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Synthesis of curcumin derivatives as potential P-glycoprotein inhibitors

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AUTHOR'S DECLARATION

Under the terms of the Decree-Law nº 216/92, of October 13th, is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work and interpretation of the results included in this dissertation. Under the terms of the referred Decree-Law, is hereby declared that the following articles/communications were prepared in the scope of this thesis.

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Abstract

Curcumin (**1**) is a secondary metabolite isolated from the turmeric of *Curcuma longa*. Many molecular targets have been identified for this compound, hence corroborating its diverse biological activities. Particularly, curcumin (**1**) has been shown to act as multidrug resistance (MDR) modulator in various cancer cell models by downregulating the *MDR1* gene expression and, therefore, decreasing the cellular levels of P-glycoprotein (P-gp). Thus, the structure of curcumin (**1**) may represent an important basis for development of effective therapeutic agents to overcome the problem of MDR in cancer. However, curcumin (**1**) has low chemical and photostability, which severely limits its application.

In order to improve stability and P-gp inhibitory effects we proposed to synthesize three stable curcumin derivatives as building blocks and several curcumin derivatives derived from the synthesized building blocks with enhanced P-gp inhibitory activity. Several synthetic strategies were used to successfully synthesize the target compounds: Claisen-Schmidt condensation reactions, S_N2 reactions, Mannich reaction, and click chemistry. Two building blocks (**117** and **33**) were successfully synthesized and, on the basis of our current state of knowledge seven of the ten derivatives (**117**, **33**, **119**, **120**, **121**, **122**, **123**, **124**, **125**, and **126**) synthesized are being described for the first time.

The structure elucidation of the synthesized derivatives (**117**, **33**, **119**, **120**, **121**, **122**, **123**, **124**, **125**, and **126**) was established on the basis of IR, UV-Vis, MS, HRMS, and NMR techniques.

Stability and photostability studies were conducted by comparing curcumin (**1**) with the enone (**117**) and dienone (**33**) building blocks. Different assays were performed in order to evaluate the compounds stability subjected to several pH buffers, biological medium buffer, temperatures and storage times.

The main focus of the biological tests was to evaluate the tumor cell growth inhibitory activity in two lung cancer cell lines (resistant and non-resistant) and to determine P-gp inhibitory activity in a P-gp overexpressing leukemia cell line. Five of the synthesized compounds (**117**, **119**, **120**, **121**, e **125**) and curcumin (**1**) were tested according to the procedure for the sulforhodamine B (SRB) assay (cytotoxicity evaluation), and the rhodamine 123 efflux assay (P-gp inhibition assay). Preliminary results obtained as a collaboration are presented.

Keywords: Curcumin, Multidrug resistance, Curcumin derivatives, P-glycoprotein inhibition.

Resumo

A curcumina (**1**) é um metabolito secundário isolado do açafrão-da-índia, da planta *Curcuma longa*. Muitos alvos moleculares foram identificados para este composto, corroborando assim com as suas diversas atividades biológicas. Particularmente, a curcumina (**1**) mostrou ser um modulador da resistência múltipla a fármacos em vários modelos de células cancerígenas através da diminuição da expressão do gene *MDR1* e, consequentemente, diminuição dos níveis celulares de glicoproteína-P (P-gp). Assim sendo, a estrutura da curcumina (**1**) pode representar uma base importante para o desenvolvimento de agentes terapêuticos eficazes para superar o problema da resistência múltipla a fármacos no cancro. Contudo, a curcumina (**1**) têm baixa estabilidade química e fotoquímica, o que limita seriamente a sua utilização.

A fim de melhorar a estabilidade e efeito inibidor da P-gp propusemos a síntese de três derivados estáveis da curcumina como blocos construtores e de vários derivados da curcumina produzidos a partir dos blocos construtores sintetizados, de forma a possuírem maior actividade inibidora da P-gp. Várias estratégias sintéticas foram utilizadas para sintetizar com sucesso os compostos desejados: reacções de condensação de Claisen-Schmidt, reacções S_N2 , reacção de Mannich, e química “click”. Dois blocos construtores (**117** e **33**) foram sintetizados com sucesso e, com base no nosso conhecimento actual, sete dos dez compostos sintetizados (**117**, **33**, **119**, **120**, **121**, **122**, **123**, **124**, **125**, e **126**) estão a ser descritos pela primeira vez.

A elucidação estrutural dos derivados sintetizados (**117**, **33**, **119**, **120**, **121**, **122**, **123**, **124**, **125**, e **126**) foi estabelecida com base em IV, UV-Vis, MS, HRMS e técnicas de RMN.

Os estudos de estabilidade e fotoestabilidade foram realizados comparando a curcumina (**1**) com os blocos construtores sintetizados, enona (**117**) e dienona (**33**). Diferentes ensaios foram realizados de forma a avaliar a estabilidade dos composto quando sujeitos a tampões com diferentes pHs, o meio biológico das células, diferentes temperaturas e tempos de armazenamento.

O principal objectivo dos ensaios biológicos era avaliar a actividade inibidora do crescimento de células tumorais em duas linhas de cancro no pulmão (resistente e não-resistente) e determinar a actividade inibidora da P-gp numa linha celular leucémica que superexpressa P-gp. Cinco dos compostos sintetizados (**117**, **119**, **120**, **121**, e **125**) e a curcumina (**1**) foram testados de acordo com o procedimento para o ensaio da sulforodamina B (SRB) (avaliação da citotoxicidade), e para o ensaio de efluxo da

rodamina 123 (avaliação da inibição da P-gp). Os resultados preliminares obtidos como colaboração são apresentados.

Palavras-Chave: Curcumina, Resistência múltipla a fármacos, Derivados da curcumina, Inibição da glicoproteína-P.

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ABBREVIATIONS

^{13}C NMR	Carbon nuclear magnetic resonance
^1H NMR	Proton nuclear magnetic resonance
ABC	ATP-binding cassette
Ar	Aromatic
BCRP	Human breast cancer resistance protein
BDMC	Bisdemethoxycurcumin
Boc	<i>tert</i> -butoxycarbonyl
<i>brs</i>	Broad singlet
<i>d</i>	Doublet
DAD	Diode array detector
<i>dd</i>	Double doublet
<i>ddd</i>	Doublet of doublets of doublets
DMC	Demethoxycurcumin,
DMSO	Dimethyl sulfoxide
<i>dt</i>	doublet of triplets
EI-MS	Electron impact-mass spectrometry
eq.	Equivalents
EtOAc	Ethyl acetate
EtOH	Ethanol
FBS	Fetal bovine serum

GC-MS	Gas chromatography-mass spectrometry
GI ₅₀	Half maximal inhibition of cell growth
h	Hours
HPLC	High-performance liquid chromatography
HMBC	Heteronuclear multiple bond correlation
HRMS-ESI	High resolution mass spectrometry-Electrospray ionization
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
IC ₅₀	Half maximal inhibitory concentration
IR	Infrared spectroscopy
<i>J</i>	Coupling constant
k	Retention factor
Lit.	Literature
<i>m</i>	Multiplet
MDR	Multidrug resistance
MeOH	Methanol
MRP1	Multidrug resistance-associated protein 1
MRP5	Multidrug resistance-associated protein 5
min	Minutes
mp	Melting point
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MW	Microwave
PBS	Phosphate buffered saline

P-gp	P-glycoprotein
r.t.	Room temperature
s	Singlet
SAR	Structure-activity relationship
Sec	Secondary
siRNAs	Small interfering RNAs
SRB	Sulforhodamine B
SXR	Steroid and xenobiotic receptor
<i>t</i>	Triplet
<i>tert</i>	Tertiary
THC	Tetrahydrocurcumin
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UV-Vis	Ultraviolet-visible

OUTLINE OF THE THESIS

The present dissertation consists of five chapters:

Chapter I: INTRODUCTION

Chapter I provide the research background of the work done with special emphasis on curcumin and its biological activities, multidrug resistance in cancer and some of the curcumin derivatives that have already been described as tumor cell growth inhibitors and/or multidrug resistance modulators. A more intensive study on curcumin derivatives and respective biological activities was submitted as a review manuscript..

Chapter II: RESULTS AND DISCUSSION

This chapter is divided into three subchapters emphasizing the results obtained concerning the synthesis and structure elucidation, the stability and photostability studies, and the biological activity of the synthesized derivatives. Herein, some structure-activity relationships (SAR) considerations are presented. Biological activity studies of the synthesized compounds were performed by Vanessa Rodrigues under supervision pf Professor Helena Vasconcelos at IPATIMUP..

Chapter III: MATERIAL AND METHODS

In this chapter the synthetic and purification methodologies are described for the synthesized compounds. Information concerning the procedures for the stability and photostability studies are also described.

Chapter IV: CONCLUSIONS

Chapter IV points out the general conclusions of this dissertation, on the basis of the proposed aims.

Chapter V: REFERENCES

Herein is presented a list of the references as well as the web pages used in this dissertation. The references followed the American Chemical Society style. The main bibliographic research motors were ISI Web of Knowledge, from Thomson Reuters, Scopus and Google.

CHAPTER I:

INTRODUCTION

1. Curcumin

1.1. Curcumin, general features

Curcumin (**1**, Figure 1) is a secondary metabolite of the well-known Indian spice turmeric, derived from the rhizomes of *Curcuma longa*, of the Zingiberaceae family ¹. Curcumin (**1**) has been used in Ayurvedic medicine for thousands of years, as a treatment for inflammatory diseases and wounds ²⁻³. Curcumin (**1**) is a polyphenol, and it is the main component of the yellow-pigmented fraction of turmeric ⁴. Besides curcumin (**1**), this fraction of turmeric also contains demethoxycurcumin (DMC, **2**) and bisdemethoxycurcumin (BDMC, **3**), collectively called curcuminoids (Figure 1) ⁵. Hence, DMC (**2**) and BDMC (**3**) are naturally-occurring analogues of curcumin (**1**). Curcuminoids represent 3-5% of turmeric. Commercially available extract contains different percentages of these compounds: 77% curcumin (**1**), 17% DMC (**2**), and 3% BDMC (**3**) ⁵. Traditionally, curcumin (**1**) has been used as a coloring agent in foods, medicine, and cosmetics. As a spice, it is used to provide curry with its characteristic yellow color, flavor, and aroma ⁴.

A captivating feature of curcumin (**1**), and one of the main reasons why it has been extensively studied and is still used in traditional medicine, is the extremely good safety profile. To date, no studies in either animals or humans have discovered toxicity associated with the use of curcumin (**1**), even when tested at high doses ⁴. Indeed, the U.S. Food and Drug Administration has approved curcumin (**1**) as a “generally regarded as safe” compound ⁶.

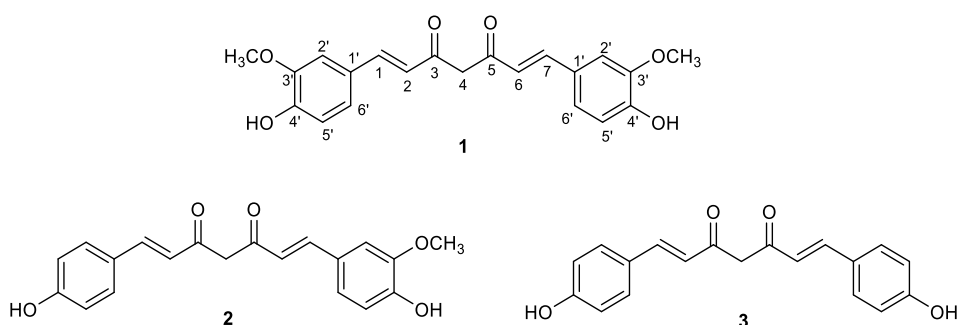


Figure 1 - Curcuminoids of the yellow-pigmented fraction of turmeric: curcumin (**1**), DMC (**2**), and BDMC (**3**).

Curcumin (**1**) or (1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene,3,5-dione, is a yellow-orange powder with a melting point of approximately 183°C. This compound exhibits keto-enol tautomerism; however, once in solution it exists mainly in its enolic form (Figure 2)⁴⁻⁵. The enolic form of curcumin (**1**) has three ionizable groups: the enolic group and the two phenolic groups. The pK_a value for this compound is 8.54¹.

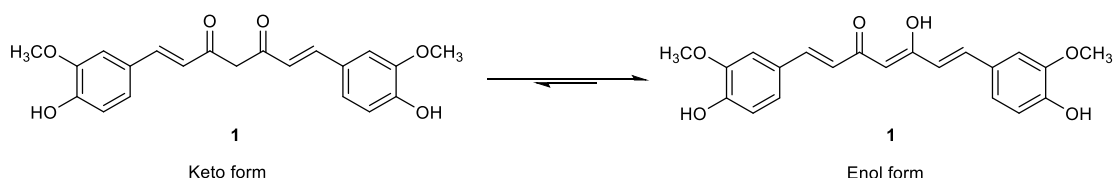


Figure 2 - The keto-enol tautomerism of curcumin (**1**).

Poor bioavailability of curcumin (**1**) can be explained by its poor water solubility and extensive metabolism. Curcumin (**1**) could only be orally effective as a drug if it could be absorbed by the gut and enter the bloodstream; thus solubility would be essential in this process. However, curcumin (**1**) is excreted from the gut, and if patients were to be subjected to curcumin (**1**) as an oral therapy, they would have to ingest 12 to 20 g of curcumin (**1**). Such doses are however too high, since more than 10 g of curcumin (**1**) leaves an intolerable aftertaste in the mouth⁷. Nevertheless, in spite of the bioavailability problems of curcumin (**1**), this compound has recognized biological and therapeutic properties.

1.2. Biological activities of curcumin

For thousands of years curcumin (**1**) has been used as a therapeutic agent throughout the Orient. The impressive healing properties and safety of curcumin (**1**) motivated research groups all over the world to study this natural product. Curcumin (**1**) was shown to have many biological activities, such as antioxidant, cardio and neuroprotective, antidiabetic, antimicrobial, antimalarial, anti-human immunodeficiency virus (HIV), thrombosuppressive, antitumor and chemopreventive activities⁴⁻⁵. Curcumin (**1**) even seems to control numerous cancer treatment-related symptoms, such as depression⁸.

Over the past few years, many review articles have been published on the subject of curcumin (**1**) biological activities ^{4-5, 8-12}. Several studies suggest that curcumin (**1**) has a diverse range of molecular targets, which supports the idea that this molecule influences a great variety of biochemical and molecular cascades. Figure 3 displays the molecular targets of curcumin (**1**) described until now ^{4, 8, 13}.

In response to the growing interest in curcumin (**1**) and its biological activities, several clinical trials have addressed the safety, pharmacokinetics and efficacy in humans. These trials have also studied the biological activities of curcumin (**1**) that are of greatest interest and that have showed best results in *in vitro* and pre-clinical assays. Currently, about 104 trials are either ongoing or have been completed, worldwide ¹⁴.

1.3. Curcuminoids

Curcuminoids exhibited antioxidant activity both *in vitro* and *in vivo* models, even though to different extents. The curcuminoids **1-3** inhibited lipid peroxidation caused by Fenton reagent, metals, H₂O₂ and 2,2'-azo-bis(2-amidinopropane) (AAPH), in several *in vitro* models ¹⁵⁻¹⁷. Curcuminoids **1-3** also revealed other antioxidant activities, such as scavenging of reactive oxygen species like superoxide anions and hydrogen peroxide, and inhibition of low density lipoproteins oxidation ¹⁸⁻¹⁹.

In an *in vitro* assay, the individual components of the curcuminoids (compounds **1-3**) have shown acetylcholinesterase (AChE) inhibitory activity ²⁰. The most potent was BDMC (**3**), followed by DMC (**2**) and curcumin (**1**). Probably the methoxyl group, present in curcumin (**1**) and DMC (**2**), reduces the AChE inhibitory activity. The curcuminoids mixture was less effective *in vitro* than BDMC (**3**). However, in an *ex vivo* assay, the curcuminoids mixture presented better results, which could indicate that curcuminoids as a mixture might be better penetrating agents of the blood brain barrier, or that they can have synergistic interactions between them. Curcumin (**1**) by itself has shown negligible effect in the *ex vivo* assay, perhaps due to poor absorption and low penetration into the brain ²⁰.

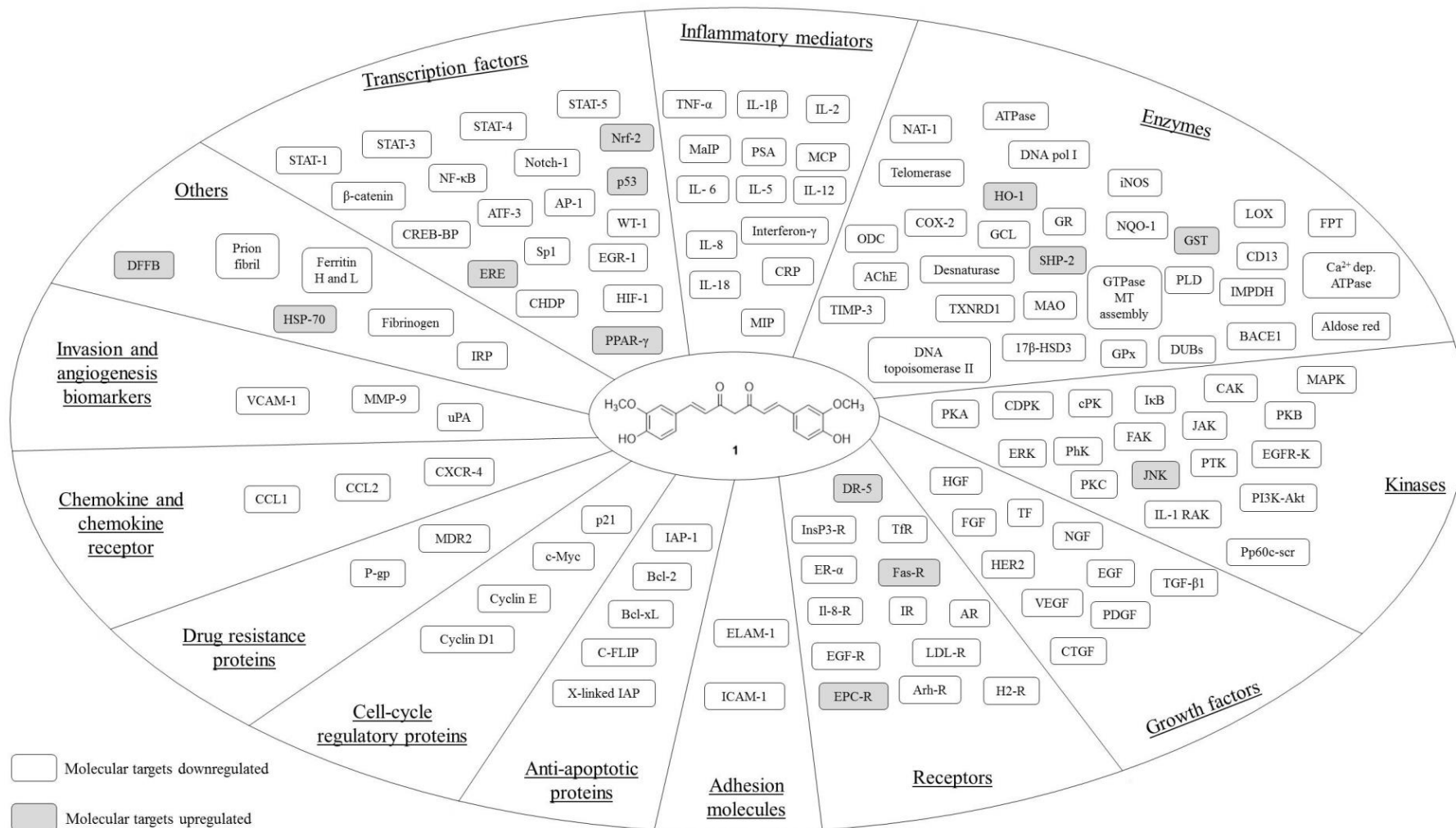


Figure 3 - Molecular targets of curcumin (**1**). Transcription factors include: signal transducers and activator of transcription-1 (STAT-1), STAT-3, STAT-4, STAT-5, β -catenin, nuclear factor-kappa B (NF- κ B), Notch-1, nuclear factor 2-related factor (Nrf-2), cAMP-response element-binding protein (CREB-BP),

activating transcription factor-3 (ATF-3), activation protein-1 (AP-1), p53, electrophile response element (ERE), specificity protein-1 (Sp1), Wilm's tumor gene (WT-1), early growth response gene-1 (EGR-1), C/EBP homologous protein (CHDP), hypoxia inducible factor-1 (HIF-1) and peroxisome proliferator-activated receptor-gamma (PPAR- γ). Inflammatory mediators include: tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), IL-2, IL-5, IL-6, IL-8, IL-12, IL-18, macrophage inflammatory protein (MIP), prostate specific antigen (PSA), monocyte chemoattractant protein (MCP), interferon- γ , C-reactive protein (CRP) and migration inhibition protein (MIP). Enzymes include: ATPase, DNA polymerase I (DNA pol I), inducible nitric oxide synthase (iNOS), glutathione-S-transferase (GST), lipoxygenase (LOX), farnesyl protein transferase (FPT), arylamine *N*-acetyltransferase-1 (NAT-1), telomerase, hemeoxygenase-1 (HO-1), glutathione reductase (GR), src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2), NAD(P)H:quinone oxidoreductase (NQO-1), phospholipase D (PLD), aminopeptidase N (CD13), inosine monophosphate dehydrogenase (IMPDH), Ca²⁺ dependent ATPase (Ca²⁺ dep. ATPase), glutamyl cysteine ligase (GCL), cyclooxygenase-2 (COX-2), ornithine decarboxylase (ODC), desaturase, monoamine oxidase (MAO), GTPase (microtubule assembly) (GTPase MT assembly), ubiquitin isopeptidases (DUBs), beta-site APP-cleaving enzyme-1 (BACE1), aldose reductase (aldose red), acetylcholinesterase (AChE), thioredoxinreductase 1 (TXNRD1), 17 β -hydroxysteroid dehydrogenase type 3 (17 β -HSD3), glutathione-peroxidase (GPx), tissue inhibitor of metalloproteinase-3 (TIMP-3) and DNA topoisomerase II. Kinase include: mitogen-activated protein kinase (MAPK), protein kinase A (PKA), protein kinase B (PKB), protein kinase C (PKC), epidermal growth factor receptor-kinase (EGFR-K), phosphatidylinositol 3-kinase/Akt (PI3K-Akt), pp60c-src tyrosine kinase (pp60c-scr), autophosphorylation-activated protein kinase (CAK), Janus kinase (JAK), protein tyrosine kinase (PTK), IL-1 receptor-associated kinase (IL-1 RAK), I κ B kinase (I κ B), focal adhesion kinase (FAK), c-jun N-terminal kinase (JNK), protamine kinase (cPK), phosphorylase kinase (PhK), Ca²⁺-dependent protein kinase (CDPK) and extracellular receptor kinase (ERK). Growth factors include: transforming growth factor- β 1 (TGF- β 1), platelet derived growth factor (PDGF), connective tissue growth factor (CTGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), human epidermal growth factor receptor-2 (HER-2), tissue factor (TF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF). Receptors include: histamine 2-receptor (H2-R), aryl hydrocarbon receptor (Arh-R), endothelial protein C-receptor (EPC-R), LDL-receptor (LDL-R), EGF-receptor (EGF-R), androgen receptor (AR), integrin receptor (IR), interleukine 8-receptor (IL-8-R), Fas receptor (Fas-R), estrogen receptor-alpha (ER- α), transferrin receptor 1 (TfR), inositol 1,4,5-triphosphate receptor (InsP3-R) and death receptor-5 (DR-5). Adhesion molecules include: endothelial leukocyte adhesion molecule-1 (ELAM-1) and intracellular adhesion molecule-1 (ICAM-1). Anti-apoptotic proteins include: X-linked inhibitor of apoptosis protein (XIAP), cellular FLICE-like inhibitor protein (C-FLIP), B-cell lymphoma protein-2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xL), and inhibitory apoptosis protein-1 (IAP-1). Cell-cycle regulatory proteins include: cyclin D1, cyclin E, c-Myc and p21. Drug resistance proteins include: P-glycoprotein (P-gp) and multi-drug resistance protein-2 (MDR2). Chemokines and chemokine receptor include: chemokine ligand 1 (CCL1), chemokine

ligand 2 CCL2 and chemokine (C-X-C motif) receptor 4 (CXCR4). Invasion and angiogenesis biomarkers include: vascular cell adhesion molecule-1 (VCAM-1), matrix metalloproteinase-9 (MMP-9), and urokinase-type plasminogen activator (uPA). Others include: DNA fragmentation factor 40kD subunit (DFFB), prion fibril, ferritin H and L, heat-shock protein 70 (HSP-70), fibrinogen and iron regulatory protein (IRP).

The curcuminoids DMC (**2**) and BDMC (**3**) also revealed cardioprotective, anti-inflammatory, antitumor, antidiabetic, nematocidal and neuroprotective activities ⁵. Among curcuminoids **1-3**, BDMC (**3**) exhibited the highest tumor cell growth inhibitory effect against the ovarian cancer cells, OVCAR-3 ²¹. BDMC (**3**) was also found to be the most effective in increasing the life span of swiss albino mice bearing Ehrlich ascites ²². DMC (**2**) and BDMC (**3**), although not as effective as curcumin (**1**), increased the KB-V1 (P-gp overexpressing) cellular sensitivity to vinblastine, blocked the efflux of fluorescent P-gp substrates and inhibited verapamil-stimulated adenosine triphosphatase (ATPase) activity in KB-V1 cells ²³. In a different study, DMC (**2**) inhibited P-gp-mediated ATP hydrolysis with lower concentration than curcumin (**1**) and BDMC (**3**) ²⁴.

1.4. Curcumin metabolites

As it was previously mentioned, curcumin (**1**) has to be absorbed by the gut and enter the bloodstream in order to be orally-effective. However, when curcumin (**1**) is orally administrated it undergoes metabolic *O*-conjugation to glucuronic acid conjugate **4** or sulfate **5** and reduction to tetrahydrocurcumin (THC, **6**), octahydrocurcumin (**7**) and hexahydrocurcumin (**8**), according to *in vivo* studies performed in rats and mice and also in studies performed in suspensions of human hepatocytes (Figure 4). Reduced metabolites of curcumin (**1**) can also be subjected to glucuronidation ²⁵. The reduction of curcumin (**1**) occurs most likely via alcohol dehydrogenase, prior to conjugation ⁴.

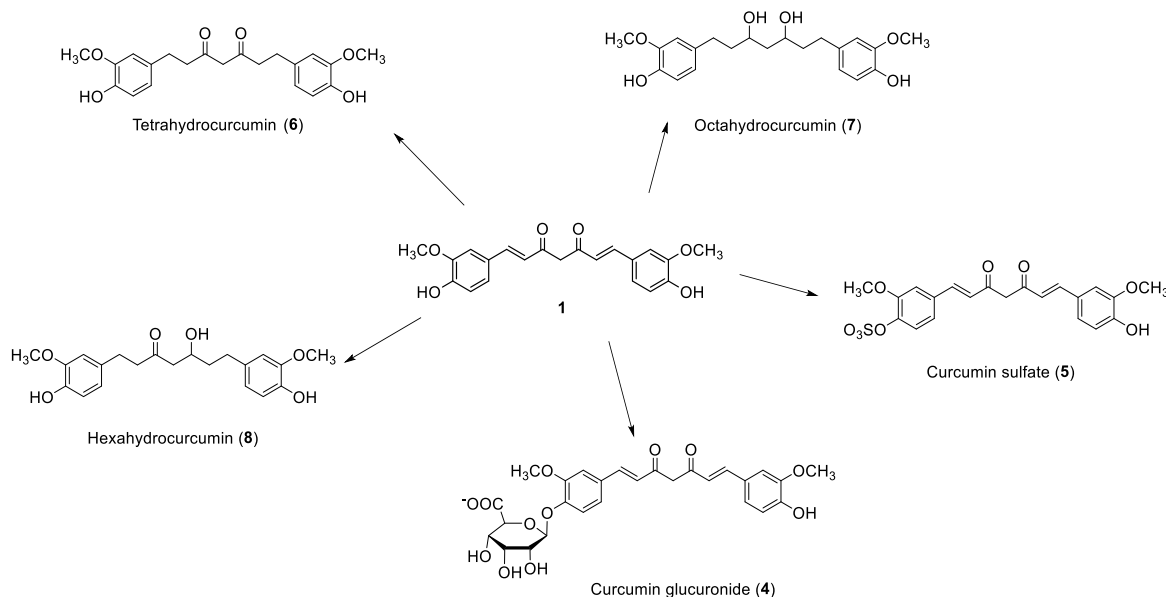


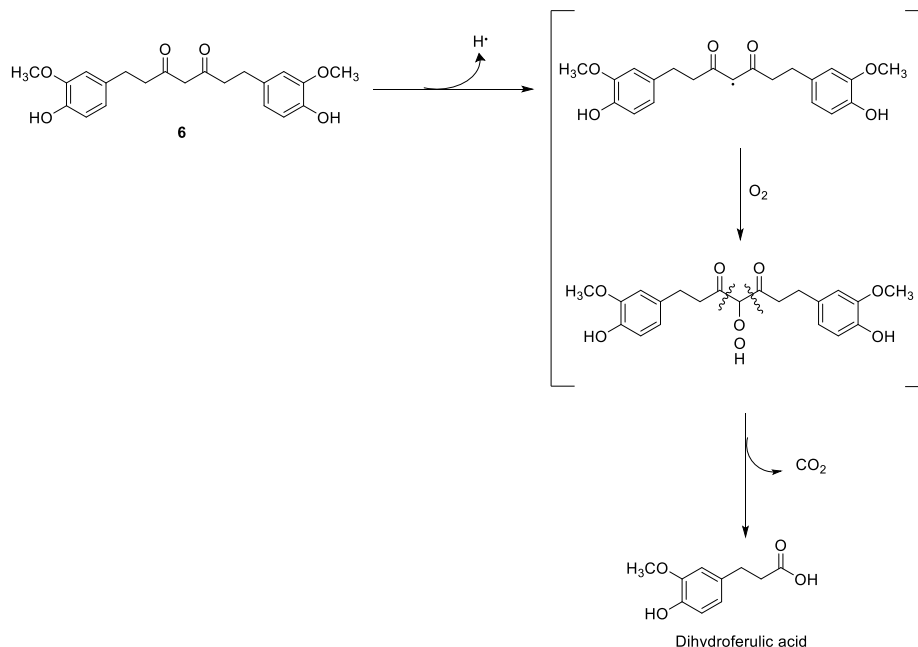
Figure 4 - Curcumin metabolites.

The curcumin (**1**) metabolism was found to be a key topic of recent research, since most studies indicate that its metabolites have biological activities. For instance, THC (**6**) revealed higher antioxidant activity than curcumin (**1**) in several assays including a linoleic acid autoxidation model, in the rabbit erythrocyte membrane ghost system and in a rat liver microsomal system ²⁶. This metabolite **6** also inhibited radiation-induced lipid peroxidation in rat liver microsomes in a concentration-dependent manner ²⁷. THC (**6**) exhibits higher antioxidant potential than curcumin (**1**) in most models and is now considered to be one of the factors responsible for the *in vivo* antioxidant activity of curcumin (**1**) ⁵. The proposed antioxidant mechanism of THC (**6**) is represented in Scheme 1 ^{26, 28}.

A study revealed that the oral administration of curcumin (**1**) and THC (**6**) could reduce aberrant crypt foci and polyps formation, although THC (**6**) showed better inhibitory effect than curcumin (**1**), in azoxymethane-induced colon carcinogenesis; both compounds, curcumin (**1**) and THC (**6**), significantly decreased azoxymethane-induced Wnt-1 and β -catenin protein expression, as well as phosphorylation of glycogen synthase kinase-3 β (GSK-3 β) in colon tissue; and reduced the protein level of connexin-43, an important molecule of gap junctions, indicating that curcumin (**1**) and THC (**6**) might affect the intracellular communication of crypt foci ²⁹.

THC (**6**) and hexahydrocurcumin (**8**) showed weaker ability to inhibit cyclooxygenase-2 (COX-2) than curcumin (**1**), in a human colon epithelial cellular models with prostaglandin E₂ production induced by phorbol ester ³⁰. Moreover, hexahydrocurcumin (**8**) as well as octahydrocurcumin (**7**) were less effective than

curcumin (**1**) in reducing the levels of inducible nitric oxide synthase (iNOS) in lipopolysaccharide-activated macrophages ³¹.



Scheme 1 - Proposed antioxidant mechanism of THC (**6**).

THC (**6**) also increases the sensitivity of HEK 293/ABCC1 cells to the multidrug resistance-associated protein 1 (MRP1) substrate etoposide ³².

2. Multidrug resistance in cancer

Cancer is one of the leading causes of death worldwide. According to the World Health Organization approximately 8.2 million deaths occurred in 2012 due to this illness. Unhealthy life style and aging are fundamental factors for the development of cancer. However, early detection and treatment can significantly increase the chances of cure ³³.

One of the causes for therapy failure is the multidrug resistance (MDR) ³⁴. MDR occurs when the disease is insensitive to treatment, and it may occur either from the begging of the treatment or be acquired during treatment ³⁵⁻³⁶. Multiple mechanisms have been identified as being responsible for MDR, and although these mechanisms have been

intensively studied, not all of them have been completely elucidated. Cancer cells can become resistant to drugs by decreasing the drug influx (when the agent enters the cell by endocytosis or associated with intracellular carriers); in cases in which drug accumulation is unchanged the cell can activate detoxifying proteins, such as cytochrome P450; cells can promote mechanisms that repair drug induced DNA damage; disrupt apoptotic signaling pathways, thus, becoming resistant to drug-induced cell death; increase the activity and/or overexpression of efflux pumps, such as the ATP-binding cassette (ABC) transporters (Figure 5)³⁷⁻³⁸.

