Universidade de Aveiro Departamento de Química

Ano 2016

Carlos Fábio Magalhães da Silva

Síntese e avaliação biológica de novas 2-benzilcromonas

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo de Bioquímica Clínica, realizada sob a orientação científica da Doutora Diana Cláudia Gouveia Alves Pinto, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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Palavras-chave Produtos naturais; cromonas; Condensação de Claisen; rearranjo de Baker-Venkataraman; anti-inflamatório; anti-acetilcolinesterase.

Resumo

Esta dissertação aborda a síntese de compostos do tipo cromona (4*H*-cromen-4-ona), em particular 2-benzilcromonas, com distintos padrões de substituição e também a avaliação da sua actividade anti-inflamatória e inibidora da acetilcolinesterase.

As cromonas apresentam-se como sendo uma vasta classe de compostos naturais heterocíclicos contendo oxigénio e que representam o fragmento principal de diversos grupos de flavonoides amplamente distribuídos pela Natureza. Este tipo de compostos apresenta uma ampla gama de actividades biológicas e este esqueleto de cromona tem provado ser um núcleo estrutural crucial no domínio dos produtos naturais, na química orgânica sintética e em estudos de desenvolvimento de novas drogas, devido às suas características estruturais particulares e diversas propriedades farmacológicas. Por essas razões, o interesse no estudo da bioactividade e respectivas aplicações biológicas deste tipo de compostostem vindo a estimular a síntese de novos derivados de cromona e o desenvolvimento de cada vez mais estudos de relação estrutura-actividade. O principal objectivo deste projecto passa então pela síntese de novas 2-benzilcromonas, conjugando diferentes 2'-hidroxiacetofenonas e 2-fenilacetatos de etilo de forma a obter compostos com padrões de substituição distintos, e avaliar o seu potencial como agentes anti-inflamatórios e/ou inibidores da acetilcolinesterase.

No primeiro capitulo, é realizada uma introdução, onde a estrutura química das cromonas e principais actividades biológicas são indicadas. Nesse mesmo capítulo, os métodos de síntese para a obtenção deste tipo de compostos são brevemente descritos e estudos de relação estrutura-actividade, desenvolvidos ao longo dos últimos anos, descritos de forma a perceber a influencia de diferentes substituintes.

No segundo capítulo, os métodos de síntese realizados ao longo do projecto são descritos, seguidos das respectivas explicações mecanísticas e da completa caracterização estrutural dos compostos sintetizados, utilizando técnicas espectroscópicas como Ressonância Molecular Nuclear de ^{1}H e ^{13}C, *Heteromolecular Single Quantum Coherence* (HSQC) e *Heteronuclear Multiple Bond Correlation* (HMBC), e espectrometria de massa.

No terceiro capítulo, os resultados da avaliação biológica dos compostos serão apresentados, nomeadamente as suas actividades anti-inflamatória e inibidora da acetilcolinesterase. Através dos resultados obtidos, torna-se possível entender o efeito dos diferentes substituintes nessas actividades, sendo o composto 2-benzil-5-(benziloxi)-4*H*-cromen-4-ona (**86.j**) o mais promissor composto anti-inflamatório. Relativamente à actividade anti-acetilcolinesterase, o composto 2-(3,4-di-hidroxibenzil)-5-hidroxi-4*H*-cromen-4-ona (**86.m**) é o mais promissor.

No quarto capítulo, uma extensa descrição dos procedimentos experimentais desenvolvidos, ao longo do nosso projecto, é apresentada, enquanto no último capítulo (Capítulo V) são apresentadas as conclusões finais sobre o trabalho realizado e as perspectivas futuras.

Keywords Natural products; chromones; Claisen condensation; Baker-Venkataraman rearrangement; anti-acetylcholinesterase; anti-inflammatory.

Abstract

This dissertation approaches the synthesis of chromone (4*H*-chromen-4-one) type compounds, particularly 2-benzylchromones, with distinct substitution patterns and the evaluation of their anti-inflammatory and anti-acetylcholinesterase activities.

Chromones are a wide class of naturally occurring oxygen-containing heterocyclic compounds with a benzoannelated γ-pyrone and represent the core fragment of several groups of flavonoids widely distributed in nature. This type of compounds presents a wide range of biological activities and the chromone ring system has proven to be a crucial core in the domain of natural products, in synthetic organic chemistry and in drug discovery studies because of its particular structural features and diverse pharmacological properties. For these reasons, the interest in the study of their bioactivity and related biological applications has stimulated the synthesis of novel chromone derivatives and development of more and more structure-activity relationships. The main objective of this project concerns the synthesis of new 2-benzylchromones, conjugating different 2'-hydroxyacetophenones and ethyl 2-phenylacetates to obtain compounds with distinct substitution patterns, and evaluate their potential as anti-inflammatory and/or anti-acetylcholinesterase agents.

In the first chapter, an introduction is made, where chromones chemical structure and their principal biological activities are indicated. In the same chapter, the most common synthetic methods for the obtention of chromones are briefly depicted and structure-activity relationship (SAR) studies, developed throughout the years, are described, highlighting the influence of different substituents.

In the second chapter, the synthetic methods performed are described, followed by a mechanistic explanation and a complete structural characterization of the synthesized compounds, using spectroscopic techniques, like ^{1}H and ^{13}C Nuclear Molecular Resonance (NMR), Heteromolecular Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC), and mass spectrometry (MS).

In a third chapter, the biological evaluation of our compounds is depicted, namely their anti-inflammatory and anti-acetylcholinesterase activities. From the results obtained, it is possible to understand the effect of different substituents in it, being compound 2-benzyl-5-(benzyloxy)-4*H*-chromen-4-one (**86.j**) the most promising anti-inflammatory one. Regarding the anti-acetylcholinesterase activity, compound 2-(3,4-dihydroxybenzyl)-5-hidroxy-4*H*-chromen-4-one (**86.m**) is the most promising.

In chapter IV, an extensive description of the experimental procedures developed throughout our project are displayed, while in the final chapter (chapter V) the conclusions are drawn and some future perspectives presented.

ABBREVIATIONS

Aβ – Amyloid β

AA – Arachidonic acid

ACh – Acetylcholine

AChE – Acetylcholinesterase

AChEI – Acetylcholinesterase inhibitors

AD – Alzheimer's disease

ADP – Adenosine diphosphate

APP – Transmembrane protein amyloid precursor protein

ATChI – Acetylthiocholine iodine

BACE-1 – β-secretase

BH4 – (6*R*)- 5,6,7,8-tetrahydro-L-biopterin

Bn – Benzyl group

BuChE – Butyrylcholinesterase

CAS – Catalytic anionic site

CNS – Central Nervous System

COX – Cyclooxygenase

DMA – Dimethylacetamide

DMEM – Dulbecco's Modified Eagle's Medium

DMF – Dimethylformamide

DMS – Dimethyl sulfide

DMSO – Dimethyl sulfoxide

DNA-PK – DNA-dependent protein kinase

DTNB – 5,5-dithio-bis-(2-nitrobenzoic acid)

ESI – Electrospay ionization

Et – Ethyl group

EWG – Electron withdrawing group

FAD – Flavin-Adenosine dinucleotide

FBS – Fetal Bovine Serum

FDA – US Food and Drug Administration

FMN – Flavin mononucleotide

HMBC – Heteronuclear Multiple Bond Correlation

HSQC – Heteromolecular Single Quantum Coherence

IL – Interleukin

IFN-γ – Interferon-γ

LOX – Lipoxygenase

LPS – Lipopolissacaride

LT – Leukotriene

MAO – Monoamine oxidase

MAPK – Mitogen Activated Protein Kinases

MS – Mass Spectrometry

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NADPH – Nicotinamide adenine dinucleotide phosphate

NFT –Neurofibrillar tangles

NF-kβ – Nuclear factor kappa-light-chain-enhancer of activated B cells

NHC – *N*-heterocyclic carbenes

NMDA – *N*-methyl-D-aspartate

NMR – Nuclear Molecular Resonance

NO – Nitric oxide

eNOS – endotelial Nitric oxide synthase

iNOS – inducible Nitric oxide synthase

nNOS – nuclear Nitric oxide synthase

PAS – Peripheral anionic site

PG – Prostaglandin

PI3K – Phosphoinositide 3-kinase

PMNL – Polimononuclear leukocytes

mRNA – messenger RNA

SAR – Structure-activity relationships

SEM – Standard error of mean

SP – Senile plaques

STAT – Signal Transducers and Activators of Transcription

TBDMS – tert-butyldimethylsilyl

THF – Tetrahydrofuran

TLC – Thin layer chromatography

TMS – Tetramethylsilane

TNB – 2-nitro-5-thiobenzoate

TNF-α – Tumor necrosis factor alpha

TPA – Tissue plasminogen activator

p-TSA – *p*-toluenesulfonic acid

UV – Ultra-violet

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Chapter I: Introduction

1. Introduction

The search for novel natural products with promising pharmacological applications is an ongoing process.[1] Much effort goes not only into the search for these compounds from natural sources, but also into their synthesis, with the aim of obtaining derivatives with enhanced biological activities and in large quantities to allow in-depth pharmacological studies and ultimately clinical applications.

1.1. Flavonoids

Flavonoids are a class of plant secondary metabolites which are ubiquitous in photosynthesizing cells.[2] Due to structural particularities, this class of compounds is divided in several smaller subclasses of compounds, such as flavones, isoflavones, chalcones, flavanones, anthocyanidins, and others (Figure 1).

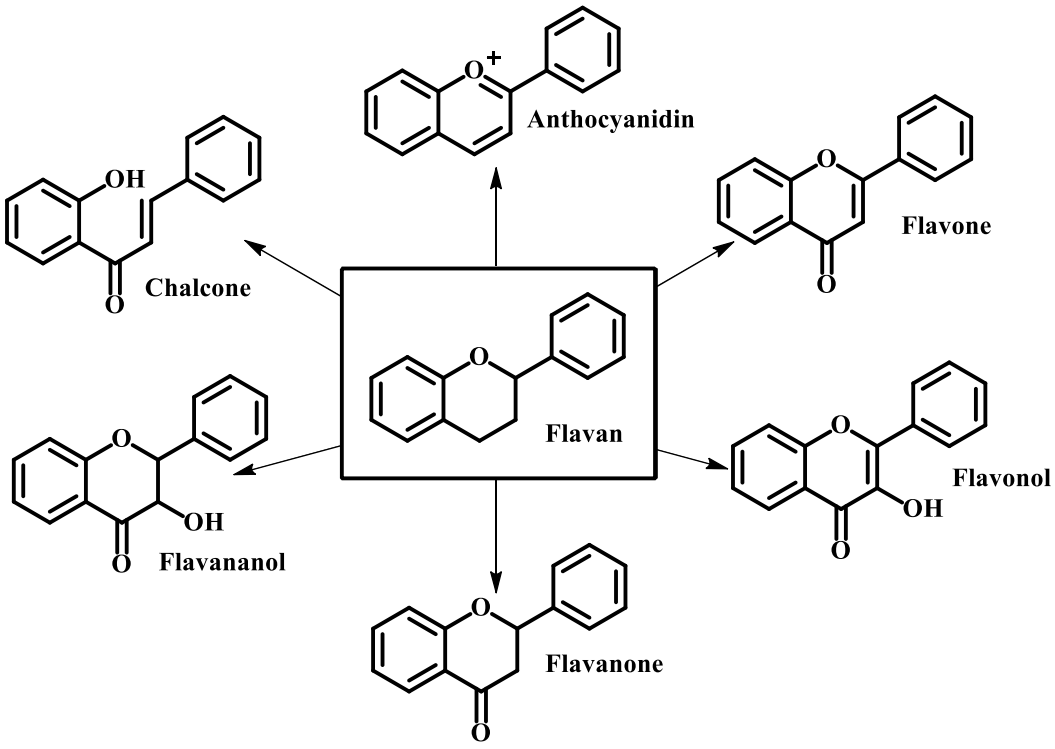


Figure 1. Core structure of flavonoids and some subclasses.

Due to the flavonoid type compounds diverse biological activities, preparations containing them as the main physiological active component have been used to treat many human diseases, since ancient times. This kind of compounds play a vast number of physiological functions depending on the plant part. In flowers, they act by providing attractive colors to plant pollinators, while in leaves it is believed that they might promote the plant's survival by protecting it from, for example, fungal pathogens and UV-radiation.[2,3] Additionally, flavonoids are also involved in photosensitization, energy transfer, the actions of plant growth hormones and growth regulators, the control of respiration and photosynthesis, morphogenesis and sex determination.

1.2. Chromones

Chromones (*4H*-chromen-4-one; *4H*-1-benzopyran-4-one; benzo-*γ*-pyrone), a group of naturally occurring compounds ubiquitous in nature, particularly in plants,[4] are a broad class of compounds where several groups of flavonoids, such as flavones (**2**), flavonols (**3**) and isoflavones (**4**) are included (Figure 2).[5,6] The interest in this type of compounds has been increasing as molecules containing the chromone scaffold have presented a wide range of biological activities.

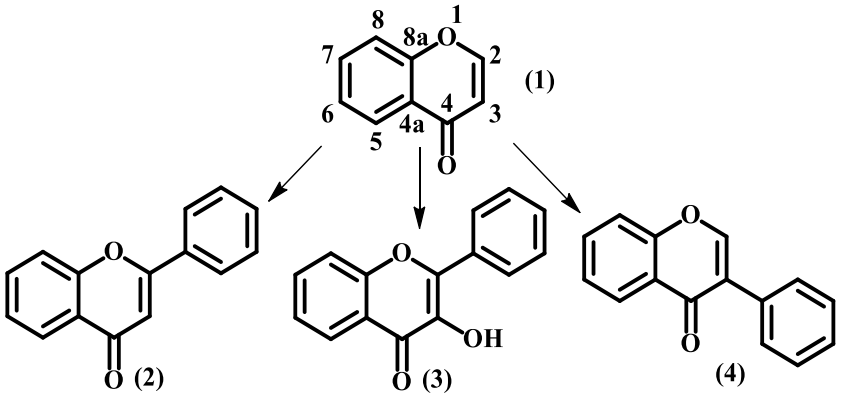


Figure 2. Chromone scaffold and respective classes of flavonoids.

The chromone ring system, a benzoannelated γ-pyrone, has proven to be an important core in the domain of natural products, synthetic organic chemistry and specially in drug discovery studies because of its particular structural features and diverse pharmacological

properties.[7] In fact, the chromone scaffold has emerged as a privileged structure for drug research because of the versatile pharmacological profile and good drug-like properties. The versatile functionalization of the chromone core allows for a great chemical diversity suitable in either improving the pharmacological profile or discovering different biological applications.[7,8]

As mentioned before, molecules containing the chromone scaffold have a wide range of biological activities, namely anti-fungal, anti-microbial, anti-allergenic, antitubulin, anti-viral, anti-hypertensive, antitumoral and also the capacity to inhibit several enzymes, such as phosphatidylinositol-3-kinase (PI3K), DNA-dependent protein kinase (DNA-PK), β-secretase (BACE-1), tyrosinase, monoamine oxidases A and B (MAO A and B) or AChE and BuChE, among others.[9,10] However, chromone synthesis is not only focused in the development of more diverse bioactive compounds and/or in structure-activity relationship (SAR) studies. For instance, chromones may be used in the preparation of fluorescent probes due to its photochemical properties, being some radioiodinated derivatives already tested as amyloid imaging agents with high binding affinity for amyloid-β (Aβ) aggregates and neurofibrillary tangles, presenting low cytotoxicity.[11]

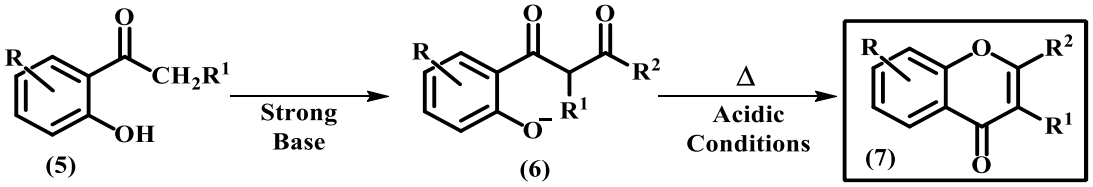
1.2.1. Synthetic methods

Due to chromones pharmacological potencial, numerous methodologies for their synthesis have been developed. Chromones my be synthesized using several distinct starting materials, such as 2'-hydroxyacetophenones, phenols or salicylic acid and their derivatives. In this topic, the different synthetic methods for attaining the chromone scaffold will be briefly described and schematized in order to summarize the numerous reagents and reaction mediums used in the design of this type of compounds.

1.2.1.1. Chromones from 2'-hydroxyacetophenones

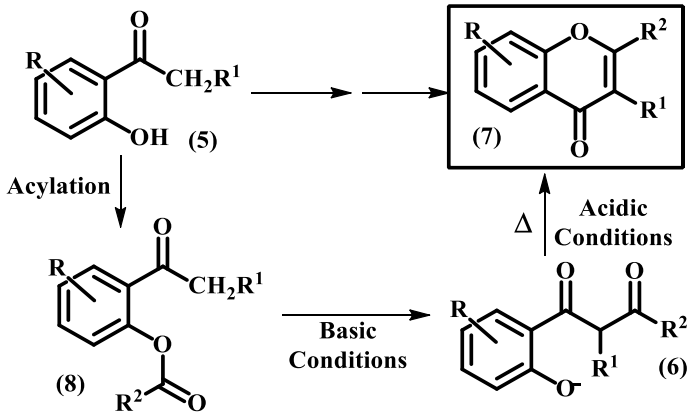
Among the previously mentioned starting materials, the most commonly used precursors are 2'-hydroxyacetophenones (**5**). In 1901, Kostanecki, Paul and Tambor applied, for the first time, the Claisen condensation as a synthetic methodology towards chromones

(Scheme 1).[12] This formation of the chromone scaffold from 2'-hydroxyacetophenones consists in a two-phase reaction where, firstly, a strong base is used to generate an enolate that will then react with a carboxylic ester, which leads to the formation of a 1,3-dioxophenoxy intermediate (**6**). The second phase of the reaction comprises a cyclization process of this intermediate by submitting it to acidic conditions and conventional heating (Scheme 1).[13]



Scheme 1. Classic Claisen Condensation.

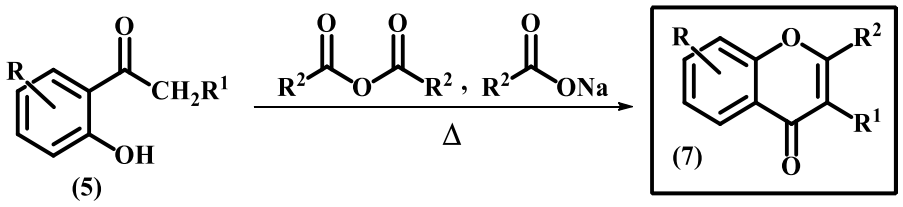
A variation of the Claisen condensation previously mentioned was described for the synthesis of chromones, namely the Baker-Venkataraman rearrangement (Scheme 2).[14,15] This variation only appears to be different in the formation of the 1,3-dioxophenoxy intermediate (**6**), since it initially involves the acylation of 2'-hydroxyacetophenone (**5**) with acyl chlorides. Then, in the presence of a strong base, it suffers a further intramolecular rearrangement, the Baker-Venkataraman rearrangement, leading to the formation of the 1,3-dioxophenoxy intermediate (**6**). This 1,3-dioxophenoxy intermediate (**6**) is then isolated and a cyclization process occurs under harsh acidic conditions. It is important to remark that 2-styrylchromones are classically obtained through Baker-Venkataraman rearrangement.[14,16]



Scheme 2. Baker-Venkataraman rearrangements.

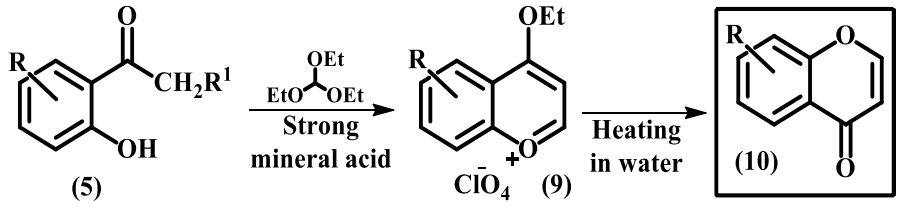
Another variation of the Claisen condensation was also advanced by Kostanecki and co-workers, by developing a procedure where resacetophenone (2',4'-dihydroxyacetophenone) and 4'-ethoxy-2'-hydroxyacetophenone reacted with refluxing acetic anhydride in the presence of sodium acetate.[17] This reaction allows the formation of the chromone nucleus without the need of the acidification process for ring closure since, after the acylation of 2'-hydroxyacetophenone (**5**), an aldol intramolecular cyclization occurs to form the chromone core.

The previous work was further elaborated by Allan and Robinson, in 1924, when they described the formation of a flavone by reacting a ω-methoxyresacetophenone with benzoic anhydride in the presence of sodium benzoate.[18] Although the formation of chromones/coumarins is usualy reffered to as Kostanecki-reaction and the synthesis of flavones as the Allan-Robinson reaction , it is frequently chosen to merge the two reactions and refer to both as the Kostanecki-Robinson reaction (Scheme 3).



Scheme 3. Kostanecki-Robinson reaction.

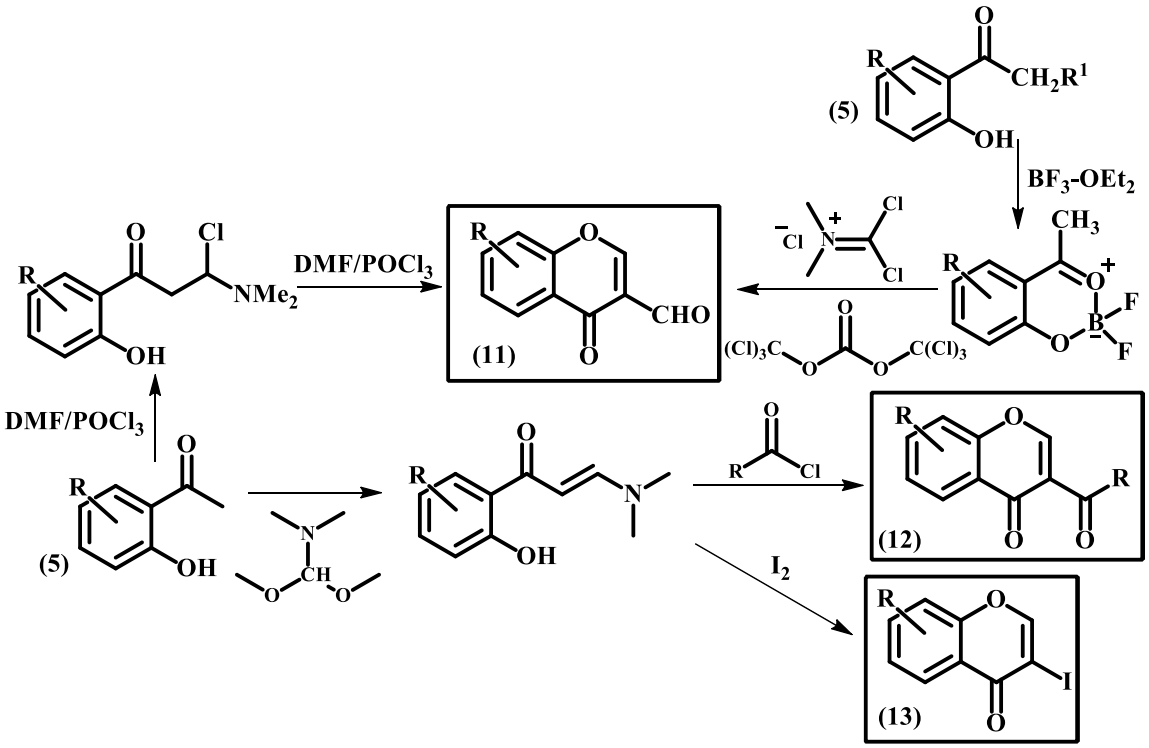
A much more recent methodology, yet with more than 40 years since its description, was developed by Dorofeenko and Tkachenko in 1972 (Scheme 4).[19] This methodology permits the obtention of 2-nonsubstituted chromones (**10**) through benzopyrylium salt intermediates, by making a 2'-hydroxyacetphenone (**5**) react with triethyl ortho formate in the presence of a strong mineral acid. The resulting benzopyrylium salt intermediate (**9**) is afterwards submitted to heating, in water, in order to attain the desired chromone (**10**).



Scheme 4. Chromone synthesis via Benzopyrylium salts.

The last procedure for the formation of the chromone nucleus from 2'-hydroxyacetophenones was named Vilsmeier-Haack reaction and it was performed with the usage of a formylating reagent, known as Vilsmeier-Haack reagent.[20] In this reaction, a chloroiminium ion is formed *in situ* through the reaction between a *N*,*N*-disubstituted formamide (Ex: DMF) and phosphorus oxychloride. This ion then reacts with the 2'-hydroxyacetophenone leading to the formation of a chromone which, due to a second attack from the previously formed chloroiminium ion, culminates in the formation of a 3-formylchromone (**11**) (Scheme 5).

Moreover, due to several downsides of this reaction, such as long reaction time, undesired side reactions or unsatisfactory yields, several modifications of the method has been developed, particularly the usage of a boron trifluoride diethyl etherate and variants of the Vilsmeier-Haack reagent or the use of DMF/DMA (Scheme 5).

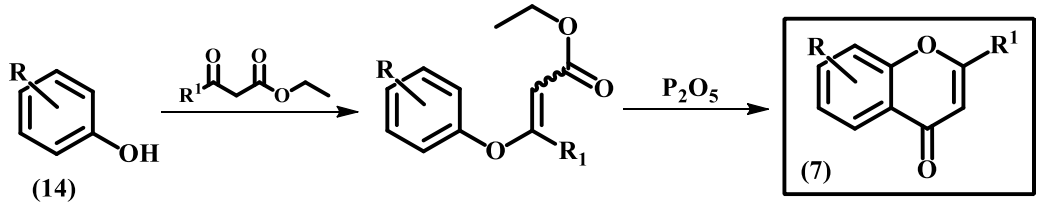


Scheme 5. Vilsmeier-Haack reaction and modifications.

