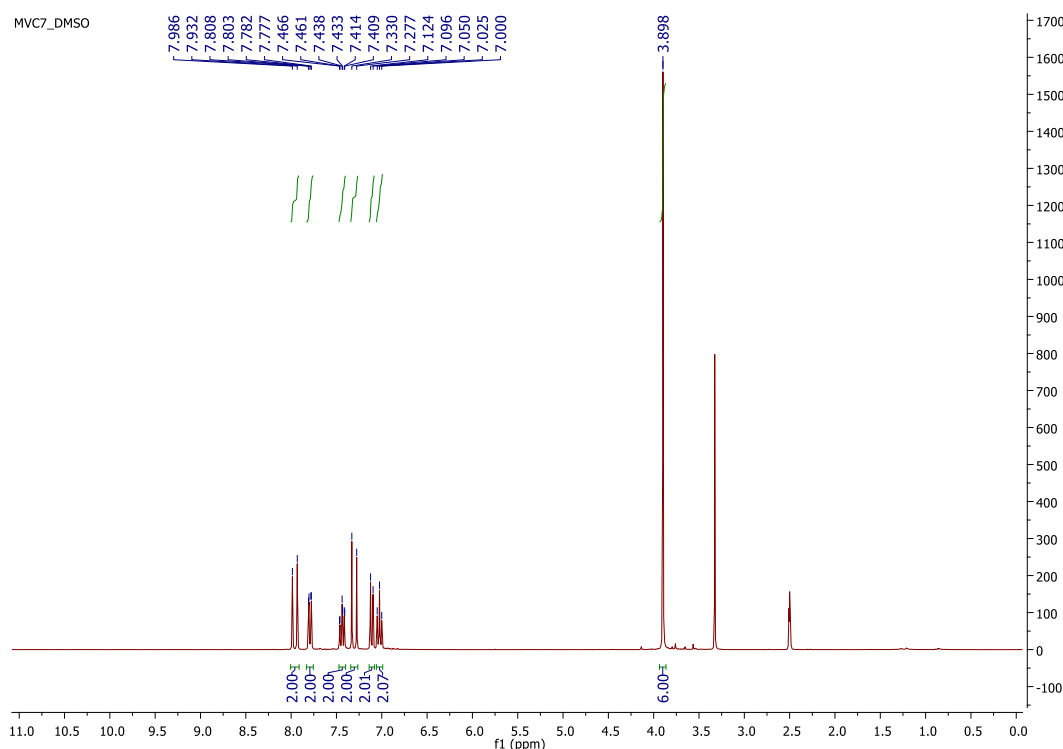


Figure 3.3.2.4 – ^1H NMR spectrum of compound **1g** in DMSO.

The spectrum in figure 3.3.2.4 was analysed and the signals were attributed to respective hydrogens (table 3.3.2.1).

Table 3.3.2.1 – ^1H NMR chemical shift for compound **1g**.

Compound	δ (ppm)					
	H_a	H_b	$\text{R}_1 = \text{OMe}$	$\text{R}_2 = \text{H}$	$\text{R}_3 = \text{H}$	$\text{R}_5 = \text{H}$
1g (C1)	7.96 (2 H, d, $J=16$ Hz)	7.31 (2 H, d, $J=16$ Hz)	3.90 (6 H, s)	7.11 (2 H, d, $J=8$ Hz)	7.44 (2 H, td, $J=2$, 8 Hz)	7.79 (2 H, dd, $J=2$, 8 Hz)

The reaction of **1g** could occur in the carbonyl group or in the β -carbon. However, the ESI $^+$ -MS analysis of the product formed when the kinetic thiol assay was monitored by UV-spectrometry and for compound **1a**, showed that the adduct originated is a result of the reaction of one mole of cysteamine with the β -carbon, adduct **a2**. Consequently, it is expected that the adduct originated by the reaction of compound **1g** with cysteamine correspond also to adduct **a2** presented in figure 3.3.2.5.

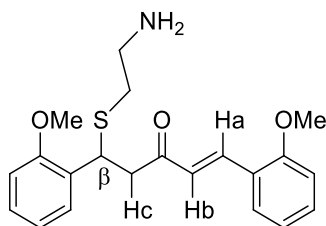


Figure 3.3.2.5 – Chemical structure of the expected adduct formed (**a2**).

To perform the assay, ^1H NMR spectra were recorded right after the addition of cysteamine (figure 3.3.2.6) and then 24 and 48 hours later.

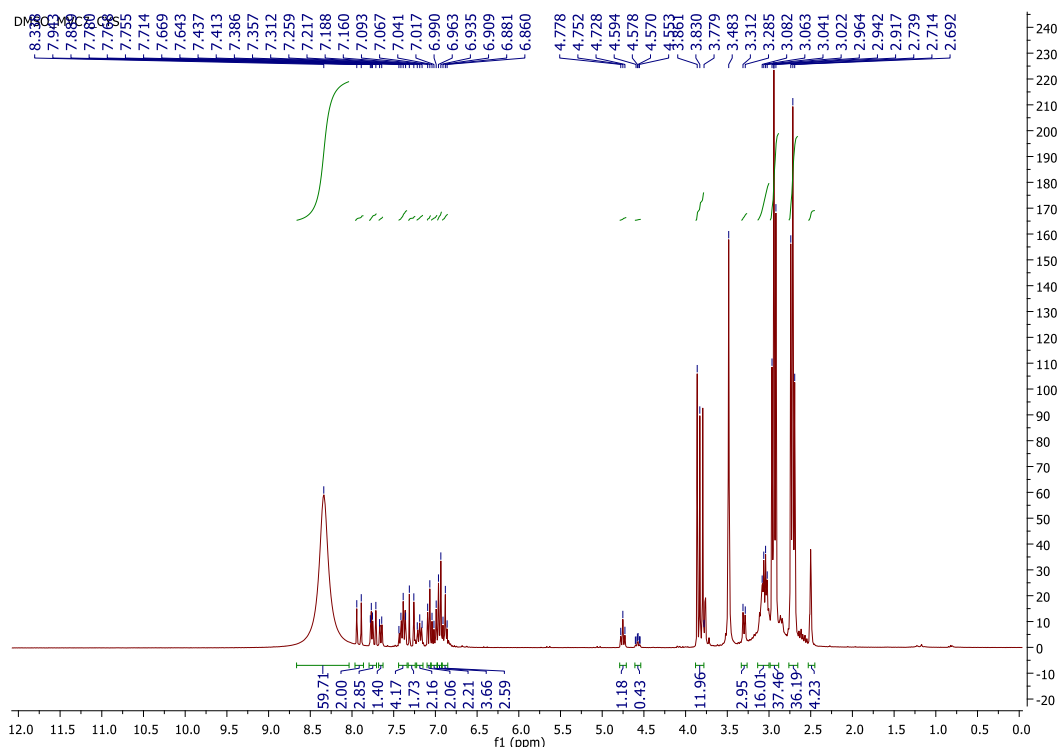


Figure 3.3.2.6 – ^1H NMR spectrum of compound **1g** after the addition of cysteamine.

Analysing this spectrum on the beginning of the reaction, it is possible to identify signals that result of the presence of cysteamine, compound **1g** and the adduct formed. Expansions on figure 3.3.2.7, allows us to identify the presence of the adduct between compound **1g** and cysteamine.

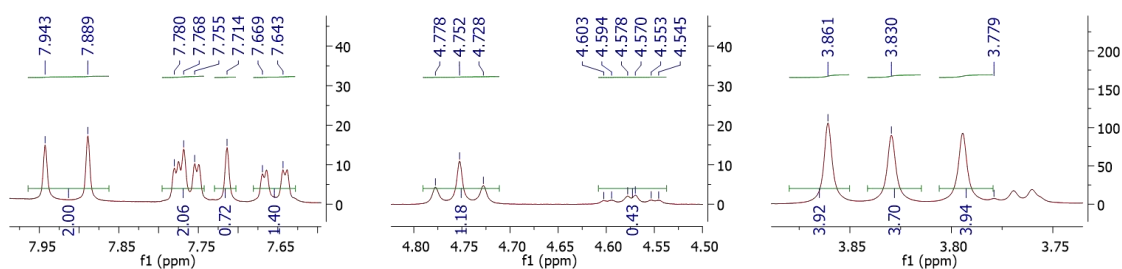


Figure 3.3.2.7 – Expansions of ^1H NMR spectrum of compound **1g** after the addition of cysteamine.

The first thing that denounced the formation of the adduct **a2** was the presence of a doublet ($J = 16$ Hz) at 7.768-7.714 ppm, which is overlapped with another signal but the total of both signals integrate to 2.85 H, deducing that the doublet correspond to 1 H, it means, it is verified the loss of one vinylic hydrogen. Also, the presence of a triplet (4.752 ppm) and a triple doublet (4.574 ppm), that integrate for 1.18 and 0.43 H, respectively, corresponding to the adduct. Additionally, there are three singlets, at 3.861,

3.830 and 3.779, which the sum of the integration corresponds to 12 H, and appear due to the methoxy groups presented on the compound **1g** (6 H) and on the adduct (6 H). So, the split of the singlet (on spectrum of compound **1g**) into three singlets is a prove that the adduct is formed, once contrary to the initial compound, the adduct is not a symmetrical molecule and the presence of the cysteamine influence the methoxy groups to have different chemical shifts.

In order to facilitate the identification of the signals, a ^1H NMR spectrum of cysteamine in DMSO was recorded (figure 3.3.2.8).

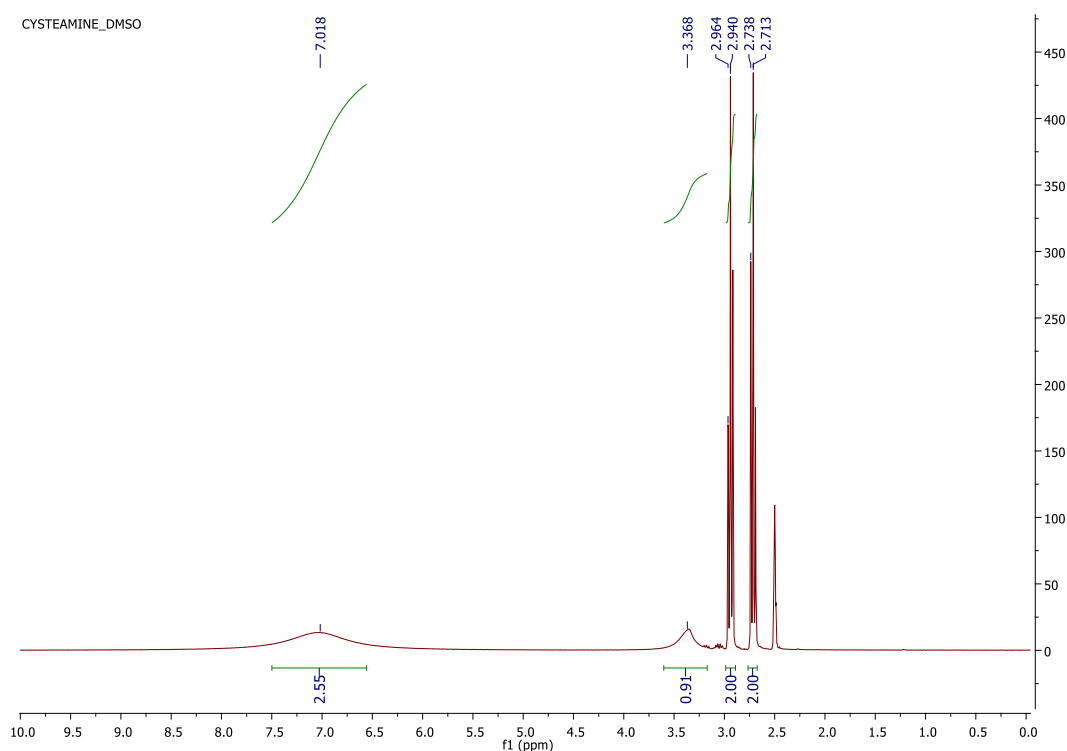


Figure 3.3.2.8 – ^1H NMR spectrum of cysteamine.

The spectrum presented in figure 3.3.2.8, showed that cysteamine presented two broad signals, one at 7.018 ppm that corresponds to NH_2 group and another at 3.368 ppm that corresponds to SH group. Also, the CH_2 groups of the molecule produce two triplets at 2.940 and 2.713 ppm.

After 24 hours of reaction, another ^1H NMR spectrum was recorded, being presented in figure 3.3.2.9.

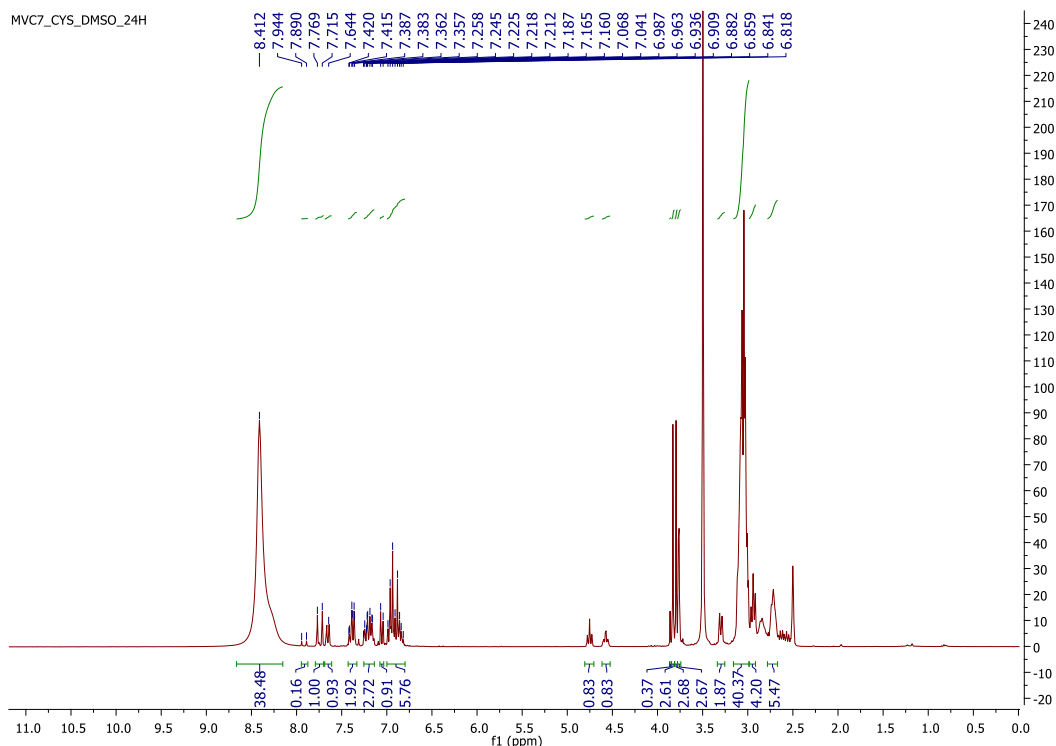


Figure 3.3.2.9 – ^1H NMR spectrum recorded 24 hours after the beginning of the assay.

After 24 hours of reaction, in the spectrum are detected signals corresponding to the adduct, cysteamine and only trace elements of the compound **1g**. Another spectrum was recorded at 48 hours of assay (figure 3.3.2.10).

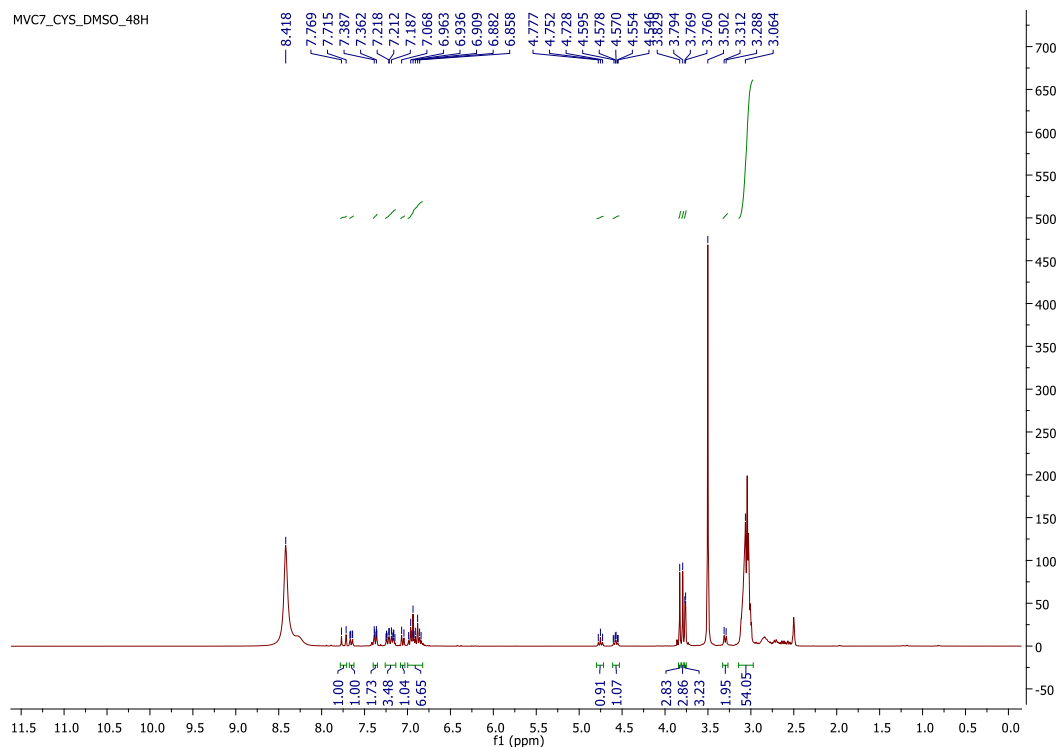


Figure 3.3.2.10 – ^1H NMR spectrum recorded 48 hours after the beginning of the assay.

After 48 hours of reaction, the signals detected correspond to the cysteamine and to the adduct formed, not observing the presence of initial monocarbonyl analogue of curcumin, which means that over 48 hours the reaction is complete. At this time of reaction, it was also recorded a ^{13}C NMR spectrum, which is presented in figure 3.3.2.11.

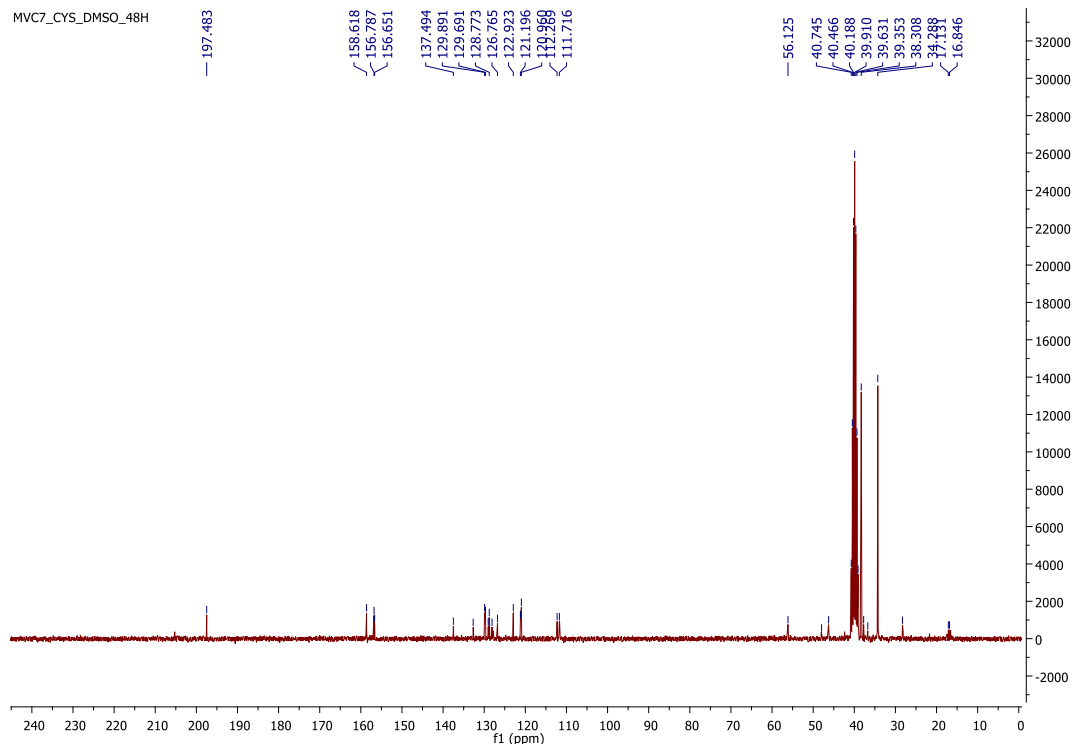


Figure 3.3.2.11 – ^{13}C NMR spectrum recorded 48 hours after the beginning of the assay.

^{13}C NMR spectrum allows us to confirm that the reaction occurred in the β -carbon instead of in the carbonyl group, once it is present a signal at 197.483 ppm, that corresponds to carbonyl group.

The reversibility of the reaction was tested by a dilution (1:10) of the reaction mixture of compound **1g** and cysteamine in CDCl_3 . The protocol related by Avonto *et al.* [124], suggests the dilution with chloroform deuterated of the adduct originated by the reaction of monocarbonyl analogue **1g** with cysteamine hydrochloride in $\text{DMSO}-d_6$, as a test for reversibility of Michael adduct formation. This is explained by a change of the polarity in the equilibrium, that would reverse the reaction, and lead to the observation of the reappearance of signals correspondent to the vinylic hydrogens, previously lost during the addition reaction in $\text{DMSO}-d_6$. The adduct stability is determinant for the biological profile of thiol-trapping agents, since a fast reversibility is associated with low toxicity. The stability is also a criterion for the potential application of such agents in medicinal chemistry [91,124]. However, when the dilution is performed, it was observed the precipitation of a powder. Previously, when trying to obtain a ^1H NMR spectrum of cysteamine, we observe that cysteamine was not soluble in CDCl_3 , so we suspected that

the solid that precipitate is cysteamine. To be sure of that, the powder was filtrated and then dissolved in DMSO, and a ^1H NMR spectrum was performed. Comparing this spectrum with the one of the cysteamine it was confirmed that the powder corresponds to cysteamine (spectrum presented in appendices section). Although it wasn't found information about solubility of cysteamine in CDCl_3 or in acetone, we verified that it isn't soluble in these solvents. The information available about solubility of cysteamine is that it is slightly soluble in water, DMSO, ethanol and methanol and freely soluble in alkaline medium [126,127]. However, the procedure for the reversibility of the reaction was performed. For that, it was diluted in one NMR tube and then the same tube was analysed, at time zero, 24 and 48 hours. The first spectrum is presented in figure 3.3.2.12.

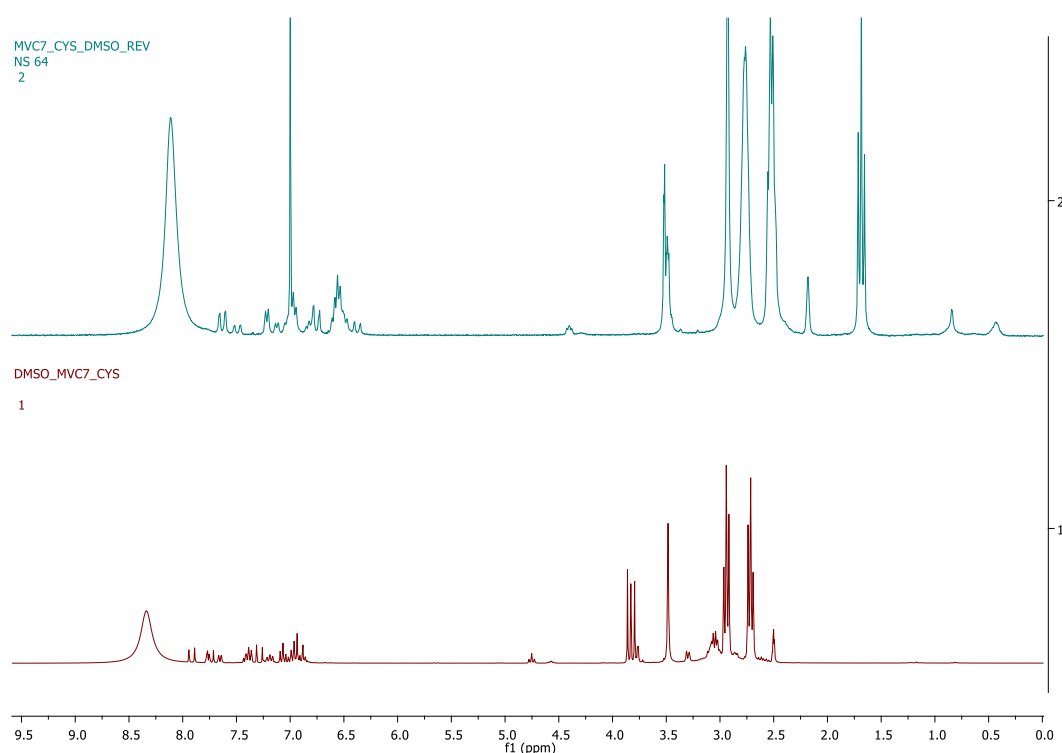


Figure 3.3.2.12 – Spectra recorded at time zero. In blue, the first spectrum of reversibility test and in red, the first spectrum recorded after the addition of cysteamine.

Observing the spectra, the signals maintain from the initial spectrum to the reversibility test, which means that the dilution with CDCl_3 didn't promote the reappearance of the initial product.

Another ^1H NMR spectrum was recorded, past 24 hours. The comparison of both spectra performed at 24 hours of reaction is presented at figure 3.3.2.13.

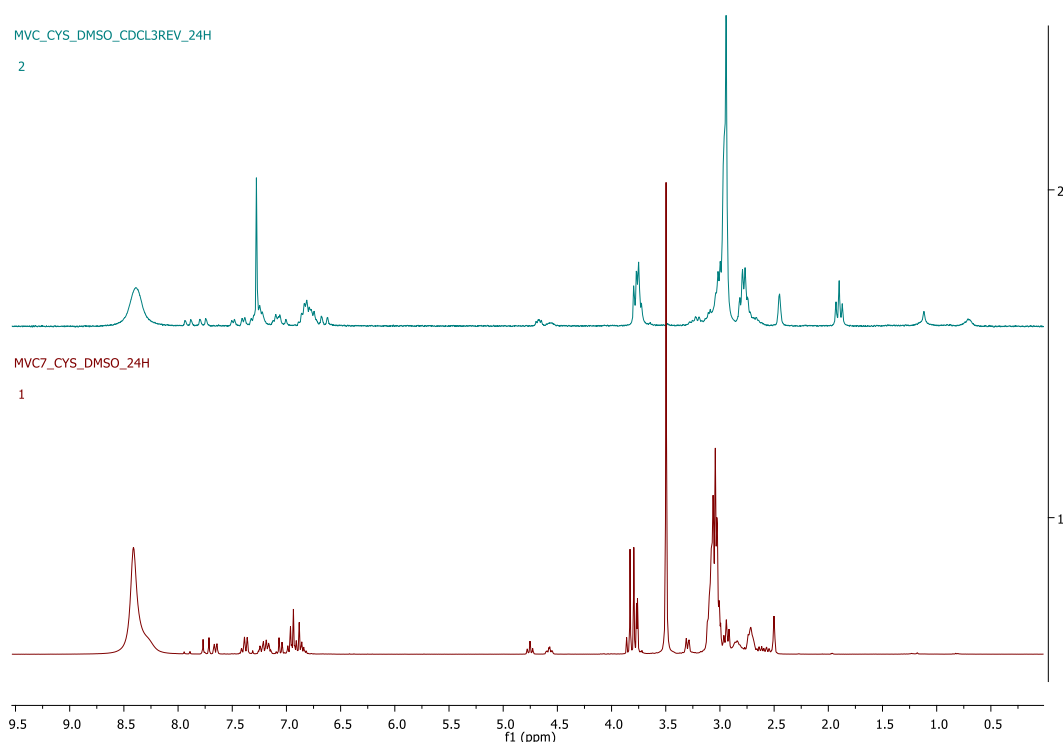


Figure 3.3.2.13 – Spectra recorded 24 hours after the beginning of the assay. In blue, the spectrum of reversibility test and in red, the spectrum recorded 24 hours after the addition of cysteamine.

Observing the ^1H NMR spectra recorded at 24 hours of assay, it is possible to see the intensification of the signals between 5 and 4.5 ppm, being this intensification also noted in the reversibility spectrum, which means that diluting with in CDCl_3 didn't stop the reaction neither promote the reversibility of it.

For the last, a new ^1H NMR spectrum recorded at 48 hours of assay. Along the time, a precipitate is originated but it was filtered and then the spectra were performed (figure 3.3.2.14).

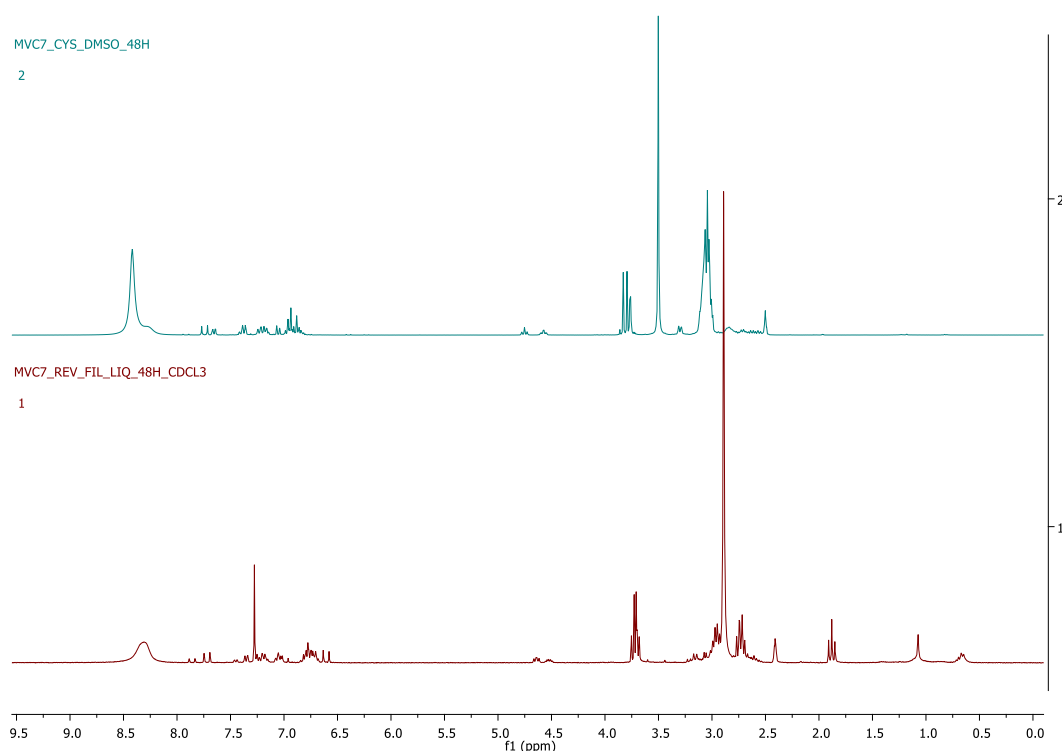


Figure 3.3.2.14 – Spectra recorded 48 hours after the beginning of the assay. In blue, the spectrum of reversibility test and in red, the spectrum recorded 48 hours after the addition of cysteamine.

Observing the ^1H NMR spectra recorded at 48 hours of assay is possible to conclude that the reaction had continued occurring in the tube with the reaction medium diluted with CDCl_3 . It means that the reversibility of the reaction was not verified. Although, we can't strongly claim that the adduct formed is stable and the reaction is not reversible, once we verified that the procedure of reversibility test is not reliable due to the low solubility of cysteamine in CDCl_3 . Additionally, it was expected that the precipitation of cysteamine would favour the reappearance of the initial monocarbonyl analogue of curcumin, because removing the reagent would move the equilibrium in order to form the cysteamine and the initial compound, which was not verified, once the reaction continue to occur. Therefore, due to the low solubility of cysteamine hydrochloride in chloroform we were unable to perform correctly this experiment, which prevented us from classifying the adduct **1g-cysteamine** at either reversible or irreversible. However, considering that the reaction didn't even stopped, probably the adduct is stable, the changing in the polarity of the solvent didn't promote the reversibility of the reaction.

Lastly, as verified in biological assays, both compounds **C1** (compound **1g**) and **C2** (compound **1a**) are activators of Nrf2, an electrophile-sensitive transcription factor. When inactive, this factor is retained in cytoplasm by Keap-1, which contains two cysteine moieties. When interaction with reactive molecules happens, Keap-1 is

degraded and occurs the translocation of Nrf2 to the nucleus, activating there Nrf2-dependent genes [128]. Therefore, the electrophilic character of these compounds might be related with its biological activity.

In summary:

In terms of drug likeness properties, it was verified that the synthesized monocarbonyl analogues of curcumin present good lipophilic properties. Also, these compounds are very stable in physiological conditions, both in PBS (pH 7.4) and in human plasma, with exception of compound **1f**, which presented a half-life of about 8 hours.

Regarding the electrophilic assays performed to evaluate Michael addition behaviour, our compounds react easily with cysteamine but not with N-acetylcysteine, forming adduct **a2**, that was confirmed by mass spectroscopy and then by the NMR assay. Although, the reactivity with N-acetylcysteine cannot be completely excluded, because the reactivity of the thiol group depends on the protein environment.

The assay of electrophilicity determination, using the NMR method, allow to understand that this method is useful, simple and fast to identify Michael acceptor sites in the compounds, which is even more important when there is more than one reactive site. In theory, the method also distinguish the adducts in reversible and irreversible, and even if it was not possible to do in our work, it has a high importance, since the stability of the adduct is related with its biological profile of thiol-trapping agents and fast reversibility is associated with low toxicity. Additionally, these studies allow to evaluate the compounds as potential false positives in High-throughput screening (HTPS) assays.

