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Project / Internship Report

SYNTHESIS OF CHIRAL AMINO ACID DERIVATIVES OF CHRYSIN AS POTENTIAL ANTI-TUMOR AGENTS

ANA SOFIA DA COSTA ALMEIDA

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SYNTHESIS OF CHIRAL AMINO ACID DERIVATIVES OF CHRYSIN AS POTENTIAL ANTI-TUMOR AGENTS

Supervisor: Professora Doutora Carla Sofia Fernandes

Faculdade de Farmácia da Universidade do Porto / Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto

Co-supervisor: Professora Doutora Maria Elizabeth Tiritan

Faculdade de Farmácia da Universidade do Porto/IINFACTS – Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde/Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto

Natural Products and Medicinal Chemistry – Laboratory of Organic and Pharmaceutical Chemistry – Department of Chemical Sciences – Faculty of Pharmacy – University of Porto.

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ABSTRACT

Biological systems, such as receptors, enzymes and other binding macromolecules, can recognize and discriminate between enantiomers leading to different responses (enantioselectivity). Chirality plays an essential role in pharmacodynamic, pharmacokinetic and toxicological events. Thus, the development of enantiomerically pure drugs has been growing over the years.

Chrysin is a flavone that presents a wide range of biological and pharmacological activities. Due to the poor solubility of chrysin, low intestinal absorption and a rapid metabolism of glycosylation, the synthesis of derivatives of chrysin has been investigated as an attempt to obtain compounds with increased bioavailability, selectivity, efficiency and permeability.

The aim of this work was the synthesis of new amino acid derivatives of chrysin in their enantiomerically pure form.

Four new compounds were synthesized by coupling reaction with chrysin with both pure enantiomers of commercially available amino esters of tryptophan and tyrosine. Four other compounds were then obtained via hydrolysis of the ester groups. Structure elucidation was achieved by spectroscopic methods such as ^{1}H-NMR and ^{13}C-NMR.

The potential of the eight chiral derivatives of chrysin for grow inhibition of human tumor cell lines will be further investigated.

Key words: Chirality; Enantiomers; Chrysin; Amino acid derivatives.

RESUMO

Sistemas biológicos como recetores, enzimas e outras macromoléculas podem reconhecer e distinguir enantiómeros resultando em diferentes respostas biológicas (enantiosseletividade). A quiralidade apresenta um papel essencial em eventos farmacodinâmicos, farmacocinéticos e toxicológicos. Assim, o desenvolvimento de fármacos na forma enantiomericamente pura tem crescido ao longo dos anos.

A crisina é uma flavona que apresenta uma vasta gama de atividades biológicas e farmacológicas. Devido à baixa solubilidade da crisina, assim como, fraca absorção intestinal e um metabolismo de glicosilação rápido, a síntese de derivados da crisina tem sido investigada com o objetivo de obter compostos com uma biodisponibilidade, seletividade, eficiência e permeabilidade superiores.

Este trabalho teve como objetivo a síntese de novos derivados de aminoácidos da crisina na sua forma enantiomericamente pura.

Quatro novos compostos foram sintetizados por acoplamento de um bloco construtor da crisina com ambos os enantiómeros de aminoésteres do triptofano e da tirosina disponíveis comercialmente. Quatro outros novos compostos foram obtidos por hidrólise dos grupos éster. A elucidação estrutural foi obtida por métodos espetroscópicos como ^{1}H-RMN e ^{13}C-RMN.

O potencial destes oito derivados quirais da crisina na inibição do crescimento de linhas celulares de tumores humanas será posteriormente investigado.

Palavras-chave: Quiralidade; Enantiómeros; Crisina; Derivados de aminoácidos.

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ABBREVIATIONS AND SYMBOLS

^{13}C-NMR – Carbon nuclear magnetic resonance

^{1}H-NMR – Hydrogen nuclear magnetic resonance

Bcl-2 – B-cell lymphoma 2

CDKs – Cyclin-dependent kinases

CIP – Cahn-Ingold-Prelog

CLL – Chronic lymphocytic leukemia

COMU – 1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate

CSPs – Chiral stationary phases

d – Doublet

DCM – Dichloromethane

dd – Double doublet

ddd – Double double doublet

DMBA – 7,12-Dimethylbenz(a)anthracene

DMSO-d6 – Hexadeuterodimethyl sulfoxide

FDA – Foods and Drugs Administration

HRMS – High resolution mass spectrometry

IR – Infrared

IUPAC – International Union of Applied Chemistry

MeOH – Methanol

MMP – Proteins of the matrix metalloproteinase

NMEs – New molecular entities

PCNA – Proliferating cell nuclear antigen

ROS – Reactive oxygen species

rt – Room temperature

s – Singlet

STAT3 – Signal transducer and activator of transcription 3

TBTU – *O*-(Benzotriazol-1-yl-)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate

TEA – Triethylamine

THF – Tetrahydrofuran

TIMP – Tissue inhibitors of metalloproteinases

TLC – Thin layer chromatography

TNF – Tumor necrosis factor

TRAIL – TNF-related apoptosis-inducing ligand

UV – Ultraviolet

VEGF – Vascular endothelial growth factor

1. INTRODUCTION

1.1. General concepts of chirality

Chirality is a geometric property of asymmetric objects and it is originated from the Greek word “cheir” which means hand (1). At molecular level nature is asymmetric as most natural molecules and secondary metabolites are chiral and appear in enantiomerically pure form. Chirality plays an essential role not only in the life of plants and animals but also in our daily life concerning, for example, chiral compounds such as pharmaceuticals, agrochemicals, fragrances and flavors. A molecule is considered chiral if its structure is nonsuperposable on its mirror image (**Figure 1**). Each form is called an enantiomer (2, 3).

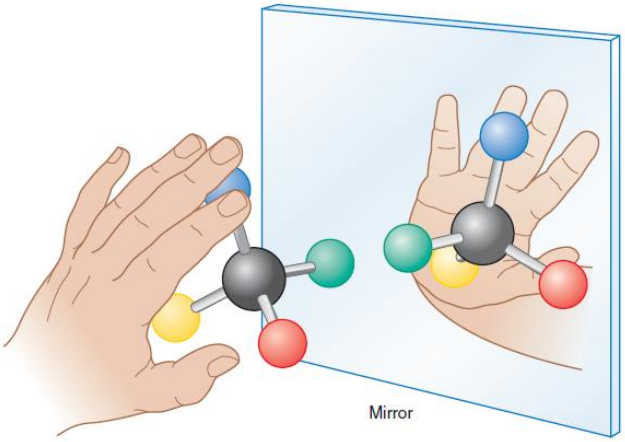


Figure 1 - Analogy between nonsuperimposable mirror images of a molecule and of a hand as an explanation to chirality (4).

The asymmetrical structure can be created by a stereogenic center being the most common a sp^3 carbon atom with four different substituents. Examples of drugs comprising this type of stereogenic center are atenolol, propranolol and fluoxetine (**Figure 2**).

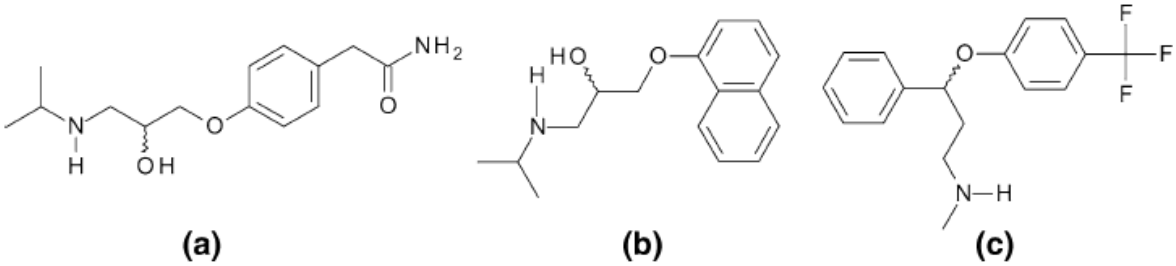


Figure 2 - Drugs with a carbon as stereogenic center: atenolol **(a)**, propranolol **(b)** and fluoxetine **(c)**.

However, other atoms such as sulphur, nitrogen or phosphorus can also be stereogenic centers (5). Nevertheless, apart from central chirality, there are other types of chirality such as axial ((*aS*) (P) and (*aR*) (M)-gossypol), planar ((*pS*) and (*pR*) paracyclophane carboxylic acid), and helical (M(-) and P(+)-hexahelicen-7yl acetic acid) (3).

Enantiomers present the same physiochemical properties such as melting point, solubility and reactivity if placed in an achiral environment. The exception is the ability to rotate plane-polarized light meaning chiral molecules are optically active (6). An enantiomer is called dextrorotatory, (+), when the rotation is for the right (clockwise) and levorotatory, (-), when for the left (counter-clockwise). An equimolar mixture of the two enantiomers of a chiral molecule is designated a racemate and does not exhibit optical activity (3, 7).