1.2.1.2. Chromones from Phenols

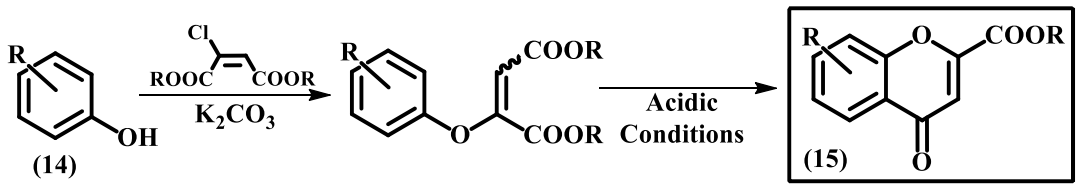
Besides 2'-hydroxyacetophenones (**5**), a few other starting materials have been used for the synthesis of chromones, like phenols. The first procedure using a phenol derivative (**14**)

as a precursor of chromones (**7**) was developed by Simonis, in 1913 (Scheme 6).[21] This reaction, Simonis reaction, consists in a condensation between a phenol or its derivatives with β-ketoesters, in the presence of phosphorus pentoxide (P_2O_5), allowing to obtain chromones as final products. Furthermore, Simonis reaction is known for having greater outcomes when the phenol used as precursor has electron-withdrawing substituents, such as halogens or nitro groups. Moreover, the presence of alkyl groups on the α-position of the β-ketoester can favor the chromone formation.



Scheme 6. Simonis reaction.

In order to attain chromones from phenols, a phenol derivative (**14**) may also react with acetylenic dicarboxylic acids or esters or with chlorofuramic acid, or other analogues, in basic conditions, the Ruhemann reaction (Scheme 7). During this reaction, the resulting intermediate suffers a subsequent cyclization process, in acidic conditions, leading to the chromone formation. This method was mainly applied to the synthesis of chromone-2-carboxylic acids (**15**) and its derivatives but also, with proper modifications, for the synthesis of flavones and styrylchromones.[22]

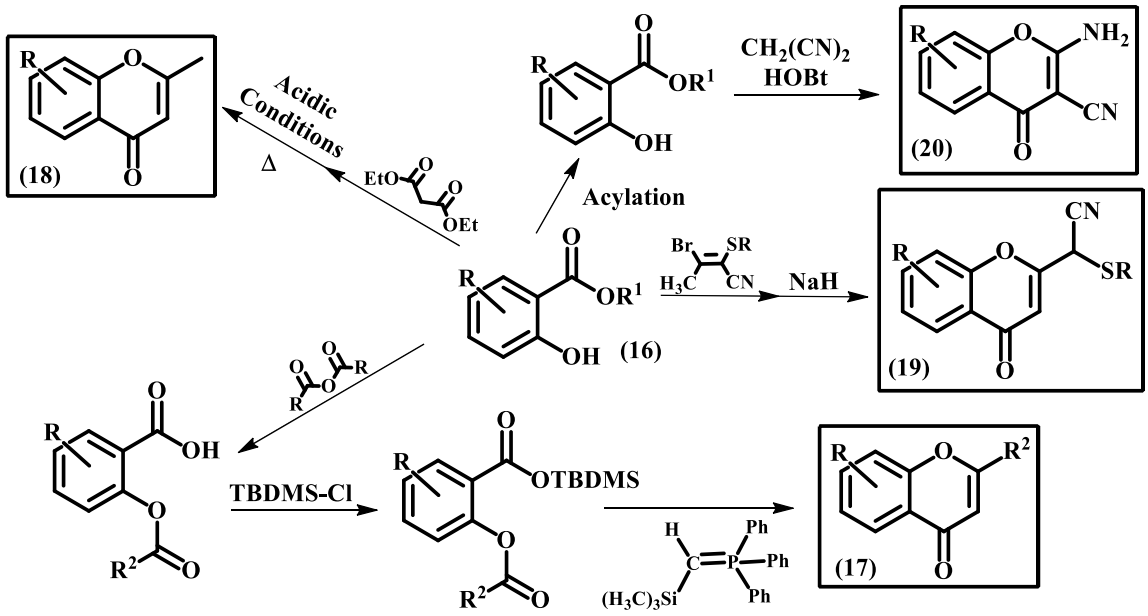


Scheme 7. Ruhemann reaction.

1.2.1.3. Chromones from Salicylic acid and derivatives

One final starting material for obtaining chromones is salicylic acid (**16**) or its derivatives, through a few distinct approaches (Scheme 8). In one of these procedures, *o*-acyl(aroyl) derivatives are attained and converted to the corresponding silyl esters, due to

the action of *tert*-butyldimethylsilyl chloride and imidazole. This intermediate is then converted into a chromone (**17**) through an intramolecular Wittig ester carbonyl olefination, caused by (trimethylsilyl)methylenetriphenylphosphorane. A different approach consists in the reaction between activated salicylic derivatives and diethyl malonate leading to the formation of intermediates that suffer further hydrolysis and decarboxylation culminating in the formation of 2-methylchromones (**18**).[23] Furthermore, chromones (**19**) may also be synthesized through a condensation of methyl salicylate with bromocrotononitrile derivatives followed by cyclization of the vinyl ether intermediate in basic conditions.[24]

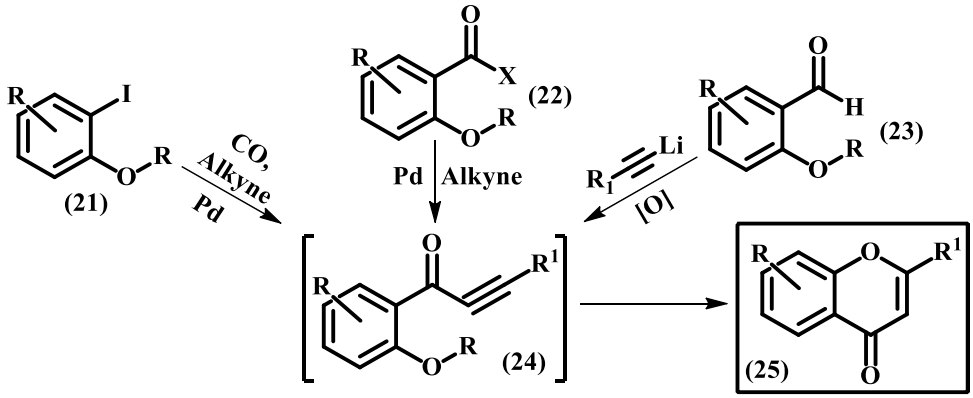


Scheme 8. Chromone synthesis from Salicylic Acids and Derivatives.

1.2.1.4. Chromones via C-C cross coupling reactions

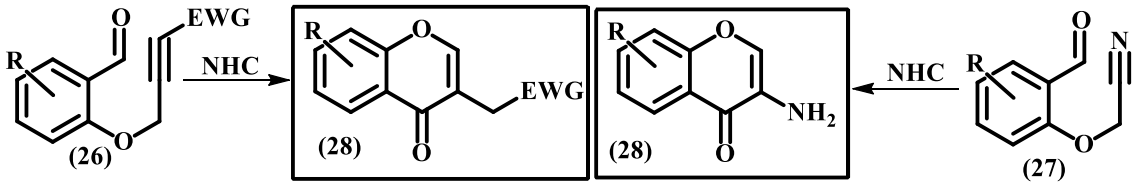
Transition-metal-catalyzed reactions are becoming one of the most important methodologies for the formation of C-C and C-heteroatom bonds. Following this tendency, a synthetic procedure for the obtention of chromones has been developed (Scheme 9). This procedure consists in a reaction between an *ortho*-iodophenol (**21**) and a terminal acetylene, in the presence of a palladium complex, culminating in the formation of an orthohydroxyarylalkynylketone intermediate (**24**). In order to obtain the final product, a cyclocarbonylation process occurs *in situ*, in the presence of carbon monoxide, culminating in the formation of the desired chromone (**25**).[25] Furthermore, the formation of chromone,

via Sonogashira coupling with palladium catalysis, may also be performed using an *ortho*-methoxybenzoyl chloride with a terminal acetylene or an *ortho*-methoxybenzaldehyde with a lithium acetylide.[26]



Scheme 9. Chromone synthesis via Pd-mediated C-C Cross Coupling.

An alternative to the previous palladium-catalyzed reactions are the organocatalyzed reactions, which consist in the use of *N*-heterocyclic carbenes (NHCs) as catalysts for the formation of C-C bonds, leading to the formation of mainly 3-substituted chromones (**28**) (Scheme 10).[27] The main purpose of these particular catalysts is to promote intramolecular cross coupling between the aldehyde and the nitrile groups or to improve a similar reaction between the aldehyde and an activated alkyne, known as intramolecular Stetter reaction.



Scheme 10. Chromone synthesis via organo-mediated C-C Cross Coupling.

1.3. Alzheimer's disease

Alzheimer's disease (AD), firstly described by Alois Alzheimer in 1906, is a progressive neurodegenerative disorder responsible for the great majority of dementias related with age, affecting the daily living through memory loss and cognition impairment.[28] This multifactorial neurodegenerative disease affects, actually, approximately 46 million people worldwide, being the majority over age 65. However, due to the increase of life expectancy, it is estimated the increase of these numbers up to 131.5 million people worldwide by 2050, affecting particularly people from undeveloped countries.[29]

The main features observed in the brains of AD patients include neuronal loss in memory and/or cognition-associated regions, particularly the loss of cholinergic neurons, neurotransmitter depletion and synaptic dysfunction.[30] From a molecular point of view, the most common neuropathological characteristics found in AD patients are abnormal protein deposits, particularly senile plaques (SP) and neurofibrillary tangles (NFT).

Senile plaques (SP) consist in the extracellular accumulation of insoluble aggregates of amyloid-β protein, a small peptide (36-43 aminoacids) originated from an abnormal cleavage of the transmembrane protein amyloid precursor protein (APP), a protein essential to neuronal growth and survival and also the repair of neuritic damage.[31] This abnormal proteolytic process is due to the sequential actions of a β-secretase and a γ-secretase, which leads to the formation of Aβ monomers. These monomers, after a conformational change, spontaneously self-aggregate to create fibrils, which culminate into the formation of insoluble fibers of advanced amyloid plaques (Figure 3).

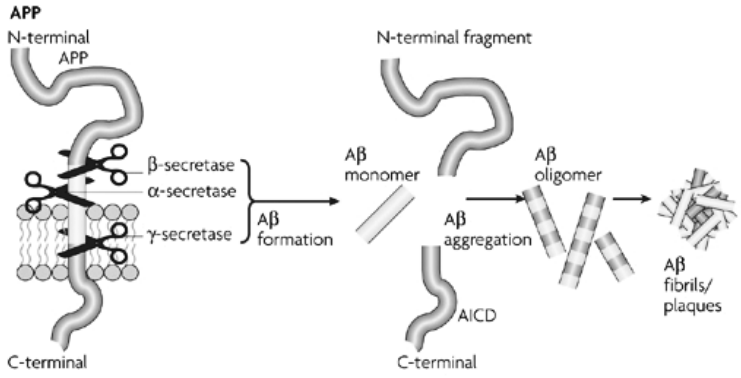


Figure 3. Formation of senile plaques from APP. (Adapted fromGötz et. al(2008)[32])

In turn, neurofibrillary tangles (NFT) are intracellular filamentous inclusions composed by an abnormally hyperphosphorylated and aggregated form of tau protein. In healthy conditions, tau protein appears in a soluble form and promotes assembly and stability of microtubules and vesicle transport in axons. However, hyperphosphorilated tau lacks affinity for microtubules, is insoluble and self-aggregates into paired helical filament structures, which in turn assemble into masses inside nerve cell NFT (Figure 4).[33] A third characteristic symptom of AD, reported by Davis and Maloney in 1976, is a decrease in the number of cholinergic neurons and consequential loss of the enzyme that synthesizes acetylcholine (ACh), reducing the levels of ACh in the brain areas connected with learning, memory, behavior and emotional responses.[34]

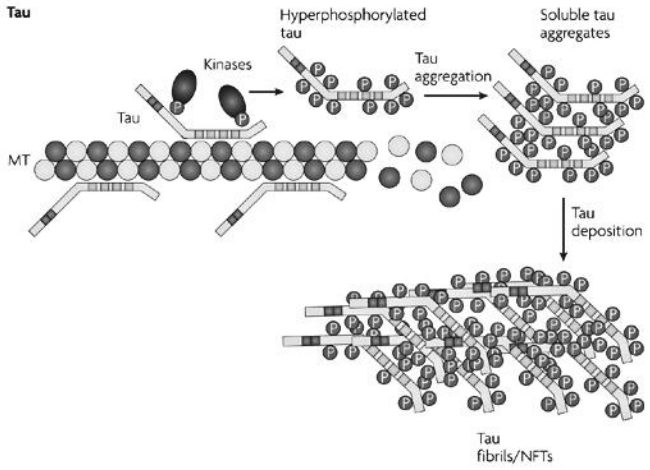


Figure 4. Formation of neurofibrillary tangles. (Adapted from Götz et. al(2008)[32])

Although there is still not a definite conclusion about the cause for the development of AD, several hypotheses have been considerate. Amongst them, the "*Amyloid hypothesis*" and "*Cholinergic hypothesis*" are the most widely accepted in the scientific world. Accordingly, the only approved treatments by US Food and Drug Administration (FDA) are used to treat the cognitive manifestations of AD, namely acetylcholinesterase inhibitors (AChEI) and NMDA receptor antagonists, acting in distinct manners to delay the breakdown of ACh. Following this principle, the "*Cholinergic hypothesis*" will be further described and the potential role of chromone derivatives as anti-acetylcholinesterase agents highlighted throughout the next topic.

1.3.1. Cholinergic hypothesis

The rise of some of the evidences earlier mentioned led to the formulation of one of several theories that try to explain the origin and main cause for the development of AD, the "*Cholinergic hypothesis*", a theory postulated by Whitehouse and his colleagues in 1982 (Figure 5).[35] This hypothesis states that a serious loss of the cholinergic function by the Central Nervous System (CNS) is closely related to the cognitive symptoms verified in the elderly, particularly in patients with AD.[36]

It has been postulated that the degeneration of basal forebrain cholinergic neurons, related to the loss of cholinergic neurotransmition in cerebral cortex and other regions of the brain, may contribute to the, already referred, deterioration of cognitive function in AD patients.[37] This cholinergic depletion in synaptic regions might be promoted by the failure in this neurotransmitter synthesis, the decrease of choline uptake by neuronal cells and, predominantly, due to the action of acetylcholinesterase (AChE). This enzyme acts by cleaving the neurotransmitter acetylcholine into choline and acetate, compromising the cholinergic neurotransmition. Because of that, this enzyme was and continues to be one of the main targets for the development of new treatments that may contradict the loss of cognitive function suffered by AD patients.[38]

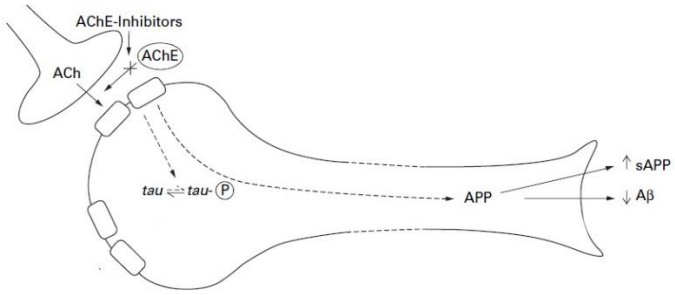


Figure 5. The Cholinergic Hypothesis: The neuronal loss gives rise to a reduced release of ACh which culminates in a shift of tau into hiperphosphorylated-tau and an increase in the production of Aβ. AChEI * would lead to the reduction of ACh breakdown and, as consequence, reduce the levels of hiperphosphorylated-tau and Aβ. (*Hypothesized consequences) (Adapted from Francis et. al(1999)[39])

Furthermore, some studies have suggested that AChE plays other roles in addition to its so called "classical" function in terminating impulse transmission at cholinergic synapses.

For instance, Inestrosa and colleagues have shown that AChE accelerates the assembly of Aβ by interacting with Aβ and promoting amyloid fibril formation through a few amino acids located in the proximity of its peripheral anionic site (PAS).[40,41] Moreover, it has also been revealed that the complexes formed by AChE and Aβ present enhanced neurotoxicity when compared with fibrils containing only Aβ, proving that several AChEI do not only facilitate cholinergic transmission but are also capable of interfering with the synthesis, deposition and aggregation of toxic Aβ.[42]

Chromones, as mention earlier, are natural compounds possessing a wide range of pharmacological properties related to Alzheimer's disease[43], such as neuro-protective effect, AChE inhibitory activities and Aβ fibril formation inhibitory activity, among others. Following this principle, various series of chromones have been synthesized and evaluated for their AChE inhibitory activity.

In 2009, Sheng and colleagues synthesized two series of chromone derivatives, namely chromone-benzylpiperidine hybrids.[44] The data obtained from this research work allowed formulating some preliminary structure-activity relationships. This SAR study showed that 3-phenylchromone series (**29**) generally demonstrated more potent inhibitory activity against AChE than 2-phenylchromone series (**30**). Furthermore, it appeared that the type of linker chain between the flavonoids and benzylpiperidine moieties have an important role in the inhibitory potency against AChE, being more potent those with only an oxygen atom as linker when compared with groups like OCH_2, $O(CH_2)_2$ or $O(CH_2)_3$ (Figure 6).

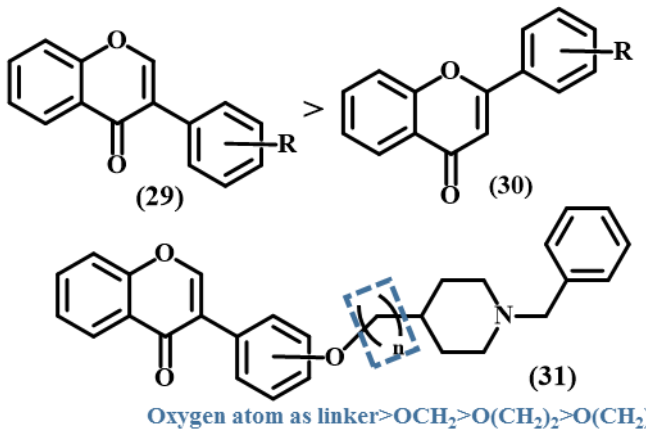


Figure 6.Structure-AChE inhibitory activity of flavonoid-benzylpiperidine hybrids.

Following the previous work, the same research group performed the synthesis of a new series of chromones (Figure 7).[45] The evaluation of its inhibitory activity towards AChE resulted in a further SAR study where it was possible to conclude that the presence of an aminomethyl group in the *para*-position of ring B is most suitable to this type of activity than when in *meta*-position. Furthermore, the substitution pattern of the aminomethyl group also influence the activity of AChE inhibition, since compounds containing pyrrolidine or piperidine showed better activity than those with methylethylamino or diethylamino group. This evidence may indicate that the enzyme favors an hydrophobic moiety with a conformational constraint in this position.

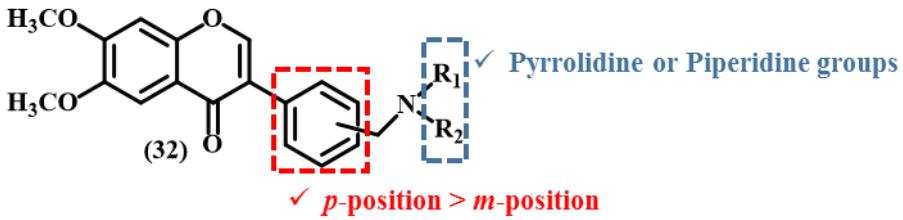


Figure 7. Structure-AChE inhibitory activity of novel series of chromone derivatives.

Following the structural knowledge of AChE, another research group decided to connect the chromone moiety with terminal amine groups through carbon spacers with different dimensions.[46] These chromone derivatives (**33**) might occupy both catalytic and peripheral active sites (CAS and PAS) via cation-π interaction and aromatic stacking interactions, respectively. Therefore, this research group designed a series of chromone derivatives and evaluated them towards their AChE inhibitory activity, as well as anti-Aβ42 aggregation and metal-chelating ability, and structure-activity relationships were delineated based on the pharmacological activities (Figure 8). The results obtained showed that the introduction of aminoalkyl-substituted groups might significantly increase the inhibitory activity of these derivatives. Moreover, it was possible to understand that the optimal chain lengths of the carbon spacer for inhibiting AChE are four and five carbons. In order to conclude this SAR study, several terminal amine groups were introduced into the chromone scaffold. This data gave rise to the understanding that both cyclic and chain monoamine groups present the ability to occupy the CAS of AChE, showing high inhibitory activity against AChE, while compounds containing additional nitrogen and oxygen atom in the terminal group showed reduced AChE inhibitory activities.

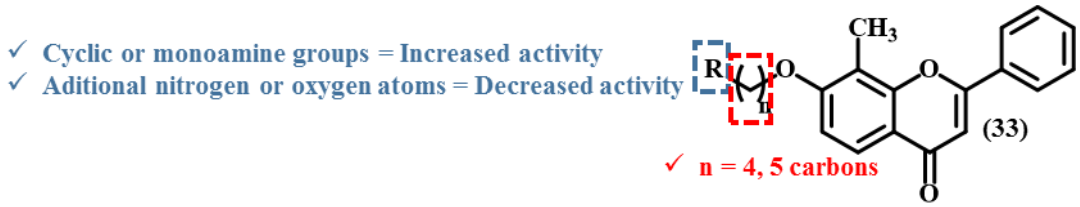


Figure 8. Structure-AChE inhibitory activity of new chromone derivatives.

In the same year, Luo and colleagues designed a new series of chromone derivatives, *N,N*-dimethylated 2-phenylchromones (**34**), by conjugating one of these compounds with a terminal amine using a long chain linker (Figure 9).[47] The results obtained throughout this work allowed to verify that these 4-dimethylamine 2-phenylchromone derivatives present considerable inhibitory potency for AChE at levels similar to or better than those of the inhibitor used for comparison, namely Rivastigmine. In this research work it was possible to understand that compounds with diethylamine or pyrrolidine groups present more potent activities that those with piperidine or benzyl methylamine groups.

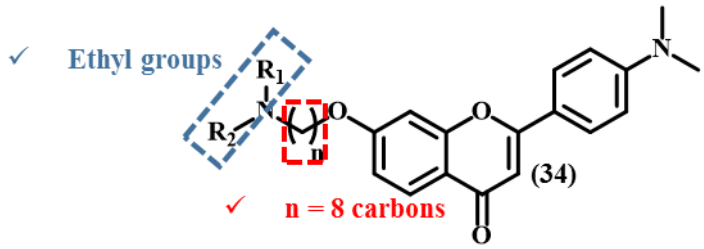


Figure 9. Structure-AChE inhibitory activity of N,N-dimethylated 2-phenylchromones.

Following the studies with chromone derivatives, neuroprotective effects and learning and memory improvements have been associated to genistein.[48,49] Based on these previous evidences, and the fact that quinolone-polyamine conjugates exhibited considerable AChE inhibition activity, several genistein conjugates modified with polyamine were designed (**35**) (Figure 10).[50] The results of this research work revealed that all of the tested compounds presented AChE inhibitory activity and also confirmed that the conjugation of polyamines with genistein led to an increase in the inhibition activity of AChE. Moreover, it was possible to understand that AChE inhibitory potency of these novel compounds stays closely related to the length and the end group of the polyamine chain, being those modified by diamine more actives than compounds conjugated with other types of amines.

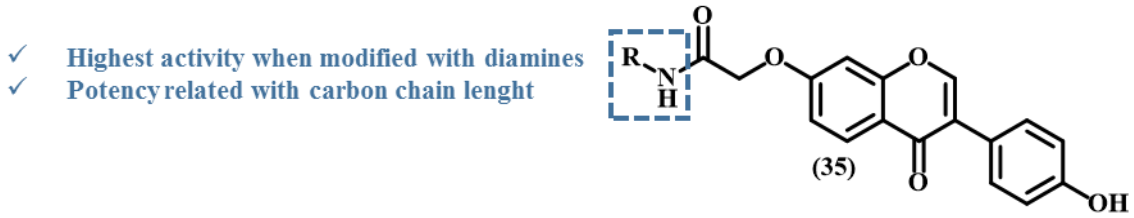


Figure 10. Structure-AChE inhibitory activity of genistein-polyamine conjugates.

Another research group based their studies in the possibility of combine scutellarein, a chromone with numerous pharmacological properties related to neurological disorders, with Rivastigmine, a carbamate compound actually used as AChE and BuChE inhibitor for the treatment of AD.[51] In this work, a series of scutellarein-rivastigmine hybrids (**36**) were designed and evaluated towards several pharmacological activities, from which is important to highlight AChE and BuChE inhibitory activity (Figure 11). From the data obtained in their studies it was possible to confirm that the insertion of carbamates leads to an increase of AChE inhibitory activity with the most potent AChE inhibitory activity being observed in compounds containing the *N*,*N*-diethylcarbamate moiety. Furthermore, it was also possible to comprehend that substitutions at 5-position of the chromone scaffold might have considerable effects on the previously mentioned activity, since 5-methoxy substituted derivatives presented better activity. The results also showed that the position of the carbamate moiety in the B-ring might have a great influence in the AChE inhibitory potency, as the compounds with this group at 4'-position presented higher activities than those with the carbamate group at 3'-position. To conclude, it was also verified that the substitution pattern of the carbamate moiety has an enormous influence in the potency of this inhibitory activity, being *N*,*N*-diethylamine and *N*-ethyl-*N*-methylamine groups the most suitable to increase the AChE inhibitory activity.

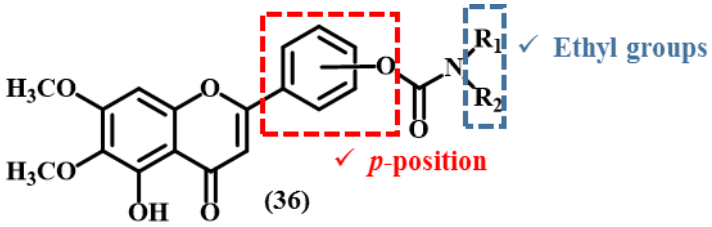


Figure 11. Structure-AChE inhibitory activity of scutellarein-rivastigmine hybrids.

Early this year, the same research group focused their efforts in the synthesis of novel chromone derivatives that may possess high AChE inhibitory capacity and be used for the treatment of neurological disorder like AD.[52] For this purpose, several new 7-aminoalkyl-substituted 2-phenylchromone derivatives (**37**), with B-ring modifications, were synthesized and evaluated for their AChE inhibitory activity (Figure 12). The results of this work revealed that the introduction of a methoxy group in 2'- or 3'-position on the B-ring of the 2-phenylchromone nucleus promoted a considerable improvement of the inhibition against AChE. Additionally, it became clear that presence of groups like benzyloxyl or hydroxyl in any of the already mentioned positions led to a decrease in the AChE inhibitory activity.

