IPAP18 Innovations in Pharmacy ADVANCES & PERSPECTIVES

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Cinnamic acid derivatives: a contribution for the study of cyclooxygenase inhibition

João JANELA
Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal
jlsjanela@gmail.com

AN INTRODUCTION TO CYCLOOXYGENASE (COX)

General perspective

- Key enzime in the biosynthesis of prostanoids (prostaglandins, prostacyclin and thromboxane)
- Main co-substrates: arachidonic acid and O_2
- Inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs)
- A second isoform of COX was discovered in the early 1990s, leading to the distinction between COX-1 and COX-2

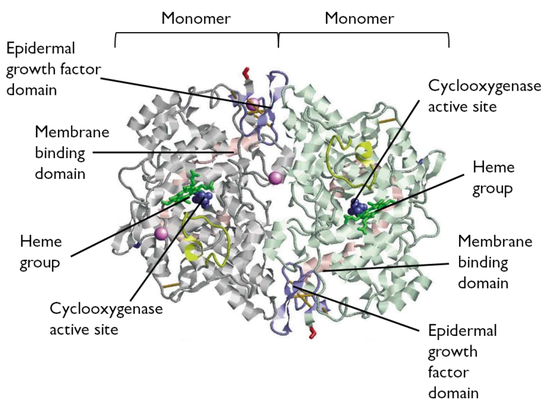

Figure 1: Structure model of COX-1. Adapted from Kulmacz *et al.*

Structural features

- Homodimeric enzyme
- Three domains per monomer
- Two distinct active sites per monomer: a cyclooxygenase and a peroxidase
- Each monomer contains a ferric heme group and a few oligosaccharides

Catalytic function

- Oxidation of the ferric heme group, catalyzed by the peroxidase active site, leading to the activation of a tyrosine residue in the cyclooxygenase active center
- Conversion of arachidonic acid into prostaglandin G_2 (PGG_2) in the cyclooxygenase active center, through a mechanism involving radical intermediates
- Conversion of PGG_2 into prostaglandin H_2 (PGH_2) in the peroxidase active center

COX isoforms and their functions

COX-1 expression is constitutive. This isoform leads to the biosynthesis of prostanoids with homeostatic functions in many organs and tissues.
COX-2 expression is mostly induced by inflammatory and proliferative stimuli. This isoform is responsible for the biosynthesis of prostanoids with physiopathological functions related with inflammation:
- PGD_2 (prostaglandin D_2), PGE_2 (prostaglandin E_2) and PGI_2 (prostacyclin) induce vasodilation;
- PGE_2 increases vascular permeability and the release of pro-inflammatory cytokines, besides being fever-inducing;
- PGD_2 attracts leukocytes to the inflammation site;
- PGE_2 and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulate pain.

COX-2 is also constitutively expressed in some organs and tissues, without inflammation, such as the kidneys, the brain and the gastric mucosa.

Nonsteroidal annti-inflammatory drugs (NSAIDs)

NSAIDs are drugs which inhibit COX and they can be divided in two subgroups: first generation NSAIDs and coxibs.
First generation NSAIDs were discovered before the existence of a second COX isoform was known. They inhibit both COX-1 and COX-2, with each drug having a specific selectivity. Most of these NSAIDS exhibit a relative selectivity towards COX-1. These drugs are associated with many adverse effects resulting from the inhibiton of homeostatic processes related mostly with COX-1, but also with COX-2.
Coxibs were specifically developed to selectively inhibit COX-2. These drugs managed to decrease the frequency of some adverse effects associated with first generation NSAIDs, but they have been shown to increase cardiovascular risk.