The International Union of Applied Chemistry (IUPAC) uses the *R/S* system to determine the spatial position of the different substituents around the stereogenic center (configuration) (8). The *R/S* system from the latin *rectus* (right) and *sinister* (left) is based in the Cahn-Ingold-Prelog (CIP) convention which assigns different priorities to the substituents of the stereogenic center. Since enantiomers differ in the spatial position of these substituents, the direction counting from the highest priority substituent to the lowest will be different, being designated *R* when clockwise and *S* when counter-clockwise (3, 7). This system has no direct relation to the optical rotation (+)/(-) meaning *R* and *S* isomers can be either dextrorotatory or levorotatory (2).

1.1.1. Enantioselectivity

Chirality is a very important feature in pharmacodynamic, pharmacokinetic and toxicological events. Since biological structures such as receptors, enzymes and other binding molecules are chiral they can recognize and discriminate between enantiomers by selective interactions leading to different biological responses (enantioselectivity). Thus, enantiomers can express differences in pharmacokinetic (absorption, distribution, metabolism and excretion) and pharmacodynamic (drug-biotarget interaction) properties. This happens due to enantioselective interactions with these chiral macromolecules (**Figure 3**) (7, 9) and it can be explained, for example, by picturing a glove-like cavity in the enzyme allowing the substrate to bind. If right-handed one of the enantiomers will fit perfectly and bind to the enzyme while the other enantiomer will have a weak bond (10).

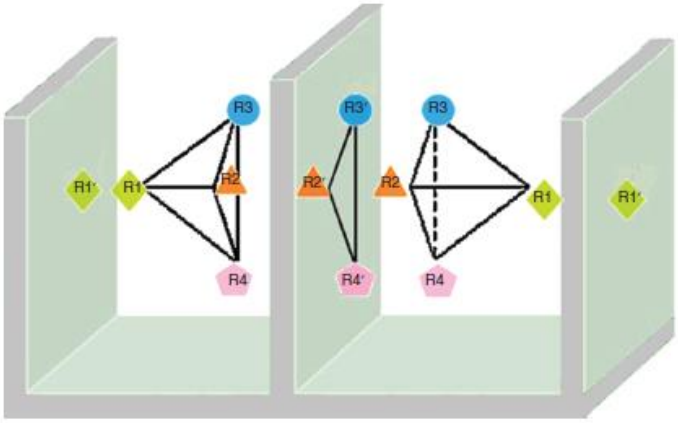


Figure 3 - Four-point location model for enantioselectivity.

Ideally, the therapeutic activity of a drug would remain in just one enantiomer and the adverse effects in the other (11). In reality, a pair of enantiomers can present a range of activity scenarios: both enantiomers can present similar pharmacological/biological activity; both can have the same activity but one can more active than the other, for instance, in propranolol, a β-blocker; they can have qualitatively different activities (levothyroxine, a synthetic thyroid hormone, and dextrothyroxine, a drug used in hypercholesterolemia); one of the enantiomers can have no activity (methyldopa, α-adrenergic antagonist) or even be highly toxic ((*S*)-thalidomide) (3). For a given biological activity, when an enantiomer of a compound is preferred exclusively or mainly over the other is designated as eutomer being the other the distomer (7).

1.1.2. Development of enantiomerically pure drugs

Many decades ago, most pharmaceuticals were either obtained from natural sources or semi-synthetized from natural compounds and since only one enantiomer was frequently present, chiral drugs were used as single enantiomers. Synthetic pharmaceuticals quickly started to represent a large part of new therapeutic agents and dominate the market. These were frequently prepared, tested and used as racemates (10).

However, due to the tragic thalidomide incident, the use of racemates was drastically reduced. Thalidomide (**Figure 4**) was a drug released in the late 1950s and was mostly used to treat morning sickness in pregnant women becoming one of the world's biggest selling drugs. Soon, after its release reports of severe malformations in children started to flare up being, later on, the cause identified as the teratogenic nature of thalidomide's (*S*)-enantiomer (10, 12).

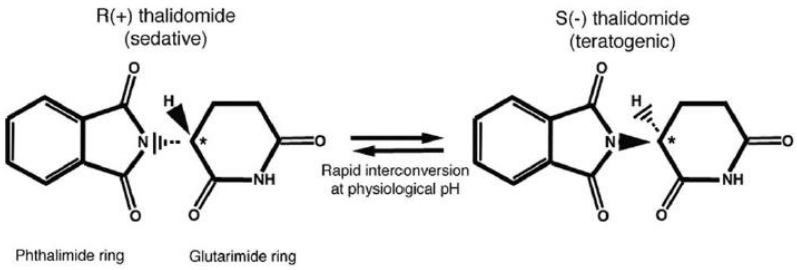


Figure 4 – Structure of thalidomide's enantiomers.

After that, many drugs previously marketed as racemates started being developed as single enantiomers being this phenomenon designated as "chiral switch". However, not all the re-evaluations resulted in the expected therapeutic properties (10).

Thalidomide's case had a great impact and stimulated new regulatory requirements for racemic drugs. In addition, the advances in synthetic methodologies, analytic and preparative separation techniques of racemates lead to an increase in the number of enantiomerically pure drugs (13). The annual distribution of Foods and Drugs Administration (FDA) approved drugs according to chirality in 2002-2016 showed that racemates represented the minority while single enantiomers overtook achiral compounds (**Figure 5**) (14). Furthermore, in 2018, only two racemic mixtures (tafenoquine and lofexidine) were approved as new molecular entities (15).

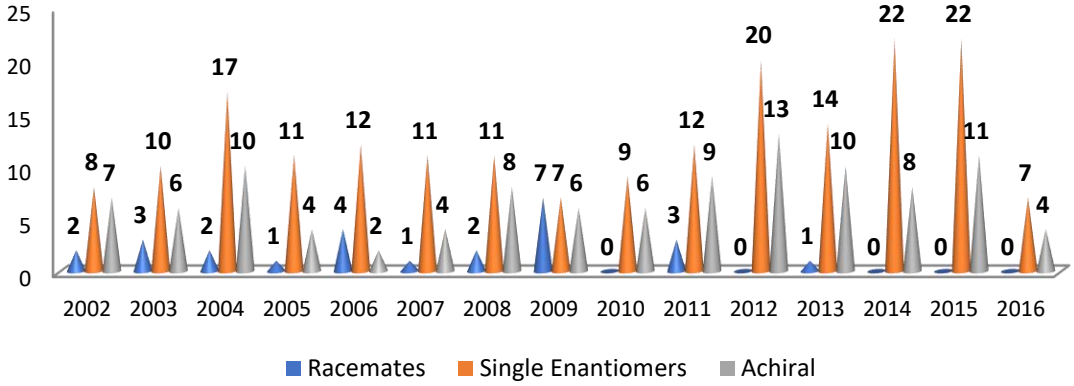


Figure 5 - New molecular entities (NMEs) approved by the U.S. Foods and Drugs Administration according to chirality in 2002-2016.

Despite this, there is still an ongoing debate about whether enantiomerically pure pharmaceuticals are more favorable than racemates. Nonetheless, enantiomerically pure drugs were found to present decreased therapeutic doses, increased safety margin, fewer drug interactions, less secondary effects such as toxicity, among other advantages (3).

To obtain enantiomeric pure drugs, two different approaches can be pursued. Firstly, the “racemic approach” consists in the preparation of the racemate followed by resolution into the individual enantiomers. This can be achieved by a range of methodologies being preparative chromatography on chiral stationary phases (CSPs) considered the most effective, when both enantiomers are needed, since it can result in both enantiomers with high enantiomeric purity.

Secondly, the “chiral approach” consists in enantioselective synthesis using chiral synthons and auxiliaries, stereoselective catalysts or enzymes and it is mostly used when a large quantity of only one enantiomer is wanted (3, 16). On the other hand, if both enantiomers are needed, two independent synthesis are required. An interesting method is the use of commercially available enantiomerically pure reagents as building blocks which also results in both enantiomers with high enantiomeric purity (17). This method, known as the “chiral pool strategy”, is widely used, being renewable and sometimes more cost-effective than others. It uses chiral reagents such as amino acids, hydroxy acids, terpenes and alkaloids, which are incorporated into the target structure and can be changed to achieve the required chiral features. Amino acids are easily available with high enantiomeric purity being considered one of the oldest sources of enantiopure compounds. A large number of important drugs, such as oxytocin (used for inducing labor) and carbapenem antibiotics, are synthetic peptides (18).

1.2. Flavonoids

Natural sources produce an immense diversity of secondary (or specialized) metabolites that present a variety of ecological and physiological roles (19).

Flavonoids are polyphenolic plant secondary metabolites originated from the amino acids phenylalanine, tyrosine and malonate, which generates the phenylbenzopyrone structure (C6-C3-C6) that identifies these compounds (**Figure 6**) (20).

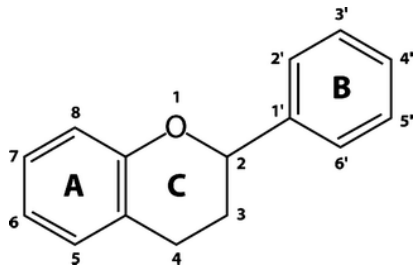


Figure 6 - Basic structure of flavonoids.