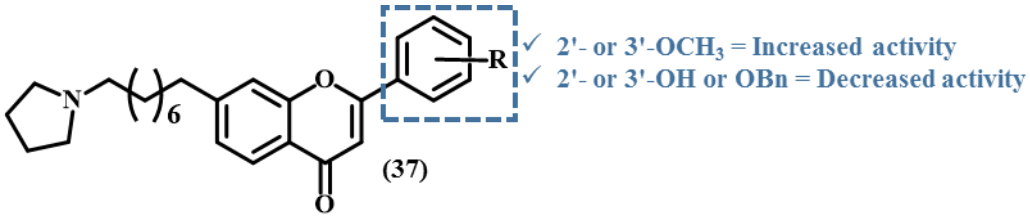


Figure 12. Structure-AChE inhibitory activity of 7-aminoalkyl-substituted 2-phenylchroone derivatives.

1.3.2. Inflammation

Inflammation is a defensive response against external stimuli, inducing physiological adaptations to minimize tissue damage or remove pathogenic infections. In its initial stage it is not considered a disease but, under chronic conditions, it may lead to a number of disorders, including rheumatoid arthritis, asthma and even cancer.[53] Given those chronic inflammatory effects, several enzymes have been studied and associated with the inflammatory processes. Therefore, these enzymes became crucial targets for the development of drugs for the treatment of a variety of inflammatory disorders, like bronchial asthma, inflammation, autoimmune diseases and cancer.

As mentioned before, chromones constitute an important class of natural compounds and have been reported to exhibit anti-inflammatory activity, for example nedocromil (**38**) and cromoglycate (**39**) (Figure 13). As anti-inflammatory agents, chromones may exert their effects by inhibiting numerous mechanisms which will be further developed.

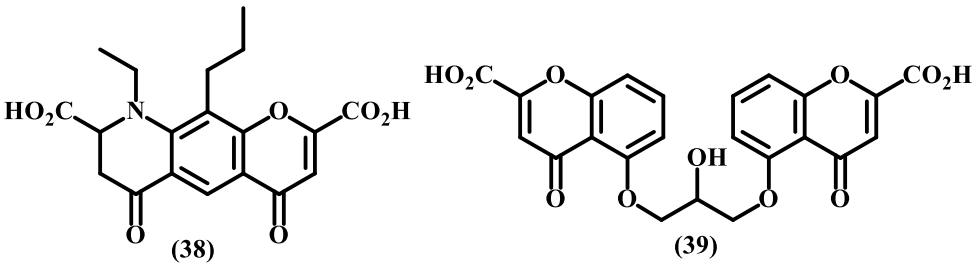


Figure 13. Examples of already marketed chromones.

1.3.2.1. Cyclooxygenase (COX) inhibitors

An understanding of the arachidonic acid (AA) metabolism led to the systematic treatment of inflammatory diseases by regulating the enzymatic actions of phospholipase, cyclooxygenases and/or lipoxygenases (Figure 14).

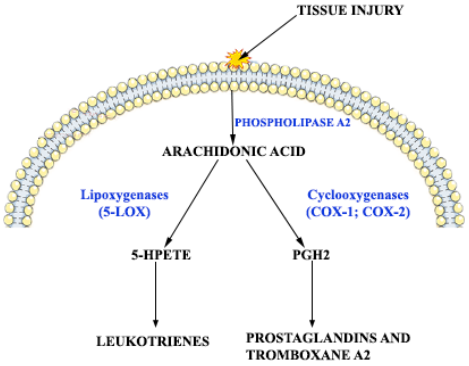


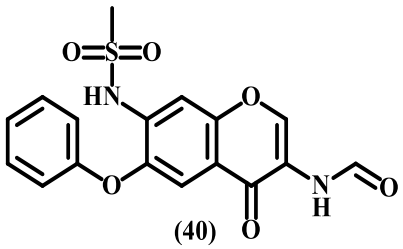
Figure 14. Metabolism of Arachidonic acid: After the release of Arachidonic acid, in response to stimuli like tissue damage, it can be metabolized by Cyclooxygenases 1 and 2 (COX-1 and COX-2) or by 5-Lipoxygenase (5-LOX). The cyclooxygenase pathway leads to the production of prostaglandins and thromboxane A2, while the lipoxygenase produces leukotrienes.

Cyclooxygenases are crucial enzymes in the synthesis of prostaglandin H_2 (PGH_2) which is an intermediate in the biosynthesis of different eicosanoids, such as prostaglandins, prostacyclins and thromboxanes.[54,55] These enzymes appear in at least two isoforms, namely cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2).[56,57] COX-1, constitutively expressed in almost every cell type, is responsible for the physiological production of prostaglandins (PGs), in several organs, and thromboxane A_2, in platelets. COX-1 is therefore critical for platelet aggregation, protection of gastric mucosa and renal blood flow. On the other hand, COX-2 is constitutively expressed in just a few organs, such as kidney,

brain and gravid uterus. However, its expression may be promptly induced by several stimuli, such as growth factors, pro-inflammatory cytokines like interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), lipopolysaccharides (LPS), and tissue plasminogen activator (TPA), and tumor promoters, during inflammation or oncogenesis. Thus, COX-2 has been indicated as an important intervenient in both acute and chronic inflammatory disorders as well as in the carcinogenesis and tumor progression. [58,59]

Several compounds have been isolated, from terpenoid, alkaloid and flavonoid categories, and their activity as COX inhibitors evaluated, amongst which chromone based compounds have shown promising inhibitory activities toward COX-1 and COX-2 enzymes.[60] In the last few years, several new compounds containing the chromone nucleus, isolated from different types of plants or synthesized, have demonstrated the ability to inhibit the referred enzymes. [61,62] For that reason, the chromone scaffold has been used as basis for the synthesis of new anti-inflammatory agents. [63,64]

The first chromone with COX inhibitory activity was *N*-(3-Formamido-4-oxo-6-phenoxy-4*H*-chromen-7-yl)methanesulfonamide (**40**), also named iguratimod (Figure 15). Some biochemical studies on the AA cascade have shown that this compound was able to selectively inhibit COX-2, meaning that it was able to successfully inhibit the production of prostaglandin E2 (PGE_2) and reduce the PGE_2 contents in inflammatory exudates without decreasing the gastric mucosal levels of prostaglandins. Moreover, it has been found that this compound is able to reduce the expression of the COX-2 mRNA induced with inflammatory stimuli in cultured fibroblasts.[65]



(40)

Figure 15. First chromone with COX inhibitory activity.

Discovery of the anti-inflammatory activity of stellatin, the major constituent of *Dysophylla stellata* (Lour.) Benth. ex Wall. as a COX inhibitor,[61] functioned as inspiration for the development of chromone analogues. Structure-activity relationship (SAR) studies

of chromones on the inhibition of COX allowed the establishment of a basic pharmacophore responsible for activity, in which the C2=C3 double bond, the 4-carbonyl group, and the 5-hydroxy substituent are essential features for the COX inhibitory activity (Figure 16).[63] This study also suggested that ether substituents with a chain length up four carbon atoms or a benzyl group at the 8-position results in decreases inhibitory activity. However, the introduction of a 5-isoprenyl group led to an improvement in COX-2 inhibition, whereas COX-1 inhibitory activity remained unaffected.

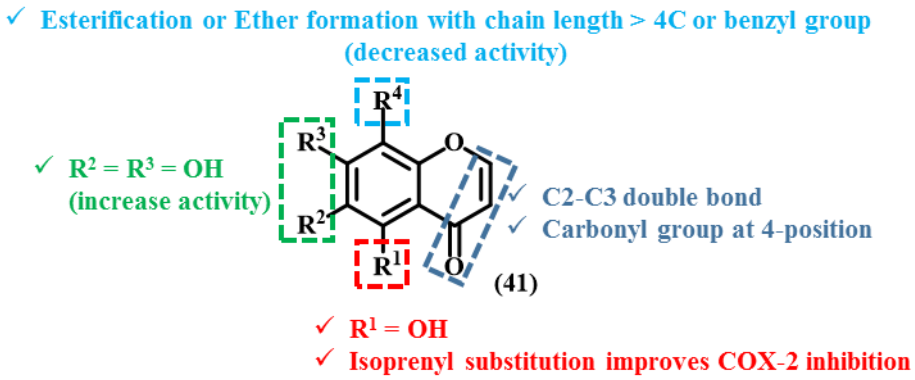


Figure 16. Structure-COX inhibitory activity relationship of chromones.

Currently, the design of new COX inhibitors considers the synthesis of hybrid molecules, obtained through the combination of different biologically active molecules. Along with chromones, indoles and pyrazoles are other naturally occurring groups that are part of numerous drugs in clinical use, such as indomethacin (**42**) and celecoxib (**43**) (Figure 17). Hybrids might exhibit higher biological activities than their individual components alone, as has been reported for some indole-pyrazole and indole-pyrimidine conjugates. [66,67]

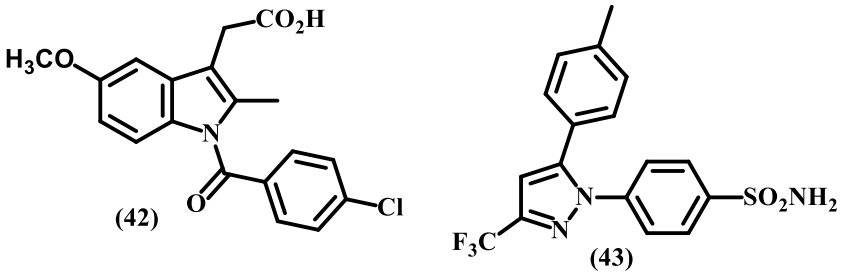


Figure 17. Examples of clinically used nitrogen heterocycles.

Following this idea, several hybrids containing chromone and indole groups were synthesized, and their COX inhibitory activities were evaluated (Figure 18).[68] Relative to

chromone- and indole-based drugs, the combination of chromone and oxindole (e.g. compounds (**44**), Figure 18) resulted in considerable inhibitory activity and selectivity for COX-2 over COX-1. Moreover, these compounds showed higher inhibition toward COX-2 and better selectivity for COX-2 over COX-1 than the parent compound, wogonin (**45**) (Figure 18) and indomethacin (**42**) (Figure 17), which demonstrates the potential of the design of these hybrids. This study by *Shaveta et al.* has proven that hybrids, obtained by combining two bioactive groups, exhibit significantly higher biological activity than their individual components.[68] In a subsequent study by Shaveta and coworkes, the biologically significant group chrysin was combined with indole and pyrazole rings. By the appropriate combination of these three moieties, a series of compounds were synthesized and evaluated for COX inhibitory activity (Figure 18).[69] The results revealed that one of derivatives, compound (**46**) (Figure 18), shows considerable COX inhibitory activity, similar to that of indomethacin (**42**), and higher selectivity for COX-2 over COX-1 than that of indomethacin (**42**), which may be a desirable characteristic. In summary, these results indicate that compounds obtained by combination of chrysin, indole and pyrazole have better COX-2 inhibitory activity than the drugs based in these three individual components alone.

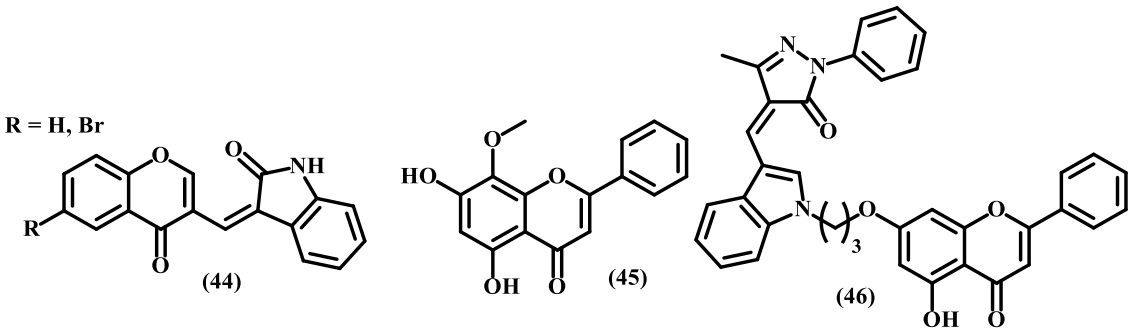


Figure 18. Hybrid molecules containing chromone and nitrogen-containing moieties.

1.3.2.2. Lipoxygenase inhibitors

Lipoxygenases (LOXs) are a family of closely related iron-containing dioxygenases, which are responsible for the production of inflammatory mediators through the addition of molecular oxygen into poly-unsaturated fatty acids. From all the mammalian lipoxygenases, 5-LOX, which is particularly expressed in polymorphonuclear leukocytes (PMNL), mast-cells, macrophages, eosinophils, monocytes, basophils and B lymphocytes, is the most involved in inflammatory and allergic disorders.[70]

The metabolites generated by LOX enzymes, particularly in the 5-LOX pathway, have been described as important mediators of chronic inflammation processes by acting as potent chemotactic agents, increasing microvascular permeability, modulating pain induced by inflammation reactions and inducing cell adhesion.[71] For instance, the leukotriene B_4 (LTB_4), produced by PMNL, has been reported as a potent chemoattractant mediator of inflammation. LTB_4 acts by stimulating neutrophil chemotaxis, enhancing neutrophil-endothelial interactions and stimulating neutrophil activation, originating degranulation and the release of superoxide radicals ($O_2^{\bullet -}$), mediators and enzymes.[72]

Although it has been thought that bronchial asthma was the principal pathophysiological implication of LTs, these eicosanoids favor the pathogenesis of many other human inflammatory diseases like rheumatoid arthritis, inflammatory bowel disease, Crohn's disease and psoriasis, as well as atherosclerosis, osteoporosis and certain types of cancer.[73–75] Consequently, LOX inhibitors might be of interest for the resolution of a wide range of inflammatory processes.

Several plants containing chromone derivatives have been used in traditional medicine.[76] For example, flavocoxid, marketed as Limbrel®, is an extract containing the naturally occurring baicalin (**47**) and catechin (**48**) (Figure 19). This extract can act as a dual inhibitor of COX enzymes and 5-LOX.[77]

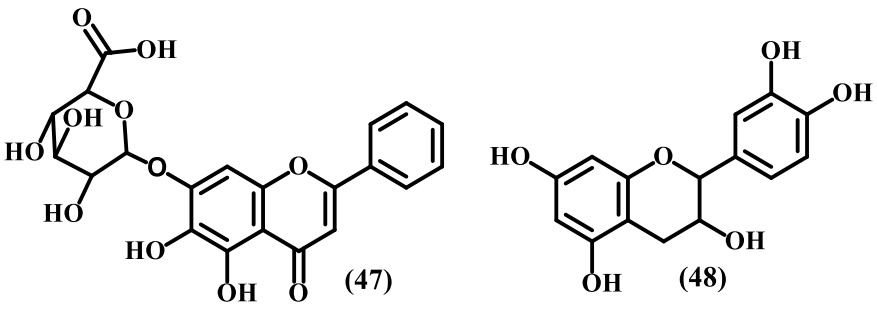


Figure 19. Flavocoxid constituents.

More recent studies have reinforced the role of flavonoids as effective inhibitors of LT production in human neutrophils and, consequently, as LOX inhibitors. Based on this evidence, a SAR study was carried out with flavones (Figure 20).[78] This study demonstrated that one of the main structural features required for considerable LOX inhibitory activity is the catechol group in the B-ring, whereas the number of hydroxy groups in the A-ring did

not seem to be relevant for the same activity. Moreover, it was verified that 7-hydroxyflavones seem to be more active than those that lack this substituent. Alternatively, it became clear that the presence of a 3-hydroxy group contributes to decrease the activity. This SAR study also confirmed the conclusions of Sadik and colleagues[79] that the vital structural characteristics for inhibition of pro-oxidants enzymes, such as lipoxygenases, are the presence of a C2=C3 double bond conjugated with a 4-carbonyl group and the presence of a catechol group in the B-ring (Figure 20). The activity of luteolin (**50**), which is higher than that of quercetin (**51**), confirms this observation (Figure 20) and also indicates that an excessive number of hydroxy groups, which lowers the compound's hydrophobicity, may restrain the access to the active site of the enzyme.

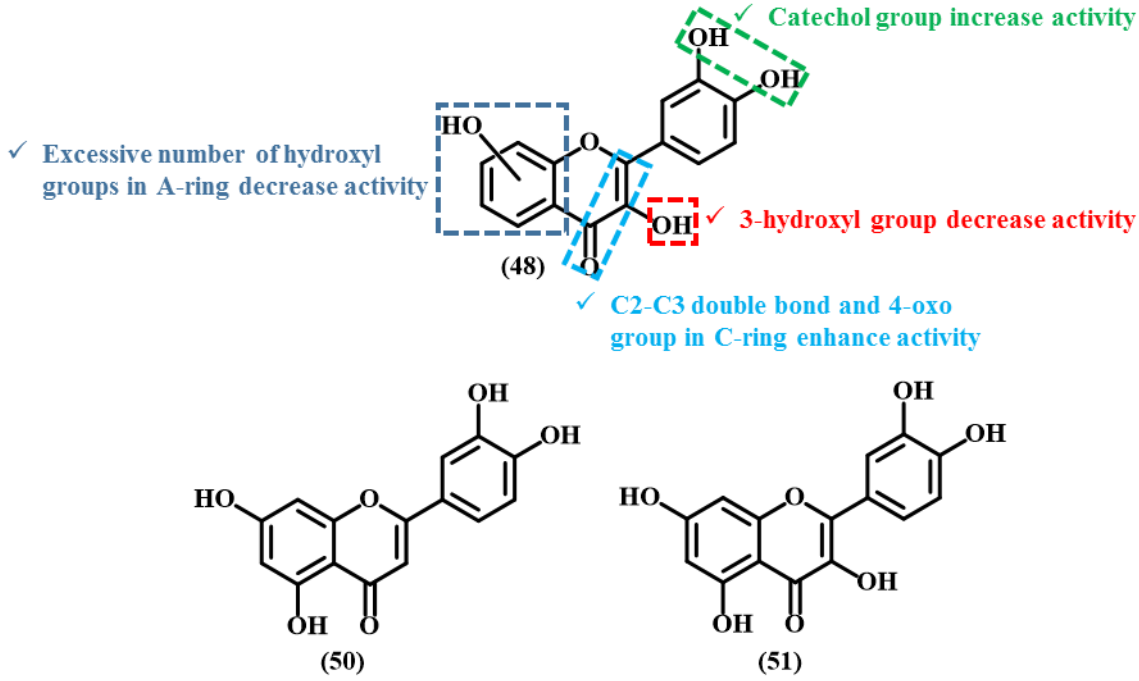


Figure 20. Structure-lipoxygenase inhibitory activity relationship of flavones.

Earlier this year, Zhao and colleagues developed a study in which the major components of four selected Chinese medicinal herbs were evaluated towards their 5-LOX inhibitory activity.[80] Among these constituents, several chromone derivatives presented promising activities, particularly daidzin (**52**), daidzein (**53**) and 3'-hydroxy-puerarin (**54**) (Figure 21).

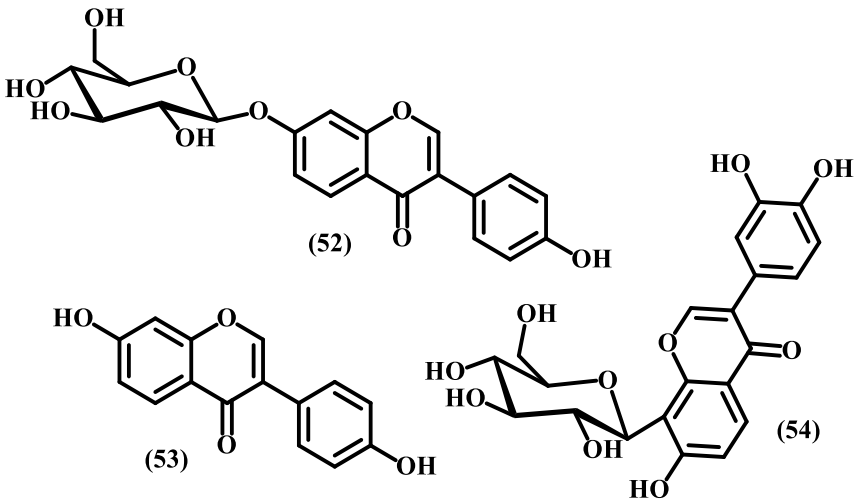


Figure 21. Structures of daidzin, daidzein and 3'-hydroxypuerarin.

Along with the inhibition of LT production, another way to prevent inflammation might be to antagonize the action of LTs for the treatment of various inflammatory diseases. Numerous chromones have emerged over the years as being able to act as LT receptor antagonists. The first example of a chromone with this activity was FPL 55712 (**55**), a compound structurally similar to LTD_4 (**56**) (Figure 22).[81] Using compound **55** as a lead, it was found that the incorporation of the chromone carboxylic acid segment increases potency. Another way to improve LT receptor antagonist properties is to replace the carboxylic acid by a tetrazole ring, yielding compound **57** (Figure 22). Further optimization of the lipid backbone then led to the discovery of pranlukast (**58**), the first LT receptor antagonist approved for the market (Figure 22).[82]

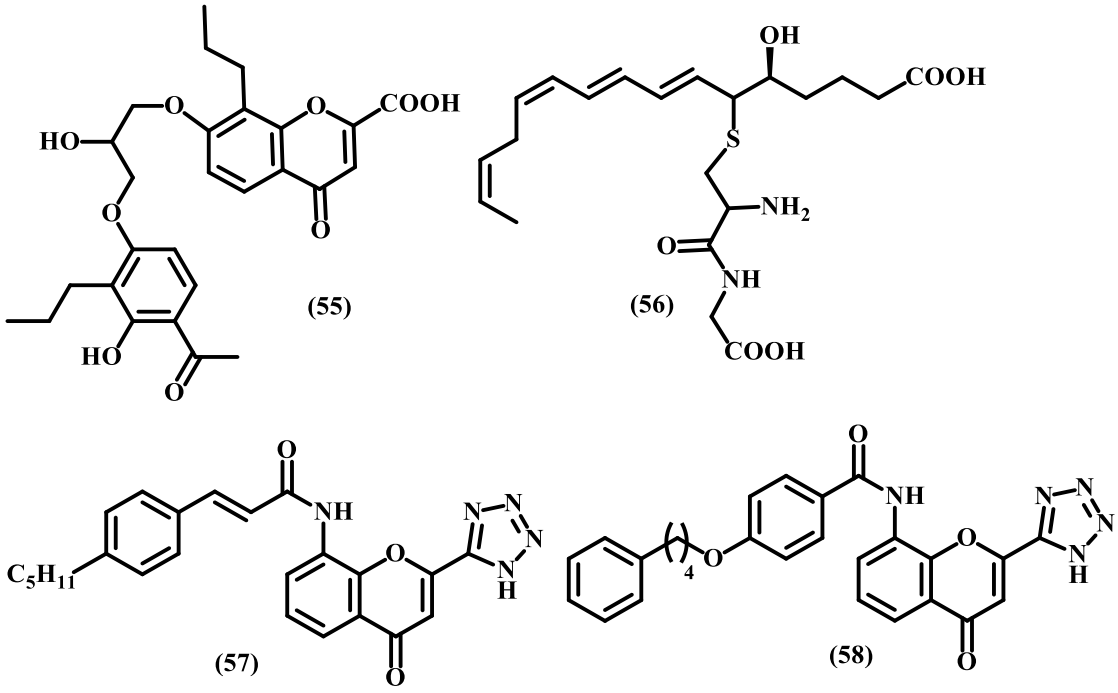


Figure 22. Compounds involved in the discovery of Pranlukast, the first marketed leukotriene receptor antagonist.

Combination of the chromone moiety with the (2-quinolinylmethoxy)phenyl group led to the discovery of the most potent LT receptor antagonist reported so far, RG 12553 (**59**) (Figure 23), which has even higher affinity than the natural ligand LTD_4 (**56**) (Figure 22).[83] Through SAR studies it was possible to verify that the presence of the chromone moiety itself is not enough for high binding affinity. Furthermore, structural features like the tetrazole group can be regarded as an acidic function that improves receptor affinity.

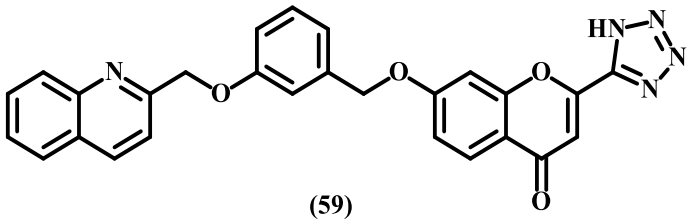
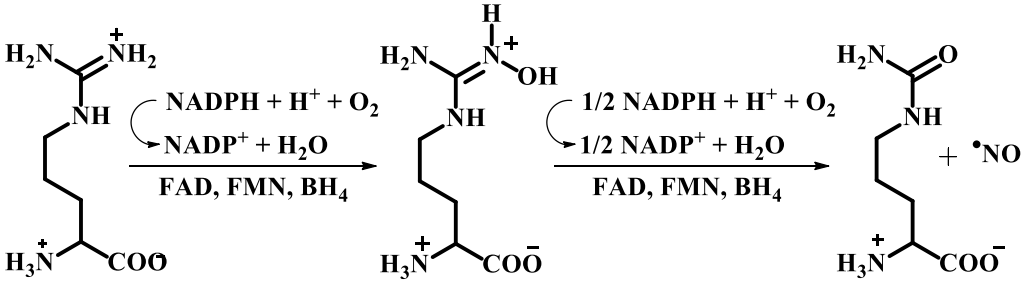


Figure 23. Structure of RG 12553

1.3.2.3. Nitric oxide (˙NO) production inhibitors

Since the discovery of its role as a biologically active molecule, in the late 1980s, nitric oxide (˙NO) has been found to be an endogenous free radical that plays an important role as a signaling molecule in many parts of the organism, as well as cytotoxic or regulatory effector molecule of the innate immune response.[84] It is now understood that ˙NO controls numerous functions, such as neurotransmission or vascular tone, by stimulating ˙NO-sensitive guanylyl cyclase, gene transcription and translation, by binding to iron-responsive elements, and post-translational modifications of proteins, for example by the action of ADP ribosylation.[85]

The generation of ˙NO in mammals ,from L-arginine, is brought by nitric oxide synthase (NOS), which is present as endothelial NOS (eNOS or NOS I), inducible NOS (iNOS or NOS II) and neuronal NOS (nNOS or NOS III).[86] This family of enzymes catalyzes NO biosynthesis via a reaction involving the conversion of L-arginine to L-citruline.[87] The reaction involves an initial hydroxylation of L-arginine, leading to the formation of *N*-hydroxy-L-arginine, which also acts as a substrate for NOS. This step is then followed by oxidation of the intermediate, using a single electron from NADPH, to form L-citruline and NO. Besides the usage of L-arginine as a substrate, molecular oxygen (O_2) and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) are also used as co-substrates, as well as flavin-adenine-dinucleotide (FAD), flavin-mononucleotide (FMN) and (*6R*)-5,6,7,8-tetrahydro-L-biopterin (BH4) are utilized as cofactors for all isozymes (Scheme 11).[88,89]

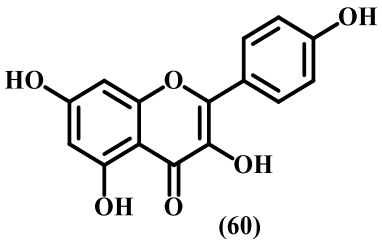


Scheme 11. Production of NO by the enzyme NOS.

eNOS and nNOS are usually referred to as constitutively expressed Ca^{2+}-dependent enzymes, producing low levels of ˙NO. On the other hand, iNOS is not constitutively

expressed, but its expression can be rapidly induced by several immunological stimuli, such as LPS, interferon-γ (IFN-γ), and a variety of pro-inflammatory cytokines, becoming essentially unregulated once expressed.[85] The vast majority of inflammatory and autoimmune lesions are characterized by an abundance of activated macrophages and neutrophils, leading to the secretion of enormous amounts of ˙NO which causes damage to the surrounding tissues.[90,91] Moreover, under inflammatory conditions, macrophages can increase the production of both ˙NO and superoxide anion ($O_2^{\bullet -}$) at the same time, which quickly react with each other to form peroxynitrite anion ($ONOO_2^-$) and play a role in inflammation and possibly also in the multi-phased process of carcinogenesis.[92] In turn, the peroxynitrite anion can activate both the constitutive and inducible forms of cyclooxygenase, COX-1 and COX-2 respectively, which are crucial enzymes during the inflammatory process, as described earlier.