CINNAMIC ACID DERIVATIVES AS MOLECULES WITH PHARMACOLOGICAL ACTIVITY

Cinnamic acid hexylamides with antioxidant activity

A 2010 study by Roleira *et al.* focused on the study of antioxidant activity of ferulic acid (4-hydroxy-3-methoxycinnamic acid) and caffeic acid (3,4-dihydroxycinnamic acid), as well as their respective hydrocinnamic acids and their corresponding hexylamides. Activity studies were based on liposome membrane lipid peroxidation. Some of the main conclusions were that:
- a cinnamic acid derivative is more active than its hydrocinnamic counterpart;
- a cinnamic acid hexylamide is more active than its respective cinnamic acid;
- a caffeic acid derivative is more active than its ferulic acid counterpart, which means that swapping a 3-methoxyl for a 3-hydroxyl group increases activity.

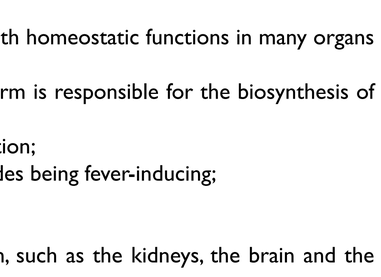

Figure 2: General strucutre of cinnamic acids.

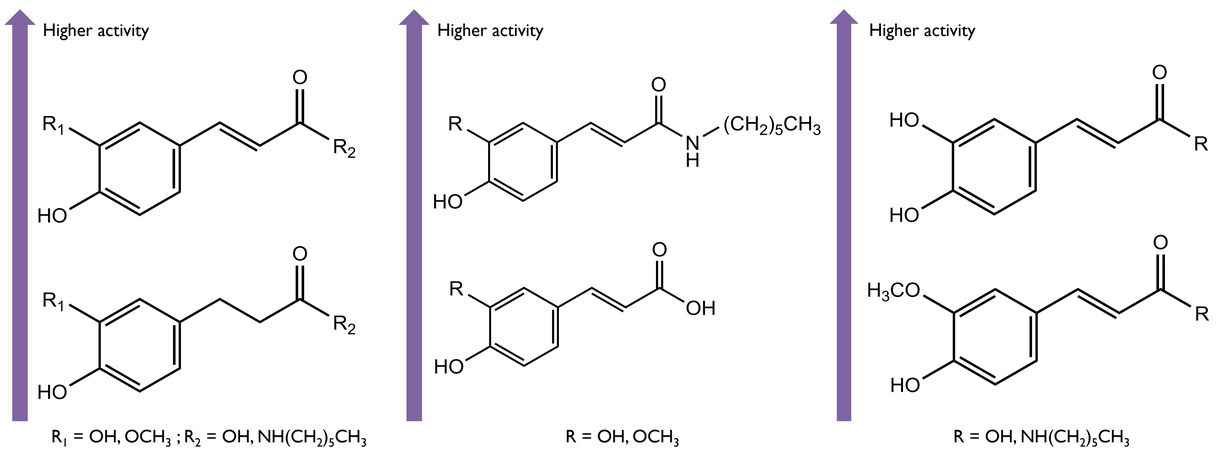

Figure 3: Structure-activity relationships of cinnamic acids and their derivatives concerning their antioxidant activity.

Cinnamic acid hexylamides with antitumoral activity

A 2016 study by Tavares da Silva *et al.* focused on the study of antitumoral activity of ferulic acid (4-hydroxy-3-methoxycinnamic acid) and caffeic acid (3,4-dihydroxycinnamic acid), as well as their respective hexylamides. Activity studies were based on neutrophil oxidative burst inhibition and anti-proliferative effect on two tumoral colorectal cell lines. Some of the main conclusions were that:
- a cinnamic acid hexylamide is more active than its respective cinnamic acid;
- a caffeic acid derivative is more active than its ferulic acid counterpart, which means that swapping a 3-methoxyl for a 3-hydroxyl group increases activity.