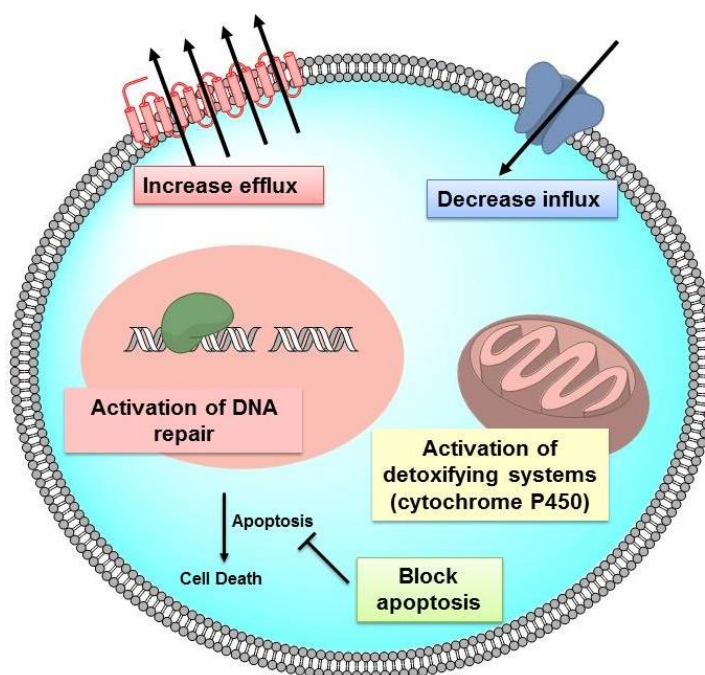


Figure 5 - Cellular factors responsible for drug resistance.

The overexpression of ABC transporters is currently one of the most studied processes of MDR. ABC transporters are active transporter proteins that use the energy derived from the hydrolysis of ATP to ADP to transport their substrates across the cell membrane, maintaining cellular homeostasis and detoxifying the cell from potentially toxic substances³⁹. Currently, 49 members of the ABC transporter family have been isolated and identified⁴⁰. Three pumps that are commonly found to confer chemoresistance in cancer are P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and human breast cancer resistance protein (BCRP)⁴¹. Thus, these transporters have been the target of several studies to identify novel compounds to counteract MDR.

P-gp (encoded by *MDR1* gene) consists of two homologous halves, each one with six hydrophobic transmembrane α -helices (which form the pathway through which the compounds cross the membrane) and one nucleotide binding domains located on the

cytoplasmic side of the membrane (which binds and hydrolyses ATP) ³⁹. P-gp is highly expressed in apical surface of epithelial cells of the intestine, liver bile ductules and kidney proximal tubules, pancreatic ductules, adrenal gland, placenta, blood-testis barrier, and blood-brain barrier ³⁹.

MRP1 transporter (encoded by *ABCC1* gene) is normally present on the basolateral surface of the epithelial membrane⁴¹. This transporter consists of seventeen transmembrane segments and two nucleotide binding domains located on the cytoplasmic side of the membrane³⁷. MRP1 transporter is a mediator of MDR in breast, lung, and prostate cancer ⁴².

BCRP (encoded by *ABCG2* gene) is highly expressed in the apical surface of small intestines, colon epithelium, liver canalicular membrane, luminal surfaces of microvessel endothelium of human brain and in the veins and capillaries of blood vessels ⁴¹. BCRP transporter has only six transmembrane domains and one nucleotide binding domain on the cytoplasmic side of the membrane ³⁷. BCRP is responsible for MDR in breast, colon, small cell lung, ovarian, gastric and intestinal cancers ⁴¹.

Altogether, the tissue location of these transporters corroborates with the physiological role they play in protecting the susceptible organs from toxic xenobiotics.

Many anticancer drugs are substrates of MDR transporters, conferring the cancer cells resistance to such treatments. Figure 6 depicts several anticancer drug substrates of P-gp, MRP1 and BCRP transporters. The promiscuity of these proteins is evident, since they are able to transport structurally and mechanistically unrelated classes of anticancer drugs ⁴³.

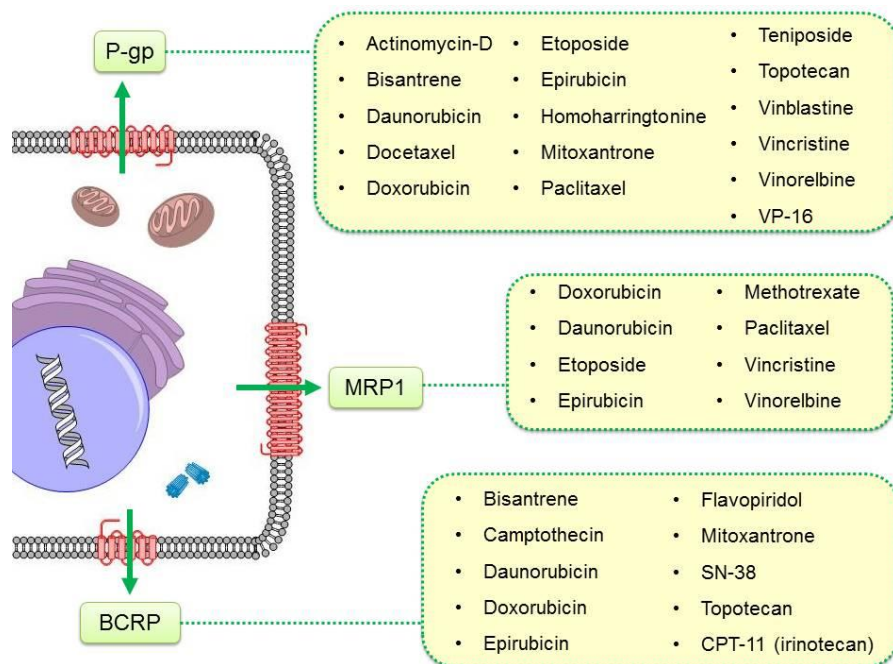


Figure 6 –P-gp, MRP1, and BCRP located on the cell surface and respective anticancer drug substrates.

2.1. MDR transporters inhibitors

One way to overcome resistance to anticancer drugs is the administration of ABC transporters inhibitors that are not toxic themselves and are able to reverse resistance against anticancer drugs. P-gp inhibition has been extensively studied and may occur through: i) direct interaction with one or multiple drug-binding sites on P-gp; ii) inhibition of the binding of ATP to its binding site on P-gp; iii) interaction with an allosteric residue important for P-gp activity; iv) or even an interaction with the lipid membrane altering the drug-membrane interaction⁴⁴⁻⁴⁶ (Figure 7). Many compounds have been identified as MDR modulators over the years.

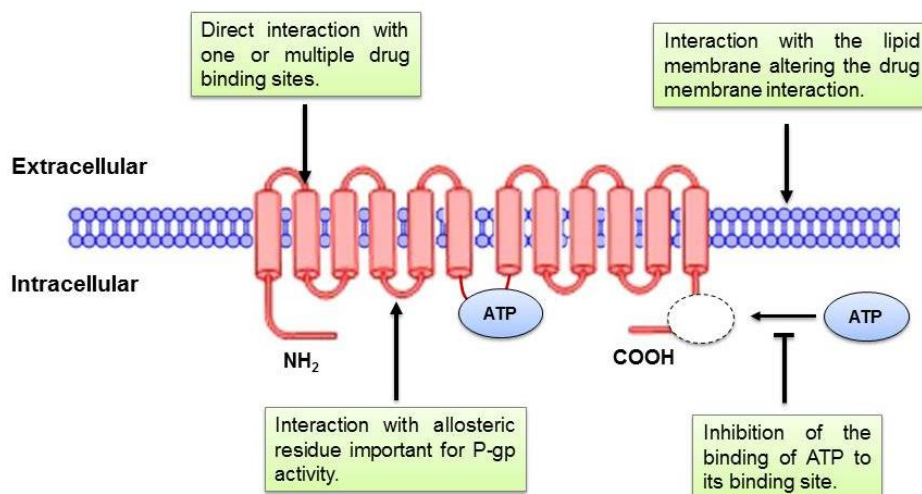


Figure 7 – Different mechanisms for P-gp inhibition.

The first-generation of MDR inhibitors was not specifically developed for this purpose, but had other pharmacological activities; also, these drugs exhibited low affinity for ABC transporters and higher doses were needed. Higher doses were associated with high toxicity levels, limiting their application. Many agents can be included in this first-generation of MDR inhibitors: calcium channel blockers, such as verapamil; calmodulin antagonists; steroidal agents; protein kinase C inhibitors; immunosuppressive drugs, such as cyclosporine A; antibiotics, like erythromycin; antimalarials, such as quinine; psychotropic phenothiazines and indole alkaloids; steroid hormones and anti-steroids (e.g. progesterone and tamoxifen); detergents and surfactants^{43, 47}.

Clinical trials with first-generation MDR modulators often failed due to side effects or the fact that these compounds were also substrates for ABC transporters, competing with the cytotoxic drug for efflux, and thus requiring high serum concentrations⁴⁷.

Second-generation MDR modulators were designed in an attempt to reduce the side effects of the first-generation MDR drugs. These compounds showed better pharmacological profile than the first generation but they failed in clinical trials. Their affinity to ABC transporters was lower than expected at tolerable doses; co-administration of MDR chemosensitizers with anticancer drugs often interfered with clearance or inhibited their metabolism and excretion, leading to high plasma concentration of the anticancer drug, and consequently toxicity; the competition between anticancer drugs and MDR inhibitor for cytochrome P450 3A4 activity resulted in unpredictable pharmacokinetic interactions⁴⁷. *R*- Verapamil and dextniguldipine are examples of this second-generation MDR modulators⁴³.

The third-generation compounds were designed to circumvent the problems associated with first and second-generation MDR inhibitors, based on quantitative

structure-activity relationships (QSAR). These inhibitors are suggested to be more potent and more specific than their precursors. Particularly, third generation chemosensitizers do not affect cytochrome P450 3A4 at relevant concentrations; they do not alter the pharmacokinetics of the co-administrated anticancer drug, compared to the previous generations extension, and so, do not require chemotherapy dose reduction ³⁵. Some examples of such compounds are: elacridar (GF120918), laniquidar (R101933) zosuquidar (LY335979) and tariquidar (XR9576) ⁴¹. However, these inhibitors exhibited toxic effects in clinical trials ³⁵.

Although most of the compounds did not reach the purpose for which they were develop these three generations of MDR inhibitors helped to set ground knowledge for the development of novel inhibitors. In the last decade several studies have reported natural compounds (such curcumin, **1**), marine compounds, tyrosine kinase inhibitors and phosphodiesterase-5 inhibitors as inhibitors of ABC transporters, which are mechanistically and structurally different from the three generations of inhibitors ⁴¹. The dual ligands approach has also been considered in order to develop molecules with dual antitumor and anti-Pgp activity. Indeed, by merging a scaffold with antitumor activity with a P-gp inhibitor scaffold, novel P-gp inhibitors with concomitant antitumor activity have been recently developed in the group ⁴⁸.

New strategies are being investigated in order to overcome MDR without the use of small molecules as pharmacological inhibitors. Downregulation of MDR transporters by antisense oligonucleotides is one of the strategies being studied currently. Target physiological mechanisms involved in regulation the of MDR proteins is another possibility. For example, *MDR1* and cytochrome P450 3A4 gene expression are stimulated by certain anticancer drugs by activation of nuclear steroid and xenobiotic receptor (SXR) leading to drug resistance. The development of SXR antagonists that would be co-administrated with anticancer drugs could counteract the induction of the mentioned genes. MDR phenotype is sometimes associated with upregulation of glucosylceramide, since glucosylceramide synthase contributes to attenuate drug-induced formation of apoptotic ceramide. Hence antisense suppression of glucosylceramide synthase could significantly improve anticancer drugs pharmacological action ³⁸. Anticancer drug delivery using particles and polymers could represent an interesting and elegant way to overcome MDR, this strategy offers a great variety of advantages: enables tissue specific targeting; “optical” agents for imaging and tracking; multiple payloads (internalization of several drugs or agents that cause tumor cell death).

It is important to understand that multidrug resistance is a double-edged sword. On one hand cancer cells need to lose their drug resistance for chemotherapy to be effective. On the other hand, chemotherapy-sensitive non-cancerous cells need to be protected

from the effects of chemotherapeutic agents. Besides that, MDR modulation faces many other challenges. For example: the existence of other unknown multidrug resistance-inducing molecules in cancer; the inhibition of MDR transporters could impair the normal function of certain organs where these MDR transporters are present and play an essential role in the body's defense mechanism (e.g. liver, kidneys, gastrointestinal gland, adrenal gland, and blood-brain barrier). Hence, MDR inhibitors should target MDR transporters in a way that is tissue and/or cancer specific ³⁴. A recently discovered challenge is the cells ability to release microvesicles, more specifically, microparticles that could carry various bioactive molecules such as proteins, like P-gp and MRP1. Thus, microparticles can confer and transfer MDR in cancer cells by intercellular transfer of functional resistance proteins, leading non-resistant cancer cells to become resistant⁴⁹.

Concluding, MDR in cancer is a complex process that needs to be overcome, having in mind the biological importance of MDR transporters.

3. Curcumin as a multidrug resistance modulator

Curcumin (**1**), and some other curcuminoids, have been shown to down-regulate the *MDR1* gene expression and decrease P-gp function ^{23, 50-51}.

A study of the modulation of P-gp function in a MDR human cervical carcinoma cell line, KB-V1, showed very promising results ²³. Indeed, the half maximal inhibitory concentration (IC₅₀) values for curcumin (**1**) were similar in the KB-V1 cells (from a drug resistant cell line, expressing high levels of P-gp) and in the KB-3-1 cells (from the parental drug-sensitive cell line).

These results indicate that curcumin (**1**) is not likely to be transported by P-gp. In addition, curcumin (**1**) increased the KB-V1 cellular sensitivity to vinblastine, an anticancer drug. It also blocked the efflux of fluorescent P-gp substrates and inhibited verapamil-stimulated Pgp-ATPase activity in KB-V1 cells. These results may suggest that the binding site of curcumin (**1**) on P-gp overlaps with that verapamil. Therefore, binding of curcumin (**1**) could affect the binding of other drug substrates by changing the conformation of P-gp ²³.

One of the undertaken studies tested the ability of curcumin (**1**) to revert MDR in a P-gp overexpressing human osteosarcoma cell line (MNNG/HOS/MTX) and also in a xenograft model of those cells in nude-mice ⁵¹. Curcumin (**1**) proved to be effective in down-regulating the expression of P-gp and also inhibiting the function of P-gp *in vitro* and

in vivo. To investigate these effects *in vitro*, several assays were performed in cells in culture such as determinations of alterations in the IC₅₀ of antitumor drugs, resistance index (RI), P-gp expression and intracellular concentration of a fluorescent P-gp substrate. In addition, real time-polymerase chain reaction (real time-PCR) and Western blot assays showed that curcumin (**1**) reversed MDR of MNNG/HOS/MTX xenografted cells in nude mice, by down-regulating the expression of P-gp and therefore improving the sensitivity of osteosarcoma MDR cells to the effect of anticancer drugs (regarding inhibition of the xenografted cells proliferation and metastasis)⁵¹.

The effect of curcumin (**1**) on the MDR of the human gastric carcinoma cell line SGC7901/VCR (which is resistant to vincristine) has also been tested⁵⁰. Curcumin (**1**) has been shown to decrease the IC₅₀ values of vincristine and to promote vincristine-mediated apoptosis in a dose dependent manner. Additionally, curcumin (**1**) increased the intracellular concentrations of a fluorescent P-gp substrate, but did not change the accumulation and efflux of such substrates in the SGC7901 drug-sensitive cells. Furthermore, when the resistant cell line was treated with vincristine in combination with curcumin (**1**), cells showed an increase in the activation of caspase-3 (indicative of apoptosis), when compared to the cells treated with vincristine alone⁵⁰.

Another study tested the effect of sulfinosine and curcumin (**1**) combined administration in a non-small cell lung carcinoma cell line overexpressing P-gp (NCI-H460/R, a drug-resistant cell line). The drugs exerted a synergistic tumor cell growth inhibitory effect and the expression of MDR-related genes (such as *mdr1*, *gst-π* and *topo IIα*) was decreased. Therefore, sulfinosine and curcumin (**1**) overcame MDR in the referred cell line in spite of the presence of a mutated *p53* gene⁵².

Co-delivery of curcumin (**1**) and doxorubicin by lipid nanoparticles revealed a significant liver damage decrease in mice with diethylnitrosamine-induced hepatocellular carcinoma. Compared with doxorubicin loaded nanoparticles, doxorubicin/curcumin (**1**) nanoparticles decreased mRNA levels of *MDR1* and protein levels of P-gp, indicating that curcumin (**1**) might reverse MDR. Increased cytotoxicity and decreased IC₅₀ and resistance index (RI) also corroborate the hypothesis of synergistic effects of doxorubicin with curcumin (**1**)⁵³.

Co-administration of curcumin (**1**) with gramidicin or ouabain selectively killed BCRP expressing-cells, also indicating that the synergistic effect of these compounds could be beneficial for MDR overcome⁵⁴.

An *in silico* study has already been made, with molecular modeling and docking simulations, in order to study the inhibitory binding mode of curcumin (**1**)⁵⁵. Molecular docking studies inferred the binding of curcumin (**1**) into the substrate binding site of P-gp, with a binding energy of -7.66 kcal/mol, by establishing bonded and non-bonded

interactions with Leu32, Met35, Met36, Phe39, Phe161, Leu299, Thr300, Phe303, Ile307, Ser311, Met916 and Phe945 located in the membrane region (aminoacid residues which were proven to have drug interactions in this protein) ⁵⁵. In spite of all of these findings, the exact molecular mechanism by which curcumin (**1**) exerts its effects on MDR is yet to be elucidated.

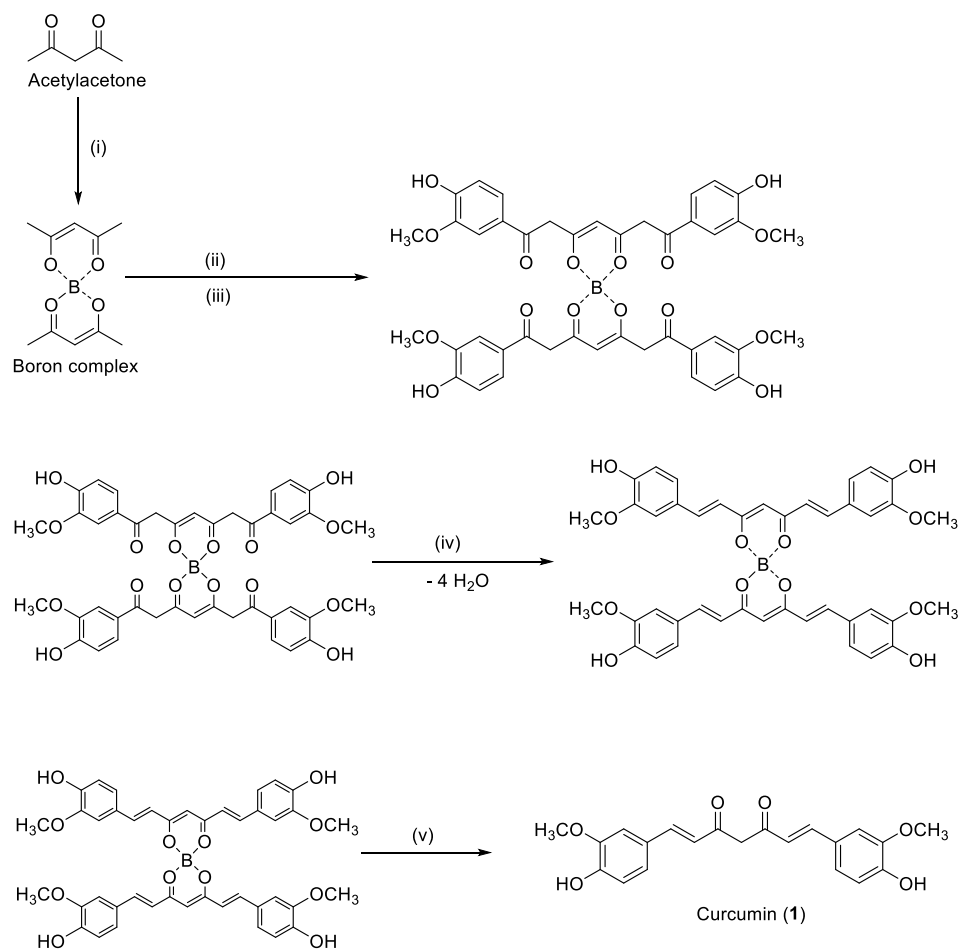
4. Synthesis and biological activities of curcumin derivatives

4.1. Curcumin synthesis

Curcumin (**1**) was isolated from turmeric in 1815 ⁵⁶, obtained in crystalline form in 1870, but it was not until 1910 that Lampe and Milobedzka elucidated the structure of curcumin (**1**), which was later confirmed by synthesis ^{4, 57-58}. The method of synthesis of curcumin (**1**) is depicted in Scheme 2 ⁵⁹. Later, Srinivasan separated and quantified the curcuminoids by chromatography ⁶⁰.

The synthesis of curcumin (**1**) is achieved by mixing acetylacetone (1 eq.) with vanillin (2 eq.). Before the addition of vanillin, boric anhydride is mixed with acetylacetone. Boric anhydride avoids Knoevenagel condensation of the reactive methylene group (C-3), by allowing the lateral methyl groups of acetylacetone to react with the aldehyde group of vanillin. The complex formed then reacts with vanillin. The solvent of the reaction is ethyl acetate (EtOAc). The *n*-butylamine, C₄H₉NH₂, is a proton extracting reagent which activates the methyl group in acetylacetone. Tributyl borate, [CH₃(CH₂)₃O]₃B, is used to absorb the water produced during the reaction. The acid, HCl, decomposes the boron complex leading to the release of the final product, curcumin (**1**) ⁵⁹.

The syntheses of the curcuminoids, DMC (**2**) and BDMC (**3**), are similar to the synthesis of curcumin (**1**). The main difference lies on the precursor aldehydes that should be used – one molar equivalent of 4-hydrobenzaldehyde and one molar equivalent of vanillin to synthesize DMC (**2**), and two molar equivalents of 4-hydrobenzaldehyde to synthesize BDMC (**3**).



Scheme 2 - Synthesis of curcumin (**1**). Reaction conditions (i): B₂O₃, EtOAc, 40°C; (ii): *n*-butylamine, 15 min; (iii): vanillin, 40°C, 24 h; (iv): tributyl borate; (v): HCl.

4.2. Synthetic derivatives of curcumin

Several curcumin (**1**) derivatives have been synthesized since the interest for this natural product and its biological activities were disclosed. The generalized representation of the pharmacophoric main structural regions of curcumin (**1**) is shown in Figure 8. The molecular structure of curcumin (**1**) is divided into three main regions: two substituted aromatic rings (A and C) linked together by a conjugated diketone (B) ⁶¹. The produced derivatives exhibit molecular modifications in one or more of these regions.

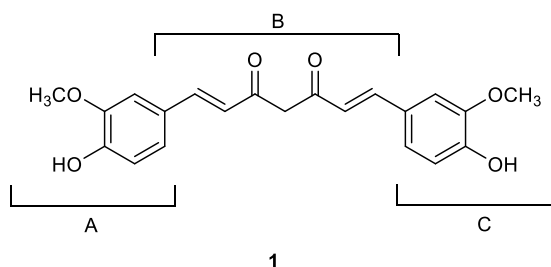


Figure 8 - Main structural moieties of curcumin (**1**).

In some cases the synthetic strategy was different from the one depicted in Scheme 2. A very common synthesis method is the Claisen-Schmidt condensation (described in the next section). The majority of the analogues were obtained by total synthesis, although some analogues were obtained from curcumin (**1**), by molecular modifications under conditions which do not cause its degradation such as acidic medium.

The syntheses of the curcuminoids **1-3** and of some other derivatives, such as carbocyclic curcumin analogues and heteroaryl hydrazinocurcumins, can be microwave assisted⁶²⁻⁶³. Microwave assisted organic reaction in such cases showed to have higher yields than conventional reactions, and decreased significantly the time of reaction⁶²⁻⁶³.

Some curcumin derivatives synthesis and respective biological activities are presented in the next section. The presented derivatives are some of the curcumin derivatives that have already been described as tumor cell growth inhibitors and multidrug resistance modulators. A more intensive study on curcumin derivatives and respective biological activities was submitted in the format of a revision manuscript.

The curcumin derivatives were divided according to their B structural region.

4.2.1. Derivatives with a β -dicetone moiety

The synthesis and biological evaluation of a compound designated as GO-Y025 (**9**) (Figure 9) was performed by a research group, which provided evidence that the methyl modification of the *p*-hydroxyl group relative to the α,β -unsaturated ketone moiety leads to considerable enhancement in the growth-suppressive activity in a colon cancer cell line (DLD-1), in comparison to curcumin (**1**)⁶⁴. The synthesis of compound **9** is similar to the curcumin (**1**) synthesis, by using an appropriate aldehyde.

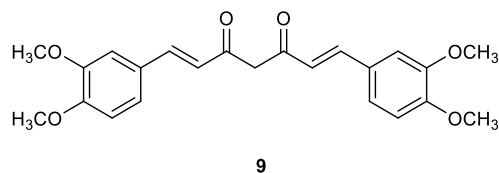
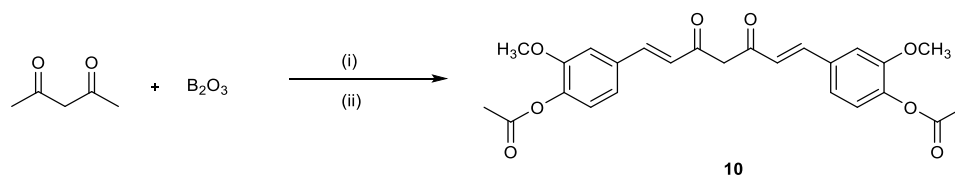


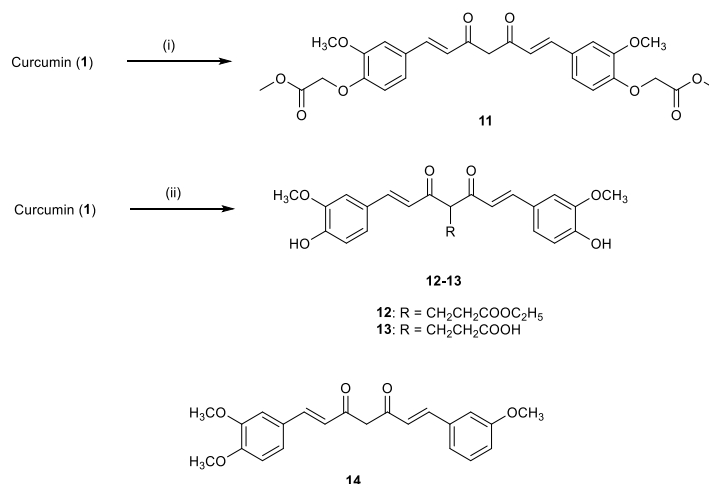
Figure 9 - Structure of GO-Y025 (1,7-bis(3,4-dimethoxyphenyl)-1,6-heptadiene-3,5-dione, **9**).

Diacetylcurcumin (**10**) was obtained, and showed to be more stable than curcumin (**1**) in physiological medium ⁶⁵. Diacetylcurcumin (**10**) was able to impair spindles formation and induced a p53- and p21^{CIP1/WAF1}-independent mitotic arrest, in a human colon cancer cell line. Then, a p53/p21^{CIP1/WAF1}-dependent inhibition of G1 to S transition was triggered by diacetylcurcumin (**10**) as a consequence of a mitotic slippage, avoiding post-mitotic cells from re-entering the cell cycle ⁶⁵. The synthesis and structure of derivative **10** is shown in Scheme 3.



Scheme 3 - Synthesis protocol of diacetylcurcumin (4-[(1E,6E)-7-(4-acetoxy-3-methoxyphenyl)-3,5-dioxohepta-1,6-dienyl]-2-methoxyphenyl] acetate, **12**). Reaction conditions (i): dimethylformamide, 30 min, 80°C; (ii): tributylborate, 30 min, 80°C and then addition of *n*-butylamine and 3-acetylbenzaldehyde, 4 h.

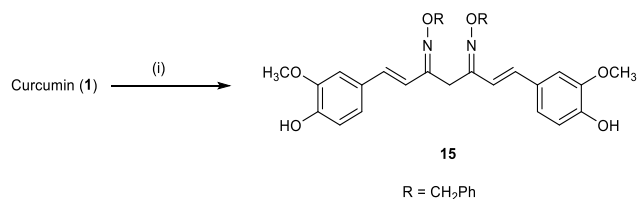
A series of curcumin analogues was synthesized and evaluated as androgen receptor antagonists against two human prostate cancer cell lines, PC-3 and DU-145. Analogues **9** and **11-14** showed higher antiandrogen activity than hydroxyflutamide, which is the currently available drug for the treatment of this type of cancer. SAR studies indicated that the co-planarity of the β -diketone moiety and the presence of a strong hydrogen bond donor group are crucial features for the antiandrogenic activity ⁶⁶⁻⁶⁷. The synthesis of compound **14** is similar to that of curcumin (**1**) (Scheme 2), consisting in using appropriate aldehydes to achieve the products of interest; synthesis of compounds **11-13** is shown in Scheme 4.



Scheme 4 - Structure of analogue **14** and synthesis of analogues **11-13**. Reaction conditions (i): acetone, methyl chloroacetate, sodium iodide, reflux for 24h; (ii): acetone, RI (R= **12**: C₂H₅COOCH₂CH₂I; **13**: COOCH₂CH₂I), K₂CO₃, reflux for 48h. **11**: dimethyl 2,2'-(((1*E*,6*E*)-3,5-dioxohepta-1,6-diene-1,7-diyl)bis(2-methoxy-4,1-phenylene))bis(oxy))diacetate; **12**: ethyl (*E*)-7-(4-hydroxy-3-methoxyphenyl)-4-((*E*)-3-(4-hydroxy-3-methoxyphenyl)acryloyl)-5-oxohept-6-enoate; **13**: (*E*)-7-(4-hydroxy-3-methoxyphenyl)-4-((*E*)-3-(4-hydroxy-3-methoxyphenyl)acryloyl)-5-oxohept-6-enoic acid; **14**: (1*E*,6*E*)-1-(3,4-dimethoxyphenyl)-7-(3-methoxyphenyl)hepta-1,6-diene-3,5-dione.

4.2.2. Derivatives with a β-dicetone modified moiety

Some curcumin derivatives were synthesized using the concept of bioisoterism⁶⁸. The ability of such derivatives to inhibit cell growth and induce apoptosis was analyzed by *in vitro* assays in the hepatocellular carcinoma HA22T/VGH cell line, breast cancer MCF-7 cell line, and in its MDR variant MCF-7R cell line. The dioxime derivative **15** showed higher potency, about twice that of curcumin (**1**), both in the MCF-7 and MCF-7R cells. Indeed, derivative **15** also showed marked pro-apoptotic activity, matching its ability to inhibit nuclear factor-kappa B (NF-κB) activation as well as the expression of anti-apoptotic factors (such as survivin, XIAP, Bcl-2 and Bcl-xL)⁶⁸. Synthesis of this compound is shown in Scheme 5.

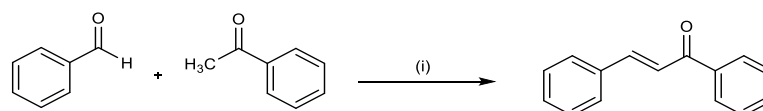


Scheme 5 - Synthesis of compound **15** ((1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione *O,O*-diphenyl dioxime). Reaction conditions (i): *O*-benzyl-hydroxylamine hydrochloride in 25% K₂CO₃:H₂O/EtOH, reflux for 25 min.

4.2.3. Derivatives with an enone moiety

The strategy followed for this type of derivatives was to eliminate the β -diketone moiety present in curcumin (**1**), and thus, to reduce the instability associated with the methylene group.

In a series of studies, some molecular modifications of curcumin (**1**) were performed and the antiangiogenic activity was tested in the resulting derivatives. The general method of synthesis followed the Claisen-Schmidt condensation is represented in Scheme 6 and some of the compounds are represented in Figure 10. The biological efficacy of the compounds as *in vitro* inhibitors of endothelial cell proliferation was evaluated in SVR cells, an immortalized endothelial cell line ⁶¹. Out of the numerous compounds synthesized, compounds **16-20** showed the best antiangiogenic effects (SVR growth inhibition in the order of 92-98% at 3 μ g/mL concentration and 94-98% at 6 μ g/mL concentration). Some tetralone analogues were also synthesized, and the one with the best antiangiogenic activity was derivative **21**, although the values observed for this compound were lower than for compounds **16-20**. Curcumin (**1**) was also tested, for a comparative purpose, and presented a considerable endothelial cell growth inhibition of approximately 37.8% at 3 μ g/mL, and 56.2% at 6 μ g/mL). In order to verify the requirement for the enone moiety for achieving antiangiogenic activity, compound **22** was also synthesized and evaluated. Its relatively low level of activity (SVR growth inhibition of approximately 11.5% at 6 μ g/mL) led to the suggestion that the enone moiety is in some way important for the potency of compounds ^{61, 69}.



Scheme 6 - Generalized synthetic scheme for the preparation of curcumin derivatives with enone moieties. Reaction conditions (i): KOH, ethanol (EtOH), 5-10°C, and 4 h.