In 1930, a new compound was isolated from oranges believed to be part of a new class of vitamins and it was designated as vitamin P. Later, it was found to be rutin, a flavonoid. Its discovery initiated the research to find and isolate new flavonoids as well as to study their mechanism of action (21).

There are more than 10000 flavonoids, known so far, and they are divided in the following different subgroups according to their structure: chalcones, flavanones, flavones, isoflavones, flavonols, flavandiols, anthocyanidins and aurones (**Figure 7**) (22).

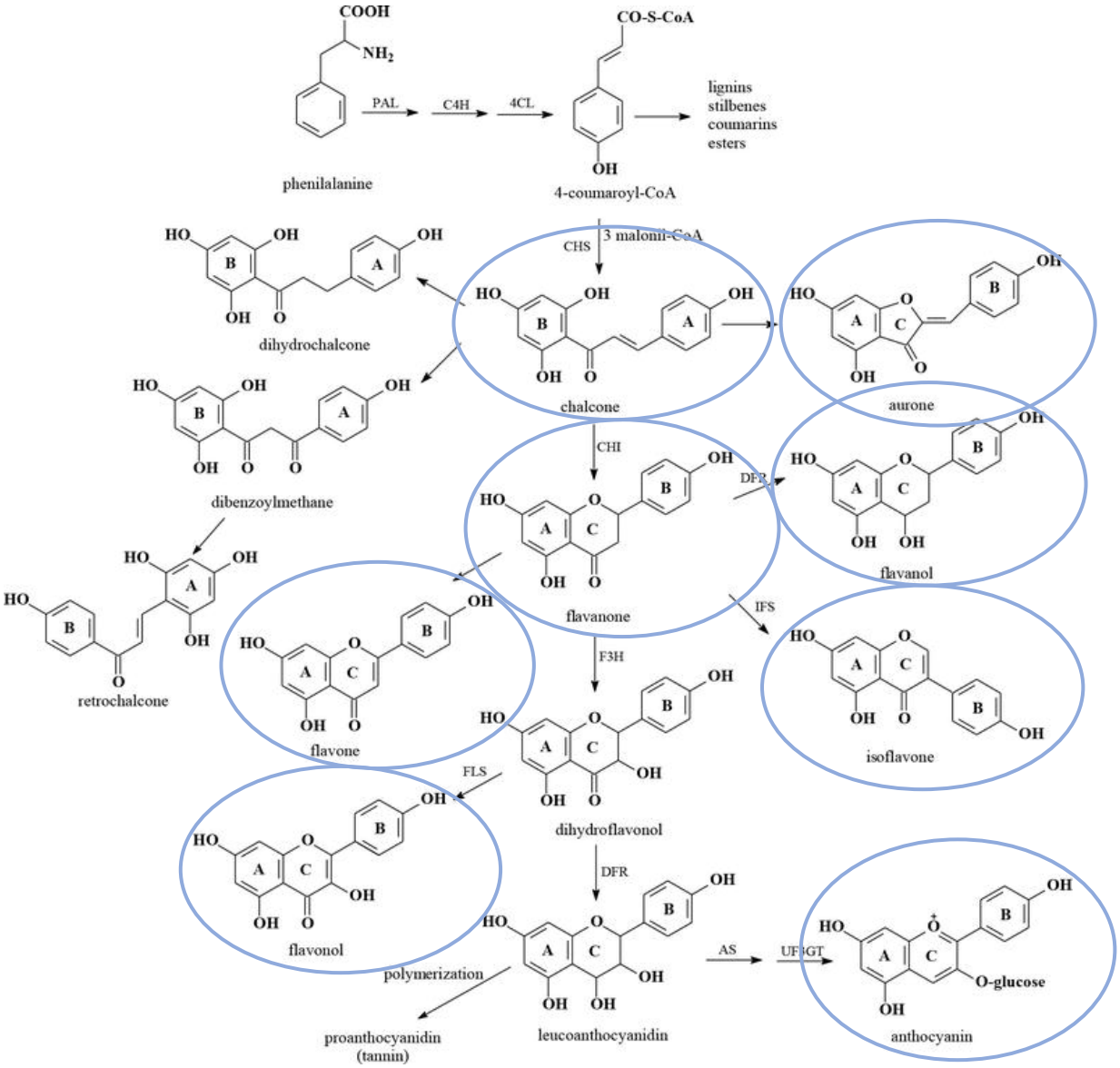


Figure 7 – Biosynthesis of flavonoids and its subgroups.

Flavonoids likely exist in nature for over one billion years. They are important in flowers being responsible for the autumnal shades of red, orange and yellow and in the growth, development and defense of plants acting, for instance, as enzyme inhibitors,

precursors of toxic substances and a defense against ultraviolet radiation. They can also present crucial functions in photosensitization, levels of respiration and photosynthesis, growth hormones and regulators (23, 24).

Additionally, these metabolites are part of the human diet being present in foods and beverages such as vegetables, fruits, cocoa, wine and tea (25). Flavonoids have been found in several medicinal plants used in traditional medicine all around the world. Thus, they are essential not only for plants, but also for animals, including humans. This ongoing interaction between flavonoids and humans associated with the health benefits found in them has attracted interest to investigate their biochemical and physiological activities (24, 26).

Flavonoids present a vast range of biological properties such as anti-mutagenic, anti-inflammatory, antioxidant, anti-carcinogenic, ability to modulate essential cellular enzyme functions, among others. Therefore, flavonoids are now a crucial part of many pharmaceutical, medicinal and cosmetic applications (25).

1.2.1. Chrysin

As previously mentioned, all subgroups of flavonoids present a variety of biological activities. Furthermore, the flavones, the largest subgroup, have been greatly explored. The "flavone scaffold" allows a great diversity of structures and derivatives associating this class of compounds with many biological activities (27).

Chrysin (**Figure 8**) is a flavone found in many natural sources such as propolis, honey, some fruits, passion flowers and even mushrooms. The IUPAC name of chrysin is 5,7-dihydroxy-2-phenyl-4*H*-chromen-4-one and 5,7-dihydroxyflavone (28).

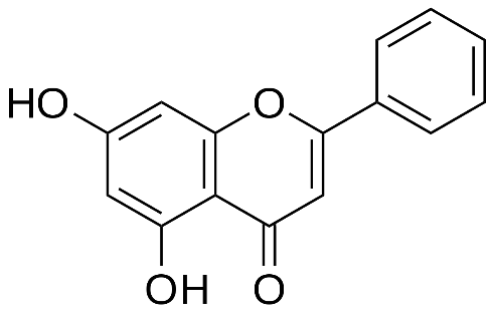


Figure 8 – Chrysin's structure (5,7-dihydroxyflavone).

Over the years, chrysin has been widely investigated in order to discover its biological and pharmacological properties being found to possess anti-bacterial (29), anti-

inflammatory (30), antioxidant (31), anxiolytic (32), anti-diabetic (33) activities and hepatoprotective effects (34). Chrysin also presents neuroprotective (35) and anti-aging effects (36).

Chrysin's anticancer properties have been notably investigated with a diversity of cancer cell lines and animal tumor models becoming the most promising activity of chrysin both as chemopreventive and chemotherapeutic. It was found that chrysin could kill cancer cells of hematological, liver, cervical, lung, prostate, breast, colon, pancreatic, nasopharyngeal, leukemia, glioblastoma, and thyroid cancers (37, 38).

Chrysin is a pro-apoptotic agent in many cell lines by different mechanisms, such as permeabilization of outer mitochondrial membrane, production of reactive oxygen species (ROS) and modulation of proteins of the B-cell lymphoma 2 (Bcl-2) family. It also inhibits tumor growth and cell proliferation by alteration of proliferating cell nuclear antigen (PCNA), cyclins, cyclin-dependent kinases (CDKs) and p53 levels and inhibits angiogenesis through inhibition of a signal transducer and activator of transcription, STAT3 and vascular endothelial growth factor (VEGF) release mediated by hypoxia through Akt signaling pathway (38). Additionally, chrysin can act in metastasis and cancer progression by increasing the expression of the tissue inhibitors of metalloproteinases TIMP-1 and 2 and reducing the expression of the proteins of the matrix metalloproteinase MMP-2 and 9 in breast tumor (39) and inhibit cells lines of colon cancer through apoptosis which reduces the volume of tumors (40).

Furthermore, it presents protective effects against 7,12-dimethylbenz(a)anthracene (DMBA) induced breast and two stage skin carcinomas by modulating phase I and phase II enzymes (41), promotes cell death and degradation of caspases 3 and 8 by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (42). Chrysin also inhibits complex II and ATPases in cancerous mitochondria and induces apoptosis in chronic lymphocytic leukemia (CLL) B-lymphocytes (43).

Consequently, chrysin blocks many cancer-related pathways and presents a wide range of anticancer related properties such as anticarcinogenic, antimetastatic, antiangiogenic, proapoptotic, antimutagenic, immunomodulatory and antioxidant properties (38).