4 Biological assessment

Curcumin has a plethora of biological properties, that include antioxidant activity, being capable of scavenging reactive oxygen species (ROS) and anticancer activity [32,45]. Also, has already been reported that curcumin improves retinal function in retinal degeneration and that enhances the autophagy [1,37,54]. Therefore, the action of monocarbonyl analogues of curcumin were evaluated in RPE cells and in colorectal cancer stem cells.

The studies in RPE cells were performed in CEDOC, Nova Medical School (UNL), in collaboration with Dr. Ana Sofia Falcão.

Age-related macular degeneration (AMD) is a disease associated with defects in autophagic pathways, as so happens with several neurodegenerative diseases. In order to understand this degenerative disease, some efforts have been focused on the role of autophagy. In AMD, vision loss occurs due to the degeneration of important functional parts of the retina, which is a result from the death of retinal pigmented epithelium (RPE) cells, photoreceptors and choriocapillaris (figure 4.1) [129–131].

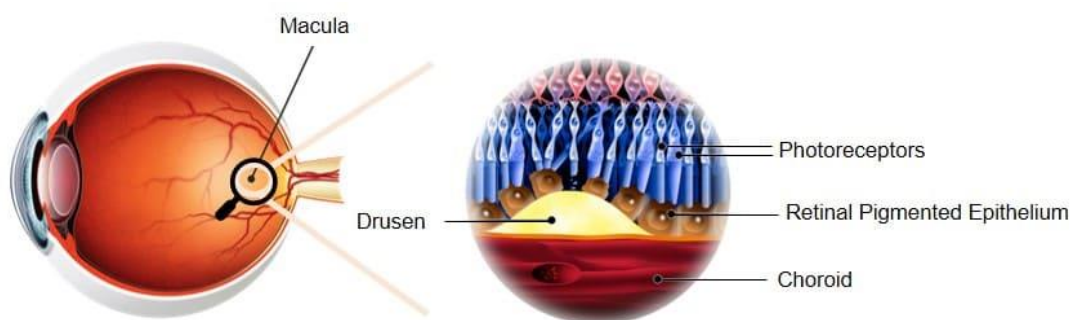


Figure 4.1 – Anatomy of eye in AMD. Adapted from [132,133].

The RPE is formed by a single monolayer of non-regenerating post-mitotic cells and it appears to be involved in the origin of the AMD [129]. Autophagy has an important role in the homeostasis of RPE cells, thus, disfunctions in this process can lead to drusen formation, which allows to associate the autophagy with AMD [131]. Lysosomes of RPE cells are crucial in the degradation of phagocytosed photoreceptor outer segments (POS), assuring optimal neuronal activity during the visual cycle. With aging, the heavy phagocytic load and the oxidative environment RPE lysosomes can lead to accumulation of autofluorescent undigested aggregates, named lipofuscin. This process leads to lysosome dysfunction, which affects other cellular functions and ultimately leads to retinal degeneration and blindness [13]. The photoreceptors are responsible for the

phototransduction, and RPE is its nutritional support element. The RPE is also responsible for the digestion of POS, which also makes pressure on their lysosome, leading to the accumulation of indigestible autofluorescent material, as observed in AMD [129].

Regarding the potential anticancer activity, a primary screening of the analogues in colorectal cancer stem cells lines was performed.

Cancer stem cells (CSCs) or tumour-initiating cells (TICs) are a subpopulation of cancer cells involved in tumour formation, metastases and recurrence due to their long-lasting properties and resistance to chemotherapy. Indeed, these cells have the ability to self-renew and turn into heterogeneous lineages of cancer cells in response to chemotherapeutic agents [134,135]. Furthermore, previous studies have identified that analogues of curcumin as potential agents to treat cancer [73,136]; for example, Difluorinated-curcumin together with conventional chemotherapeutic agents could be effective in the elimination of CSCs, preventing the emergence of chemoresistant colon cancer cells [137].

4.1 Assays in RPE cells

4.1.1 Cell viability

The cell viability of RPE cells was evaluated after 24 hours of incubation in DMEM 1% with the monocarbonyl analogues of curcumin **C1** (compound **1g**) and **C2** (compound **1a**), performing an MTS assay.

The results obtained were expressed as a percentage of control, which was considered as 100% and are presented in figure 4.1.1.1.

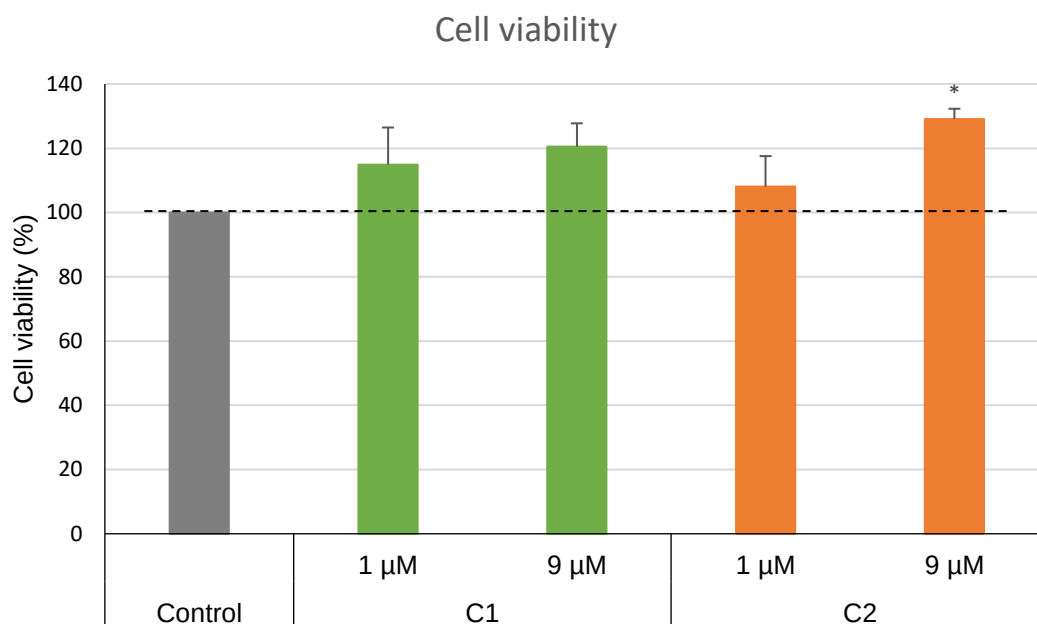


Figure 4.1.1.1 – Cell viability of RPE cells incubated with analogues **C1** and **C2** for 24 hours. * $p < 0.05$ vs. control. $n = 2$.

Observing the graph presented in figure 4.1.1.1 it is possible to conclude that both compounds allow the cellular growth as compared with the control. The major cell viability is obtained for compound **C2** in the concentration of 9 µM ($p < 0.05$), but there is no significative difference between both compounds in this concentration. Comparing the analogues in the concentration of 1 µM, it is verified that compound **C1** promotes a better ambient for cells to develop. However, both compounds' results in the concentration of 1 µM and compound **C1** in the concentration of 9 µM, revealed a significative difference relatively to control values ($p > 0.05$, $n = 2$), therefore, the results are not significative. Concluding, both monocarbonyl analogues of curcumin, **C1** and **C2**, aren't toxic to the RPE cells, favouring its growth.

4.1.2 Autofluorescence assays

The assays for biological evaluation of monocarbonyl analogues of curcumin **C1** (compound **1g**) and **C2** (compound **1a**) (figure 4.1.2.1) were performed in human RPE cells, where lysosomal dysfunction was induced by feeding cells with POS. Two different assays were done, one where the POS were normal and another with POS irradiated with UV light, making the POS more difficult to digest. A set of cells incubated with POS is exposed to curcumin analogues, at different concentrations and then POS intracellular accumulation and POS-dependent autofluorescence is measured by flow cytometry

(detection of the percentage of 488-positive cells), evaluating the RPE ability to digest the internalized POS.

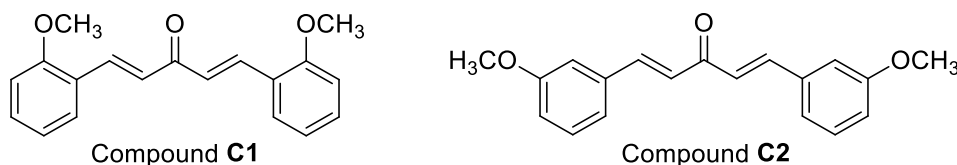


Figure 4.1.2.1 – Chemical structure of compounds **C1** and **C2**, which action are evaluated in the RPE cells.

It is expected that the compounds reduce the autofluorescence, which means that occurs the recovery of lysosome function, observed by the reduction of POS intracellular accumulation, and consequently, the reduction of the autofluorescence. The results obtained for the assay where the compounds **C1** and **C2** were evaluated with the normal POS are in the figure 4.1.2.2.

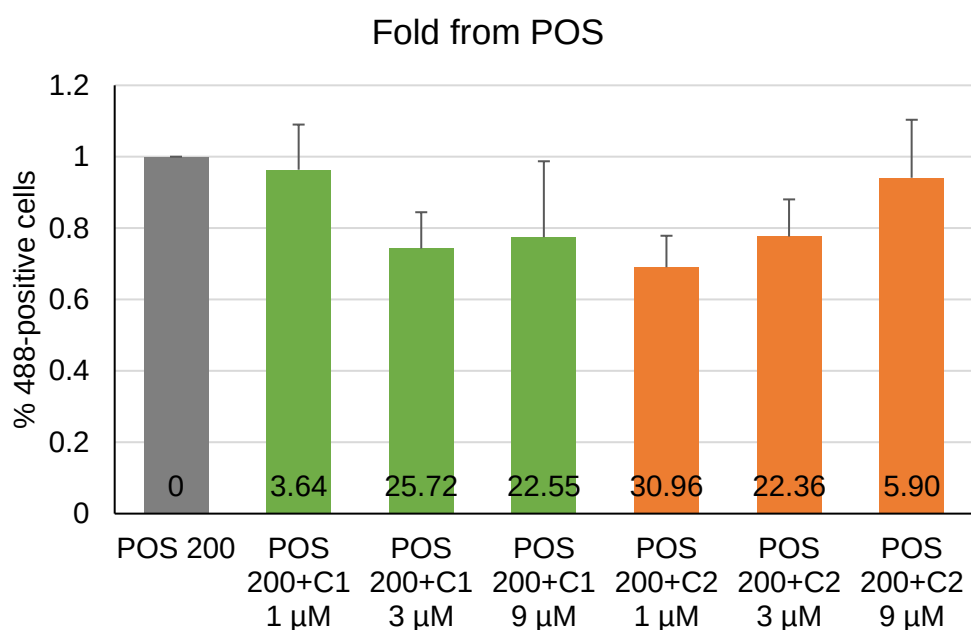


Figure 4.1.2.2 – Effect of **C1** and **C2** in POS-dependent autofluorescence of RPE cells fed with POS, with percentage of reduction of autofluorescence.

The results presented in figure 4.1.2.2 shows that both compounds reduce the POS-dependent autofluorescence. The best results are shown for the compound **C2**, in the concentration of 1 μM, which reduces the autofluorescence in 30.96%. It means that both compounds, in all concentrations tested, are capable to reduce the autofluorescence comparing with the control, in the range of 3.64%-30.96%. However, the results are not significative once it is revealed a significative difference face to control values ($p > 0.05$, $n=2$).

For this assay, it is observed that for compound **C2**, the reduction of autofluorescence diminishes when the concentration of compound increases, once it is verified that the lysosomes digest better the POS when the concentration is lower. Regarding compound **C1**, it is not observed a correlation of the concentration with the reduction of the autofluorescence, being that there is no significant difference in the reduction of autofluorescence when compound **C1** is in the concentration of 3 and 9 μM .

The results obtained for the assay where the compounds **C1** and **C2** were evaluated on RPE cells feed with POS irradiated with UV light are presented in the figure 4.1.2.3.

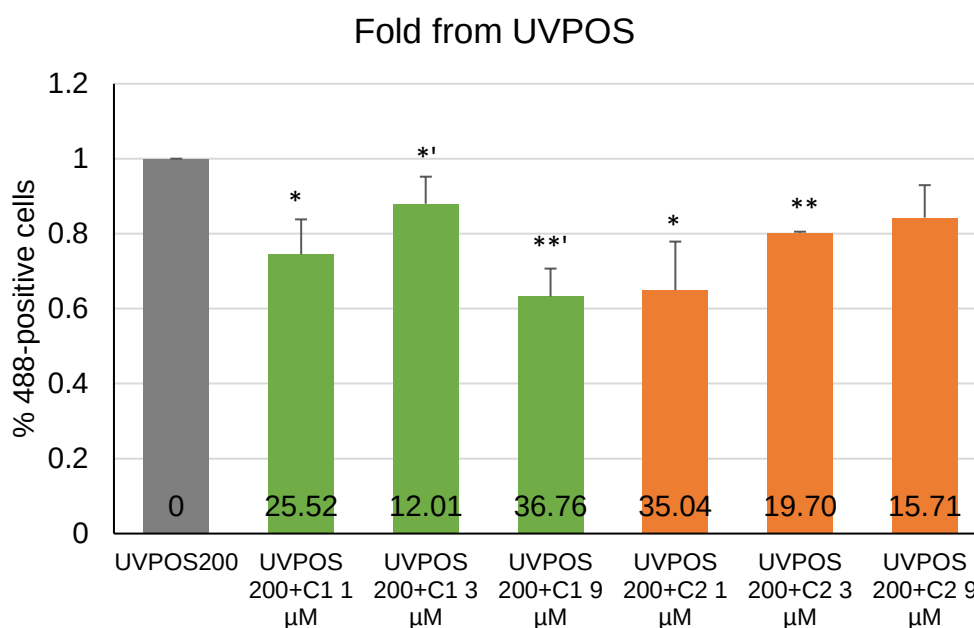


Figure 4.1.2.3 – Effect of **C1** and **C2** in UVPOS-dependent autofluorescence of RPE cells fed with POS irradiated with UV. * $p < 0.05$ vs. control $n = 3$. *' $p < 0.05$ vs control and ** $p < 0.01$ vs. control. $n = 2$. **' $p < 0.01$ vs. control. $n = 4$.

An observation of figure 4.1.2.3 allows us to verify that both compounds have the ability of reduce the autofluorescence in RPE cells fed with POS irradiated with UV light. This assay is more relatable with what occurs in the AMD. Both compounds, in all of test concentrations, are capable to reduce the autofluorescence comparing with the control, in the range of 12.01%-36.76%.

It is verified that compound **C1**, specifically in the concentration of 9 μM , is the one that reduces more the autofluorescence, diminishing it in 36.76% ($p < 0.01$). However, there is no significant difference in the reduction by **C1** in the concentration of 9 μM and **C2** in the concentration of 1 μM .

Comparing the reduction of autofluorescence in both assays with POS and POS irradiated with UV light, it is possible to note that for compound **C2**, the correlation is the

same for normal POS and UV-POS. It is, the lower concentration of compound **C2** is responsible for better response of the lysosomes. For compound **C1**, there is still no correlation dose-response, as observed for normal POS, but this time, the reduction in autofluorescence is higher when the concentration of **C1** is higher.

In order to understand the mechanism of action by which the compounds **C1** and **C2** induce the autophagic pathway, the group of investigation of CEDOC performed a Western blot of both compounds **C1** and **C2** in the concentrations of 1 and 9 μ M (figure 4.1.2.4). To be sure that the alterations observed in the Western blot in the concentration of the proteins tested are due to the action of compounds, β -Actin was used as the immunoblotting loading control.

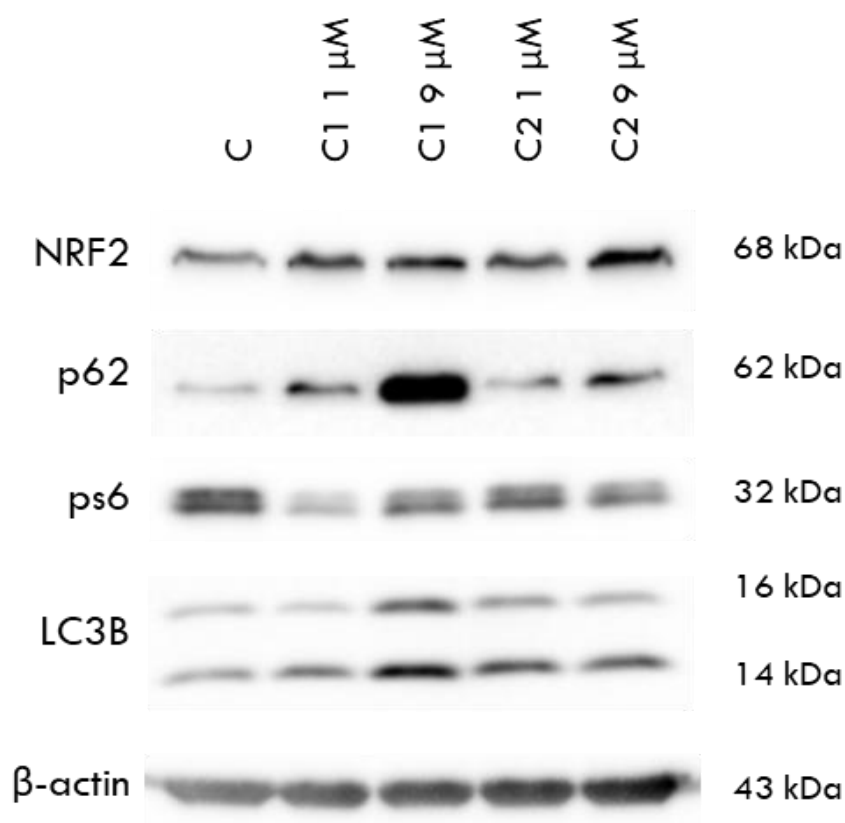


Figure 4.1.2.4 – Results of western blot to understand the mechanism of action of compounds **C1** and **C2**.

Analysing the results presented in the figure 4.1.2.4 and comparing the results for analogues **C1** and **C2** with the control, it is verified that both compounds are activators of Nrf2, by the intensification of the band correspondent to this factor. Also, **C1** increases p62 (a receptor of autophagy involved in several signal transduction pathways, such as Keap1-Nrf2 pathway [138]) and LC3B-II (the LC3 subfamily is a marker molecule of autophagy [139]), being this more evidenced in the concentration of 9 μ M.

Relatively to ps6 (a mTORC1 activity indicator [140]), it is observed a lower intensity of the band for compound **C1** (1 μ M), comparing with the control, which means that **C1** decreases ps6 levels, and therefore we may suggest that it is a mTOR-independent activator of TFEB, which is in agreement with what was reported by Song *et al.* [1].

It was observed that **C1** increases LC3B-II but **C2** hasn't that ability. This and the fact that **C1** increases the levels of p62, are in agreement with the results obtained by Song *et al.* [1].

Our results are also in accordance with Magini *et al.*, that have already tested the analogue **C1** in the concentration of 1 μ M and reported that **C1** activates TFEB without effects on mTORC1 activity and described a decrease on phospho-S6 (ps6) ribosomal protein and an increase on LC3B-II [79].

Deck *et al.* reported that analogues of curcumin activate Nrf2 by inhibition of GSK-3 or activation of kinases, including Akt, independently of Keap 1. Among the analogues of curcumin tested by Deck, it was verified that **C1** is a potent Nrf2 activator [61].

4.2 Assays in cancer stem cells

4.2.1 Cell viability

The cell viability of HT29 human colorectal adenocarcinoma cells was evaluated after seven days of incubation with the monocarbonyl analogues of curcumin, performing a measurement of adenosine triphosphate (ATP) depletion assay. Table 4.2.1.1 presents the results obtained for the cell viability after incubation of each compound.

Table 4.2.1.1 – Cell viability in cancer stem cells.

Compound	Substituents			Cell viability (%)
	R ₁	R ₂	R ₃	
1a (C2)	H	OCH ₃	H	90.79
1b	OCH ₃	OCH ₃	OCH ₃	83.57
1c	H	H	OCH ₃	110.21
1d	Br	H	H	52.68
1e	H	H	H	112.76
1f	NO ₂	H	H	0.007
1g (C1)	OCH ₃	H	H	78.10
1h	OCH ₂ CH ₃	H	H	50.73
1i	CF ₃	H	H	17.64
1j	CH ₃	H	H	89.57
1k	F	H	H	93.90
1l	OCF ₃	H	H	128.48

Analysing the results and considering that the compounds only proceed to other studies if the cell viability is lower than 30%, meaning that the value of ATP depletion is higher than 70%, thus, it is verified that majority of the compounds are not a hit for anti-cancer activity. Only compounds **1f** and **1i** presented cell viability lower than 30%. However, compound **1f**, which has a nitro group in the *ortho* position, presented a cell viability of 0.007%, but is not a hit because it may possess excessive toxicity. Therefore, only compound **1i** (figure 4.2.1.1), which has trifluoromethyl group in *ortho* position and has 17.64% of cell viability, is considered a hit and proceed to studies of dose-response.

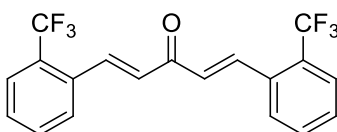


Figure 4.2.1.1 – Chemical structure of compound **1i**.

It was already reported that the position of the substituents is crucial for cytotoxicity against adenocarcinoma cells: substituents in the *ortho* position to the five carbons spacer raise the cytotoxicity, regardless if the substituent is electron donating or electron withdrawing. However, the electronic effects also affect the activity, even if less than the position, it was reported that electron withdrawing groups increase the anticancer activity [77].

Comparing the results for compounds **1a**, **1c** and **1g**, which all have the same substituent (methoxy group) in different positions on the ring, it is possible to verify this *ortho* effect since the cell viability is lower for the compound that presents the methoxy group in the *ortho* position. Additionally, observing the structure of compound **1i**, the only compound that proceed to further studies, its substituent is a trifluoromethyl group, an electron withdrawing group, which is in agreement with the fact that electron withdrawing groups raises the anticancer activity.

Furthermore, analogues of curcumin have already shown to be effective against cancer. The compound GO-Y030 (figure 4.2.1.2), a monocarbonyl analogue of curcumin, which has two methoxymethoxy substituents, one in position 3 and another in position 5 of the phenyl group, inhibited the phosphorylation of STAT3, which results in the decrease of tumour formation and growth, by induction of apoptosis [141].

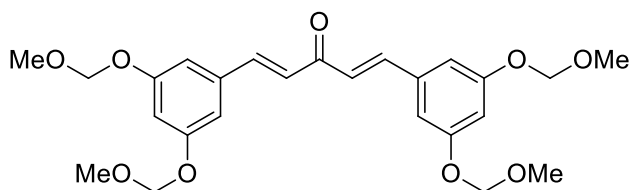


Figure 4.2.1.2 – Chemical structure of the GO-Y030, an analogue of curcumin.

Another study proved that curcumin analogue B19 (figure 4.2.1.3), which have methoxy groups in *ortho* and *meta* positions, can induce endoplasmic reticulum stress and autophagy and by this pathways, induce apoptosis in ovarian cancer cell lines *in vitro* [51] .

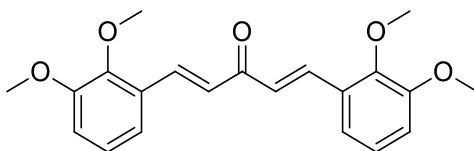


Figure 4.2.1.3 – Chemical structure of curcumin analogue B19.

Considering the reported results, it was expected that our monocarbonyl analogues of curcumin also have action in the CSCs cells. However, in general, the results are not good in the point of view of using the compounds as agents for cancer, but our initial goal is to use them as agents for neurodegenerative diseases, where contrary of cancer cells, the aim is to stop the cell death rather than promote cell death. Therefore, these results may be positive for our main purpose. Also, if the viability in cancer cells is high, perhaps in the RPE cells is also high.

4.2.2 IC₅₀ Determination

Since the cell viability of HT29 human colorectal adenocarcinoma cells incubated with compound **1i** was 17.64%, this compound proceeded other assays, in order to determine the half maximal inhibitory concentration, the IC₅₀ value.

To assess whether the compound **1i** has a generic effect on colorectal CSCs or whether this is a cell line dependent effect, compound **1i** was tested on CSC-enriched tumourspheres from other colorectal cell lines (HCT116, SW480 and SW620). Additionally, to ensure compound **1i** specificity in targeting CSC-enriched spheroids, compound **1i** inhibitory potency on adherent cell lines was also evaluated. The IC₅₀ values obtained for each colorectal cell line are presented in table 4.2.2.1.

Table 4.2.2.1 – IC₅₀ determination in both CSC-enriched tumourspheres (3D; n= 3-5) and under adherent conditions (2D; n=1-4). Concentrations units: µM.

	HT29		HCT116		SW620		SW480	
	3D IC ₅₀	2D IC ₅₀	3D IC ₅₀	2D IC ₅₀	3D IC ₅₀	2D IC ₅₀	3D IC ₅₀	2D IC ₅₀
Compound 1i	0.9295	≈2.9790	0.8100	1.7860	0.7621	1.6850	0.51570	2.3270

Considering the assay in CSC-enriched tumourspheres, it is verified that compound **1i** presents different activity in the four colorectal cell lines, being more active

in SW480 colorectal cells. Additionally, analysing the IC_{50} values it is observed that compound **1i** is more active in CSC-enriched tumourspheres than under adherent conditions. Under adherent conditions, the lower IC_{50} determined occur for SW620 colorectal cells.

The dose-response curves that lead to the IC_{50} value determination, for each cell line in CSC-enriched tumourspheres are presented in figures 4.2.2.1.

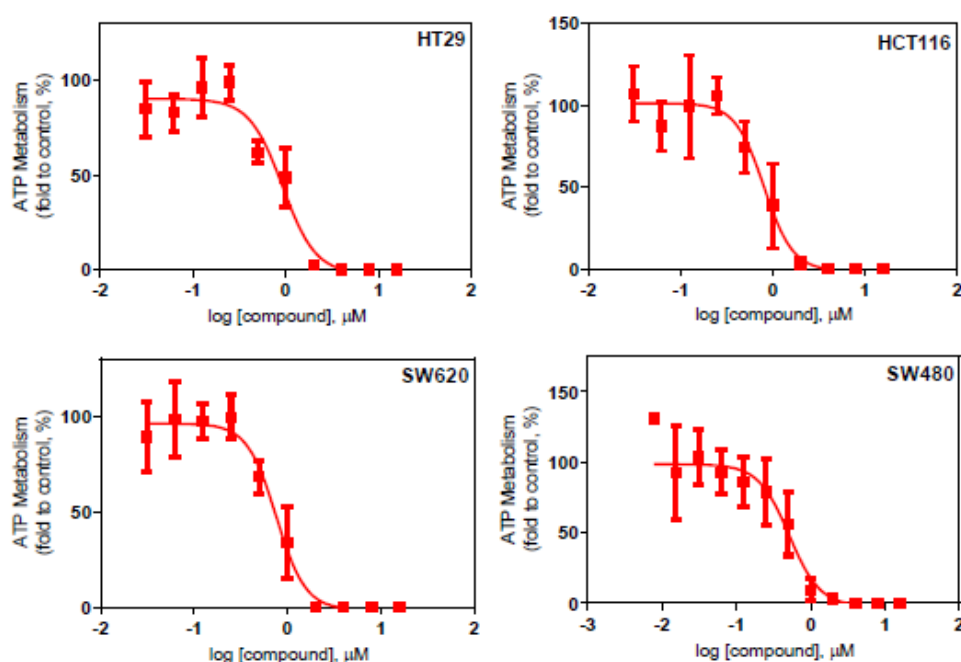


Figure 4.2.2.1 – Dose-response curves in CSC-enriched tumourspheres (n= 3-5).

Regarding the CSC-enriched tumourspheres cellular model, compound **1i** showed adequate dose-response curves for all four tested cell lines, presenting IC_{50} values under 1 μM .

Under adherent conditions, compound **1i** also showed adequate dose-response curves (figure 4.2.2.2). To complete IC_{50} determination under adherent conditions, compound **1i** samples are required at concentrations higher than 10 mM. The IC_{50} values for compound **1i** in adherent conditions are, for all the cell lines, higher than in CSC-enriched tumourspheres, appearing to be less active under these conditions when compared to CSC-enriched spheroids.

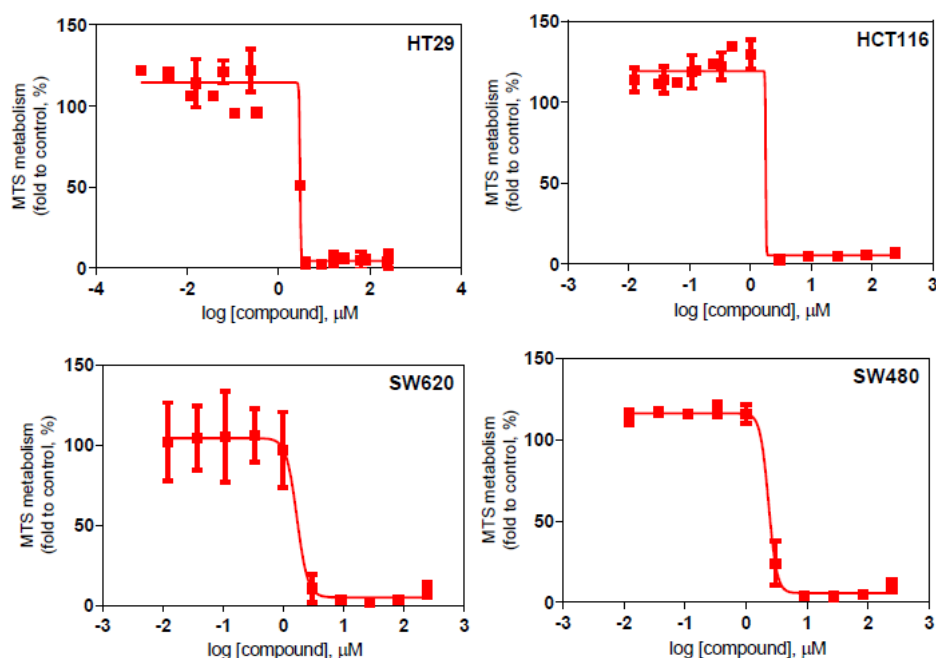


Figure 4.2.2.2 – Dose-response curves under adherent conditions (n=1-4).

Considering this results, compound **1i** showed to be promisor and proceed to further studies where it will be assessed the phenotypic effect of this compound on tumoursphere formation, evaluating the quantity and the size of tumourspheres,

In summary:

Regarding the potential protective effect in neurodegenerative diseases, the biological activity of the compounds **C1 (1g)** and **C2 (1a)** was evaluated in retinal pigmented epithelium cells. The cell viability assessment allows to verify that in the minor concentration tested (1 μM), compound **C1 (1g)** promotes a better ambient for cells to growth, while in the major concentration (9 μM) there is no significative difference between the results for both compounds.

The autofluorescence assays indicate that both compounds in all tested concentrations are capable to reduce the POS and UV-POS-dependent autofluorescence, meaning that these compounds may improve the lysosomal function.

Western blot is useful in the investigation of the mechanism of action by which the compounds **C1 (1g)** and **C2 (1a)** induce the autophagic pathway. **C1 (1g)** increases p62 and lower ps6 levels, being a mTOR-independent TFEB activator. **C1 (1g)** contrarily to **C2 (1a)** increases LC3B-II. Both compounds are Nrf2 activators, which may be a potential mechanism to explain the neuroprotector effect of curcumin and, consequently, of these compounds too.

About the effect of these compounds in cancer stem cells, the cell viability showed that only compound **1i** may be promisor, while compound **1f** may have overstated toxicity. IC₅₀ values were determined for compound **1i** in four types of cancer cell lines both in CSC-enriched tumourspheres and under adherent conditions, concluding that this compound is more active in CSC-enriched tumourspheres than under adherent conditions. However, in general, the results are promising and compound **1i** will proceed to more studies, where the phenotypic effect of this compound on tumourspheres formation will be studied.

5 Conclusions and future perspectives

Curcumin has an ample range of biological activity and low toxicity, although, the clinical use is limited due to pharmacological and pharmacokinetic defects. Consequently, in the past few years, interest has been focused in the development of analogues of curcumin with pharmacological and pharmacokinetic profiles improved.

This work reports the synthesis, assessment of drug-likeness properties and biological evaluation in retinal pigmented epithelium cells and in cancer stem cells of the monocarbonyl analogues of curcumin. Compounds were obtained by an aldol condensation reaction, with yields varying between 9.9 and 77.5%. The analogues were characterized by NMR techniques, IR spectroscopy, ESI⁺ mass spectroscopy, elemental analysis and UV spectroscopy.

Partition coefficients and stability in physiological conditions were both assessed by HPLC. Synthesized monocarbonyl analogues of curcumin assure Lipinski's rule of five, presenting good lipophilic properties. Additionally, the analogues are very stable in physiological conditions, both in PBS (pH 7.4) and in human plasma, with exception of the analogue with a nitro group in *ortho* position, which presented a half-life of about 8 hours.

Monocarbonyl analogues of curcumin consist in α,β -unsaturated carbonyl compounds, and even if this class of compounds is avoided due to its relation with cell damage and cytotoxicity, they are useful in drug design due to its electrophilic properties. The electrophilicity determination was performed using two different methods, following the reaction by UV and by ¹H NMR. NMR method demonstrated to be simple and fast to identify Michael acceptor sites in the compounds, also distinguishing the adducts as reversible or irreversible, but due to the insolubility of cysteamine in CDCl₃ it was not possible to identify the reversible nature of the adducts formed. Monocarbonyl analogues of curcumin react easily with cysteamine but not with N-acetylcysteine. Still, the reactivity with N-acetylcysteine cannot be completely excluded, since the reactivity of the thiol group depends on the protein environment. Consequently, it is pretentious to transfer the results obtained into biological systems. However, the electrophilicity assessment may be a useful way to predict toxicity from the structure of the compounds, optimizing the product development, eliminating toxic compounds early and, thus, reducing costs.