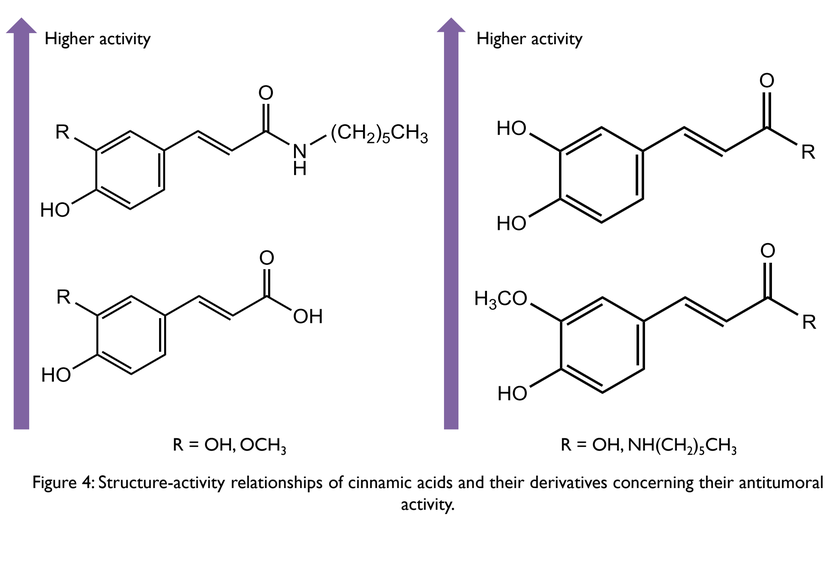

Figure 4: Structure-activity relationships of cinnamic acids and their derivatives concerning their antitumoral activity.

Cinnamic acid derivatives with COX-inhibiting activity

A 2015 study by Silva et al. with different cinnamic acids (p-cumaric, caffeic and 3,4,5-trihydroxycinnamic) and their respective ethyl esthers and ethyl diesthers revealed that those cinnamic acid derivatives are able to inhibit both COX-1 and COX-2 (with different selectivities) in an *in vitro* human whole blood assay.

SYNTHESIS, PURIFICATION AND CHARACTERIZATION OF CINNAMIC ACID HEXYLAMIDES WITH POTENTIAL COX-INHIBITING ACTIVITY

Theoretical grounds and compound selection

In the forementioned study of the antioxidant activity of cinnamic acid derivatives, the logarithm of the octanol-water partition coefficient (log P) was determined for the assessed compounds. It was revealed that cinnamic acid hexylamides had a higher log P than their respective cinnamic acids, due to the incorporation of a hexyl group in their structure. which led to a higher lipophilicity of those compounds. It was also noticed that the tested hexylamides fulfilled all the criteria of the Lipinski Rule, which means that they are theoretically predicted to be able to cross biological membranes and to reach their intracellular or membranal pharmacological targets. These factors were pointed out as the reasons for the higher activity of the hexylamides. The previously mentioned study on the antitumoral activity of cinnamic acid derivatives also refers the higher lipophilicity of hexylamides as the reason for their higher activty compared to their respective cinnamic acids. Therefore, it appears that the conversion of a cinnamic acid (or a derivative) into an hexylamide could be a way to preserve or increase its activity. The known evidence for cinnamic acid derivatives with COX-inhibiting activity suggests that there could be cinnamic acid hexylamides with this type of activity.
The laboratorial work of this project mainly consisted of the synthesis and COX-inhibiting activity assessment of the hexylamides of three different cinnamic acids: 3,4-dimethoxycinnamic acid, 3-hydroxy-4-methoxycinnamic acid and 3,4-(methylenedioxy)cinnamic acid.
Since the cinnamic acid unit of the hexylamides seems to be critical to the activity of such compounds, an attempt was made to synthesize an hexylamide-related compound with two cinnamic acid units (two amide functions). The chosen cinnamic acid to pursue this approach was 3,4-(methylenedioxy)cinnamic acid, since it has no reactive hydroxyl groups in the aromatic ring which could lead to secondary reactions.