Flavonoids, as mentioned before, are naturally occurring polyphenolic compounds broadly found in the plant kingdom, that display a wide variety of effects, such as antioxidant, antitumor, and anti-inflammatory activities.[93] One moleculas mechanism implicated in the anti-inflammatory activity of flavonoids is the inhibition of iNOS expression and ˙NO production.[93,94] Through the evaluation of several SAR of naturally occurring flavonoids on ˙NO production, using LPS-activated RAW 264.7 macrophages, it was possible to verify that luteolin (**50**), quercetin (**51**) (Figure 20) and apigenin (**60**) (Figure 24) were able to inhibit ˙NO production.[95]



(60)

Figure 24. Structure of apigenin, an inhibitor of ˙NO production.

These results indicated that, along with the substitution pattern, the presence of a C2=C3 double bond might be an important structural feature for the inhibitory activity. The optimal structural features for inhibition of ˙NO production in LPS-stimulated RAW 264.7 macrophages were found to be 5,7-dihydroxyflavones bearing 2',3'-dihydroxy or 3',4'-dihydroxy groups, with the most potent inhibition observed for

3',4',5,7-tetrahydroxyflavone, which provides a clue that increasing the number of hydroxy groups in the chromone skeleton might improve the ˙NO inhibitory activity.

Supported by the advantage of having multiple hydroxy groups in the chromone scaffold, numerous dihydroxychromone derivatives have been synthesized in order to understand how different 3-substituents might influence the ˙NO inhibitory activity of this compound type (Figure 25).[96] This work revealed that esterification at 3-position improves the inhibitory effect. It was also found that the fatty acid chain length had only little effect on inhibitory activity; however extremely long chain length might decrease the activity. In addiction, aromatic esters show better activity than fatty acid derivatives. In contrast, with a slightly different substituent (CH_2OR), the length of the carbon chain seemed to be closely related to inhibitory activity: that is, increasing chain lengthled to a decrease in activity. In summary, this work revealed that a 3-CH_2OH group on the chromone skeleton, an appropriate carbon chain length and/or aromatic esterification might give rise to new leads for improving the ˙NO inhibitory activity of this compound class.

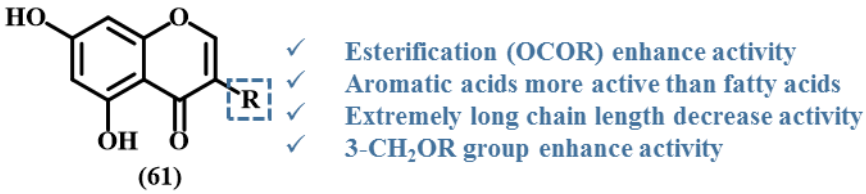


Figure 25. Dihydroxychromone derivatives with ˙NO inhibitory activity.

The potent anti-inflammatory activity of wogonin (**45**) (Figure 18) and its structural similarity with oroxylin A (**62**) (Figure 26) motivated the synthesis and evaluation of several oroxylin A derivatives as ˙NO production inhibitors.[97] The results provided confirmation that simple modifications to the oroxylin A core can enhance activity. It became clear that the anti-inflammatory activity of these derivatives depends on the substituent position and less on the functional group itself. Moreover, single substitutions on the B-ring, mainly in 3'- or 4'-position, produced the most active compounds.

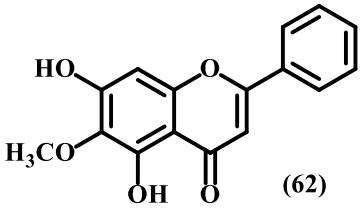
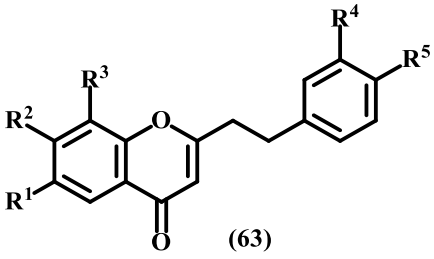


Figure 26. Oroxylin A structure

Chen et al. evaluated five 2-(2-phenylethyl)chromone derivatives (**63**) isolated from the resinous wood of *Aquilaria sinensis* (Lour.) Spreng. (Figure 27).[98] Although these compounds showed considerable inhibitory activity, no clear SAR was found, as all of them exhibited approximately the same level of activity, regardless the presence of various methoxy and hydroxy groups or their distinct substitution sites.



R^1 = R^5 = OH, R^2 = R^4 = OMe, R^5 = H;
R^1 = OMe, R^2 = R^3 = R^4 = H, R^5 = OH;
R^1 = R^4 = OH, R^2 = R^3 = H, R^5 = OMe;
R^1 = R^4 = OH, R^2 = R^5 = OMe, R^3 = H;
R^1 = R^3 = R^4 = OH, R^2 = H, R^5 = OMe;

Figure 27. 2-(2-Phenylethyl)chromone derivatives.

Early in 2014, four new natural chromone derivatives extracted from the roots of *Baeckea frutescens* L., baeckin F (2*S*,3*S*) and baeckin G (2*R*,3*R*) (**64**) and baeckin H (2*S*,3*S*) and baeckin I (2*R*,3*R*) (**65**) (Figure 28), were studied for their anti-inflammatory effects through inhibition of ˙NO production.[99] The results revealed that the 2*R*,3*R* isomers of these compounds have higher ˙NO inhibitory activity than the 2*S*,3*S*-configured isomers. Moreover, for these compounds it became clear that the presence of a sugar residue improves inhibitory activity, as compounds with a β-D-glucose moiety were found to be more active than those without. However, the influence of the absolute configuration seems to be much greater than the presence of a β-D-glucose unit.

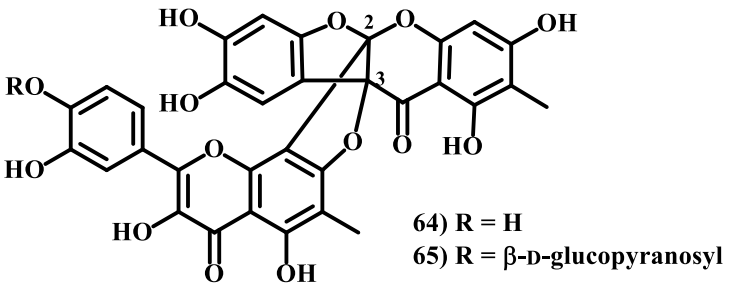


Figure 28. Novel C-methylated biflavonoids from the roots of Baeckea frutescens L.

In 2014, Gao and colleagues isolated the components of Zhuyeqing Liquor, a famous traditional Chinese functional health liquor.[100] Among all the constituents present, it is important to individualize two of them, namely kaempferide (**66**) and 5-hydroxy-3',4',7,8-tetramethoxyflavonol (**67**), which revealed the most potent ˙NO inhibitory activity (Figure 29).

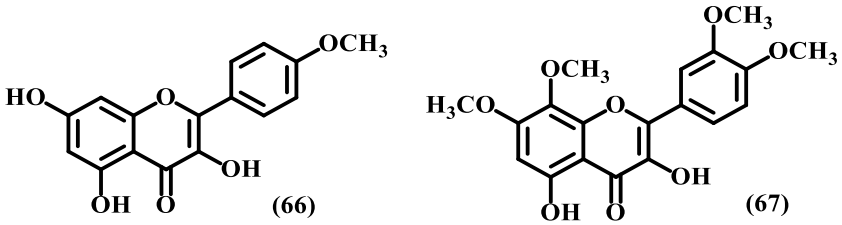


Figure 29. Structure of kaempferide and 5-hydroxy-3',4',7,8-tetramethoxyflavonol.

Last year, Ma et al. focused their efforts onto the study of the flavonoids from a Tradicional Chinese medicine formula known as Baoyuan Decoction (Figure 30).[101] In their work, two particular chromones (**68** and **69**) could be highlighted owing to their significant inhibitory activity. A preliminary SAR study revealed that the presence of hydroxy groups is important to enhance the activity.

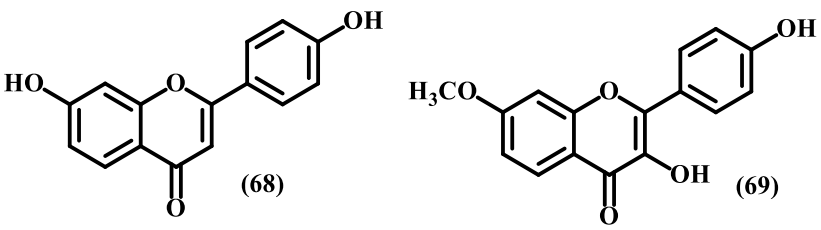
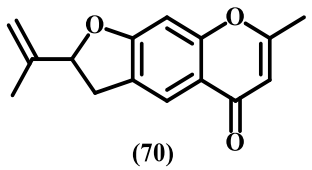


Figure 30. Chromone derivatives isolated from Baoyuan Decoction.

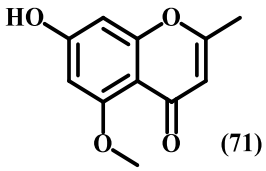
The anti-inflammatory effect of three natural chromones, isolated from the plant *Corydalis heterocarpa* Siebold & Zucc. was assessed by measuring ˙NO production in LPS-stimulated RAW 264.7.[102] The results showed that only one of the tested chromones, heterocarpin (**70**) (Figure 31), inhibited ˙NO production by decreasing iNOS protein expression.



(70)

Figure 31. Heterocarpin structure.

Isoeugenin (**71**) (Figure 32), a chromone isolated from the rizomes of *Imperata cylindrical* (L.) P.Beauv., was evaluated towards its iNOS inhibitory activity.[103] The results of this study verified that this compound exhibits not only the capacity to inhibit iNOS expression, with consequent decrease of ˙NO production, but is also able to inhibit COX-2 expression.



(71)

Figure 32. Structure of isoeugenin.

Early this year, a new chromone glycoside known as frachromone C (**72**) (Figure 33), isolated from *Dryopteris fragrans* (L.) Schott, was found to inhibit ˙NO production.[104] In another recent research effort, a chloride substituted 2-(2-phenylethyl)-chromone, GYF-17 **(73)**, showed inhibitory activity toward ˙NO synthesis (Figure 33).[105] The results also showed that this inhibitory activity is due to the suppression of iNOS expression, with consequent decrement in ˙NO production.

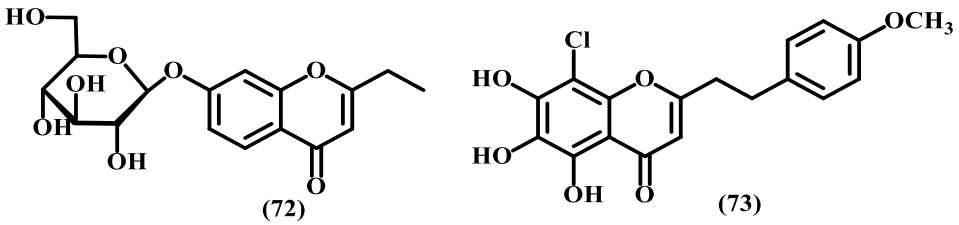


Figure 33. Structure of frachromone C and GYF-17.

1.3.2.4. Interleukin-5 inhibitors

Eosinophils are recognized as a pro-inflammatory granulocyte involved in numerous allergic reactions, such as in bronchial asthma or atopic dermatitis.[106] Moreover, eosinophils are the major effector cells in allergic inflammation which are produced by several eosinophil-activating cytokines, such as granulocyte macrophage GSF (GM-GSF), IL-3 and IL-5, the latter being the most predominant chemokine.[107]

IL-5 was originally found as the "T-cell replacing factor", secreted from T lymphocytes to promote antibody production by activated B cells.[108] This 115-residue cytokine is produced by T_h2 cells, after stimulation by pathogens or allergens, and by mast cells upon stimulation with allergen-immunoglobulin E (IgE) complex. The effects of IL-5 on eosinophils largely fall into four main categories: differentiation, migration, activation and survival (Figure 34).[109].

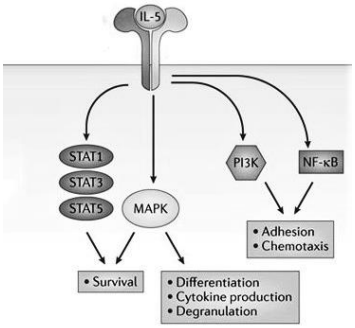
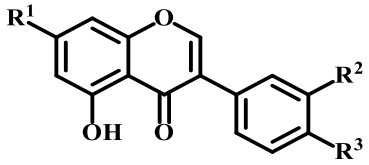


Figure 34. Schematic representation of IL-5-mediated signaling pathway in eosinophils: After the binding of IL-5 to IL-5 receptor, a signal transduction is initiated by stimulating the STATs pathway, which results in the expression of genes that promote eosinophil survival. The activation of mitogen-activated protein kinase (MAPK) results in eosinophil differentiation and degranulation, as well as cytokine production. IL-5 also activates the phosphoinositide 3-kinase (PI3K) and nuclear factor-κB (NF-κB) pathways, which promotes the adhesion and chemotaxis of eosinophils. (Adapted from Fulkerson et. al(2013)[109])

Eosinophilia is then associated with a wide variety of inflammatory conditions, including asthma, atopic diseases, and helminth infections. Therefore, eosinophil appear to be an ideal target for selectively preventing the tissue damage triggered by allergic diseases, without inducing immunosuppressive consequences that can emerge from systemic use of glucocorticoids.[110] It is possible to conclude that the exclusive inhibition of IL-5 may be enough to inhibit one of the alleged causes of asthma, i.e., tissue damage due to eosinophil accumulation during pulmonary inflammation.

Throughout the years, some small organic compounds have been shown to inhibit the activity of IL-5.[111] In 1999, *Sophora japonica* L., a plant of Leguminosae family, was found to have an inhibitory effect in the IL-5 bioassays; some isoflavones, namely sophoricoside (**74**), genistein (**75**), orobol (**76**) and genistin (**77**) (Figure 35), were identified as the active principles.[111] These isoflavones are unrelated to the structural unit of IL-5, in contrast to the IL-5 antagonists isothiozolones, and are the first natural products to show inhibitory activity against IL-5 bioactivity.



74) R^1 = OH, R^2 = H, R^3 = OGlc;
75) R^1 = OH, R^2 = H, R^3 = OH;
76) R^1 = OH, R^2 = OH, R^3 = OH;
77) R^1 = OGlc, R^2 = H, R^3 = OH.

Figure 35. Isoflavones that were the first natural compounds found to exhibit inhibitory activity against IL-5.

Based on these results, a SAR study was developed to better understand the structural requirements of isoflavones for IL-5 inhibitory activity (Figure 36).[112] From this study it was possible to conclude that the structural requirements for IL-5 inhibitory activity of sophoricoside analogs fall into three main features: the planar nature of the chromone scaffold, the presence of a 4'-hydroxy group, and the introduction of a 5-benzyloxy group. Moreover, the glucopyranosyl moiety of sophoricoside (**74**) may not be crucial for the intrinsic activity of these analogs, as the weak glycosidic linkage of isoflavone glycoside is chemically cleaved under metabolic conditions.

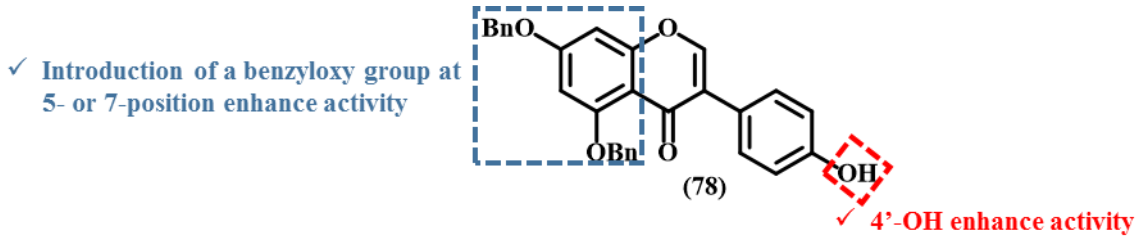


Figure 36. Structure-IL-5 inhibitory activity relationship of isoflavones.

With the purpose of continuing the effort to find a more detailed SAR for isoflavones as IL-5 inhibitors, another study was designed to determine the role of B-ring substituents of isoflavones on this activity.[113] The results confirmed that the B-ring substituents of isoflavone need a strong electron-donating group with hydrogen bond acceptor properties. Nevertheless, the dimensions of this electron-donating group also seems to be important, attesting that the small size of hydrogen binding group like the 4'-hydroxy might be a crucial factor for activity, as the introduction of bulkier groups such as $–SO_2NH_2$ or $–CO_2H$ decrease inhibitory activity. Moreover, it was also verified that the optimum 5-alkoxy group for enhancing IL-5 inhibitory activity is a cyclohexylmethoxy group, as this increases both hydrophobicity and cell permeability. Furthermore, replacement of the 3-aryl group with hydroxylmethyl, chloromethyl, carboxylic or oxime groups leads to a decrease in the inhibitory activity of these derivatives,once more confirming the importance of a 4'-substituted B-ring with an electron-donating group.

Following the principle of the SAR study discussed above, a series of amino alcohol derivatives were designed, and their capacity to inhibit IL-5 evaluated (Figure 37).[114] To obtain a deeper understanding of the influence of the B-ring (the 3-aryl group) in the activity of isoflavone-type compounds, an amino alcohol unit at position 3 was introduced in place of an aryl ring. Furthermore, determining the optimal size of the amino alcohol moiety led to the addition of one methylene unit between amino and hydroxy function, which was found to decrease inhibitory activity. Consequently, it appears that the chain length of the amino alcohol plays a critical role in this activity. Moreover, the results of this study also revealed that the presence of a bulky hydrophobic group as a side chain in amino ethanol moiety is crucial for IL-5 inhibitory activity (Figure 37).

Having understood some important features that enhanced the inhibitory activity toward IL-5, numerous *N*-substituted hydroxyethylaminomethylchromenone derivatives were

investigated as IL-5 inhibitors.[115] It was found that by increasing the bulkiness or hydrophobicity at amino group the inhibitory activity against IL-5 is intensified (Figure 37). Furthermore, the fact that all benzyl analogs presented the same level of activity, regardless the type of substituents at *para*-position of the phenyl ring, might lead one to conclude the presence of a particular structure in the presumed receptor for accepting these side chains. Moreover, based on conformational analysis, the insertion of a hydrophobic group on the nitrogen seems to enforce the more effective conformation of hydroxyethylaminomethyl side chain, thereby increasing the activity of these analogs.

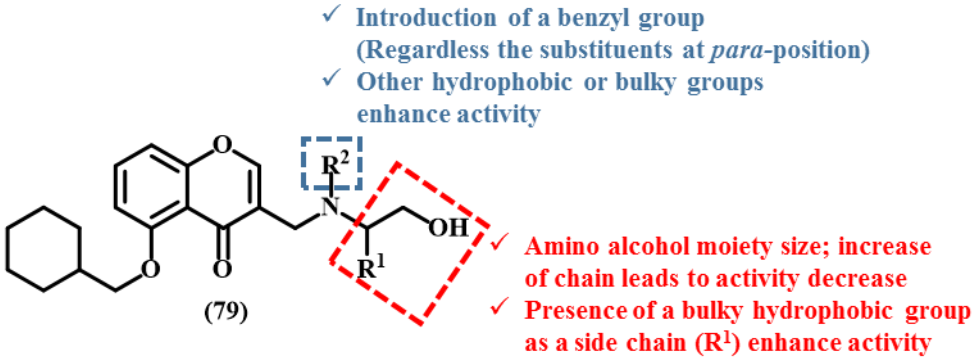


Figure 37. Structure-IL-5 inhibitory activity of chromone amino alcohol derivatives.

Recently, two new studies by Jung and coworkers[116,117], in which the structural characteristics of chromones with a chalcone analogue (**80**) (Figure 38), were reported. However, these hybrids (**80**) have not improved the inhibitory activity of IL-5.[116] Given this observation, another set of hybrids were synthesized, in which the α,β-unsaturated ketone of (*E*)-3-(3-phenyl-3-oxoprop-1-enyl)-4*H*-chromen-4-ones (**80**) was reduced to the racemic allylic alcohols (**81**) (Figure 38). Evaluation of the inhibitory activity of these analogs confirmed both the importance of bulky hydrophobic groups at 5-position (Figure 38), already demonstrated in another paper[113], and the relevance of hydrogen bond characteristics of the allylic hydroxyl group for IL-5 inhibitory activity. The enhanced activity of these latter compounds may be due to the effective location of the allylic hydroxyl group, since these analogs appear to present a folded conformation similar to the accurate conformation required to bind the predicted receptor. Furthermore, several electron-withdrawing groups, namely chloro, fluoro, trifluoro and cyano, were introduced at the *para*-position of B-ring (Figure 38) leading to an increase in the hydrogen bonding strength (involving the allylic hydroxyl group) and consequently to an increase in the activity of these analogs.

In the second work, Jung and colleagues synthesized a series of 5-(cyclohexylmethoxy)-3-[3-(4-phenyl)-3-oxopropyl]-4*H*-chromen-4-ones (**82**) and 5-(cyclohexylmethoxy)-3-[3-hydroxy-3-(4-phenyl)propyl]-4*H*-chromen-4-ones (**83**) (Figure 38) and studied their SAR.[117] Comparing these two series of compounds with each other and also with those mentioned above, it was possible to observe that the inhibitory activity of the saturated ketones (**82**) was considerably weaker, whereas a few examples of the alcohols (**83**) showed potent inhibitory activity. These results allowed the authors to conclude that structural modification into saturated ketones is not sufficient for the design of IL-5 inhibitors. Furthermore, in the case of the alcohols, it again became clear that the presence of the hydroxypropyl linkage and a small electron-donating group with hydrogen bonding capacity at the *para*-position of the B-ring are quite important for the IL-5 inhibitory activity.

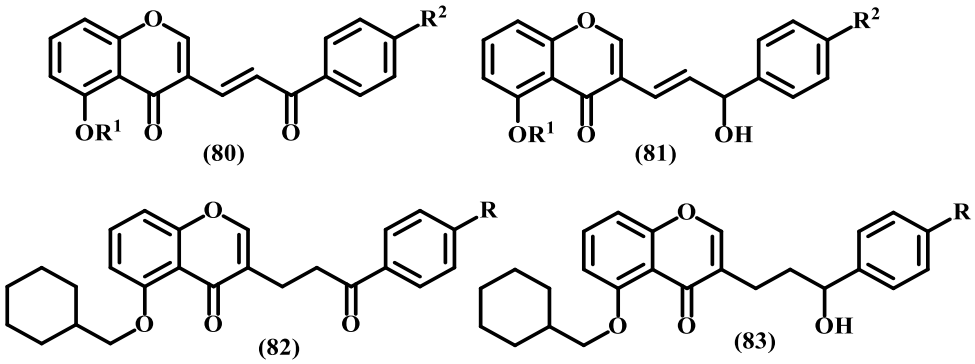


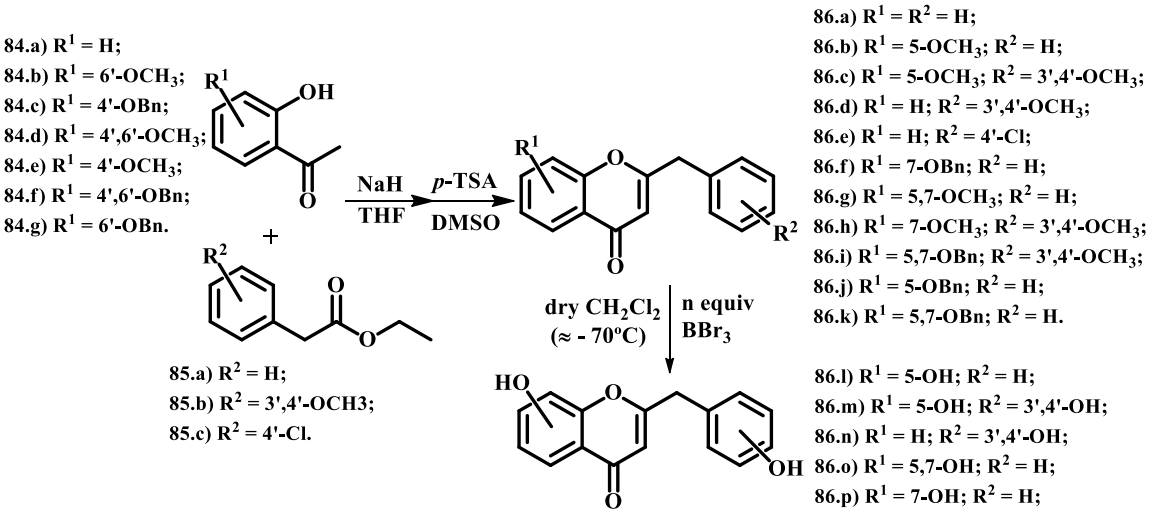
Figure 38. Hybrids containing a chromone core.

Chapter II: Synthesis and structural characterization

1. Work perspectives and synthetic strategy

Throughout the years as stated in the previous chapter, chromones have appeared as biologically active compounds with numerous relevant properties, from which can be highlighted the anti-inflammatory and anti-colinesterase activities. These biological properties, along with their good bioavailability and low rates of side-effects, turn the chromone scaffold into a crucial basis for the design and development of novel drugs.