There is no doubt that chrysin is a bioactive small molecule like many other flavones. However, it can be difficult to extrapolate biological activities observed *in vitro* to actual *in vivo* studies. Although previous *in vivo* studies may have shown that chrysin easily penetrates lipid membranes, others have shown low oral bioavailability (44, 45).

Additionally, chrysin was found to present poor solubility, low intestinal absorption and a rapid metabolism of glycosylation. To overcome these problems, the synthesis of derivatives of chrysin have been investigated as an attempt to increase its biological properties by obtaining compounds with improved efficacy and selectivity (46).

Amino acids (**Figure 9**) are essential compounds in life presenting important physiological functions such as good biocompatibility and cell affinity. Considering their functionality and the properties also originated from chirality, they are extremely valuable biochemically and for the chemical industry. Therefore, the coupling of chrysin with amino acids could lead to an increase in interaction and selectivity to the target-cells, improve the permeability of cell walls and enhance bioavailability (47, 48).

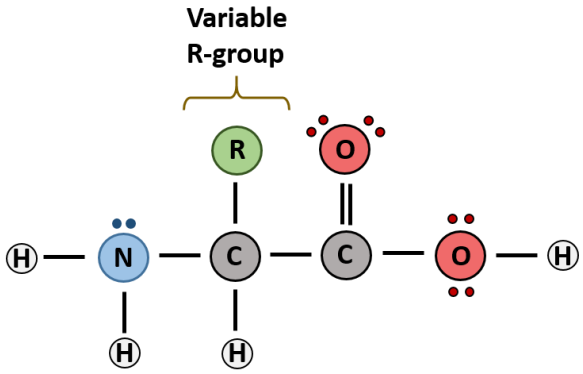


Figure 9 - Basic structure of an amino acid.

Several approaches can be followed to synthesize amino acid derivatives, as for instance, the method of activated esters, which is vastly used in peptide chemistry and has been used before to successfully synthesize amino acid derivatives of chrysin (48-51).

Nevertheless, no studies have been reported concerning the role of chirality in the biological and pharmacological properties of these synthetic chiral derivatives. Thus, this project aims the synthesis and structure elucidation of new promising bioactive amino acid derivatives of chrysin in their enantiomeric pure form for evaluation of anti-tumor activity and the influence of the stereochemistry.

2. AIMS

The principal aims of this project were:

- Synthesis of a suitable building block of chrysin for further coupling reaction with amino esters;

- Synthesis of new chiral amino ester derivatives of chrysin by coupling reaction with commercially available enantiomerically pure amino esters;

- Synthesis of new chiral amino acid analogues by hydrolysis of the ester groups;

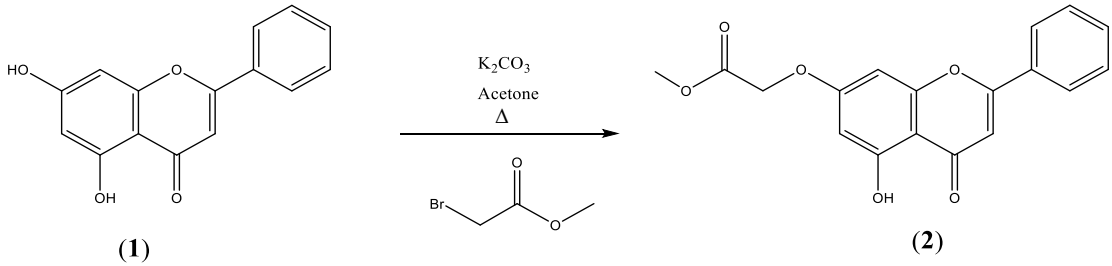
- Structure elucidation of all the obtained compounds by spectroscopic methods such as ^{1}H NMR and ^{13}C NMR;

The enantiomeric purity evaluation and anti-tumoral activity of the synthesized chiral derivatives of chrysin will be further evaluated.

3. RESULTS AND DISCUSSION

3.1. Synthesis of a building block of chrysin

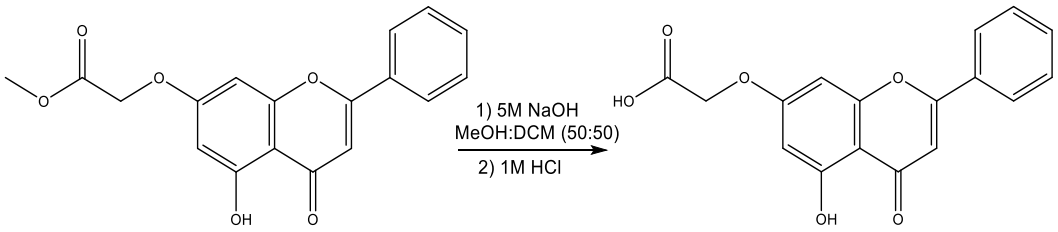
In order to react with the amine group of an amino ester, a functional group such as a carboxylic acid group was necessary to introduce in the structure of chrysin (**1**). Firstly, a reaction was performed at 60 °C under reflux, in nitrogen atmosphere and K_2CO_3 to provide an alkaline medium. K_2CO_3 removed the proton of the phenol group and allowed the reaction with methyl bromoacetate resulting in the formation of methyl 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetate, an ester derivative of chrysin (**2**) (**Figure 10**).



(**1**) (**2**)

Figure 10 - Synthesis of methyl 2-((5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)acetate, an ester of chrysin (**2**).

Secondly, to obtain the desired carboxylic acid group, an alkaline hydrolysis reaction was performed in methanol (MeOH):dicloromethane (DCM) (50:50 *v/v*) in the presence sodium hydroxide (5M NaOH) and further regeneration with hydrochloric acid (1M HCl) resulting in the formation of 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetic acid (**3**) (**Figure 11**).



(**2**) (**3**)

Figure 11 - Synthesis of 2-((5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)acetic acid (**3**).

The mechanism of the hydrolysis can be found in **Figure 12**. The hydroxide ion present in NaOH acts as a nucleophile attacking the carbonyl of the carboxylic acid group in

a nucleophilic acyl substitution reaction. Then the newly formed carboxylate anion is protonated by HCl.

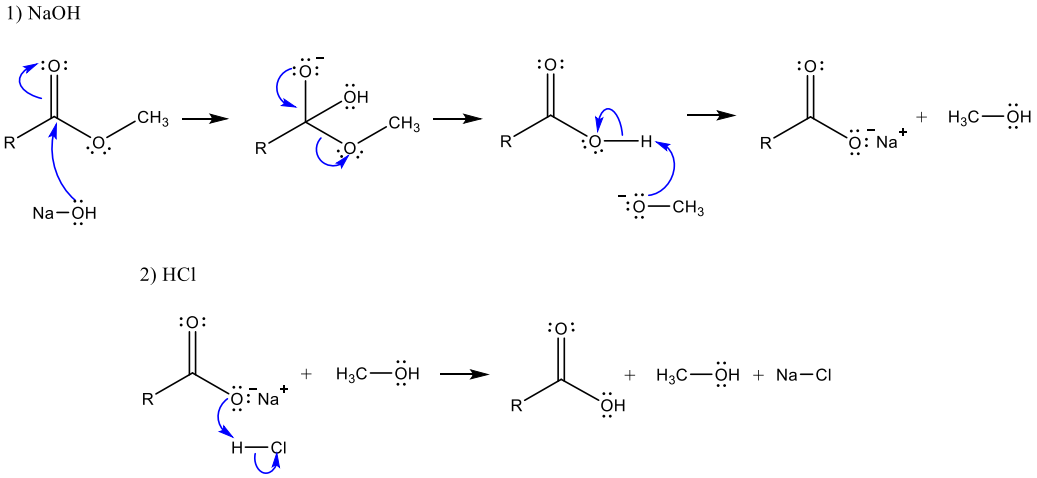
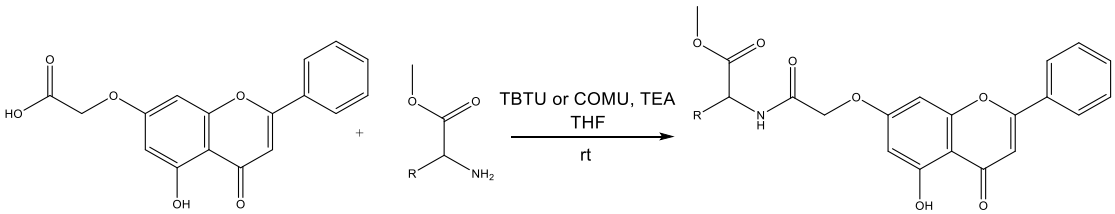


Figure 12 - Mechanism of alkaline hydrolysis.

3.2. Synthesis of the chiral amino esters derivatives of chrysin (4-7)

Four new chiral amino acid derivatives of chrysin (**4-7**) were obtained by coupling reaction of 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetic acid (**3**) with both commercially available enantiomerically pure amino esters of tryptophan (**8** and **9**) and tyrosine (**10** and **11**) (**Figure 13)**.

The reactions were performed at room temperature in tetrahydrofuran (THF) with compound **3**, the selected amino ester, *O*-(benzotriazol-1-yl-)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate (TBTU) or (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU), as coupling reagents, and a catalytic amount of triethylamine (TEA).