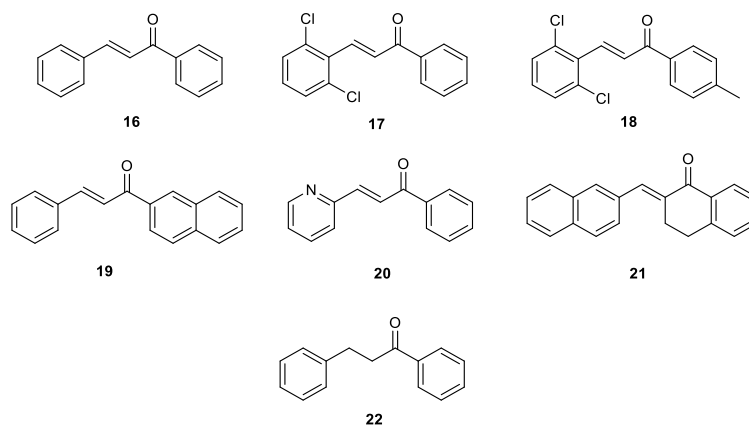
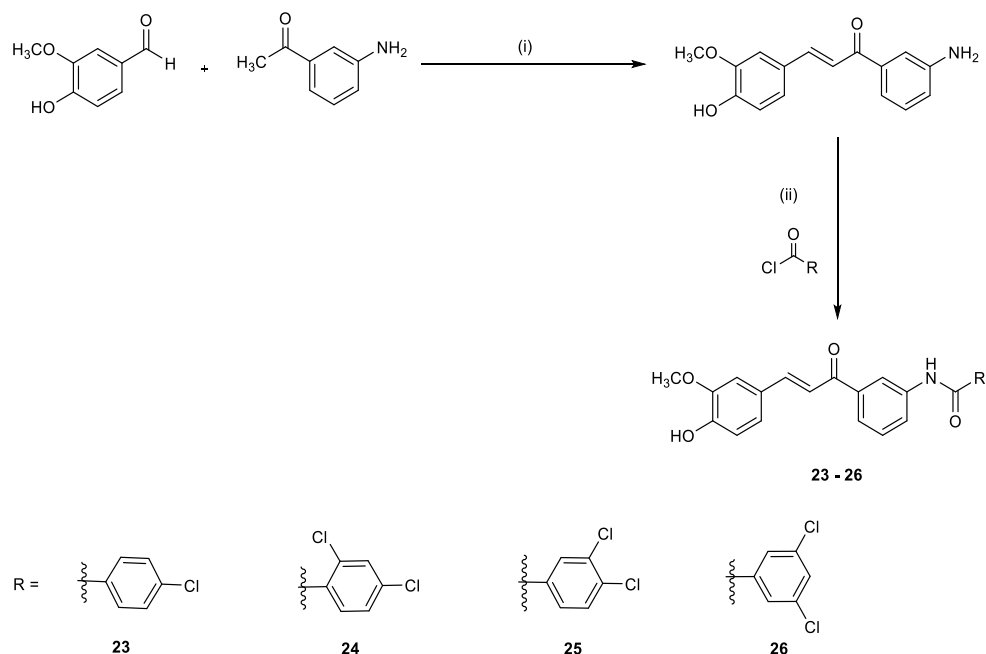


Figure 10 - Derivatives **16-22**. **16**: chalcone; **17**: 2,6-dichlorochalcone; **18**: 2,6-dichloro-4'-methylchalcone; **19**: 2-naphthalene-3-phenylprop-2-en-1-one; **20**: 1-phenyl-3-(pyridin-2-yl)prop-2-en-1-one; **21**: 2-(naphthalene-2-ylmethylene)-tetralone; **22**: 1,3-diphenyl-1-propanone.

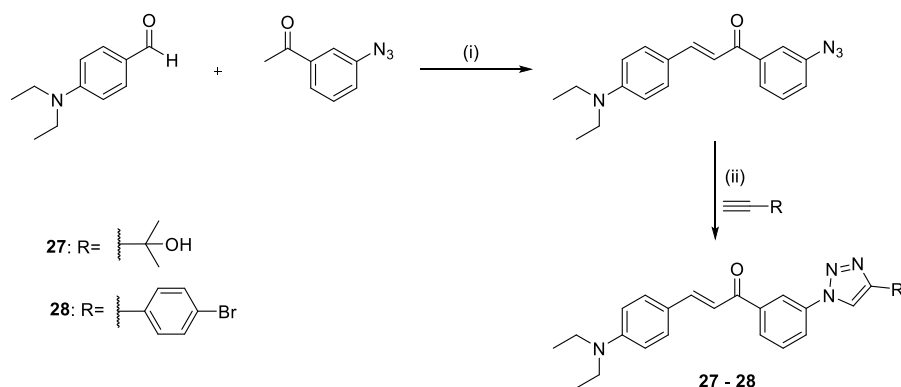
Interestingly, compounds with a chlorobenzamide moiety were found to exhibit MDR reversal activity, by inhibiting the drug efflux function of P-gp; based on these considerations, other molecular modifications of curcumin (**1**) were attempted⁷⁰. The derivatives **23-26**, synthesized according to Scheme 7, did not present cytotoxicity either against KB cells (not-expressing P-gp) or KBV20C cells (expressing P-gp). Nevertheless, they significantly improved the cytotoxicity of vincristine and paclitaxel, in KBV20C cells. According to the obtained results, chlorobenzamides **23-26** were shown to have the strongest MDR reversal activity. A structure-activity relationship could be established: apparently one chlorine group at the *meta*- or *para*- position on benzamide increases the MDR reversal activity; indeed, a bulkier group at the *meta*- or *para*- position on benzamide may be the reason why these compounds presented promising results. To confirm that the MDR reversal activity of these curcumin analogues **23-26** was due to the inhibition of the drug efflux function of P-gp, the effect of compounds **23-26** on intracellular accumulation of rhodamine 123, a fluorescent P-gp substrate, was examined. Indeed, treatment with these compounds was enough to inhibit P-gp function and increase the intracellular concentration of rhodamine 123. The authors proposed that the derivatives **23-26** were interesting starting points for the synthesis of new compounds capable of

causing the intracellular accumulation of anticancer drugs (thus enabling them to exert their cytotoxic effects)⁷⁰.



Scheme 7 - Synthesis of compounds **23-26**. Reaction conditions (i): KOH, EtOH, 40°C, r.t., 10 h; (ii): Dioxane/water (1:1), the appropriate benzoyl chloride, 0°C, 5-7 h. **23** (4-chloro-*N*-(3-(3-(4-hydroxy-3-methoxyphenyl)-acryloyl)-phenyl)-benzamide); **24** (2,4-dichloro-*N*-(3-(3-(4-hydroxy-3-methoxyphenyl)-acryloyl)-phenyl)-benzamide); **25** (3,4-dichloro-*N*-(3-(3-(4-hydroxy-3-methoxyphenyl)-acryloyl)-phenyl)-benzamide); **26** (3,5-dichloro-*N*-(3-(3-(4-hydroxy-3-methoxyphenyl)-acryloyl)-phenyl)-benzamide).

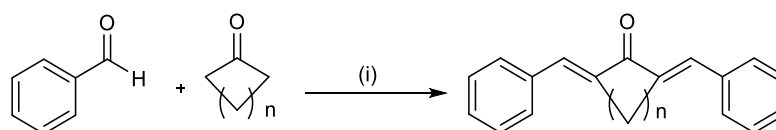
An interesting set of derivatives with 2-ethylamino groups in a chalcone structure were synthesized (**27-28**), with the aim of stimulating the cytotoxicity of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) using TRAIL-resistant human CRT-MG astroglioma cells⁷¹. The synthetic strategy followed was the Huisgen 1,3-cycloaddition between several alkynes and an intermediate. The two compounds depicted in Scheme 8 showed the most promising results as TRAIL sensitizers. Glioblastoma multiforme is a human malignant brain tumor known to be very aggressive and has a high mortality rate. Since co-treatment of glioblastoma multiform cells with a chemo-sensitizer and TRAIL is more efficient than TRAIL alone, these two compounds have the potential to be used in combination therapy for brain tumors⁷¹⁻⁷².



Scheme 8 - Synthesis of compounds **27-28**. Reaction conditions (i): 40% KOH, EtOH, r.t., 10 h; (ii) the correspondent alkynes, sodium ascorbate (2.5 eq.), CuSO₄ (1 eq.), chloroform/EtOH/H₂O (5:3:1), r.t., 5 h. **27** ((*E*)-3-(4-(diethylamino)phenyl)-1-(3-(4-(2-hydroxypropan-2-yl)-1*H*-1,2,3-triazol-1-yl)phenyl)prop-2-en-1-one) and **28** ((*E*)-1-(3-(4-(4-bromophenyl)-1*H*-1,2,3-triazol-1-yl)phenyl)-3-(4-(diethylamino)phenyl)prop-2-en-1-one).

4.2.4. Derivatives with a dienone moiety

The strategy followed to obtain this type of derivatives was also the elimination of the β -diketone moiety in order to reduce curcumin (**1**) instability. The compounds in this category were synthesized by Claisen-Schmidt condensation, according to Scheme 9, which represents one of the most popular methods to obtain curcumin analogues.



Scheme 9 - Generalized synthetic scheme for the preparation of curcumin derivatives with a dienone moiety. The ketone could be a cyclohexanone ($n=3$), a cyclopentanone ($n=2$), or acetone ($n=0$). Reaction conditions (i): KOH, EtOH, 5°C, 10 h.

Compounds **29-32** were obtained according to Scheme 9 and their efficacy as *in vitro* inhibitors of endothelial cell proliferation was evaluated with the SVR cells, an immortalized endothelial cell line. Compounds **29-31** presented interesting results (SVR growth inhibition in the order of 90-94% at 3 μ g/mL concentration and 96-98% at 6 μ g/mL

concentration). Additionally, derivative **32** with a simpler structure was produced, which showed similar results to compounds **29-31** (SVR growth inhibition of 96.6% at 3 $\mu\text{g/mL}$ concentration and 97.7% at 6 $\mu\text{g/mL}$ concentration). Derivatives **29-32** structures are represented in Figure 11. In another study, derivative **31** also showed antioxidant activity

⁷³.

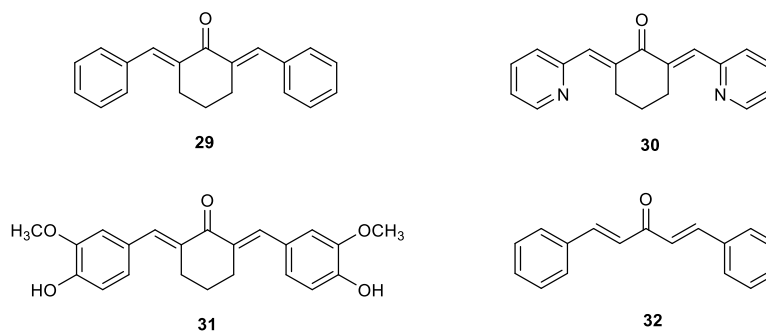


Figure 11 - Derivatives **29-32**. **29**: 2,6-di-benzylidenecyclohexan-1-one; **30**: 2,6-bis(pyridin-2-ylmethylene)cyclohexan-1-one; **31**: 2,6-bis(4-hydroxy-3-methoxybenzylidene)cyclohexan-1-one; **32**: 1,5-diphenylpenta-1,4-dien-3-one.

A dienone derivative designated GO-035 (**33**) was found to have a stronger effect than curcumin (**1**) in suppressing the growth of the cancer cell line DLD-1 ⁶⁴. The growth-suppressive activity of this compound was tested in other cancer cell lines, such as the ones derived from tumors of the stomach (GCIY, SH10TC), lung (LK87), breast (MCF7), ovary (OVK18), prostate (PC3), pancreas (PK9), bile duct (HuCCT1), thyroid gland (8505c), skin (A431), kidney (ACHN), liver (HepG2) and melanoma (G361). Curcumin (**1**) and its derivative **33** presented growth-suppressive activity in all types of the cancer cell lines tested, with IC_{50} values ranging from 4.0 to 9.0 $\mu\text{mol/L}$, and from 0.6 to 7.0 $\mu\text{mol/L}$, respectively. Compound **33** also showed a stronger potential than curcumin (**1**) for causing downregulation of some oncoproteins, such as β -Catenin, ErbB-2-, c-Myc, Cyclin D1 and K-Ras ⁶⁴. Consecutively, compounds designated GO-Y078, 030 and 098 (derivatives **34-36** respectively, Figure 12) were developed and revealed at least 10 times higher growth-suppressive potential than curcumin (**1**) in several cell lines such as the ones derived from tumors of the stomach (GCIY, SH10TC), colon (HCT116, DLD1 and SW680), lung (A549), pancreas (PK1), kidney (ACHN), liver (HUH7), ovary (OVK18), breast (MCF7), skin (A431), bile duct (HuCCT1), thyroid (8505c), melanoma (G361), and prostate (PC3) ⁷⁴. It is known that, in these cancer cell lines, high activation levels of NF- κ B can lead to protection from apoptosis and that NF- κ B activation can occur in response

to certain inflammatory signals, such as tumor necrosis factor- α (TNF- α)⁷⁴⁻⁷⁶. Since it had previously been shown that curcumin (**1**) inhibited NF- κ B transactivation⁷⁷, in this work it was shown that the derivatives GO-Y078 (**34**), 030 (**35**) and 098 (**36**) decreased TNF α -induced NF- κ B transactivation at a concentration of 2 μ M⁷⁴. Synthesis of such derivatives is similar to the one depicted in Scheme 9.

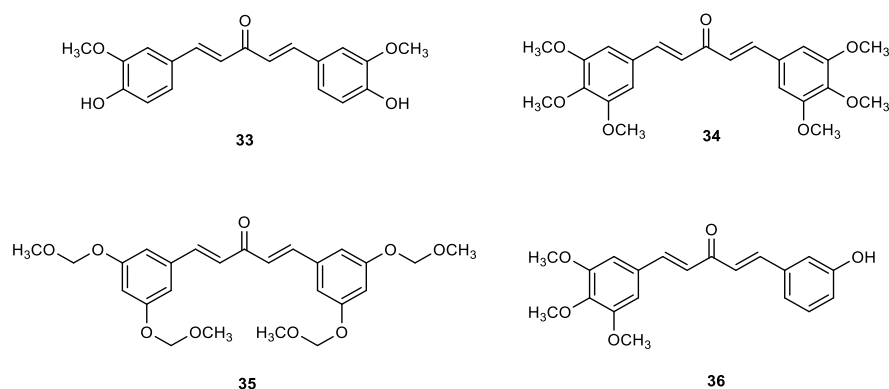


Figure 12 - Structure of GO-035 (1,5-bis(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one, **33**), GO-Y078 (1,5-bis(3,4,5-trimethoxyphenyl)penta-1,4-dien-3-one, **34**), GO-Y030 (1,5-Bis(3,5-bis(methoxymethoxy)phenyl)-1,4-pentadien-3-one, **35**), GO-Y098 (1-(3-hydroxyphenyl)-5-(3,4,5-trimethoxyphenyl)penta-1,4-dien-3-one, **36**).

Compound **33** was investigated in an *in silico* study for its properties as a possible multidrug resistance associated protein 5 (MRP5) inhibitor for the efflux of hyaluronan from fibroblasts. Hyaluronan is overproduced in many pathologies, including, inflammation, metastasis, and ischemia. Currently, there is no drug able to attenuate hyaluronan production. Virtual docking identified compound **33** (from among 120 compounds) as the best hyaluronan export inhibitor⁷⁸.

A compound designated GL63 (Figure 13, **37**) was studied for its ability to induce apoptosis in the human hepatocellular carcinoma cell line HepG2. Derivative **37** appeared to be more potent than curcumin (**1**) in the inhibition of HepG2 cellular proliferation and induction of cellular apoptosis⁷⁹. Its synthesis is similar to the one shown in Scheme 9.

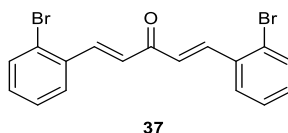
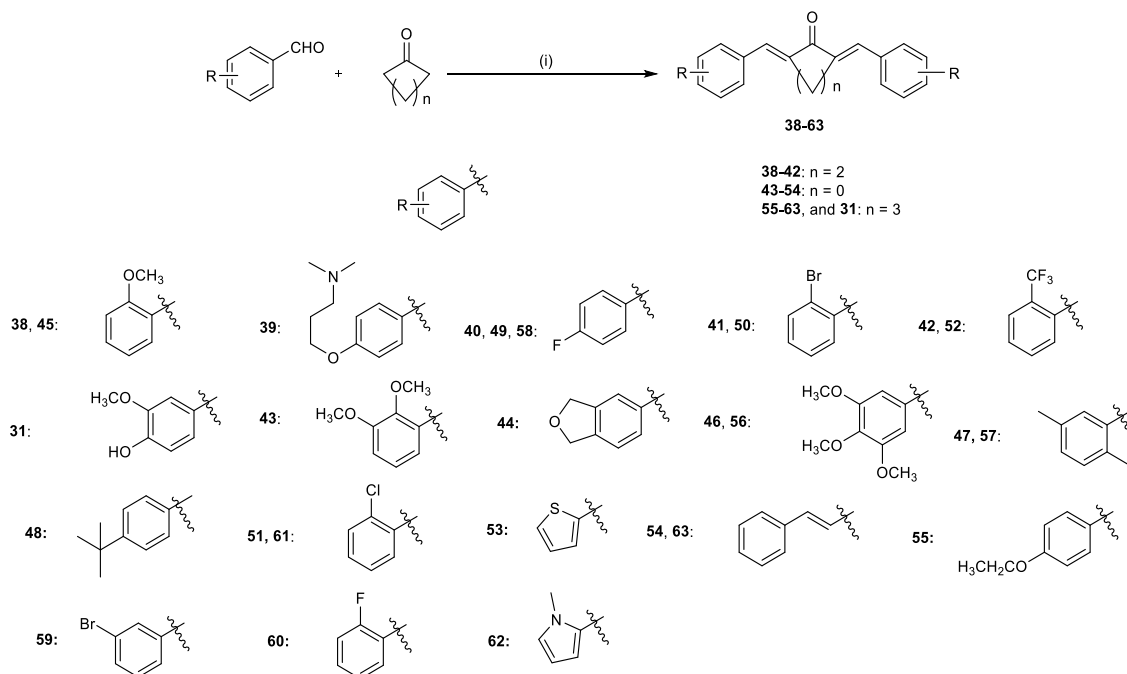


Figure 13 - Structure of compound **37** ((1*E*,4*E*)-1,5-bis(2-bromophenyl)penta-1,4-dien—
one).

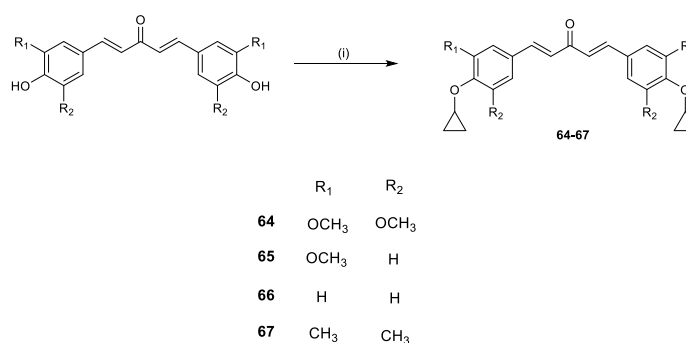
Many compounds were synthesized and studied for their tumor cell growth inhibitory activity against BGC823, CNE, HL-60, KB, LS174T, PC3 and HeLa human tumor cell lines (Scheme 10, compounds **38-64**, and **31**)⁸⁰. This study proposed some ideas concerning SAR: i) curcumin derivatives with an acetone or cyclohexanone linker exhibited much higher activities than the compounds with a cyclopentanone linker; ii) particularly at the 2'-position, strong electron-withdrawing substituents may increase bioactivity; iii) at the 4'-position, weak electron-donating substituents may increase the tumor cell growth inhibitory activity of the compound, and strong electron-withdrawing ones may reduce it; iv) the introduction of an heteroatomic ring does not cause significant changes in the ability of the compound to inhibit tumor cell growth⁸⁰.



Scheme 10 - Synthesis of derivatives **38-63**. Reaction conditions (i): NaOCH₃, methanol (MeOH), r.t., 20 min. **38**: 2,5-bis(2-methoxybenzylidene)cyclopentan-1-one; **39**: 2,5-bis((*E*)-4-(3-(dimethylamino)propoxy)benzylidene)cyclopentan-1-one; **40**: 2,5-bis((*E*)-4-fluorobenzylidene)cyclopentan-1-one; **41**: 2,5-bis((*E*)-2-bromobenzylidene)cyclopentan-1-one; **42**:

2,5-bis((*E*)-2-(trifluoromethyl)benzylidene)cyclopentan-1-one; **43**: 1,5-bis(2,3-dimethoxyphenyl)penta-1,4-dien-3-one; **44**: 1,5-bis(1,3-dihydroisobenzofuran-5-yl)penta-1,4-dien-3-one; **45**: 1,5-bis(2-methoxyphenyl)penta-1,4-dien-3-one; **46**: 1,5-bis(3,4,5-trimethoxyphenyl)penta-1,4-dien-3-one; **47**: 1,5-bis(2,5-dimethylphenyl)penta-1,4-dien-3-one; **48**: 1,5-bis(4-(*tert*-butyl)phenyl)penta-1,4-dien-3-one; **49**: 1,5-bis(4-fluorophenyl)penta-1,4-dien-3-one; **50**: 1,5-bis(2-bromophenyl)penta-1,4-dien-3-one; **51**: 1,5-bis(2-chlorophenyl)penta-1,4-dien-3-one; **52**: 1,5-bis(2-(trifluoromethyl)phenyl)penta-1,4-dien-3-one; **53**: 1,5-di(thiophen-2-yl)penta-1,4-dien-3-one; **54**: 1,9-diphenylnona-1,3,6,8-tetraen-5-one; **55**: 2,6-bis((*E*)-4-hydroxy-3-methoxybenzylidene)cyclohexan-1-one; **55**: 2,6-bis((*E*)-3,4,5-trimethoxybenzylidene)cyclohexan-1-one; **57**: 2,6-bis((*E*)-2,5-dimethylbenzylidene)cyclohexan-1-one; **58**: 2,6-bis((*E*)-4-fluorobenzylidene)cyclohexan-1-one; **59**: 2,6-bis((*E*)-3-bromobenzylidene)cyclohexan-1-one; **60**: 2,6-bis((*E*)-2-fluorobenzylidene)cyclohexan-1-one; **61**: 2,6-bis((*E*)-2-chlorobenzylidene)cyclohexan-1-one; **62**: 2,6-bis((1-methyl-1*H*-pyrrol-2-yl)methylene)cyclohexan-1-one; **63**: 2,6-bis((*E*)-3-phenylallylidene)cyclohexan-1-one.

Other authors synthesized four dienone cyclopropoxy curcumin derivatives (compounds **64-67**, Scheme 11) and evaluated their antitumor and antiangiogenic effects *in vivo*, on mouse Ehrlich ascites tumor. These compounds increased the life span of tumor-bearing mice, with a corresponding significant reduction in ascites volume and cell number and increase in apoptotic bodies in Ehrlich ascites tumor cells. Compounds **64-67** also reduced microvessels density, associated with angiogenesis⁸¹.



Scheme 11 - Synthesis of four cyclopropoxy curcumin derivatives **64-67**. Reaction conditions (i): dimethylformamide, K₂CO₃, cyclopropyl bromide, 60°C, 6 h. **64**: 1,5-bis(4-cyclopropoxy-3,5-dimethoxy-phenyl)-penta-1,4-dien-3-one; **65**: 1,5-bis(4-cyclopropoxy-3-methoxyphenyl)-penta-1,4-dien-3-one; **66**: 1,5-bis(4-cyclopropoxy-phenyl)-penta-1,4-dien-3-one; **67**: 1,5-bis(4-cyclopropoxy-3,5-dimethylphenyl)-penta-1,4-dien-3-one.

In a different study some novel curcumin derivatives **68-77** (Figure 14 and Scheme 12) were synthesized and their ability to inhibit tumor cell growth together with antiangiogenic activity was evaluated. Most of the analogues exhibited a moderate tumor cell growth inhibitory activity⁸². Derivatives **73**, **74** and **75** presented high inhibition of tumor cell growth in an *in vitro* anticancer cell line screen. This screen based on evaluation of cell growth inhibition revealed that these compounds were more potent than cisplatin. In an independent *in vitro* screen, the same derivatives and analogues **69-72** and **75** also demonstrated high degree of cytotoxicity to tumor cells. Derivatives that were effective in the anticancer screens, which were based on tumor cell growth inhibition, were also effective in the *in vitro* antiangiogenesis assays⁸². Compounds **69**, **72** and **74-76** were the most potent in this assay. Compound **76** was almost as potent as the TNP-470, an antiangiogenic drug. This compound effectively reduced the size of human breast tumors grown in mice and presented little toxicity. These data suggest that the symmetrical α,β -unsaturated ketone structure found in these novel compounds shows increased *in vitro* tumor cell growth inhibitory activity and antiangiogenic activity, when compared to the β -diketone structure of curcumin (**1**). *ortho*-Substitution such as in derivatives **69**, **73**, **74**, **75** and **76**, seems to favor the activity of these analogues. Compounds with substitutions on *meta*- or *para*-positions, such as **68** and **70**, are less active. The presence of a heteroatom in the cyclic ketone portion seems to increase the tumor cell growth inhibitory activity and antiangiogenic activity⁸². Synthesis of such derivatives is similar to the one depicted in Scheme 9, except for derivative **77**, which resulted from the reduction of curcumin (**1**) and which synthesis is shown in Scheme 12.

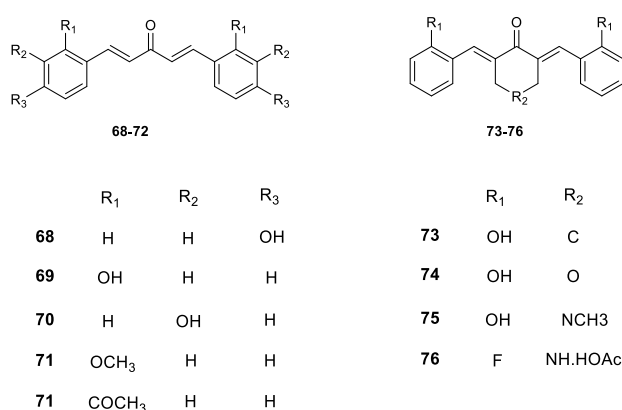
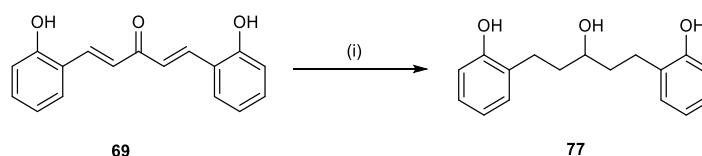


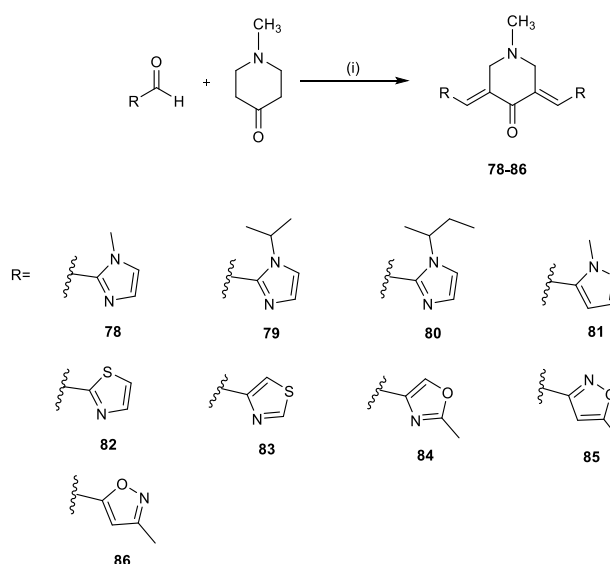
Figure 14 - Derivatives 68-76. **68**: 1,5-bis(4-hydroxyphenyl)-penta-1,4-dien-3-one; **69**: 1,5-bis(2-hydroxyphenyl)-penta-1,4-dien-3-one; **70**: 1,5-bis(3-hydroxyphenyl)-penta-1,4-dien-3-one; **71**: 1,5-bis(2-methoxyphenyl)-penta-1,4-dien-3-one; **72**: 1,5-bis(2-acetylphenyl)-penta-1,4-diene-3-one; **73**: 2,6-bis(2-hydroxybenzylidene)-cyclohexanone; **74**: 3,5-bis(2-hydroxybenzylidene)-tetrahydro-4-

H-pyran-4-one; **75**: 3,5-bis(2-hydroxybenzylidene)-1-methyl-4-piperidone; **76**: 3,5-bis(2-fluorobenzylidene)-piperidin-4-one, acetic acid salt.



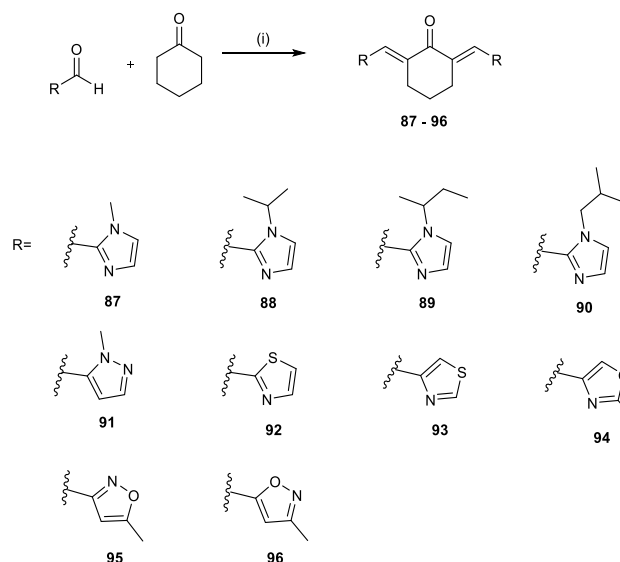
Scheme 12 - Synthesis of derivative **77** (2,2'-(3-hydroxypentane-1,5-diyl)diphenol). Reaction conditions (i): EtOH, Raney nickel as a catalyst, 45 psi, 4h.

A total of thirty two heteroatomic analogues with dienone linkers were synthesized and their cytotoxicity against two human androgen-independent-prostate cancer cell lines (PC-3 and DU-145) was shown to be higher than curcumin (**1**)⁸³. The synthesis of these compounds is similar to the one represented in Scheme 9 and is depicted in Scheme 13, Scheme 14, and Scheme 15, according to the dienone linker.

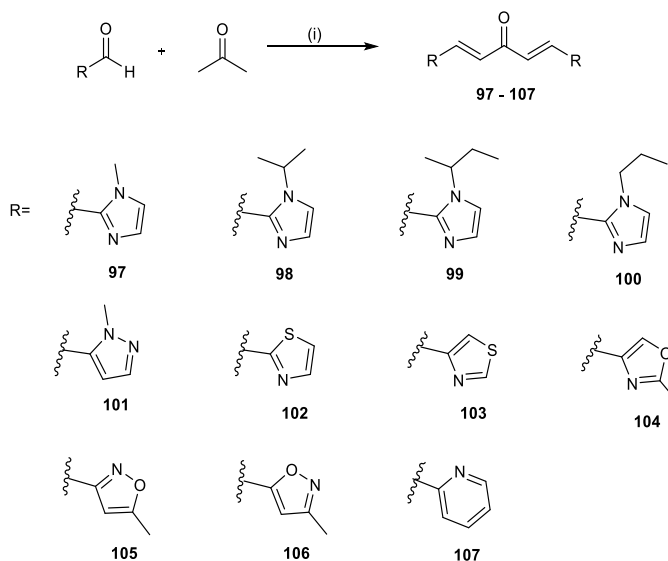


Scheme 13 - Synthesis of compounds **78-86**. Reaction conditions (i): NaOCH₃, MeOH, r.t., 4h. **78**: (3*E*, 5*E*)-1-methyl-3,5-bis((1-methyl-1*H*-imidazole-2-yl)methylene)piperidin-4-one; **79**: (3*E*, 5*E*)-1-methyl-3,5-bis((1-isopropyl-1*H*-imidazole-2-yl)methylene)piperidin-4-one; **80**: (3*E*, 5*E*)-3,5-bis((1-(*sec*-butyl)-1*H*-imidazol-2-yl)methylene)-1-methylpiperidin-4-one; **81**: (3*E*, 5*E*)-1-methyl-3,5-bis((1-methyl-1*H*-pyrazol-5-yl)methylene)piperidin-4-one; **82**: (3*E*, 5*E*)-1-methyl-3,5-bis((thiazole-2-yl)methylene)piperidin-4-one; **83**: (3*E*, 5*E*)-1-methyl-3,5-bis((thiazole-4-yl)methylene)piperidin-4-

one; **84**: (3*E*, 5*E*)-1-methyl-3,5-bis((2-methyloxazol-4-yl)methylene)piperidin-4-one; **85**: (3*E*, 5*E*)-1-methyl-3,5-bis((5-methylisoxazol-3-yl)methylene)piperidin-4-one; **86**: (3*E*, 5*E*)-1-methyl-3,5-bis((3-methylisoxazol-5-yl)methylene)piperidin-4-one.