The main goal of our project involves the synthesis of a specific type of chromones, 2-benzylchromones, aiming the obtention of new active compounds with anti-inflammatory and anti-colinesterase effects. Having consolidated the synthetic method for attaining this type of compounds, several distinct substitution patterns will be added to the 2-benzylchromone aiming for a deeper understanding of their influence in the method's efficiency, as well as their importance for the improvement of the anti-inflammatory and anti-acetylcholinesterase activities of this type of compounds. Firstly, the substitution patterns will be chosen by comparison with other biological active chromone types already described in literature. These consist mainly in methoxy and benzyloxy groups, placed at specific positions of the 2-benzylchromone scaffold, which might confer distinct effects in the evolution of the synthetic procedure. Furthermore, the methoxy- and benzyloxy-substituted chromones are subjected to a deprotection process, attaining the correspondent hydroxylated 2-benzylchromone. For the accomplishment of the main objective of our project, it will also be needed to perform the synthesis of a few starting materials, particularly the esters used for the formation of the chromone scaffold. These compounds will then act as a precursor of our desired 2-benzylchromones, alongside with a few selected 2'-hydroxyacetophenones.



Scheme 12. Synthetic strategy.

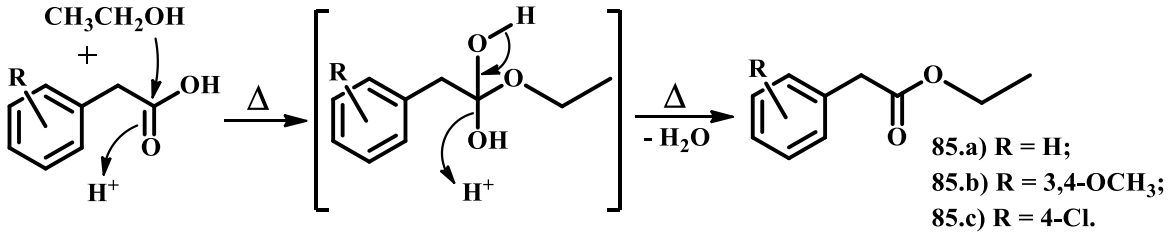
During our project, the structural characteriation of the compounds will be performed based on monodimensional (^{1}H NMR and ^{13}C NMR) and bidimensional (HSQC and HMBC) tecniques. In order to obtain a more complete characterization, the synthesized compounds will also be analised using mass spectrometry (MS) tecniques, namely electrospray ionization (ESI).

Regarding the monodimensional NMR spectra, these are influenced by the action of two specific effects: the inductive deshielding effect promoted by the electronegativity of the atoms involved in covalent bonds, and the mesomeric effect which is caused by the capability of a molecule to delocalize its electrons. Moreover, in ^{1}H NMR, another effect might influence the chemical shifts of protons, the anisotropic effect. This effect is characterized by the movement of π-electrons, composing double bonds and carbonyl groups, which induce magnetic fields that, depending on its direction, may apply a shielding or deshielding action towars the protons.

The remaining tecniques consist in 2D NMR tecniques, where HSQC correspond to the direct correlation between proton and the carbon at just one bond of distance and HMBC correspond to the correlation between protons and carbons at long distances, mainly at two or three bonds of distance, allowing a more complete determination of the molecular structure of the compounds.

2. Synthesis of esters

The synthesis of the necessary esters was accomplished through a simple esterification where a carboxylic acid, with a desired substitution pattern, reacts with ethanol under reflux temperature, in the presence of catalytic quantities of sulphuric acid (H_2SO_4), for approximately 12h (Scheme 13). This process permited the obtention of these esters with very good yields, over 80%, which also confirms the efficiency of this type of synthesis.



Scheme 13. Schematic representation of the esterification condition and mechanism.

From the ^{1}H NMR spectrum, it is possible to verify the presence of some characteristic chemical shifts of this type of compounds, ethyl 2-phenylacetates. Some of these characteristic signals correspond to the ethyl group and are located at δ 1.23 ppm (triplet, CH_3) and δ 4.13 ppm (quartet, CH_2), having the later one a particular high chemical shift, for a CH_2 group, due to the inductive deshielding effect promoted by the electronegativity of the oxygen atom directly bond to it. Another characteristic signal of ethyl 2-phenylacetates corresponds to H-2, located at δ 3.59 ppm (singlet, CH_2), whose chemical shift is influenced by the dishielding effect promoted by the carbonyl group and the aromatic ring in the proximity of these protons. Moreover, the signals corresponding to the phenyl group appear at δ 7.21-7.33 ppm, consisting in a multiplet since it is not possible to distinguish the signals individually (Figure 39).

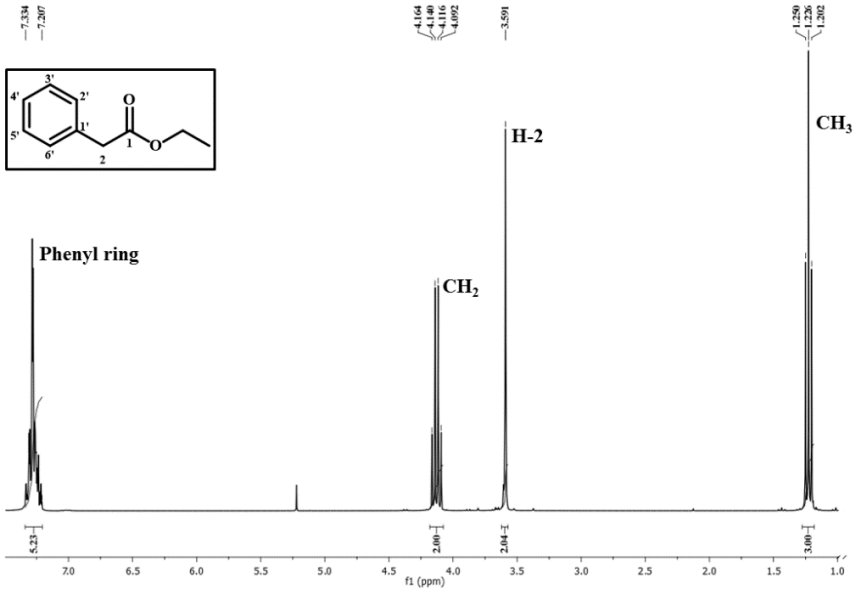


Figure 39. ^{1}H NMR spectrum of ethyl 2-phenyacetate (85.a) given as an example.

With the exception of the anisotropic effect, all the effects previously mentioned for ^{1}H NMR are also verified in ^{13}C NMR. Following this principle, it is possible to identify the signals assigned to the three aliphatic carbons at δ 14.2 ppm (CH_3), δ 41.45 ppm (C2) and δ 60.9 ppm (CH_2), and also the signal at δ 171.71 ppm, corresponding to the carbonyl group (C1). In order to distinguish the aromatic signals, some 2D NMR tecniques were used. These comprehend HSQC where the correlation between protons and the correspondent carbon atoms is made and HMBC which showns the correlation between proton and carbon at long distances, allowing the complete identification of the molecular structure of the analysed compound (Figure 40). However, the presence of distinct substituents, particularly in the phenyl ring, might cause some modifications in the NMR signals both at multiplicity and chemical shift levels, which in the case of ^{13}C NMR were resolved using the HMBC technique (Figure 42 and 43).

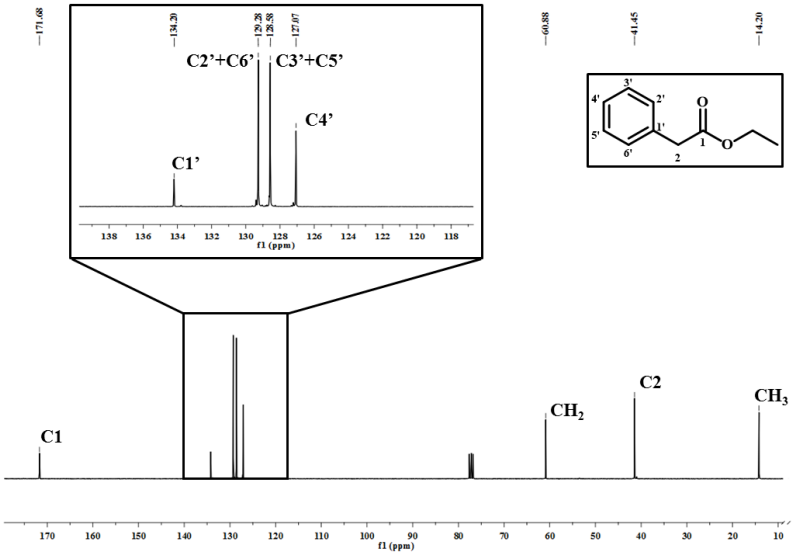


Figure 40. ^{13}C NMR spectrum of ethyl 2-phenyacetate (85.a).

Ethyl 2-phenylacetate (85.a):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.23 (3H, t, *J* = 7.1Hz, CH_3), 3.59 (2H, s, H-2), 4.13 (2H, q, *J* = 7.1Hz, CH_2), 7.21-7.25 (1H, m, H-4'), 7.25-7.33 (4H, m, H-2'; H-3'; H-5'; H-6').

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 14.2 (CH_3), 41.5 (C2), 60.9 (CH_2), 127.1 (C4'), 128.6 (C3', C5'), 129.3 (C2', C6'), 134.2 (C1'), 171.7 (C1).

MS (ESI) m/z (rel. int.): 187.1 $[M+Na]^+$ (5.0 %).

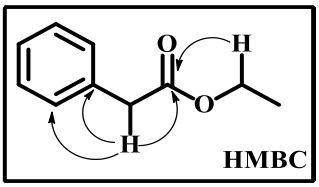


Figure 41. Structure of ethyl 2-phenylacetate.

Ethyl 2-(3,4-dimethoxyphenyl)acetate (85.b):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.25 (3H, t, *J* = 7.1Hz, CH_3), 3.55 (2H, s, H-2), 3.86 (3H, s, 3'-OCH_3)*, 3.88 (3H, s, 4'-OCH_3)*, 4.15 (2H, q, *J* = 7.1Hz, CH_2), 6.82 (1H, s, H-2'), 6.82-6.83 (2H, m, H-5'; H-6').

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 14.2 (CH_3), 40.9 (C2), 55.8 (3'-CH_3)*, 55.9 (4'-OCH_3)*, 60.8 (CH_2), 111.2 (C5'), 112.4 (C2'), 121.4 (C6'), 126.6 (C1'), 148.1 (C3')*, 148.9 (C4')*, 171.9 (C1).

* *This identification may be switched.*

MS (ESI) m/z (rel. int.): 224.1 $[M+H]^+$; (20.0%) 247.1 $[M+Na]^+$ (75.0%).

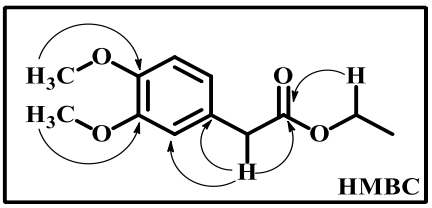


Figure 42. Structure of ethyl 2-(3,4-dimethoxyphenyl)acetate.

Ethyl 2-(4-chlorophenyl)acetate (85.c):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.25 (3H, t, *J* = 7.1Hz, CH_3), 3.57 (2H, s, H-2), 4.14 (2H, t, *J* = 7.1Hz, CH_2), 7.19-7.23 (2H, m, H-2'; H-6'), 7.26-7.31 (2H, m, H-3'; H-5').

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 14.2 (CH_3), 40.7 (C2), 61.0 (CH_2), 128.7 (C3'; C5'), 130.7 (C2'; C6'), 132.6 (C1'), 133.0 (C4'), 171.2 (C1).

MS (ESI) m/z (rel. int.): 171.0 $[(M-Et)+H]^+$ (15.0%); 221.0 $[M+Na]^+$ (10.0%).

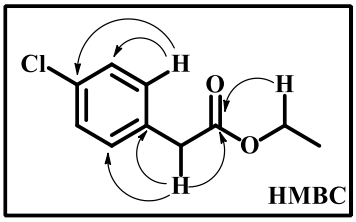
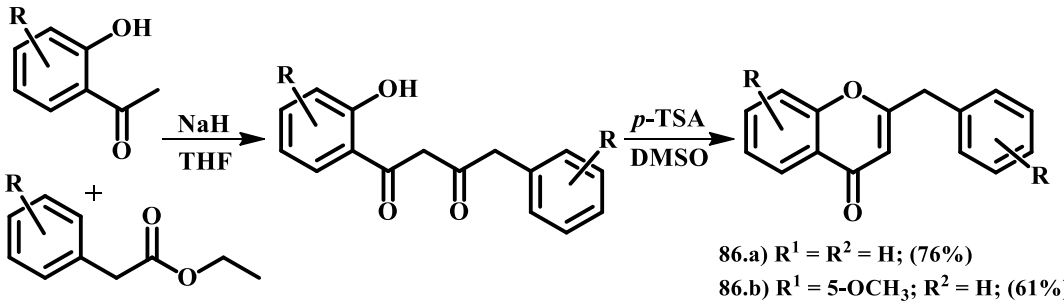


Figure 43. Structure of ethyl 2-(4-chlorophenyl)acetate.

3. Synthesis of 2-benzylchromones

After the synthesis of the esters, and having all the desired 2'-hydroxyacetophenones, the next stage of our project comprises a new synthetic route which culminates in the formation of a specific type of chromones, 2-benzylchromones (Scheme 14). The formation of 2-benzylchromones is achieved through a Claisen condensation, being the procedure divided in two separate phases. In the first step, our method consists in a reaction between a 2'-hydroxyacetophenone and an ethyl 2-phenylacetate, both with the appropriate substitution pattern. This phase takes place in dry THF, for 3 hours, in the presence of a strong base (NaH) under inert atmosphere conferred by N_2. The second phase occurs by adding *p*-toluenesulfonic acid to a solution of the crude resulting from the previous step, in DMSO over-night, culminating with the formation of the desired chromone (Scheme 14).

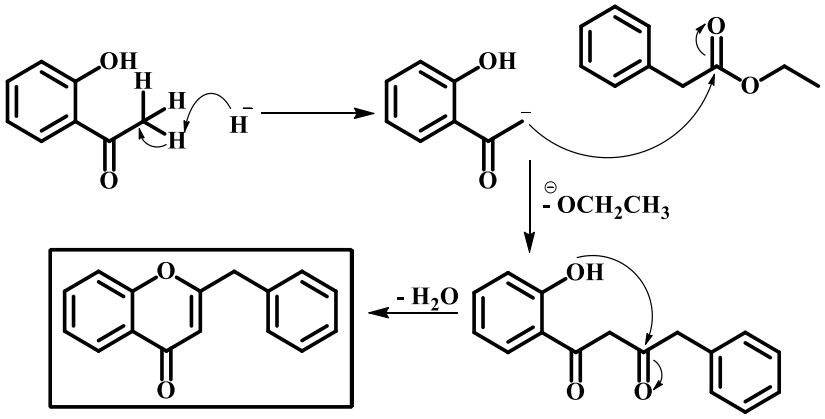


86.a) $R^1 = R^2 = H$; (76%)
86.b) $R^1 = 5\text{-}OCH_3$; $R^2 = H$; (61%)
86.c) $R^1 = 5\text{-}OCH_3$; $R^2 = 3',4'\text{-}OCH_3$; (72%)
86.d) $R^1 = H$; $R^2 = 3',4'\text{-}OCH_3$; (71%)
86.e) $R^1 = H$; $R^2 = 4'\text{-}Cl$; (37%)
86.f) $R^1 = 7\text{-}OBn$; $R^2 = H$; (50%)
86.g) $R^1 = 5,7\text{-}OCH_3$; $R^2 = H$; (31%)
86.h) $R^1 = 7\text{-}OCH_3$; $R^2 = 3',4'\text{-}OCH_3$; (14%)
86.i) $R^1 = 5,7\text{-}OBn$; $R^2 = 3',4'\text{-}OCH_3$; (20%)
86.j) $R^1 = 5\text{-}OBn$; $R^2 = H$; (22%)
86.k) $R^1 = 5,7\text{-}OBn$; $R^2 = H$. (30%)

Scheme 14. Synthesis of 2-benzylchromones.

Mechanistically, this reaction involves the formation of a carbanion in the 2'-hydroxylacetophenone, due to the action of a strong base (NaH). The obtained carbanion performs a nucleophilic attack to the ester's carbonyl group, leading to the formation of a diketonic intermediate, with the expulsion of ethanol. The second phase of this reaction, the cyclization, occurs through a new nucleophilic attack involving the hydroxyl group of the

2'hydroxyacetophenone and the carbonyl group that previously belonged to the ester, concluding the chromone formation (Scheme 15).

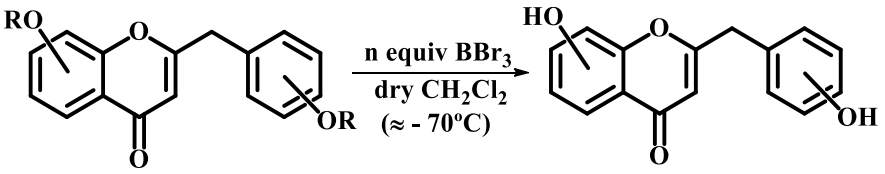


Scheme 15. Mechanistic explanation for the formation of 2-benzylchromones.

Based on the results of the synthetic process, it is possible to understand the influence of distinct substituent groups in the efficiency of the method applied for synthesizing 2-benzylchromones. In this project, it is verified that the most efficient one corresponded to the synthesis of the non-substituted 2-benzylchromone followed by those with the catechol group in the B-ring whose influence is almost minimal to the reactional efficiency. Some B-ring substituents, such as the weakly electron-withdrawing group 4'–Cl, may cause a considerable decrease in the reaction yield and might even preclude the reaction outcome, like in the case of the strongly electron-withdrawing group 4'–NO_2. However, the substituent groups that most contribute for the efficiency of this method are those in the A-ring of the chromone scaffold. Briefly, although the presence of 5–OCH_3 or 5–OBn groups does not significantly influence the outcome of this type of reaction, the existence of the same kind of substituent groups at 7-position revealed to be crucial for the final yield of our synthetic method. Furthermore, it is possible to dispose these groups by level of influence (–OCH_3 > –OBn > –H), from the most to the least decrease caused in the outcome of the synthetic method applied (Figure 67).

The remaining 2-benzylchromones, namely the hydroxylated 2-benzylchromones, were attained through a deprotection process, originating hydroxyl groups. In our project, this procedure consists in a reaction between methoxy- or benzyloxy-substituted 2-benzychromones with BBr_3, at approximately –70ºC, in dry dichloromethane (Scheme 16). Due to the high efficiency of this reaction and, usually, the absence of need to purify

the final residues (hydroxylated chromones), the reaction yields were not calculated since the product losses were almost insignificant.

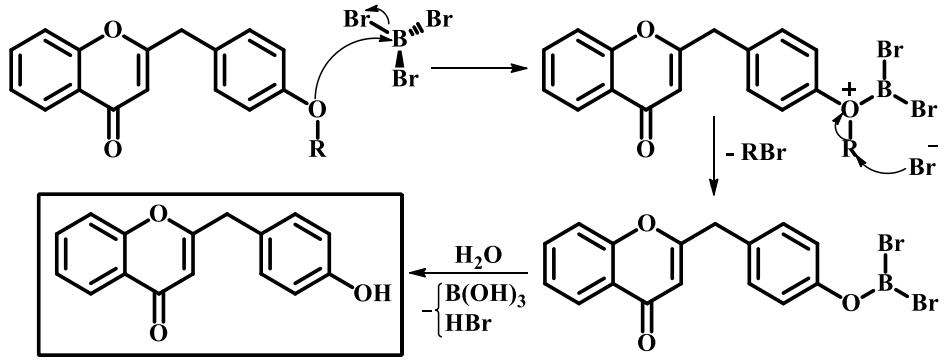


86.b) R^1 = 5-OCH_3; R^2 = H;
86.c) R^1 = 5-OCH_3; R^2 = 3',4'-OCH_3;
86.d) R^1 = H; R^2 = 3',4'-OCH_3;
86.f) R^1 = 7-OBn; R^2 = H;
86.g) R^1 = 5,7-OCH_3; R^2 = H;

86.l) R^1 = 5-OH; R^2 = H;
86.m) R^1 = 5-OH; R^2 = 3',4'-OH;
86.n) R^1 = H; R^2 = 3',4'-OH;
86.o) R^1 = 5,7-OH; R^2 = H;
86.p) R^1 = 7-OH; R^2 = H;

Scheme 16. Deprotection process.

The mechanism of the deprotection process consist in a nucleofilic attack by the –OR group to BBr_3 originating the liberation of a $^-$Br ion and leaving the oxygen atom positively charged. Then, to regenerate the charge of the oxygen atom, the –R group is caught by the previously expelled $^-$Br ion, culminating in the formation of a RBr molecule. To conclude the deprotection process, it is necessary to add water into the system, promoting a hydrolization process that leads to the release of HBr and $B(OH)_3$, and originating hydroxylated compounds (Scheme 17).



Scheme 17. Mechanistic explanation for the demethylation process.

From the ^{1}H NMR spectrum, it is possible to verify the existence of some 2-benzylchromone characteristic signals. One of them corresponds to the vinilic hydrogen H-3 which presents a singulet located at δ 6.23 ppm, due to the anisotropic deshielding effect caused by the C2=C3 double bond, and also by the influence of the carbonyl group. Another one of these characteristic signals belongs to H-α, a singulet that appears located at

δ 3.88 ppm because of the deshielding effects provoqued by the electron withdraw actions of the carbonyl group and the aromatic B-ring. Finally, the signals from A-ring may also present peculiar characteristics since this protons suffer, by resonance, the shielding effect of the oxygen atom from C-ring and the deshielding effect of the carbonyl group. The proton H-5 appears as the one with the highest chemical shift also due to the anisotropic deshielding effect caused by the carbonyl group (Figure 44).

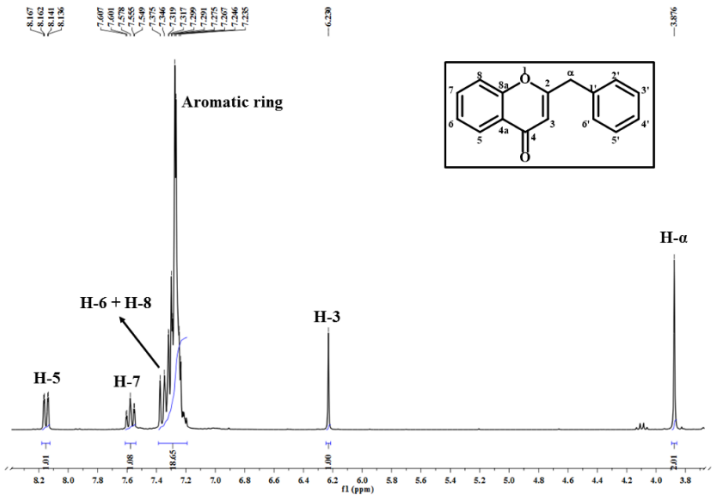


Figure 44. ^{1}H NMR spectrum of 2-benzyl-4H-chromen-4-one (86.a) given as an example.

For the identification of the carbon signals is more practical to use 2D NMR tecniques, like the already mentioned HSQC and HMBC. However, by using only the ^{13}C NMR spectrum is possible to identify some particular signals, namely those corresponding to the carbonyl group (C4) and Cα. These signals are promply distinguished since the carbonyl group appears at δ 179.1 ppm, being the most deshielded carbon, and Cα at δ 40.7 ppm, being the only signal existent in the aliphatic area. The remaining carbon signals can only be distinguished by using the previously indicated 2D NMR tecniques originating a complete molecular characterization of the compound (Figure 45). Like it was indicated in the case of ethyl 2-phenylacetates, the presence of different substitution patterns might cause some modifications in the NMR signals both at multiplicity and chemical shift levels (Figures 47–61).

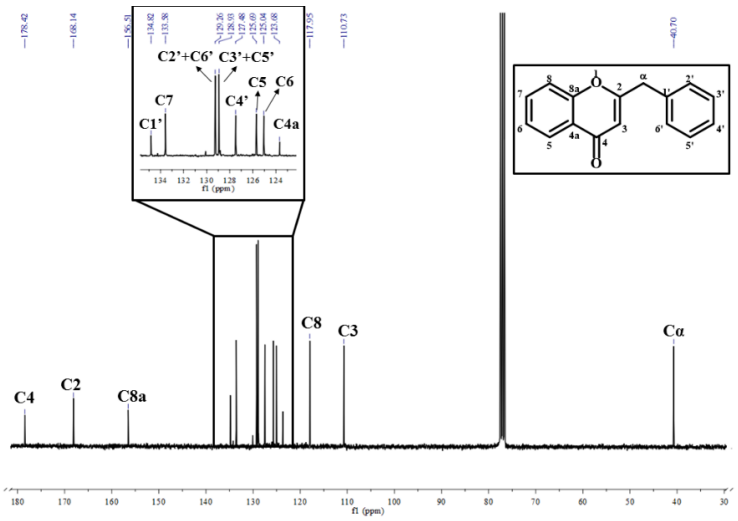


Figure 45. ^{13}C NMR spectrum of 2-benzyl-4H-chromen-4-one.

2-benzyl-4*H*-chromen-4-one (86.a):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 3.88 (2H, s, H-α), 6.23 (1H, s, H-3), 7.21-7.23 (1H, m, H-4'), 7.23-7.28 (2H, m, H-2'; H-6'), 7.28-7.32 (2H, m, H-3'; H-5'), 7.29-7.35 (1H, m, H-6), 7.35-7.38 (1H, m, H-8), 7.58 (1H, dt, *J* = 1.6Hz; 7.8Hz, H-7), 8.15 (1H, dd, *J* = 1.6Hz; 8.0Hz, H-5).

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 40.7 (Cα), 110.7 (C3), 118.0 (C8), 123.7 (C4a), 125.0 (C6), 125.7 (C5), 127.5 (C4'), 128.9 (C3', C5'), 129.3 (C2', C6'), 133.6 (C7), 134.8 (C1'), 156.5 (C8a), 168.1 (C2), 178.4 (C4).

MS (ESI) m/z (rel. int.): 237.1 $[M+H]^+$ (12.0%); 259.1 $[M+Na]^+$ (100%).

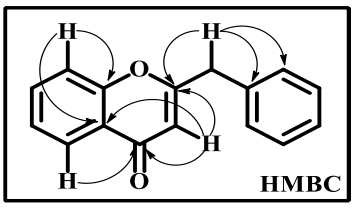


Figure 46. Structure of 2-benzyl-4H-chromen-4-one.