Synthesis methodologies and reaction mechanisms

In order to synthesize an hexylamide, it must be considered that an amidation reaction involving a carboxylic acid is difficult to carry out. Therefore, an activated derivative of the reacting cinnamic acids was obtained, using a base (triethylamine) and BOP (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate). Such activated derivative is able to react with hexylamine through a nucleophilic acyl substitution reaction.

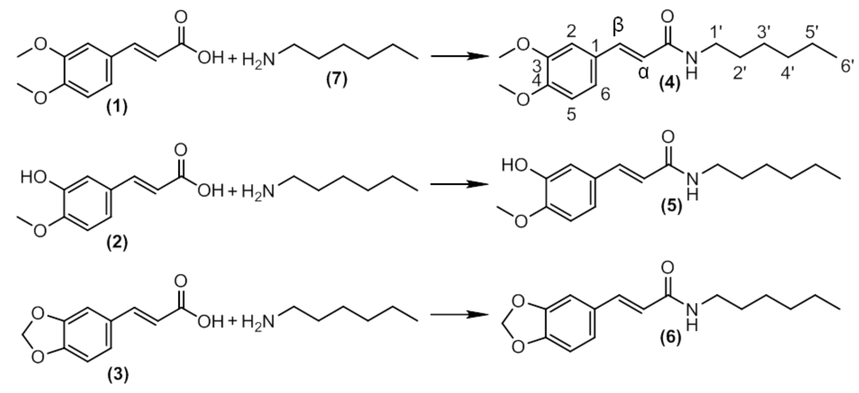

Figure 5: Performed reactions in order to synthesize cinnamic acid hexylamides.

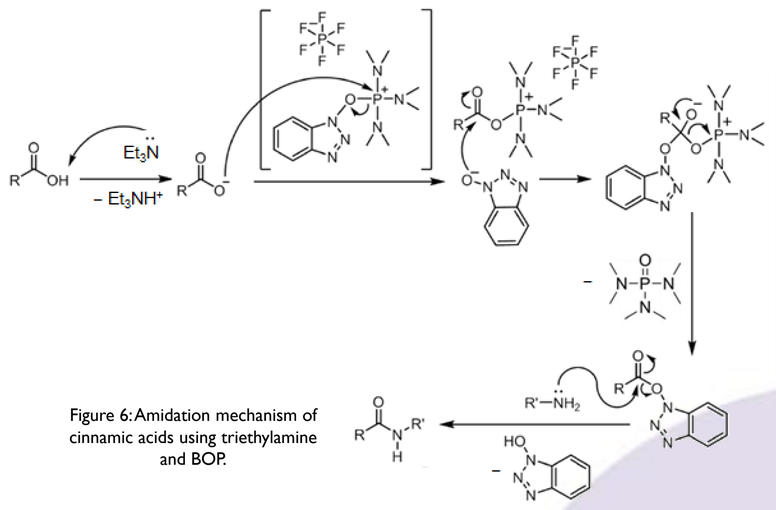

Figure 6: Amidation mechanism of cinnamic acids using triethylamine and BOP.

Table 1: Numerical identification of the compounds involved in the current work.

#	Compound name
1	3,4-dimethoxycinnamic acid
2	3-hydroxy-4-methoxycinnamic acid
3	3,4-(methylenedioxy)cinnamic acid
4	3,4-dimethoxycinnamic acid hexylamide
5	3-hydroxy-4-methoxycinnamic acid hexylamide
6	3,4-(methylenedioxy)cinnamic acid hexylamide
7	hexylamine
8	*N*-(3,4-(methylenedioxy)cinnamoyl)-*N'*-*tert*-butyloxycarbonyl-1,6-diaminohexane
9	*N*-(3,4-(methylenedioxy)cinnamoyl)-1,6-diaminohexane
10	*N,N'*-di(3,4-(methylenedioxy)cinnamoyl)-1,6-diaminohexane
11	*N*-*tert*-butyloxycarbonyl -1,6-diaminohexane