(**3**) (**8**) and (**9**) R = $CH_2C_8H_5N$ (**4**) and (**5**) R = $CH_2C_8H_5N$

(**10**) and (**11**) R = CH_2PhOH (**6**) and (**7**) R = CH_2PhOH

Figure 13 – Synthesis of chiral amino esters derivatives of chrysin (**4-7**) by coupling reaction.

COMU contains in its structure (**Figure 14**) an excellent leaving group (oxyma) and a very reactive dimethylmorpholino core. Additionally, it presents advantages in comparison

with other typical coupling reagents: it is non-explosive since there is no benzotriazole moiety that is present in many of this type of reagents; it is suitable for solution phase and solid phase peptide synthesis; it presents high solubility and stability in commonly used solvents and its by-products and easily removed by water (52).

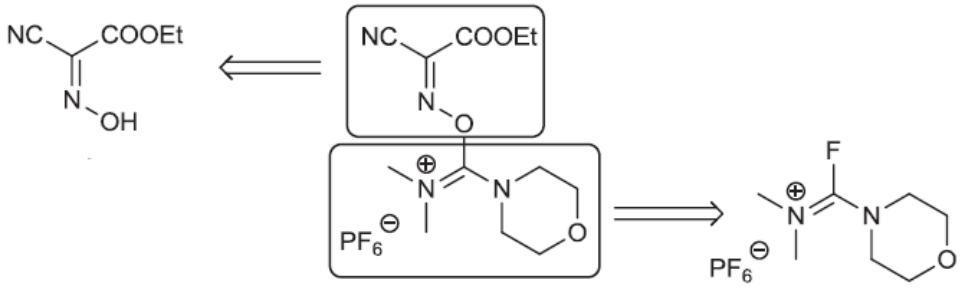


Figure 14 – COMU's structure.

TBTU is an uronium salt with two tautomeric forms (**Figure 15**). Like COMU, it is also widely used due to its many advantages: TBTU conducts reactions with good yields; it presents stability and long shelf-time solubility in typical organic solvents; the coupling reaction results in the generation of HOBt which is a water-soluble by-product; and the by-products present low toxicity (53).

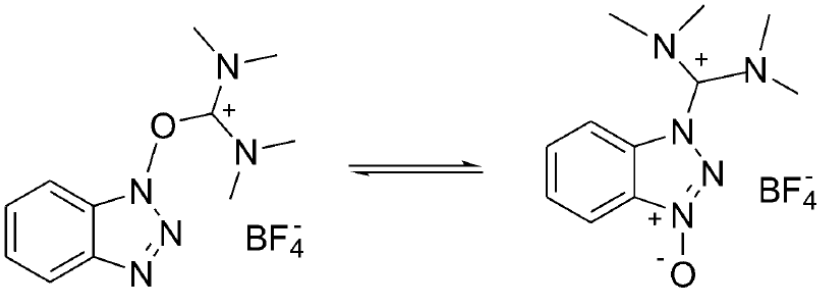


Figure 15 – Tautomeric forms of TBTU.

The proposed mechanisms of the coupling reaction of COMU and TBTU are present in **Figure 16** and **Figure 17**, respectively. The mechanisms of both coupling reagents are similar. TEA is a base used to deprotonate the carboxylic acid. The resulting carboxylate anion attacks the coupling reagent, which results in an activated carboxylic acid derived intermediate and an anion. The anion reacts with the activated carboxylic acid derived intermediate to form an activated ester. Finally, the amine of the amino ester reacts with the activated ester to form the amide bound.

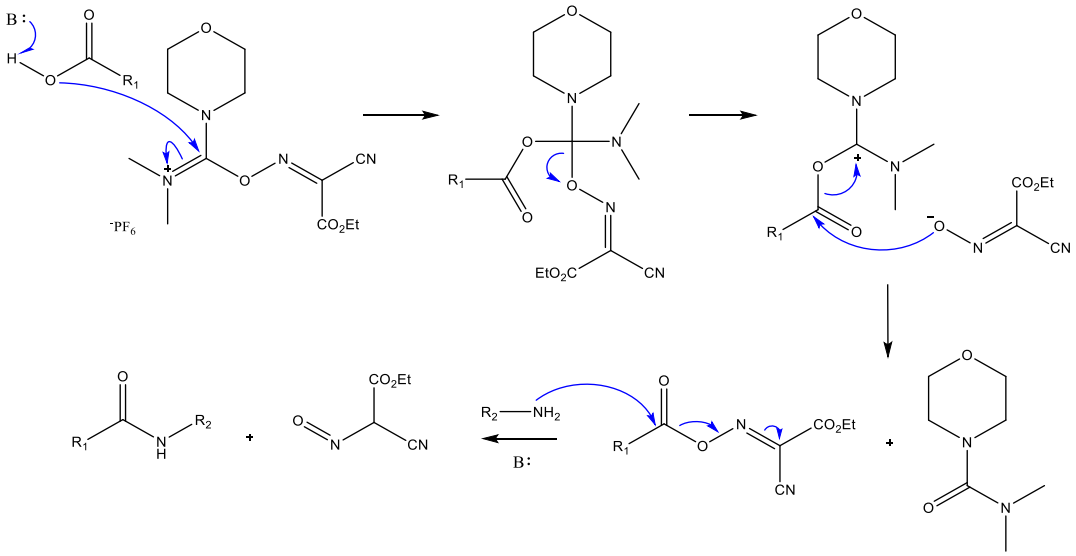


Figure 16 – Mechanism of COMU coupling reaction (54).

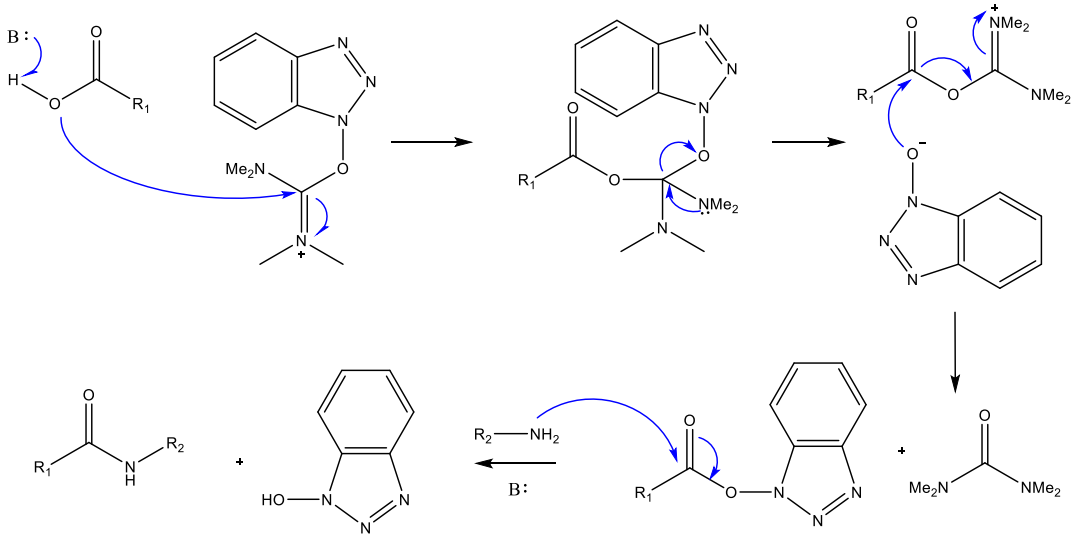


Figure 17 – Mechanism of TBTU coupling reaction.

The coupling reactions were performed with both pure enantiomers of amino esters of tryptophan and tyrosine resulting in four chiral derivatives of chrysin (**Table 1**).

All the reactions were followed via TLC with appropriate mobile phases. Most reactions took around 3 hours. For the reactions that showed slightly impure products, recrystallization was attempted.

3.3. Synthesis of the chiral amino acid derivatives of chrysin (12-15)

A portion of each of the four compounds **4-7** was afterwards used for a hydrolysis reaction of the ester groups (**Figure 18**). The hydrolysis was performed with a low

concentration of sodium hydroxide (0.25M) to prevent any possible racemization. These reactions took around 24 hours to finish.