2-benzyl-5-methoxy-4*H*-chromen-4one (86.b):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 3.65 (2H, s, H-α), 3.88 (3H, s, 5-OCH_3), 6.08 (1H, s, H-3), 6.76 (1H, d, *J* = 8.4Hz, H-6), 6.95 (1H, d, *J* = 8.4Hz, H-8), 7.26-7.30 (2H, m, H-2'; H-6'), 7.28-7.30 (1H, m, H-4'), 7.32-7.36 (2H, m, H-3', H-5'), 7.49 (1H, t, *J* = 8.4Hz, H-7).

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 40.1 (Cα), 53.5 (5-O CH_3), 106.3 (C6), 110.0 (C8), 112.1 (C3), 114.2 (C4a), 127.4 (C4'), 128.9 (C3', C5'), 129.3 (C2', C6'), 133.7 (C7), 134.8 (C1'), 158.6 (C8a), 159.7 (C5), 165.9 (C2), 178.5 (C4).

MS (ESI) m/z (rel. int.): 267.1 $[M+H]^+$ (35.0%); 289.1 $[M+Na]^+$ (82.0%); 555.2 $[2M+Na]^+$ (100%).

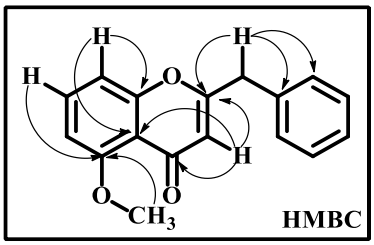


Figure 47. Structure of 2-benzyl-5-methoxy-4H-chromen-4-one.

2-(3,4-dimethoxybenzyl)-5-methoxy-4*H*-chromen-4-one (86.c):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 3.79 (2H, s, H-α), 3.86 (3H, s, 4'-OCH_3), 3.87 (3H, s, 3'-OCH_3), 3.95 (3H, s, 5-OCH_3), 6.03 (1H, s, H-3), 6,77 (1H, d, *J* = 8.3Hz, H-6), 6.78 (1H, large s, H-2'), 6.81-6.86 (2H, m, H-5'; H-6'), 6,96 (1H, dd, *J* = 8.3Hz, H-8), 7,50 (1H, t, *J* = 8.3Hz, H-7).

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 39.7 (Cα), 55.9 (4'-OCH_3), 56.0 (3'-OCH_3), 56.5 (5-OCH_3), 106.3 (C6), 110.0 (C8), 111.4 (C5'), 112.0 (C3), 112.3 (C2'), 114.3 (C4a), 121.5 (C6'), 127.2 (C1'), 133.6 (C7), 148.4 (C3'), 149.2 (C4'), 158.6 (C8a), 166.0 (C2), 178.3 (C4).

MS (ESI) m/z (rel. int.): 327.1 $[M+H]^+$ (66.0%); 349.1 $[M+Na]^+$ (20.0%); 675.2 $[2M+Na]^+$ (100%)

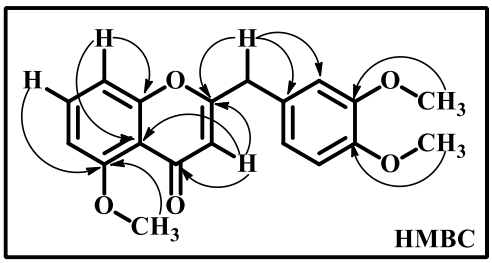


Figure 48. Structure of 2-(3,4-dimethoxybenzyl)-5-methoxy-4H-chromen-4-one.

2-(3,4-dimethoxybenzyl)-4*H*-chromen-4-one (86.d):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 3.87 (8H, s, 3',4'-OCH_3, H-α), 6.12 (1H, s, H-3), 6.79 (1H, large s, H-2'), 6.82-6.87 (2H, m, H-5',6'), 7.36 (1H, t, *J* = 7.5Hz, H-6), 7,40 (1 H, d, *J* = 8,8Hz, H-8), 7,62 (1H, ddd, *J* = 1,7Hz; 7.5Hz; 8,8Hz, H-7), 8,15 (1H, dd, *J* = 1,7Hz; 8,0Hz, H-5).

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 40.3 (Cα), 55.9 (4'-O CH_3), 56.0 (3'-O CH_3), 110.5 (C3), 111.5 (C5'), 112.3 (C2'), 117.9 (C8), 121.5 (C6'), 123.7 (C4a), 125.0 (C6), 125.7 (C5), 127.1 (C1'), 133.6 (C7), 148.4 (C3'), 149.2 (C4'), 156.5 (C8a), 168.5 (C2), 178.4 (C4).

MS (ESI) m/z (rel. int.): 297.1 $[M+H]^+$ (100%); 319.1 $[M+Na]^+$ (100%); 615.1 $[2M+Na]^+$ (87.0%).

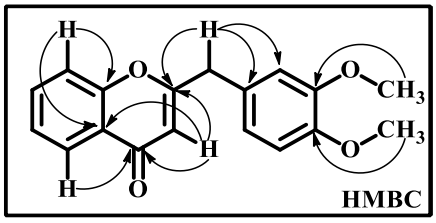


Figure 49. Structure of 2-(3,4-dimethoxybenzyl)-4H-chromen-4-one.

2-(4-chlorobenzyl)-4*H*-chromen-4-one (86.e):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 3.90 (2H, s, H-α), 6.12 (1H, s, H-3), 7.22-7.26 (2H, m, H-3',5'), 7.31-7.35 (2H, m, H-2',6'), 7.37-7.40 (1H, m , H-6), 7.38-7.41 (1H, m , H-8), 7.63 (1H, ddd, *J* = 1.7Hz; 7.1Hz; 8.7Hz, H-7), 8.16 (1H, dd, *J* = 1.7Hz;7.9Hz, H-5).

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 40.0 (Cα), 110.8 (C3), 117.9 (C8), 123.6 (C4a), 125.1 (C6), 125.7 (C5), 129.1 (C2', C6'), 130.6 (C3', C5'), 133.3 (C1'), 133.5 (C4'), 133.7 (C7), 156.4 (C8a), 167.4 (C2), 178.2 (C4).

MS (ESI) m/z (rel. int.): 271.1 $[M+H]^+$ (90.0%); 293.1 $[M+Na]^+$ (100.0 %)

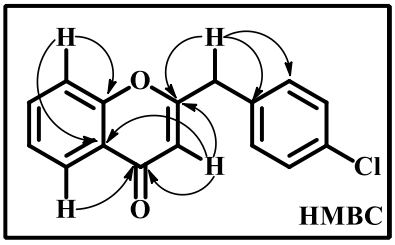


Figure 50. Structure of 2-(4-chlorobenzyl)-4H-chromen-4-one.

2-benzyl-7-(benzyloxy)-4*H*-chromen-4-one (86.f):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 3.90 (2H, s, H-α), 5.08 (2H, s, H-β) 6.12 (1H, s, H-3), 6.85 (1H, d, *J* = 2.4Hz, H-8), 6.98 (1H, dd, *J* = 2.4Hz; 8.9Hz, H-6), 7.23-7.28 (2H, m, H-2'; H-6'), 7.29-7.37 (4H, m, H-3'; H-5'; β-3; β-5), 7.38-7.42 (4H, m, H-4'; β-2; β-4; β-6), 8.06 (1H, d, *J* = 8.9Hz, H-5).

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 40.6 (Cα), 70.5 (Cβ), 101.4 (C8), 110.6 (C3), 114.8 (C6), 117.7 (C4a), 127.1 (C5), 127.5 (C4'), 127.6 (β-2, β-6), 128.4 (β-4), 128.8 (β-3, β-5), 128.9 (C3', C5'), 129.3 (C2', C6'), 135.0 (C1'), 135.8 (β-1), 158.2 (C8a), 163.1 (C7), 167.7 (C2), 177.9 (C4).

MS (ESI) m/z (rel. int.): 343.1 $[M+H]^+$ (100%); 707.1 $[2M+Na]^+$ (40.0%).

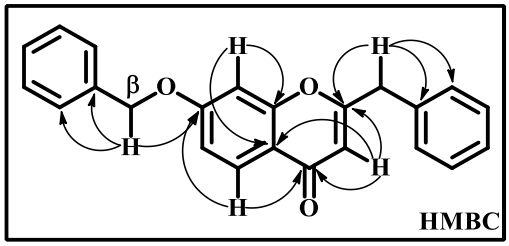


Figure 51. Structure of 2-benzyl-7-(benzyloxy)-4H-chromen-4-one.

2-benzyl-5,7-dimethoxy-4*H*-chromen-4-one (86.g):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 3.78 (2H, s, H-α), 3.80 (3H, s, 7-OCH_3), 3.85 (3H, s, 5-OCH_3), 5.95 (1H, s, H-3), 6.28 (1H, d, *J* = 1.4Hz, H-6), 6.35 (1H, d, *J* = 1.4Hz, H-8), 7.23-7.26 (2H, m, H-2'; H-6'), 7.24-7.26 (1H, m, H-4'), 7.28-7.33 (2H, m, H-3'; H-5').

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 39.9 (Cα), 55.7 (7-O CH_3), 56.3 (5-O CH_3), 92.7 (C8), 96.0 (C6), 108.9 (C4a), 112.0 (C3), 127.3 (C4'), 128.8 (C3', C5'), 129.2 (C2', C6'), 135.0 (C1'), 160.2 (C8a), 160.8 (C5), 163.9 (C7), 165.0 (C2), 177.6 (C4).

MS (ESI) m/z (rel. int.): 297.0 $[M+H]^+$ (90.0%); 319.0 $[M+Na]^+$ (50.0%); 615.1 $[2M+Na]^+$ (100%)

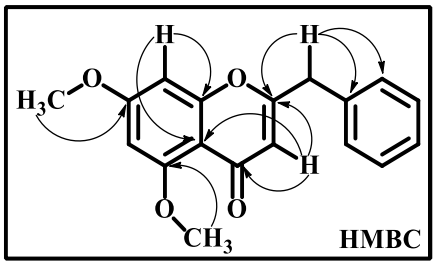


Figure 52 Structure of 2-benzyl-5,7-dimethoxy-4H-chromen-4-one.

2-(3,4-dimethoxybenzyl)-7-methoxy-4*H*-chromen-4-one (86.h):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 3.85 (2H, s, H-α), 3.87 (3H, s, 3'-OCH_3), 3.88 (3H, s, 4'-OCH_3), 3.89 (3H, s, 7-OCH_3), 6.08 (1H, s, H-3), 6.79 (1H, large s, H-2'), 6.80 (1H, d, *J* = 2.4Hz, H-8), 6.83-6.87 (2H, m, H-5',6'), 6.94 (1H, dd, *J* = 2.4Hz; 8.9Hz, H-6), 8.07 (1H, d, *J* = 8.9Hz, H-5).

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 40.2 (Cα), 55.8-55.9 (7,3',4'-OCH_3), 100.3 (C8), 110.5 (C3), 111.5 (C5'), 112.3 (C2'), 114.2 (C6), 117.5 (C4a), 121.5 (C6'), 127.1 (C5), 127.3 (C1'), 148.4 (C4'), 149.2 (C3'), 158.2 (C8a), 164.0 (C7), 167.8 (C2), 177.9 (C4).

MS (ESI) m/z (rel. int.): 327.0 $[M+H]^+$ (100%); 349.1 $[M+Na]^+$ (27.0%); 675.1 $[2M+H]^+$ (55.0%).

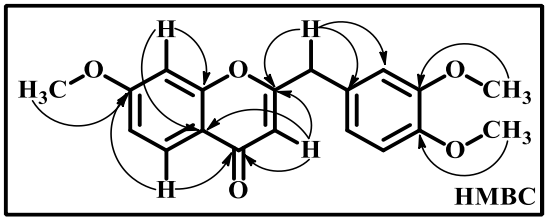


Figure 53. Structure of 2-(3,4-dimethoxybenzyl)-7-methoxy-4H-chromen-4-one.

5,7-bis(benzyloxy)-2-(3,4-dimethoxybenzyl)-4*H*-chromen-4-one (86.i):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 3.77 (2H, s, H-α), 3.86 (3H, s, 3'-OCH_3), 3.88 (3H, s, 4'-OCH_3), 5.06 (2H, s, H-γ), 5.20 (2H, s, H-β), 5.99 (1H, s, H-3), 6.45 (1H, d, *J* = 2.3Hz, H-6), 6.48 (1H, d, *J* = 2.3Hz, H-8), 6.77 (1H, large s, H-2'), 6.81-6.86 (2H, m, H-5',6'), 7.26-7.31 (1H, m, γ-4), 7.35-7.41 (2H, m, β-2; β-6), 7.35-7.42 (3H, m, β-4; γ-3; γ-5) , 7.37-7.41 (2H, m, β-3; β-5).

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 39.6 (Cα), 55.9 (3'-OCH_3), 56.0 (4'OCH_3), 70.4 (Cγ), 70.7 (Cβ), 109.6 (C4a), 111.4 (C5'), 112.1 (C2'), 112.2 (C3), 121.4 (C6'), 126.5 (C2γ, C6γ), 127.4 (C1'), 127.6 (β-2; β-6; γ-4), 128.5 (β-4), 128.6 (β-3; β-5), 128.8 (γ-3; γ-5), 135.7 (β-1), 136.4 (γ-1), 148.3 (C4'), 149.2 (C3'), 159.7 (C7), 160.1 (C8a), 162.8 (C5), 165.2 (C2), 177.4 (C4).

MS (ESI) m/z (rel. int.): 509.1 $[M+H]^+$ (100%); 531.1 $[M+Na]^+$ (20.0%).

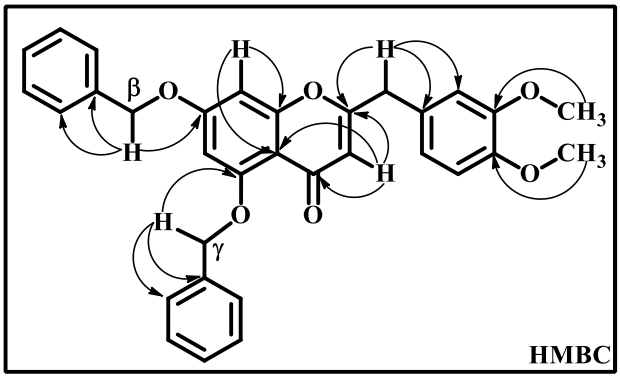


Figure 54. Structure of 5,7-bis(benzyloxy)-2-(3,4-dimethoxybenzyl)-4H-chromen-4-one.

2-benzyl-5-(benzyloxy)-4*H*-chromen-4-one (86.j):

^{1}H NMR (300.13 MHz, $(CH_3)_2CO$): δ 3.84 (2H, s, H-α), 5.23 (2H, s, H-β), 6.11 (1H, s, H-3), 6.76 (1H, dd, *J* = 8.4Hz, H-6), 6.94 (1H, dd, *J* = 0.9Hz; 8.4Hz, H-8), 7.23-7.29 (2H, m, H-4'; γ-4), 7.24-7.29 (2H, m, H-2'; H-6'), 7.31-7.38 (4H, m, H-3'; H-5'; γ-3; γ-5), 7.42 (1H, t, *J* = 8.4 Hz, H-7), 7.53-7.55 (2H, m, γ-2; γ-6)

^{13}C NMR (75.47 MHz, $(CH_3)_2CO$): δ 40.1 (Cα), 70.8 (Cβ), 108.4 (C6), 110.3 (C8), 112.2 (C3), 114.7 (C4a), 126.7 (γ-2; γ-6), 127.4 (C4'), 127.7 (γ-4), 128.6 (γ-3, γ-5), 128.9 (C3', C5'), 129.2 (C2', C6'), 133.6 (C7), 134.9 (C1'), 136.6 (γ-1), 158.5 (C8a), 158.6 (C5), 165.9 (C2), 178.5 (C4).

MS (ESI) m/z (rel. int.): 343.1 $[M+H]^+$ (60.0%); 365.1 $[M+Na]^+$ (100%); 707.2 $[2M+Na]^+$ (70.0%).

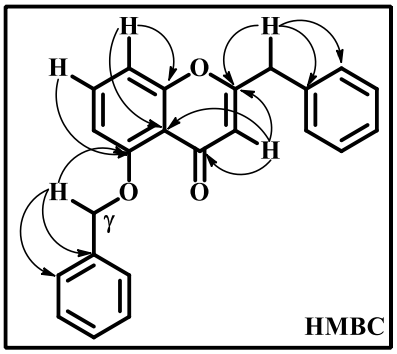


Figure 55. Structure of 2-benzyl-5-(benzyloxy)-4H-chromen-4-one.

2-benzyl-5,7-bis(benzyloxy)-4*H*-chromen-4-one (86.k):

^{1}H NMR (300.13 MHz, $(CH_3)_2CO$): δ 3.83 (2H, s, H-α), 5.01 (2H, s, H-β), 5.19 (2H, s, H-γ), 6.09 (1H, s, H-3), 6.45 (1H, d, *J* = 2.3Hz, H-6), 6.48 (1H, d, *J* = 2.3Hz, H-8), 7.26-7.33 (3H, m, β-3; β-5; γ-4), 7.28-7.33 (4H, m, C2';C3'; C5'; C6') , 7.30-7.33 (1H, m, H-4'), 7.34-7.39 (2H, m, β-3; β-5), 7.34-7.42 (2H, m, γ-3; γ-5), 7.58-7.60 (2H, m, γ-2; γ-6).

^{13}C NMR (75.47 MHz, $(CH_3)_2CO$): δ 40.0 (Cα), 70.5 (Cβ), 70.6 (Cγ), 94.2 (C8), 98.3 (C6), 109.4 (C4a), 112.1 (C3), 126.6 (γ-2, γ-6), 127.4 (C4'), 127.7 (γ-4, β-2, β-6), 128.6 (β-4), 128.6 (β-3, β-5), 128.8 (γ-3, γ-5), 128.9 (C3', C5'), 129.2 (C2', C6'), 135.1 (C1'), 135.7 (β-1), 136.4 (γ-1), 159.7 (C8a), 160.1 (C5), 163.0 (C7), 165.3 (C2), 177.8 (C4).

MS (ESI) m/z (rel. int.): 449.2 $[M+H]^+$ (100%); 471.2 $[M+Na]^+$ (35.0%); 919.3 $[2M+Na]^+$ (70.0%).

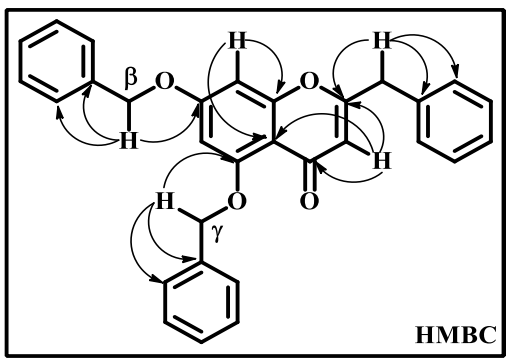


Figure 56. Structure of 2-benzyl-5,7-bis(benzyloxy)-4H-chromen-4-one.

2-benzyl-5-hydroxy-4*H*-chromen-4-one (86.l):

^{1}H NMR (300.13 MHz, $CDCl_3$): δ 3.88 (2H, s, H-α), 6.02 (1H, s, H-3), 6.73 (1H, d, *J* = 8.3Hz, H-6), 6.81 (1H, d, *J* = 8.3Hz, H-8), 7.24-7.30 (2H, m, H-2'; H-6'), 7.28-7.34 (1H, m, H-4'), 7.34-7.39 (2H, m, H-3'; H-5'), 7.43 (1H, t, *J* = 8.3Hz, H-7), 12.47 (1H, s, OH).

^{13}C NMR (75.47 MHz, $CDCl_3$): δ 40.6 (Cα), 107.0 (C8), 109.2 (C3), 110.5 (C4a), 111.3 (C6), 127.6 (C4'), 129.0 (C3', C5'), 129.3 (C2', C6'), 134.4 (C1'), 135.3 (C7), 156.7 (C8a), 160.8 (C5), 169.7 (C2), 183.7 (C4).

MS (ESI) m/z (rel. int.): 253.1 $[M+H]^+$ (95.0%); 275.1 $[M+Na]^+$ (100%).

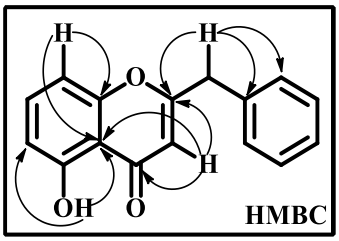


Figure 57. Structure of 2-benzyl-5-hydroxy-4H-chromen-4-one.

2-(3,4-dihydroxybenzyl)-5-hydroxy-4*H*-chromen-4-one (86.m):

^{1}H NMR (300.13 MHz, $(CH_3)_2CO$): δ 3.91 (2H, s, H-α), 6.16 (1H, s, H-3), 6.74 (1H, dd, *J* = 2.1Hz; 8.1Hz, H-6'), 6.75 (1H, dd, *J* = 0.9Hz; 8.4Hz, H-8), 6.83 (1H, d, *J* = 8.1Hz, H-5'), 6.89 (1H, d, *J* = 2.1Hz, H-2'), 6.97 (1H, dd, *J* = 0.9Hz; 8.4Hz, H-6), 7.61 (1H, t, *J* = 8.4Hz, H-7)

^{13}C NMR (75.47 MHz, $(CH_3)_2CO$): δ 40.1 (Cα), 107.9 (C6), 109.3 (C3), 111.0 (C4a), 111.6 (C8), 116.3 (C5'), 117.1 (C2'), 121.5 (C6'), 127.4 (C1'), 136.3 (C7), 145.2 (C4'), 146.1 (C3'), 157.7 (C8a), 161.4 (C5), 172.0 (C2), 184.32 (C4).

MS (ESI) m/z (rel. int.): 285.0 $[M+H]^+$ (100%); 307.0 $[M+Na]^+$ (15.0%); 591.0 $[2M+Na]^+$ (15.0%)

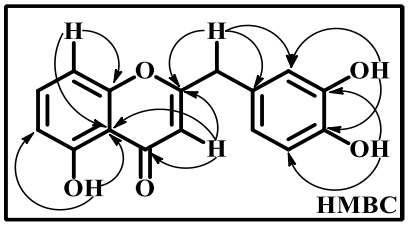


Figure 58. Structure of 2-(3,4-dihydroxybenzyl)-5-hydroxy-4H-chromen-4-one.

2-(3,4-dihydroxybenzyl)-4*H*-chromen-4-one (86.n):

^{1}H NMR (300.13 MHz, $(CH_3)_2CO$): δ 3.88 (2H, s, H-α), 6.13 (1H, s, H-3), 6.74 (1H, dd, *J* = 2.1Hz; 8.1Hz, H-6'), 6.83 (1H, d, *J* = 8.1Hz, H-5'), 6.89 (1H, d, *J* = 2.1Hz, H-2'), 7.45 (1H, ddd, *J* = 1.1Hz; 7.2Hz; 8.0Hz, H-6), 7.53 (1H, dd, *J* = 1.1Hz;8.6Hz, H-8), 7.76 (1H, ddd, *J* = 1.7Hz; 7.2Hz; 8.6Hz, H-7), 7.98 (1H, s, 3'-OH), 7.99 (1H, s, 4'-OH), 8.07 (1H, dd, *J* = 1.7Hz; 8.0Hz, H-5).

^{13}C NMR (75.47 MHz, $(CH_3)_2CO$): δ 40.1 (Cα), 110.6 (C3), 116.3 (C5'), 117.0 (C2'), 117.1 (C8), 118.9 (C6'), 121.5 (C4a), 125.8 (C6), 125.9 (C5), 127.9 (C1'), 134.5 (C7), 145.2 (C4'), 146.1 (C3'), 157.3 (C8a), 169.9 (C2), 177.8 (C4).

MS (ESI) m/z (rel. int.): 269.0 $[M+H]^+$ (100%); 291.0 $[M+Na]^+$ (60.0%); 559.0 $[2M+Na]^+$ (40.0%).

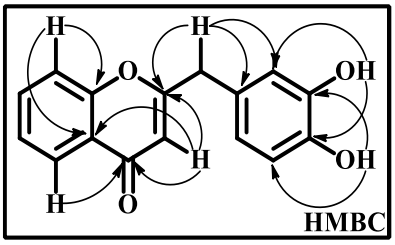


Figure 59 Structure of 2-(3,4-dihydroxybenzyl)-4H-chromen-4-one.

2-(3,4-dihydroxybenzyl)-4*H*-chromen-4-one (86.o):

^{1}H NMR (300.13 MHz, $(CH_3)_2CO$): δ 4.01 (2H, s, H-α), 6.06 (1H, s, H-3), 6.24 (1H, large s, H-6), 6.36 (1H, large s, H-8), 7.29-7.32 (1H, m, H-4'), 7.33-7.42 (4H, m, H-2'; H-3'; H-5'; H-6'), 9.94 (1H, s, 7-OH), 12.81 (1H, s, 5-OH).

^{13}C NMR (75.47 MHz, $(CH_3)_2CO$): δ 40.5 (Cα), 94.5 (C8), 99.7 (C6), 105.0 (C4a), 109.1 (C3), 128.1 (C4'), 129.6 (C3', C5'), 130.1 (C2', C6'), 136.4 (C1'), 159.2 (C8a), 163.3 (C5), 165.0 (C7), 170.1 (C2), 183.1 (C4).

MS (ESI) m/z (rel. int.): 269.0 $[M+H]^+$ (100%); 291.0 $[M+Na]^+$ (35.0%).

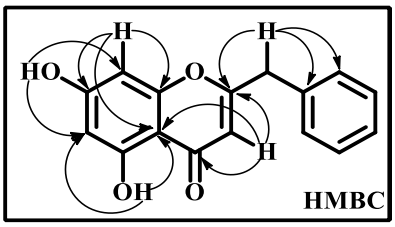


Figure 60. Structure of 2-(3,4-dihydroxybenzyl)-4H-chromen-4-one.

2-benzyl-7-hydroxy-4*H*-chromen-4-one (86.p):

^{1}H NMR (300.13 MHz, $(CH_3)_2CO$): δ 4.00 (2H, s, H-α), 6.03 (1H, s, H-3), 6.86 (1H, d, *J* = 2.3Hz, H-8), 6.95 (1H, dd, *J* = 2.3Hz; 8.7Hz, H-6), 7.28-7.33 (1H, m, H-4'), 7.35-7.39 (2H, m, H-3'; H-5'), 7.40-7.44 (2H, m, H-2'; H-6'), 7.92 (1H, d, *J* = 8.7Hz, H-5), 9.73 (1H, s, 7-OH)

^{13}C NMR (75.47 MHz, $(CH_3)_2CO$): δ 40.6 (Cα), 103.2 (C8), 110.7 (C3), 115.3 (C6), 117.6 (C4a), 127.7 (C5), 128.0 (C4'), 130.0 (C3', C5'), 130.0 (C2', C6'), 136.8 (C1'), 159.1 (C8a), 163.3 (C7), 168.4 (C2), 177.2 (C4)

MS (ESI) m/z (rel. int.): 253.1$[M+H]^+$ (100%); 275.2$[M+Na]^+$ (20.0%);

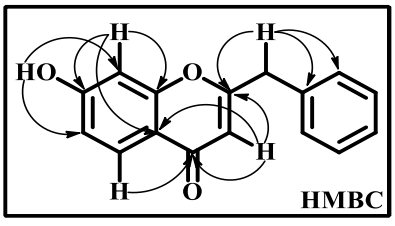


Figure 61. Structure of 2-benzyl-7-hydroxy-4H-chromen-4-one.