Regarding the viability in cancer stem cells results, compound **1i** is the only one considered a hit and proceed to studies of dose-response in cancer stem cells, while compound **1f** is not a hit because it may possess excessive toxicity. The results of dose-response studies for compound **1i** in four types of cancer cell lines both in CSC-enriched

tumourspheres and under adherent conditions are promising and this compound proceed to more studies, where the phenotypic effect of this compound on tumourspheres formation will be studied.

Assays in retinal pigmented epithelium cells, revealed that both compounds **C1 (1g)** and **C2 (1a)** may induce the autophagic pathway. The western blot assay proved that both compounds are activators of Nrf2, which results in the upregulation of antioxidative and detoxifying enzymes, being the Nrf2/ARE signal pathway one of the potential mechanisms that explains the neuroprotector effect of curcumin and therefore, of these monocarbonyl analogues. Therefore, the drug-likeness properties of **C1** and **C2** and the biological results are encouraging and even if further studies are required, the compounds have potential to treat age-related macular degeneration.

6 Experimental procedure

6.1 Reagents and Solvents

The reagents and solvents used along the project were purchased from Merck, Sigma-Aldrich, Alfa Aesar and Fischer Chemical.

For NMR analysis, samples were prepared in deuterated solvents, such as chloroform, acetone, dimethyl sulfoxide and methanol, all of them with purity degree higher than 99% and all were purchased from Sigma-Aldrich.

For HPLC experiments, deionized water and acetonitrile (Honeywell | Riedel-de-Haën™), with analytical grade, were used. For the UV experiments, the same acetonitrile was used. The water used in the procedures of synthesis, extractions and kinetical studies was distilled and purified using a MilliQ system (Millipore). The Phosphate-buffered saline (pH 7.4, 0.01 M) used in the assays was prepared through the dissolution of one tablet in 200 mL of deionized water.

6.2 Equipment

The NMR spectra (mono and bidimensional) were recorded on a Bruker Ultra-Shield 300 MHz spectrometer. Chemical shifts (δ) are presented in parts per million (ppm) relative to tetramethylsilane (TMS) as internal standard and coupling constants (J) are expressed in Hertz (Hz). The multiplicities of the peaks are given as: singlet (s), doublet (d), doublet of doublets (dd), doublet of doublets of doublets (ddd), doublet of quartets (dq), triplet (t), triplet of doublets (td), quartet (q) and multiplet (m).

To perform the infrared (IR) spectra, an IRAffinity-1 FTIR Spectrophotometer (Shimadzu) was used and for spectra acquisition and processing it was used an IRsolution software. The samples were prepared in tablets with KBr. The absorption bands were reported, expressing the wavenumber in reciprocal centimetres (cm^{-1}).

Mass spectra analysis were performed using Electrospray Ionization technique, in a Micromass Quattro Micro API spectrometer (Micromass Quattromicro® API Spectrometer, with Waters® Alliance 2695 HPLC Module). The elemental analysis was performed in a "FLASH 2000 Series CHNS/O Analyzers" by Thermo Scientific™ equipped with a thermal conductivity detector and for data acquisition and processing it was used an Eager Xperience software.

The melting points of the synthesized compounds were determined using a Kofler Bock-Monoscop <M>.

UV spectra were recorded in Shimadzu UV-1603 and UV-1280 UV-spectrophotometer and for data acquisition and processing it was used an UV-1601PC and UVProbe 2.43 software, respectively.

High-performance liquid chromatography (HPLC) was performed in an Elite LaChrom (VWR Hitachi) system, composed by a L-2130 pump, a L-2400 UV detector, a LiChrospher® 100 RP-18 (5 µm) column and a Rheodyne® manual sample injector, which has a 20 µL sample loop. It was used a Hamilton microliter syringe to inject the samples. For data acquisition and processing it was used an EZChrom Elite software.

Other equipment were also used, including an ultrasonic bath (Bandelin Sonorex TK52), a vortex mixer (Heidolph), a thermostatic bath (Julabo F12), an UV light with wavelengths of 254 nm and 366 nm (CAMAG), magnetic stirrers (Velp Scientifica and IKA-Combimag RCT), analytical scales (Mettler Toledo AG135 and Ohaus Adventurer Pro), a centrifuge (Eppendorf 5430) and rotative evaporators (VWR).

6.3 Chromatography

Thin layer chromatography (TLC) was required to monitoring the reactions. It was performed in silica-gel aluminium sheets (Merck Kieselgel 60 F₂₅₄, thickness = 0.25 mm) and a UV light with wavelengths of 254 nm and 366 nm (CAMAG) was utilized to visualise the spots. As all the compounds have an aromatic ring, there was no need to use developers.

The purification of the monocarbonyl analogues of curcumin sometimes required preparative thin layer chromatography but most of the time it was done by column chromatography. The preparative thin layer chromatography was carried out on glass plates coated with silica-gel (Merck Kieselgel 60 GF₂₅₄) and column chromatography was performed using silica-gel (Merck Kieselgel 60, particle size = 0.040 - 0.063 mm) and the most suitable eluent, which is a mixture of n-hexane and ethyl acetate.

6.4 Synthetic procedure

6.4.1 General procedure

The monocarbonyl analogues of curcumin were synthesized according to what is described by Dai *et al.* [77]. The synthesis consists in an aldol condensation of modified benzaldehyde and ketone, in a proportion of two for one, in presence of Sodium Hydroxide (NaOH), using ethanol as solvent for the reaction (figure 6.4.1.1).

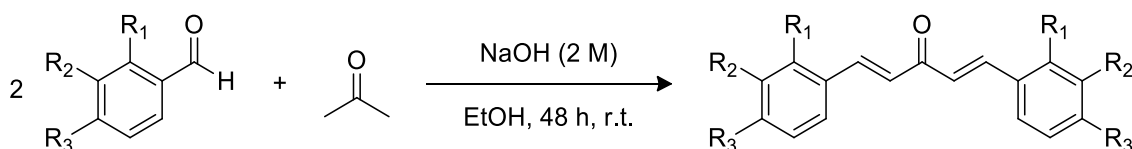


Figure 6.4.1.1 – General reaction for the synthesis of monocarbonyl analogues of curcumin.

Methodology:

Firstly, ethanol (4 mL) is put in a reaction round bottom flask and then the ketone (25 mmol; 183 μ L) is added, followed by the modified benzaldehyde (51 mmol), in the ratio 1:2, and the solution is vigorously stirred for 48 hours, at room temperature. The NaOH 2 M (1.25 mL) is added dropwise for about half an hour. After 48 hours, it is joined distilled water to the reaction mixture and then it is neutralized with HCl (37%). Mostly, it is observed a formation of a solid precipitate, that is filtered, washed with distilled water and dried under vacuum. When it isn't observed a solid precipitate, an extraction is performed, normally with dichloromethane (DCM). Then, the products are charged into a silica gel column and eluted with a mixture of ethyl acetate and hexane, in a right proportion according to what better separates the product. Solid compounds were posteriorly recrystallized using DCM and petroleum ether.

Modifications to general methodology:

For compound **1e**, **1k** and **1l**, the column doesn't separate properly, so it was necessary to resort to preparative thin layer chromatography. The eluent utilized in the column or preparative thin layer chromatography (compounds **1e**, **1k** and **1l**) is described in table 6.4.1.1.

Table 6.4.1.1 – Solvents used in column chromatography separation of the synthesized compounds **1a-l**.

Compound	Hexan: Ethylacetate proportion
1a (C2)	7:3
1b	7:3
1c	7:3
1d	9:1
1e	8:2
1f	7:3
1g (C1)	7:3
1h	8:2
1i	8:2
1j	9:1
1k	6:4
1l	8:2

Compounds **1k** and **1l** weren't obtained in a totally pure state. Synthesis of these compounds were performed several times in order to understand a way to purify them. For both compounds, ^1H RMN demonstrates "noise" on the aromatic region, making it impossible to identify the signals of compounds in this region.

To compound **1k**, which has a Fluor group in the *ortho* position, five synthesis attempts were performed and are resumed in figure 6.4.1.2.

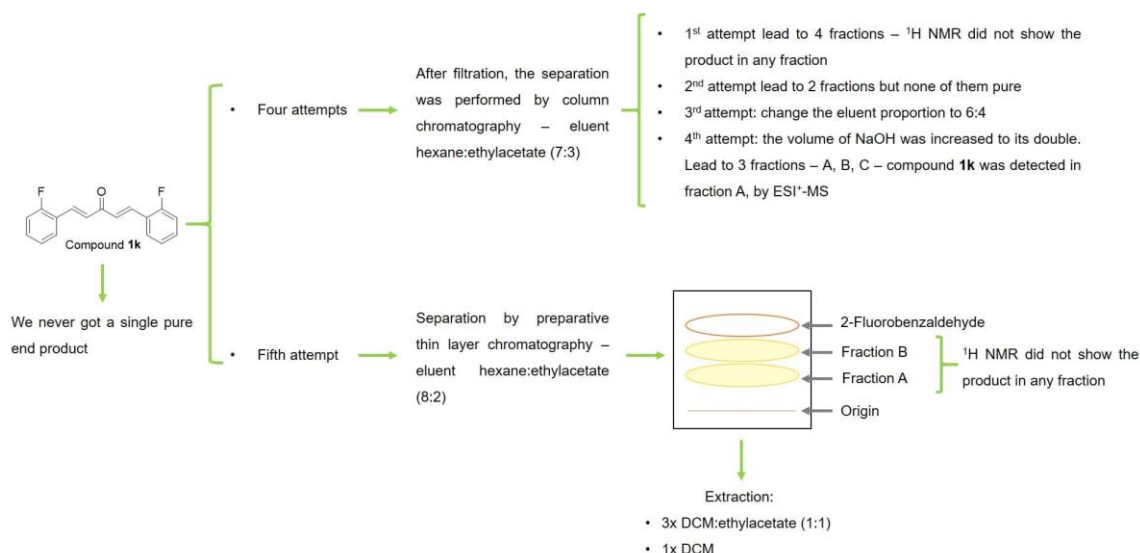


Figure 6.4.1.2 – Resume of attempts to synthesize compound **1k**.

Two attempts were performed to synthesize compound **1l**, which has a trifluoromethoxy group in the *ortho* position. The efforts are resumed in figure 6.4.1.3.

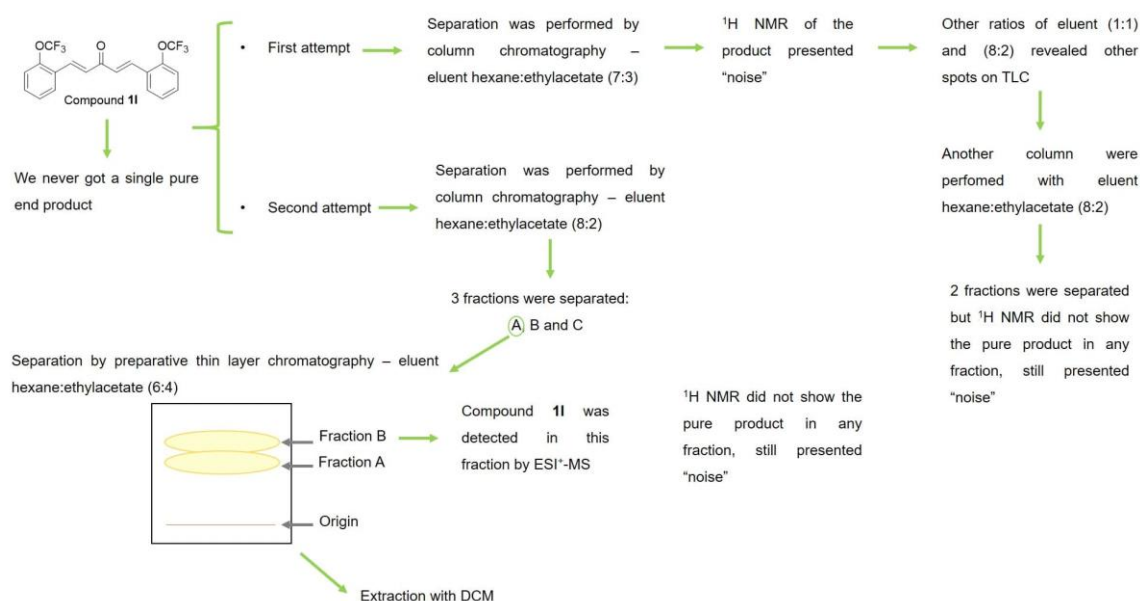


Figure 6.4.1.3 – Resume of attempts to synthesize compound **1l**.

Several attempts to synthesize another monocarbonyl analogue of curcumin, which has a hydroxyl group in the *ortho* position, were performed but none of them successful.

In the first attempt and following the general procedure, it was observed that an alteration of the colour of the solution from light orange to yellow with the addition of the base and then the reactional mixture solidified. More solvent (ethanol) was added, changing the appearance to powder in suspension. The TLC allows us to see the spot of the initial salicylaldehyde and tailing. For the next hours, the reaction maintained the same appearance and the TLC remained equal. So, the starting reagent was not consumed. After 48 hours, when the distilled water was added, it was verified the dissolution of the powder. An extraction with DCM was performed and the two phases were saved. The TLC revealed the same product in both phases and the ^1H RMN spectra of both phases didn't correspond to the desired product.

In the second attempt, it was observed the same alterations in the appearance of the reactional mixture, and it was also added more ethanol. This time, after the 48 hours, the solvent was evaporated, saving the solid. The ^1H RMN didn't also correspond to the expected product. It was thought that maybe the NaOH deprotonate the 2-hydroxy-benzaldehyde, forming a salt that precipitate and blocking the aldol condensation to occur.

In the third attempt, the order of addition was modified: ketone and the base were joined, and then added dropwise to the ethanol and salicylaldehyde, previously mixed. However, the precipitation also occurred, and it was necessary to add more ethanol. The TLC showed only one spot corresponding to the salicylaldehyde. After 24 hours, it was added more NaOH, and even without precipitation this time, the TLC didn't change, presenting also only one spot. After about 3 hours, another TLC was done, where it was possible to see tailing and it was thought that maybe the reaction occurs, but the product may have the same retention factor (R_f) than the salicylaldehyde. Different eluents were used, but none of them showed two different spots on TLC. To finish the reaction and inspired by Rani *et al.*, we proceeded to the addition of HCl (37%) to promote precipitation and then filter and wash with ethanol [142]. Nevertheless, neither the solid that were filtered, nor the liquid phase presented the desired product, which were confirmed by ^1H RMN.

The try that followed up, consisted in the same order of addition as in the third attempt but not adding more base after 24 hours. This time, in the initial TLC there are two defined spots, with tailing, showing that the reaction is occurring. However, after 24 and 48 hours the TLC showed only the spot corresponding to the 2-hydroxy-benzaldehyde and tailing. After the 48 hours stirring, it was dropwise added HCl (37%).

An extraction was performed with DCM, observing the dissolution of the powder. The solvent was evaporated. Then, it was tested in different *eppendorfs* whose solvent promotes a better dissolution, testing DCM, ethylacetate, hexan, ethylic ether and petroleum ether. It was observed that DCM and ethylacetate were the ones that dissolved better, but not totally. By TLC, the one that only presented one spot, without tailing is DCM. Therefore, it was added DCM to dried and solid residue and then the remaining suspension was filtered. ^1H RMN spectra didn't corresponds to the desired compound.

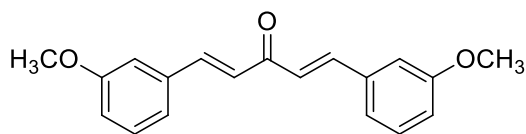
The fifth attempt was partially equal to the first one, the initial TLC and the final one showed the spot of the salicylaldehyde and tailing. The TLC of the reaction mixture was done facing the evaporated solid and the part dissolved in DCM. Both were equal. So, all the solvent was evaporated. Next, DCM was added but the precipitate didn't all dissolve. So, the solid were filtered and saved and the liquid was extracted with DCM. In the end, ^1H RMN proved that it wasn't the expected compound.

In the last effort, when the mixture of NaOH and ketone was added dropwise, the reaction mixture solidified and removing a little quantity of the solid, while dissolving it in DCM and then by TLC facing the salicylaldehyde, it was confirm that is the starting material that precipitate. Then, it was observed that MeOH dissolved the solid, but when added not all the solid were dissolved, even if it was added more MeOH. Next, water was added, but in the end, turbidity was still observed. Furthermore, an alteration in the colour, from yellow to dark brown, was observed. By TLC, it was observed that the benzaldehyde was still not reacting. So, we never obtained the monocarbonyl analogue with a hydroxy group in *ortho* position.

Another monocarbonyl analogue of curcumin, was also attempted to synthesize, using 2-cyanobenzaldehyde as the starting material. The synthesis was performed accordingly to the general procedure. In the end of the reaction, it was filtered but the liquid fraction was still showing a yellow colouration, so it was supposed that the product was still there, even if only in part. Therefore, it was done a TLC from both phases, and it was observed that there were no spot in TLC of the precipitate, and two spots in the liquid phase. Several eluents were tested to see which separates better and then a column chromatography was performed with the eluent hexan:ethylacetate (9:1). However, after the separation and ^1H RMN characterization it was proved that none of these fractions corresponds to the desired compound.

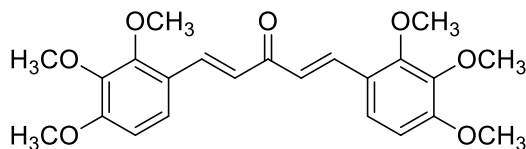
6.4.2 Product structural characterization

6.4.2.1 1,5-Bis(3-methoxyphenyl)-1,4-pentadien-3-one (1a)



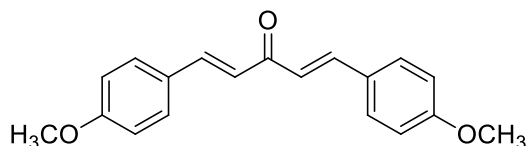
Yellow oil; Yield: 21.6%; ^1H NMR (300 MHz, CDCl_3) δ 7.69 (2 H, d, $J = 16$ Hz), 7.31 (2 H, t, $J = 8$ Hz), 7.19 (2 H, d, $J = 8$ Hz), 7.12 (2 H, t, $J = 2$ Hz), 7.06 (2 H, d, $J = 16$ Hz), 6.94 (2 H, dq, $J = 8$ Hz), 3.82 (6 H, s); ^{13}C NMR (75 MHz, CDCl_3) δ 188.76, 159.93, 143.17, 136.13, 129.91, 125.63, 121.06, 116.30, 113.30, 55.25; ESI $^+$ -MS m/z 295 ($\text{M}+\text{H}^+$, 100%).

6.4.2.2 1,5-Bis(2,3,4-trimethoxyphenyl)-1,4-pentadien-3-one (1b)



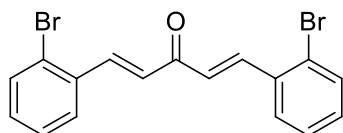
Yellow oil; Yield: 46.8%; ^1H NMR (300 MHz, CDCl_3) δ 7.85 (2 H, d, $J = 16$ Hz), 7.27 (2 H, d, $J = 9$ Hz), 7.00 (2 H, d, $J = 16$ Hz), 6.62 (2 H, d, $J = 9$ Hz), 3.86 (6 H, s), 3.80 (6 H, s), 3.79 (6 H, s); ^{13}C NMR (75 MHz, CDCl_3) δ 189.43, 155.56, 153.46, 142.26, 137.71, 124.83, 123.32, 121.82, 107.59, 61.39, 60.81, 55.97; ESI $^+$ -MS m/z 415 ($\text{M}+\text{H}^+$, 100%).

6.4.2.3 1,5-Bis(4-methoxyphenyl)-1,4-pentadien-3-one (1c)



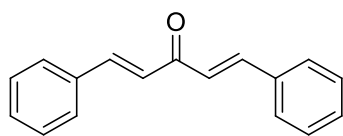
Yellow solid; Yield: 59.6%; m.p.: 124-125 $^\circ\text{C}$; IR (KBr, cm^{-1}): 1597, 1240, 1176; ^1H NMR (300 MHz, CDCl_3) δ 7.71 (2 H, d, $J = 16$ Hz), 7.57 (4 H, d, $J = 9$ Hz), 6.98 (2 H, d, $J = 16$ Hz), 6.94 (4 H, d, $J = 9$ Hz); 3.85 (6 H, s); ^{13}C NMR (75 MHz, CDCl_3) δ 188.99, 161.70, 142.81, 130.21, 127.80, 123.66, 114.56, 55.53; ESI $^+$ -MS m/z 295 ($\text{M}+\text{H}^+$, 100%); Elemental analysis (%): C (77.19), H (6.18), O (16.55).

6.4.2.4 1,5-Bis(2-Bromophenyl)-1,4-pentadien-3-one (1d)



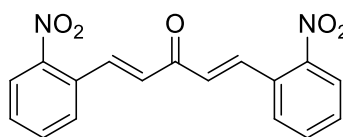
Brown solid; Yield: 20.7%; m.p.: 64-66 $^\circ\text{C}$; IR (KBr, cm^{-1}): 1647, 1192; ^1H NMR (300 MHz, CDCl_3) δ 8.09 (2 H, d, $J = 16$ Hz), 7.71 (2 H, dd, $J = 2, 8$ Hz), 7.64 (2 H, d, $J = 8$ Hz), 7.36 (2 H, t, $J = 7$ Hz), 7.26 (2 H, td, $J = 2, 8$ Hz), 7.03 (2 H, d, $J = 16$ Hz); ^{13}C NMR (75 MHz, CDCl_3) δ 188.82, 142.14, 134.96, 133.70, 131.55, 128.00, 127.92, 127.87, 126.01; ESI $^+$ -MS m/z 393 ($\text{M}+\text{H}^+$, 25%); Elemental analysis (%): C (53.25), H (3.88), O (5.38).

6.4.2.5 1,5-Bisphenyl-1,4-pentadien-3-one (1e)



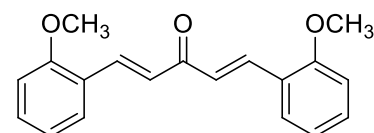
Yellow solid; Yield: 10.5%; m.p.: 58-59 °C; IR (KBr, cm^{-1}): 1647; ^1H NMR (300 MHz, CDCl_3) δ 7.75 (2 H, d, $J = 16$ Hz), 7.61-7.64 (4 H, m), 7.39-7.43 (6 H, m), 7.10 (2 H, d, $J = 16$ Hz); ^{13}C NMR (75 MHz, CDCl_3) δ 189.04, 143.44, 134.84, 130.62, 129.07, 128.50, 125.47; ESI⁺-MS m/z 235 ($\text{M}+\text{H}^+$, 100%); Elemental analysis (%): C (85.02), H (6.71), O (7.83).

6.4.2.6 1,5-Bis(2-Nitrophenyl)-1,4-pentadien-3-one (1f)



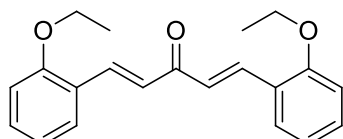
Green solid; Yield: 9.9%; m.p.: 158-159 °C; IR (KBr, cm^{-1}): 1624, 1508, 1340; ^1H NMR (300 MHz, CDCl_3) δ 8.16 (2 H, d, $J = 16$ Hz), 8.08 (2 H, d, $J = 8$ Hz), 7.67-7.74 (4 H, m), 7.58 (2 H, td, $J = 2, 8$ Hz), 6.97 (2 H, d, $J = 16$ Hz); ^{13}C NMR (75 MHz, CDCl_3) δ 188.46, 148.67, 139.57, 133.82, 131.10, 130.69, 129.36, 125.23; ESI⁺-MS m/z 325 ($\text{M}+\text{H}^+$, 10%); Elemental analysis (%): C (63.06), N (8.52), H (4.05), O ($\approx$ 24.37 – to be repeated).

6.4.2.7 1,5-Bis(2-methoxyphenyl)-1,4-pentadien-3-one (1g)



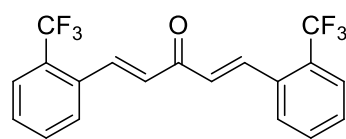
Yellow solid; Yield: 77.5%; m.p.: 123-124 °C; IR (KBr, cm^{-1}): 1614, 1332, 1252; ^1H NMR (300 MHz, CDCl_3) δ 8.07 (2 H, d, $J = 16$ Hz), 7.62 (2 H, dd, $J = 2, 8$ Hz), 7.37 (2 H, td, $J = 2, 8$ Hz), 7.18 (2 H, d, $J = 16$ Hz), 6.99 (2 H, t, $J = 8$ Hz), 6.94 (2 H, d, $J = 8$ Hz), 3.91 (6 H, s); ^{13}C NMR (75 MHz, CDCl_3) δ 190.13, 158.71, 138.33, 131.68, 128.84, 126.40, 124.13, 120.87, 111.33, 55.65; ESI⁺-MS m/z 295 ($\text{M}+\text{H}^+$, 100%); Elemental analysis (%): C (77.44), H (6.30), O (16.50).

6.4.2.8 1,5-Bis(2-Ethoxyphenyl)-1,4-pentadien-3-one (1h)



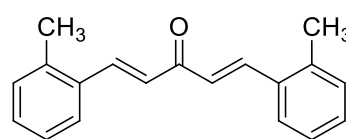
Yellow solid; Yield: 35.3%; m.p.: 83-84 °C; IR (KBr, cm^{-1}): 1670, 1333, 1250, 556; ^1H NMR (300 MHz, CDCl_3) δ 8.05 (2 H, d, $J = 16$ Hz), 7.60 (2 H, dd, $J = 2, 8$ Hz), 7.34 (2 H, ddd, $J = 1, 2, 8$ Hz), 7.23 (2 H, d, $J = 16$ Hz), 6.98 (2 H, td, $J = 1, 8$ Hz), 6.92 (2 H, d, $J = 8$ Hz), 4.14 (4H, q, $J = 7$ Hz), 1.51 (6 H, t, $J = 7$ Hz); ^{13}C NMR (75 MHz, CDCl_3) δ 190.32, 158.19, 138.65, 131.58, 129.41, 126.56, 124.15, 120.76, 112.23, 64.13, 14.98; ESI⁺-MS m/z 323 ($\text{M}+\text{H}^+$, 100%); Elemental analysis (%): C (77.97), H (6.98), O (15.07).

6.4.2.9 1,5-Bis(2-Trifluorophenyl)-1,4-pentadien-3-one (1i)



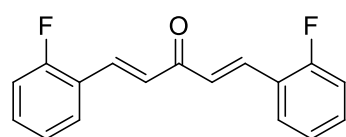
Yellow solid; Yield: 49.7%; m.p.: 131-132 °C; IR (KBr, cm^{-1}): 1591, 1155-1198; ^1H NMR (300 MHz, CDCl_3) δ 8.09 (2 H, dd, $J = 2, 16$ Hz), 7.79 (2 H, d, $J = 8$ Hz), 7.74 (2 H, d, $J = 8$ Hz), 7.61 (2 H, t, $J = 7$ Hz), 7.51 (2 H, t, $J = 8$ Hz), 7.01 (2 H, d, $J = 16$ Hz); ^{13}C NMR (75 MHz, CDCl_3) δ 188.44, 139.39, 133.85, 132.33, 129.96, 129.56, 129.16, 129.02, 128.09, 126.45, 124.12; ESI $^+$ -MS m/z 371 ($\text{M}+\text{H}^+$, 70%); Elemental analysis (%): C (61.52), H (3.47), O (6.99).

6.4.2.10 1,5-Bis(2-methylphenyl)-1,4-pentadien-3-one (1j)



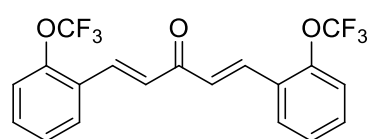
Yellow solid; Yield: 70.1%; m.p.: 90-91 °C; IR (KBr, cm^{-1}): 2945-2924, 1612; ^1H NMR (300 MHz, CDCl_3) δ 8.05 (2 H, d, $J = 16$ Hz), 7.66 (2 H, d, $J = 8$ Hz), 7.00 (2 H, d, $J = 16$ Hz), 7.22-7.34 (6 H, m), 2.49 (6 H, s); ^{13}C NMR (75 MHz, CDCl_3) δ 189.06, 141.04, 138.34, 133.96, 131.03, 130.34, 126.85, 126.56, 126.50, 19.99; ESI $^+$ -MS m/z 263 ($\text{M}+\text{H}^+$, 100%); Elemental analysis (%): C (86.68), H (7.06), O (6.21).

6.4.2.11 1,5-Bis(2-fluorophenyl)-1,4-pentadien-3-one (1k)



Orange oil; Yield: 82.4%; ESI $^+$ -MS m/z 271 ($\text{M}+\text{H}^+$, 100%).

6.4.2.12 1,5-Bis(2-trifluoromethoxyphenyl)-1,4-pentadien-3-one (1l)



Orange oil; Yield: 11.8%; ESI $^+$ -MS m/z 403 ($\text{M}+\text{H}^+$, 45%).

6.5 High-Performance Liquid Chromatography

The solvents used for mobile phase, such as acetonitrile and water were firstly sonicated to degass, preventing bubble formation.

Initially, it is performed an elution in a gradient of acetonitrile (ACN) and water, to determine the most suitable mobile phase to choose the better method for each compound, based on the retention time and the correspondent proportion of ACN and

water. Then the method is tested, applying an isocratic elution, where the composition of the mobile phase remained constant throughout the run.

The flow rate of the mobile phase was set in 1.0 mL/min.

The maximum of absorption of each compound was determined by UV spectroscopy and the UV detector from HPLC system was set accordingly to it. The aqueous phase (deionized water) and organic phase (acetonitrile) ratio, the wavelength as well as the retention time (Rt) of each compound in the determination of partition coefficient and in PBS and human plasma assays are shown in table 6.5.1.

Table 6.5.1 – Specifications for HPLC studies for compounds **1a-l**.

Compound	Retention time (min)			Mobile phase		Wavelength (nm)
	ACN	Plasma	PBS	ACN (%)	Water (%)	
1a (C2)	2.760	2.711	2.878	70	30	316
1b	2.192	2.422	2.473	70	30	340
1c	4.460	4.150	4.209	60	40	356
1d	3.463	3.290	3.386	80	20	295
1e	4.148	4.529	4.134	60	40	303
1f	2.574	2.373	2.403	65	35	273
1g (C1)	2.210	2.391	2.065	75	25	280
1h	2.883	2.212	1.993	85	15	358
1i	2.886	3.164	3.163	80	20	300
1j	1.847	1.785	1.744	90	10	329
1k	1.823	–	–	85	15	285
1l	2.153	–	–	95	5	285

6.6 UV-Visible Spectroscopy

The determination of maximum absorption of each compound was performed in a UV-Visible Spectrometer, using quartz cells with an optical path length of 1 cm.

For this, it was prepared a mother solution in acetonitrile of each compound with concentration of 1 mg per 1 mL.

In the UV-Vis spectrometer, it was firstly performed a lecture of the blank from 400 to 200 nm, which consists in the lecture of 3 mL of acetonitrile. Then, 30 µL of the compound mother solution (10^{-2} M) previously prepared were added and the spectra was recorded from 400 to 200 nm. Analysing the spectra, the maximum of absorbance of each compound is determined.

6.7 Partition coefficients determination

A solution of octanol/phosphate buffer was prepared. In a separating funnel, 40 mL of PBS and 40 mL of octanol were added, the mixture was shaken for 30 minutes and left to rest for 48 hours, the separating funnel was closed and covered with aluminium paper. After that, the two fractions were separated and stored in dark glass bottles, at room temperature (r.t.).

For each compound, the quantification is performed by HPLC-UV, in the same conditions as the stability studies.

Firstly, it was performed a calibration curve experiment.

For the Log P experimental determination, around 1 mg of each compound was dissolved in 2.5 mL of octanol saturated with PBS. In an *eppendorf*, 500 μ L of this solution plus 500 μ L of PBS saturated with octanol were mixed, shaken in a vortex for 2 minutes and then centrifugated in a microcentrifuge for 10 minutes at 5000 rpm. Afterwards, the mixture rested for 15 minutes and, only after that, the phases were finally separated.

The octanol phase was diluted 20 times in ACN, and the PBS phase is directly injected in the HPLC without any dilution.

6.8 Stability in physiological conditions

6.8.1 Stability studies in PBS

To study the stability of the monocarbonyl analogues of curcumin **1a-j** in Phosphate-buffered saline, a flask with PBS and 20% of ACN (total volume 3 mL) was incubated at 37 °C. Then, 10 to 100 μ L of the mother solution (10^{-2} M) of each compound are added and left in the thermostated bath for 48 hours. The samples were collected during the 48 hours, directly analysed and quantified by HPLC.

6.8.2 Stability studies in human plasma

To study the stability of the monocarbonyl analogues of curcumin **1a-j** in human plasma, firstly, a plasma sample (2.5 mL) is defreeze and 0.5 mL of PBS 0.01 M (pH 7.4) is added, so the human plasma is diluted to 80%(v/v). The human plasma was incubated at 37 °C for 5 to 10 minutes and then 10 to 40 μ L of the mother solution (10^{-2} M) of each compound were added. The incubated solution was shaken, and the first sample is collected (time zero) by collecting 200 μ L to an *eppendorf* that has 400 μ L of cold ACN. The sample was shaken three times on vortex and then it was centrifugated for 10

minutes at 5000 rpm. For each sampling time, the protocol is repeated, stopping the reaction by ACN precipitation, sample centrifugation, and quantification of products in the supernatant by HPLC. The assay is followed for 48 hours.

6.9 Electrophilicity assessment

6.9.1 Electrophilicity assessment by a kinetic thiol assay

To study the electrophilicity of the monocarbonyl analogues, a kinetic thiol assay was performed by a protocol adapted from Dai *et al.* and Amslinger *et al.* [77,114]. The assay was performed in a UV-Visible Spectrometer, using quartz cells with an optical path length of 1 cm.