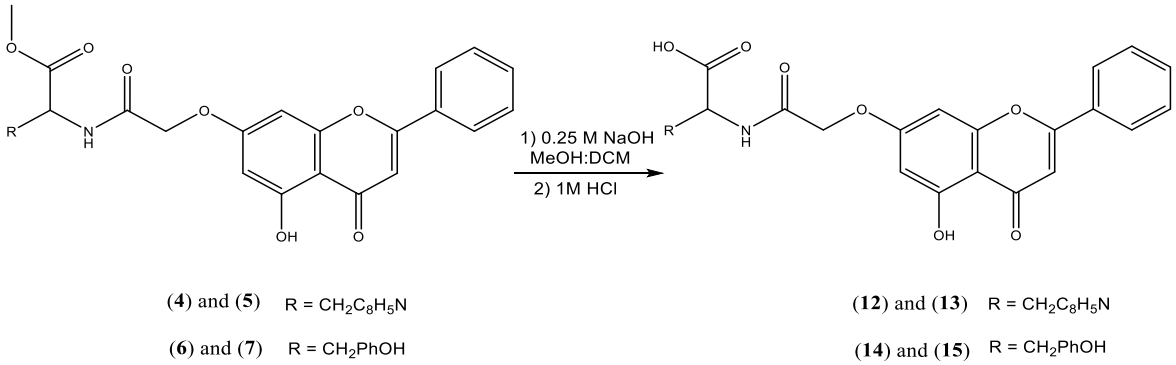
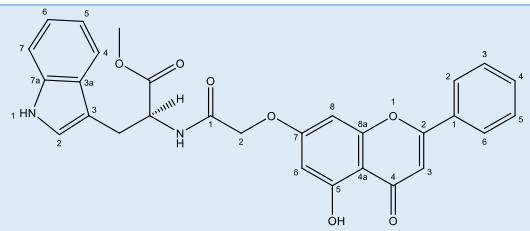
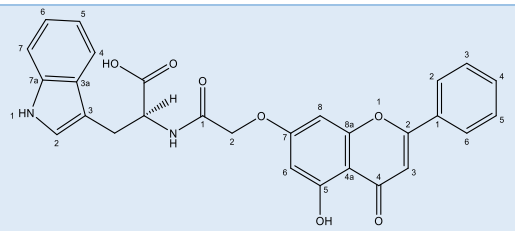
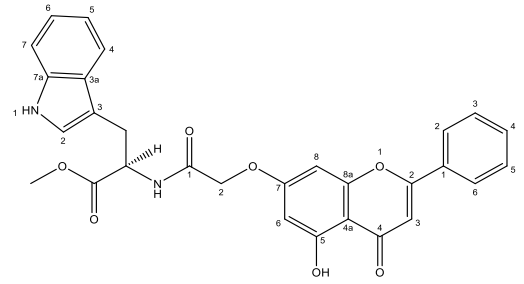
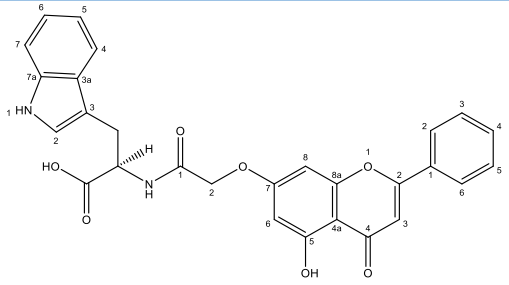
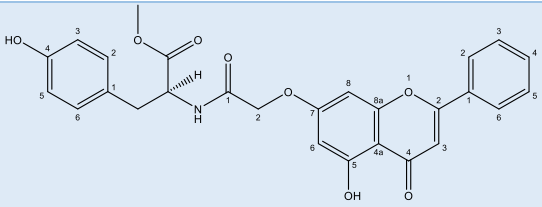
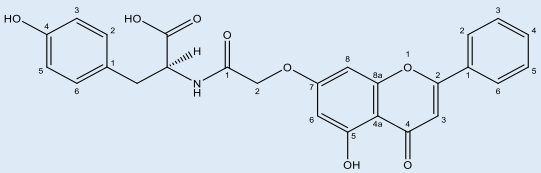
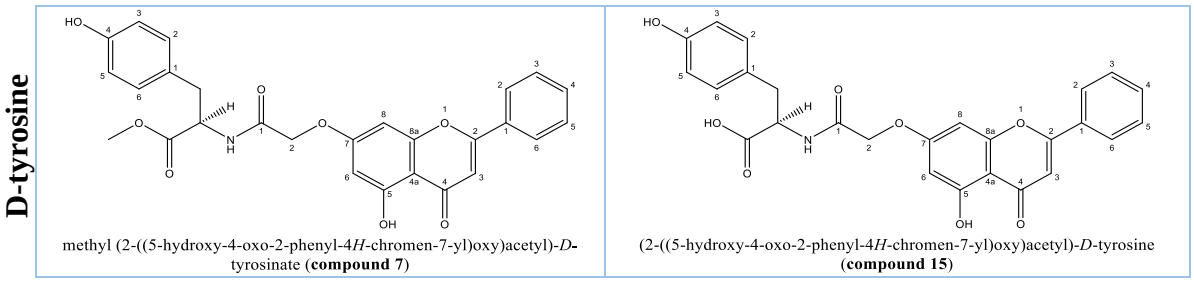


Figure 18 - Synthesis of chiral amino acid derivatives of chrysin (**12-15**) by hydrolysis of the ester groups.

From this reaction, other four chiral derivatives of chrysin were also obtained. The structures and names of the eight obtained compounds are found in **Table 1**.

Table 1 - Structure of the eight chiral derivatives of chrysin synthesized (**4-7** and **12-15**).

	Amino ester derivatives	**Amino acid derivatives**
L-tryptophan	 methyl (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tryptophanate (**compound 4**)	 (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tryptophan (**compound 12**)
D-tryptophan	 methyl (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*D*-tryptophanate (**compound 5**)	 (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*D*-tryptophan (**compound 13**)
L-tyrosine	 methyl (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tyrosinate (**compound 6**)	 (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tyrosine (**compound 14**)



3.4. Structure Elucidation

3.4.1. NMR analysis

The ^{1}H-NMR spectrum obtained for methyl 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetate (**2**) (**Figure 19**) shows chemical shifts expected for a derivative of chrysin. Signals at 6.69 ppm (s, 1H), 5.93 ppm (s, 1H) and 6.22 ppm (s, 1H) are typical of protons at C-3, C-6 and C-8 (**Figure 6**) of a flavone skeleton. Furthermore, the chemical shifts at 8.00 ppm (dd, 2H) and 7.56 ppm (m, 3H) suggest that there is no substitution in the B ring as expected for chrysin. Signals at 4.81 ppm (s, 2H) and 3.71 ppm (s, 3H) confirm the formation of chrysin's ester via methyl bromoacetate. No signal for the C-5 hydroxyl was detected which can happen due to an exchange of the proton of the hydroxyl with the deuterium of the used solvent causing the signal to shrink or disappear. This can also happen to N-H protons. No ^{13}C-NMR spectrum was obtained for this compound.

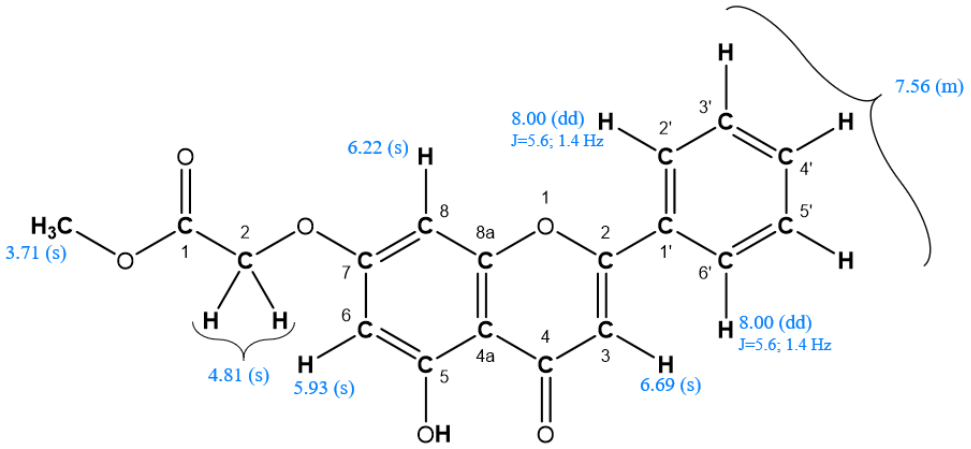


Figure 19 - ^{1}H-NMR spectral data of methyl 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetate (**2**).

The ^{1}H-NMR spectrum obtained for 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetic acid (**3**) (**Figure 20**) shows similar signals for the chrysin skeleton as obtained in the previous spectrum. Signals at 6.36 ppm (d, 1H) and 6.73 ppm (d, 1H) of protons at C-

6 and C-8 present the same coupling constant of 2.4 Hz which shows coupling between these two protons. Additionally, the signal at 3.71 (s, 3H) found in the spectrum of methyl 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetate (**2**) is absent in this spectrum confirming the hydrolysis of the ester group of the starting material (**2**). Again, no signals were obtained for both hydroxyls of the compound. The ^{13}C-NMR spectrum shows the expected signals for all the carbons in 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetic acid confirming its structure.