4. Synthesis of 2-benzoylchromones

Throughout the synthesis of the numerous 2-benzylchromones already describe, several secondary products were also encountered, consisting in 2-benzoylchromones. Even though there is no conclusive justification for the formation of this type of compounds, during the synthesis of the 2-benzylchromones, a few explanations emerge. One possible explanation might be the oxidation of the chromone scaffold at α-position by the action of DMSO. Since this solvent has been already proven to be capable of promoting oxidation processes[118] and, due to the high reactivity of this particular position of the chromone core, it becames the most provable cause for the appearance of these compounds (Figure 62).

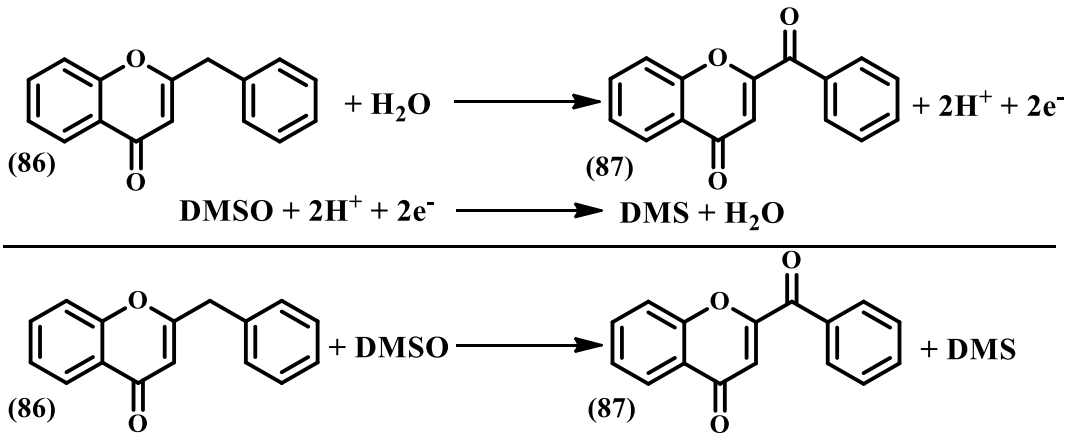


Figure 62.Equations of the Redox reactions.

In the ^{1}H NMR spectrum, this type of compounds is particularly characterized by the disappearance of the signal correspondent to H-α. Moreover, it is possible to verify the deshielding effect, provoqued by the new carbonyl group existent at α-position, upon the B-ring protons. This effect leads to higher chemical shifts than those observed for 2-benzylchromones, as well as distinct multiplicities for these particular signals (Figure 63).

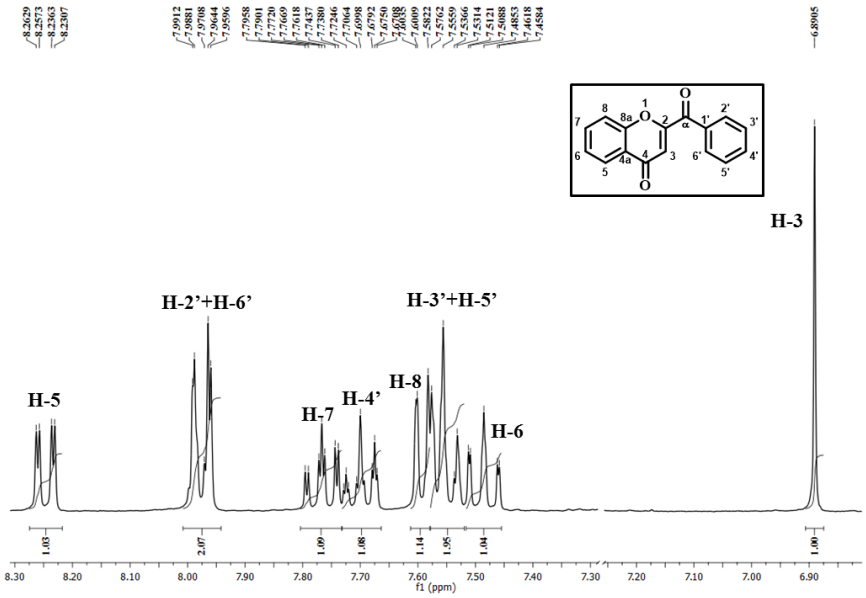


Figure 63. ^{1}H NMR spectrum of 2-benzoyl-4H-chromen-4-one (87.a) given as an example.

Using the ^{13}C NMR spectrum it is also possible to identify a few characteristics that are particular to this type of compounds. These include the disappearance of the signal usually correspondent to the CH_2 group at α-position, as already described for ^{1}H NMR, and also the existence of another signal corresponding to a carbonyl group (Figure 64).

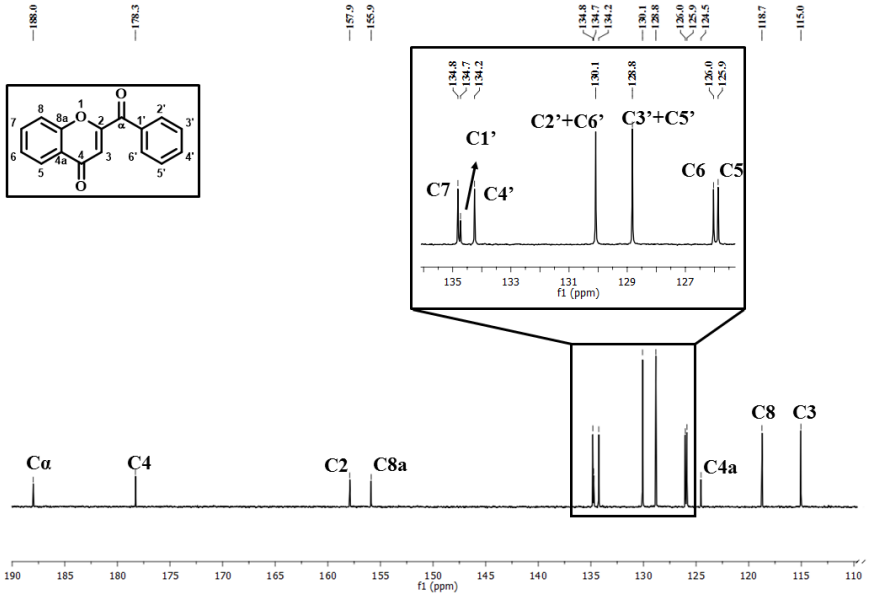


Figure 64. ^{13}C NMR spectrum of 2-benzoyl-4H-chromen-4-one.

2-benzoyl-4*H*-chromen-4-one (87.a):

^{1}H NMR (300,13 MHz, $CDCl_3$): δ 6.89 (1H, s, H-3), 7.49 (1H, ddd, *J* = 1.0Hz; 7.5Hz, H-6), 7.53-7.58 (2H, m, H-3',5'), 7.59 (1H, d, *J* = 8.2Hz, H-8), 7.70 (1H, tt, *J* = 1.6Hz; 7.5Hz, H-4'), 7.77 (1H, ddd, *J* = 1.7Hz; 7.1Hz; 8.7Hz, H-7),7.96-8.00 (2H, m, H-2', 6'), 8.25 (1H, dd, *J* = 1.7Hz; 8.0Hz, H-5).

^{13}C NMR (75,47 MHz, $CDCl_3$): δ115.0 (C3), 118.7 (C8), 124.5 (C4a), 125.9 (C5), 126.0 (C6), 128.8 (C3', C5'), 130.1 (C2', C6'), 134.2 (C4'), 134.7 (C1'), 134.8 (C7), 155.9 (C8a), 157.9 (C2), 178.3 (C4), 188.0 (Cα).

MS (ESI) m/z (rel. int.): 251.1 $[M+H]^+$ (45.0%); 273.1 $[M+Na]^+$ (100%); 523.2 $[2M+Na]^+$ (45.0%)

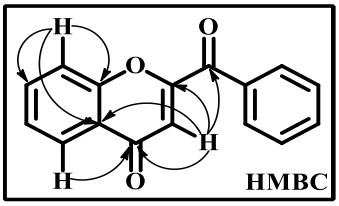


Figure 65. Structure of 2-benzoyl-4H-chromen-4-one.

2-(3,4-dimethoxybenzoyl)-5-methoxy-4*H*-chromen-4-one (87.b):

^{1}H NMR (300,13 MHz, $CDCl_3$): δ 3,92 (3H, s, 3'-OCH_3), 3,95 (3H, s, 4'-OCH_3), 3,96 (3H, s, 5-OCH_3), 6,70 (1H, s, H-3), 6,83 (1H, d, *J* = 8,3Hz, H-6), 6,91 (1H, d, *J* = 8,5Hz, H-5'), 7,09 (1H, d, *J* = 8,3Hz, H-8), 7,51 (1H, d, *J* = 2,0Hz, H-2'), 7,59 (1H, t, *J* = 8,3Hz, H-7), 7,61 (1H, dd, *J* = 2,0Hz; 8,5Hz, H-6').

^{13}C NMR (75,47 MHz, $CDCl_3$): δ 56,1 (3'-OCH_3), 56,3 (4'-OCH_3), 56,5 (5-OCH_3), 106,9 (C6), 110,1 (C5'), 111,7 (C2'), 115,2 (C4a), 116,2 (C3), 125,7 (C6'), 127,5 (C1'), 134,7 (C7),149,3 (C3'), 154,5 (C4'), 156,5 (C2), 157,9 (C8a), 159,8 (C5), 177,8 (C4), 186,0 (Cα).

MS (ESI) m/z (rel. int.): 341,1 $[M+H]^+$ (62,0%); 361,1 $[M+Na]^+$ (25,0%); 703,2 $[2M+Na]^+$ (100%).

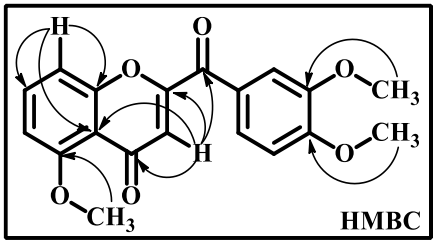


Figure 66. Structure of 2-(3,4-dimethoxybenzoyl)-5-methoxy-4H-chromen-4-one.

2-(4-chlorobenzoyl)-4*H*-chromen-4-one (87.d):

^{1}H NMR (300,13 MHz, $CDCl_3$): δ 6.87 (1H, s, H-3), 7.47 (1H, ddd, *J* = 0.9Hz; 7.1Hz; 8.0Hz, H-6), 7.50-7.54 (2H, m, H-2',6'), 7.55 (1H, dd, *J* = 0.9Hz; 8.8Hz, H-8), 7.76 (1H, ddd, *J* = 1.7Hz; 7.1Hz; 8.8Hz, H-7), 7.91-7.96 (2H, m, H-3',5'), 8.22 (1H, dd, *J* = 1.7Hz; 8.0Hz, H-5).

^{13}C NMR (75,47 MHz, $CDCl_3$): δ 114,8 (C3), 118.6 (C8), 124.5 (C4a), 125.9 (C5), 126.1 (C6), 129.2 (C2', C6'), 131.4 (C3', C5'), 133.0 (C1'), 134.9 (C7), 140.9 (C4'), 155.7 (C8a), 157.7 (C2), 178.0 (C4), 186.6 (Cα).

MS (ESI) m/z (rel. int.): 285.1 $[M+H]^+$ (40.0%); 307.1 $[M+Na]^+$ (45.0%).

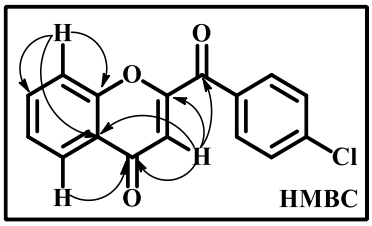


Figure 67. Structure of 2-(4-chlorobenzoyl)-4H-chromen-4-one.

2-benzoyl-7-(benzyloxy)-4*H*-chromen-4-one (87.e):

^{1}H NMR (300,13 MHz, $CDCl_3$): δ 5.18 (2H, s, H-β), 6.82 (1H, s, H-3), 7.05 (1H, d, *J* = 2.4Hz, H-8), 7.12 (1H, dd, *J* = 2.4Hz; 8.9Hz, H-6), 7.34-7.39 (1H, m, β-4), 7.41-7.44 (2H, m, β-3; β-5) 7.41-7.46 (2H, m, β-2; β-6) 7.55 (2H, t, *J* = 7.6Hz, H-3',5'), 7.69 (1H, dt, *J* = 1.6Hz;7.6Hz, H-4'), 7.95 (2H, m, H-2',6'), 8.16 (1H, d, *J* = 8.9Hz, H-5).

^{13}C NMR (75,47 MHz, $CDCl_3$): δ 70.7 (Cβ), 101.7 (C8), 115.7 (C3), 116.2 (C6), 118.6 (C4a), 127.3 (C5), 127.6 (β-2, β-6), 128.5 (β-4), 128.8 (C3', C5', β-3, β-5),

130.0 (C2', C6'), 134.1 (C4'), 134.9 (β-1), 135.4 (C1'), 157.4 (C2), 157.8 (C8a), 164.0 (C7), 177.5 (C4), 188.2 (Cα).

MS (ESI) m/z (rel. int.): 357.1 $[M+H]^+$ (100%); 379.1 $[M+Na]^+$ (35.0%); 735.2 $[2M+Na]^+$ (45.0%).

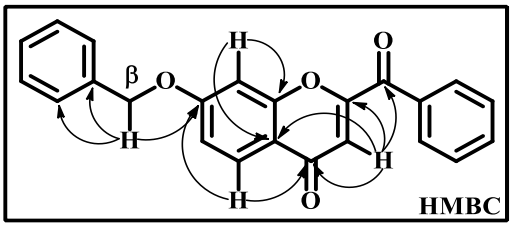


Figure 68. Structure of 2-benzoyl-7-(benzyloxy)-4H-chromen-4-one.

2-(3,4-dimethoxybenzoyl)-7-methoxy-4*H*-chromen-4-one (87.f):

^{1}H NMR (300,13 MHz, $CDCl_3$): δ 3.93 (3H, s, 7-OCH_3), 3.97 (3H, s, 3'-OCH_3), 4.00 (3H, s, 4'-OCH_3), 6.78 (2H, s, H-α), 6.95 (1H, d, *J* = 8.5Hz, H-5'), 6.97 (1H, d, *J* = 2.4Hz, H-8), 7.04 (1H, dd, *J* = 2.4Hz; 8.9Hz, H-6), 7.56 (1H, d, *J* = 2.0Hz, H-2'), 7.63 (1H, dd, *J* = 2.0Hz; 8.5Hz, H-6'), 8.15 (1H, d, *J* = 8.9Hz, H-5).

^{13}C NMR (75,47 MHz, $CDCl_3$): δ 56.0 (7-OCH_3), 56.2 (3'-OCH_3), 56.3 (4'-OCH_3), 100.6 (C8), 110.1 (C5'), 111.7 (C2'), 114.9 (C3), 115.5 (C6), 118.5 (C4a), 125.8 (C6'), 127.2 (C5), 127.6 (C1'), 149.5 (C3'), 154.6 (C4'), 157.9 (C8a), 158.1 (C2), 164.9 (C7), 177.4 (C4), 186.3 (Cα).

MS (ESI) m/z (rel. int.):341.1 $[M+H]^+$ (100%); 361.1 $[M+Na]^+$ (60.0%); 703.2 $[2M+Na]^+$ (95.0%).

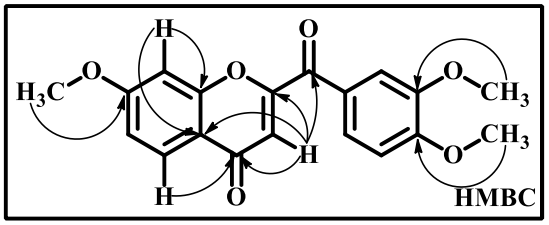


Figure 69. Structure of 2-(3,4-dimethoxybenzoyl)-7-methoxy-4H-chromen-4-one

Chapter III: Biological evaluation

1. Cell viability/Citotoxicity

In order to evaluate the influence of the synthesized compounds in cell viability, the resazurin reduction assay was performed.[119] Resazurin (**88**) (Figure 70) consists in a cell permeable redox indicator that may be used to assess viable cell number by following protocols similar to those using the tetrazolium compounds, like the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

Resazurin (**88**) is then a deep blue colored dye, weakly fluorescent, until being irreversively reduced, by the action of viable cells with active metabolism, into a pink colored and with high red fluorescence product named resorufin (**89**) (Figure 70). Since the quantity of resorufin (**89**) produced is directly proportional to the number of viable cells, it becomes possible to quantify them by measuring the absorvance of resorufin (**89**) at 570nm (Abs_{max} for resorufin) and 600nm (Abs_{max} for resazurin).

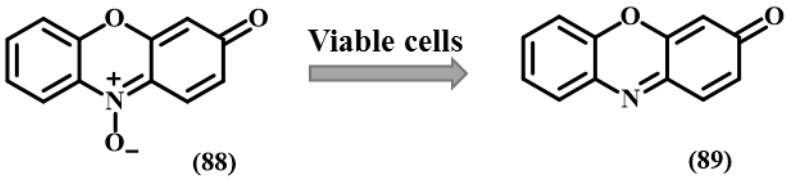


Figure 70. Structure of resazurin and resorufin, the product resulting from reduction in viable cells

The preliminary results for the cytotoxicity of our compounds are depicted in Figure 71, with the exception of the compounds **86.f**, **86.g** and **86.i** since it was not possible to solubilize them in suitable quantities of DMSO. The range of analyzed concentrations is not the same for all the evaluated compounds, mainly due to quantity limitations. However, it was possible the evaluation of any adverse effect caused by the compounds and also to perform it in triplicate.

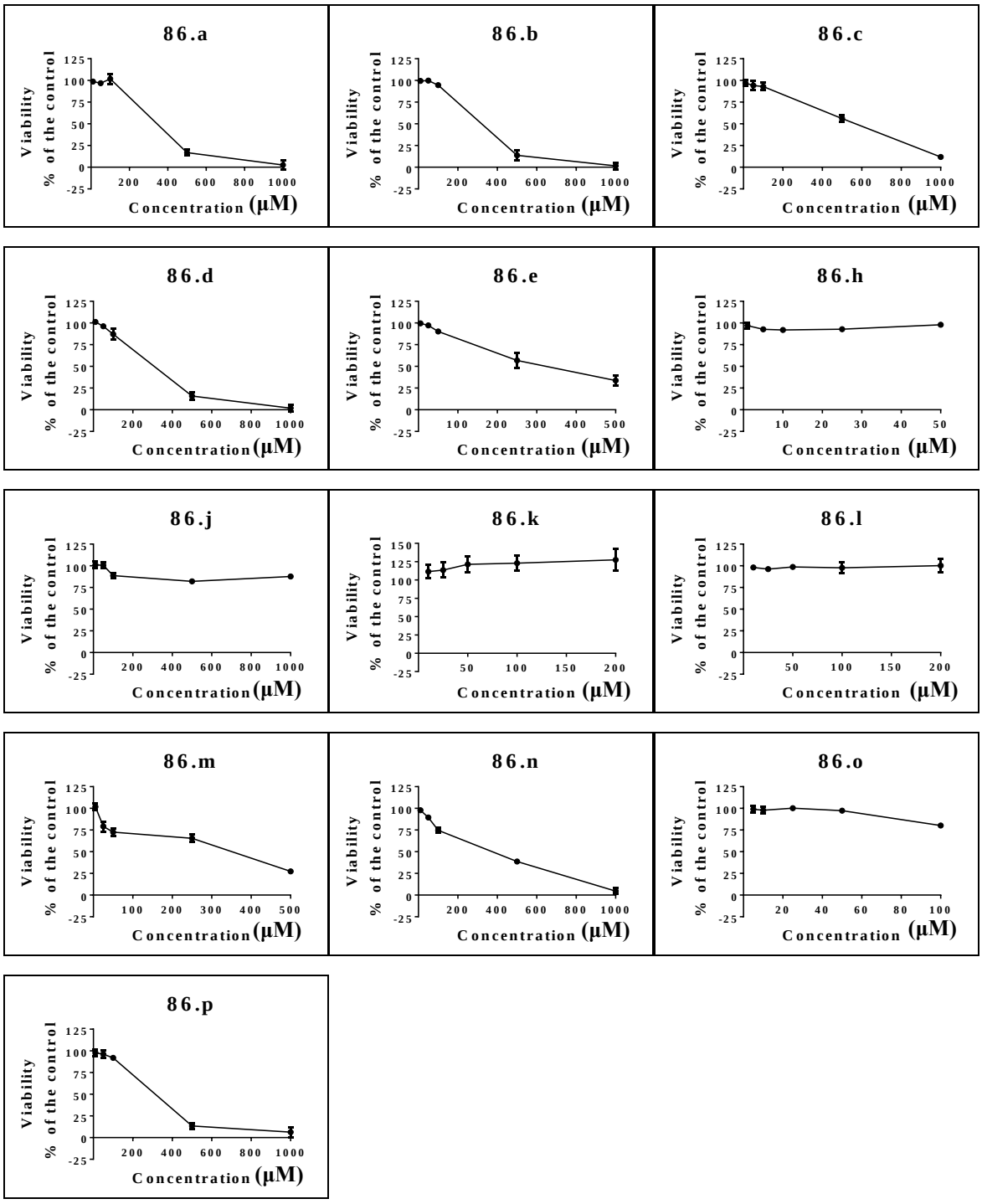


Figure 71. Cell viability of synthesized compounds

These results show that at low concentrations (<50μM), practicaly all the compounds show no significant effects, since they maintain the cell viability close to 100%, while at high concentrations (>50μM) a considerable decrease in cell viability is observed, with many of them being capable of reducing the cell viability levels to nearly 0%. Based on this

evidence, two particular concentrations have been chosen to be used in the development of the coming biological assay, a screening assay for anti-inflammatory activity.

2. Anti-inflammatory activity

Inflammation, as described above, is an extremely complex process in which numerous molecular mechanisms are involved. Thus, in order to evaluate the anti-inflammatory effects of the synthesized compounds, a screening assay was performed by measuring the production of the inorganic free radical ˙NO. Firstly, the influence of the tested compounds was compared with a negative control, without the addition of LPS to the system (Figure 72).

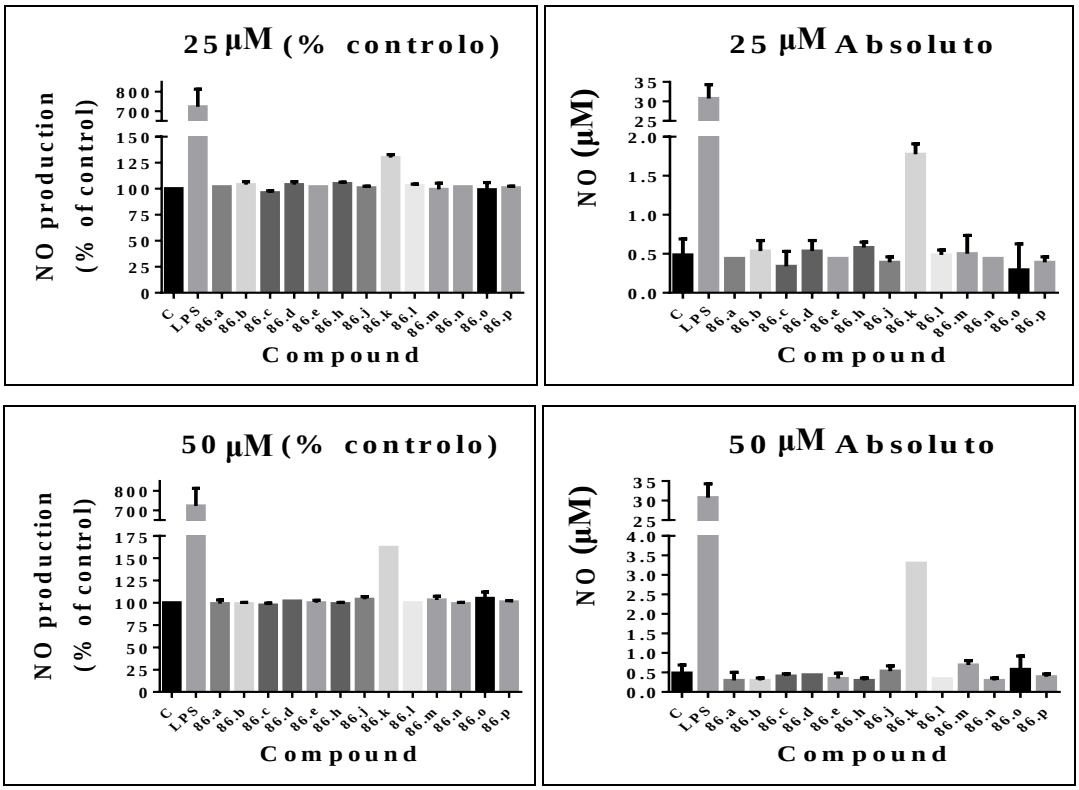


Figure 72. Production of ˙NO, without LPS-stimulation.

This comparision allows to verify that, with the exception of the compound **86.k**, all the evaluated compounds have no significant stimulating effect in ˙NO production. This exception, compound **86.k**, is characterized by a slight increase in ˙NO production when

compared with the remaining compounds, which indicates a stimulative effect of this compound. However, this effect might not be direcly related with a pro-inflammatory action since, in absolute terms, the difference of ˙NO production between treated and untreated cells is almost irrelevant. Moreover, the results of the cellular viability assay show that this particular compound (**86.k**) promotes cellular proliferation or, at least, an increment of their metabolic activity, which might also explain the apparent stimulation of ˙NO production.

After that, the compounds were added to previously LPS- stimulated macrophages. The effects of the evaluated compounds were then compared with the positive control, in which only LPS was present (Figure 73).