Scheme 14 - Synthesis of compounds **87-96**. Reaction conditions (i): NaOCH₃, MeOH, r.t., 4h. **87**: (2*E*, 6*E*)-2,6-bis((1-methyl-1*H*-imidazol-2-yl)methylene)cyclohexanone; **88**: (2*E*, 6*E*)-2,6-bis((1-isopropyl-1*H*-imidazol-2-yl)methylene)cyclohexanone; **89**: (2*E*, 6*E*)-2,6-bis((1-(*sec*-butyl)-1*H*-imidazol-2-yl)methylene)cyclohexanone; **90**: (2*E*, 6*E*)-2,6-bis((1-isobutyl-1*H*-imidazol-2-yl)methylene)cyclohexanone; **91**: (2*E*, 6*E*)-2,6-bis((1-methyl-1*H*-pyrazol-5-yl)methylene)cyclohexanone; **92**: (2*E*, 6*E*)-2,6-bis((thiazole-2-yl)methylene)cyclohexanone; **93**: (2*E*, 6*E*)-2,6-bis((thiazol-4-yl)methylene)cyclohexanone; **94**: (2*E*, 6*E*)-2,6-bis((2-methyloxazol-4-yl)methylene)cyclohexanone; **95**: (2*E*, 6*E*)-2,6-bis((5-methylisoxazol-3-yl)methylene)cyclohexanone; **96**: (2*E*, 6*E*)-2,6-bis((3-methylisothiazol-5-yl)methylene)cyclohexanone.

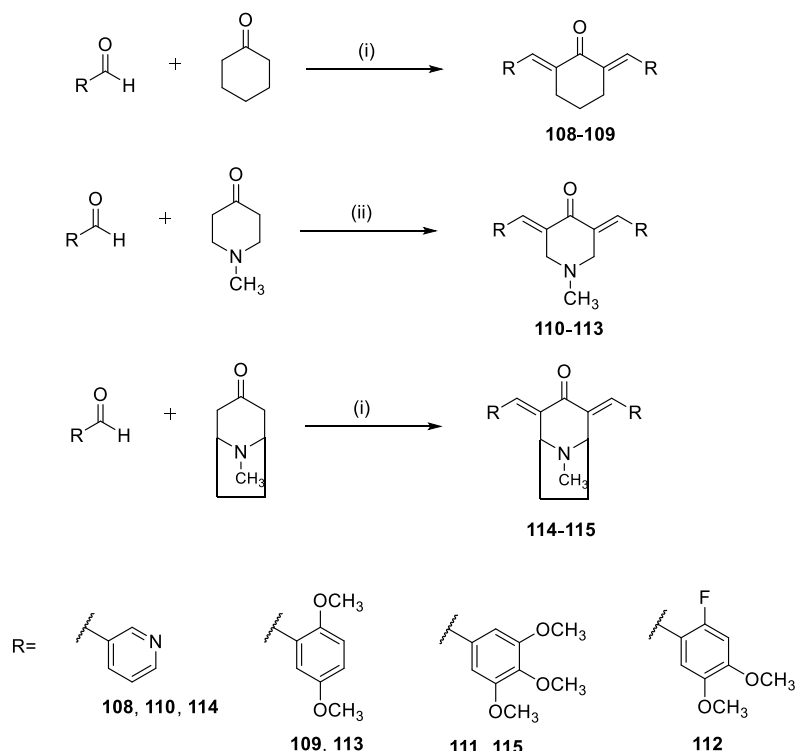


Scheme 15 - Synthesis of compounds **97-107**. Reaction conditions (i) for compounds **97** and **98**: NaOCH₃, MeOH, 4-18 h. Reaction conditions (i) for compounds **99-107**: K₂CO₃, toluene-EtOH-water (4:4:2), 70°C, 12 h. **97**: (1*E*,4*E*)-1,5-bis(1-methyl-1*H*-imidazol-2-yl)penta-1,4-dien-3-one; **98**: (1*E*,4*E*)-1,5-bis((1-isopropyl-1*H*-imidazol-2-yl)penta-1,4-dien-3-one; **99**: (1*E*,4*E*)-1,5-bis(1-(sec-butyl)-1*H*-imidazol-2-yl)penta-1,4-dien-3-one; **100**: (1*E*,4*E*)-1,5-bis(1-isobutyl-1*H*-imidazol-2-yl)penta-1,4-dien-3-one; **101**: (1*E*,4*E*)-1,5-bis(1-methyl-1*H*-pyrazol-5-yl)penta-1,4-dien-3-one; **102**: (1*E*,4*E*)-1,5-di(thiazol-2-yl)penta-1,4-dien-3-one; **103**: (1*E*,4*E*)-1,5-di(thiazol-4-yl)penta-1,4-dien-3-one; **104**: (1*E*,4*E*)-1,5-bis(2-methyloxazol-4-yl)penta-1,4-dien-3-one; **105**: (1*E*,4*E*)-1,5-bis(5-methylisoxazol-3-yl)penta-1,4-dien-3-one; **106**: (1*E*,4*E*)-1,5-bis(3-methylisoxazol-5-yl)penta-1,4-dien-3-one; **107**: (1*E*,4*E*)-1,5-di(pyridin-2-yl)penta-1,4-dien-3-one.

From these compounds, all showed higher effect than curcumin (**1**) against PC-3 cells, except for compound **80**. In addition, compounds **78-80**, **82**, **83**, **84**, **88**, **89**, **92**, **93**, **95**, **97-103**, and **105-107** exhibited higher cytotoxicity than curcumin (**1**) towards DU-145 cells. Moreover, analogues **99**, **100**, **107** also showed impressive cytotoxic effects against three other tumor cell lines: MDA-MB-231, HeLa and A549, with IC₅₀ values between 50 nM and 390 nM. These three potent curcumin analogues were tested for their cytotoxic effect towards MCF-10A normal mammary epithelial cells and were shown to have no apparent cytotoxicity⁸³. From this work, the authors concluded that nitrogen-containing heteroatomic rings seem to be promising bioisosteres of the methoxyphenol motif in curcumin (**1**)⁸³.

Recently, during the elaboration of this thesis a set of heterocyclic cyclohexanone derivatives of curcumin (**1**) was synthesized and tested for their cytotoxicity and MDR transporters inhibitory activity. Compound **109**, **101**, **111**, **113**, and **114** exhibited potent cytotoxicity towards MDA-MB-231 cells and inhibition of NF-κB activity in K562 cells.

Compound **110**, **111**, and **114** exhibited strong cytotoxicity not only in MBA-MB-231 cell line, but also MDA-MB-468 and SkBr3 cell lines. Particularly, derivative **111** caused 43% MDA-MB-231 cells to undergo apoptosis after 18 h exposure⁸⁴. Analogue **109**, **31** (Scheme 10 or Figure 11), and **162** were able to inhibit BCRP transporters. Instead, analogue **115** inhibited P-gp and analogue **31** inhibited MRP1 and MRP5⁸⁵. Synthesis of compounds **108-115** is depicted in Scheme 16.



Scheme 16 – Synthesis of derivatives **108-115**. Reaction conditions (i): EtOH, NaOH 1M, 18 h, r.t.; (ii): MeOH, NaOCH₃, 5M, 18 h, r.t. **108**: (2*E*,6*E*)-2,6-bis(pyridin-3-ylmethylene)cyclohexan-1-one; **109**: 2,6-bis((*E*)-2,5-dimethoxybenzylidene)cyclohexan-1-one ; **110**: (3*E*,5*E*)-1-methyl-3,5-bis(pyridin-3-ylmethylene)piperidin-4-one; **111**: 1-methyl-3,5-bis((*E*)-3,4,5-trimethoxybenzylidene)piperidin-4-one; **112**: 3,5-bis((*E*)-2-fluoro-4,5-dimethoxybenzylidene)-1-methylpiperidin-4-one; **113**: 3,5-bis((*E*)-2,5-dimethoxybenzylidene)-1-methylpiperidin-4-one; **114**: (2*E*,4*E*)-8-methyl-2,4-bis(pyridin-3-ylmethylene)-8-azabicyclo[3.2.1]octan-3-one; **115**: 8-methyl-2,4-bis((*E*)-3,4,5-trimethoxybenzylidene)-8-azabicyclo[3.2.1]octan-3-one.

EF24 (**116**, Figure 13) is a curcumin derivative that greatly inhibits cisplatin-resistant human ovarian cancer cellular proliferation⁸⁶. The inhibitory effect of EF24 (**116**) on cellular proliferation is associated with G₂/M checkpoint protein levels. The tumor cell growth inhibitory effect of EF24 (**116**) in breast cancer cells has also been partially attributed to redox-mediated induction of apoptosis. Also, EF24 (**116**) activated the

phosphorylated phosphatase and tensin homolog (PTEN) and up-regulated total PTEN expression through the inhibition of ubiquitin-mediated PTEN degradation. Suppression of PTEN with small interfering RNAs (siRNAs) resulted in a significant reduction of p53 and p21 levels and activated Akt phosphorylation at Ser⁴⁷³ and Thr³⁰⁸, resulting in decreased apoptosis and increased cell survival. In contrast, the overexpression of PTEN significantly induced apoptosis. In this study, it was suggested that EF24 (**116**) induced a substantial increase in PTEN expression. The up-regulation of PTEN inhibited Akt and MDM2, which increased the levels of p53, thus inducing G₂/M arrest and apoptosis⁸⁶. EF24 (**116**) synthesis is similar to the one depicted in Scheme 9.

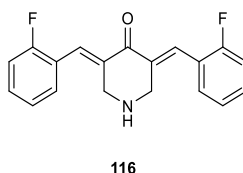


Figure 15 - Structure of compound **116** (3,5-bis((*E*)-2-fluorobenzylidene)piperidin-4-one, EF24).

Overall, several dienone analogues such as anelar isosters or halogenated derivatives were shown to be promising compounds in several bioactivities.

Heterocycles are a privileged chemical source of bioactive compounds. Several compounds were synthesized with heterocyclic moieties instead of the carbonyl group in curcumin (**1**) displaying promising results in several activities such as free radical scavenging activity, antioxidant, and antitumor activity⁸⁷; antibacterial activity⁸⁸; antimalarial activity⁸⁹, among others.

Over the past few years, many research groups have focused their efforts in improving curcumin (**1**) bioavailability by slowing its metabolism, increasing solubility or cellular uptake by synthesizing curcumin bioconjugates. This strategy was based on merging curcumin (**1**) with aminoacids⁹⁰, nucleosides⁹¹, dipeptides, fatty acids and folic acid⁹². The synthesized compounds showed a vast range of bioactivities such as antiproliferative, antimicrobial, and antiviral.

In addition to the synthetic analogues, several other strategies have been successful in the improvement of curcumin (**1**) bioavailability. Adjuvants, nanoparticles, liposomes, micelles and phospholipids are some of the attempted strategies^{5, 93-101}.

CHAPTER II:

RESULTS AND DISCUSSION

1. Synthesis and structure elucidation

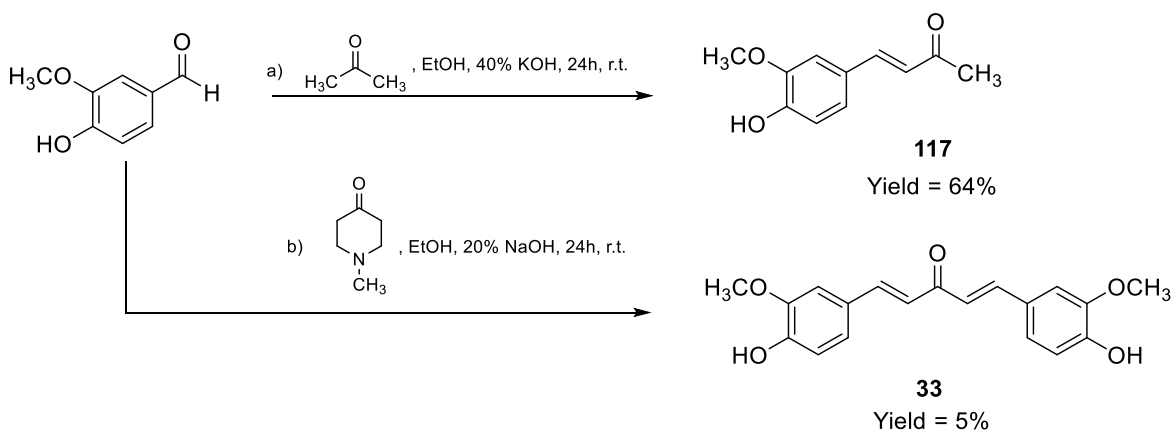
Curcumin (**1**) has shown to be a promising compound in therapeutics; however, the chemical stability and extensive metabolism are substantial limitations to its potential use. Therefore, we aimed to synthesize three mono-carbonyl building blocks chemically more stable, eliminating the unstable portion of the molecule. The selected building blocks had already been synthesized and showed improved stability comparing to curcumin (**1**)⁸⁰. In order to improve the P-gp modulatory activity different substitution patterns were introduced in the phenolic groups. The substitution patterns include alkylated groups (with an alkene and alkyne terminal portion), a sugar, amines, carbamates and an ester. These substitution patterns were selected considering the chemical diversity they represent. Specifically the amines are well known for their drug-like properties, and the nitrogen atom, charged at physiological pH, has often been considered to be a symbol of P-gp substrates and inhibitors¹⁰².

1.1. Synthesis of 4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one (**117**) and 1,5-bis(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**33**)

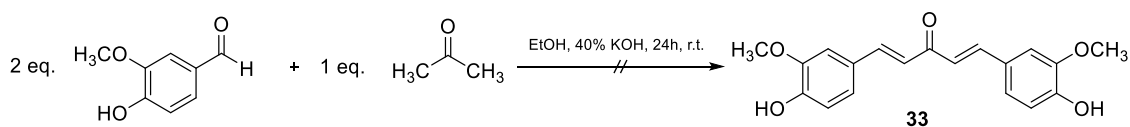
The synthetic approach used for the synthesis of the mono-carbonyl analogues of curcumin, was the Claisen-Schmidt condensation. The synthesis of these building blocks, 4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one (**117**) and 1,5-bis(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**33**), has been previously described^{69, 82}. One equivalent of vanillin was allowed to react one equivalent with acetone to afford compound **117** (Scheme 17a) and two equivalent of vanillin with one equivalent of *N*-methyl-piperidone to afford compound **33** (Scheme 17b). Scheme 17 summarizes the reaction conditions and the results obtained in the synthesis of compound **117** and **33**. Regarding compound **117** synthesis, originally, the goal was to obtain a building block with two aromatic rings (**33**, Scheme 18). With the conditions depicted in Scheme 17a, the major product formed was compound **117**, however, other by-product was formed. The major by-products were isolated by preparative chromatography, and analysis by ¹H NMR lead to the conclusion that this by product was not the expected 1,5-bis(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**33**, Scheme 18), and no further investigation was performed. Due to the satisfactory yield in obtaining compound **117** and the convenient

purification by crystallization (without need of chromatographic purification) the enone **117** was selected as a suitable model for further molecular modifications.

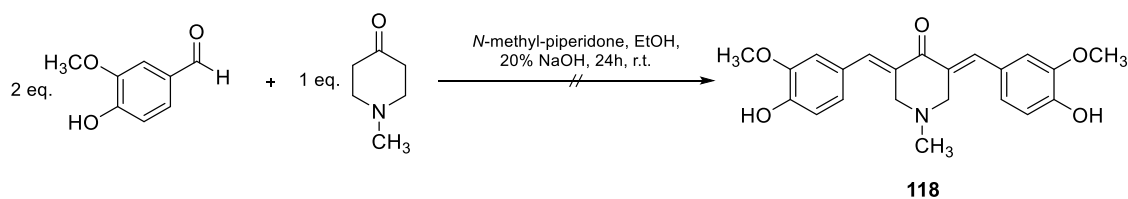
Regarding compound **33** synthesis, originally, the goal was to obtain the building block depicted in Scheme 19. Although the purification process followed for compound **117** was exactly as the one described in the literature for compound **33**⁷³, the results were slightly different from the one described in the literature for compound 3,5-bis((*E*)-4-hydroxy-3-methoxybenzylidene)-1-methylpiperidin-4-one (**118**)⁸², since after neutralizing the solution, could not be observed precipitate formation. Thus, the crude product was submitted to a liquid-liquid extraction, and solid-liquid anionic exchange followed by a column chromatography which can also explain the low isolated yield for compound **33**. During the purification process no acetone was used, which could have triggered the reaction leading to the synthesis of compound **33**. However, the purified compound was not the expected one and its synthesis could have been the outcome of base and acid usage through the purification process.



Scheme 17 – Reaction conditions and results for the synthesis of compounds **117** and **33** (r.t.= room temperature).



Scheme 18 – Described reaction conditions for the synthesis of compound **33**⁷³.



Scheme 19 – Described reaction conditions for the synthesis of **118**⁸².

Comparing the yields obtained for the compounds **117** and **33** building blocks it is possible to infer that the reaction between vanillin and *N*-methyl-piperidone occurred with difficulties, probably due to steric hindrance. Due to low yielding reaction to obtain compound **33**, the synthesis of derivatives was not further pursuit with this building block.

Thus, from the above obtained results, compound **117** was selected as a potential stable model of curcumin (**1**) to pursuit the synthesis of derivatives.

Structure characterization of the two synthesized compound **117** and **33** was performed by melting point, UV-Visible (UV-Vis), infrared (IR), ¹H and ¹³C NMR spectroscopy techniques and mass spectrometry (MS) techniques. In the case of compound **33**, further analysis by HRMS was performed to unequivocally establish the structure. Melting point can be consulted in page 91 and UV-Vis data in page 77.

The IR data of these two compounds were in accordance with the molecular modifications performed (**Table 1**). The presence of bands between 960-990 cm⁻¹ typical of the sp² C-H bond of a *trans* disubstituted alkene (bend pattern) corroborate with the hypothesis of the reaction occurring successfully. The aldehyde band for the C-H bond at around 2800-2700 cm⁻¹ (bend pattern) was only observed for the precursor compound vanillin which is also in accordance with the performed molecular modifications for compound **117** and **33**. Additionally, IR spectra revealed the presence of a large band corresponding to stretching vibration of hydroxyl groups at, 3200-3500 cm⁻¹.

Table 1 - IR data of compound **117** and **33**.

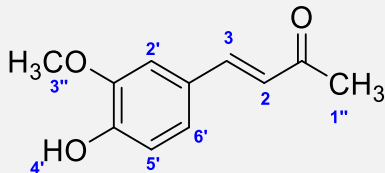
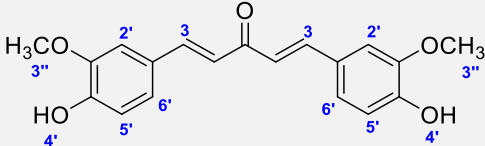
Group	ν (cm ⁻¹)		
	Vanillin	117	33
OH	3000-3400	3200-3500	3200-3600
C-H (Stretch sp ²)	-----	3002	2934
C-H (Aldehyde stretch)	2857	-----	-----
C=O	1665	1637	1639
C=C (Stretch Ar)	1588, 1509, 1465	1583, 1519, 1452	1587, 1511, 1449
C-O (Acyl)	1299, 1266, 1199, 1171, 1154	1297, 1267, 1226, 1188	1267, 1159
C-O (Alcoxy)	1124, 1039	1124, 1026	1104, 1029
C-H (Bend alkene sp ² - <i>trans</i>)	-----	981	981, 852, 811

The ¹H and ¹³C NMR data of compounds **117** and **33** are depicted in Table 2 and Table 3, respectively.

According to the ¹H NMR spectra of compounds vanillin (data not shown)¹⁰³, compounds **117** and **33** (Table 2) only the precursor vanillin showed signals in the aldehyde region (δ_{H} 10-9 ppm), indicating that the bond between the aldehyde and the ketones was formed successfully in the synthesized compounds **117** and **33**. Also, the signals corresponding to vinyl hydrogens are in accordance with the molecular modifications introduced. In compound **117**, the signals corresponding to two vinyl protons H-2 and H-3 split each other into two doublets, one centered at δ_{H} 7.45 ppm more deshielded than the phenyl protons and the other at δ_{H} 6.59 ppm more shielded than the phenyl protons. The H-3 proton, attached to the carbon adjacent to the phenyl ring, shows a higher chemical shift when compared to H-2 proton, as it should be in a deshielding area of the anisotropic field generated by the π electrons of the aromatic ring. The coupling constant observed for the signal corresponding to the H-3 doublet was of 16.2 Hz, a common value for *trans* proton-proton coupling across a double bond¹⁰⁴. Multiplicities and chemical shifts of signals corresponding to protons H-2 and H-3 in compound **33** are similar to the same signals of compound **117**. Similarly to the precursor vanillin¹⁰³, ¹H NMR spectra of compounds **117** and **33** showed signals corresponding to the aromatic protons: H-2', H-5', and H-6' with the expected multiplicities (*d*, *d* and *dd*), as well as the hydroxyl group (H-4') and methoxyl group (H-3''). The signal at δ_{H} 2.37 ppm integrating for three protons present in compound **117** spectra was not in agreement with

the originally proposed structure (compound **33**) but the latter proposed structure of compound **117**. This signal was crucial to determinate compound **117** structure.

Table 2 - ^1H NMR data of compounds **117** and **33**.

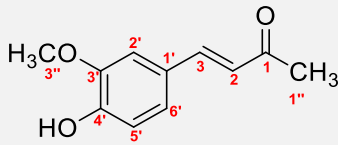
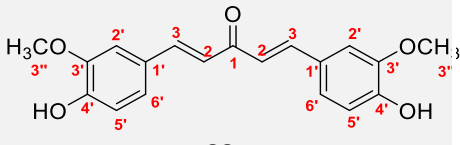
 117		 33	
Proton*	δ_{H} (values in ppm relative to Me_4Si as an internal reference)**		
H-3	7.45, 1H, <i>d</i> , $J = 16.2$	7.68, 2H, <i>d</i> , $J = 15.5$	
H-6'	7.09, 1H, <i>dd</i> , $J = 8.1$ and 1.9	7.18, 2H, <i>dd</i> , $J = 8.2$ and 1.8	
H-2'	7.05, 1H, <i>d</i> , $J = 1.9$	7.11, 2H, <i>d</i> , $J = 1.8$	
H-5'	6.93, 1H, <i>d</i> , $J = 8.1$	6.92, 2H, <i>d</i> , $J = 8.2$	
H-2	6.59, 1H, <i>d</i> , $J = 16.2$	6.94, 2H, <i>d</i> , $J = 15.5$	
H-4'	6.00, 1H, <i>brs</i>	5.99, 2H, <i>brs</i>	
H-3''	3.92, 3H, <i>s</i>	3.95, 6H, <i>s</i>	
H-1''	2.37, 3H, <i>s</i>	-----	

* Numbering of atoms was attributed for NMR assignments.

** J values are presented in Hz.

^{13}C NMR assignments of the hydrogenated carbons were determined by 2D heteronuclear single quantum correlation (HSQC) and the chemical shifts of the carbon atoms not directly bonded to hydrogen atoms were deduced by heteronuclear multiple bond correlation (HMBC) experiments (Table 3 and Figure 16). HMBC spectra also corroborated with the proposed structure and that the compound synthesized was in fact the derivative **117**. The NMR spectroscopic data are in accordance with the described in literature for compound **117**¹⁰⁵ and **33**¹⁰⁶.

Table 3 - ^{13}C NMR data of compounds **117** and **33**.

	 117	 33
Carbon*	δ_{C} (values in ppm)	
C-1	198.7	188.9
C-4'	148.4	148.2
C-3'	147.0	147.2
C-3	144.0	143.3
C-1'	126.8	127.5
C-2	124.9	123.4
C-6'	123.6	123.3
C-5'	114.9	114.9
C-2'	109.4	109.8
C-3''	56.0	56.0
C-1''	27.3	-----

* Numbering of atoms was attributed for NMR assignments.

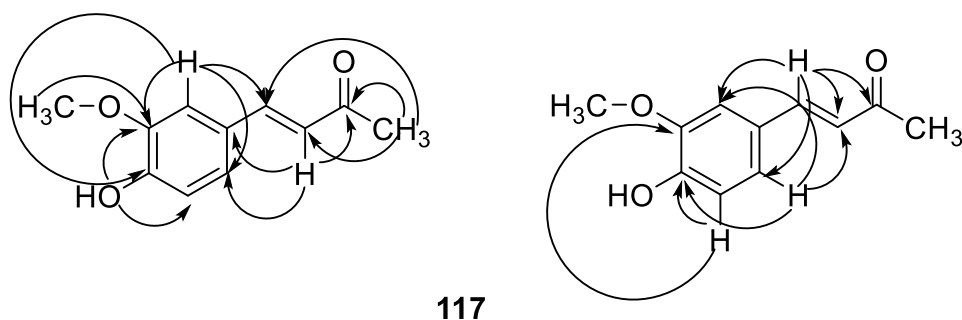


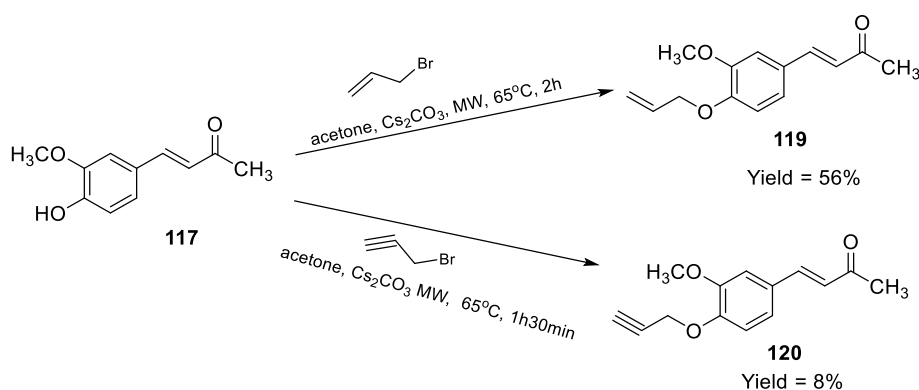
Figure 16 - Connectivities found in the HMBC spectra of compound **117**.

The electron impact-mass spectrometry (EI-MS) spectra were obtained in a gas chromatography-mass spectrometry (GC-MS) equipment and the molecular ion peak and base peak for the trimethylsilyl derivative of **117** was found to be 264 m/z and 233 m/z, respectively. Considering that the compound had to be derivatized for GC-MS analysis,

which involved the reaction of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) with the phenol group, the correspondent value subtracting the molecular weight of the trimethylsilyl group would be 192 and 161, respectively. Therefore, the molecular ion peak is in agreement with the molecular weight of the proposed compound **117**. The HRMS-ESI analysis of compound **33** gave the accurate molecular mass of 327.12265 and the molecular formula of C₁₉H₁₉O₅.

1.2. Synthesis of alkylated derivatives of compound **117**, compounds **119** and **120**.

The synthesis of the two alkylated derivatives of compound **117**, 4-(4-(allyloxy)-3-methoxyphenyl)but-3-en-2-one (**119**) and 4-(3-methoxy-4-(prop-2-yn-1-yloxy)phenyl)but-3-en-2-one (**120**), followed a synthetic microwave (MW) assisted approach based on a nucleophilic substitution bi-molecular (S_N2) reaction¹⁰⁷. One equivalent of allyl bromide and propargyl bromide reacted with one equivalent of **117** to afford compound **119** or **120**, respectively. Scheme 20 summarizes the reaction conditions and the results obtained. Both compounds were purified by column chromatography, and compound **120** was further crystallized from a mixture of diethylether:petroleum ether in order to isolate the compound.



Scheme 20 – Reaction conditions and results for the synthesis of compounds **119** and **120**.

In these two reactions, the major products formed were compound **119** and **120**. However, other by-products could be observed by thin layer chromatography (TLC) analysis.

Although compound **119** structure is protected in a patent application for the treatment of hypertension ¹⁰⁸, to our knowledge the synthesis and structure elucidation is described herein for the first time.

Structure elucidation was established by IR, ¹H and ¹³C NMR and MS techniques.

The IR data of these two compounds was in accordance with the performed molecular modifications (Table 4). The presence of alkyl groups was suggested by the bands appearing at 2960-2851 cm⁻¹ (aliphatic C-H). Comparing the IR spectra of compounds **119** and **120** with the precursor **117**, the band corresponding to hydroxyl group was only observed for compound **117**. Additionally, the IR spectra revealed the presence of bands at 1639-1624 cm⁻¹ (C=C alkene) and 3258 (C-H alkyne), in compounds **119** and **120** spectra, respectively.

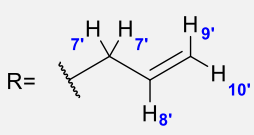
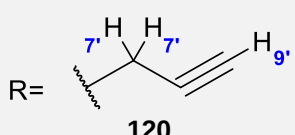
Table 4 - IR data of **119** and **120**.

Group	ν (cm ⁻¹)	
	119	120
C-H (Stretch sp)	----	3258
C-H (Stretch sp ³)	2960, 2924	2922, 2851
C=O	1664	1673
C=C (Stretch alkene)	1639, 1624	-----
C=C (Stretch Ar)	1593, 1511	1597, 1515
C-H (bend sp ³)	1418, 1362	1424, 1372
C-O (Acyl)	1252	1272, 1247, 1225
C-O (Alcoxy)	1164, 1134	1168, 1145, 1034, 1006
C-H (Ar sp ²)	-----	984, 805, 723
C-H (Bend alkene sp ²)	997, 969, 800	-----
C-H (Bend alkyne sp)	-----	682

The ¹H and ¹³C NMR data of compounds **119** and **120** are depicted in Table 5 and Table 6, respectively. ¹H NMR spectra of both compounds (Table 5) showed signals with integration and multiplicities corresponding to the aromatic protons: H-2', H-5', and H-6'; the methoxyl group protons (H-3''); the vinyl protons H-2 and H-3; and the methylic proton H-1'', similarly to the precursor **117**. Comparing the ¹H NMR spectra of compounds **119** and **120** with the precursor **117**, the signal corresponding to a hydroxyl proton was only

observed for compound **117**. Instead, derivatives **119** and **120** both showed a set of peaks characteristic of the molecular modifications performed, signals for protons H-7', H-8', H-9', H-10' and H-7', H-9' for compounds **119** and **120**, respectively.

Table 5 - ^1H NMR data of compounds **119** and **120**.

	 119	 120
Proton*	δ_{H} (values in ppm relative to Me_4Si as an internal reference)**	
H-3	7.46, 1H, <i>d</i> , $J = 16.2$	7.46, 1H, <i>d</i> , $J = 16.2$
H-6'	7.11, 1H, <i>d</i> , $J = 2.0$	7.13, 1H, <i>d</i> , $J = 8.2$
H-2'	7.08, 1H, <i>s</i>	7.09, 1H, <i>s</i>
H-5'	6.88, 1H, <i>d</i> , $J = 8.1$	7.04, 1H, <i>d</i> , $J = 8.2$
H-2	6.61, 1H, <i>d</i> , $J = 16.2$	6.62, 1H, <i>d</i> , $J = 16.2$
H-8'	6.15-6.02, 1H, <i>m</i>	----
H-9'	5.46-5.39, 1H, <i>m</i>	2.53, 1H, <i>s</i>
H-10'	5.35-5.30, 1H, <i>m</i>	----
H-7'	4.67-4.65, 2H, <i>dt</i> , $J = 5.4$ and 1.4	4.81, 2H, <i>s</i>
H-3''	3.92, 3H, <i>s</i>	3.91, 3H, <i>s</i>
H-1''	2.38, 3H, <i>s</i>	2.37, 3H, <i>s</i>

* Numbering of atoms was attributed for NMR assignments. Protons of the building block **117** maintained their assigned numbers.

** J values are presented in Hz.

Considering compound **119**, the spectra showed a complex multiplicity of signals that could be explained considering the allylic coupling, a coupling between protons substituted on carbons α to the double bond and those on the opposite end of the double bond, a four-bond coupling (^4J)¹⁰⁹. The signals corresponding to the methylenic protons (H-7') appear in the spectra as a doublet of triplets (*dt*). This pattern is the result of H-7' being split by H-8' (vicinal coupling, ^3J), H-10' (*trans* allylic coupling, ^4J), and H-9' (*cis* allylic coupling, ^4J). In theory, this would lead to a doublet of doublets of doublets (*ddd*), but because some peaks overlap the result observed is a doublet of triplets. The signal corresponding to proton H-10', considered a multiplet is actually a doublet of quartets. The multiplicity observed is the result of H-10' being split by H-8' (*cis* coupling, ^3J), H-9'

(geminal coupling, ^2J), and H-7' (allylic geminal coupling, ^4J). The expected final pattern would be a doublet of doublet of triplets, but due to peaks overlap the spectra shows a doublet of quartets. The signal corresponding to proton H-9' exhibits a similar pattern for the same reasons explained for proton H-10'. Assigning the two terminal vinyl hydrogens relies on the difference of J values for *cis* and *trans*-coupling constants. The signal corresponding to proton H-9' has a wider pattern than proton H-10' because proton H-9' has a *trans* coupling to proton H-8', while proton H-10' has a lower coupling constant, typical of *cis* coupling (Figure 17). Proton H-8' is split by H-9' (*trans* coupling, ^3J), H-10' (*cis* coupling, ^3J), and H-7' (^3J), which would translate in a doublet of doublet of triplets. However, due to overlaps, the spectra exhibits what appears to be two quintets¹⁰⁹.