A solution of cysteamine hydrochloride (9.8×10^{-2} M) in PBS with 20% methanol (MeOH) was prepared. The procedure consists in varying the concentration of cysteamine maintaining the quantity of the compound and follow the absorbance along the time, in the maximal wavelength of each compound. Firstly, it is necessary to do a scan from 400 to 200 nm with the more diluted concentration of cysteamine to determine the maximum wavelength. The pseudo-first-order decay (k_{obs}) was obtained by the decay of each compound and then the second-order rate constants (k) were obtained by plotting the pseudo-first-order constants vs concentration of cysteamine, corresponding to the slope.

6.9.2 Electrophilicity assessment by NMR assay

The electrophilicity assay in ^1H NMR was performed accordingly to a procedure adapted from Avonto *et al.* [124] and Autelitano *et al.* [125]. Three solvents were tested: DMSO- d_6 , acetone- d_6 and methanol- d_4 . Firstly, a ^1H NMR spectrum from the compound in each solvent is performed. Then, it was added 10 molar equivalents of cysteamine hydrochloride and another ^1H NMR spectrum is performed. After that and in order to test the reversibility of the Michael addition, it was performed a dilution with CDCl_3 (1:10) and a ^1H NMR spectrum was performed.

It was observed that cysteamine isn't soluble in acetone and that the studied compound is not soluble in methanol. Therefore, the reaction was not verified in these two solvents, being the DMSO the only solvent useful for this study. Thus, after 24 and 48 hours of assay, other ^1H NMR spectra of the reaction and the dilution in CDCl_3 were assessed.

In order to facilitate the interpretation of all the spectra, a ^1H NMR spectrum of cysteamine was recorded, firstly using CDCl_3 as solvent, but it was verified that

cysteamine isn't soluble in CDCl_3 , so it was necessary to do the spectrum in DMSO. The problem of non-solubility of cysteamine in CDCl_3 was crucial when testing the reversibility of the solution. When the dilution with CDCl_3 (1:10) occurred, an appearance of powder in suspension was observed but in a small quantity, supposing that the powder was the cysteamine that precipitate. However, past 24 hours, the tube with this mixture has precipitate in a large quantity. Thus, was necessary to filtrate the solution and both phases were analysed by ^1H NMR, dissolving the powder in DMSO.

6.10 Biological assays

6.10.1 RPE cells assays

6.10.1.1 Cell viability

Cell viability was determined assessing the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), which forms a coloured formazan product that is released to the culture medium. The CellTiter 96® Aqueous One Solution Reagent (Promega) was freshly prepared. After the cell treatment, supernatants were removed and cells were incubated with this reagent for 60 min, at 37 °C. At the end of incubation, the absorbance of the medium was read at 490 nm using an ELISA plate reader PR 2100 (Bio-Rad Laboratories). Results were expressed as percentage of control, which was considered as 100%.

6.10.1.2 Autofluorescence assays

In order to evaluate the synthesized monocarbonyl analogues of curcumin in RPE cells, firstly, the lysosomal dysfunction of RPE cells were induced, feeding the cells with POS and UV-POS at concentrations higher than the physiological ones, inducing a phagocytic burden in RPE, resembling the accumulation of indigestible autofluorescent material, as observed in AMD. For the assays, commercially available human primary RPE cells, obtained from Lonza, were used. Cells are cultured in RPE Growth media (from Lonza), were incubated for 4 hours with POS and POS-UV (200 $\mu\text{g}/\text{mL}$) in DMEM supplemented with 10% of fetal bovine serum (FBS). After POS incubation, the medium is removed and substituted by DMEM medium supplemented with 1% FBS. The autofluorescence is only read after 72 hours, in that time the culture is incubated at 37 °C with 5% of CO_2 . After 48 hours, our compounds (9000 μM in DMSO, diluted in DMEM 1% FBS at the final concentration) and the control (DMSO) are administered. After the

72 hours, the autofluorescence (% of 488-positive cells) is read by Flow cytometry using the BD FACSCanto™, using a 488 nm excitation wavelength.

6.10.2 Cancer stem cells

6.10.2.1 Cell viability

To evaluate the monocarbonyl analogues of curcumin in colorectal cancer stem cells, firstly HT29 colorectal adenocarcinoma were plated in 96-well plates, performing 100 cells for well, using an undifferentiated medium in ultra-low attachment conditions in order to form spheres. Then, a positive control was done, using salinomycin 1 μ M, which is a CSC-targeting agent. The cells were also treated with the monocarbonyl analogues of curcumin or with DMSO (vehicle controls).

Cells were incubated for 7 days and the cell viability were evaluated, measuring the ATP, using CellTiter-Glo™ Luminescent Cell Viability Assay (Promega).

6.10.2.2 IC₅₀ Determination

To quantitatively assess the inhibitory potency, the IC₅₀ value was determined for compound **1i**. Briefly, HT29 human colorectal adenocarcinoma cells were plated in 96-well plates (100 cell/well), in undifferentiated medium, in ultra-low attachment conditions for sphere formation. Then, a positive control was done, using salinomycin 1 μ M, which is a CSC-targeting agent. The cells were also treated with compound **1i** or with DMSO (vehicle controls). After 7 days of incubation, cell viability was assessed, based on measurement of ATP, using the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega). All data analysis and IC₅₀ value calculations were performed using GraphPad Prism version 6.01.

To evaluate if compound **1i** has a generic effect on colorectal CSCs or if it is a cell line dependent effect, this compound was tested on CSC-enriched tumourspheres from other colorectal cell lines (HT116, SW480 and SW620) in the same experimental setting previously described.

To guarantee compound **1i** specificity in targeting CSC-enriched spheroids, compound **1i** inhibitory potency on adherent cell lines was also evaluated. Hence, HT29, HCT116, SW620 and SW480 cell lines were plated in 96-well plates (0.5 x 10⁴ cell/well) in appropriate cell culture media supplemented with 10% FBS and 1% antibiotic/antimycotic. After 24 h of plating, cells were treated with compound **1i** (concentrations ranging from 256 to 9.31x10⁻¹⁰ μ M), salinomycin (1 μ M), 5-fluoruracil (10 μ M) or DMSO (vehicle control). After 72 h of incubation, cell viability was assessed based

on measurement of MTS metabolism, using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega). All data analysis and IC₅₀ value calculations were performed using GraphPad Prism version 6.01.

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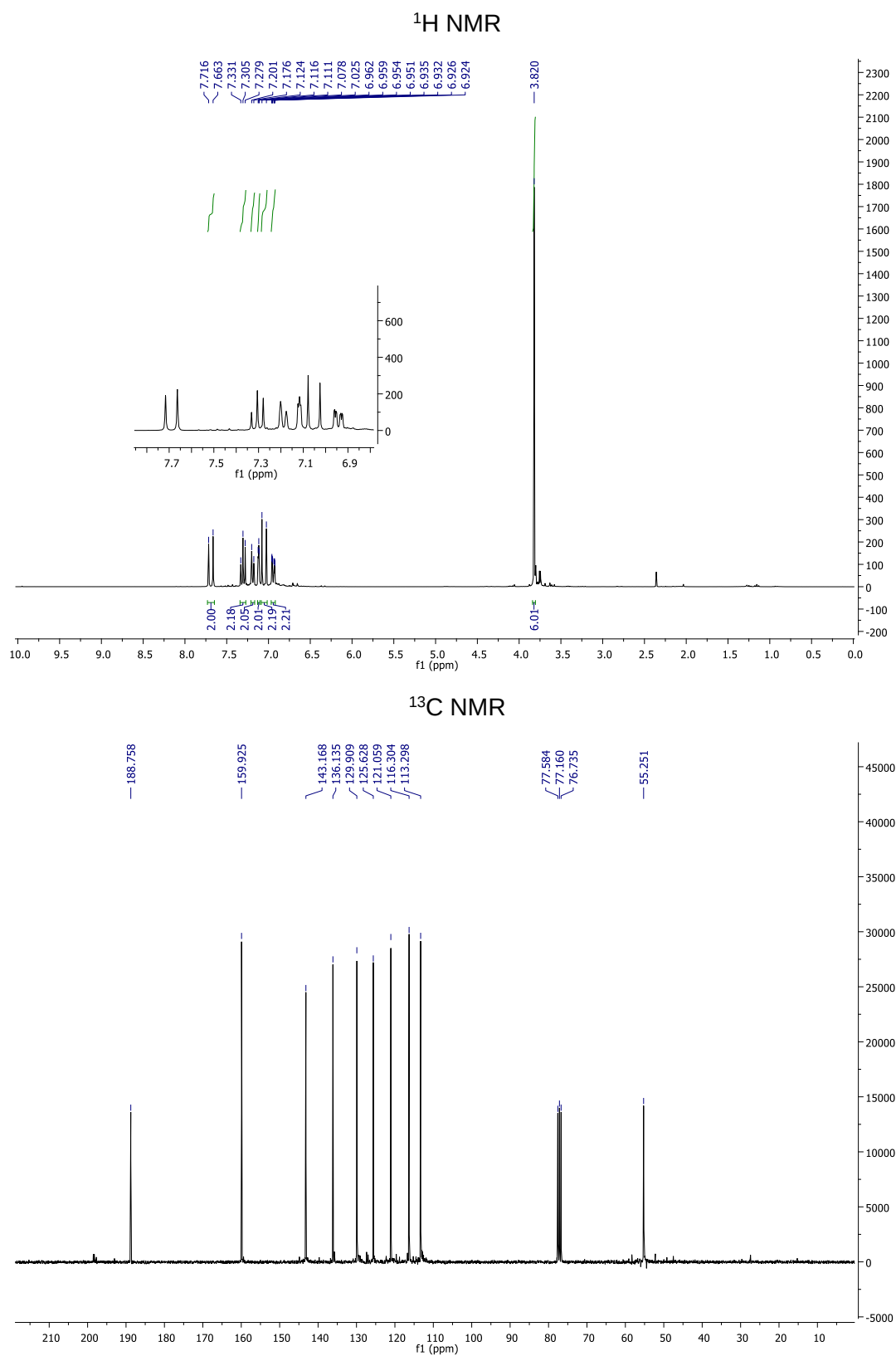
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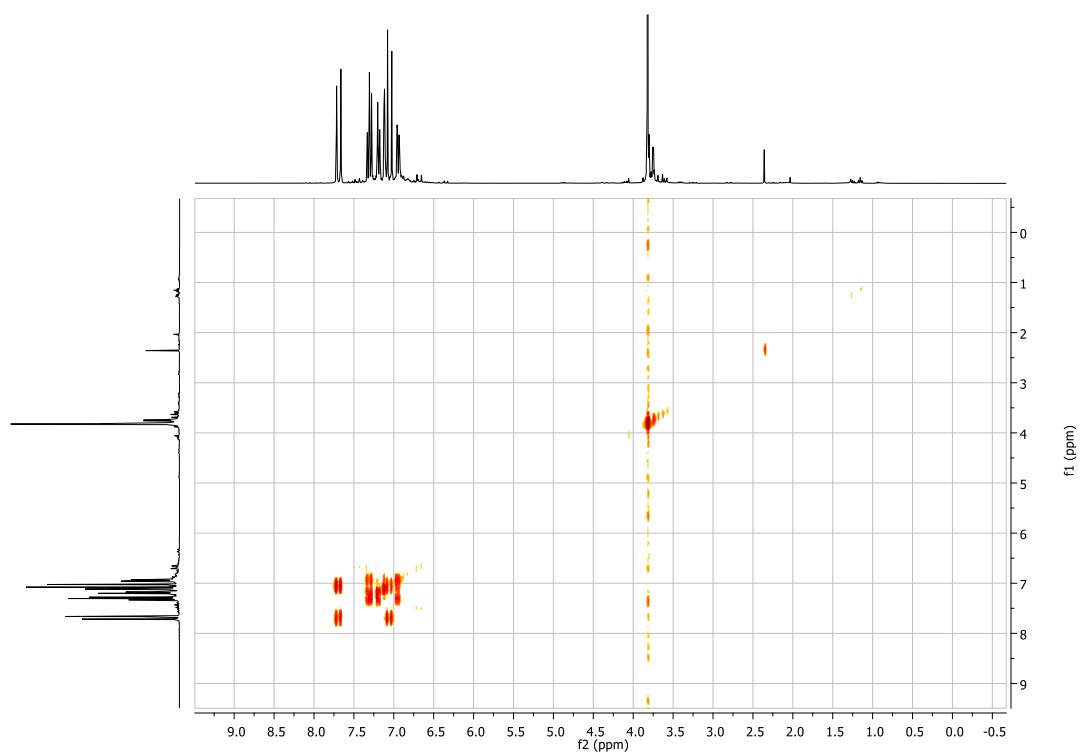
Appendices

Appendix I – Structural characterization spectra

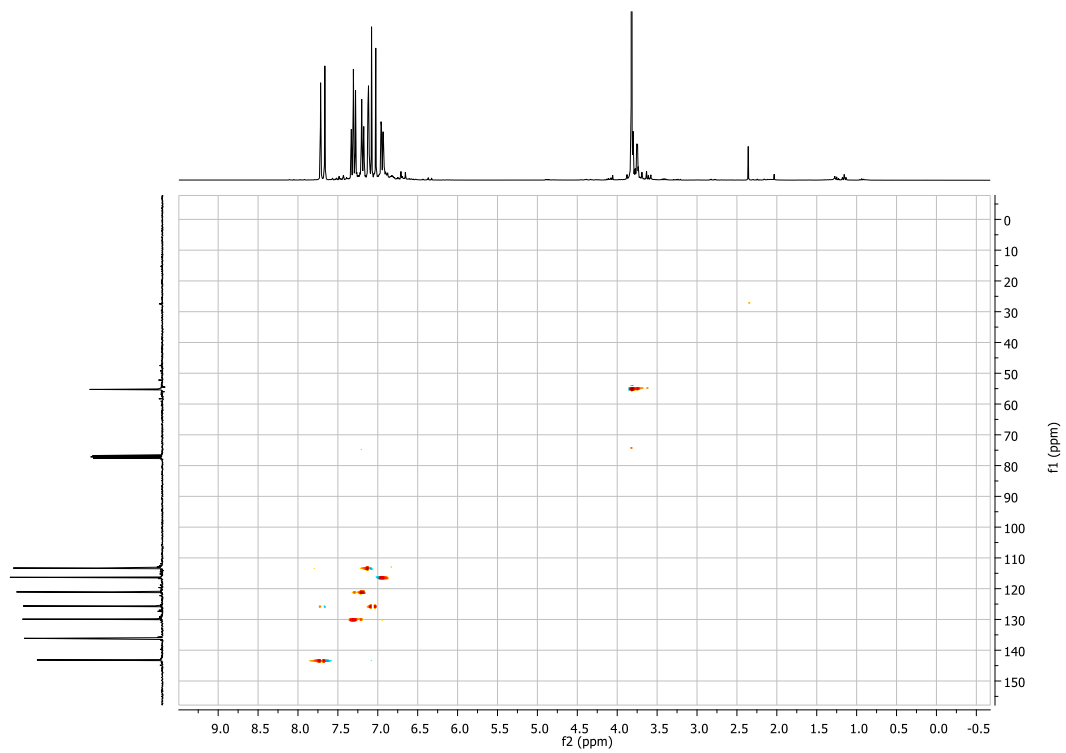
I.I Compound 1a

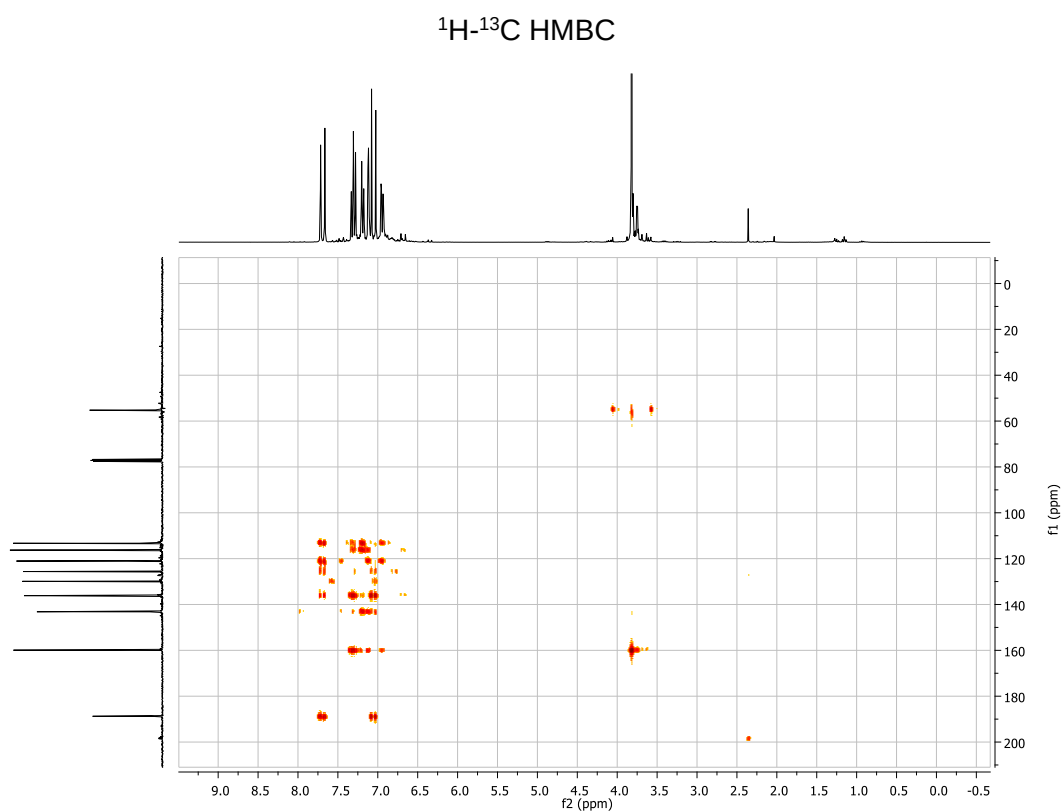


^1H - ^1H COSY



^1H - ^{13}C HMQC



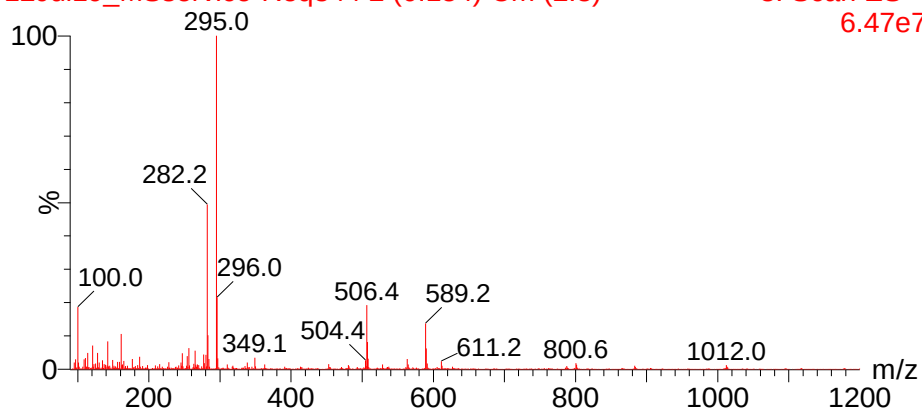


ESI⁺ mass spectrum

Req 344/2019 - MVC C2

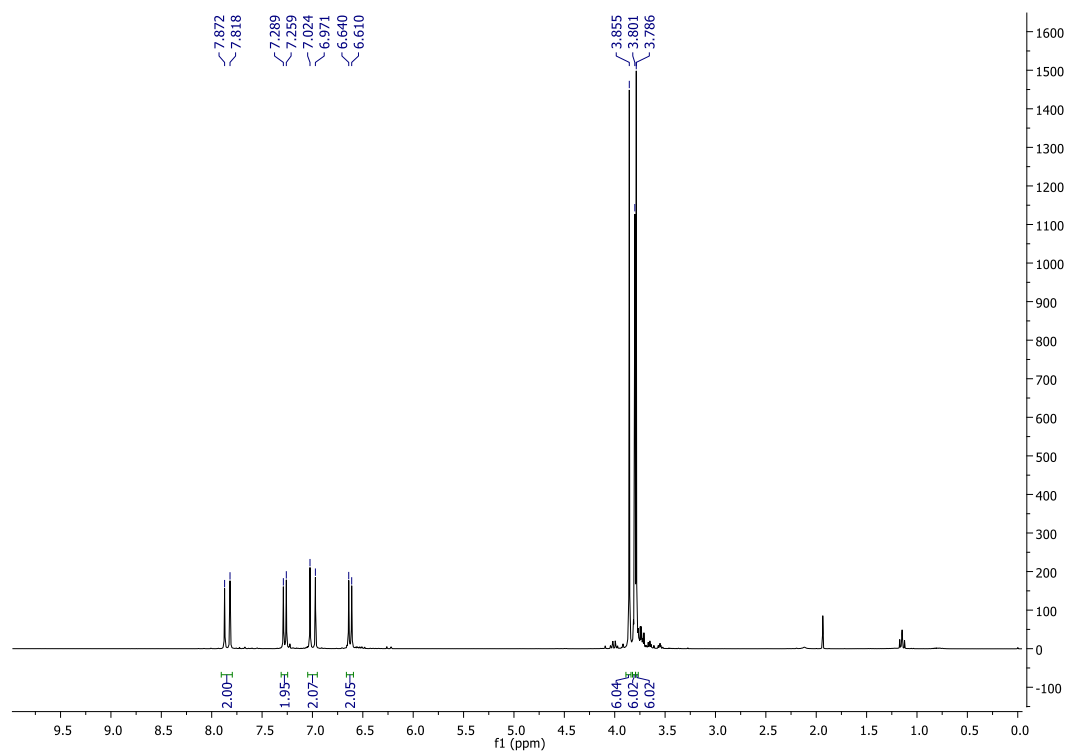
12Jul19_MSservico-Req344 2 (0.154) Cm (2:3)

3: Scan ES+
6.47e7

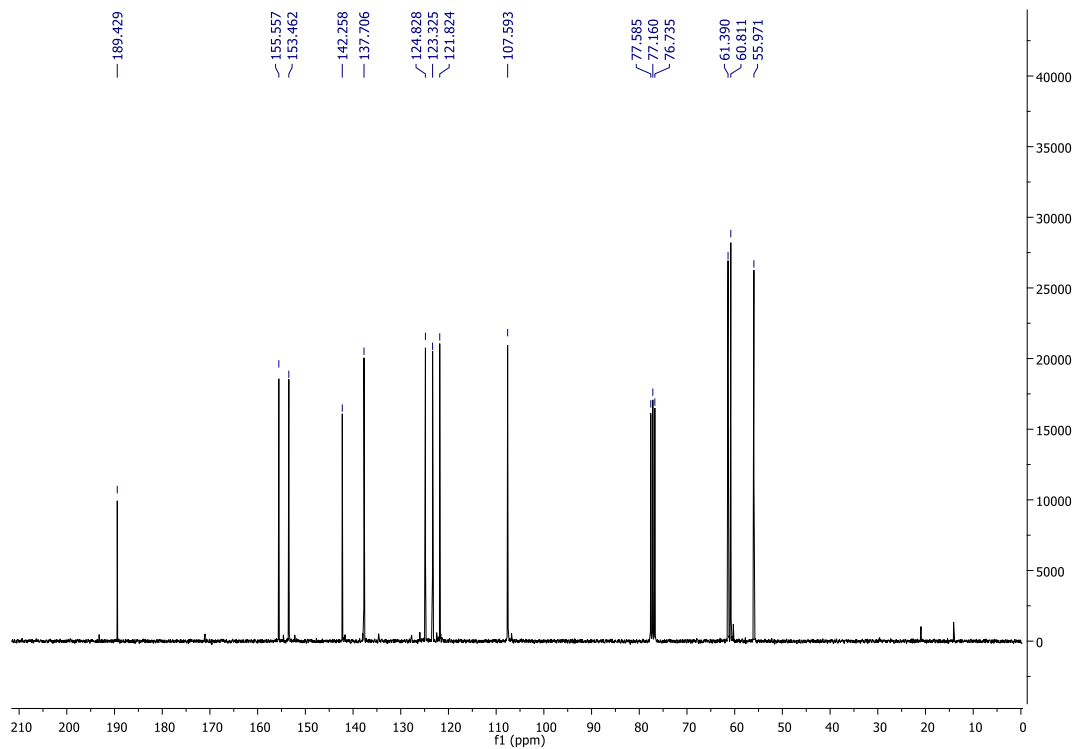


I.II Compound 1b

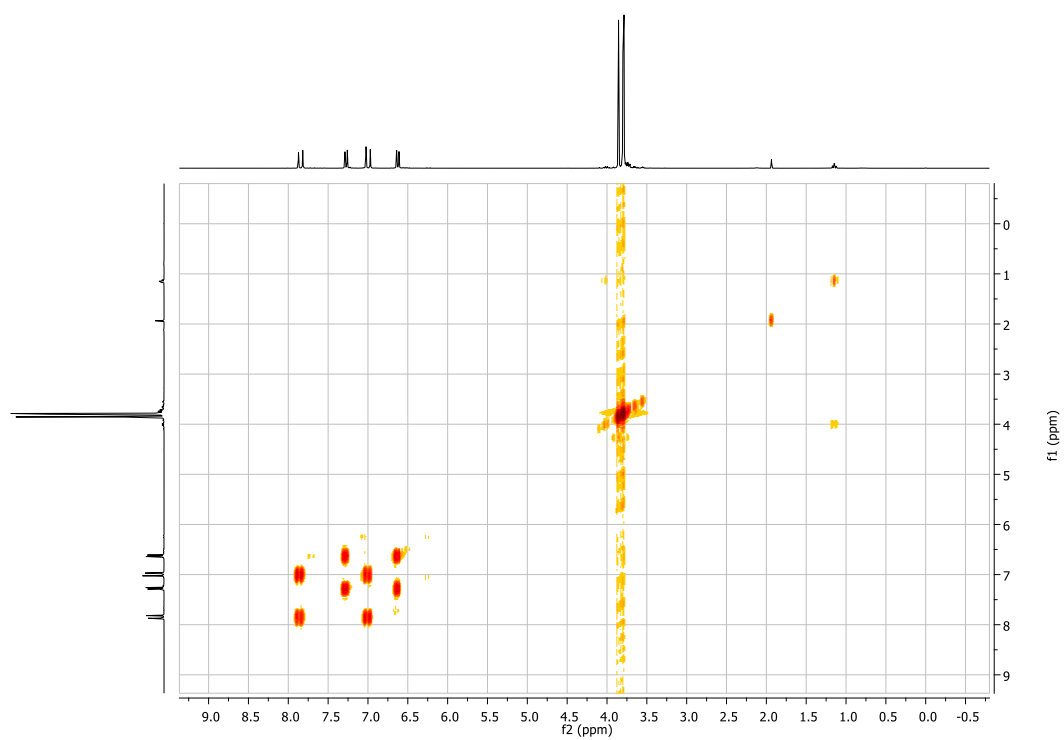
^1H NMR



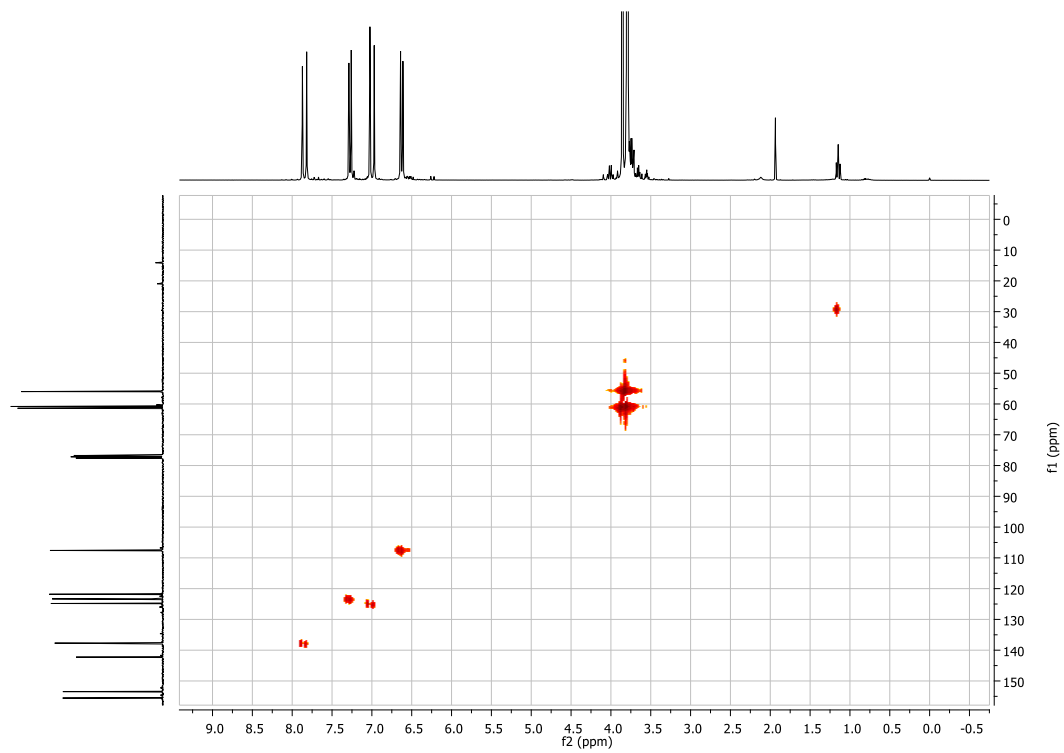
^{13}C NMR

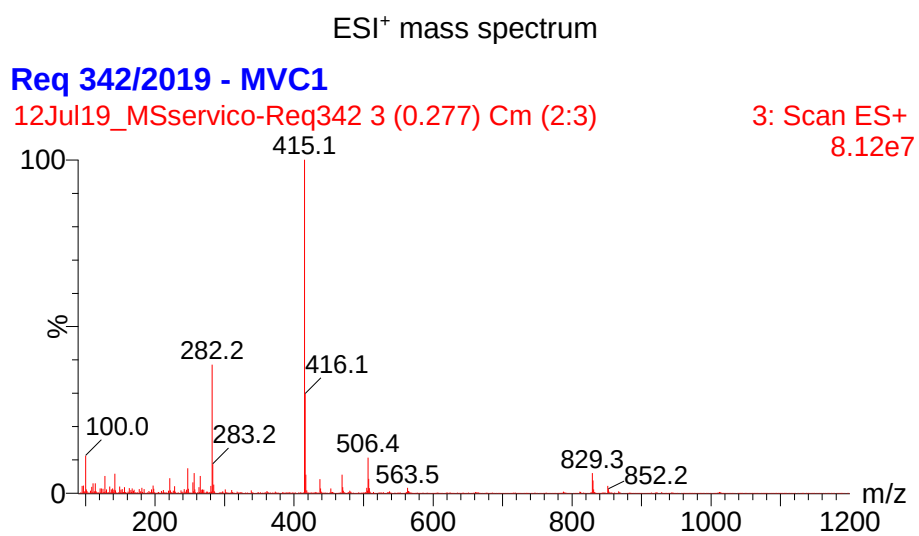
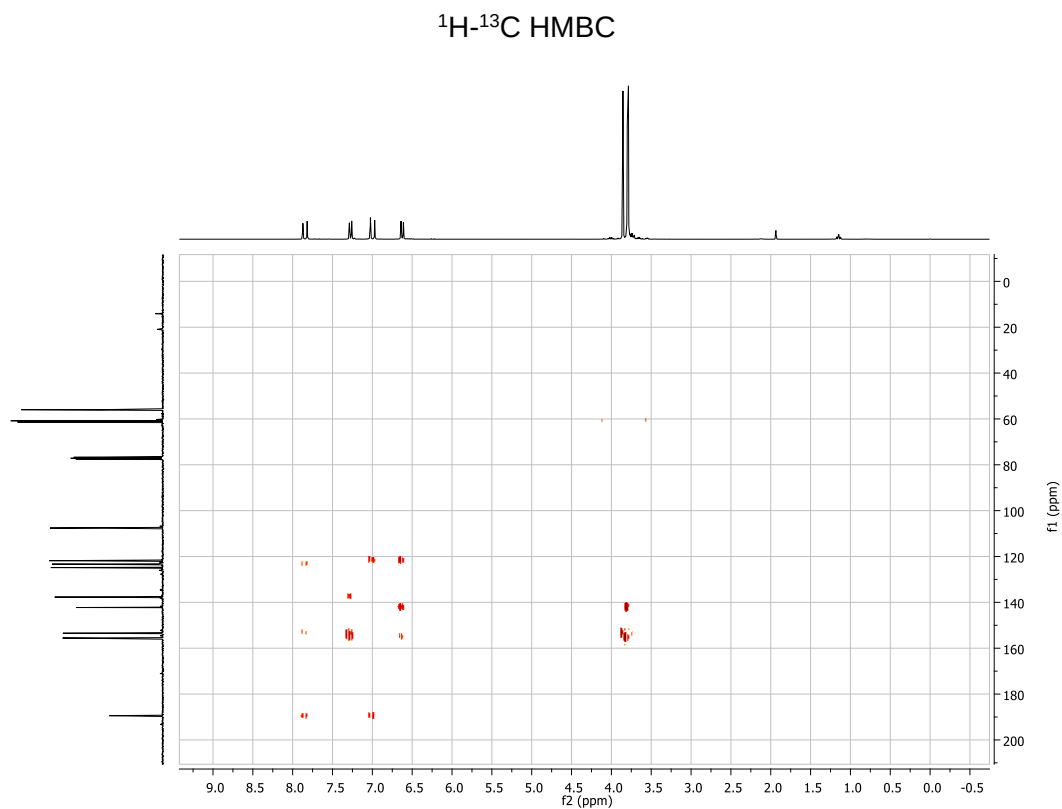