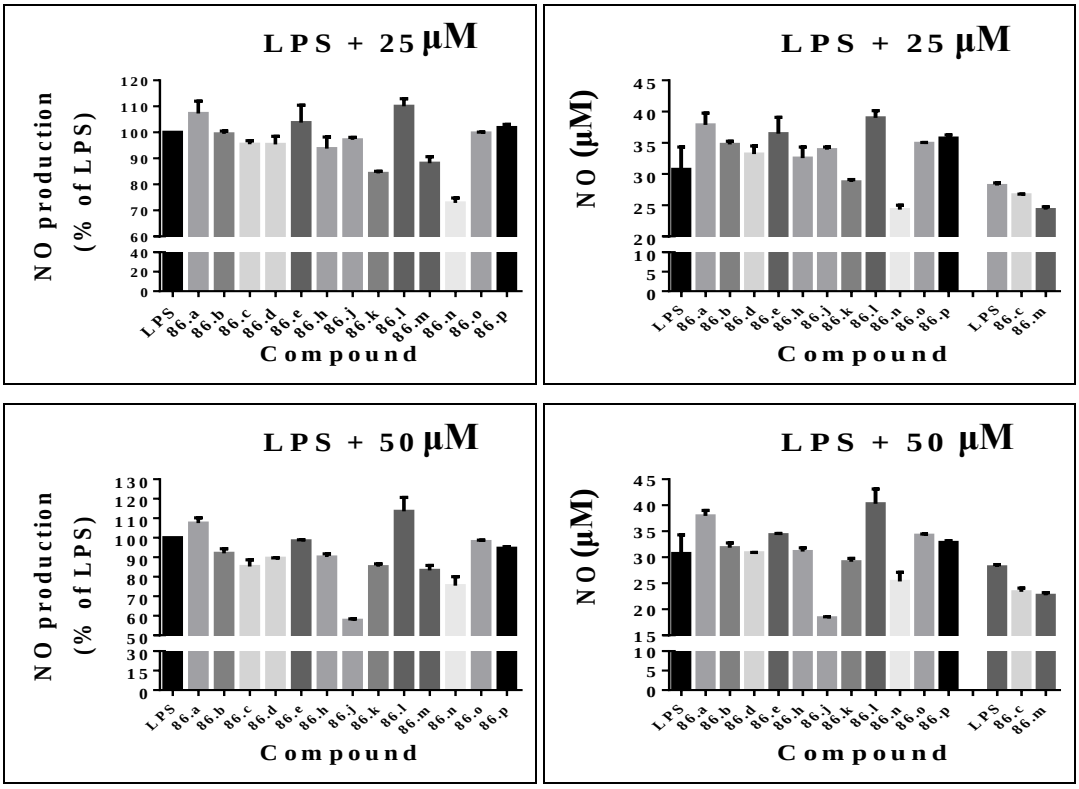


Figure 73. Inhibition of ˙NO production in LPS-stimulated macrophages.

The results of this inhibitory assay show that several compounds reveal minimal levels of inhibition. Based on these results, it is possible to understand that only two compounds show moderate levels of inhibition (**86.j** and **86.n**), from which **86.j** should be highlighted. Moreover, by comparing the results for the different concentrations of these compounds, it becomes clear that the inhibition promoted by **86.n** is practically independent from the

concentration, while compound **86.j** clearly provoques a concentration-dependent type of inhibition.

On the other hand, the compounds **86.a** and **86.l** reveal a slight increase in the ˙NO production when applied in both tested concentrations. However, since none of them promotes the same effect without LPS-stimulation, they do not appear to induce iNOS expression which implies that NF-kB is not activated by them. Based on this, the LPS-stimulation seems to be potentiated by these two particular compounds explaining the increase verified in Figure 73.

3. Inhibition of Acetylcholinesterase

In order to measure the activity of AChE, an addaptation of the Ellman`s[120] method is applied using acetylthiocholine iodine(ATChI), a thiol analogue of the natural substrate, acetylthiocholine iodine (ATChI). In this method, the thiocholine resulting from the enzymatic catalysis of ATChI reacts with 5,5`-dithiobenzoic acid (DTNB), also known as Ellman`s reagent, originating 5-thio-2-nitrobenzoic acid (TNB). The resulting TNB is then strongly yellow and is promptly quantified by measuring the absorvance at 415 nm (Figure 74).

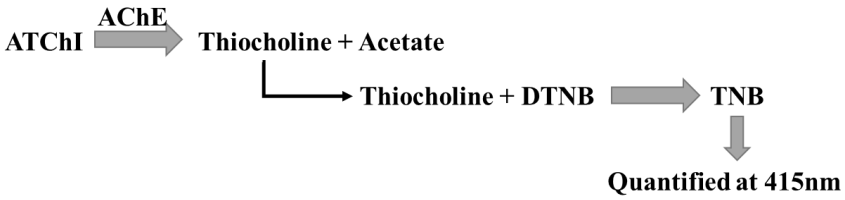


Figure 74. Schematic representation of the anti-colinesterase assay.

The results of this assay are represented in Table 1, where the concentrations used in it and the percentage of inhibition are indicated. The percentages of inhibition were all calculated with MeOH/phosphate buffer pH 8.0 as control and with Donepezil as a positive reference for this type of activity.

Table 1. Acetylcholinesterase inhibitory activity.

Compound	[Compound] (μm)	% inhibition (mean ± sem)
86.a	200 μM	34.29 ± 0.92
86.b	200 μM	18.69 ± 1.66
86.c	200 μM	22.94 ± 2.18
86.d	200 μM	30.40 ± 0.70
86.e	200 μM	24.22 ± 1.86
86.j	100 μM	4.15 ± 1.62
86.l	200 μM	————
86.m	200 μM	40.05 ± 1.27
86.n	200 μM	19.65 ± 1.49
86.p	200 μM	10.00 ± 1.58

*** Donepezil with 51.14 ± 5.82 % at 0.029 μM[44];**
*** Values expressed as mean ± SEM.**

As well as in anti-inflammatory assay, some compounds could not be tested due to solubility problems, namely compounds **86.f**, **86.g**, **86.h**, **86.i**, **86.k** and **86.o**. Also due to solubility issues, compound **86.j** was tested at a lower concentration than the other compounds. Based on the results obtained, it is possible to verify that several compounds (**86.b**, **86.c**, **86.e** and **86.n**) reveal similar inhibitory activities. Important to refer that three compounds present minimal (**86.j** and **86.p**) or no inhibitory activity (**86.l**). Compounds **86.a**, **86.d** and **86.m**, are those with the higher activities being compound **86.m** the most promising one.

Chapter IV: Experimental procedures

1. Equipment and reagents

During the development of our project, some aspects regarding the conditions and quality of the reagents and equipment were considered:

- The commercial reagents were used without any previous purification;
- Solvents used for reactions or purification procedures were analytically pure or were, wherever necessary, purified through distillation processes.
- Tetrahydrofuran (THF) was dry at reflux temperature, in the presence of metallic sodium, until the obtention of a blue colored solution, due to the action of benzophenone, followed by proper distillation.
- The evolution of our reaction was controlled by thin-layer chromatography (TLC), using laminated plates covered by gel silica 60 F_{254} (Merck), with 0.25mm of thickness and incorporated indicator. The plates were observed using a 254/366nm ultra-violet (UV) lamp.
- The purifications by preparative thin-layer chromatography were carried out by using glass plates (20 x 20 cm), previously covered with gel silica 60 GF_{254} from Merck, with 0.5mm of thickness and activated during 12h at 120ºC. After the elution of the mixtures of compounds, the plates were observed at the wavelengths (λ) of 254 nm and 366nm.
- In the purifications through column chromatography it was used gel silica 60 from Merck, with 0.063-0.2 mm of granulometry. The recovered fractions were monitored by TLC and visualized in a UV lamp.
- The spectrums of nuclear magnetic resonance of proton (H^1 NMR), carbon (C^{13} NMR) and bidimensional studies of heteronuclear spectroscopic correlation (HSQC and HMBC) were obtained in an Bruker Avance 300 spectrometer (300.13 MHz and 75.47 MHz for H^1 NMR and C^{13} NMR, respectively). For the analysis of our compounds, deuterated solvents like chloroform and acetone were used, having tetramethylsilane (TMS) as internal standard. The chemical shifts (δ, ppm) indicated were obtained at room temperature in the deuterated solvents referred on the corresponding characterizations.

- The spectra of mass spectrometry by electrospray ionization were acquired using a Q-TOF 2 instrument. Solutions of the samples with a concentration of 1mg/mL were prepared by dissolving the compound with a 1:1 mixture of MeOH/$CHCl_3$. The samples of ESI analysis were prepared by diluting 2 μL of the sample solutions with 200 μL of methanol/formic acid (0.1%). Nitrogen was used as nebulizer gas and argon was used as collision gas. Samples were introduced into the mass spectrometer at a flow rate of 10 μL/min, the needle voltage was set at 3000 V, with the ion source at 80°C and desolvation temperature of 150°C. The spectra were acquired for a cone voltage of 30 V. This study was performed by the mass spectrometry group of the Chemistry Department from the University of Aveiro.

2. Chemistry

2.1. Synthesis of esters

To a round bottom flask with the appropriate phenylacetic acid, an excessive amount of ethanol and 2 mL of sulphuric acid were added (Table 2). The reactional mixture was then kept in permanent magnetic stirring at approximately 90℃ during the night. In the next day, the solution was poured into ice and water and the pH adjusted to approximately 5-6, using potassium carbonate (K_2CO_3).

A liquid-liquid extaction is then performed using dichloromethane as organic solvent and the organic phase is completely dryed, using sodium sulphate anidre (Na_2SO_4). The exceding solvent was evaporated to dryness and the final residue consists in the desired ester. Depending on the efficacy of the reaction, purification tecniques might be necessary in order to obtain the ester without any contamination from the starting materials. In our case, the yields of the reaction stand around 80-90% since the losses of compound are almost exclusively due to the presence of the starting marerials, which probably may be overcomed by slightly higher times of reaction.

Table 2. List of synthesized esters, with correspondent reagent quantities.

compound	m (acid)	V (ethanol)
ethyl 2-phenylacetate (85.a)	30.0g	70 mL
ethyl 2-(3,4-dimethoxyphenyl)acetate (85.b)	30.0g	50 mL
ethyl 2-(4-chlorophenyl)acetate (85.c)	15.0g	50 mL

2.2. Synthesis of 2-benzylchromones

The appropriate ethyl 2-phenylacetate (2 equiv.) was added to a solution of a selected acetophenone, in previously dryed THF, with the strong base NaH (Table 3). The solution was stirred at reflux temperature for approximately 3h, under N_2 atmosphere. After that, the solution was poured into ice and water and the pH adjusted near to 2-3 with sulphuric acid (98%). The two-phased solution was then purified by liquid-liquid extraction, using dichloromethane as organic solvent, and dryed, using sodium sulphate anidre. The solvent was evaporated to dryness and the residue, consisting in the diketonic intermediate, isolated.

In a second stage, for every 1.30g of obtained residue, 1g of *p*-toluenosulphonic acid (*p*-TSA) was added. These reagents were then dissolved in the minimum amount possible of DMSO and the solution heated to approximately 90 ºC, over the night. In the next day, the solution was again poured into ice and water and another liquid-liquid extraction was performed, using the most appropriate organic solvent (usualy dichloromethane). The organic solvent was then completely evaporated to dryness and the final residue purified by column and preparative thin-layer chromatography, using the most appropriate eluent. The solvent was, once again, evaporated to dryness obtaining the desired chromone with moderate to high yields (Table 3).

Table 3. List of synthesized chromones, with correspondent reagent quantities.

Compound	Acetophenone	Ester	m (NaH)	Yield
86.a	0.5 mL (0.004154 mol)	2 equiv (≈ 1.364g)	0.300g (≈3 equiv)	76 %
86.b	0.690g (0.004154 mol)	2 equiv (≈ 1.364g)	0.300g (≈3 equiv)	61 %
86.c	0.690g (0.004154 mol)	2 equiv (≈ 1.863g)	0.300g (≈3 equiv)	72 %
86.d	0.5 mL (0.004154 mol)	2 equiv (≈ 1.863g)	0.300g (≈3 equiv)	71 %
86.e	1 mL (0.008307 mol)	2 equiv (≈ 3.300g)	0.600g (≈3 equiv)	37 %
86.f	0.950g (0.004154 mol)	2 equiv (≈ 1.364g)	0.300g (≈3 equiv)	50 %
86.g	0.850g (0.004154 mol)	2 equiv (≈ 1.364g)	0.450g (≈4.5 equiv)	31 %
86.h	0.690g (0.004154 mol)	2 equiv (≈ 1.863g)	0.450g (≈4.5 equiv)	14 %
86.i	1.450g (0.004154 mol)	2 equiv (≈ 1.863g)	0.300g (≈ 3 equiv)	20 %
86.j	1.036g (≈0.004154 mol)	2 equiv (≈ 1.364g)	0.450g (≈4.5 equiv)	22 %
86.k	1.031g (≈0.002959 mol)	2 equiv (≈ 0.970g)	0.450g (≈4.5 equiv)	30 %

2.3. Deprotection reaction

To a solution of a methoxylated chromone in dry dichloromethane, 1.5 equiv/OCH_3 group of BBr_3 (Table 4) was added at approximately -70ºC, temperature guaranteed by a cryostat. After the addition of the BBr_3, the solution is left in permanent magnetic stirring at room temperature for 2-3h. Note that this whole procedure was kept under N_2 atmosphere.

To conclude this methodology, the reactional mixture was poured into ice and water and left stirring for a few minutes so the hydrolysis process was completed. After that, a liquid-liquid extraction was performed, using dichloromethane as organic solvent, and the solution was completely dryed by Na_2SO_4. The solvent was the evaporated to dryness obtaining the desired hydroxylated chromone. Due to the high efficacy verified in this reaction, purification processes are almost not even needed. However, when necessary, the isolated residue was purified by preparative thin-layer chromatography, using the most suitable eluent.

Table 4. List of hydroxylated chromones, with correspondent reagent quantities.

Compound	m (chromone)	Nº of OR	V (BBr_3)
86.l	0.291g	1 (5-OCH_3)	1.64mL
86.m	0.229g	3 (5,3',4'-OCH3)	3.30mL
86.n	0.193g	2 (3',4'-OCH_3)	1.95mL
86.o	0.100g	2 (5,7-OCH_3)	1.00mL
86.p	0.213g	2 (7-OBn)	0.75mL

3. Biological assays

3.1. Cell Culture

Raw 264.7, a mouse leukaemic monocyte macrophage cell line from American Type Culture Collection, were cultured in Dullbecco's Modified Eagle Medium (DMEM) supplemented with 10% non-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Cells were subcultured every two days and used until passage 30. Along the experiments, they were monitored by microscope observation in order to detect any morphological change.

3.2. Cell Viability assays

In order to investigate the influence of the synthesized chromones, cell viability was assessed by a resazurin assay. Briefly, $0.05x10^6$ cells/well in a 96-well plate were exposed to indicated concentrations of chromones for 24h. Two hours before the end of exposure, resazurin solution was added to each well to a final concentration of 50 μM. Finally, a sample form each condition was transferred to a 96 wells plate and the absorbance of resorufin (product originated in resazurin reduction) was then measured at 570 and 600 nm in a standard spectrophotometer BioTek Synergy HT (Biotek Instruments, Winooski, VT, EUA).

3.3. Screening for anti-inflammatory activity

The potential anti-inflammatory activity of the synthesized chromones was evaluated by analyzing their capacity to inhibit NO production in LPS-stimulated Raw 264.7 macrophages. The production of NO was measured by the accumulation of nitrite in the culture supernatants, using a colorimetric reaction with the Griess reagent. The cells were plated at 3 × 105 cells/well in 48-well culture plates, allowed to stabilize for 12 hours and then incubated with two concentrations (25μM and 50μM) of the chromones during 1h. Following this, lipopolysaccharide (LPS) 1μg/mL was added and cells incubated during 24 hours. For NO measurement, 100 μL of culture supernatants were collected and diluted with equal volume of the Griess reagent [0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 1% (w/v) sulfanilamide containing 5% (w/v) H_3PO_4] during 30 minutes, in the dark. The absorbance at 550 nm was measured using a Synergy HT spectrophotometer.

3.4. Inhibition of acetylcholinesterase

Acetylcholinesterase (AChE, E.C. 3.1.1.7, from *Electrophorus electricus*), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodine (ATChI) were purchased from SigmaAldrich. Due to solubility issues, test compounds were prepared, in methanol/sodium phostphate buffer pH 8.0 (1:1 proportion), to give solutions with the concentrations indicated in Table 1. In order to minimize the quantity of aqueous buffer in the final solution, the volumes to be added were slightly adapted, maintaining the quantities of each reagent in the final solution. To each well of a 96 well microplate, 50 μL of the tested compounds was added with a micropipetted. Then, 100 μL of a 0.025 U/mL solution of AChE was added to all wells and the solution incubated during 5 min at 37 °C. After these 5 min and in sequence, 50 μL of a 2.5 mM solution of ATChI and 50 μL of a 0.5 mM solution of DTNB were pipetted to all the wells of the microplate. The microplate was then placed in the microplate reeder programmed to shake for 5 seconds before each reading and read Abs_{415nm} at different times (0 s, 150 s, 300 s, and 450 s) to monitor the reaction rate

Chapter V: Conclusion and future perspectives

1. Conclusion

During the synthetic process a range of yields were obtained. These distinct levels of efficiency observed in the synthetic method applied allow the elaboration of a preliminar structure-reactivity relationship study (Figure 75). Through these particular results it becomes possible to conclude that the existence of several substituent groups may lead to a decrease in the efficiency of this method, while some other substituents have minimal or no effect. Our results indicate that the presence of electron withdrawing groups in B-ring decrease the yields. On the other hand, the influence of A-ring substituents is not related with the type of group only -OR type were tested, but with the position of the group, being 7-position the one that produced more influence in the yields.

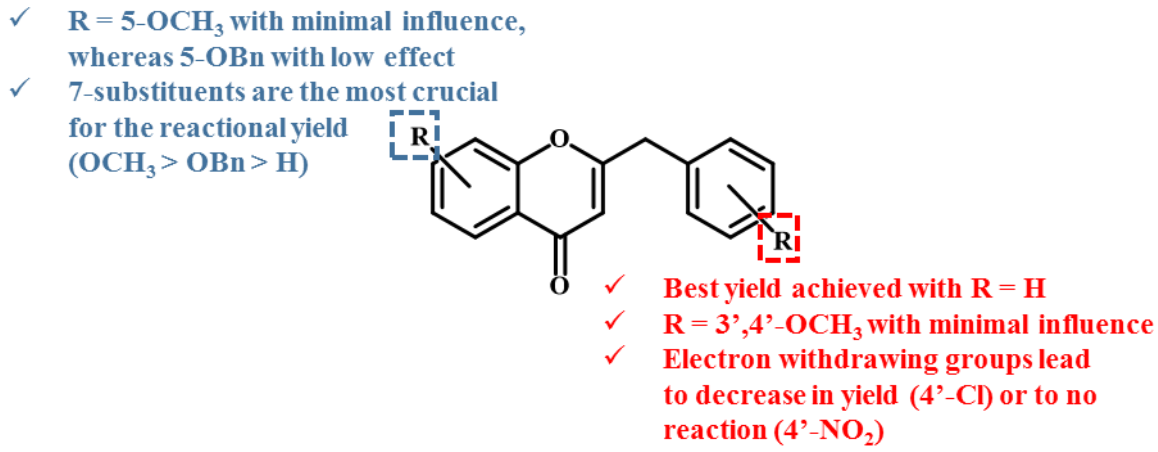


Figure 75. Structure-Reactivity relationship study of 2-benzylchromones.

The biological evaluation of our compounds consisted in two particular activities, namely anti-inflammatory activity and inhibition of acetylcholinesterase, allowing the obtention of two individual SAR studies for this particular type of compounds, the 2-benzylchromones. The anti-inflammatory evaltuation of these compounds shows that, while the majority of the substituent groups have no effect to their activity, the presence of a catechol group at B-ring promotes a higher anti-inflammatory activity. Moreover, the introduction of a 5-OBn in the A-ring seems to be the most promising substituent group, confirming some SAR studies already made for chromones, with the hydrolysis of this group culminating with a decrease in the activity of this type of compound (Figure 76).

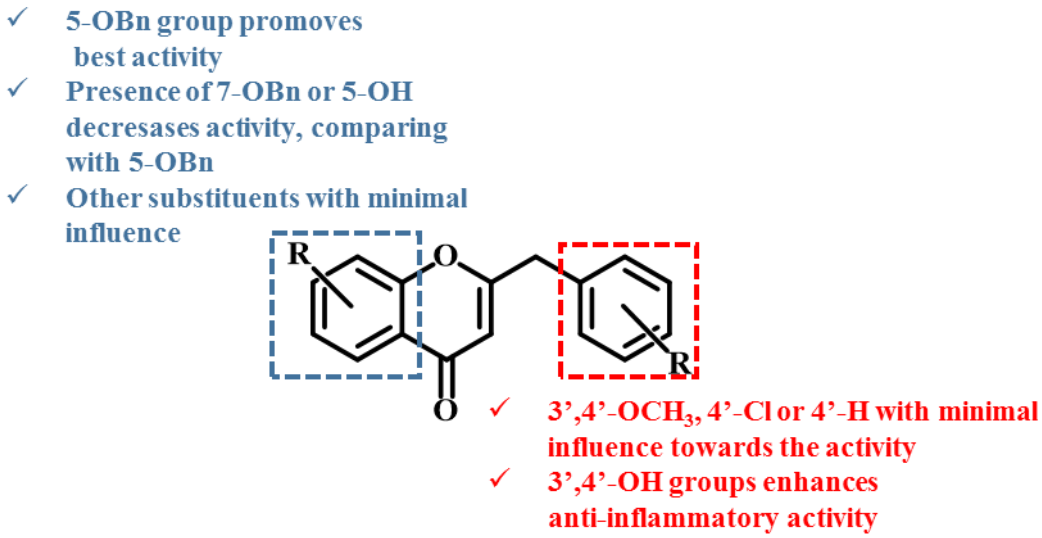


Figure 76. Structure-Anti-inflammatory activity of 2-benzylchromones.

In the case of the acetylcholinesterase inhibitory activity, it is possible to conclude that, excepting the introduction of a catechol group at B-ring conjugated with a 5-OH group, all the substintuent groups introduced to the 2-benzylchromone scaffold lead to a decrease in the activity of this type of compounds (Figure 77).

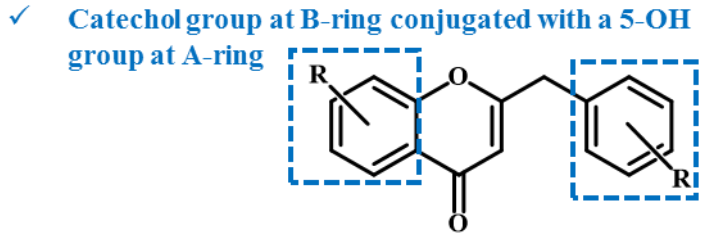


Figure 77. Structure-Anti-acetylcholinesterase relationship of 2-benzylchromones.

To summarize, the catechol group at B-ring has proven to be the most promising structural feature for the evaluated biological activities, moreover it can be achieved by our method without major losses. Furthermore, in the A-ring it is comproved that the presence of groups that increase the compound's hydrophobicity highly contributes to their biological activities. However, the introduction of substituent groups at A-ring promotes a decrease in the reaction yields, which can be a limitation for the synthesis of this type of compounds. Therefore, the combination of other substituent groups at A-ring with the catechol group at B-ring should originate compounds of easier obtention and improved activities.

2. Future perspectives

Since the yields of the applied method are directly influenced by the substituents introduced in the 2-benzylchromone scaffold, a optimization of the method by adapting reagent quantities and/or reactional medium conditions becomes crucial for the obtention of an higher efficiency. Moreover, the appearance of the by-products, 2-benzoylchromones (**87**), was not fully explored in this work and might give rise to other studies in order to understand the causes of their formation.

Regarding the biological evaluation of our compounds, it is important to also evaluate the secondary compounds formed during the synthetic process, the 2-benzoylchromones. Futhermore, new functionalizations should be performed in the already obtained compounds in order to attain molecules with improved biological activities. This functionalizations may be performed in both 2-benzylchromones and 2-benzoylchromones, allowing a wider range of synthetic routes that enable the modification of the molecules (Figure 78).

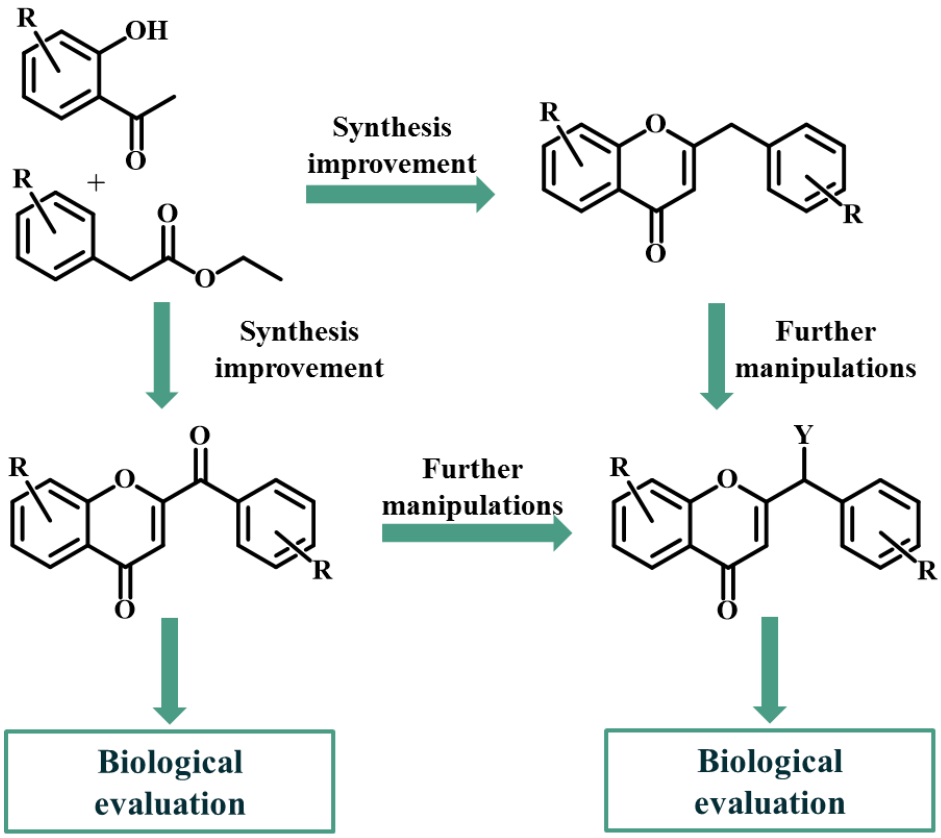


Figure 78. Future perpectives of the project.

Chapter VI: Bibliography

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