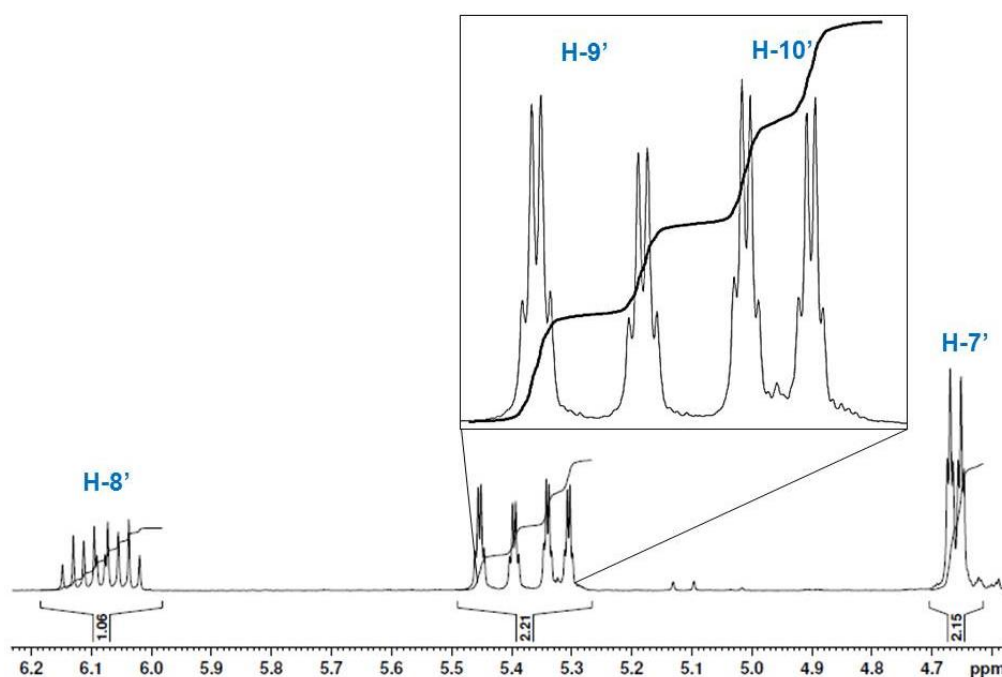
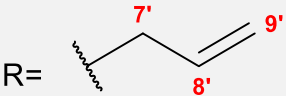
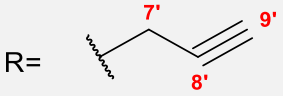


Figure 17 – Amplification of compound **119** ^1H NMR spectra peak region of protons H-7', H-8', H-9' and H-10', with a close-up of protons H-9' and H-10' peaks.

Regarding compound **120**, the signal corresponding to the acetylenic proton (H-9') was found at δ_{H} 2.53 ppm due to anisotropic shielding by the adjacent π bond. The methylenic protons (H-7') on the adjacent carbon are also shielded by this anisotropic field of the π bond and the corresponding signals were found at δ_{H} 4.81 ppm. Allylic coupling was not observed in this case.

Table 6 - ^{13}C NMR data of compounds **119** and **120**.

	 119	 120
Carbon*	δ_{C} (values in ppm)	
C-1	198.4	198.4
C-4'	150.3	149.8
C-3'	149.6	149.0
C-3	143.6	143.3
C-8'	132.7	77.9
C-1'	127.5	128.5
C-2	125.3	125.7
C-6'	122.8	122.5
C-9'	118.5	76.3
C-5'	112.8	113.6
C-2'	110.0	110.2
C-7'	69.7	56.6
C-3''	55.9	55.9
C-1''	27.4	27.4

* Numbering of atoms was attributed for NMR assignments. Carbons of the building block **117** maintained their assigned numbers.

^{13}C NMR assignments for these two derivatives were determined using spectroscopy data tables for typical ^{13}C chemical shifts, and by comparison with literature. Both compounds showed similar signals to the precursor **117**, which correspond to the carbons of this building block moiety, and new signals related with the molecular modifications performed. Compound **119** exhibit three signals at: 132.7, 118.5, and 69.7 ppm which were assigned to C-8', C-9', and C-7', respectively¹¹⁰. Compound **120** spectra also shows three peaks: 77.9, 76.3, and 56.6 ppm, assigned to C-8', C-9', and C-7', respectively¹¹¹.

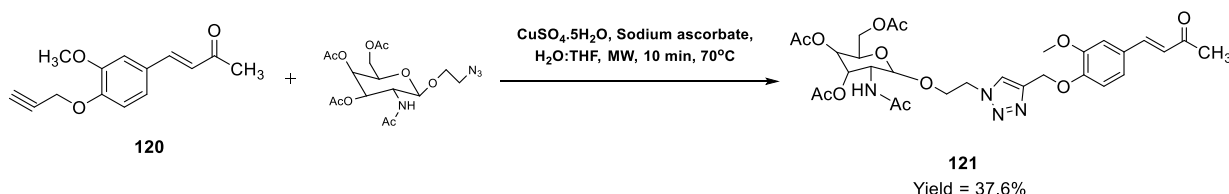
EI-MS data for these two compounds indicated that they are both in agreement with the suggested structures. Compound **119** exhibits a molecular ion peak and base peak of

232 m/z. Compound **120** shows a molecular ion peak of 230 m/z and a base peak of 191 m/z.

1.3. Synthesis of glucosamine derivative of compound **117**, compound **121**.

The synthesis of the sugared derivative (2*R*,4*S*,5*R*)-5-acetamido-2-(acetoxymethyl)-6-(2-(4-((2-methoxy-4-((*E*)-3-oxobut-1-en-1-yl)phenoxy)methyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)tetrahydro-2*H*-pyran-3,4-diyl diacetate (**121**) was accomplished using click chemistry, more precisely, the Huisgen 1,3-dipolar cycloaddition. Click chemistry is the term used to describe reactions with a specific set of criteria: modular reactions with wide scope, high yields, simple reaction conditions, use of no solvent or inoffensive ones that can be easily removed, simple product isolation, generate armless by-products that can be removed by non-chromatographic methods and be stereospecific¹¹²⁻¹¹³. The Huisgen 1,3-dipolar cycloaddition, the most popular reaction of click chemistry, occurs between an azide and a terminal alkyne affording the 2,3-triazole moiety¹¹³. To synthesize **121**, the derivative **120** was added to 2-azidoethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- β -D-galactopyranoside using a copper (II) salt (CuSO₄·5H₂O) as a catalyst in the presence of sodium ascorbate, the reducing agent (Scheme 21)¹¹³. Sodium ascorbate constantly reduced copper (II) to copper (I) maintaining significantly high levels of the catalytic specie.

In this reaction, the major product formed was compound **121**. Another by-product was formed; however, it was not isolated and identified. The major product was isolated by crystallization, without the need of chromatographic methods.



Scheme 21 - Reaction conditions and results for the synthesis of compound **121** (THF=tetrahydrofuran).

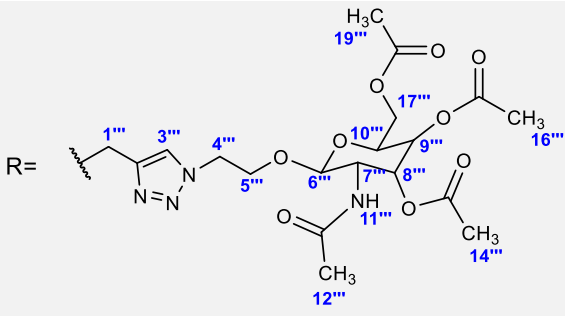
The IR data of compound **121** is shown in Table 7. Comparing the IR spectra of compound **121** with the precursor **120**, the band corresponding to the bond C-H (*sp*) was only observed for compound **120**, which is good indicator of the molecular modification occurring successfully. ¹H (Table 8) and ¹³C NMR (Table 9) allowed to confirm the molecular transformations occurred.

Table 7 – IR data of compound **121**.

Group	ν (cm ⁻¹)
	121
C=O	1748
C=C (Stretch Ar)	1667, 1596, 1513
C-H (Bend sp²)	1424
C-O (Acyl)	1371, 1165, 1140
=C-N (Stretch)	1251
C-O (Alcoxy)	1083, 1047

¹H NMR spectra of compound **121** shows signals with integration and multiplicities corresponding to the aromatic protons: H-2', H-5', and H-6', as well as the methoxyl group (H-3''); the vinyl protons H-2 and H-3; and the methylic protons H-1'', similarly to the precursors **117** and **120**. The ¹H NMR spectra of compound **121** showed a set of peaks characteristic of the molecular modifications performed, signals for protons H-1''' to H-19'''. Since this structure is very complex and possess a large number of protons the assignments were made comparing with assignments described in the literature for the building block 2-azidoethyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranoside¹¹⁴.

Table 8 - ^1H NMR data of compound **121**.

 121	
Proton*	δ_{H} (values in ppm relative to Me_4Si as an internal reference)**
H-3'''	7.83, 1H, s
H-3	7.45, 1H, <i>d</i> , $J = 16.2$
H-6', H-2', H-5'	7.12-7.08, 3H, <i>m</i>
H-2	6.61, 1H, <i>d</i> , $J = 16.2$
H-11'''	5.54, 1H, <i>d</i> , $J = 8.8$
H-5'''	5.32, 2H, <i>dd</i> , $J = 13.1$
H-1'''	5.30, 1H, s
H-4'''	5.15, 1H, <i>dd</i> , $J = 13.3$ and 3.2
H-6'', H-7'' and H-8''	4.65-4.55, 3H, <i>m</i>
H-9''	4.30-4.25, 1H, <i>m</i>
H-17'''a	4.13, 1H, <i>d</i> , $J = 1.68$
H-17'''b	4.11, 1H, <i>d</i> , $J = 1.17$
H-10'''	4.07-4.00, 1H, <i>m</i>
H-3''	3.89, 3H, s
H-1''	2.37, 3H, s
H-14'', H-16'', H-19''	2.15 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H)
H-12'''	1.85, 3H, s

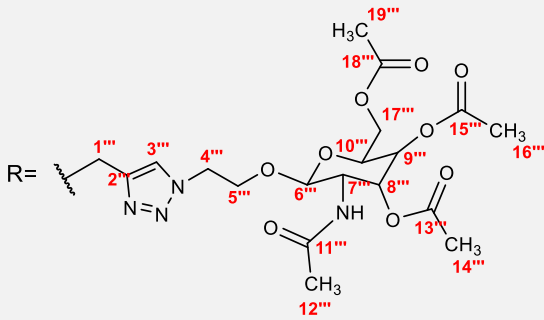
* Numbering of atoms was attributed for NMR assignments. Protons of the building block **117** maintained their assigned numbers.

** J values are presented in Hz.

Regarding ^{13}C NMR data, this derivative showed similar signals to the precursors **117** and **120**, spectra which correspond to the carbons of this moiety, and new signals

corresponding to the added molecular portion: C-1''' to C-19''' ¹¹⁴. Given the complexity of the molecular portion introduced and the large number of carbons in the molecule assignments were made comparing with data described in the literature for the reagent 2-azidoethyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranoside ¹¹⁴.

Table 9 - ¹³C data of compound **121**.



Carbon*	δ _c (values in ppm)	Carbon*	δ _c (values in ppm)
C-1	198.2	C-6'''	101.0
C-11'''	170.7	C-10'''	70.9
C-13''' and C-15'''	170.4	C-8'''	69.8
C-18'''	170.1	C-5'''	67.4
C-4'	149.9	C-9'''	66.1
C-3'	149.6	C-1'''	62.8
C-3	143.2	C-17'''	61.4
C-2'''	143.2	C-3''	55.9
C-1'	128.2	C-7'''	51.2
C-2	125.7	C-4'''	50.2
C-3'''	125.7	C-1''	27.4
C-6'	122.9	C-12'''	23.2
C-5'	113.6	C-16''' and C-14'''	20.7
C-2'	110.3	C-19'''	20.6

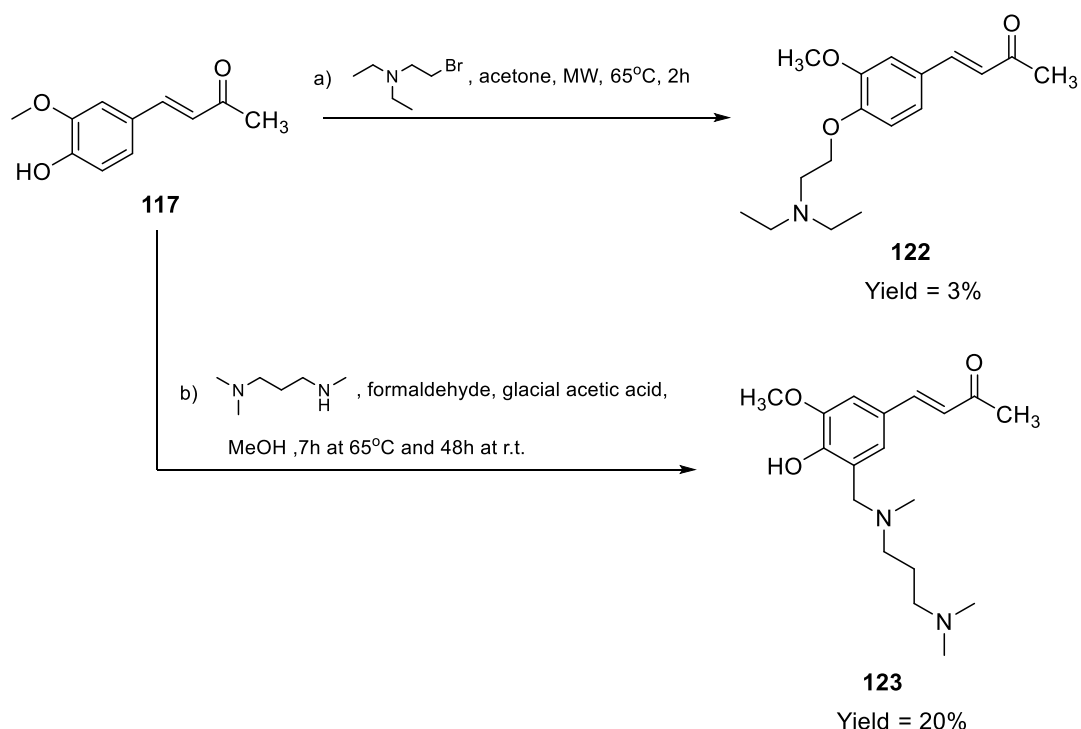
* Numbering of atoms was attributed for NMR assignments. Carbons of the building block **117** maintained their assigned numbers.

1.4. Synthesis of aminated derivatives of compound **117**, compounds **122** and **123**

The synthesis of the two aminated derivatives of compound **117**, 4-(4-(2-(diethylamino)ethoxy)-3-methoxyphenyl)but-3-en-2-one (**122**) and 4-(3-(((3-(dimethylamino)propyl)(methyl)amino)methyl)-4-hydroxy-5-methoxyphenyl)but-3-en-2-one (**123**), followed different synthetic approaches. Compound **122** was obtained by a S_N2 reaction¹⁰⁷ and compound **123** by a Mannich reaction¹¹⁵. In both cases, one equiv of the appropriate amine reacted with one equiv of compound **117** to afford the correspondent products. The strategy to synthesize compound **122** was similar to the previously section describing alkylated derivatives of compound **117**. Compound **123** was synthesized following a protocol¹¹⁵ where, primarily, the amine (*N,N,N'*-trimethyl-1,3-propanediamine) was cooled in ice bath for 5 min, then glacial acetic acid and formaldehyde were added dropwise. After 1h of stirring at room temperature the building block **117** was added, hence starting the reaction that would lead to the desirable product. Before this, another protocol¹¹⁶ was used as an attempt to synthesize compound **123** that revealed to be inefficient; the starting material compound **117** and *N,N,N'*-trimethyl-1,3-propanediamine were dissolved in MeOH in the presence of formaldehyde; this mixture was under stirring for 45 hours (h) at 50°C. There was not a specific order of adding reagents, nor acid involved in the reaction, unlike the protocol described above. The acid seems to be an essential part for the success of this reaction; it is possible to hypothesize that the acid helps the production of the Mannich base to consequently react with the building block **117**.

In these two reactions, the major products formed were compound **122** and **123**. However, other by-products could be observed by TLC analysis. Compound **123** was isolated after liquid-liquid extraction followed by column chromatography; while for compound **122**, the liquid-liquid extraction process was efficient in furnishing the desirable product, without the need of chromatographic methods.

Scheme 22 summarizes the reaction conditions and the results obtained.



Scheme 22 – Reaction conditions for the synthesis of compounds **122** and **123**.

Structure elucidation of these two aminated derivatives **122** and **123** was established by IR, ^1H and ^{13}C NMR.

The IR data compound **121** is showed in Table 10. Comparing the IR spectra of compounds **122** and **123** with the precursor **117**, the band corresponding to hydroxyl group was only observed for compound **117** and **123**, which is good indicator of the molecular modification occurring successfully. ^1H and ^{13}C NMR allowed to confirm the molecular transformations occurred (Table 11 and Table 12, respectively).

Table 10 – IR data of compounds **122** and **123**.

Group	ν (cm ⁻¹)	
	122	123
OH	-----	3500-3200
C-H (Stretch sp ²)	2959, 2925, 2855	2962
C=O	1729	1633
C=C (Stretch Ar)	1629	-----
C-H (bend sp ³)	1425	1471
C-O (Acyl)	1384, 1273	1385
C-O (Alcoxy)	1124	1126
C-H (bend Ar sp ²)	669	619

¹H NMR spectra of compound **122** shows similar signals corresponding to the protons of the building block moiety: aromatic H-2', H-5', and H-6'; methoxyl group H-3''; vinyl protons H-2 and H-3; and the methylic protons H-1''. In contrast to compound **117** and **123**, that showed a signal corresponding to the hydroxyl group ($\delta_{\text{H}} \approx 5$ ppm), derivatives **122** shows a set of peaks characteristic of the new molecular portion introduced (H-7' to H-10'). The signals of compound **122** corresponding to methylene protons H-7', H-8' and H-9' are deshielded due to the influence of electronegative oxygen and nitrogen, while H-10' are more shielded and the signals appear in the typical region of methyl protons.

¹H NMR spectra of compound **123** exhibits similar signals corresponding to the aromatic protons: H-2' and H-6'; the protons from the methoxyl H-3''; vinyl protons H-2 and H-3; and the methylic protons H-1''. The presence of only two signals integrating for two protons in the aromatic region highlights the molecular transformation occurred. The methylene protons H-7', H-9' and H-11' are slightly deshielded because of the nitrogen; the same happens for the methylic protons H-12'. Although by TLC analysis, compound **123** was considered to be pure, ¹H NMR spectrum revealed some signals at $\delta^1\text{H}$ 2.60-2.45, 2.39-2.17, and 1.81-1.73 ppm that are considered impurities resulting from the amine precursor and this compound was not further investigated concerning biological activities.

Table 11 – ^1H NMR data of compounds **122** and **123**.

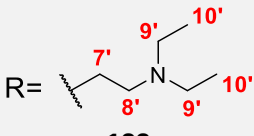
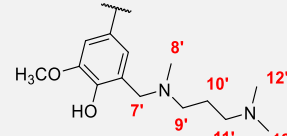
	122	123
Proton*	δ_{H} (values in ppm relative to Me_4Si as an internal reference)**	
H-3	7.46, 1H, <i>d</i> , $J = 16.2$	7.48, 1H, <i>d</i> , $J = 16.05$
H-6'	7.11, 1H, <i>dd</i> , $J = 8.3$ and 1.9	7.02, 1H, <i>d</i> , $J = 1.7$
H-2'	7.07, 1H, <i>d</i> , $J = 1.9$	6.84, 1H, <i>d</i> , $J = 1.7$
H-5'	6.91, 1H, <i>d</i> , $J = 8.3$	-----
H-2	6.61, 1H, <i>d</i> , $J = 16.2$	6.60, 1H, <i>d</i> , $J = 16.05$
H-4'	-----	5.49, 1H, <i>brs</i>
H-7'	4.24, 2H, <i>t</i> , $J = 6.2$	3.75, 2H, <i>s</i>
H-3''	3.89, 3H, <i>s</i>	3.92, 3H, <i>s</i>
H-8'	3.08, 2H, <i>t</i> , $J = 6.2$	1.99, 3H, <i>s</i>
H-9'	2.81, 4H, <i>q</i> , $J = 7.2$	2.92-2.81, 4H, <i>m</i>
H-11'	-----	
H-1''	2.38, 3H, <i>s</i>	2.36, 3H, <i>s</i>
H-10'	1.17, 6H, <i>t</i> , $J = 7.2$	1.33-1.25, 2H, <i>m</i>
H-12'	-----	2.41, 6H, <i>s</i>

* Numbering of atoms was attributed for NMR assignments. Protons of the building block **117** maintained their assigned numbers.

** J values are presented in Hertz (Hz).

^{13}C NMR assignments for these two derivatives were determined using spectroscopy data tables for typical ^{13}C chemical shifts, and by comparison with literature. These derivatives showed similar signals to the precursor **117**, which correspond to the carbons of this building block moiety, and new signals were observed corresponding to the molecular portion introduced. Compound **122** exhibit four new signals when comparing with compound **117** at $\delta^{13}\text{C}$: 66.4, 51.2, 47.7, and 11.0 ppm assigned to C-7', C-8', C-9' and C-10', respectively¹¹⁷. Compound **123** showed five new signals when comparing with compound **117** at $\delta^{13}\text{C}$: 61.1, 45.4, 55.2, 24.9, and 57.1 ppm assigned to C-7', C-8'/C-12', C-9', C-10, and C-11', respectively¹¹⁸.

Table 12 – ^{13}C NMR data of compounds **122** and **123**.

	 122	 123
Group*	δ_c (values in ppm)	
C-1	198.4	198.3
C-4'	150.3	150.6
C-3'	149.6	148.3
C-3	143.5	143.9
C-1'	127.8	125.1
C-2	125.4	124.3
C-6'	122.9	123.4
C-5'	112.7	109.8
C-2'	110.1	109.7
C-7'	66.4	61.1
C-3''	55.9	55.9
C-8'	51.2	45.4
C-12'	-----	
C-9'	47.7	55.2
C-1''	27.4	27.0
C-10'	11.0	24.9
C-11'	-----	57.1

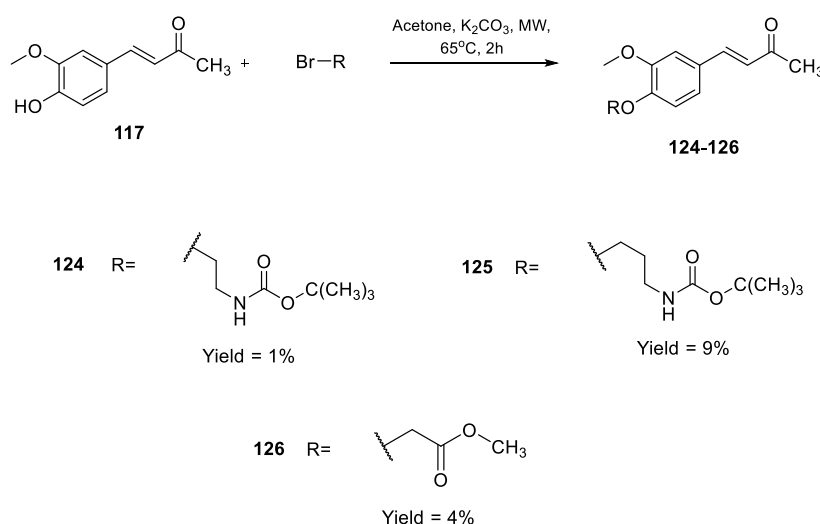
* Numbering of atoms was attributed for NMR assignments. Carbons of the building block **117** maintained their assigned numbers.

1.5. Synthesis of derivatives of compound **117** with carbamates and an ester, compounds **124**, **125** and **126**

The synthesis of the three derivatives *tert*-butyl (2-(2-methoxy-4-(3-oxobut-1-en-1-yl)phenoxy)ethyl)carbamate (**124**), *tert*-butyl (3-(2-methoxy-4-(3-oxobut-1-en-1-

yl)phenoxy)propyl)carbamate (**125**) and methyl 2-(2-methoxy-4-(3-oxobut-1-en-1-yl)phenoxy)acetate (**126**) followed a synthetic approach based on a S_N2 reaction¹⁰⁷. The reaction conditions were similar to the ones described for compounds **119** and **120**, one equivalent of 2-(boc-amino)ethyl bromide, 3-(boc-amino)propyl bromide, or methyl 2-bromoacetate reacted with one equivalent of compound **117** to afford compound **124**, **125**, and **126**, respectively. Scheme 23 summarizes the reaction conditions and the results obtained. The low yields can be due to the production of by-products during the reaction, which were not isolated and identified. These by-products could be resulting from the hydrolysis of the carbamates or ester groups.

Compound **124** and **125** were purified using chromatographic methods while compound **126** was purified by extraction.



Scheme 23 – Reaction conditions for the synthesis of compounds **124**, **125**, and **126**.

Structure elucidation of these three aminated derivatives was established by IR, ^1H and ^{13}C NMR. GC-MS analysis was also performed for the two amides, compounds **124** and **125**.

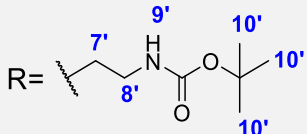
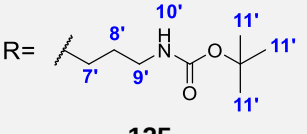
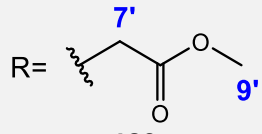
The IR data of these three compounds is shown in Table 13. The presence of bands between $3100\text{--}3500\text{ cm}^{-1}$, typical of the secondary amines N-H bond (compounds **124** and **125**), could indicate the presence of the desirable compounds; however, this band overlapped with the typical hydroxyl group band and further spectroscopic techniques were used to better characterize these structures. Regarding compound **126**, comparing the IR spectra of this compound with the precursor **117**, the band corresponding to hydroxyl group was only observed for compound **117** which is good indicator of the molecular modification occurring successfully.

Table 13 – IR data of compounds **124**, **125**, and **126**.

Group	ν (cm ⁻¹)		
	124	125	126
N-H (stretch)	3447	3377	-----
C-H (Stretch sp ³)	2920, 2852	-----	2918, 2849
C=O	1636	1686	1733
N-H (bend)	-----	1669, 1644	-----
C=C (Stretch Ar)	-----	1621, 1595, 1518	1634, 1515, 1463
C-O (Acyl)	1385	1273, 1260, 1225	1262
C-O (Alcoxy)	1099	1167, 1138, 1033	1111
C-H (bend Ar sp ²)	668	975	668

¹H and ¹³C NMR data of compounds **124**, **125**, and **126** are depicted in Table 14 and Table 15, respectively. ¹H NMR spectra of all three compounds show similar signals corresponding to the building block **117** moiety protons: aromatic H-2', H-5', and H-6'; methoxyl group H-3''; vinyl protons H-2 and H-3; and the methylic protons H-1'' Instead of the signal corresponding to the hydroxyl group, the derivatives **124**, **125**, and **126** show a set of peaks characteristic of the new molecular portion introduced. The methylene/methyl protons H-7' and H-8' for compound **124**, H-7' and H-9' for compound **125**, and H-7' and H-9' for compound **126** are deshielded due to the electronegativity of the oxygen and nitrogen and the corresponding signals appeared between 2 and 5 ppm. The signal corresponding to the proton of the secondary amine only appears in compound **125** spectrum, the absence of this signal in compound **124** could be due to chemical exchange of the -NH proton, that causes the signal to be very broad and weak.

Table 14 - ^1H NMR data of compounds **124**, **125**, and **126**.

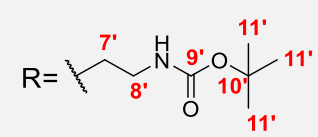
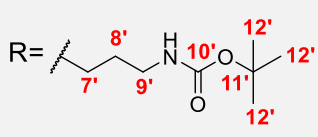
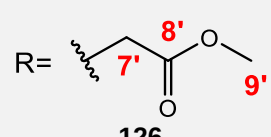
	 124	 125	 126
Proton*	δ_{H} (values in ppm relative to Me_4Si as an internal reference)**		
H-3	7.45, 1H, <i>d</i> , $J = 16.2$	7.46, 1H, <i>d</i> , $J = 16.2$	7.45, 1H, <i>d</i> , $J = 16.2$
H-6'	7.10, 1H, <i>dd</i> , $J = 8.1$ and 1.8	7.11, 1H, <i>d</i> , $J = 8.2$	7.09, 2H, <i>dd</i> , $J = 6.7$ and 1.9
H-2'	7.06, 1H, <i>d</i> , $J = 1.8$	7.07, 1H, <i>s</i>	
H-5'	6.94, 1H, <i>d</i> , $J = 8.1$	6.86, 1H, <i>d</i> , $J = 8.2$	6.80, 1H, <i>d</i> , $J = 6.7$
H-2	6.59, 1H, <i>d</i> , $J = 16.2$	6.61, 1H, <i>d</i> , $J = 16.2$	6.61, 1H, <i>d</i> , $J = 16.2$
H-7'	4.91, 2H, <i>m</i>	4.14, 2H, <i>t</i> , $J = 5.0$	4.76, 2H, <i>s</i>
H-8'	3.23-3.18, 2H, <i>m</i>	2.04, 2H, <i>t</i> , $J = 5.0$	-----
H-9'	-----	3.38, 2H, <i>d</i> , $J = 5.0$	3.81, 3H, <i>s</i>
H-3''	3.94, 3H, <i>s</i>	3.92, 3H, <i>s</i>	3.93, 3H, <i>s</i>
H-1''	2.37, 3H, <i>s</i>	2.38, 3H, <i>s</i>	2.38, 3H, <i>s</i>
H-10'	2.17, 9H, <i>s</i>	5.50, 1H, <i>brs</i>	-----
H-11'	-----	1.46, 9H, <i>s</i>	-----

* Numbering of atoms was attributed for NMR assignments. Protons of the building block **117** maintained their assigned numbers.

** J values are presented in Hz.

^{13}C NMR assignments for these three derivatives were determined using spectroscopy data tables for typical ^{13}C chemical shifts, and by comparison with literature. This derivative showed similar signals to the precursor **117**, which correspond to the carbons of this building block moiety, and new signals of the molecular portion added. Compound **123** exhibit five carbons peaks at $\delta^{13}\text{C}$: 68.2, 42.3, 160.5, 77.3, and 29.7 ppm assigned to C-7' to C-11', respectively¹¹⁹. Compound **124** shows six carbons peaks at $\delta^{13}\text{C}$: 68.2, 30.9, 38.9, 156.1, 79.0, and 28.5 ppm assigned to C-7' to C-12, respectively¹¹⁹. Compound **126** exhibits three carbon peaks at $\delta^{13}\text{C}$: 66.0, 169.0, and 52.4 ppm, corresponding to C-7' to C-9', respectively¹²⁰.

Table 15 - ^{13}C NMR data of compounds **124**, **125**, and **126**.

	 124	 125	 126
Group*	δ_{C} (values in ppm)		
C-1	198.5	198.4	198.4
C-9'	160.5	38.9	52.4
C-4'	148.2	150.5	149.7
C-3'	146.9	149.5	149.3
C-3	143.8	143.5	143.2
C-1'	127.0	127.6	128.8
C-2	125.0	125.3	125.5
C-6'	123.6	122.9	122.5
C-5'	114.8	112.2	113.4
C-2'	109.3	109.7	110.5
C-7'	68.2	68.2	66.0
C-10'	77.3	156.1	-----
C-8'	42.3	30.9	169.0
C-3''	56.0	55.8	56.0
C-11'	29.7	79.0	-----
C-1''	27.3	27.4	27.4
C-12'	-----	28.5	-----

* Numbering of atoms was attributed for NMR assignments. Carbons of the building block **117** maintained their assigned numbers.

Compounds **124** and **125** were analyzed GC-MS. MS data for these two compounds indicated that MS spectra are both in agreement with the suggested structures. Compound **124** exhibits a molecular ion peak of 334.8 m/z and base peak of 281.9 m/z. Compound **125** shows a molecular ion peak and base peak of 349.1 m/z.

1.6. Other synthetic attempts that were ineffective in obtaining curcumin derivatives

Throughout the development of the experimental work performed in this thesis other compounds were attempt to be synthesized, in order to increase the library of curcumin derivatives and for SAR studies. Although the target compounds were not isolated from the reaction conditions depicted in Table 16 and Table 17, these are listed to discuss some of the attempts and strategies used. Reagents, reaction conditions and desirable products of these attempts are depicted in Table 16 and Table 17.

Table 16 - Reaction conditions for the synthesis of curcumin derivatives using S_N2 reaction (entry 1 and 2) and the Claisen-Schmidt condensation (entry 3).