^1H - ^1H COSY



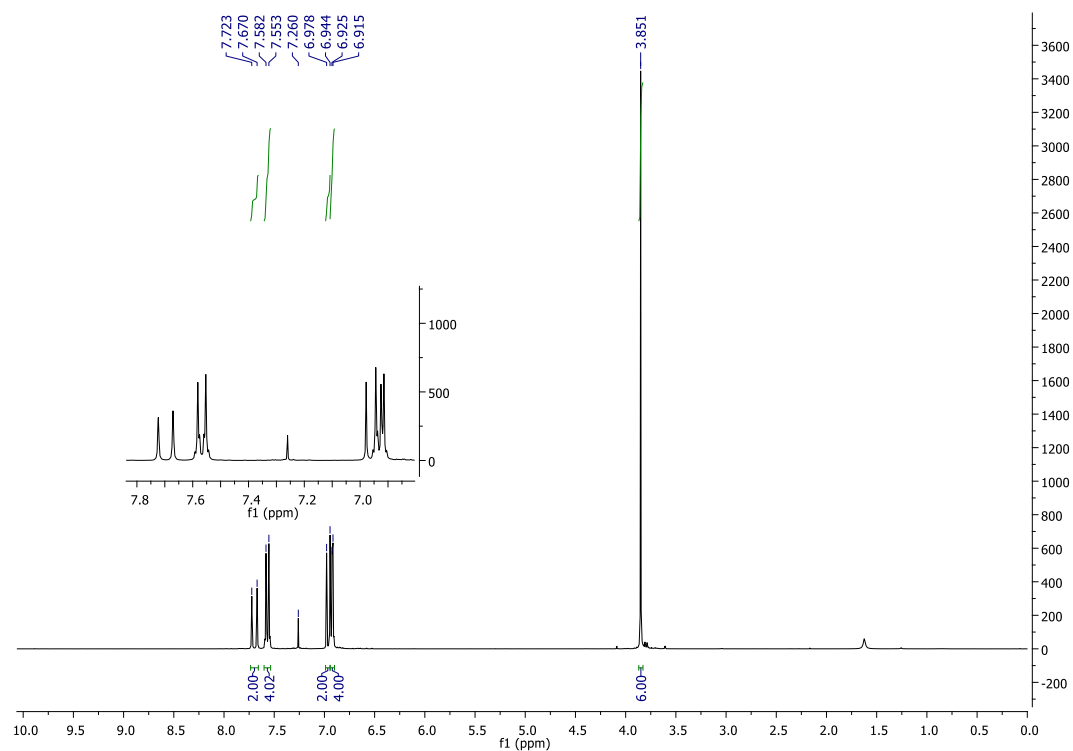
^1H - ^{13}C HMQC



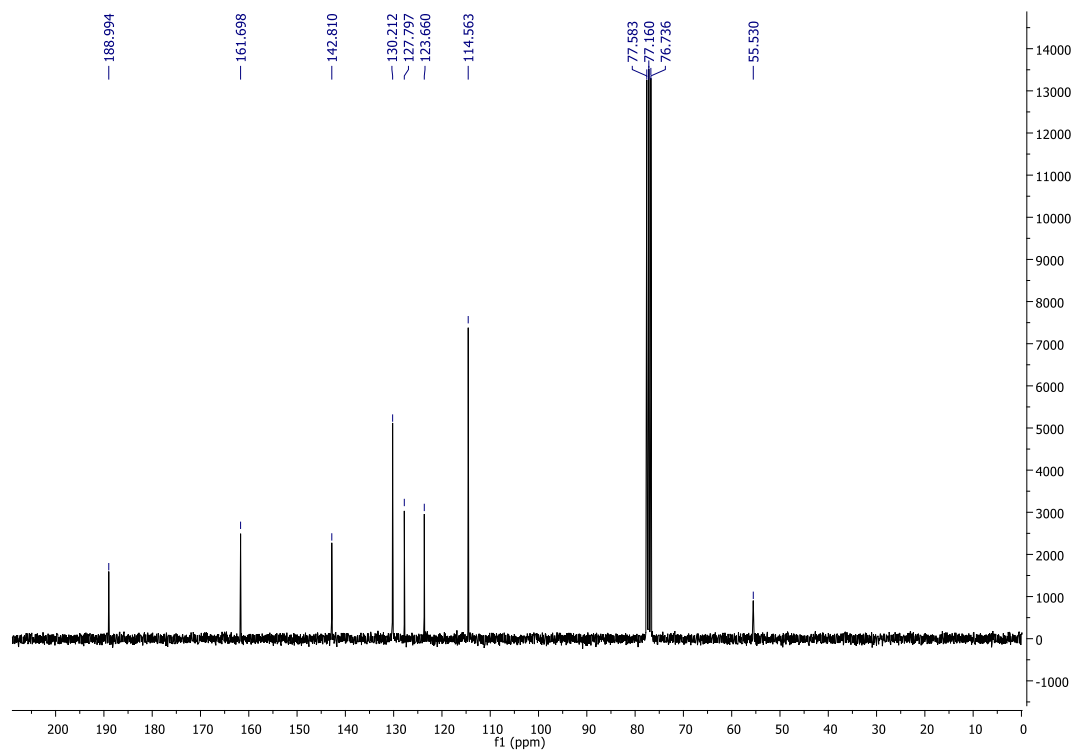


I.III Compound 1c

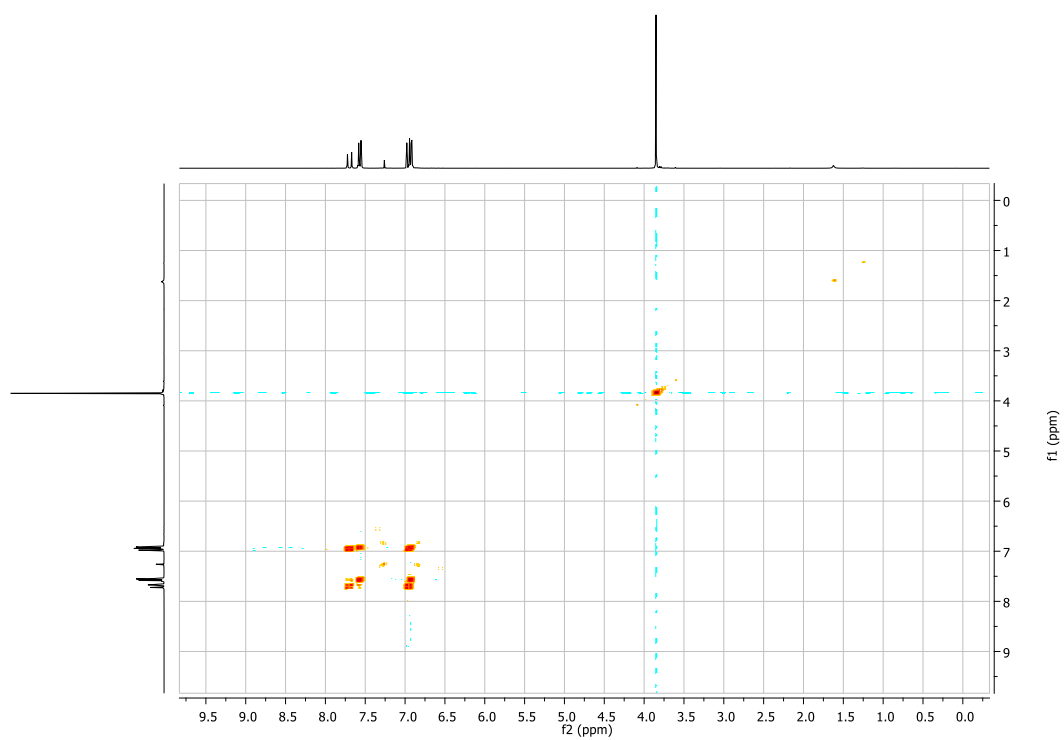
^1H NMR



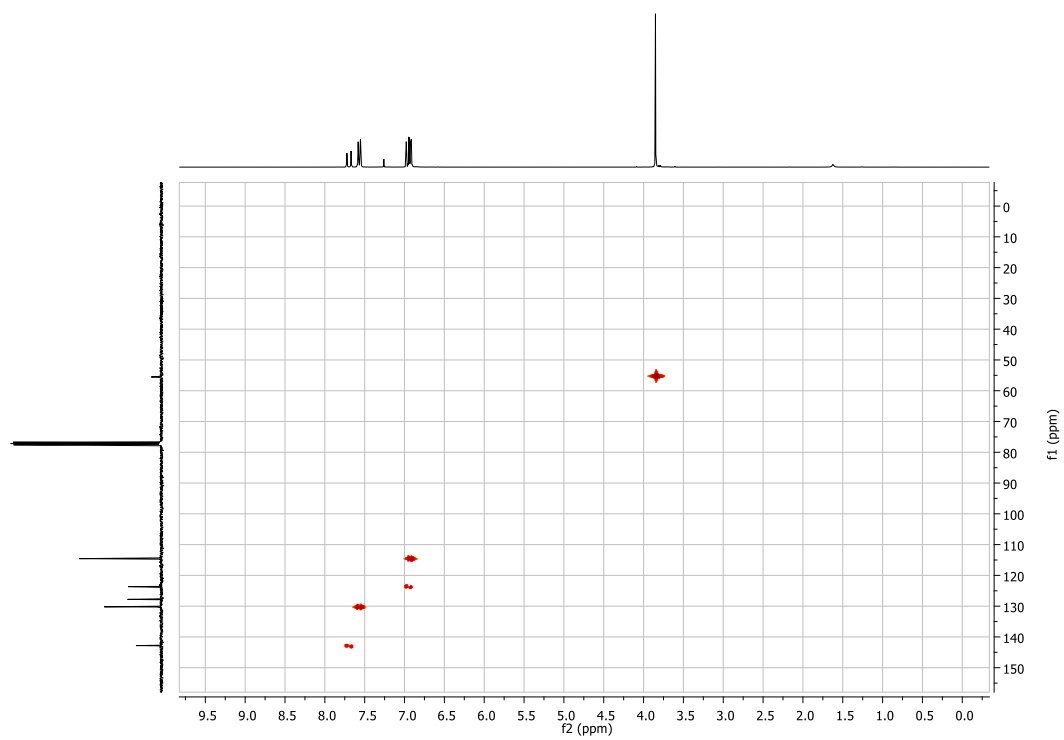
^{13}C NMR



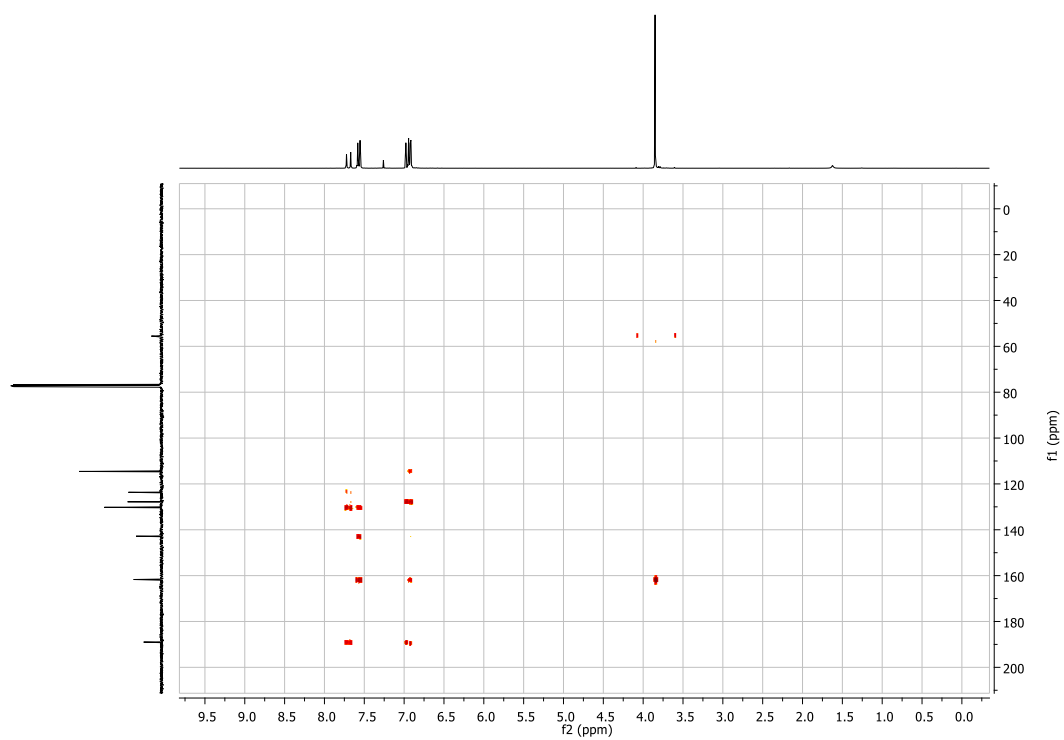
^1H - ^1H COSY



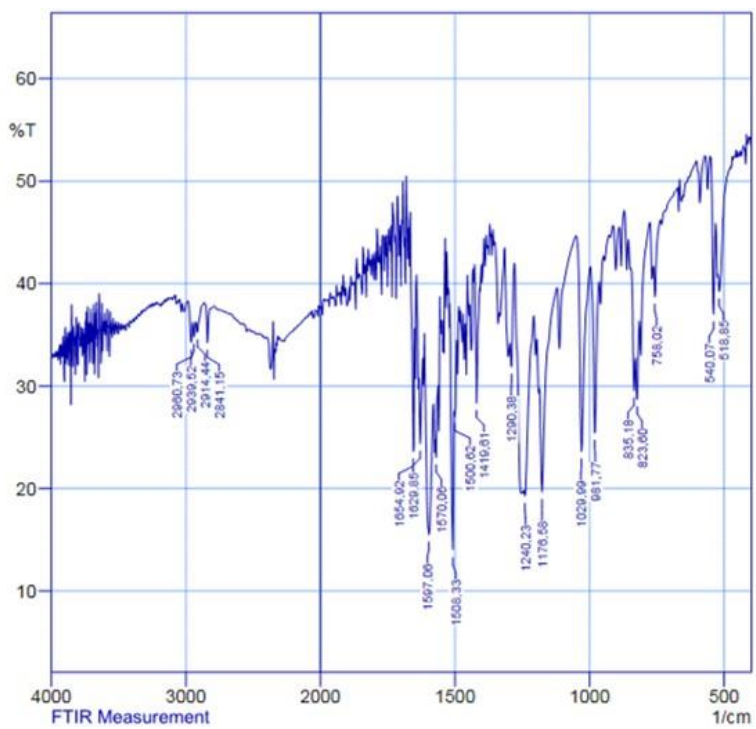
^1H - ^{13}C HMQC



^1H - ^{13}C HMBC



IR spectrum

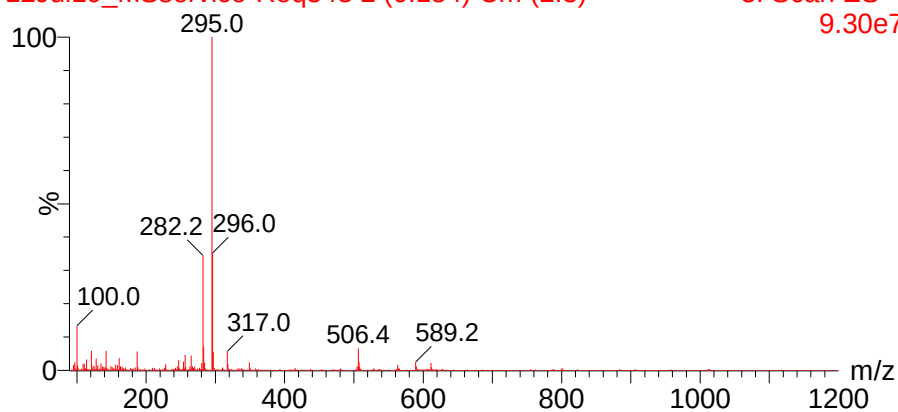


ESI⁺ mass spectrum

Req 343/2019 - MVC2

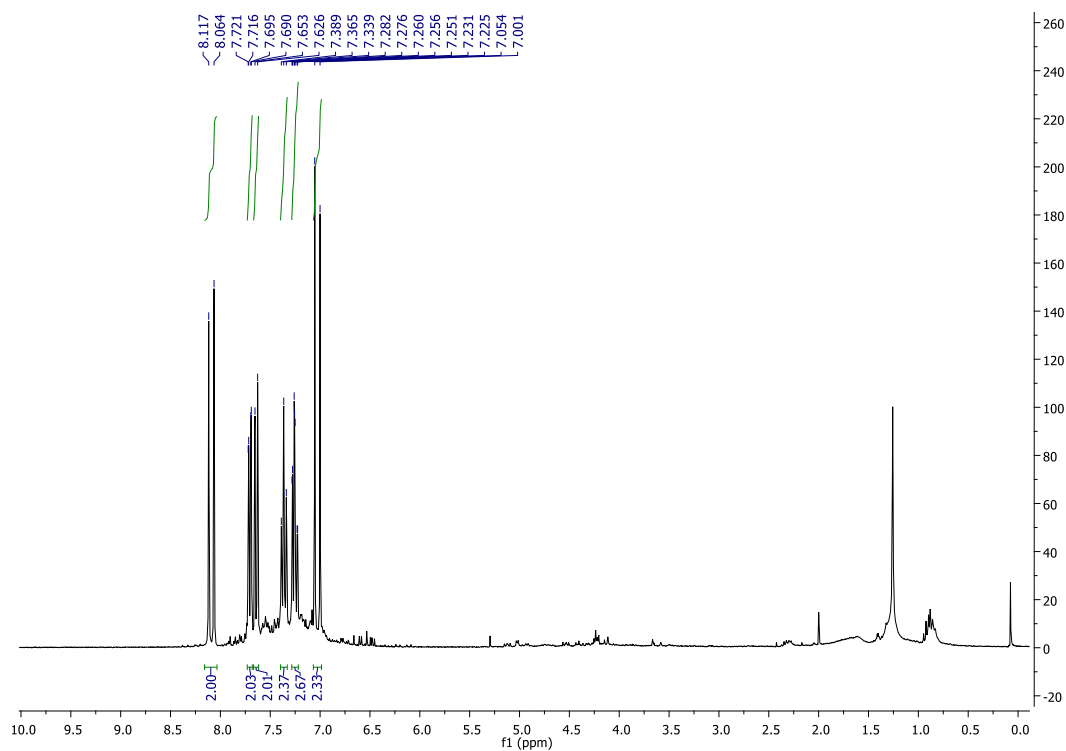
12Jul19_MSservico-Req343 2 (0.154) Cm (2:3)

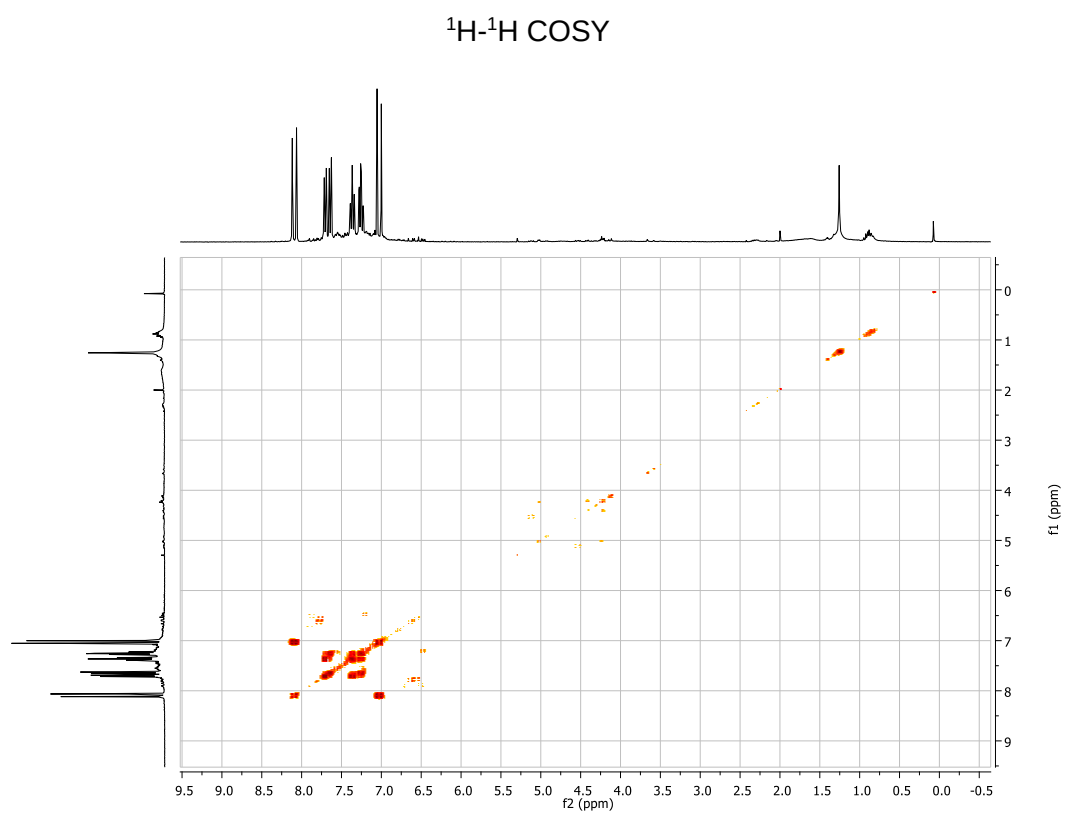
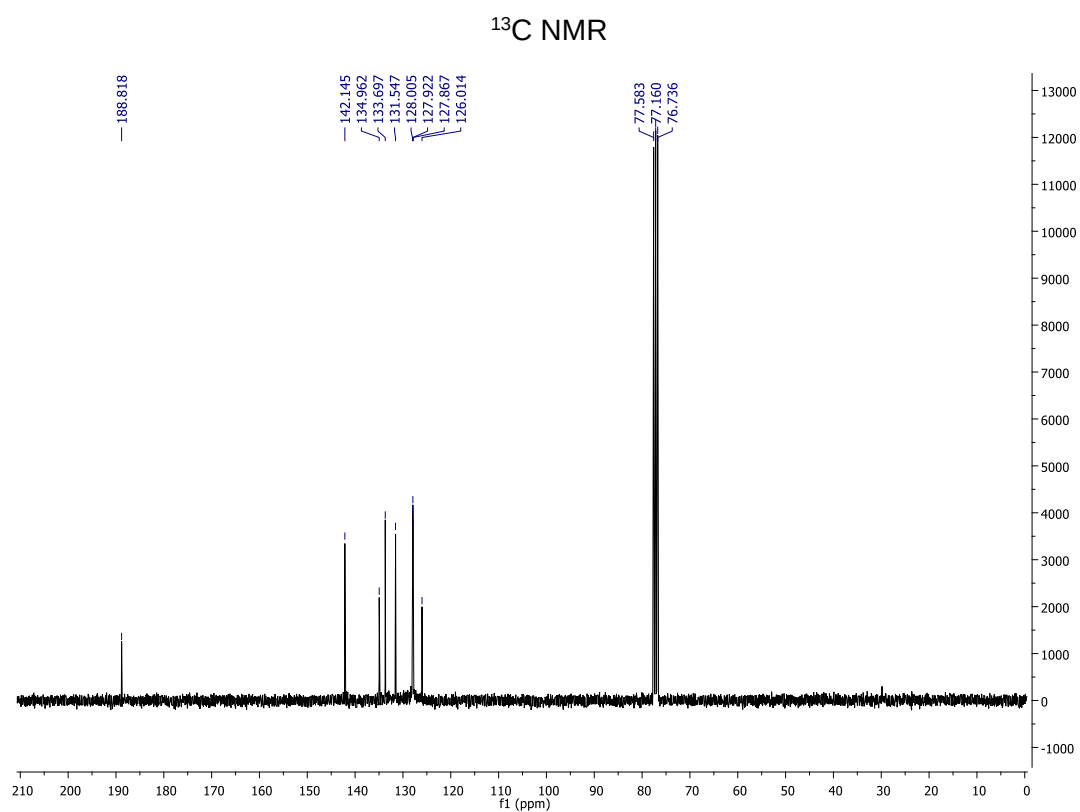
3: Scan ES+
9.30e7



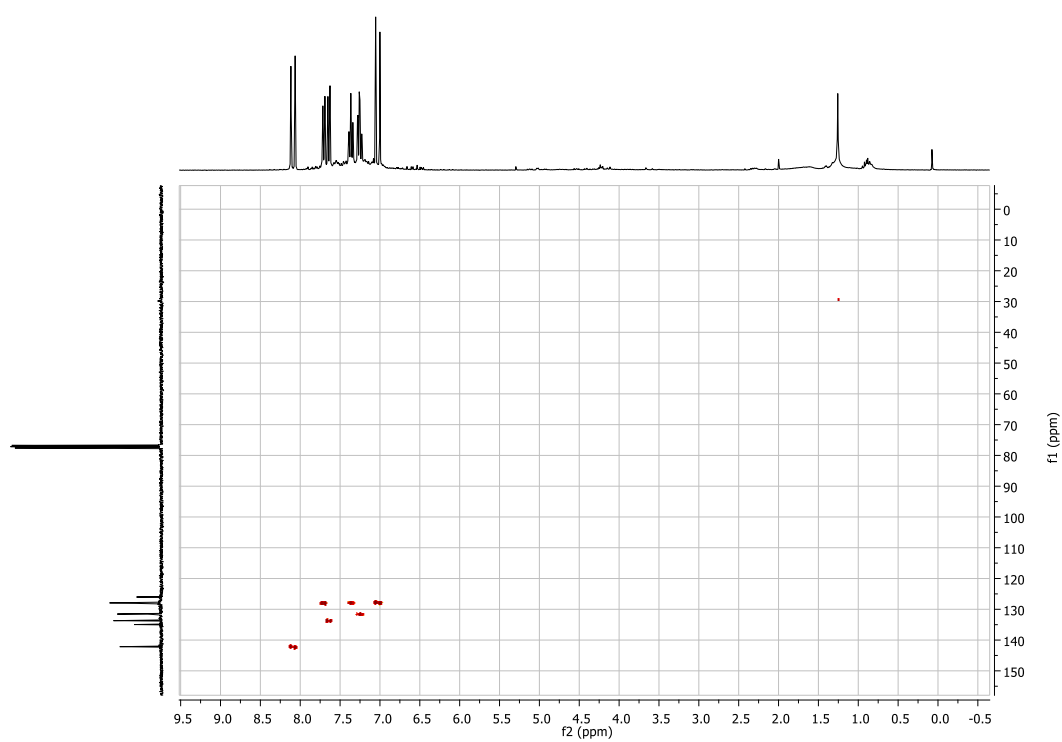
I.IV Compound 1d

¹H NMR

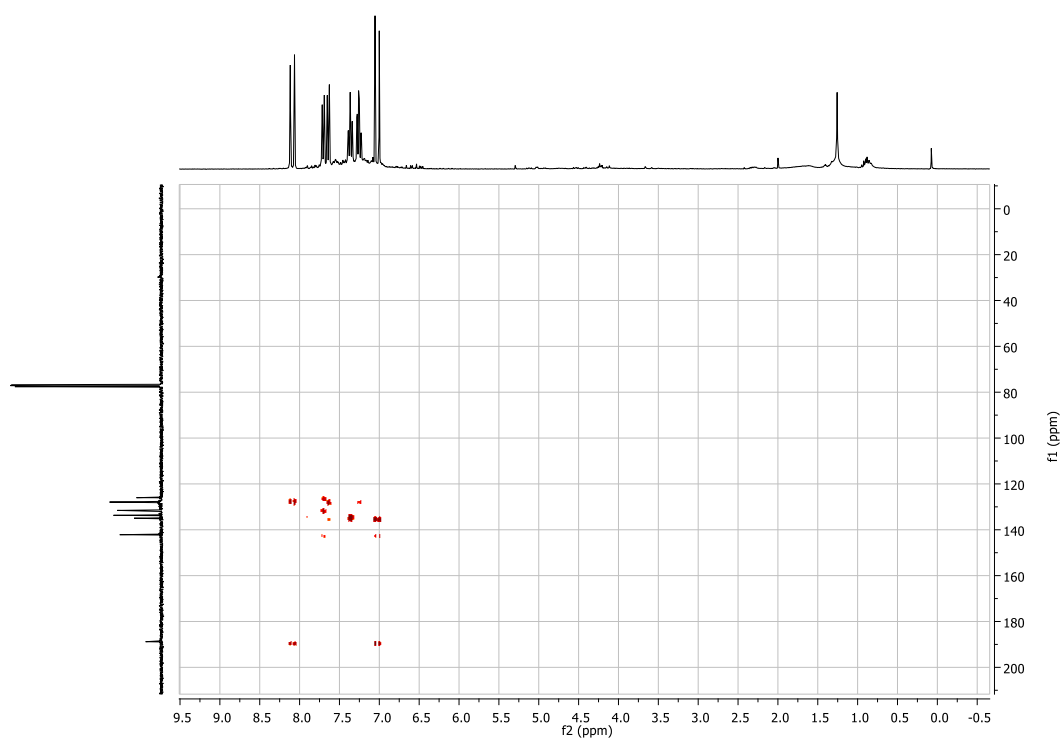




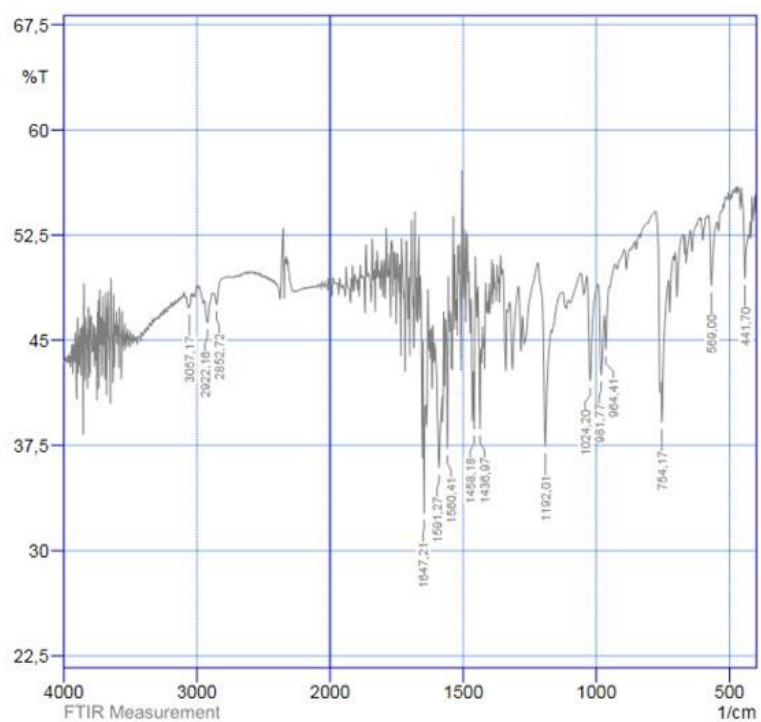
^1H - ^{13}C HMQC



^1H - ^{13}C HMBC



IR spectrum

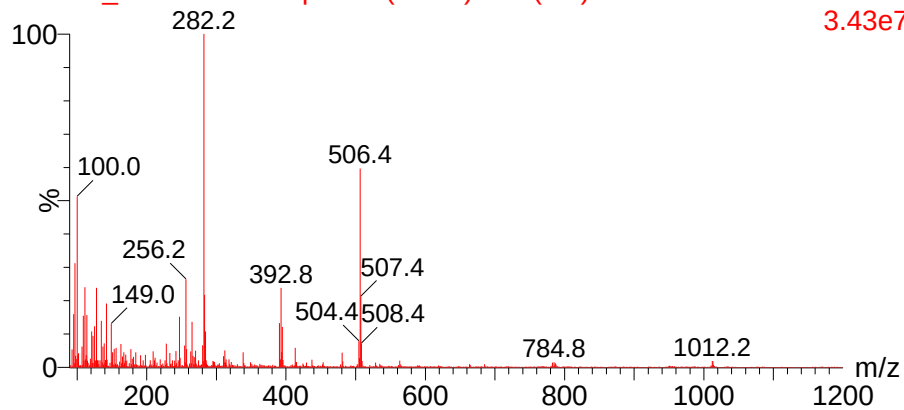


ESI⁺ mass spectrum

Req 345/2019 - MVC 3

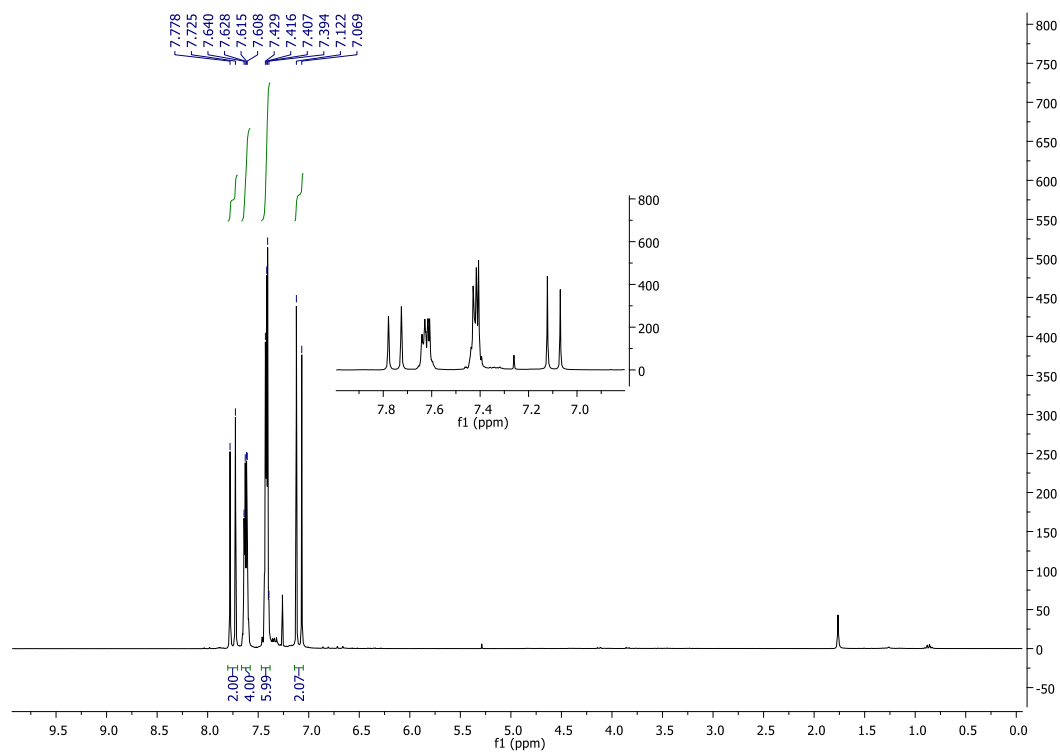
12Jul19_MSservico-Req345 2 (0.164) Cm (2:3)