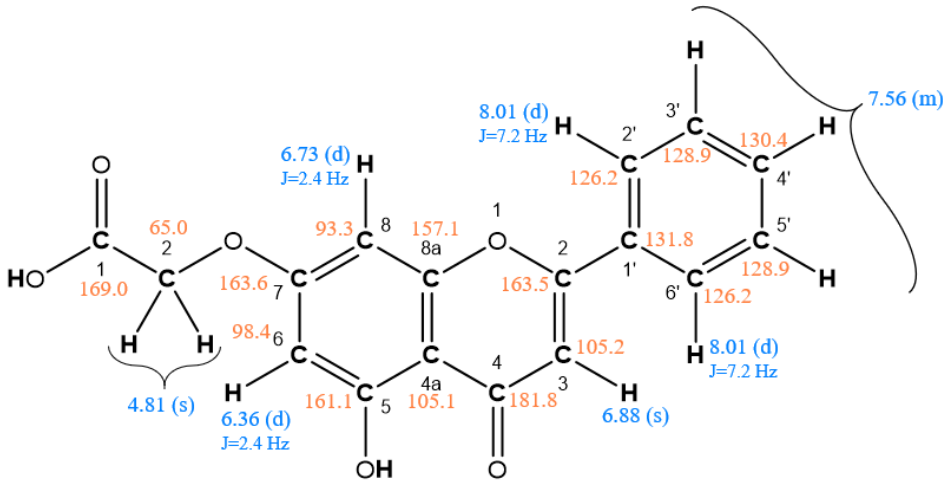


Figure 20 - ^{1}H-NMR and ^{13}C-NMR spectral data of 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetic acid (**3**).

The ^{1}H-NMR and ^{13}C-NMR data of the products of the coupling reactions were only obtained for the *L*-enantiomer since both enantiomers of a molecule present similar spectral data.

The new signals found in the ^{1}H-NMR and ^{13}C-NMR spectra of methyl (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tryptophanate (**4**) (**Figure 21**) and of methyl (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tyrosinate (**6**) (**Figure 22**) prove the success of the coupling reactions with amino esters.

For tryptophan moiety (indole group), signals at 7.51 ppm (dd, 1H), 7.33 ppm (dd, 1H), 7.06 ppm (ddd, 1H) and 7.01 ppm (ddd, 1H) are typical for the protons of the six-membered benzene and 7.18 ppm (s, 1H) and 10.89 ppm (s, 1H) for the protons of the C-2 and of the -NH, respectively, of the five-membered pyrrole ring. The coupling constants calculated for the benzylic protons confirm coupling. In this case, the ^{1}H-NMR spectrum shows signals for the protons of -OH at 12.81 ppm (s, 1H) and -NH at 8.50 ppm (d, 1H).

Additionally, it is important to refer the signal at 2.74 ppm (s, 3H) for the protons and 52.0 ppm for the carbon of the -OCH_3 group.

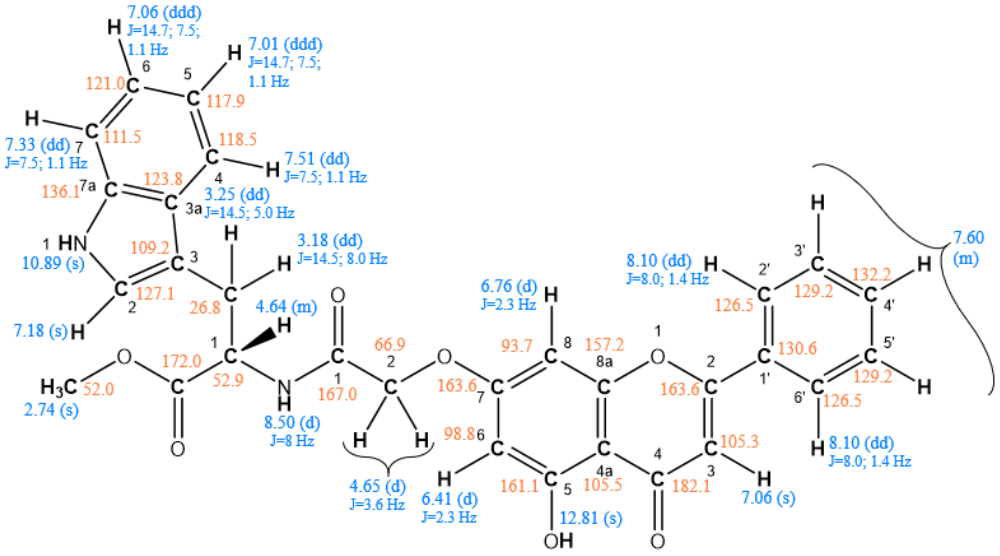


Figure 21 - ^{1}H-NMR and ^{13}C-NMR spectral data of methyl (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tryptophanate (**4**).

For tyrosine moiety (phenol group), signals at 6.63 ppm (d, 2H) and 7.00 ppm (d, 2H) for the aromatic hydrogens and at 9.23 ppm (s, 1H) for the -OH were found. The coupling constants calculated for these protons confirms coupling. In this ^{1}H-NMR spectrum, signals for the protons of -OH at 12.83 ppm (s, 1H) and -NH at 8.54 ppm (d, 1H) were also found. Again, it is important to refer the signal at 3.62 ppm (s, 3H) for the protons and 52.0 ppm for the carbon of the -OCH_3 group.

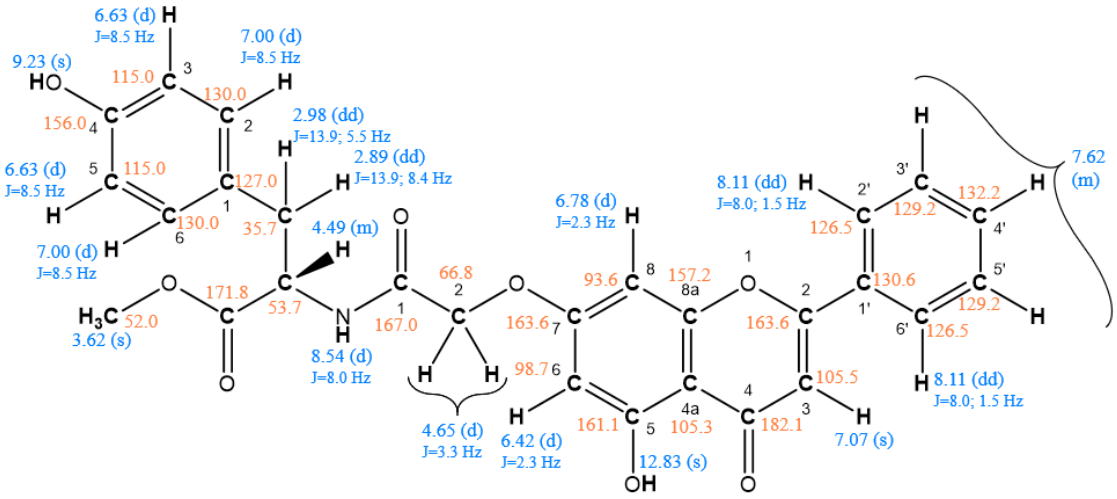


Figure 22 - ^{1}H-NMR and ^{13}C-NMR spectral data of methyl (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tyrosinate (**6**).

Finally, for the products of hydrolisys, (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tryptophan (**12**) (**Figure 24**) and (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tyrosine (**14**) (**15**) (**Figura 25**) it is important to refer the absence of the signals of the methyl group at 2.74 (s, 3Hs) in the ^{1}H-NMR spectrum and 52.0 in the ^{13}C-NMR spectrum of **Figure 21** in **Figure 23** and at 3.62 (s, 3H) in the ^{1}H-NMR spectrum and 52.0 in the ^{13}C-NMR spectrum of **Figure 22** in **Figure 24** proving the success of the hydrolysis of the methyl ester. However, no signals for the hydrogen of the new -OH group were found in both cases.

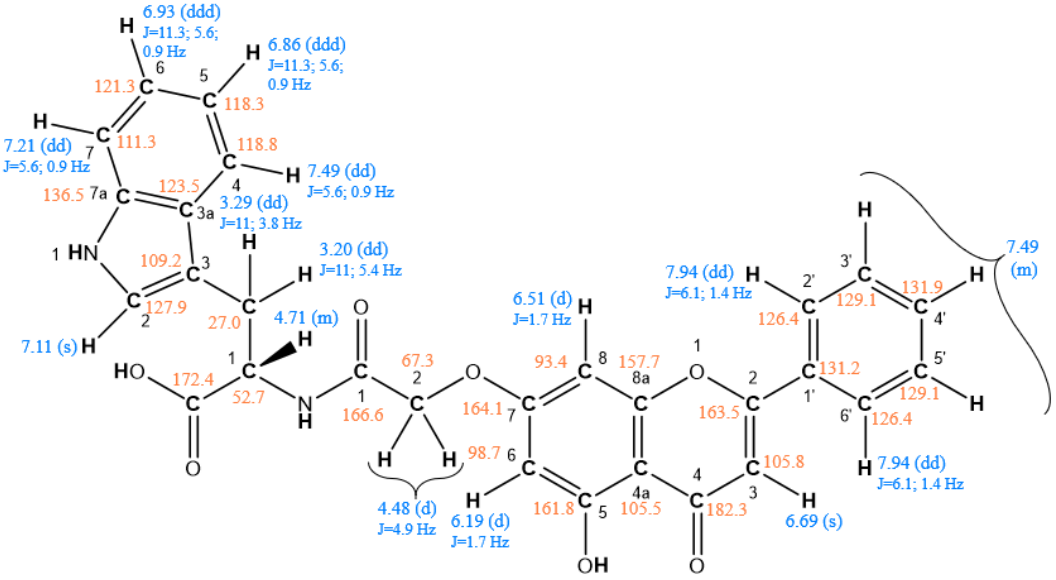


Figure 23 - ^{1}H-NMR and ^{13}C-NMR spectral data of (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tryptophan (**12**).