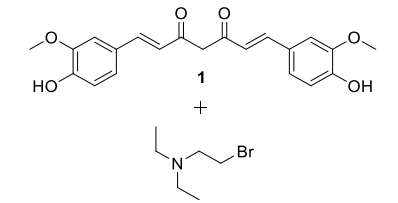
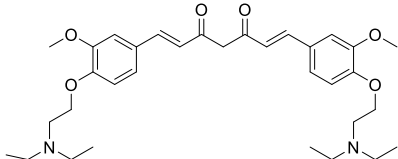
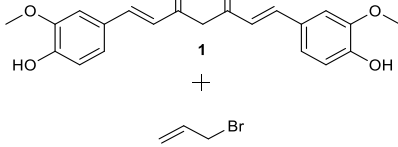
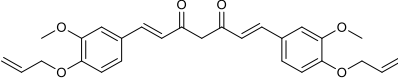
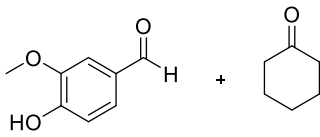
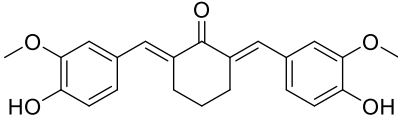
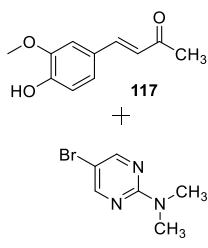
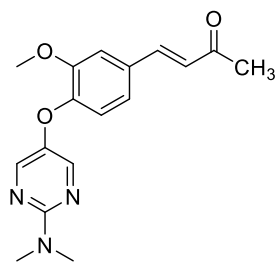
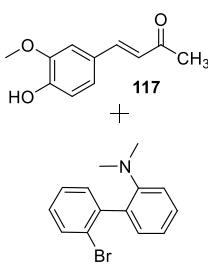
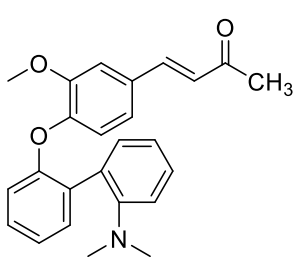
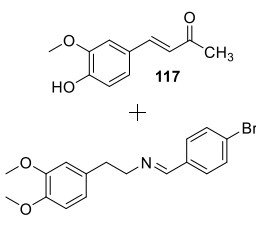
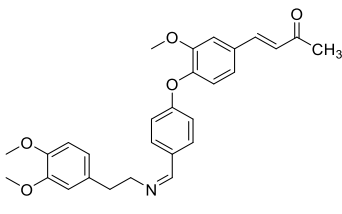
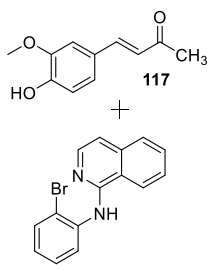
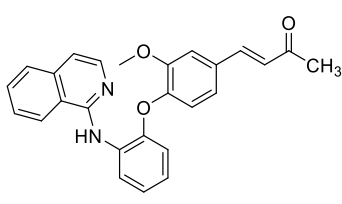
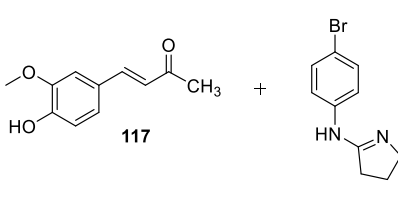
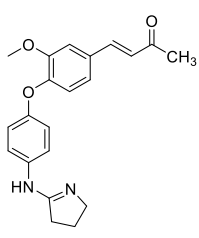
Reaction n.º	Reagents	Reaction conditions	Target molecule
1		Acetone, Cs_2CO_3 , MW 200W, 3h at 65°C ¹⁰⁷	
2			
3		EtOH, 4°C, 40% KOH. 28 h at r.t. ⁶⁹	 31

Table 17 - Reaction conditions for the synthesis of derivatives of compound **117** using Ullmann's reaction (entry 1-5).

Entry	Reagents	Reaction conditions	Target molecule
1		MeOH, K₂CO₃, CuI, <i>N, N</i>-dimethylglycine, 110°C, 2 days.*	
2			
3			
4			
5			

* Ullmann's reaction conditions were investigated previously with another scaffold in the group (unpublished results).

Entry 1 and 2 of Table 16 followed a S_N2 approach, similar to the ones described for previous compounds (**119**, **120**, **122**, **124**, **125**, and **126**), with the aim of producing the corresponding curcumin derivatives. In these reactions, the building block curcumin (**1**) was mixed with the appropriate amine or alkyne bromide and let to react in alkaline conditions. After work up, the major product was isolated and analyzed by ¹H and ¹³C NMR spectroscopy. The results led to the conclusion that the isolated compounds were not the desirable compounds and no further investigations were performed. Alkaline conditions were hypothesized to degrade curcumin, which could have been the main reason for this outcome. The target molecule of entry 2 of Table 16 has already been described as possessing antiviral activity ¹²¹, hence our aim to synthesize and test this compound in order to evaluate the cytotoxicity and P-gp inhibition.

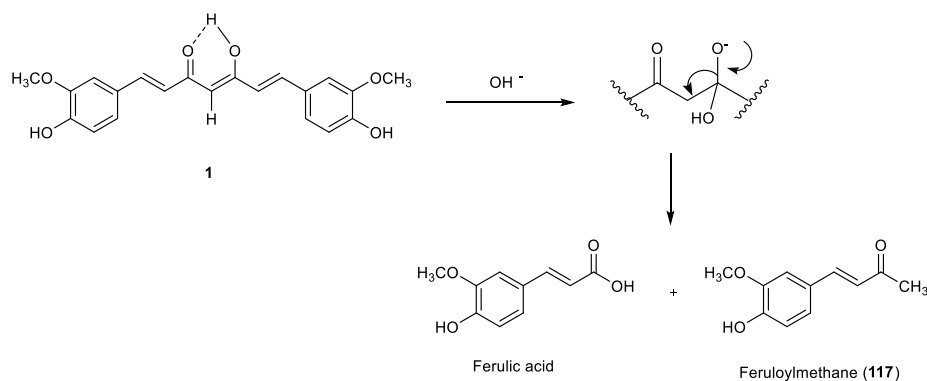
In entry 3 of Table 16 had the aim of producing a third building block and curcumin derivative. Similarly to compounds **117** and **33**, a Claisen-Schmidt condensation was the synthetic strategy; after work-up, the isolated compound was analyzed by ¹H and ¹³C NMR and since the spectra indicated that the isolated compound was not the desirable product no further investigations were performed.

Entry 1 to 5 of Table 17 followed a different synthetic strategy from the previous ones, the Ullmann's reaction. All the major products formed in the reaction were isolated using chromatographic methods and analyzed by ¹H and ¹³C NMR. The spectra indicated that the isolated compound were not the desirable product and no further investigations were performed.

2. Stability and photostability studies

Under neutral and basic pH conditions, curcumin (**1**) is very unstable, and eventually is degraded to ferulic acid and feruloylmethane, as depicted in Scheme 24^{4, 94}. Such instability is associated with the methylene moiety between the two carbonyl groups: the hydrogens of the methylene group are very acidic making this molecule only stable at acidic pH. Curcumin (**1**) is hydrophobic; therefore, it is soluble in organic solvents such as dimethyl sulfoxide (DMSO), MeOH, EtOH or acetone and poorly soluble in aqueous solvents ⁴. Curcumin (**1**) in phosphate buffer at pH 7.2 is rapidly and almost completely degraded (approximately 90%, within 30 minutes). Degradation of curcumin (**1**) is extremely slow at pH 1 to 6. Vanillin, ferulic acid, feruloylmethane (**117**) and *trans*-6-(4'-

hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal are degradation products of curcumin (**1**)⁴. Besides being prone to hydrolysis under alkaline conditions, curcumin (**1**) is also very susceptible to photochemical degradation⁹⁴.



Scheme 24 - Hydrolytic degradation of curcumin (**1**).

Given these curcumin (**1**) characteristics, were tested and compared the other two building blocks synthesized **117** and **33** to evaluate if they are more, less or as stable as curcumin (**1**).

2.1. Stability studies

Stability studies concerning different pH, solvents, temperature and storage time were performed by high-performance liquid chromatography (HPLC) and aimed to evaluate compounds stability in a quantitative manner, thus examining if compounds **117** and **33** are more, less or as stable as curcumin. Stability studies were divided in different assays each one with a different purpose: a pH stability assay, a biological buffer assay and temperature/storage time assay. The stability was evaluated comparing the challenged samples with the standard compounds dissolved in DMSO, immediately injected at room temperature. The curcumin (**1**) used was purchased, and according to our study showed a purity level of 55% (information provided by the seller company describes 65% purity by HPLC analysis). Compounds **117** and **33** used for these assays were the compounds isolated after the described purification procedures, which showed 96% and 72% purity in HPLC analysis, respectively. Figure 18 shows curcumin (**1**), compound **117** and **33** chromatograms in the chromatographic system analyzed.

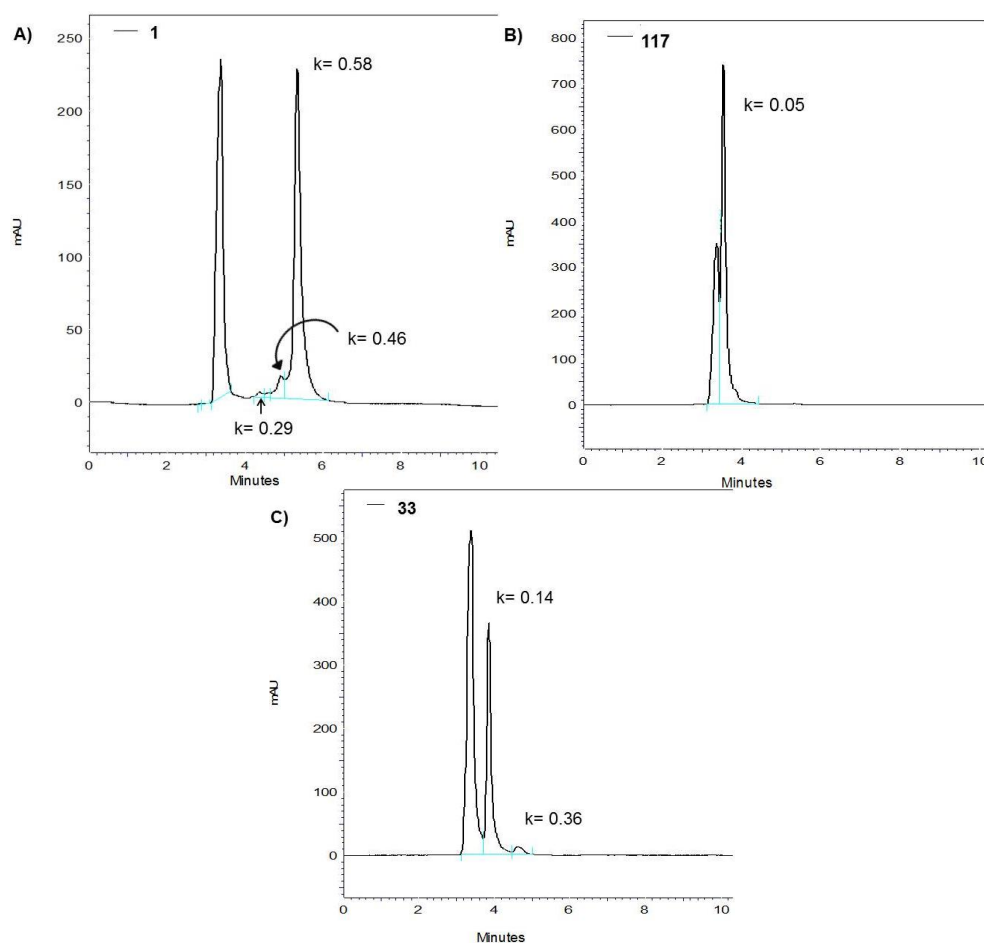


Figure 18 – Representative HPLC chromatogram of (A) curcumin (**1**), (B) compound **117** and (C) **33** [$\lambda=254$ nm, C18, isocratic solution of MeOH:H₂O:acetic acid (70:30:1)]. HPLC chromatogram of curcumin (**1**): bisdemethoxycurcumin (**3**, $k = 0.29$), desmethoxycurcumin (**2**, $k = 0.46$) and curcumin (**1**, $k = 0.58$)¹²². HPLC chromatogram of compound **117** ($k = 0.05$). HPLC chromatogram of compound **33** ($k = 0.14$).

At 254nm it is possible to observe a second major peak in all chromatograms with a retention time of 3.36-3.38 min; this peak can be assigned to DMSO which also absorb at 254 nm (data not shown). In curcumin (**1**) chromatogram three peaks were observed that were attributed to: bisdemethoxycurcumin (**3**, $k = 0.29$), desmethoxycurcumin (**2**, $k = 0.46$) and curcumin (**1**, $k = 0.58$) as described in the literature¹²². Commercialized curcumin (**1**) contains small amounts of other curcuminoids, hence the presence of more than one peak.

2.1.1. pH Stability assay

Five pH values were selected and obtained with following buffers: HCl pH 1.0, sodium acetate pH 5.0, potassium phosphate pH 6.7, phosphate buffered saline (PBS) pH 7.4 and sodium boric acid pH 9.1. The compounds were dissolved in each buffer, to the final concentration of 10^{-4} M, and let to incubate overnight at room temperature, except in HCl buffer that incubated only for 75 min¹²³.

After overnight incubation the compounds in certain buffers precipitated: curcumin (**1**) precipitated in all buffers; compound **117** precipitated in all buffers; compound **33** at pH 1, 6.7 and 7.4. The precipitate was filtrated and both filtrated and solid (dissolved in MeOH) were analyzed separately.

Figure 19 and Figure 20 shows curcumin (**1**) filtrate solution chromatogram after being subjected to pH 9.1 and curcumin (**1**) precipitate overlapped chromatograms after being subjected to different buffers, respectively.

The filtrate solution of curcumin (**1**) corresponded solely to buffer solution (data not shown). It was hypothesized that all curcumin (**1**) precipitated at lower pH values (pH 1, pH 5, pH 6.7 and 7.4) because this compound would be in a non-ionized form and, thus, not soluble in aqueous solvents; at the highest pH value (pH 9.1) curcumin (**1**) could have been degraded; the chromatogram depicted in Figure 19 is very different from the standard one; furthermore, the spectra of each peak showed no similarities with curcumin (**1**) UV spectra. These peaks could be due to degradation products as it would be expected since curcumin (**1**) degrades in alkaline conditions.

The precipitate formed after curcumin (**1**) incubation with certain buffers was dissolved and analyzed. As suspected, buffers with lower pH values (pH 1, 5, 6.7 and 7.4) are associated to the non-ionized form and thus the compound precipitate in aqueous solvents. From pH 1 to 7.4 (Figure 20), the main peak ($k = 0.66$) corresponds to curcumin. At pH 9.1 (Figure 19) is observed a different compound, possibly the same as the one present in chromatogram shown in Figure 19. The proportion of the area correspondent to curcumin (**1**) peak maintained around 55% (with small oscillations), except for pH 7.4 in which the area decreased to 49%.

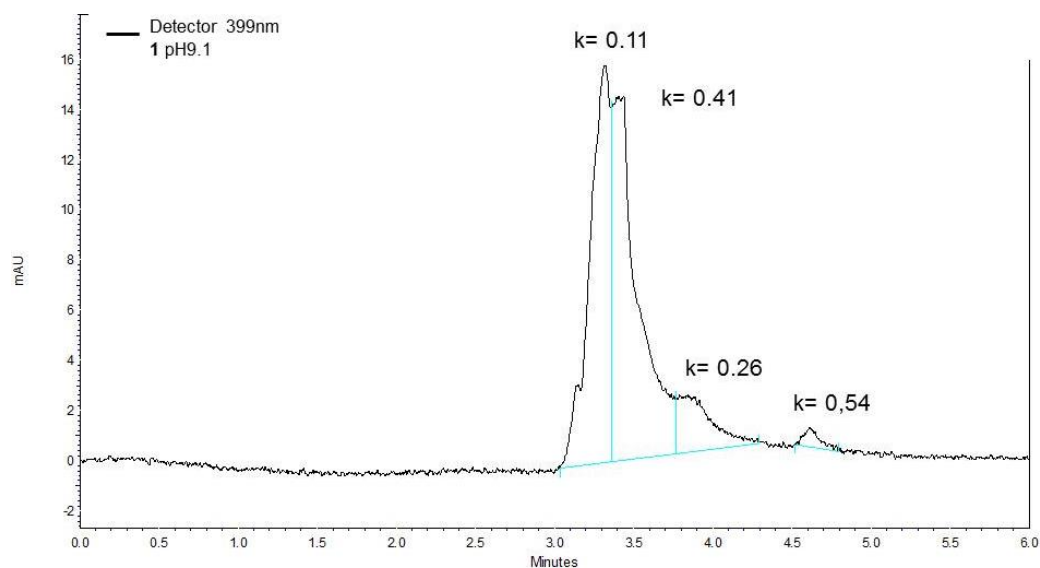


Figure 19 - Curcumin (**1**) filtrate solution chromatogram after being subjected to sodium boric acid (pH 9.1) and respective retention factor of each peak [$\lambda=399$ nm, C18, isocratic solution of MeOH:H₂O:acetic acid (70:30:1)].

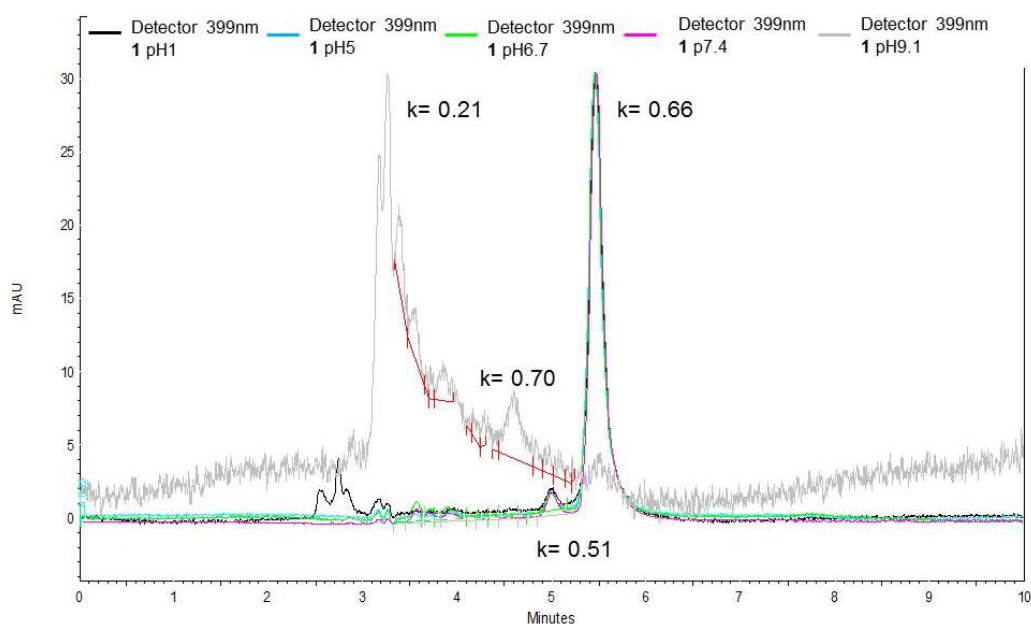


Figure 20 – Curcumin (**1**) precipitate overlapped chromatograms after being subjected to different buffers [$\lambda=399$ nm, C18, isocratic solution of MeOH:H₂O:acetic acid (70:30:1)].

Figure 21 depict compound **117** filtrate overlapped chromatograms after being subjected to different buffers. It is possible to observe that in the chromatograms for higher pH values (pH 6.7, 7.4, and 9.1) besides compound **117** another peak is present. This second peak could be due to some compound **117** degradation, caused by alkaline

conditions. The spectra of second peak showed no similarities with compound **117** UV spectra.

The precipitate chromatograms of compound **117** corresponded solely to buffer solution (data not shown). The proportion of the area correspondent to compound **117** peak maintained around 96% (with small oscillations), except for pH 9.1 in which the area decreased to 55%.

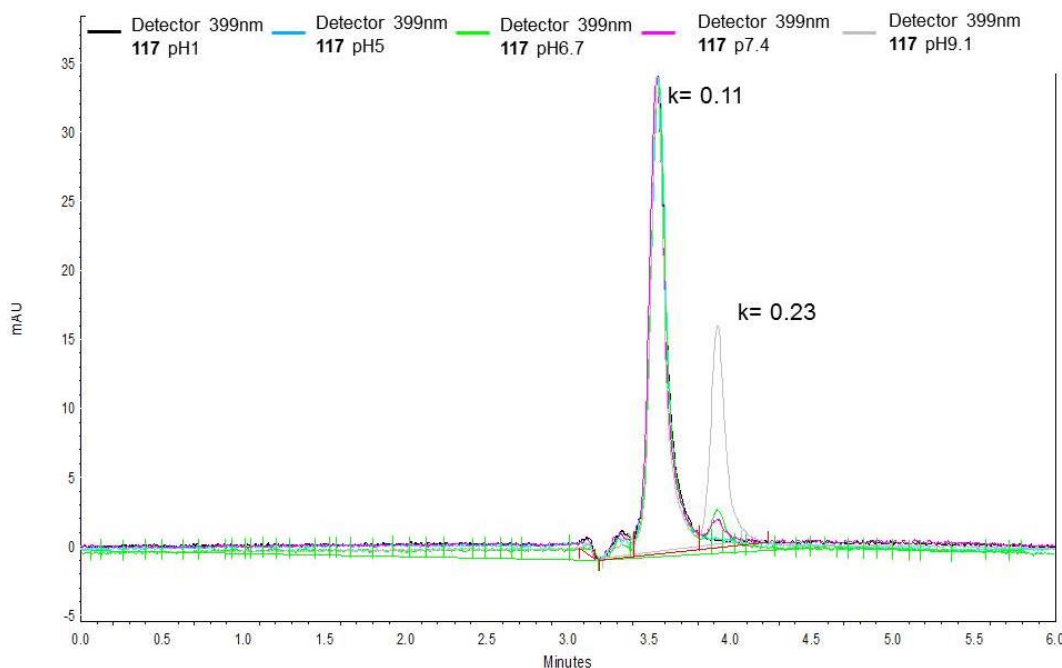


Figure 21 - Compound **117** filtrate overlapped chromatograms after being subjected to different buffers [$\lambda=399$ nm, C18, isocratic solution of MeOH:H₂O:acetic acid (70:30:1)].

Figure 22 and Figure 23 shows compound **33** filtrate solution overlapped chromatograms after being subjected to different buffers and compound **33** precipitate overlapped chromatograms after being subjected to different buffers, respectively. Compound **33** solution maintained the same composition regardless the buffer. From this result it was hypothesized that this compound under different pH conditions can be the most stable of the three.

In Figure 23, compound **33** chromatograms exhibited a main peak with a retention factor of 0.18 that corresponds to the indicated compound. No considerable differences can be observed between this and the standard chromatogram. The proportion of the area correspondent to compound **33** peak maintained around 72% (with small oscillations) in both filtrate solution and precipitate chromatograms.

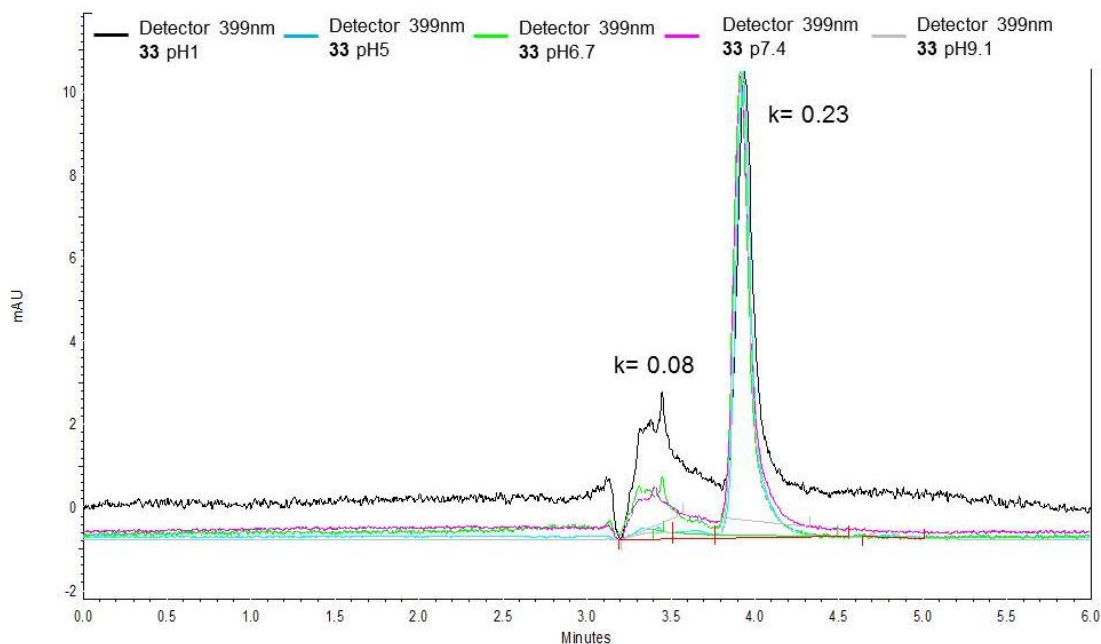


Figure 22 - Compound **33** filtrate solution overlapped chromatograms after being subjected to different buffers [$\lambda=399$ nm, C18, isocratic solution of MeOH:H₂O:acetic acid (70:30:1)].

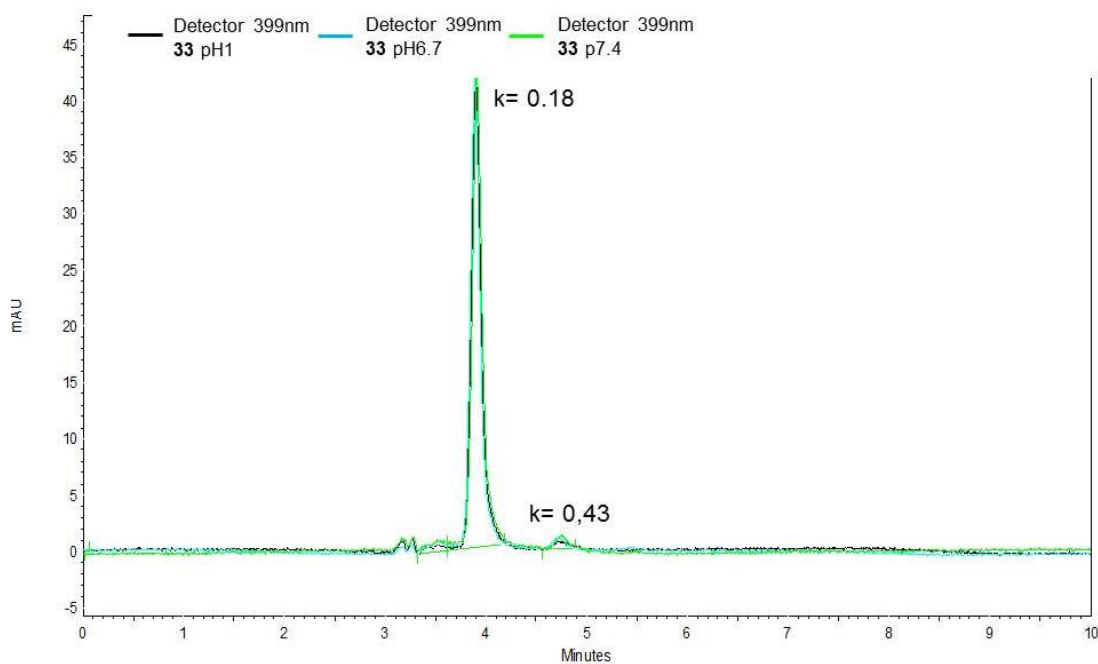


Figure 23 - Compound **33** precipitate overlapped chromatograms after being subjected to different buffers [$\lambda=399$ nm, C18, isocratic solution of MeOH:H₂O:acetic acid (70:30:1)].

By chromatograms analysis of the three compounds subjected to different buffers we can hypothesize that compound **33** is the most stable compounds, followed by

compound **117** and then curcumin (**1**), which exhibited the most prominent degradation in alkaline conditions.

2.1.2. Biological buffer assay

The biological buffer assay was selected to evaluate if the compounds showed significant degradation in the cell medium used for cell culture (see biological reagents). It is of great importance that the compounds maintain stable in this buffer in order to reach the cells as intact molecules, not as degradation products. The compounds were incubated for 5, 10, 20 and 30 min at 37°C in biological buffer, having a final concentration of $1.5 \times 10^{-3} \text{M}$, the solution were filtrated and injected. Figure 24, Figure 25 and Figure 26 exhibit curcumin (**1**), compound **117** and **33** overlapped chromatograms when subjected to biological buffer in the specified condition.

Curcumin (**1**) overlapped chromatograms show that the retention factor of the main peak are not exactly the same ($k \approx 0.55\text{—}0.70$). Nevertheless, the two main peaks observed have overlapping UV-Vis spectra and they were hypothesized to correspond both to curcumin (**1**). The same was observed for compounds **117** ($k \approx 0.26\text{--}0.37$) and **33** ($k \approx 0.34\text{--}0.46$), respectively. The presence of two distinct peaks can be due to the pKa of the compound being similar to the pH of the biological buffer, which can be translated into a protonated and deprotonated form of the same compound in the sample.

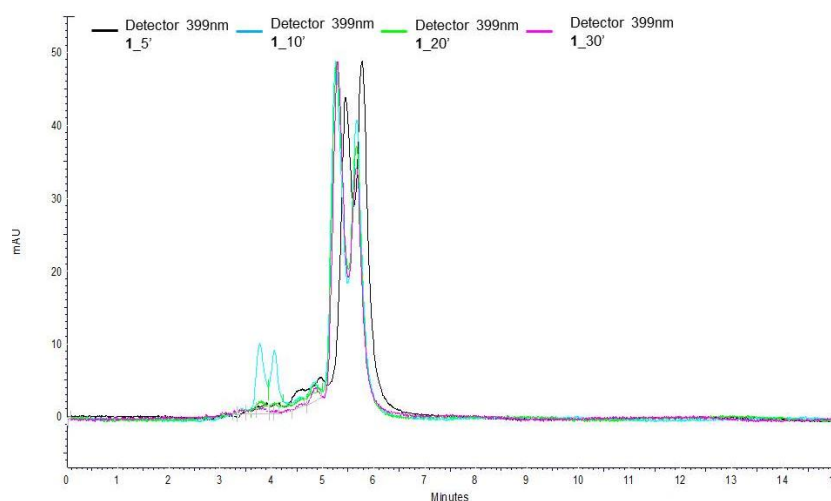


Figure 24 - Curcumin (**1**) overlapped chromatograms when subjected to biological buffer for 5, 10, 20 and 30 min at 37°C [$\lambda=399$ nm, C18, isocratic solution of MeOH:H₂O:acetic acid (70:30:1)].

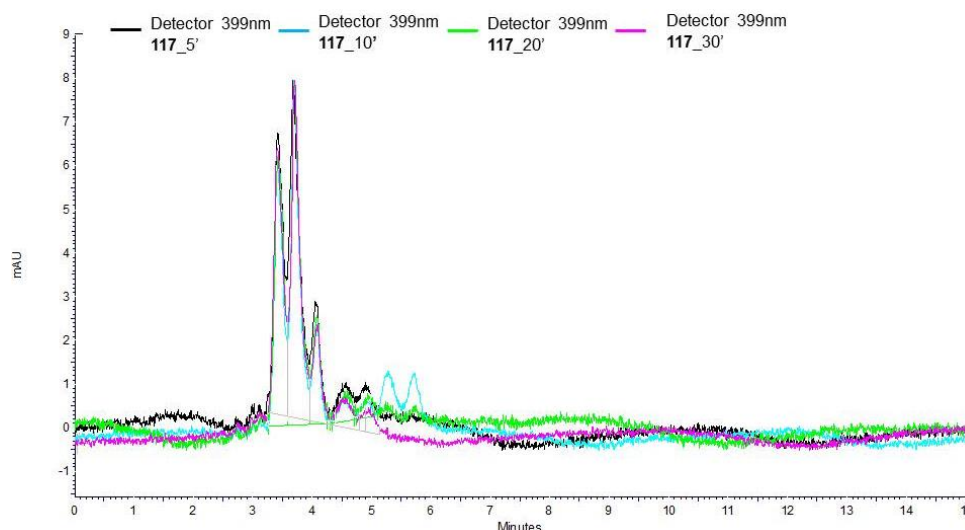


Figure 25 - Compound **117** overlapped chromatograms when subjected to biological buffer for 5, 10, 20 and 30 min at 37°C [λ =399 nm, C18, isocratic solution of MeOH:H₂O:acetic acid (70:30:1)].

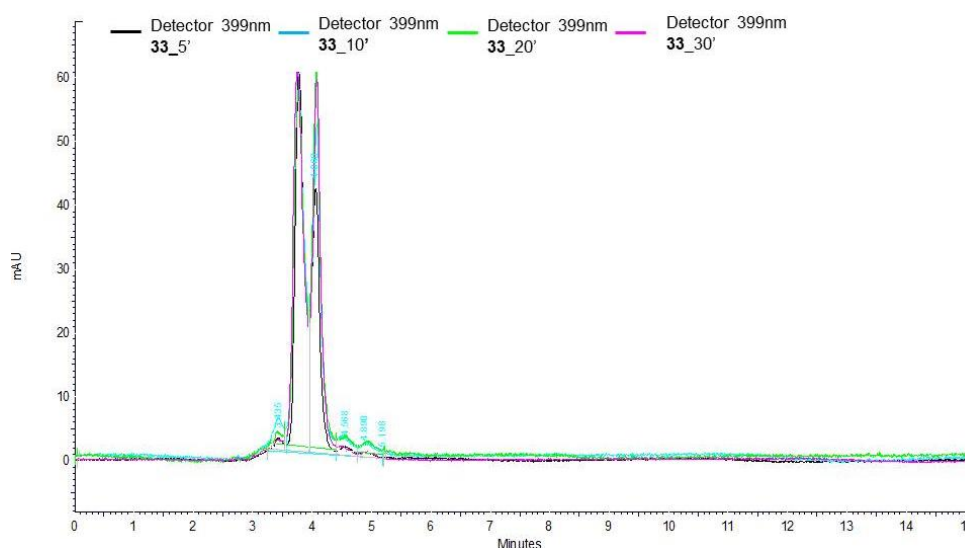


Figure 26 – Compound **33** overlapped chromatograms when subjected to biological buffer for 5, 10, 20 and 30 min at 37°C [λ =399 nm, C18, isocratic solution of MeOH:H₂O:acetic acid (70:30:1)].

When comparing with the standard solutions the compounds exhibited a similar behavior in the biological buffer and appear to be chemically stable at these conditions. Hence, the biological buffer does not degrade these compounds (**1**, **33**, and **117**).