4: Scan ES+
3.43e7

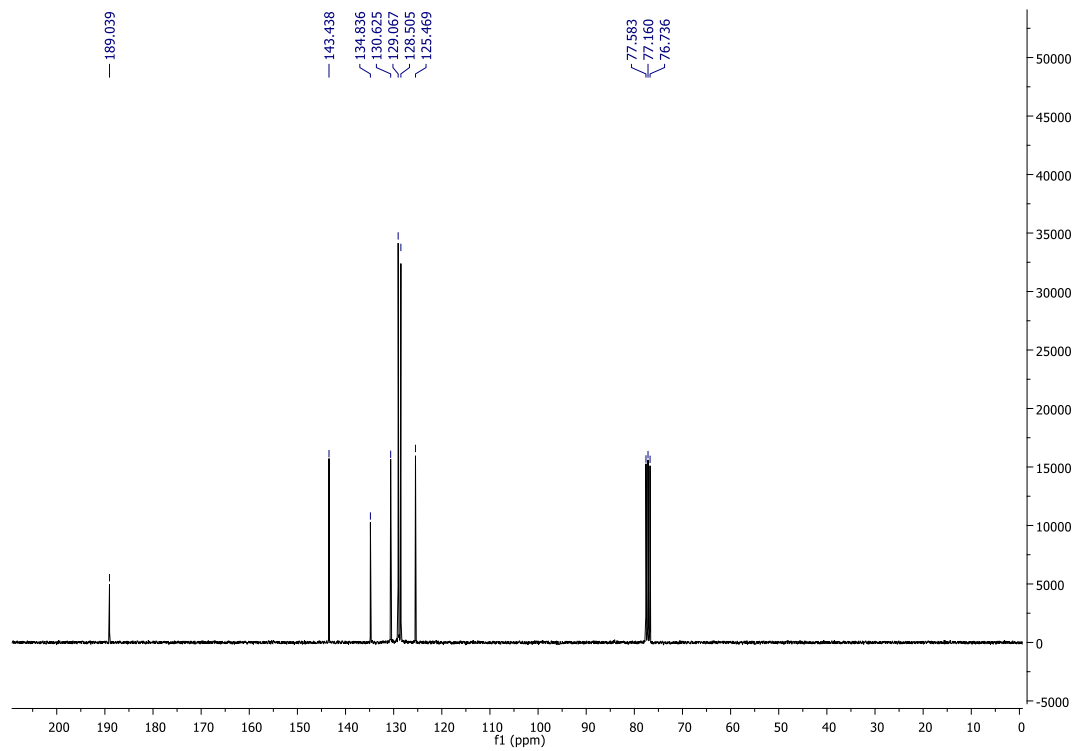


I.V Compound 1e

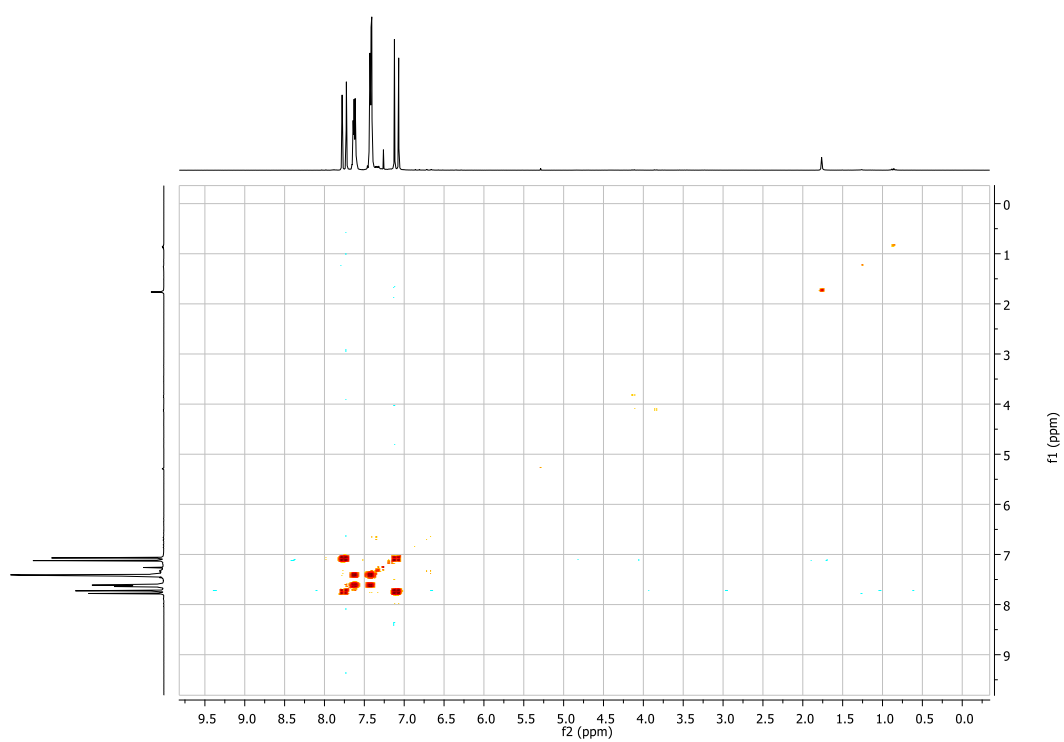
^1H NMR



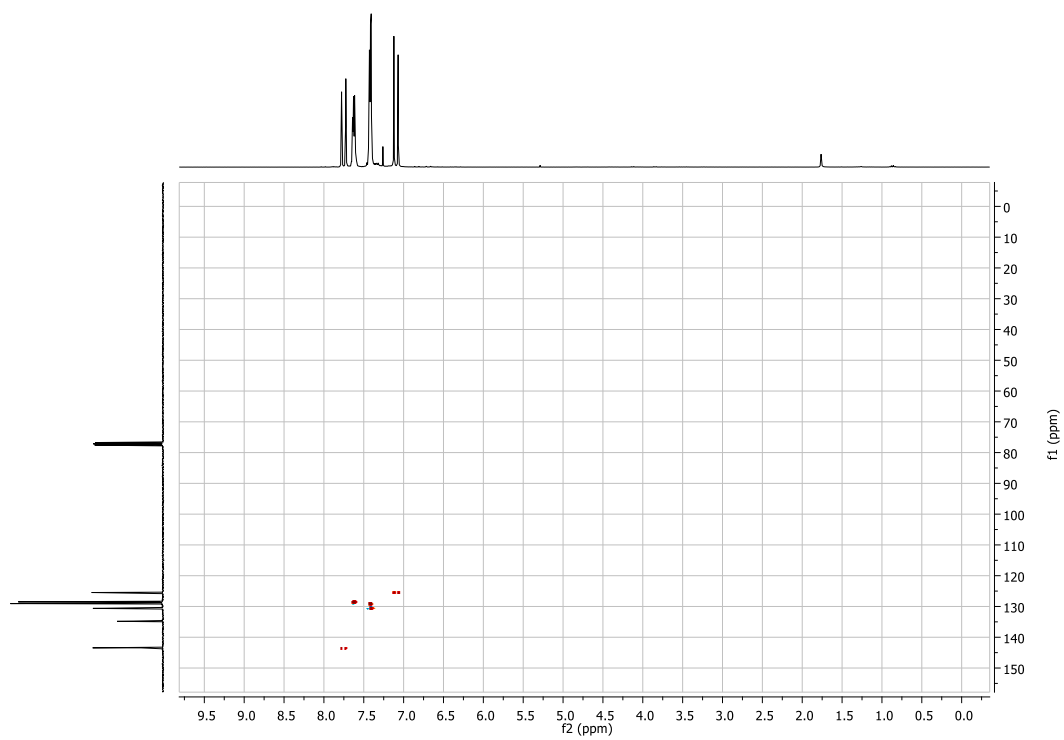
^{13}C NMR



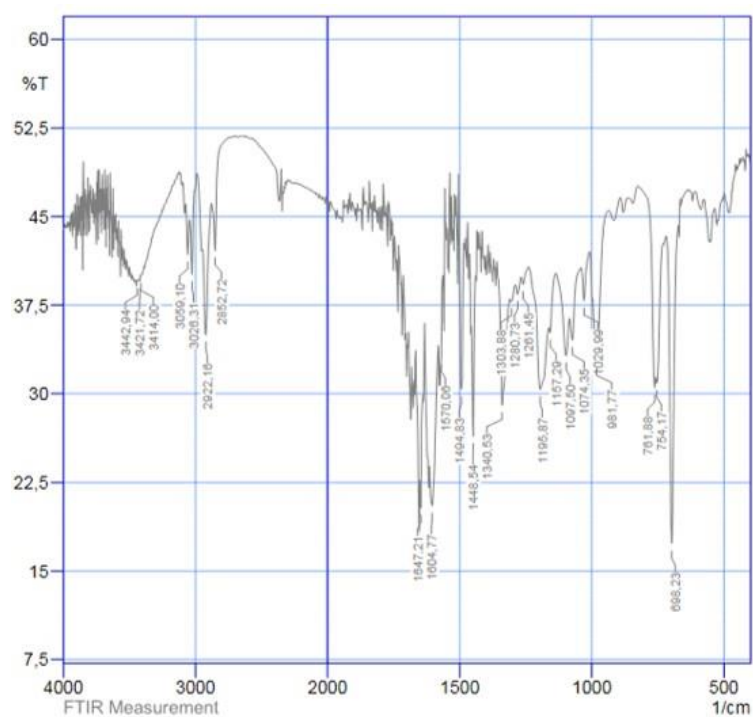
^1H - ^1H COSY



^1H - ^{13}C HSQC



IR spectrum

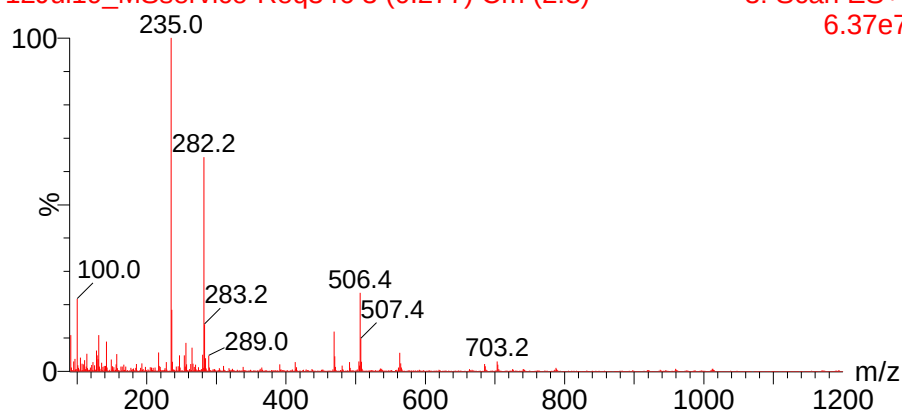


ESI⁺ mass spectrum

Req 346/2019 - MVC 4

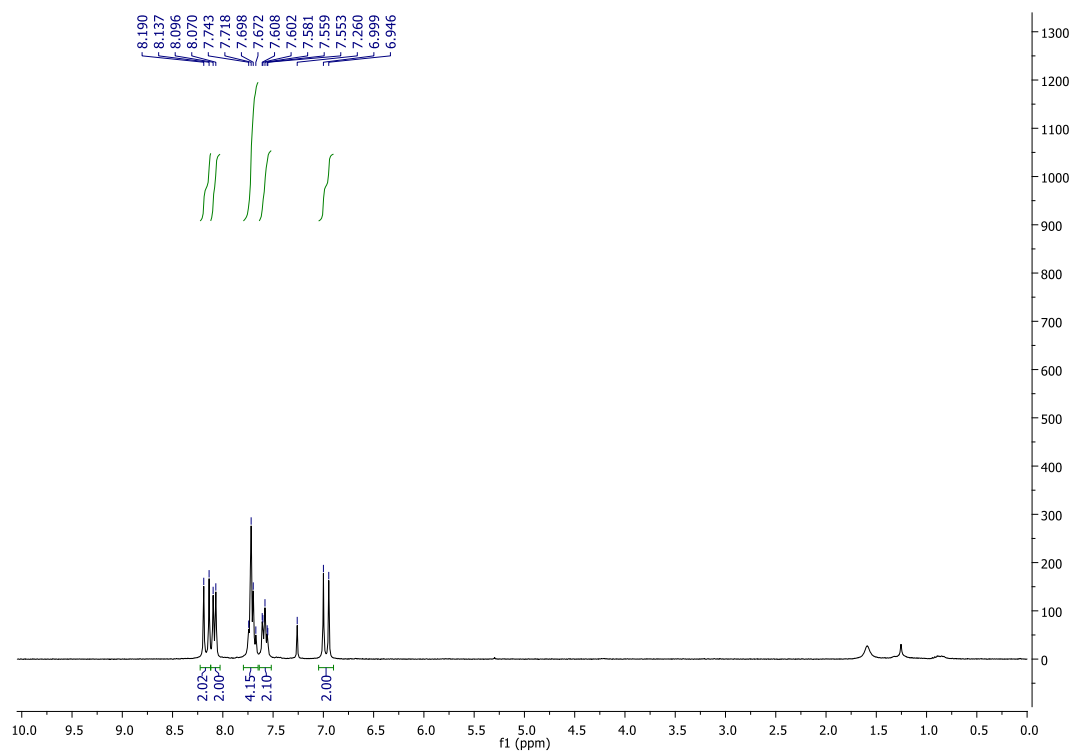
12Jul19_MSservico-Req346 3 (0.277) Cm (2:3)

3: Scan ES+
6.37e7

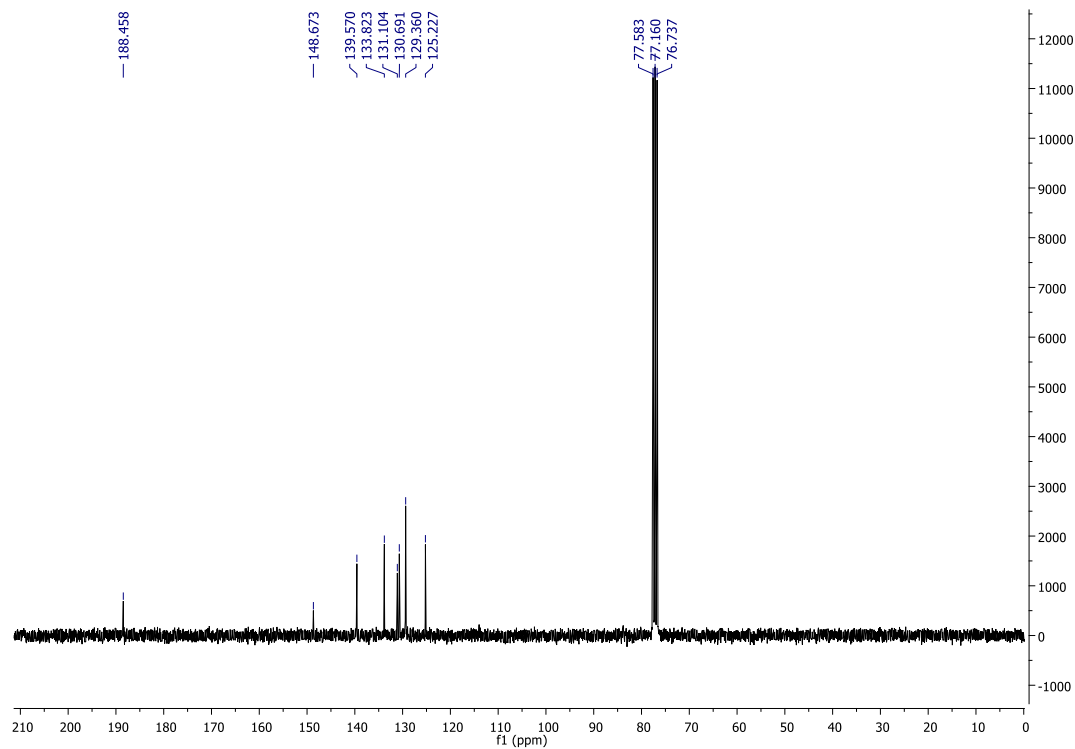


I.VI Compound 1f

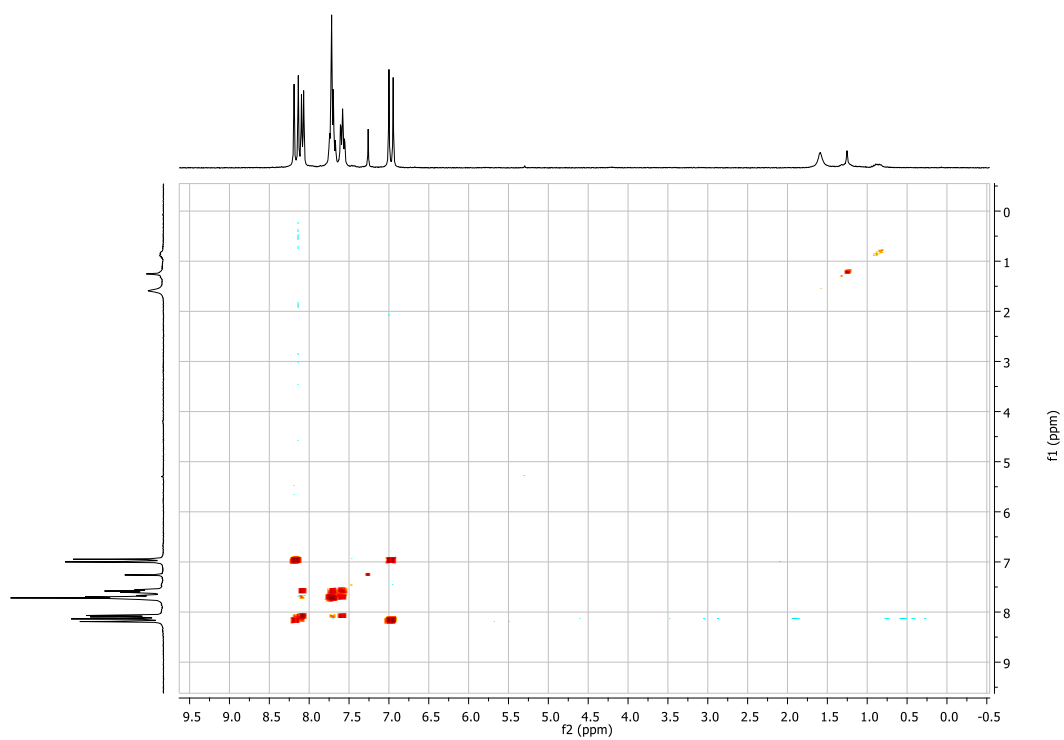
^1H NMR



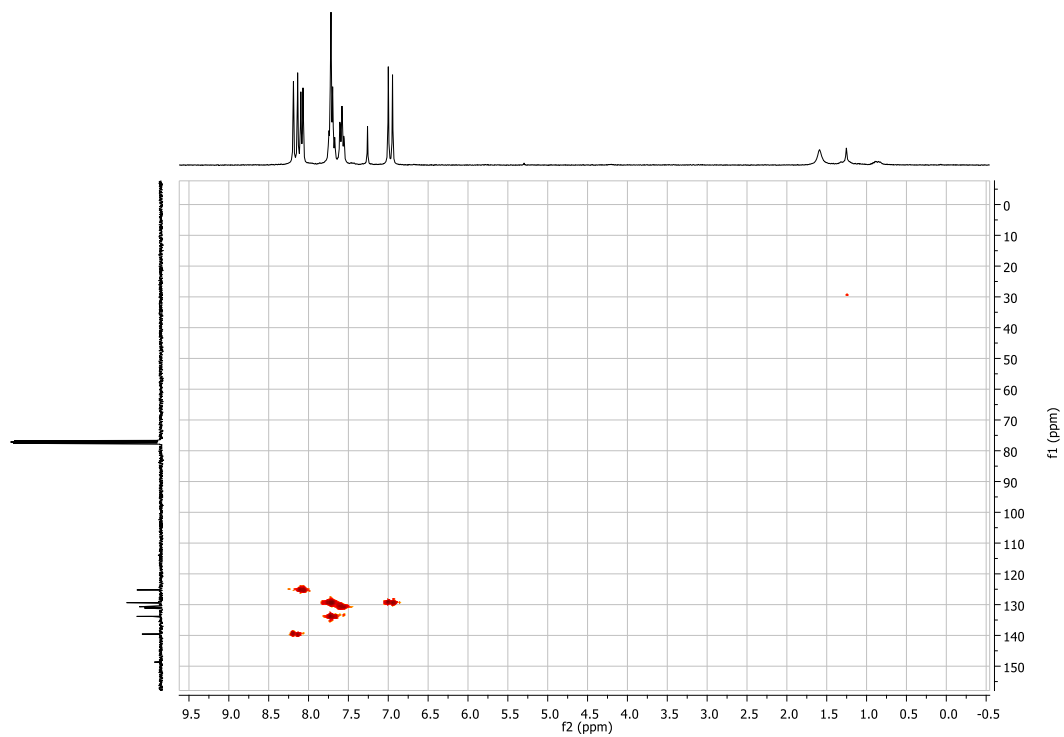
^{13}C NMR



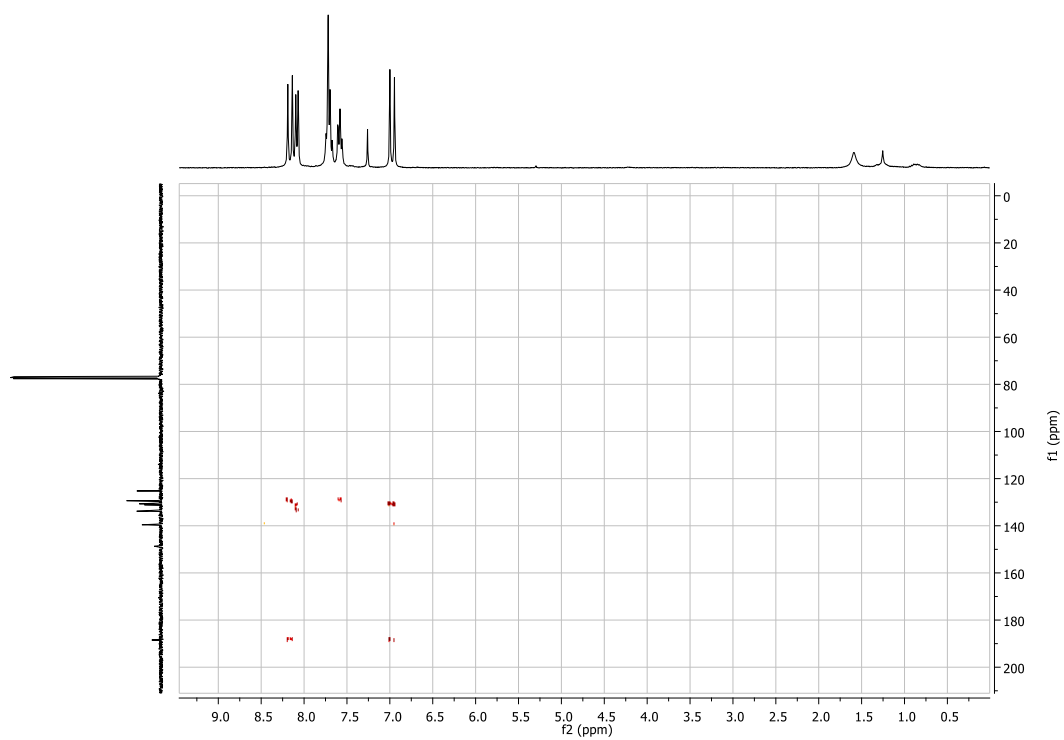
^1H - ^1H COSY



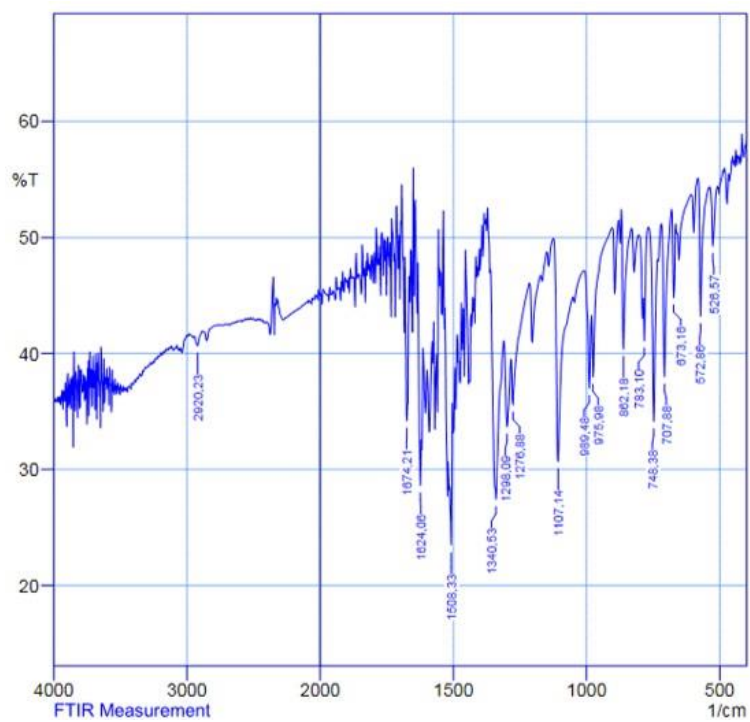
^1H - ^{13}C HMQC



^1H - ^{13}C HMBC



IR spectrum

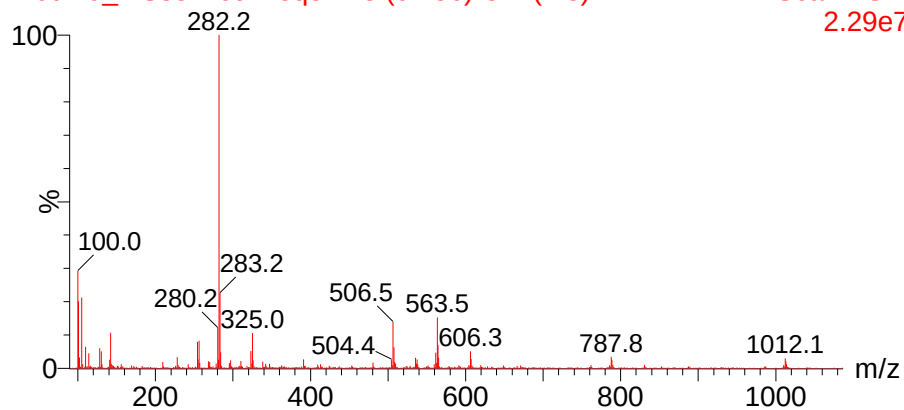


ESI⁺ mass spectrum

Req 347/2019 - MVC 5

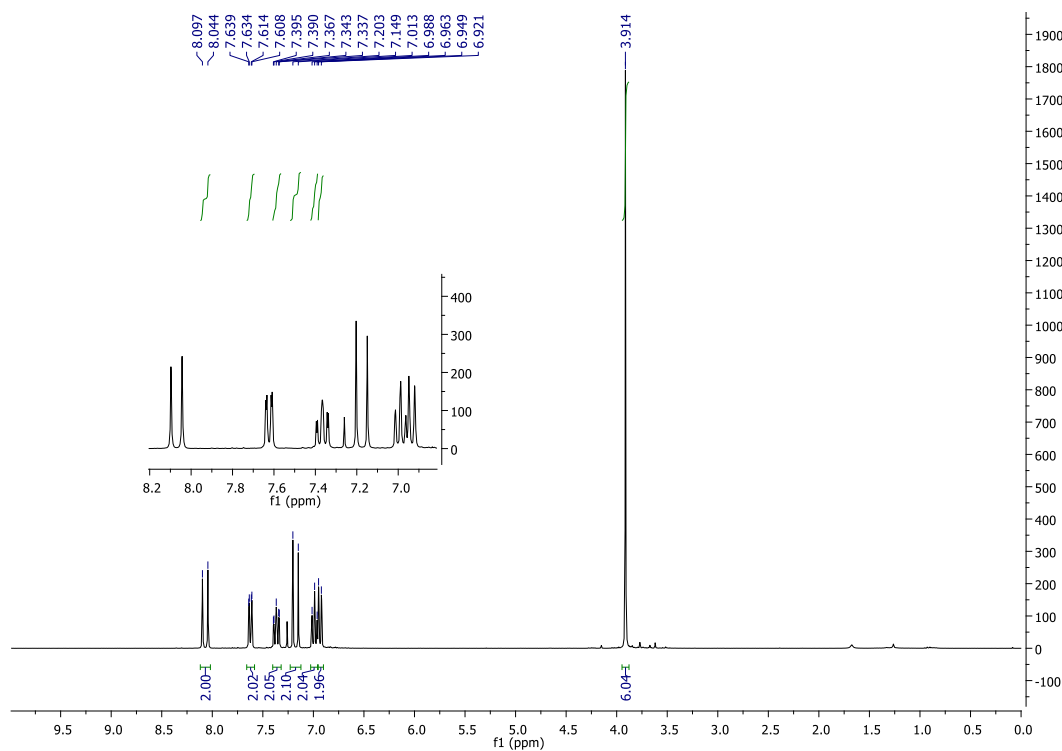
12Jul19_MSservico-Req347 3 (0.256) Cm (2:3)

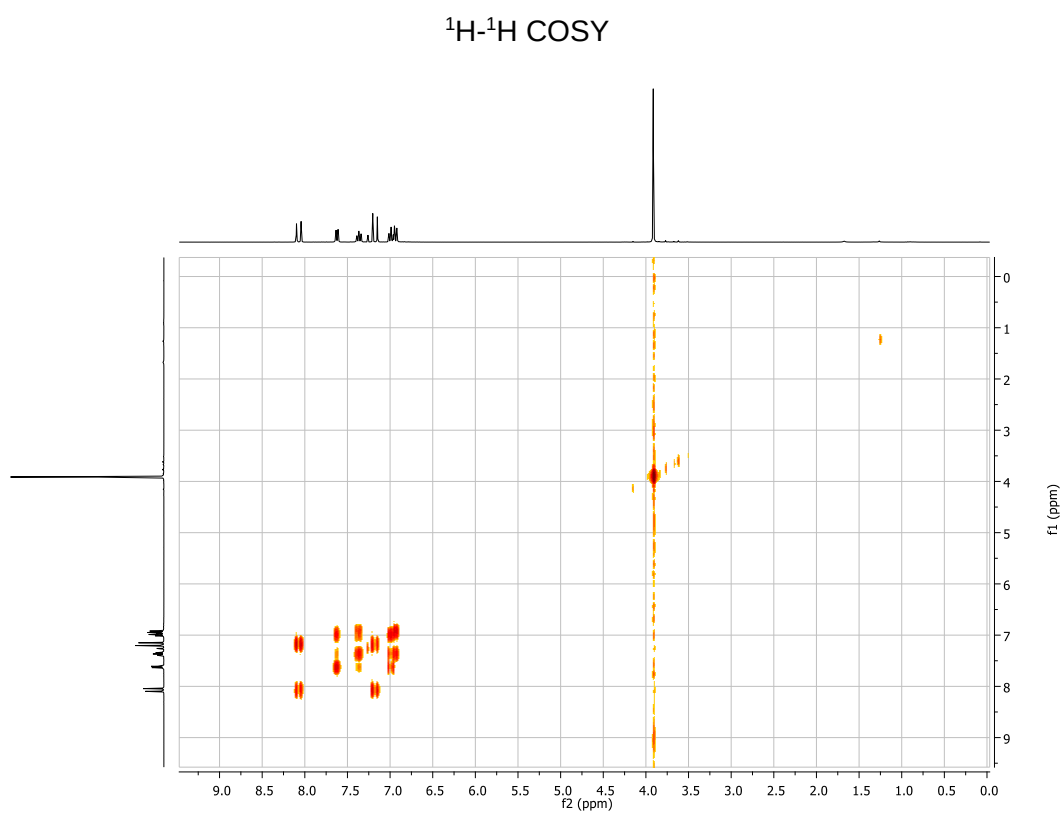
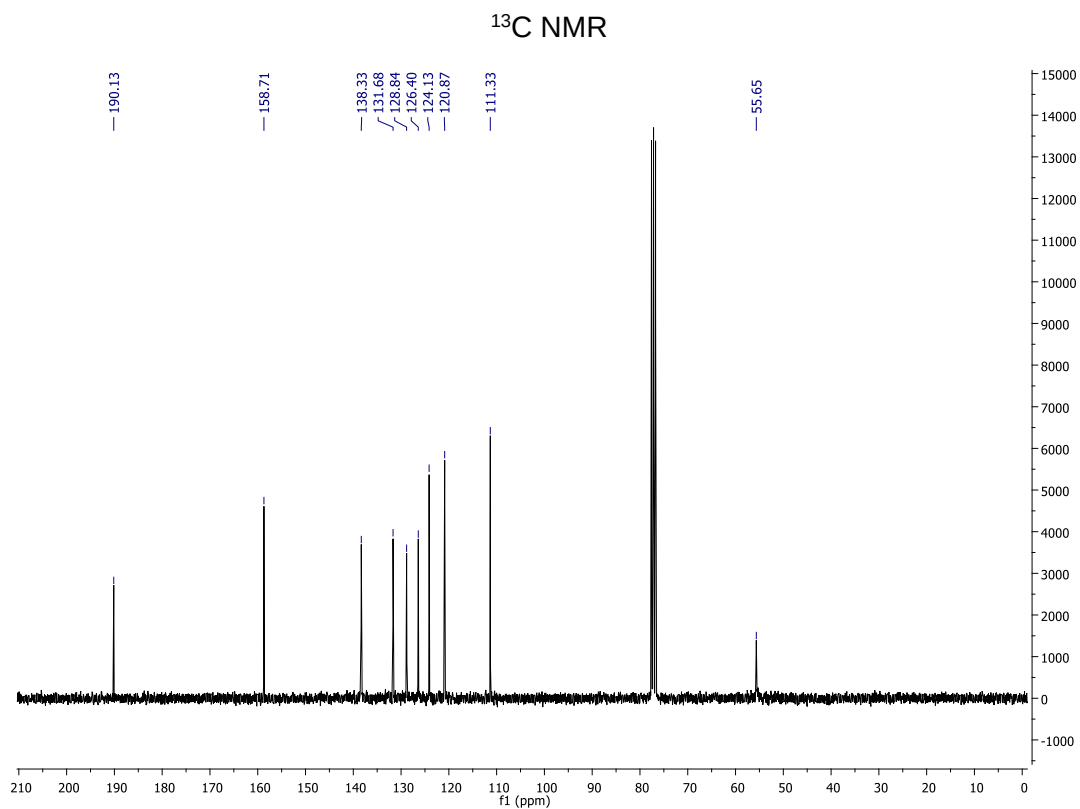
1: Scan ES+
2.29e7