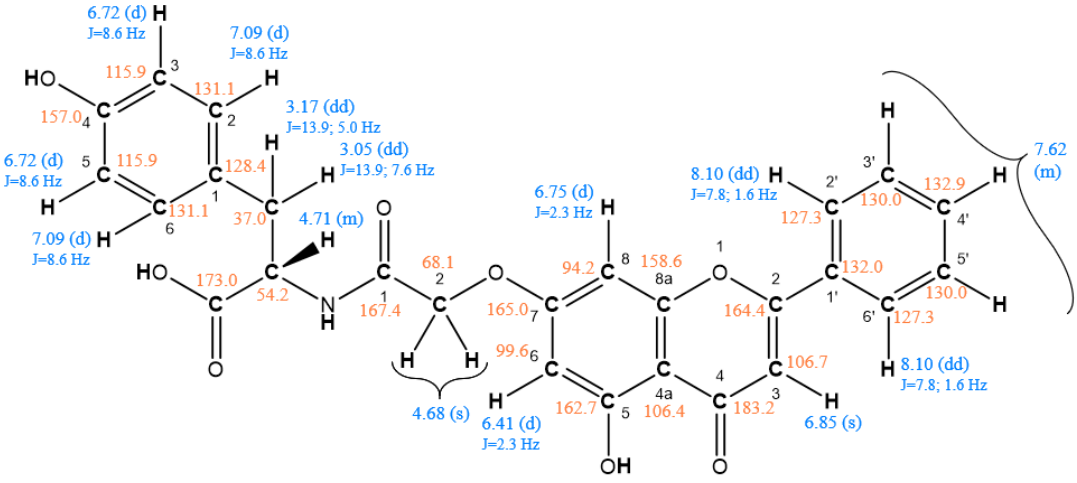


Figure 24 - ^{1}H-NMR and ^{13}C-NMR spectral data of (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tyrosine (**14**).

4. EXPERIMENTAL

4.1. General methods

The reagents and solvents were purchased from Sigma-Aldrich and used without purification.

Solvent evaporation was performed in a rotary evaporator under reduced pressure.

Reactions were controlled by thin-layer chromatography (TLC) (Merck Silica gel 60 F254 plates) with appropriate mobile phases and revealed by UV light at 245nm and 365 nm.

^{1}H-NMR and ^{13}C-NMR were performed on a Brücker Avance 300 instrument (300.13 MHz for ^{1}H-NMR and 75.47 MHz for ^{13}C-NMR) with DMSO-d6 or acetone-d6 as solvents, in the Department of Chemistry of the University of Aveiro.

4.2. Synthesis of chiral derivatives of chrysin

4.2.1. Synthesis of methyl 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetate (2)

Chrysin (**1**) (2.00 g, 7.87 mmol) was dissolved in acetone (120 mL) and treated with potassium carbonate (1.57 g). After 1h of vigorous stirring at 60 °C under reflux and nitrogen atmosphere, methyl bromoacetate (1 mL) was added with a syringe. When the reaction was finished, the mixture was cooled in an ice water bath and the precipitate was removed by filtration under reduced pressure. To recover leftover product in the filtrate, the solvent was evaporated, water was added, and the product was extracted with dichloromethane. The recovered product was recrystallized from DCM and hexane to afford methyl 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetate (**2**), as a light-yellow solid (1.99g, 77%). ^{1}H-NMR (300.13 MHz, DMSO-d6): **Figure 19**.

4.2.2. Synthesis of 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetic acid (3)

Compound **2** was dissolved in 500 mL of DCM:MeOH (50:50 *v/v*) and treated with 5M NaOH (11.5 mL) while stirred vigorously. After standing overnight the solvent was evaporated and the compound was dissolved in water, treated with 1M HCl to adjust pH to

1-2 and left overnight in an ice water bath. The precipitate was removed under reduced pressure to afford 2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetic acid (**3**) as a brownish solid (1.12 g, 61%). ^{1}H-NMR (300.13 MHz, DMSO-d6): **Figure 20**; ^{13}C-NMR (75.47 MHz, DMSO-d6): **Figure 20**.

4.2.3. Coupling reaction to obtain compounds 4-7

The starting material (**3**) (0.10 g) was dissolved in THF (30 mL) and treated with triethylamine (0.1 mL, 0.72 mmol). The coupling reagent (COMU or TBTU - 1.2 e.q.) was added and after stirred vigorously at room temperature for 30 minutes, the mixture was treated with a chiral amino acid methyl ester (**8-11**) (1.7 e.q.). When the reaction was finished (most reactions took around 3 hours to finish) the solvent was evaporated and the resulting compound was dissolved in DCM and washed with 1M HCl, saturated $NaHCO_3$ and water. The organic layer was dried with anhydrous sodium sulphate, filtered and the solvent evaporated. The obtained product was recrystallized from DCM and hexane to afford compounds **4-7**. Some of the reactions were also repeated in an anhydrous environment achieved using anhydrous THF.

Methyl (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tryptophanate (4)

Reaction time: 2h. Yield: not determined, yellow solid. ^{1}H-NMR (300.13 MHz, DMSO-d6): **Figure 21**; ^{13}C-NMR (75.47 MHz, DMSO-d6): **Figure 21**.

Methyl (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*D*-tryptophanate (5)

Reaction time: 3h. Yield: not determined, yellow solid. ^{1}H-NMR (300.13 MHz, DMSO-d6): **Figure 21**; ^{13}C-NMR (75.47 MHz, DMSO-d6): **Figure 21**.

Methyl (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tyrosinate (6)

Reaction time: 3h. Yield: not determined, yellow solid. ^{1}H-NMR (300.13 MHz, DMSO-d6): **Figure 22**; ^{13}C-NMR (75.47 MHz, DMSO-d6): **Figure 22**.

Methyl (2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*D*-tyrosinate (7)

Reaction time: 3h. Yield: not determined, yellow solid. ^{1}H-NMR (300.13 MHz, DMSO-d6): **Figure 22**; ^{13}C-NMR (75.47 MHz, DMSO-d6): **Figure 22**.

4.2.4. Hydrolysis to obtain compounds 12-15

The starting material (**4-7**) (0.05 g) was dissolved in a mixture of MeOH and DCM and treated with 0.25 M NaOH (3.75 mL). The mixture was kept at room temperature while vigorously stirring. After the reaction was finished, the solvent was evaporated and the compound was dissolved in water, acidified with 1M HCl and left in an ice water bath. The precipitate was removed by filtration under reduced pressure. Leftover product in the filtrate was extracted with ethyl acetate. The obtained product was recrystallized from methanol and water to afford compounds **12-15**.

(2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tryptophan (12)
Reaction time: 28.5h. Yield: not determined, light yellow solid. ^{1}H-NMR (300.13 MHz, Acetone-d6): **Figure 23**; ^{13}C-NMR (75.47 MHz, acetone-d6): **Figure 23**.

(2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*D*-tryptophan (13)
Reaction time: 16.5h. Yield: not determined, light yellow solid. ^{1}H-NMR (300.13 MHz, Acetone-d6): **Figure 23**; ^{13}C-NMR (75.47 MHz, acetone-d6): **Figure 23**.

(2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*L*-tyrosine (14)
Reaction time: 26h. Yield: not determined, light brown solid. ^{1}H-NMR (300.13 MHz, Acetone-d6): **Figure 24**; ^{13}C-NMR (75.47 MHz, acetone-d6): **Figure 24**.

(2-((5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yl)oxy)acetyl)-*D*-tyrosine (15)
Reaction time: 23h. Yield: not determined, light brown solid. ^{1}H-NMR (300.13 MHz, Acetone-d6): **Figure 24**; ^{13}C-NMR (75.47 MHz, acetone-d6): **Figure 24**.

5. CONCLUSIONS AND FUTURE WORK

As a result of this work, eight new chiral derivatives of chrysin were successfully synthesized by coupling reactions with commercially available enantiomerically pure amino esters of tryptophan and tyrosine and further hydrolysis of the ester groups.

The structure elucidation was achieved by spectroscopic methods: ^{1}H-NMR and ^{13}C-NMR. However, to achieve a complete structure elucidation it is important to perform IR (infrared) spectroscopy and HRMS (high resolution mass spectrometry). The determination of the melting point is also important, not only because it is a characteristic value for compounds but also because it can be used to evaluate their purity. Since the synthetized compounds are chiral, the optical activity will also be determined.

Furthermore, to determinate whether racemization happened during the synthesis, the enantiomeric purity of the new compounds will be evaluated by liquid chromatography using chiral stationary phases.

The compounds will, then, be tested for their biological activity focusing in anti-tumoral activity and other enantioselective studies will also be performed.

In a future work, the synthesis of chiral amino acid derivatives of chrysin could also be attempted with a different building block of chrysin synthetized using a reagent with a longer carbon chain than methyl bromoacetate, for instance, methyl-4-bromobutyrate.

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