2.1.3. Temperature/storage time assay

The temperature/storage time assay was performed in order to evaluate if the compounds would maintain their stability with different temperatures conditions and storage times. The compounds were dissolved in DMSO, having a final concentration of 10^{-4} M, and store at -20°C , 4°C and room temperature for 6, 15, and 21 days ¹²³. After being subjected to such conditions the stability was evaluated by HPLC. Figure 27, Figure 28, and Figure 29 exhibit curcumin (**1**), compound **117** and **33** overlapped chromatograms when subjected to -20°C , 4°C and r.t. along 6, 15, and 21 days, respectively.

Curcumin (**1**) chromatograms indicate that this compound degraded with all temperatures along all storage days defined. Between 3-4.6 min of the chromatographic run the degradation products are visible and around 5-6 min other curcuminoids, or degradation products are observed with similar UV-Vis spectra.

The proportion of the area correspondent to curcumin/curcuminoids peak gradually decreased with increased storage time from 54% (with small oscillations) to 28%. However, the proportion of the area correspondent to curcumin/curcuminoids peak was similar between temperatures with the same storage time.

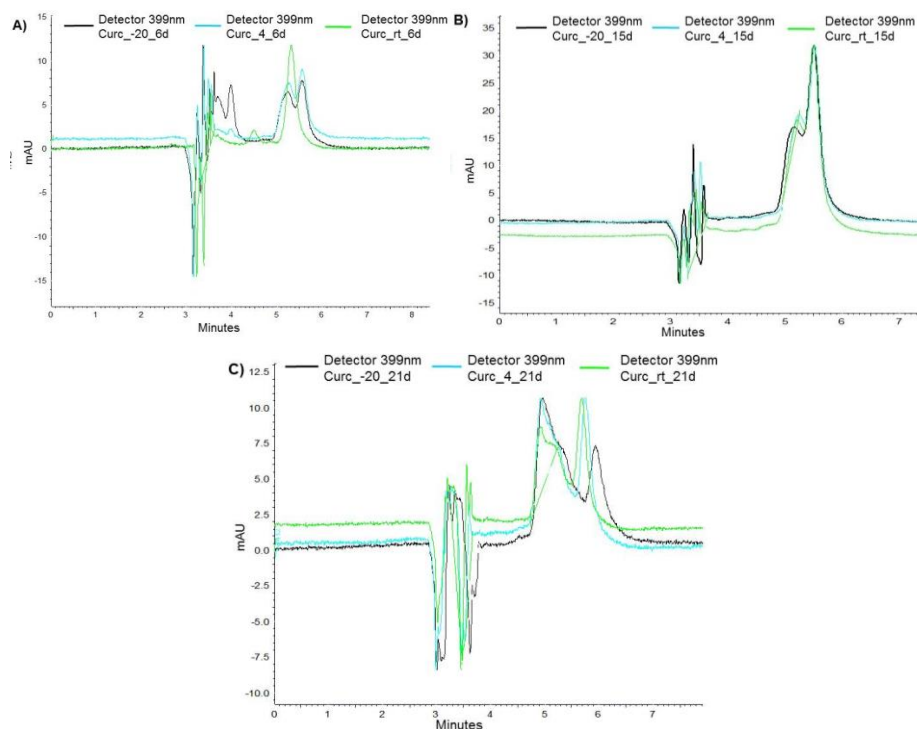


Figure 27 - Curcumin (**1**) overlapped chromatograms when subjected to -20°C , 4°C and r.t. along (A) 6 days, (B) 15 days, and (C) 21 days [$\lambda=399$ nm, C18, isocratic solution of MeOH:H₂O:acetic acid (70:30:1)].

Compound **117** also degraded with all temperatures along all storage days defined. Between 3.45-3.65 min of the chromatographic run compound **117** or a degradation product with similar UV-Vis spectra are observed, and peaks with higher retention time are degradation products. Two possible degradation products are observed after 6 days, and three possible degradation products after 15 and 21 days. The proportion of the area correspondent to compound **117** peak gradually decreased with increased storage time from 94% (with small oscillations) to 34%. However, the proportion of the area correspondent to compound **117** peak was similar between temperatures with the same storage time.

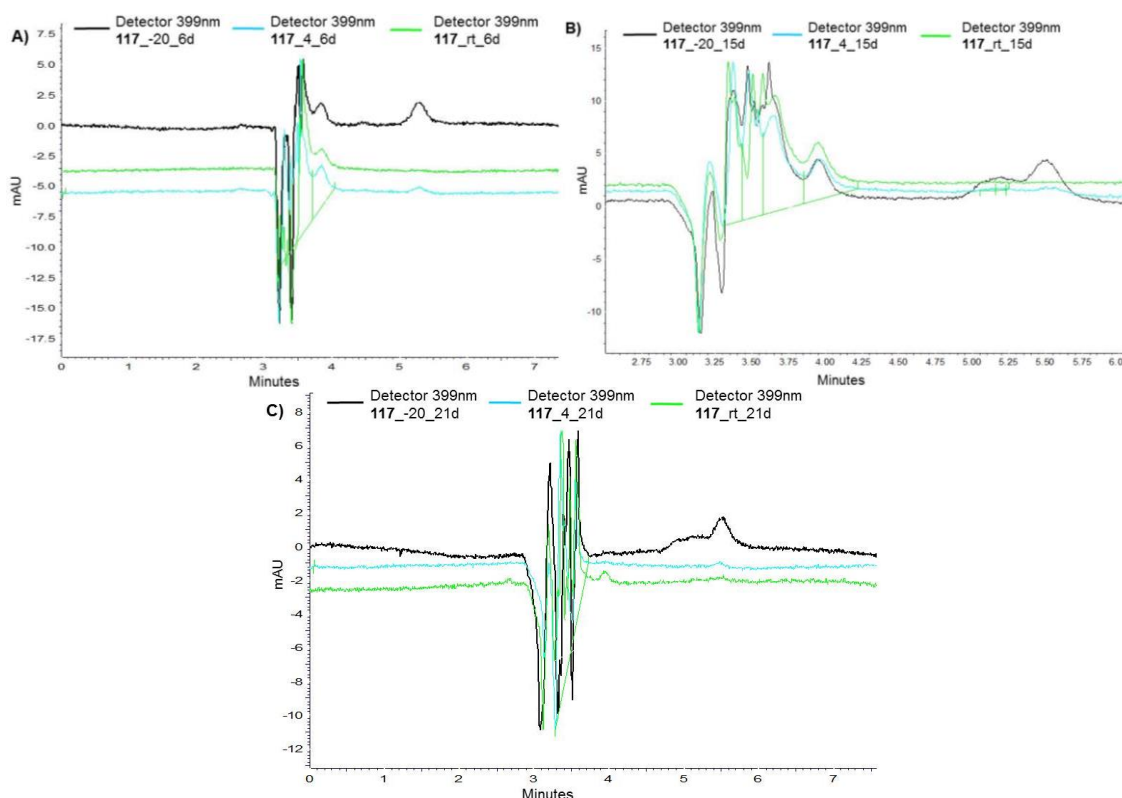


Figure 28 - Compound **117** overlapped chromatograms when subjected to -20°C, 4°C and r.t. over (A) 6 days, (B) 15 days, and (C) 21 days [$\lambda=399$ nm, C18, isocratic solution of MeOH:H₂O:acetic acid (70:30:1)].

Regarding compound **33**, after 6 days storage the chromatograms exhibit the presence of this compound between 3.86-3.88 min and impurities with lower retention time. At 15 days two peaks could be attributed to compound **33** or a degradation product with similar UV-Vis spectra, between 3.70-4min of the chromatographic run. Peaks with lower retention time are due to degradation products. After 21 days storage, compound **33**

was degraded or is present in vestigial amounts, and only degradation products are observed.

The proportion of the area correspondent to compound **33** peak gradually decreased with increased storage time from 56% (with small oscillations) to 36%. However, the proportion of the area correspondent to compound **33** peak was similar between temperatures with the same storage time.

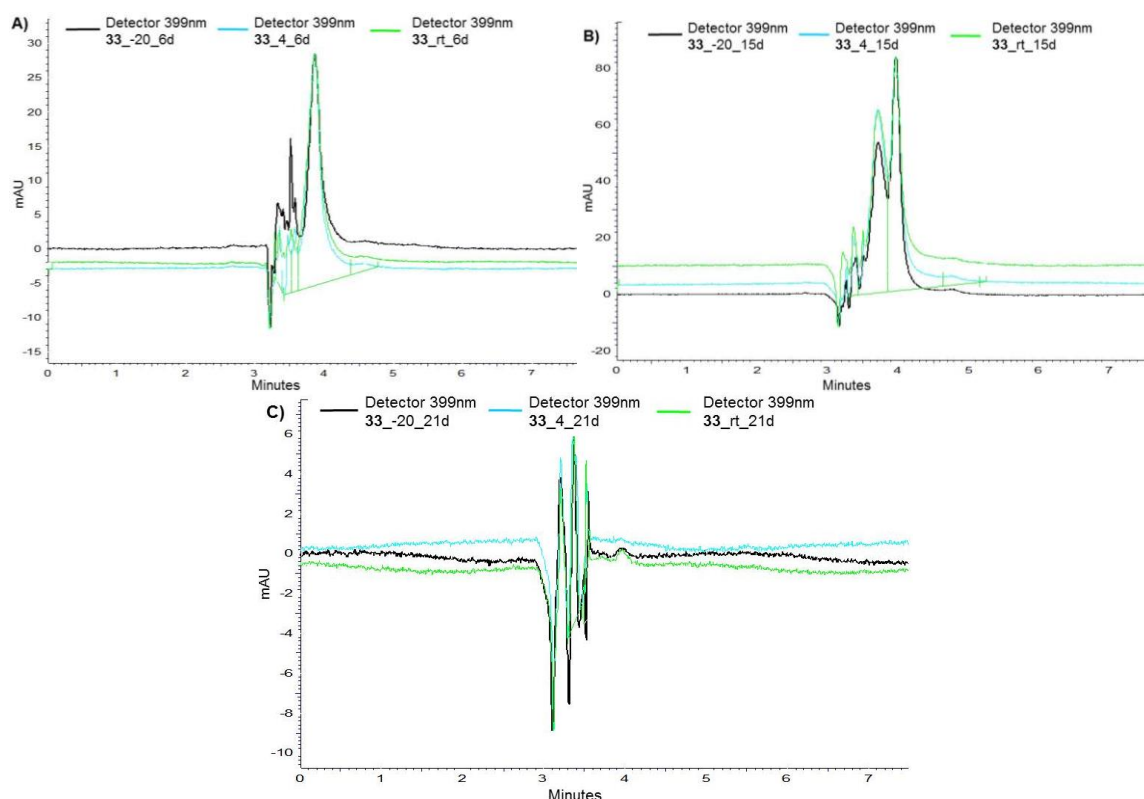


Figure 29 - Compound **33** overlapped chromatograms when subjected to -20°C, 4°C and r.t. over (A) 6 days, (B) 15 days, and (C) 21 days [$\lambda=399$ nm, C18, isocratic solution of MeOH:H₂O:acetic acid (70:30:1)].

In this assay, it was possible to observe two peaks for each compound, which could be due to the protonation form of the compounds or could be degradation products (intermediates for other degradation products) with identical spectra to the parent compound curcumin (**1**), **117** or **33**. Temperature variations seem to not influence significantly the stability. All compounds have shown to be unstable along the temperature and storage times tested. Curcumin (**1**) and compound **117** may have similar stability, after 6 days of storage they already show degradation signals. Compound **33** was proving to be the most stable, but at 21 days of storage the compound had completely decomposed.

2.2. Photostability studies

The photostability studies aimed to analyze compounds stability in a qualitative manner, thus examining the compounds degradation. Photostability studies of curcumin (**1**), compound **117**, and **33** methanolic solutions were accomplished using UV-Vis spectrophotometry. The solutions were subjected to selected times intervals of light exposure: 30 min, 1 h, 2 h, 3 h and 24 h, and compared to a standard solution, 0 min of light exposure of the respective compound. UV-Vis spectra of each standard solution are shown in Figure 30 and revealed the spectral differences between compounds in maximum absorption wavelength and intensity of absorption. Curcumin (**1**) standard solution shows three maximum absorption wavelengths at 424, 258, and 207 nm with the respective molar attenuation coefficient (ϵ) of 17400, 4800, and 10900 $\text{M}^{-1}\cdot\text{cm}^{-1}$. Compound **117** standard solution shows four maximum absorption wavelengths at 399, 243, 222, and 205 nm and ϵ of 11400, 5700, 4900, and 6000 $\text{M}^{-1}\cdot\text{cm}^{-1}$, respectively. Compound **33** standard solution shows four maximum absorption wavelengths at 387, 368, 264, and 208 nm with the respective ϵ of 11100, 10000, 6000, and 12800 $\text{M}^{-1}\cdot\text{cm}^{-1}$. To evaluate compounds susceptibility to photodegradation is essential to analyze bathochromic or hypsochromic shifts as well as hyperchromic and hypochromic effects on UV-Vis spectra of light exposed solution⁹⁴. The bands observed for the three compounds at 205-208 nm could indicate $n\rightarrow\sigma^*$ transitions, which is usually associated with alcohols. The conjugated ketone π system of the three compounds appear in bands at higher wavelengths than 300 nm due to the $n\rightarrow\pi^*$ transitions, and around 250nm due to $\pi\rightarrow\pi^*$ transitions. The bands at 424 and 399 nm, of curcumin (**1**) and compound **33** respectively, could be attributed to the aromatic chromophore conjugated with the double bond and carbonyl group of each compound¹²⁴.

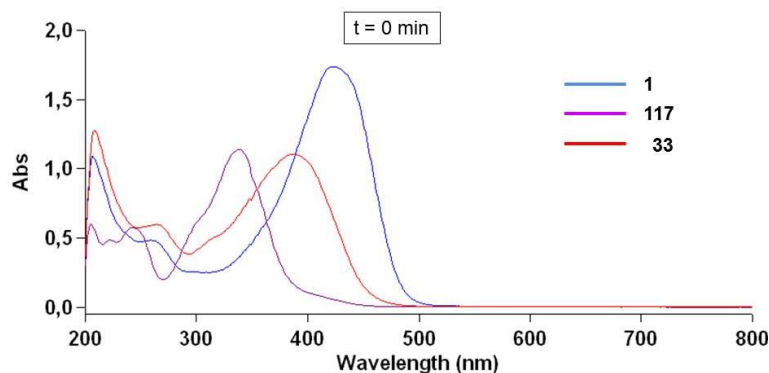


Figure 30 – UV-Vis spectra of standard solutions at 10^{-4}M concentration of curcumin (**1**), compound **117** and compound **33** ($t=0$ min).

Curcumin (**1**) UV-Vis spectra regarding different light exposure times are depicted in Figure 31. All the light exposed and protected solutions had shown no considerable bathochromic or hypsochromic shifts when compared to the standard solution. The 30 min light exposed solution show a generalized hypochromic effect (Figure 31 A); consecutive light exposed solutions show a hyperchromic effect regarding 424 nm maximum absorption and an hypochromic effect regarding 258 and 207 nm maximum absorption. After 24h of light exposure (Figure 31 B) the solution shows a generalized hypochromic effect in all maximum absorption wavelengths.

A previously described study indicated that curcumin (**1**) is more photostable than the curcuminoid BDMC (**3**) in MeOH solution⁹⁴. However, when curcumin (**1**) is in a cyclodextrin solution shows lower photostability and the curcuminoid BDMC (**3**) shows higher photostability⁹⁴.

A control solution protected from light exposure revealed no significant changes in the UV-Vis spectra when compared to the standard solution (data not shown).

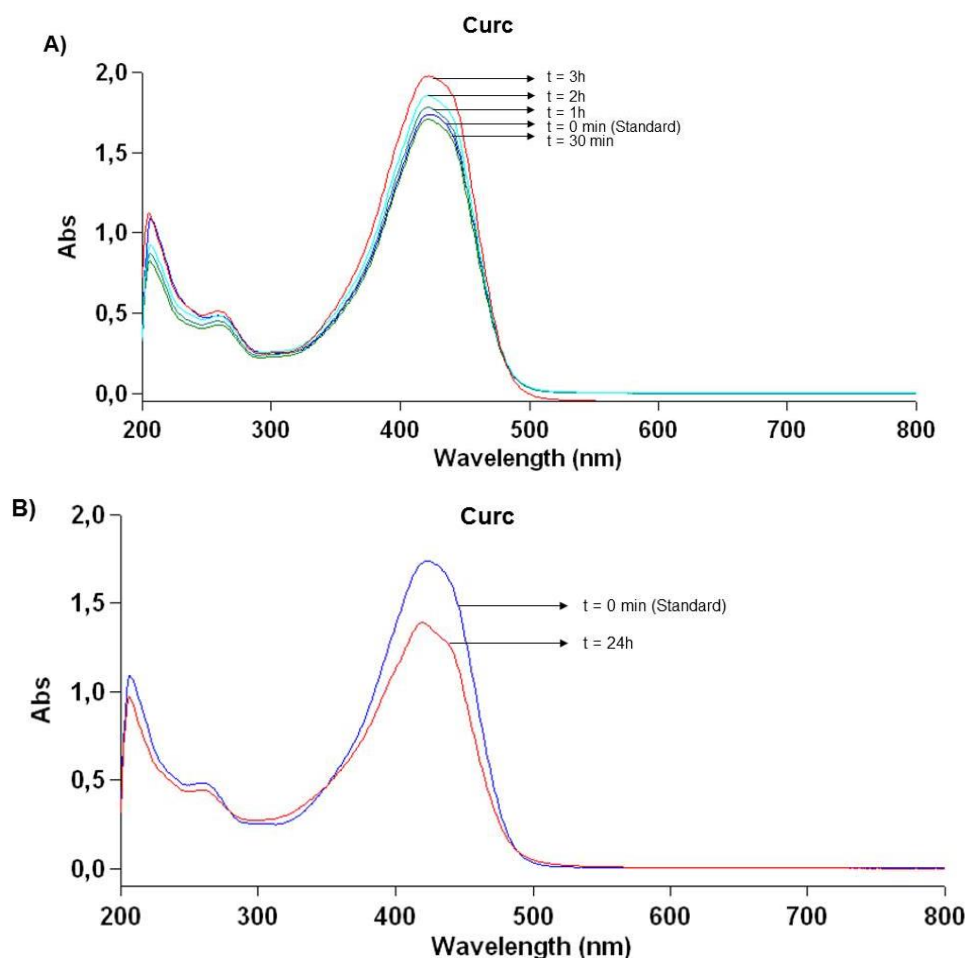


Figure 31 – A) UV-Vis spectra of curcumin (**1**) after different light exposure times, from 0 min (standard) to 3h; B) UV-Vis spectra of curcumin (**1**) after 24h light exposure and standard solution.

Compound **117** UV-Vis spectra regarding different light exposure times are depicted in Figure 32. All the light exposed and protected solutions (controls not shown) had shown no considerable bathochromic or hypsochromic shifts when compared to the standard solution. Consecutive light exposed solutions show a hyperchromic effect throughout the spectra, which is still observed after 24 h of light exposure in all maximum absorption wavelengths compared with both compound **117** UV-Vis at 0 min and the corresponding protected control solutions.

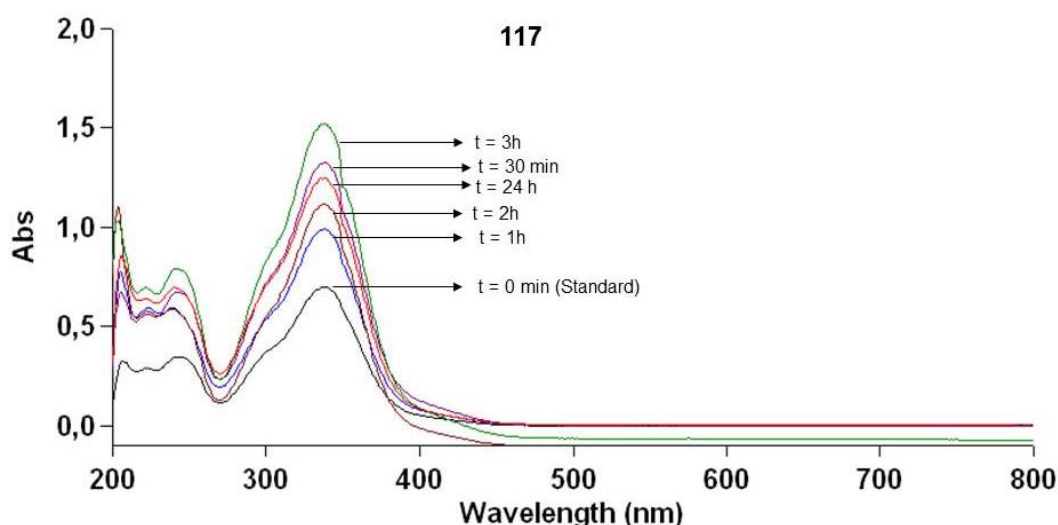


Figure 32 - UV-Vis spectra of compound **117** after different light exposure times, from 0 min (standard) to 24h.

Comparing to curcumin (**1**), it is possible to conclude that UV-Vis spectra of compound **117** are affected by light exposure but no significant hipso or bathochromic effects were observe. Nevertheless, after 24 h the alteration of compound **117** spectra revealed an increase in ϵ (when compared to standard solution) contrary to curcumin (**1**) that gave a decrease of ϵ .

Compound **33** UV-Vis spectra regarding different light exposure times are depicted in Figure 33. All the light exposed and protected solutions had shown no considerable bathochromic or hypsochromic shifts when compared to the standard solution. The standard solution, 30 min and 1 h light exposure solution shows overlapping spectra; 2 and 3 h light exposed solutions show a hyperchromic effect regarding 424 nm maximum absorption and an hypochromic effect regarding 258 and 207 nm maximum absorption.

After 24 h of light exposure the solution shows a generalized hypochromic effect in all maximum absorption wavelengths.

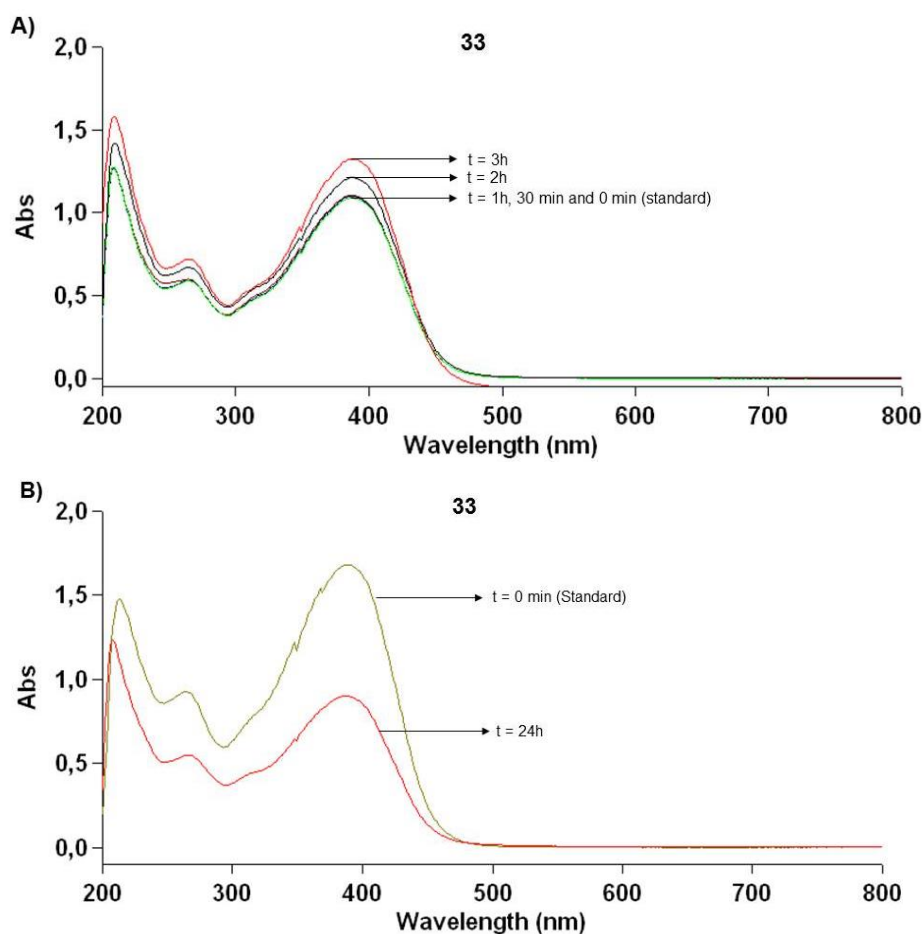


Figure 33 – A) UV-Vis spectra of compound **33** after different light exposure times, from 0 min (standard) to 3h; B) UV-Vis spectra of compound **33** after 24h light exposure and standard solution.

When comparing compound **33** with curcumin (**1**) it is possible to conclude that both UV-Vis spectra are affected by light exposure but no significant hipso or bathochromic effects were observe. After 24 h the alteration of compound **33** spectra revealed a decrease in ϵ similarly to curcumin (**1**).

After this UV-Vis spectra analysis it is not possible to conclude that compounds **117** and **33** are more or less stable than curcumin, all compounds spectra show differences from the standard solution after light exposure which could be due to some degradation. The fact that some curcumin (**1**) and compound **33** shows hyperchromic effect after 3 h of light exposure and hypochromic effect after 24h could mean that over 3h the compounds

degraded losing their chromophores, and from 3 to 24 h the degradation product also suffered degradation.

3. Biological activity studies

The synthesized compounds **117**, **119**, **120**, **121**, and **125** and curcumin (**1**) were tested in order to evaluate their cytotoxicity and P-gp inhibition. The compounds were tested by Vanessa Rodrigues as part of her PhD work. The tests were conducted at the Institute of Molecular Pathology and Immunology at the University of Porto – IPATIMUP.

The compounds cytotoxicity was evaluated with an indirect method, the sulforhodamine B colorimetric assay (SRB assay). This assay is based in cell density determination, evaluated by the measurement of cellular protein content ¹²⁵. The cell lines used for this assay were NCI-H460 (lung carcinoma cells). The tested tumor cell growth inhibitory activity of the compounds was compared to doxorubicin.

In the drug sensitive cell line, doxorubicin exhibited the lowest half maximal inhibition of cell growth (GI₅₀) value followed by curcumin; none of the derivatives showed GI₅₀ values lower than curcumin (**1**) or doxorubicin. Among the synthesized derivatives, compounds **119** and **120** were the most potent tumor cell growth inhibitors in the cell line NCI-H460. Compounds **125**, **117**, and **121** showed sequentially higher GI₅₀ values.

The detection of P-gp activity was accomplished using the rhodamine 123 assay ¹²⁶. This method is based on the fluorescent properties of rhodamine 123 and P-gp's ability to efflux this compound. Hence, by flow cytometry it is possible to detect and quantify rhodamine 123 inside the cells ¹²⁶. In this assay, the K562 cells (a chronic myeloid leukemia cell line) and K562-Dox cells (P-gp overexpressing cells derived from K562 cell line) were used. The tested compounds were compared with verapamil, a known P-gp inhibitor.

Curcumin (**1**) showed the lowest P-gp inhibitory activity. Verapamil was the most potent compound regarding P-gp inhibition, followed by compound **125** and **119**. Compounds **121**, **117**, and **120** exhibited sequentially lower P-gp inhibition. However, the latter three compounds inhibition is almost null.

Compounds **119** and **125** may act as dual ligands, since they show tumor cell growth inhibition and P-gp inhibitory activity, exhibiting a synergistic effect.

Considering these results, it is possible to hypothesize about some SAR features. Regarding tumor cell growth inhibition curcumin (**1**) and **117** both possess the same substitution pattern in the aromatic rings in contrast to compounds **119**, **120**, **121** and **125**, so the hydroxyl group may not be essential for this biological activity. The methoxyl group was maintained in every tested compounds, thus it is not possible to conclude about the importance of this substituent on the aromatic rings. Curcumin (**1**) showed to be more potent than compound **117**, which means that the presence of two aromatic rings may confer higher potency (since smaller linkers have already been proven to be beneficial for tumor cell growth inhibition ⁸⁰). Compounds **119**, **120** and **125** were the most potent tumor cell growth inhibitors when compared to the other derivatives of compound **117** in both cell lines, which could indicate that the strong electron density, confer by π electrons of the double and triple bond, and the moderate electron density, confer by the nonbonding pair of electrons of the nitrogen, may favor this activity. Studies with more derivatives are needed to corroborate these hypothesis.

Considering the P-gp inhibitory activity, compounds **125** and **119** were the most potent inhibitors when compared to the other derivatives of compound **117**. Again the nonbonding pair of electrons of the nitrogen and the π electrons of the double bond could be important to inhibit P-gp. Interestingly, compound **120** was not a good P-gp inhibitor despite the similarity to compound **119**. More studies with new derivatives and the synthesized derivative **33**, **122**, **123**, **124**, and **125** should provide more information to establish the SAR.

CHAPTER III:

CONCLUSION

CONCLUSIONS

Nature provides a large variety of molecules, with different degrees of complexity and many biological activities. Curcumin (**1**) is an example of such molecules, which has been associated with several interesting biological activities.

However, the extensive metabolism and instability prevent curcumin (**1**) from being used at its full potential. As mentioned before, we aimed to synthesize building blocks potentially more stable than curcumin, and several derivatives derived from one of the building blocks. We were able to synthesize successfully two out of three building blocks and eight out of sixteen derivatives.

The synthesized building blocks were not the originally planned target molecules. Nevertheless, the enone (**117**) and dienone (**33**) building blocks are curcumin derivatives without the base sensitive methylene moiety and the synthesis of derivatives proceeded. Due to the satisfactory yield and the convenient purification obtained for compound **117** eight derivatives were synthesized from **117**, possessing different substitution patterns in the phenolic group. Ten derivatives were synthesized and seven are herein being described for the first time. Structure elucidation was accomplished on the basis of several spectrophotometric and non-spectrophotometric techniques.

In order to evaluate the chemical and photostability of the building blocks when compared to curcumin (**1**) four assays were prepared. Regarding the pH buffers assay it can be hypothesized that compound **33** is the most stable compound, followed by compound **117**. Curcumin (**1**) showed to be most unstable of the three compounds. The biological buffer assay has shown that this medium did not degrade any of the compounds. The temperature/storage time assay showed that all of the tested compounds are unstable along the temperature and storage times tested. Curcumin (**1**) and compound **117** exhibited similar stability; and compound **33** has proven to be the most unstable in the described conditions.

Another aim of this thesis was to evaluate the ability of curcumin derivatives to inhibit tumor cell growth and P-gp activity. Compounds **119** and **125** showed the highest potency in both assays when compared to the other synthesized derivatives (**117**, **120**, and **121**). However, curcumin (**1**) was the most potent tumor cell growth inhibitor in sensitive cancer cell lines, when compared to its derivatives. On the basis of the performed biological assays it is possible to draw some conclusion and establish a SAR. Regarding tumor cell growth inhibition the hydroxyl group may not be essential for this biological activity; the presence of two aromatic rings may confer higher potency; high

electron density on the phenolic group substituents may favor this activity. The high electron density on the phenolic group substituents may also be important to inhibit P-gp. Studies with more derivatives are needed to corroborate these hypotheses.

Future studies can be proposed in order to continue and improve the present work. It would be interesting to introduce the same substitution patterns used in derivatives of compound **117** in curcumin (**1**) and compound **33**; and test the derivatives for the possible tumor cell growth and P-gp inhibitory activities. Hence, a more accurate SAR could be established. It would also be interesting to synthesize derivatives of compound **124** and **125** without the protecting group (*boc*) and allowing the hydrolysis of the carbamates, and consequent evaluation of the biological activity of the resulting carbamic acids.

Overall, the aims of this thesis were accomplished.

CHAPTER IV:

MATERIALS AND METHODS

1. General Methods

All reagents and solvents were purchased from Sigma Aldrich, and had no further purification process, except for 2-bromo-*N,N*-diethylethan-1-amine hydrobromide that was submitted to liquid extraction (NaOH, CH₂Cl₂) to be in the non-ionized form. Solvents were evaporated using rotary evaporator under reduced pressure, Buchi Waterchath B-480. All reactions were monitored by TLC carried out on precoated plates with 0.2 mm of thickness using Merck silica gel 60 (GF254). The visualization of the chromatograms was under UV light at 254 and 365 nm. Microwave (MW) reactions were performed in glassware open vessel reactors in a MicroSYNTH 1600 Microwave Labstation from Milestone (ThermoUnicam, Portugal). The internal reaction temperature was controlled by a fiber-optic probe sensor. Purification of the synthesized compounds was performed by chromatography flash and semi-flash columns using Merck silica gel 60 (0.040-0.063 mm and 0.063-0.200 mm, respectively), chromatography flash cartridge (GraceResolv®, Grace Company, Deerfield, IL, USA), Discovery® DSC-SAX SPE anionic exchange cartridge, and preparative thin layer chromatography using Merck silica gel HPLC60 RP-18 (GF254) plates purchased from Merck (Germany). Melting points (mp) were measured in a Köfller microscope and are uncorrected. Infrared (IR) spectra were obtained in KBr microplates in a Fourier transform infrared spectroscopy spectrometer Nicolet iS10 from Thermo Scientific with Smart OMNI-Transmisson accessory (Software OMNIC 8.3) (cm⁻¹). NMR spectra were performed in University of Aveiro, Department of Chemistry, and were taken in CDCl₃ (Deutero GmbH) at room temperature (r.t.) on Bruker Avance 300 spectrometer (300.13 MHz for ¹H and 75.47 for ¹³C). ¹³C NMR assignments were made by 2D (HSQC and HMBC) NMR experiments (long-range ¹³C-¹H coupling constants were optimized to 7 Hz).