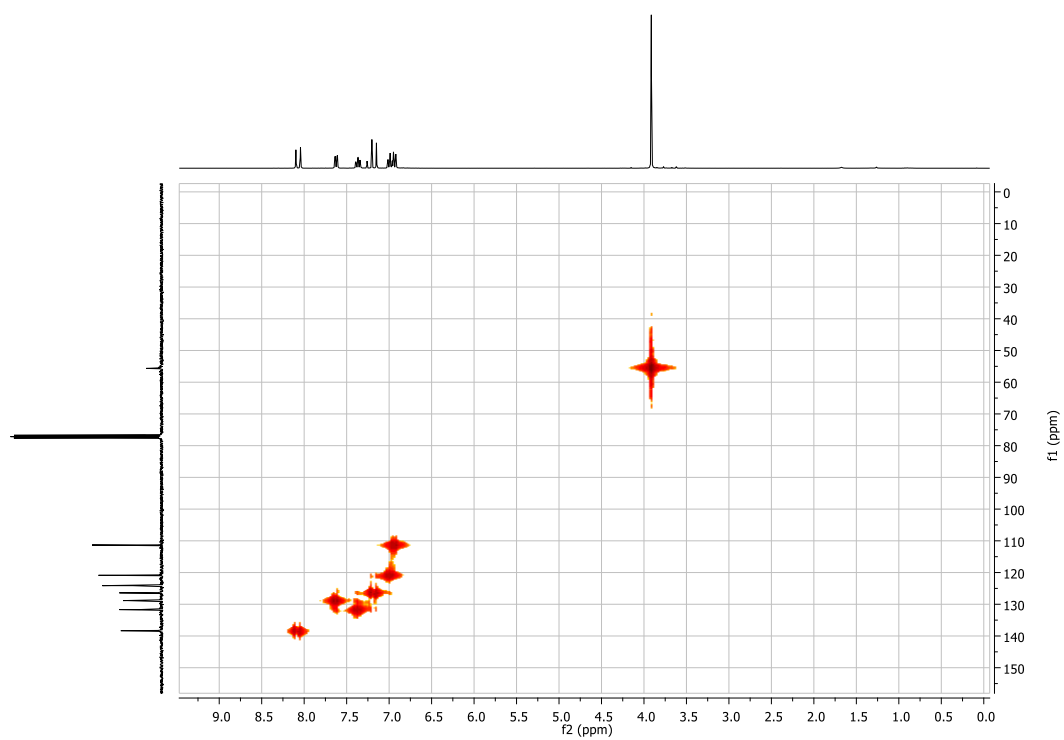
I.VII Compound 1g

¹H NMR

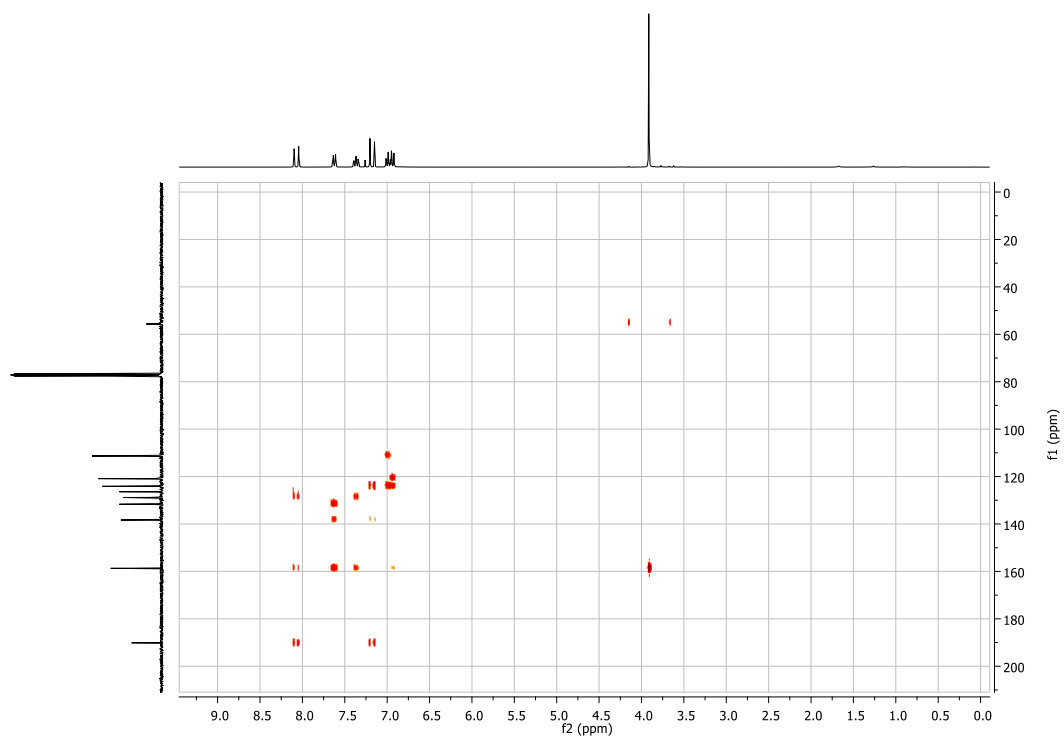




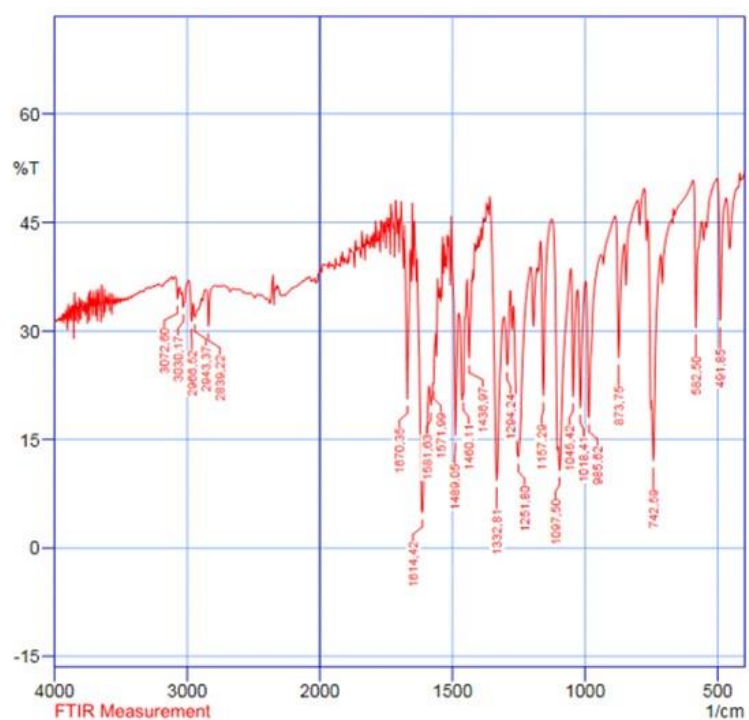
^1H - ^{13}C HMQC



^1H - ^{13}C HMBC



IR spectrum

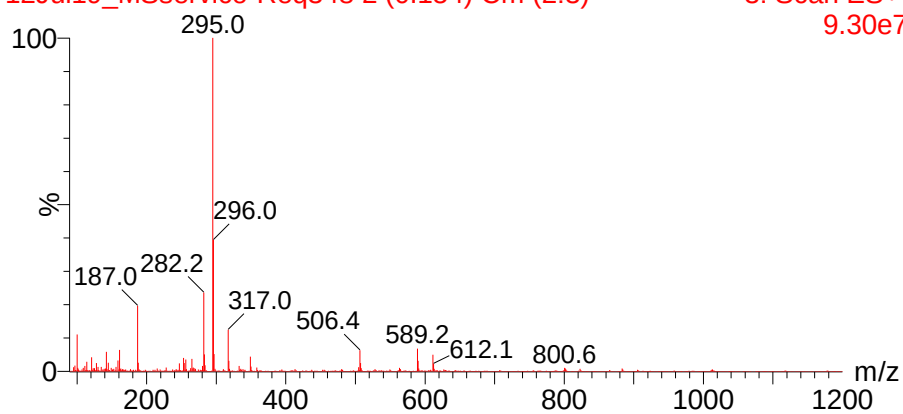


ESI⁺ mass spectrum

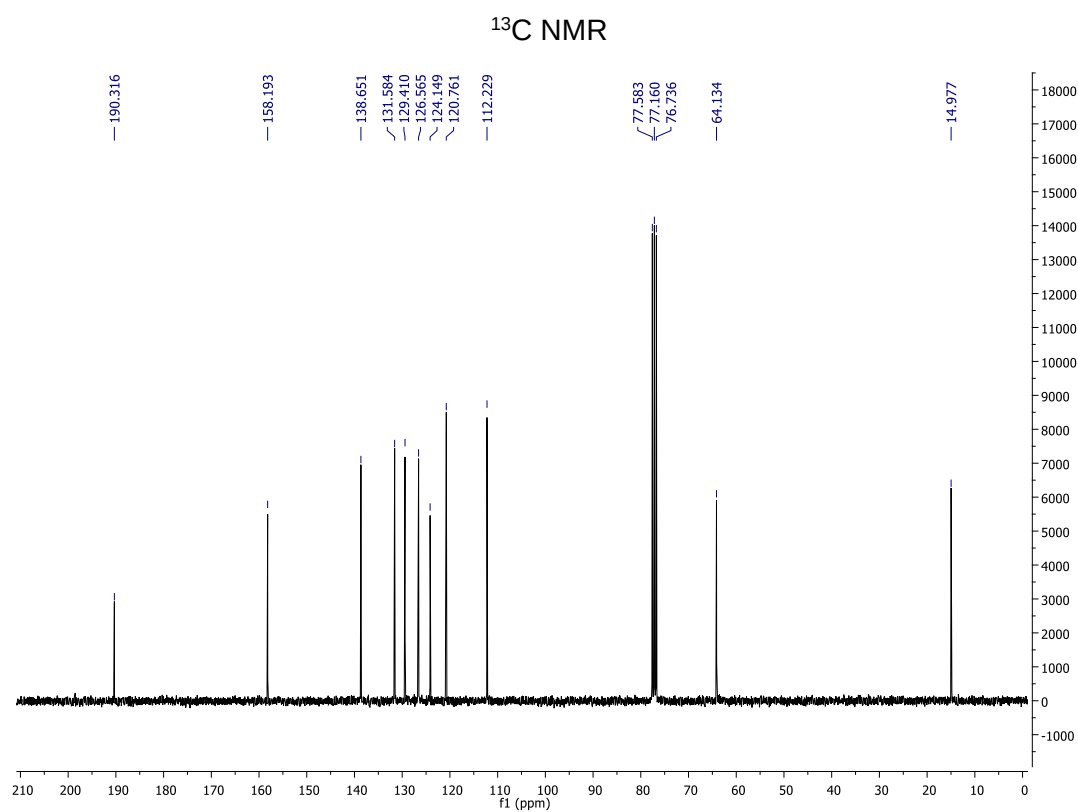
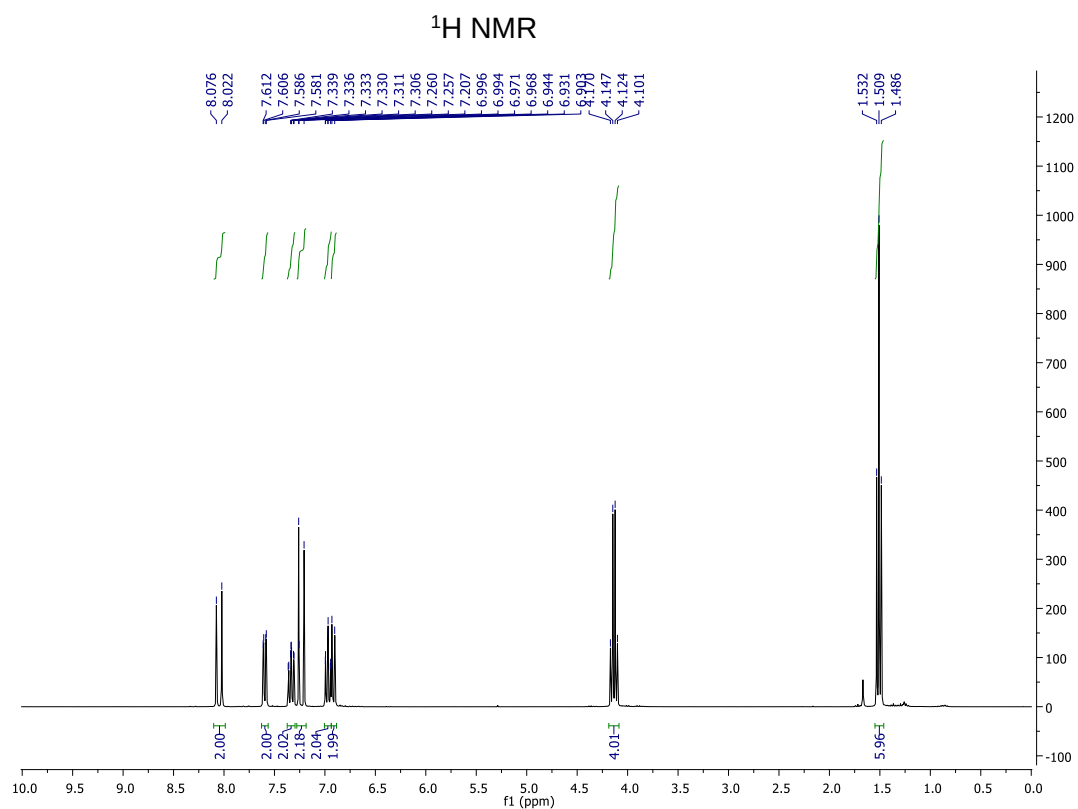
Req 348/2019 - MVC 7

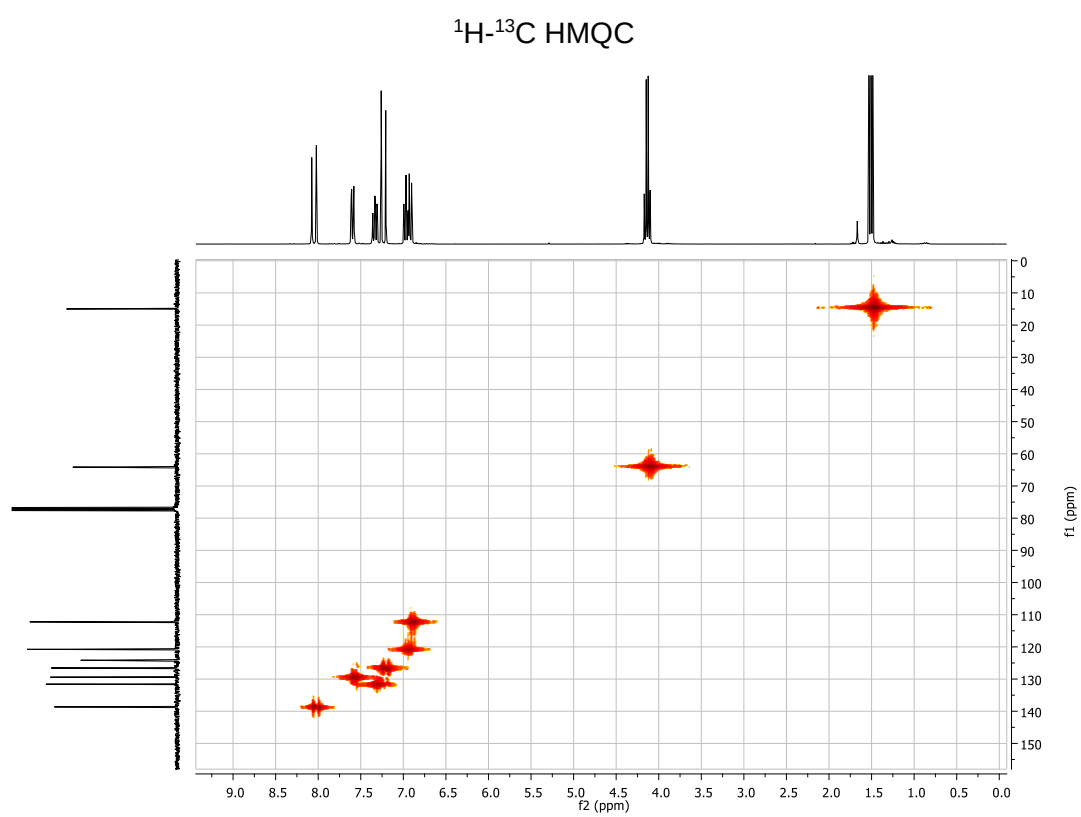
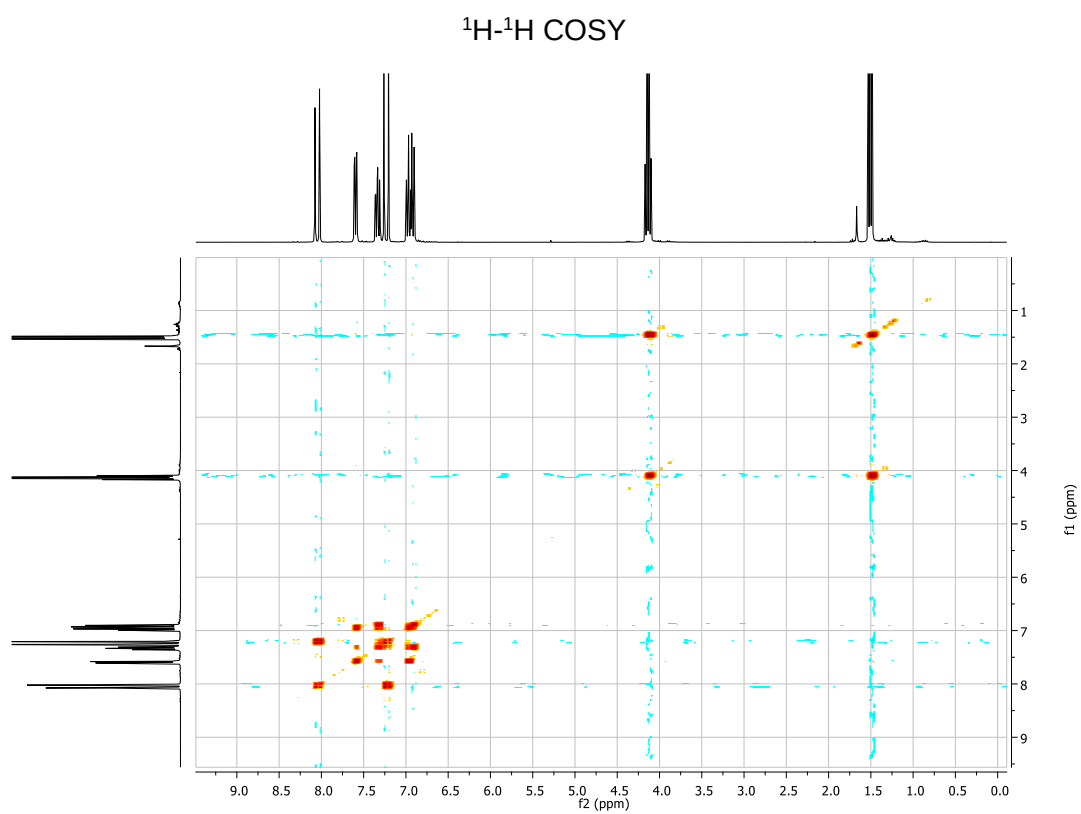
12Jul19_MSservico-Req348 2 (0.154) Cm (2:3)

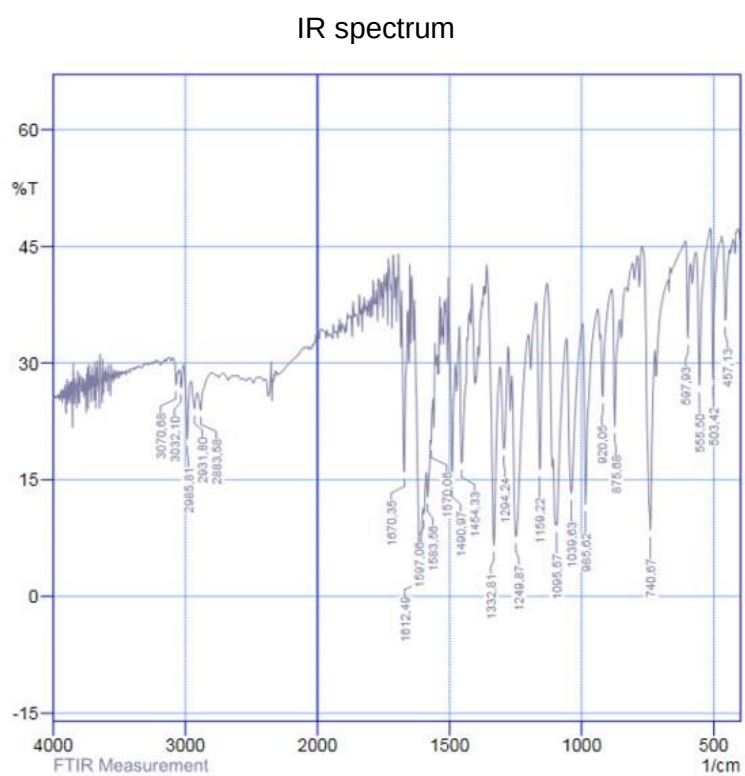
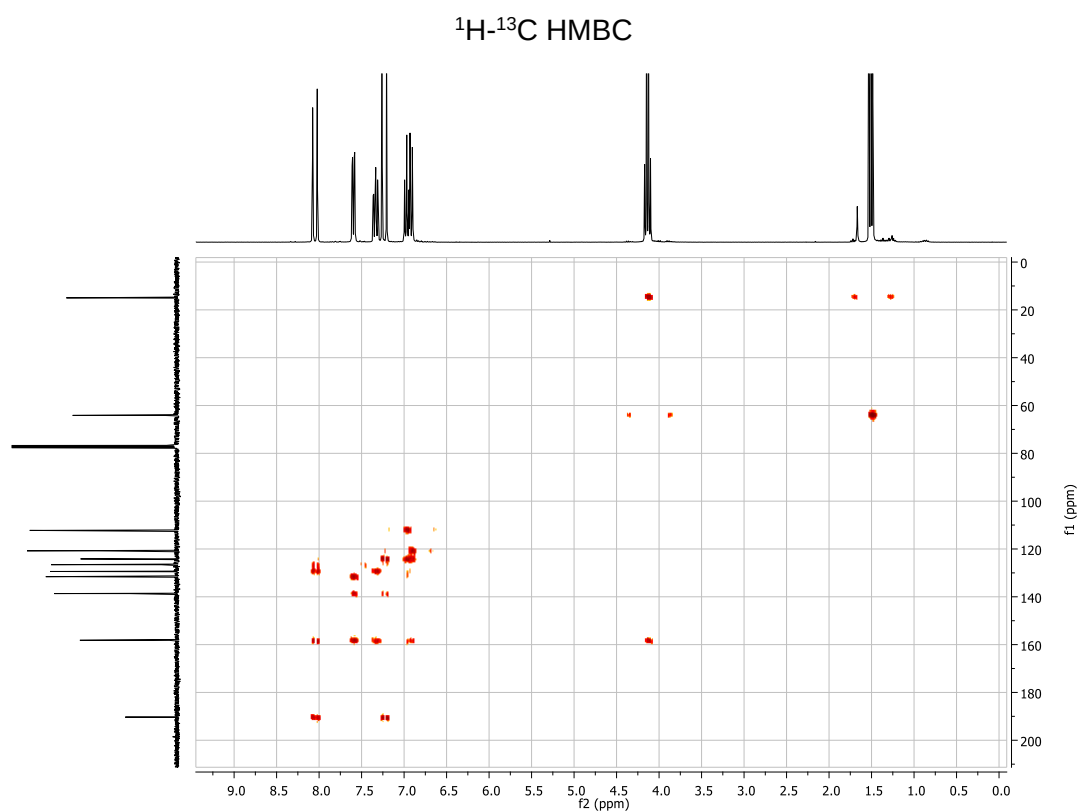
3: Scan ES+
9.30e7



I.VIII Compound 1h





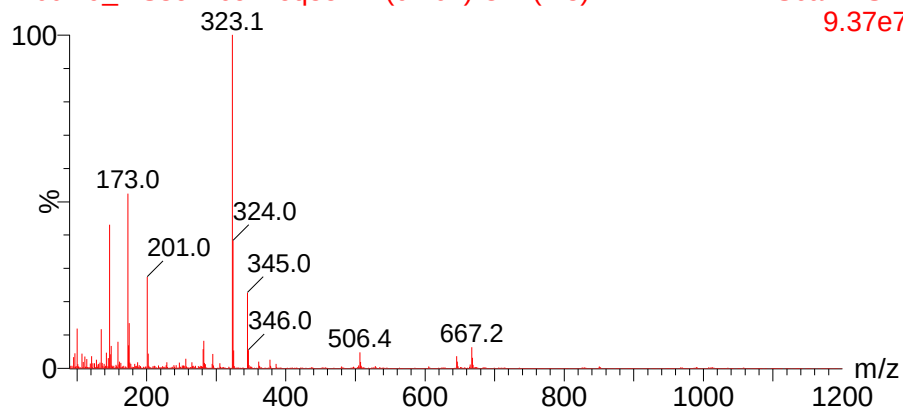


ESI⁺ mass spectrum

Req 351/2019 - MVC 11

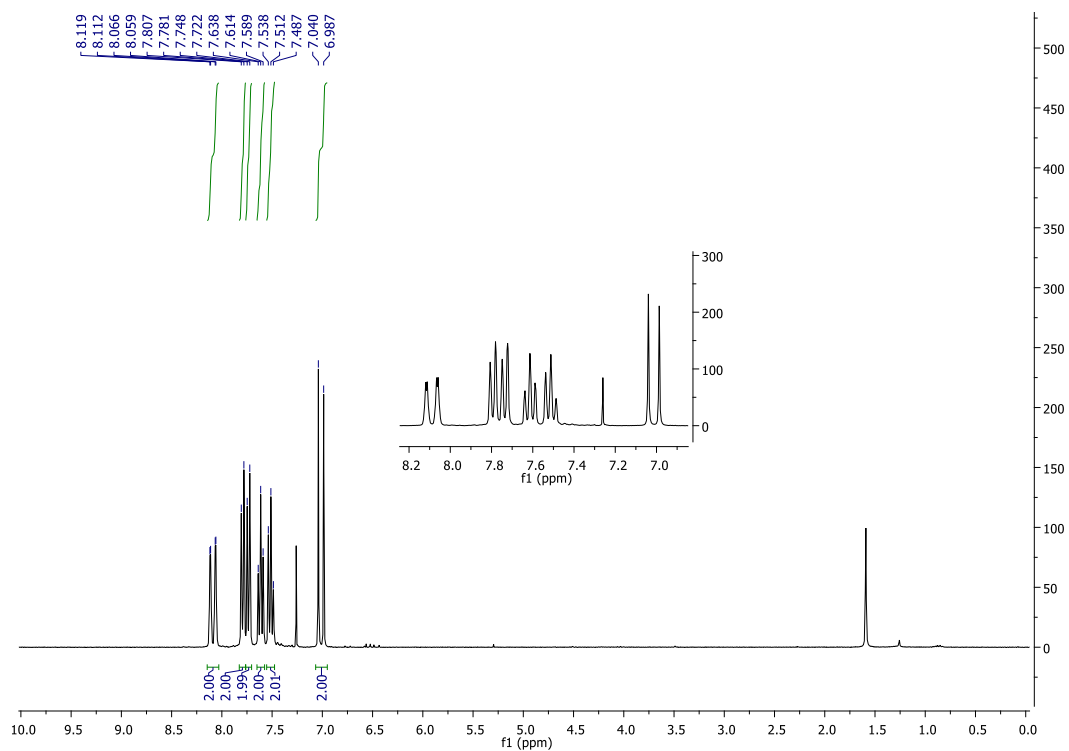
12Jul19_MSservico-Req351 2 (0.164) Cm (2:3)

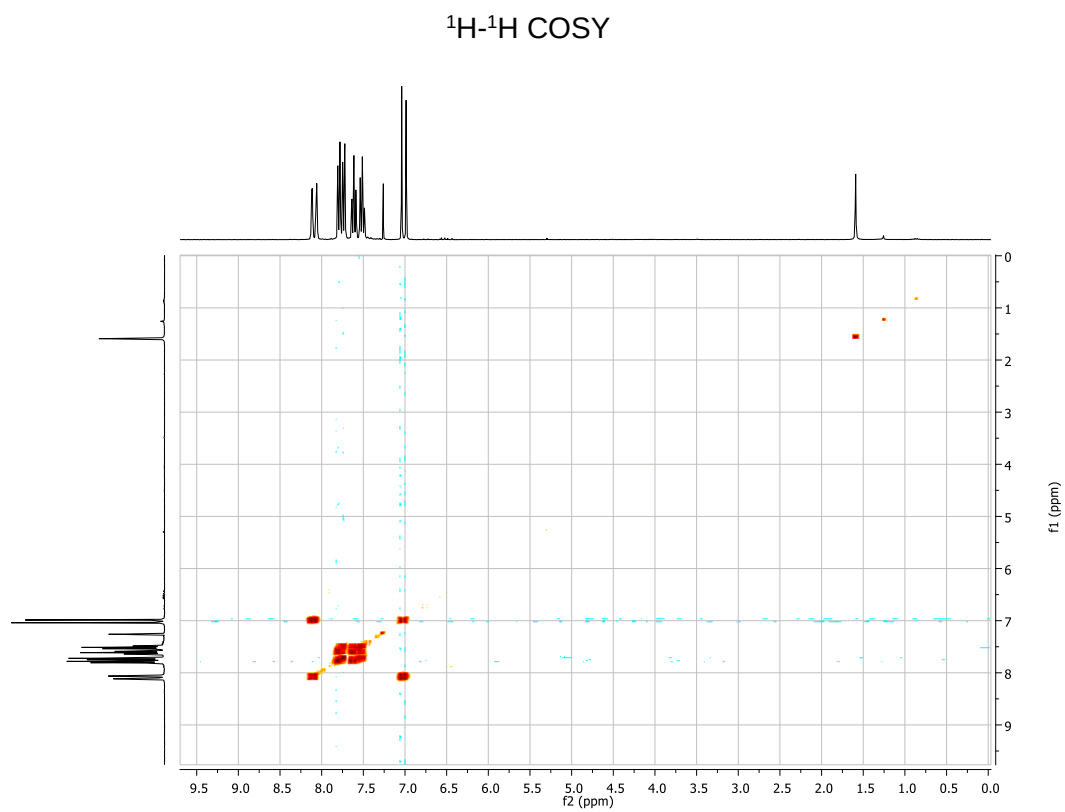
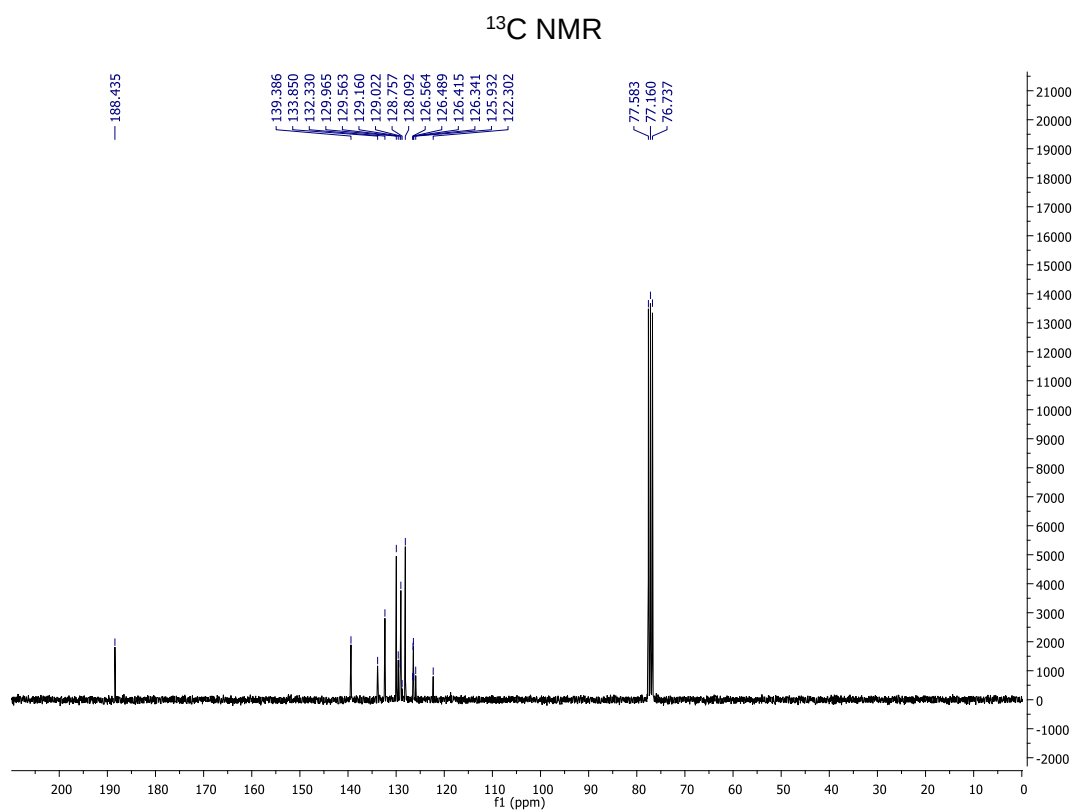
4: Scan ES+
9.37e7



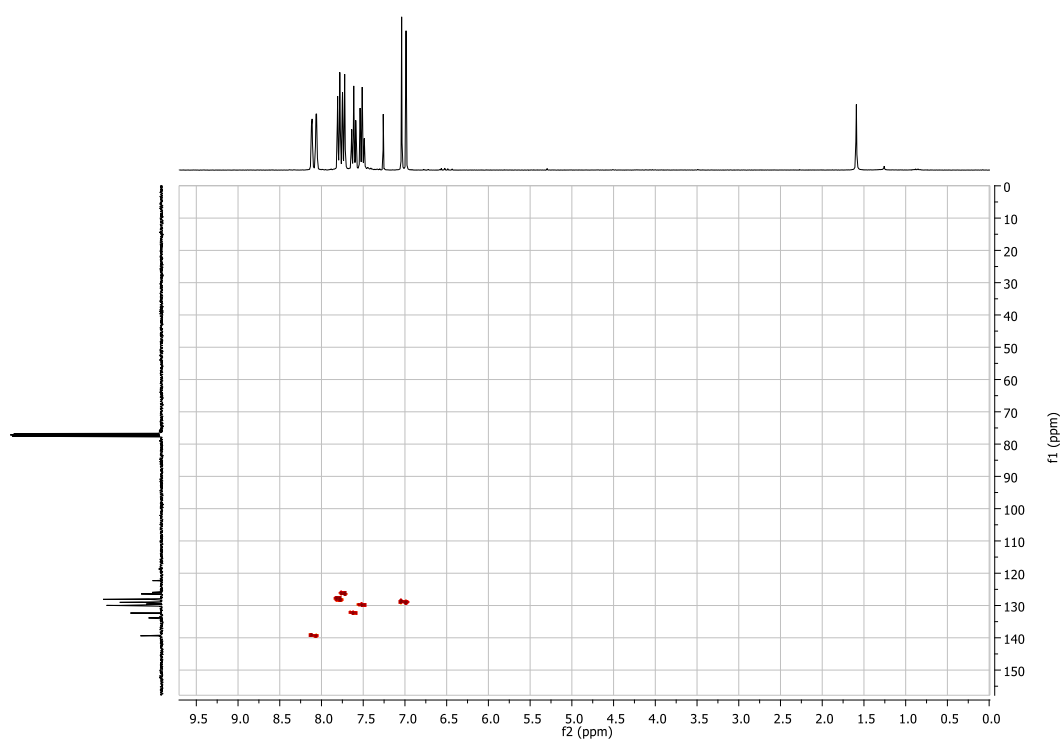
I.IX Compound 1i

¹H NMR

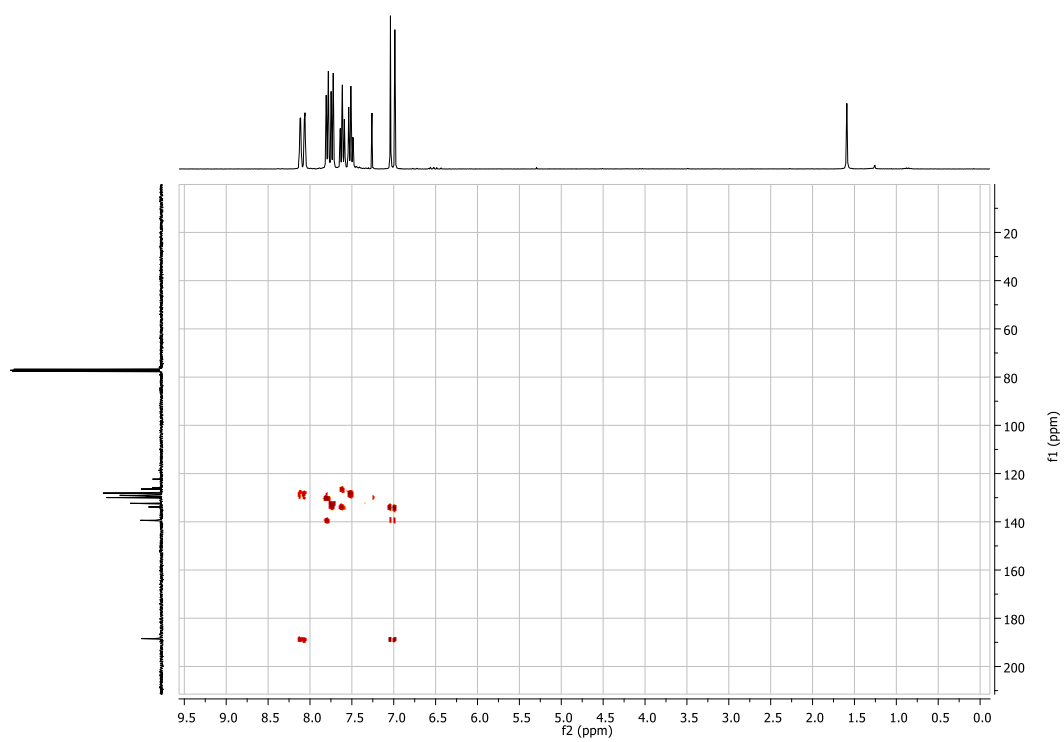




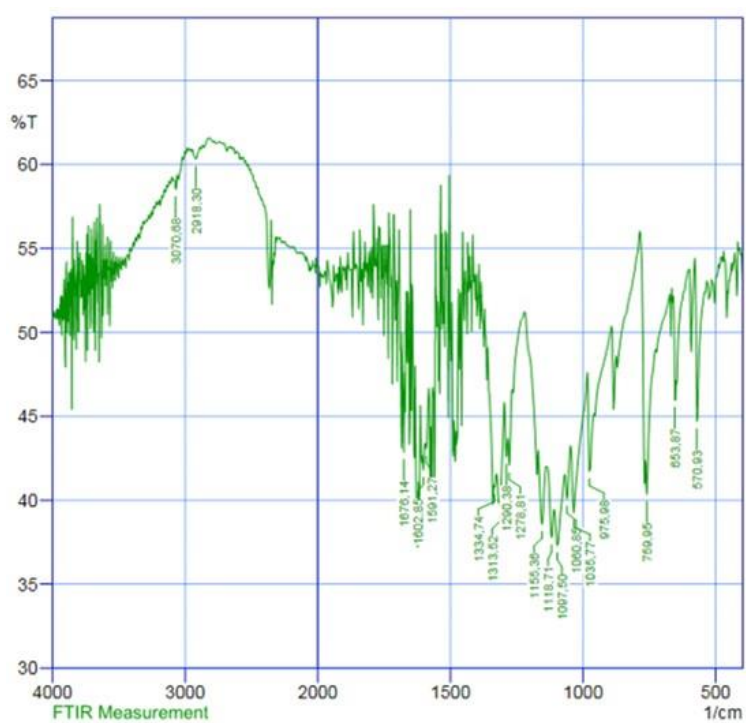
^1H - ^{13}C HMQC



^1H - ^{13}C HMBC



IR spectrum

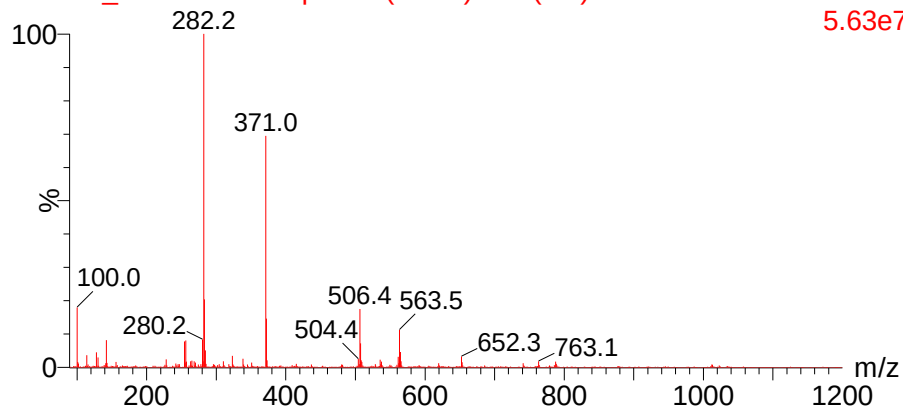


ESI⁺ mass spectrum

Req 352/2019 - MVC 12

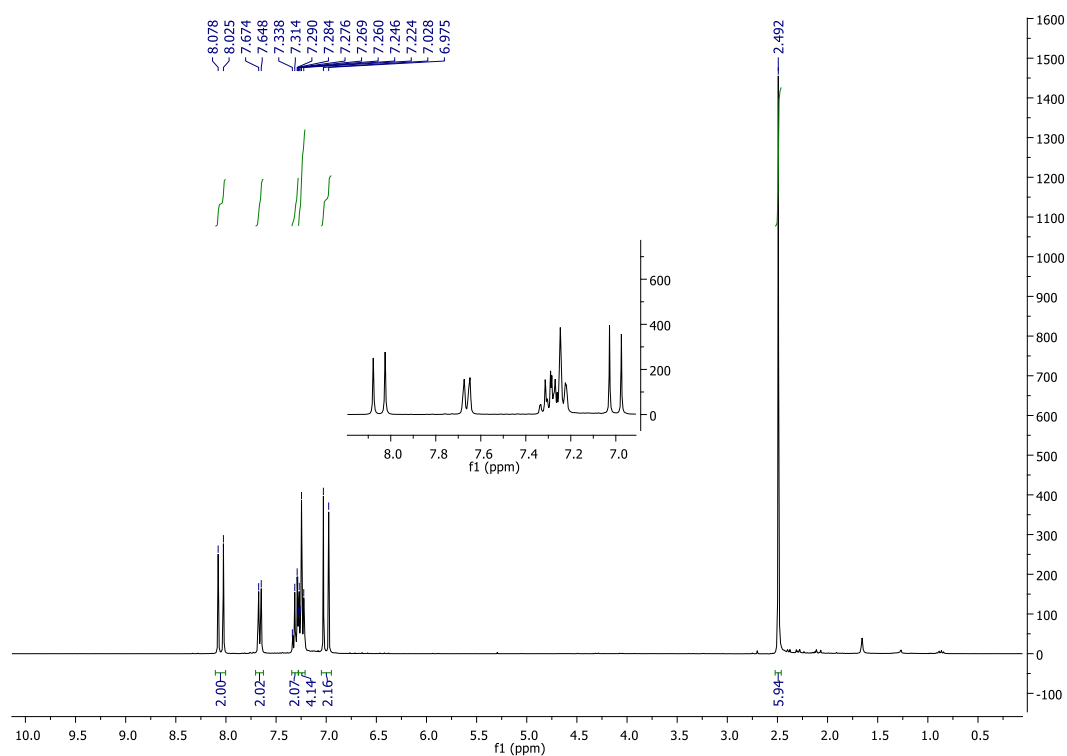
12Jul19_MSservico-Req352 3 (0.266) Cm (2:3)

2: Scan ES+
5.63e7

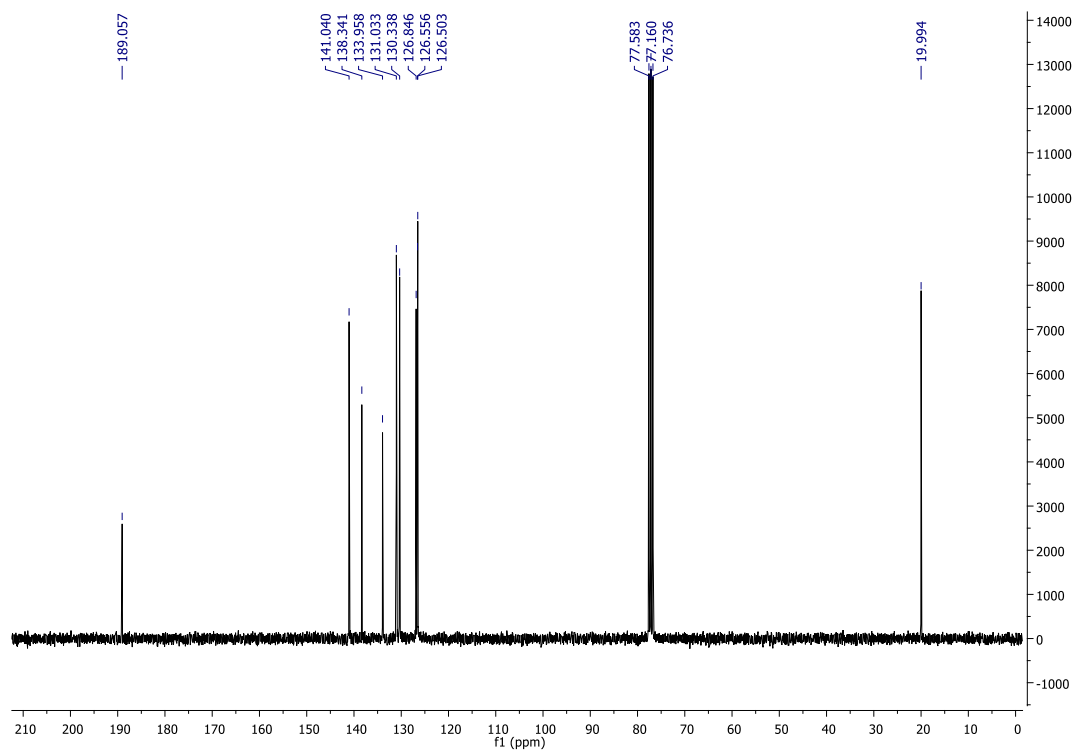


I.X Compound 1j

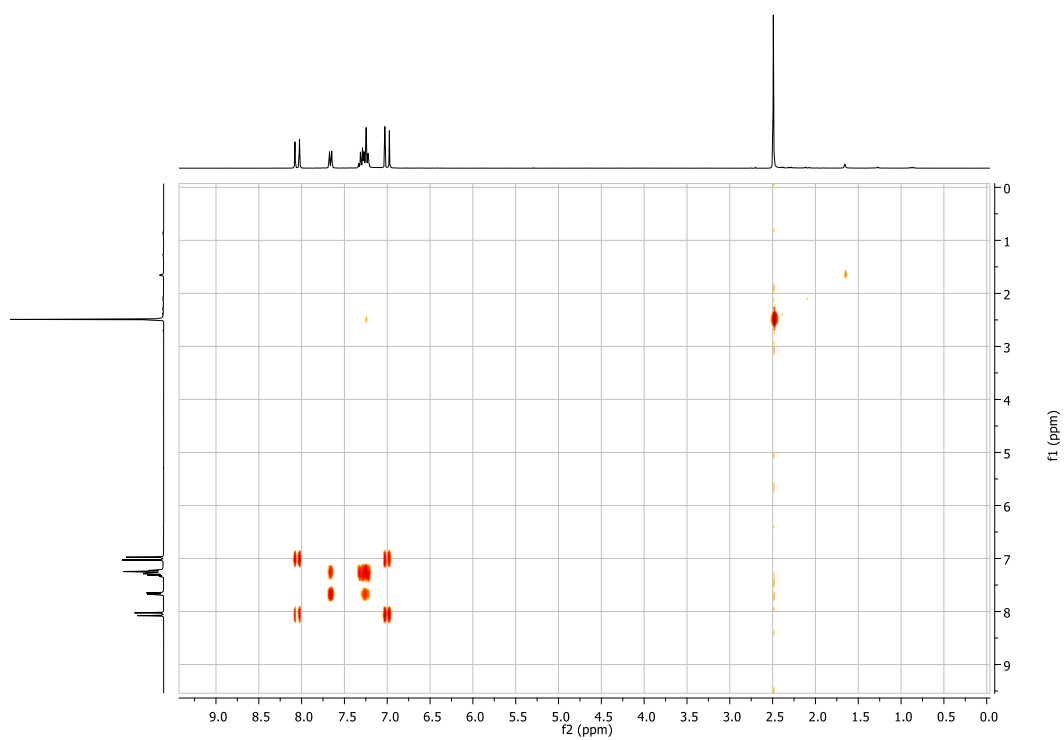
¹H NMR



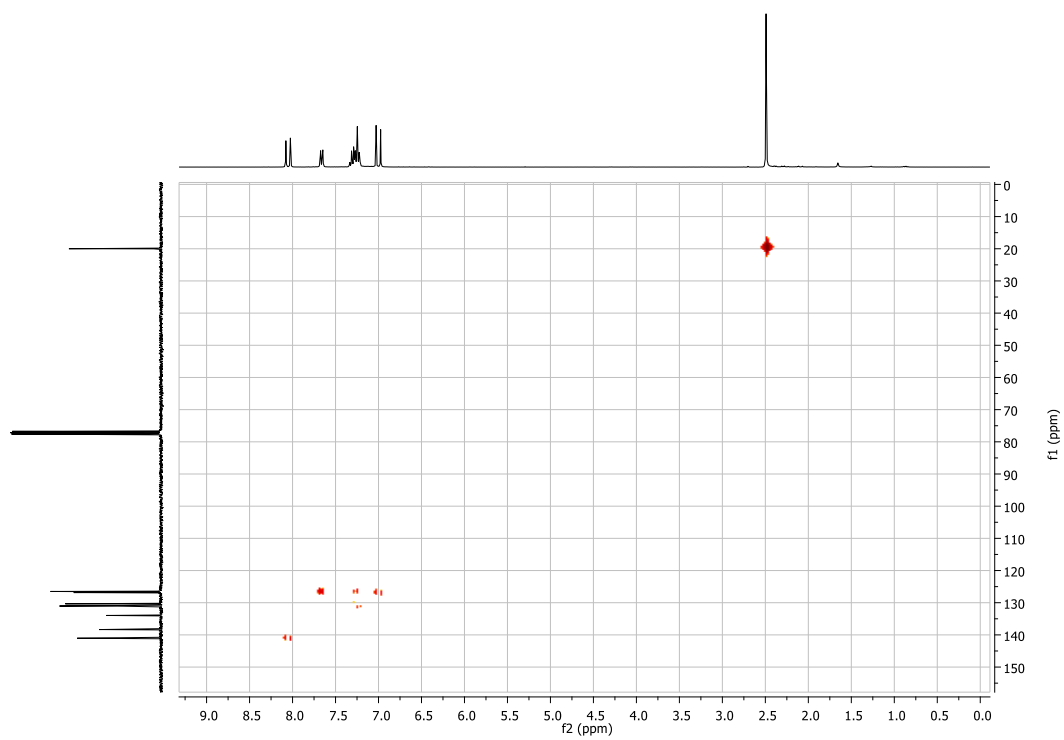
¹³C NMR



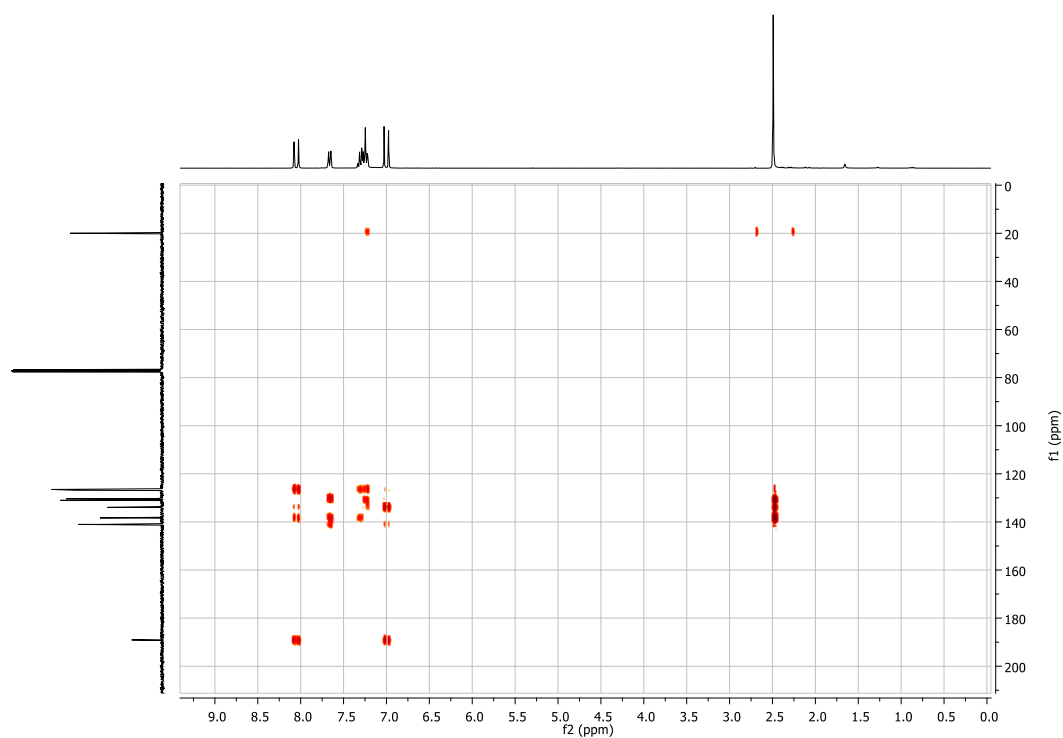
^1H - ^1H COSY



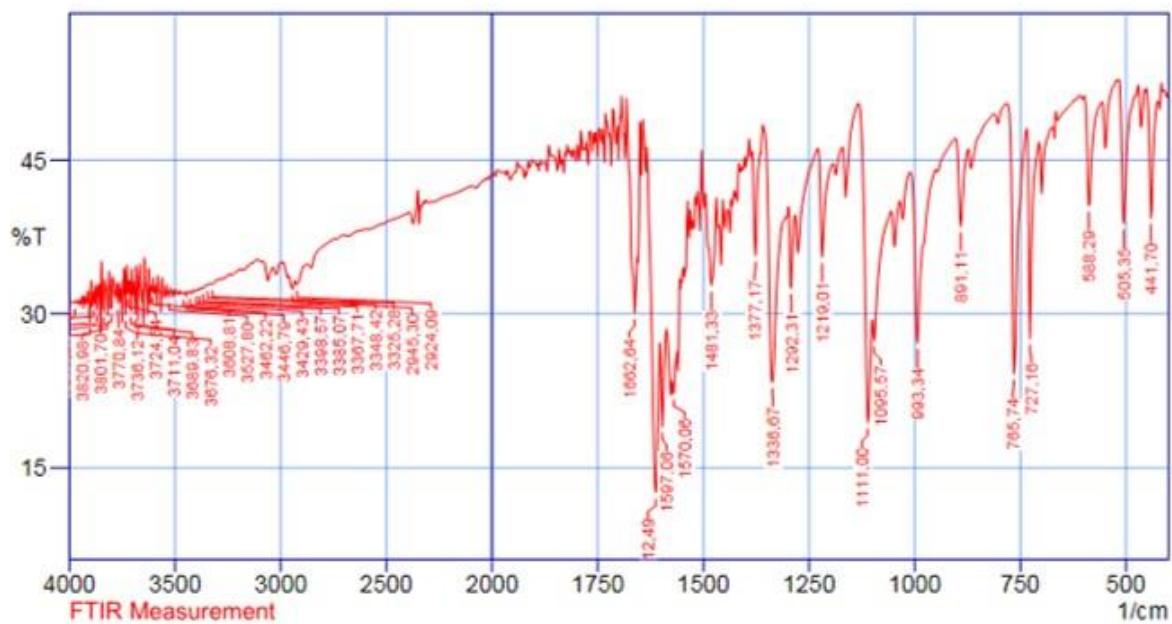
^1H - ^{13}C HMQC



^1H - ^{13}C HMBC



IR spectrum

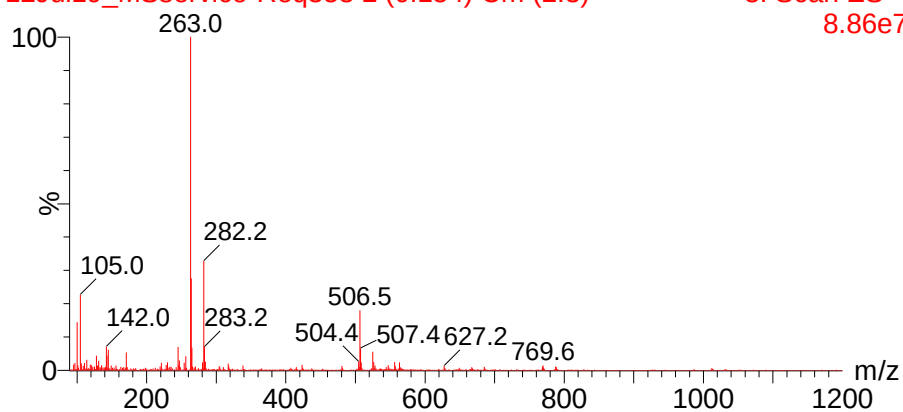


ESI⁺ mass spectrum

Req 353/2019 - MVC 13

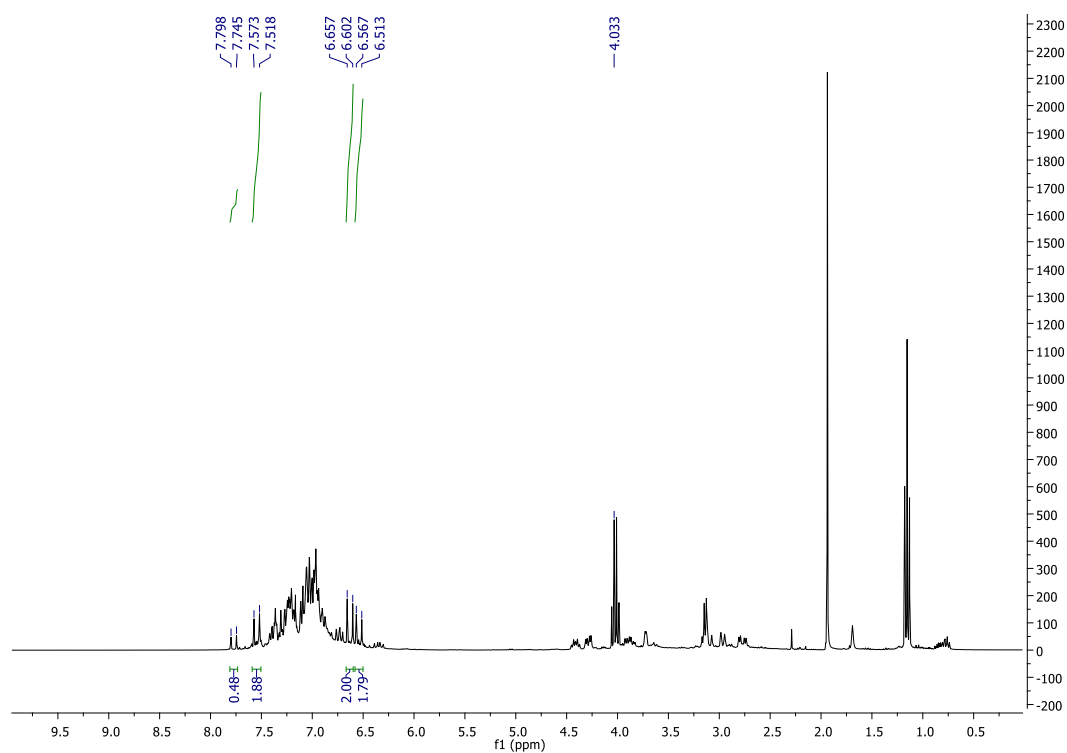
12Jul19_MSservico-Req353 2 (0.154) Cm (2:3)

3: Scan ES+
8.86e7



I.XI Compound 1k

¹H NMR impure

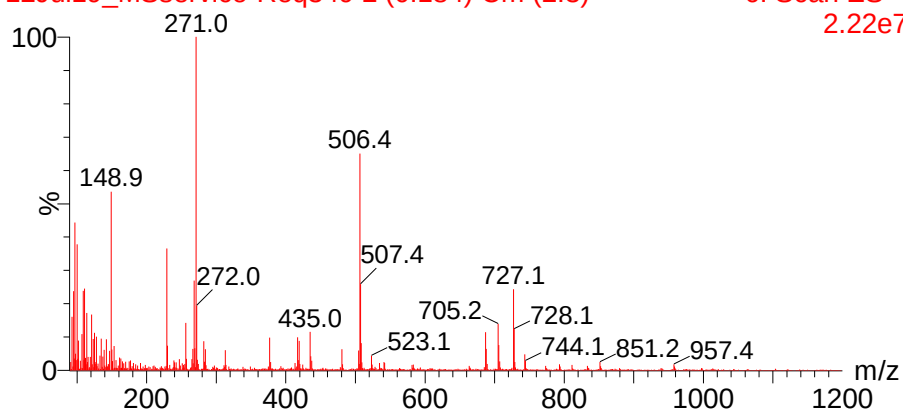


ESI⁺ mass spectrum

Req 349/2019 - MVC 8

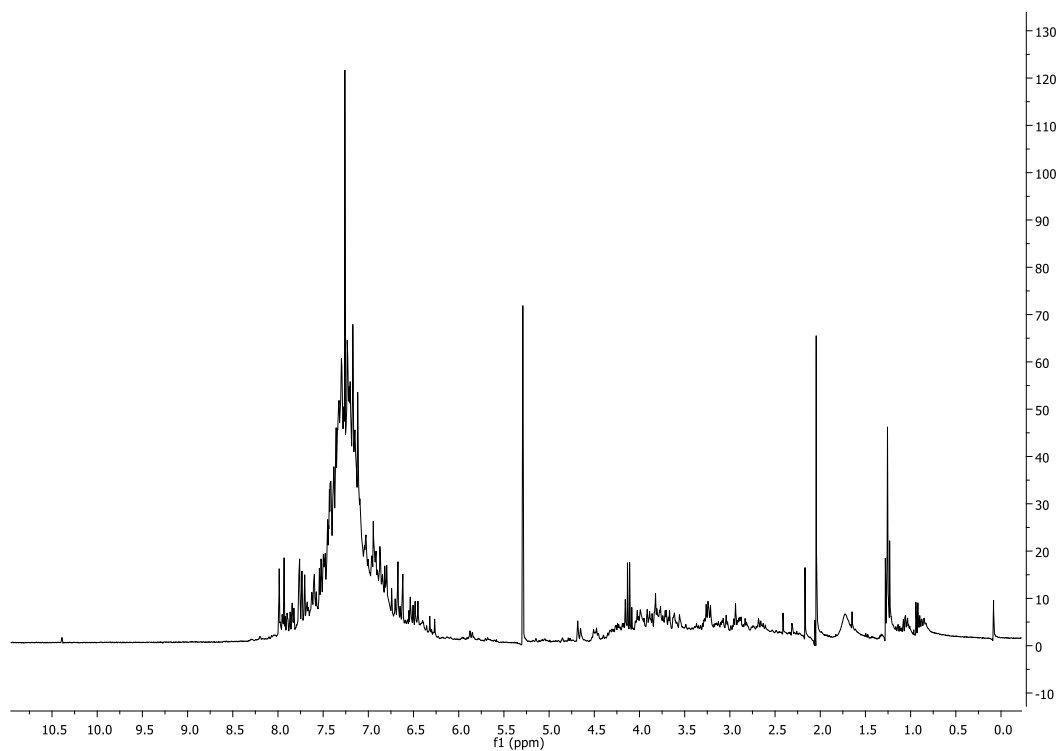
12Jul19_MSservico-Req349 2 (0.184) Cm (2:3)

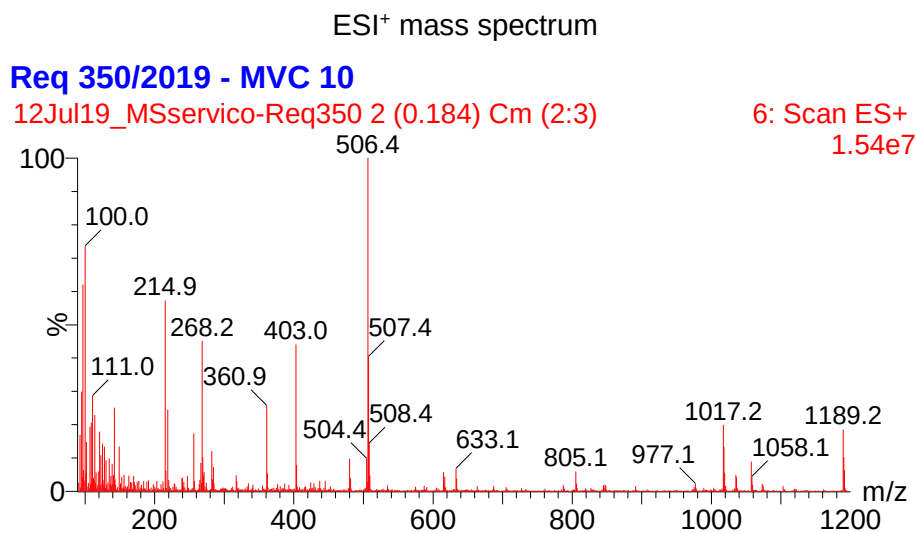
6: Scan ES+
2.22e7



I.XII Compound 1l

¹H NMR impure





Appendix II – ¹H NMR of the powder that precipitate in the test of reversibility of the reaction in the electrophilicity assessment by NMR.