Analytical HPLC-DAD (High-performance liquid chromatography-diode array detector) analyses were performed on a SpectraSYSTEM (Thermo Fisher Scientific, Inc, USA) equipped with a P4000 pump, a AS3000 autosampler and a diode array detector UV8000. The separation was carried out on a 250 x 4.6 mm i.d. FortisBIO C18 (5 µm) (Fortis™ Technologies Ltd, Cheshire, UK). ChromQuest 5.0 (version 3.2.1) software (Thermo Fisher Scientific Inc.) managed chromatographic data. Methanol (HPLC grade) was obtained from Carlo Erba Reagents, (Val de Reuil, Italy), acetic acid (HPLC grade) was obtained from Romil Pure Chemistry (Cambridge, UK) and HPLC grade water (Simplicity® UV Ultrapure Water System, Millipore Corporation, USA). Prior to use, mobile phase solvents were degassed in an ultrasonic bath for 15 min. All samples were dissolved and filtered through a PTFE syringe filter of 0.2µm pore size and 4 mm diameter

(Thermo Fisher Scientific) before injection. To determine the purity of compounds **117**, **119**, **120**, and **121**, LC analysis was performed by isocratic elution using a mixture of MeOH:H₂O:acetic acid (50:50:1), for compound **125** a mixture of MeOH:H₂O:TEA (50:50:1) as mobile phase, and for compound **33** a mixture of MeOH:H₂O:TEA (70:30:1) as mobile phase. The flow rate was set at 1 mL/ min. The injected volume was 10 µL and the eluent was monitored at 254 nm for every compound except compound **33** which was monitored at 399nm. The final concentration of the tested compounds was 100 µg/mL. The detector was set at a wavelength range of 220–500 nm with a spectral resolution of 1 nm. The purity parameters included a 95% active peak region and a scan threshold of 5 mAU. With these general conditions compounds 122 and 124 could not be detected (with different mobile phases, with and without ionic suppressors).

UV-Vis spectra were measured in a Cary IE UV-Visible spectrophotometer (Varian®) with a Cary Dual cell peltier accessory (Varian®), acquiring a spectrum in a range of 200-800nm. Software: Cary win UV, Cary 100, version 3.00 (182).

Qualitative GC-MS analyses were performed by Dr. Sara Cravo, Department of Chemistry, Laboratory of Organic and Pharmaceutical Chemistry, Faculty of Pharmacy, University of Porto. The analyses were performed on a Trace GC 2000 Series ThermoQuest gas chromatography equipped with ion-trap GCQ Plus ThermoQuest Finnigan mass detector. Chromatographic separation was achieved using a capillary column (30m × 0.25 mm × 0.25 µm, cross-linked 5% diphenyl and 95% dimethyl polysiloxane) from Thermo Scientific™ and high-purity helium C-60 as carrier gas. An initial temperature of 80°C was maintained for 1 min, increased to 310°C at 10°C/min, and held for 5 min giving a total run time of 33 min. The flow of the carrier gas was maintained at 1.5 mL/min. The injector port was set at 280°C. Analyses were performed with splitless injection in the full-scan mode in the scan range of m/z 50-700. For derivatized samples 30 µL of MSTFA were added and samples heated at 80°C for 30 min to accomplish silylation. An aliquot of 1 µL of the derivatized extract was injected into the GC-MS system. HRMS results were obtained in the services of C.A.C.T.I. (Vigo, Spain).

2. Synthesis

2.1. Synthesis of 4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one (**117**)

The reagents vanillin (10.03 g, 65.89 mmol) and acetone (3 mL, 40.6 mmol) were dissolved in EtOH (100mL). This mixture was allowed to stir for 5 min and cooled to 0°C (ice bath) before the drop wise addition of 70 mL of a 40% KOH aqueous solution. The mixture was stirred for approximately 24 h at r.t.. The reaction was neutralized by adding a 10% aqueous solution of hydrochloric acid and the precipitate formed was filtrated. The crude product was purified by recrystallization from EtOH, furnishing an yellow solid of 4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one (**117**, 3.13g, 64%).

4-(4-Hydroxy-3-methoxyphenyl)but-3-en-2-one (**117**): mp 127-129°C (EtOH) (lit. 127-128 °C *n*-hexane: EtOAc ¹⁰⁵). Purity: 96%. IR (KBr) ν_{max} cm⁻¹ 3200-500, 3002, 1637, 1583, 1519,, 1452, 1297, 1267, 1226, 1188, 1124, 1026, 981. UV maximum absorption wavelengths 399, 243, 222, and 205 nm, with respective ϵ of 11400, 5700, 4900, and 6000 M⁻¹·cm⁻¹. ¹H NMR (CDCl₃, 300 MHz) δ : 7.45 (1H, *d*, *J*= 16.2 Hz, H-3), 7.09 (1H, *dd*, *J*= 8.1 and 1.9, H-6'), 7.05 (1H, *d*, *J*= 1.9, H-2'), 6.93 (1H, *d*, *J*= 8.1 Hz, H-5'), 6.59 (1H, *d*, *J*= 16.2 Hz, H-2), 6.00 (1H, *s*, H-4'), 3.92 (3H, *s*, H-3'), 2.37 (3H, *s*, H-1''); ¹³C NMR (75.47 MHz, CDCl₃) δ : 198.7 (C-1), 148.4 (C-4'), 147.0 (C-3'), 144.0 (C-3), 126.8 (C-1'), 124.9 (C-2), 123.6 (C-6'), 114.9 (C-5'), 109.4 (C-2'), 56.0 (C-3''), 27.3 (C-1''). EI-MS (70 eV) *m/z* (rel. intensity, %): 264 (M⁺, 96), 265 (M⁺, 30), 233 (100), 219 (52), 191 (26), 102 (20).

2.2. Synthesis of 1,5-bis(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**33**)

The reagents vanillin (10.015 g, 6.67 mmol) and *N*-methyl-piperidone (4.5 mL, 37 mmol) were dissolved in EtOH (48 mL). This mixture was allowed to stir for 5 min and cooled to 0°C (ice bath) before the drop wise addition of 28 mL of a 20% NaOH aqueous solution. The mixture was stirred for approximately 24 h at room temperature, then 100 mL of distilled H₂O was added, and the resulting orange-brown solution was neutralized by adding a 10% aqueous solution of hydrochloric acid. After solvent evaporation, the crude product acidified with 20% aqueous solution of hydrochloric acid and extracted with CHCl₃ (3×100 mL). The organic phase was dried by anhydrous sodium sulfate, filtrated

and concentrated under reduced pressure to furnish a brown oil. The brown oil was dissolved in MeOH and basified with 20% methanolic solution of NH_3 . Then, the solution was applied in a Discovery® DSC-SAX SPE anionic exchange cartridge. The first yellow fraction, showed a new yellow spot on TLC plates. This fraction was concentrated and purified by flash chromatography (SiO_2 ; *n*-hexane: EtOAc several proportions). The fractions that eluted with *n*-hexane: ethyl acetate 8:2 were gathered and concentrated affording an orange-brown oil of 1,5-bis(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**33**, 57.1 mg, 5%).

3,5-bis(4-Hydroxy-3-methoxybenzylidene)-1-methylpiperidin-4-one (**33**): 74-79°C (*n*-hexane:EtOAc) (lit. mp 68-70°C, MeOH ¹⁰⁶). Purity 72%. IR (KBr) ν_{max} cm^{-1} 3200-3500, 2934, 1639, 1587, 1511, 1449, 1267, 1159, 1104, 1029, 981, 852, 811. UV maximum absorption wavelengths 387, 368, 264 and 208 nm, with respective ϵ of 11100, 10000, 6000, and 12800 $\text{M}^{-1}\cdot\text{cm}^{-1}$. ^1H NMR (CDCl_3 , 300 MHz) δ : 7.68 (2H, *d*, J = 15.5 Hz, H-3), 7.18 (2H, *dd*, J = 8.2 and 1.8 Hz, H-6'), 7.11 (2H, *d*, J = 1.8 Hz, H-2'), 6.92 (2H, *d*, J = 8.2 Hz, H-5'), 6.94 (2H, *d*, J = 15.5 Hz), 5.99 (2H, *brs*, H-4'), 3.95 (6H, *s*, H-3'); ^{13}C NMR (75.47 MHz, CDCl_3) δ : 188.9 (C-1), 148.2 (C-4'), 147.2 (C-3'), 143.3 (C-3), 127.5 (C-1'), 123.4 (C-2), 123.3 (C-6'), 114.9 (C-5'), 109.8 (C-2'), 56.0 (C-3''). HRMS- Electrospray ionization (ESI) (+) m/z : Anal. Calc. for $\text{C}_{19}\text{H}_{19}\text{O}_5$ $[\text{M}-\text{H}]^+$ 327.12; found: 327.12265.

2.3. Synthesis of 4-(4-(allyloxy)-3-methoxyphenyl)but-3-en-2-one (**119**)

To a solution of 4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one (**117**) (102.9 mg, 0.315 mmol) and allyl bromide (60 μL , 0.693 mmol) in acetone (20 mL), Cs_2CO_3 (121.2 mg, 0.37 mmol) was added and placed in a opened microwave reactor. The mixture under stirring was irradiated at 200 W for 2h, at a final temperature of 65°C. The solid was then removed by filtration with a sintered glass Buchner funnel under reduced pressure. The yellow-orange solution was evaporated and the product purified by semi-flash chromatography (SiO_2 ; petroleum ether: diethyl ether: several proportions). The fractions that eluted with petroleum ether: diethyl ether 8:2 were gathered and concentrated furnishing a yellow solid of 4-(4-(allyloxy)-3-methoxyphenyl)but-3-en-2-one (**119**, 70 mg, 56%).

4-(4-(Allyloxy)-3-methoxyphenyl)but-3-en-2-one (**119**): mp 52-55°C (petroleum ether: diethyl ether). Purity 98%. IR (KBr) ν_{max} cm^{-1} 2960, 2924, 1664, 1639, 1624, 1593,

1511, 1418, 1362, 1252, 1164, 1134, 997, 969, 800. ^1H NMR (CDCl_3 , 300 MHz) δ : 7.46 (1H, *d*, *J*= 16.2 Hz, H-3), 7.11 (1H, *d*, *J*= 2.0 Hz, H-6'), 7.08 (1H, *s*, H-2'), 6.88 (1H, *d*, *J*= 8.1 Hz, H-5'), 6.61 (1H, *d*, *J*= 16.2 Hz, H-2), 6.15-6.02 (1H, *m*, H-8'), 5.46-5.39 (1H, *m*, H-9'), 5.35-5.30 (1H, *m*, H-10'), 4.67-4.65 (2H, *dt*, *J*= 5.4 and 1.4 Hz, H-7'), 3.92 (3H, *s*, H-3'), 2.38 (3H, *s*, H-1''); ^{13}C NMR (75.47 MHz, CDCl_3) δ : 198.4 (C-1), 150.3 (C-4'), 149.6 (C-3'), 143.6 (C-3), 132.7 (C-8'), 127.5 (C-1'), 125.3 (C-2), 122.8 (C-6'), 118.5 (C-9'), 112.8 (C-5'), 110.0 (C-2'), 69.7 (C-7'), 55.9 (C-3''), 27.4 (C-1''). EI-MS (70 eV) *m/z* (rel. Intensity, %): 233 (M^{+1} , 18), 232 (M^+ , 100), 191 (95), 163 (28), 135 (22).

2.4. Synthesis of 4-(3-methoxy-4-(prop-2-yn-1-yloxy)phenyl)but-3-en-2-one (120)

4-(4-Hydroxy-3-methoxyphenyl)but-3-en-2-one (**117**) (1.00 g, 3.07 mmol) and propargyl bromide (1 mL, 11.22 mmol) were dissolved in acetone (180 mL), Cs_2CO_3 (680.03 mg, 2.08 mmol) was added and placed in an open vessel microwave reactor. The mixture under stirring was irradiated at 200 W for 1h 30 min, at a final temperature of 65°C. The solid was then removed by filtration with a sintered glass Buchner funnel under reduced pressure. The yellow solution was concentrated and the product submitted to a semi-flash chromatography (SiO_2 ; petroleum ether: diethyl ether: several proportions). The fractions that eluted with petroleum ether: diethyl ether 8:2 were gathered and after solvent evaporation a recrystallization from diethyl ether: petroleum ether (4:1) was performed providing a white solid of 4-(3-methoxy-4-(prop-2-yn-1-yloxy)phenyl)but-3-en-2-one (**120**, 101 mg, 8%).

4-(3-Methoxy-4-(prop-2-yn-1-yloxy)phenyl)but-3-en-2-one (**120**): mp 110-111°C (diethyl ether: petroleum ether). Purity 96%. IR (KBr) ν_{max} cm^{-1} 3258, 2922, 2851, 1673, 1597, 1515, 1424, 1372, 1272, 1247, 1225, 1168, 1145, 1034, 1006, 984, 805, 723, 682. ^1H NMR (CDCl_3 , 300 MHz) δ : 7.46 (1H, *d*, *J*= 16.2 Hz, H-3), 7.13 (1H, *d*, *J*= 8.2 Hz, H-6'), 7.09 (1H, *s*, H-2'), 7.04 (1H, *d*, *J*= 8.2 Hz, H-5'), 6.62 (1H, *d*, *J*= 16.2 Hz, H-2), 4.81 (2H, *s*, H-7'), 3.91 (3H, *s*, H-3''), 2.53 (1H, *s*, H-9'), 2.37 (3H, *s*, H-1''); ^{13}C NMR (75.47 MHz, CDCl_3) δ : 198.4 (C-1), 149.8 (C-4'), 149.0 (C-3'), 143.3 (C-3), 128.5 (C-1') 125.7 (C-2), 122.5 (C-6'), 113.6 (C-5'), 110.2 (C-2'), 77.9 (C-8'), 76.3 (C-9'), 56.6 (C-7'), 55.9 (C-3''), 27.4 (C-1''). EI-MS (70 eV) *m/z* (rel. intensity, %): 231 (M^{+1} , 25), 230 (M^+ , 88), 191 (100), 163 (22).

2.5. Synthesis of (2*R*,4*S*,5*R*)-5-acetamido-2-(acetoxymethyl)-6-(2-(4-((2-methoxy-4-((*E*)-3-oxobut-1-en-1-yl)phenoxy)methyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)tetrahydro-2*H*-pyran-3,4-diyl diacetate (**121**)

A mixture of 4-(3-methoxy-4-(prop-2-yn-1-yloxy)phenyl)but-3-en-2-one (**120**) (60.0 mg, 0.149 mmol), 2-azidoethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- β -D-galactopyranoside (123.6 mg, 0.297 mmol) CuSO₄·5H₂O (80.1 mg, 0.32 mmol), sodium ascorbate (131.1 mg, 0.66 mmol) and tetrahydrofuran/ H₂O solvent mixture (2:1; 7mL), were placed in a opened microwave reactor. The non-homogeneous mixture under stirring was irradiated at 200 W for 10 min, at a final temperature of 70°C. After filtration, THF was evaporated under reduced pressure and the aqueous solution was extracted with CHCl₃ (3 x 100 mL). The organic phase was dried with anhydrous sodium sulfate, filtrated and concentrated under reduced pressure to furnish a light-brown solid of (2*R*,4*S*,5*R*)-5-acetamido-2-(acetoxymethyl)-6-(2-(4-((2-methoxy-4-((*E*)-3-oxobut-1-en-1-yl)phenoxy)methyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)tetrahydro-2*H*-pyran-3,4-diyl diacetate (**121**, 63.4 mg, 38%).

(2*R*,4*S*,5*R*)-5-Acetamido-2-(acetoxymethyl)-6-(2-(4-((2-methoxy-4-((*E*)-3-oxobut-1-en-1-yl)phenoxy)methyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)tetrahydro-2*H*-pyran-3,4-diyl diacetate (**121**): mp 84-86°C (CHCl₃). Purity 97%. IR (KBr) $\nu_{\max}$ cm⁻¹ 1748, 1667, 1596, 1513, 1424, 1371, 1165, 1140, 1251, 1083, 1047. ¹H NMR (CDCl₃, 300 MHz) δ : 7.83 (1H, s, H-3'''), 7.45 (1H, *d*, *J*= 16.2 Hz, H-3), 7.12-7.08 (3H, *m*, H-6', H-2' and H-5'), 6.61 (1H, *d*, *J*= 16.2 Hz, H-2), 5.54 (1H, *d*, *J*= 8.8 Hz, H-11'''), 5.32 (2H, *dd*, *J*= 13.1 Hz, H-5'''), 5.30 (1H, s, H-1'''), 5.15 (1H, *dd*, *J*= 13.1 and 3.2 Hz, H-4'''), 4.65-4.55 (3H, *m*, H-6''', H-7''', and H-8'''), 4.30-4.25 (1H, *m*, H-9'''), 4.13 (1H, *d*, *J*= 1.68 Hz, H-17'''a), 4.07-4.00 (1H, *d*, H-17'''b), 4.07-4.00 (1H, *m*, H-10'''), 3.89 (3H, s, H-3''), 2.37 (3H, s, H-1''), 2.15, 2.04, 1.99 (3H, s, H-14''', H-16''', H-19'''), 1.85 (3H, s, H-12'''); ¹³C NMR (75.47 MHz, CDCl₃) δ : 198.2 (C-1), 170.7 (C-11'''), 170.4 (C-13''' and C-15'''), 170.1 (C-18'''), 149.9 (C-4'), 149.6 (C-3'), 143.2 (C-3), 143.2 (C-2'''), 128.2 (C-1'), 125.7 (C-2), 125.7 (C-3'''), 122.9 (C-6'), 113.6 (C-5'), 110.3 (C-2'), 101.0 (C-6'''), 70.9 (C-10'''), 69.8 (C-8'''), 67.4 (C-5'''), 66.1 (C-9'''), 62.8 (C-1'''), 61.4 (C-17'''), 55.9 (C-3''), 51.2 (C-7'''), 50.2 (C-4'''), 27.4 (C-1''), 23.2 (C-12'''), 20.7 (C-16''' and C-14'''), 20.6 (C-19''').

2.6. Synthesis of 4-(4-(2-(diethylamino)ethoxy)-3-methoxyphenyl)but-3-en-2-one (**122**)

To a solution of 4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one (**117**) (106.1 mg, 0.325 mmol) and 2-bromo-*N,N*-diethylethan-1-amine (175.1 mg, 0.972 mmol) in acetone (20 mL), K₂CO₃ (174.7 mg, 1.26 mmol) was added and placed in a opened microwave reactor. The mixture under stirring was irradiated at 200 W for 3h, at a final temperature of 65°C. When the reaction was finished, the solid was removed by filtration with a sintered glass Buchner funnel under reduced pressure. After removal of acetone under reduced pressure the crude product was dissolved in dichloromethane, acidified with an aqueous solution of HCl 5N and extracted with H₂O. The aqueous phase was basified with a 20% NaOH aqueous solution and extracted with CH₂Cl₂ (3×100 mL). The organic phase was dried with anhydrous sodium sulfate, filtrated, and concentrated under reduced pressure to furnish a yellow solid of 4-(4-(2-(diethylamino)ethoxy)-3-methoxyphenyl)but-3-en-2-one (**122**, 5.0 mg, 3%).

4-(4-(2-(Diethylamino)ethoxy)-3-methoxyphenyl)but-3-en-2-one (**122**): mp 207-209 °C (CH₂Cl₂). IR (KBr) ν_{max} cm⁻¹ 2959, 2925, 2855, 1729, 1629, 1425, 1384, 1273, 1124, 669. ¹H NMR (CDCl₃, 300 MHz) δ : 7.46 (1H, *d*, *J*= 16.2 Hz, H-3), 7.11 (1H, *dd*, *J*= 8.3 and 1.9 Hz, H-6'), 7.07 (1H, *d*, *J*= 1.9 Hz, H-2'), 6.91 (1H, *d*, *J*= 8.3 Hz, H-5'), 6.61 (1H, *d*, *J*= 16 Hz, H-2), 4.24 (2H, *t*, *J*= 6.2 Hz, H-7'), 3.89 (3H, *s*, H-3''), 3.08 (2H, *t*, *J*= 6.2 Hz, H-8'), 2.81 (4H, *q*, *J*= 7.2 Hz, H-9'), 2.38 (3H, *s*, H-1''). 1.17 (6H, *t*, *J*= 7.2 Hz, H-10'); ¹³C NMR (CDCl₃, 75.47 MHz) δ : 198.4 (C-1), 150.3 (C-4'), 149.6 (C-3'), 143.5 (C-3), 127.8 (C-1'), 125.4 (C-2), 122.9 (C-6'), 112.7 (C-5'), 110.0 (C-2'), 66.4 (C-7'), 55.9 (C-3''), 51.2 (C-8'), 47.7 (C-9'), 27.4 (C-1''), 11.0 (C-10').

2.7. Synthesis of 4-(3-(((3-(dimethylamino)propyl)(methyl)amino)methyl)-4-hydroxy-5-methoxyphenyl)but-3-en-2-one (**123**)

N,N,N'-Trimethyl-1,3-propanediamine (160 μ L, 1.04 mmol) was cooled in an ice-bath for 5min before the dropwise addition of a 37% formaldehyde solution (78 μ L) and glacial acetic acid (1.5 mL). After 1h stirring at room temperature 4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one (**117**, 200 mg, 1.04 mmol) was added to the mixture. The reaction proceeded at 65°C for 7 h and then at room temperature for 48 h. At the end of the reaction, water (10 mL) was added, and the solution was basified with a 20% NaOH aqueous solution and extracted with CH₂Cl₂ (3×100 mL). The organic phase was dried by

anhydrous sodium sulfate, filtrated, and the solvent evaporated under reduced pressure. The solid thus obtained was purified by chromatography flash cartridge using MeOH (100%) and MeOH:TEA (100:1). The target compound was eluted using the later mobile phase. The fractions were gathered and concentrated furnishing a yellow-orange solid of 4-(3-(((3-(dimethylamino)propyl)(methyl)amino)methyl)-4-hydroxy-5-methoxyphenyl)but-3-en-2-one (**123**, 65.5 mg, 20%)

4-(3-(((3-(dimethylamino)propyl)(methyl)amino)methyl)-4-hydroxy-5-methoxyphenyl)but-3-en-2-one (**123**): mp 122-125°C (MeOH). IR (KBr) ν_{max} cm⁻¹ 3500-3200, 2962, 1633, 1471, 1385, 1126, 619. ¹H NMR (CDCl₃, 300 MHz) δ : 7.48 (1H *d*, *J*= 16.05 Hz, H-3), 7.02 (1H, *d*, *J*= 1.7 Hz, H-6'), 6.84 (1H, *d*, *J*= 1.7 Hz, H-2'), 6.60 (1H, *d*, *J*= 16.05 Hz, H-2), 3.92 (3H, *s*, H-3''), 3.75 (2H, *s*, H-7'), 2.92-2.81 (4H, *m*, H-9' and H-11'), 2.41 (6H, *s*, H-12'), 2.36 (3H, *s*, H-1''), 1.99 (3H, *s*, H-8'), 1.33-1.25 (2H, *m*, H-10'). ¹³C NMR (CDCl₃, 75.47 MHz) δ : 198.3 (C-1), 150.6 (C-4'), 148.3 (C-3'), 143.9 (C-3), 125.1 (C-1'), 124.3 (C-2), 123.4 (C-6'), 109.8 (C-5'), 109.7 (C-2'), 61.1 (C-7'), 57.1 (C-11'), 55.9 (C-3''), 55.2 (C-9'), 45.4 (C-8' and C-12'), 27.0 (C-1''), 24.9 (C-10').

2.8. Synthesis of *tert*-butyl (2-(2-methoxy-4-(3-oxobut-1-en-1-yl)phenoxy)ethyl)carbamate (**124**)

4-(4-Hydroxy-3-methoxyphenyl)but-3-en-2-one (**117**) (202.5 mg, 0.621 mmol), 2-(*boc*-amino) and ethyl bromide (274 mg, 1.22 mmol) were dissolved in acetone (40 mL), K₂CO₃ (204.5 mg, 1.48 mmol) was added and placed in a opened microwave reactor. The mixture under stirring was irradiated at 200 W for 3 h, at a final temperature of 65°C. The solid was removed by filtration with a sintered glass Buchner funnel under reduced pressure. The yellow solution was purified by semi-flash chromatography (SiO₂; petroleum ether: diethyl ether: several proportions). The fractions that eluted with petroleum ether: diethyl ether 7:3 were gathered and the product purified by preparative chromatography with CHCl₃: acetone: triethylamine (9:1:0.1) affording an yellow solid of *tert*-butyl (2-(2-methoxy-4-(3-oxobut-1-en-1-yl)phenoxy)ethyl)carbamate (**124**, 4 mg, 1.1%).

tert-Butyl (2-(2-methoxy-4-(3-oxobut-1-en-1-yl)phenoxy)ethyl)carbamate (**124**): mp 89-92 °C (CHCl₃: acetone). IR (KBr) ν_{max} cm⁻¹ 3447, 2920, 2852, 1636, 1385, 1099, 668. ¹H NMR (CDCl₃, 300 MHz) δ : 7.45 (1H, *d*, *J*= 16.2 Hz, H-3), 7.10 (1H, *dd*, *J*= 8.1 and 1.8 Hz, H-6'), 7.06 (1H, *d*, *J*= 1.8 Hz, H-2'), 6.94 (1H, *d*, *J*=8.1 Hz, H-5'), 6.59 (1H, *d*, *J*= 16.2 Hz, H-2), 4.91 (2H, *m*, H-7'), 3.94 (3H, *s*, H-3'), 3.23-3.18 (2H, *m*, H-7'), 2.37 (3H, *s*, H-1''), 2.17 (9H, *s*, H-10'); ¹³C NMR (75.47 MHz, CDCl₃) δ : 198.5 (C-1), 160.5 (C-9'), 148.2 (C-96

4'), 146.9 (C-3'), 143.8 (C-3), 127.0 (C-1'), 125.0 (C-2), 123.6 (C-6'), 114.8 (C-5'), 109.3 (C-2'), 68.2 (C-7'), 56.0 (C-3''), 29.7 (C-11'), 27.3 (C-1''). EI-MS (70e V) m/z (rel. intensity, %): 335 (M^+ , 26), 281 (100), 234 (63), 74 (82).

2.9. Synthesis of *tert*-butyl (3-(2-methoxy-4-(3-oxobut-1-en-1-yl)phenoxy)propyl)carbamate (**125**)

A mixture of 4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one (**117**) (101.6 mg, 0.311 mmol), 3-(*boc*-amino) propyl bromide (137 mg, 0.575 mmol), K_2CO_3 (105.2 mg, 0.76 mmol), and acetone (30 mL), was placed in an opened microwave reactor. The mixture under stirring was irradiated at 200 W for 3 h, at a final temperature of 65°C. The solid was removed by filtration with a sintered glass Buchner funnel under reduced pressure. The yellow solution was purified by semi-flash chromatography (SiO_2 ; *n*-hexane: diethyl ether: several proportions). The fractions that eluted with *n*-hexane: diethyl ether 7.5:2.5 were gathered and after solvent evaporation a recrystallization from diethyl ether: petroleum ether (4:1) was performed providing a yellow solid of *tert*-butyl (3-(2-methoxy-4-(3-oxobut-1-en-1-yl)phenoxy)propyl)carbamate (**125**, 16.6 mg, 9%).

tert-Butyl (3-(2-methoxy-4-(3-oxobut-1-en-1-yl)phenoxy)propyl)carbamate (**125**): mp 108-110°C (diethyl ether: petroleum ether). Purity 96%. IR (KBr) ν_{max} cm^{-1} 3377, 1686, 1669, 1644, 1621, 1595, 1518, 1273, 1260, 1225, 1167, 1138, 1033, 975. ^1H NMR ($CDCl_3$, 300 MHz) δ : 7.46 (1H, *d*, J = 16.2 Hz, H-3), 7.11 (1H, *d*, J = 8.2 Hz, H-6'), 7.07 (1H, *s*, H-2'), 6.86 (1H, *d*, J = 8.2 Hz, H-5'), 6.61 (1H, *d*, J = 16.2 Hz, H-2), 5.50 (1H, *brs*, H-10') 4.14 (2H, *t*, J = 5 Hz, H-7'), 3.92 (3H, *s*, H-3''), 3.38 (2H, *d*, J = 5 Hz, H-9'), 2.38 (3H, *s*, H-1''), 2.04 (2H, *t*, J = 5 Hz, H-8'), 1.46 (9H, *s*, H-11'); ^{13}C NMR (75.47 MHz, $CDCl_3$) δ : 198.4 (C-1), 156.1 (C-10'), 150.5 (C-4'), 149.5 (C-3'), 143.5 (C-3), 127.6 (C-1'), 125.3 (C-2), 122.9 (C-6'), 112.2 (C-5'), 109.7 (C-2'), 79.0 (C-11'), 68.2 (C-7'), 55.8 (C-3''), 38.9 (C-9'), 30.9 (C-8'), 28.5 (C-12'), 27.4 (C-1''). EI-MS (70e V) m/z (rel. Intensity, %): 349 (M^+ , 96), 308 (32), 261 (38), 253 (52).

2.10. Synthesis of methyl 2-(2-methoxy-4-(3-oxobut-1-en-1-yl)phenoxy)acetate (**126**)

4-(4-Hydroxy-3-methoxyphenyl)but-3-en-2-one (**117**) (150 mg, 0.78 mmol) and methyl 2-bromoacetate (80 μ L; 0.78 mmol) were dissolved in acetone (20 mL) and K_2CO_3 (261 mg; 1.89 mmol) was added. The mixture was placed in an opened microwave

reactor where it was irradiated at 200 W for 3h, at a final temperature of 65°C with stirring. The solid was removed by filtration with a sintered glass Buchner funnel under reduced pressure. After acetone evaporation, the crude product was dissolved in a basified aqueous solution with 20% NaOH and extracted with CH₂Cl₂ (3×100 mL). The organic phase was dried by anhydrous sodium sulfate, filtrated, and concentrated under reduced pressure to furnish a yellow solid of methyl 2-(2-methoxy-4-(3-oxobut-1-en-1-yl)phenoxy)acetate (**126**, 7.9 mg, 4%).

Methyl 2-(2-methoxy-4-(3-oxobut-1-en-1-yl)phenoxy)acetate (**126**): mp 79-81°C (CH₂Cl₂). IR (KBr) ν_{max} cm⁻¹ 2918, 2849, 1733, 1634, 1515, 1463, 1262, 1111, 668. ¹H NMR (CDCl₃, 300 MHz) δ : 7.45 (1H, *d*, *J*= 16.2 Hz, H-3), 7.09 (2H, *dd*, *J*= 6.7 and 1.9 Hz, H-6' and H-2'), 6.80 (1H, *d*, *J*= 6.7 Hz, H-5'), 6.61 (1H, *d*, *J*= 16.2 Hz, H-2), 4.76 (2H, *s*, H-7'), 3.93 (3H, *s*, H-3''), 3.81 (3H, *s*, H-8'), 2.38 (3H, *s*, H-1''); ¹³C NMR (75.47 MHz, CDCl₃) δ : 198.4 (C-1), 169. (C-8'), 149.7 (C-4'), 149.3 (C-3'), 143.2 (C-3), 128.8 (C-1'), 125.5 (C-2), 122.5 (C-6'), 113.4 (C-5'), 110.5 (C-2'), 66.0 (C-7'), 56.0 (C-3''), 52.4 (C-9'), 27.4 (C-1'').

3. Stability and photostability studies

3.1. Stability study

Standard solutions of curcumin (**1**), compound **117** and **33** were prepared and injected in the HPLC equipment with a final concentration of 1mg/mL in DMSO, at room temperature and immediately after being prepared. Regarding pH stability assay, five pH values were selected and obtained with following buffers: HCl pH 1.0, sodium acetate pH 5.0, potassium phosphate pH 6.7, PBS pH 7.4 and sodium boric acid pH 9.1. A stock solution of each compound was prepared with the initial concentration of 10⁻² M in DMSO. Then the compounds were dissolved in each buffer in a final concentration of 10⁻⁴ M. The solutions were allowed to incubate overnight at room temperature, except in HCl buffer that incubated only for 75 min, protected from light exposure. After overnight or 75min of incubation certain compounds precipitated. In such cases, the precipitate was filtrated and dissolved in 4 mL of MeOH before being also injected.

The biological buffer assay was performed with cells medium. Cells medium composition was: 87.5 % RPMI medium, 2.5% *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and 10% fetal bovine serum (FBS). The compounds were incubated for 5, 10, 20, and 30 min at 37° C in biological buffer, protected from light exposure, at a final concentration of 1.5×10^{-3} M. Before being injected the samples were filtrated.

Solutions at 10^{-4} M in DMSO of each compound were prepared and store at -20°C, 4°C and r.t. for 6, 15 and 21 days for the temperature/storage time assay. After the indicated storage time, where the samples were protected from light exposure, the solutions were filtrated and injected. All samples were filtrated using 0.80 µm Minisort ® filters (Sartorius Stedim Biotech)

3.2. Photostability study

The photostability of curcumin, compound **117** and **33** was determined by UV-Vis spectrophotometry. Stock solutions of these three compounds were prepared in MeOH at a concentration of 1mg/mL (2.72×10^{-3} M for curcumin, 5.2×10^{-3} M for compound **117**, and 2.62×10^{-3} M for compound **33**). The compounds were then dissolved to a final concentration of 1×10^{-4} M. The solutions were maintained in glass vials and irradiated with a 120 Watts lamp in a VILBER LourMAT chamber, with a constant temperature of 43°C. The solutions were subjected to selected times intervals of light exposure: 30min, 1h, 2h, 3h and 24h, and compared to 0min light exposure (standard). One solution of each compound was also prepared and protected from light exposure for 3h and 24h. The experiences were carried out in duplicate. Methanol was used to define the baseline.

CHAPTER V:

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