A previous attempt to synthesize a compound with two cinnamic acid units using a diamine revealed that such approach does not lead to the desired compound. As an alternative methodology, a hexane-1,6-diamine related compound in which one of the amine groups is protected by a BOC (*tert*-butyloxycarbonyl) group was used. A first reaction consists in the insertion of the first amide function into the intended compound. This reaction is nearly identical to an hexylamidation reaction, the main difference being that *N*-BOC-1,6-diaminohexane is used instead of hexylamine. A second reaction removes the BOC protector group using a methanol / HCl reagent (1:1). A third reaction consists in the insertion of the second amide function in the remaining amine group, in a similar way to the first reaction.

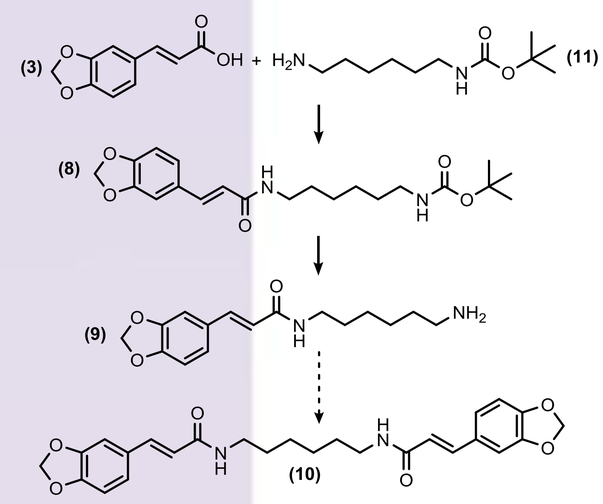

Figure 7: Synthesis methodology for a compound with two cinnamic acid units.

Laboratorial procedures

To synthesize the amides **4**, **5** and **6**, 500.0 mg of cinnamic acids **1**, **2** and **3**, respectively, were dissolved in dimethylformamide (DMF) and triethylamine (TEA). The solution was then cooled in an ice-water bath and hexylamine was added, followed by a solution of BOP in CH_2Cl_2. The mixture was stirred at 0 °C for 30 min and then at room temperature for specific periods of time. CH_2Cl_2 was removed under reduced pressure and the remaining solution was diluted with water (70 mL). The mixture was then extracted with ethyl acetate (2 x 70 mL). The extracts were washed with 1 N HCl (2 x 70 mL), water (70 mL), $NaHCO_3$ 5% (2 x 70 mL) and again with water (70 mL), dried over anhydrous $MgSO_4$, filtered and concentrated. The obtained residues were purified by recrystallization and/or column chromatography yielding the corresponding hexylamides **4**, **5** and **6**.

Table 2: Specific reaction conditions for each synthesized amide.

	Amide **4**	Amide **5**	Amide **6**	Amide **8***
V (DMF)	5.5 mL	6 mL	6 mL	3.5 mL
V (TEA)	0.34 mL	0.36 mL	0.37 mL	0.21 mL
V (hexylamine)	0.32 mL	0.34 mL	0.34 mL	0.33 mL**
m (BOP)	1.06 g	1.14 g	1.15 g	637.5 mg
V (CH_2Cl_2)	5 mL	6 mL	6 mL	4 mL
Reaction time at room temperature	4 h 45 min	5 h	5 h 30 min	4 h 30 min
Purification	Recrystallization in ethyl acetate	Column chromatography (hexane / ethyl acetate)	Recrystallization in ethyl acetate	Recrystallization in ethyl acetate and column chromatography (hexane / ethyl acetate)
Yield	76%	43%	66%	47%
Melting point	95-98 °C	102-103 °C	80-81 °C	129-130 °C

* 277.2 mg of compound 3 were used instead of 500.0 mg // ** *N*-BOC-1,6-diaminohexane was used instead of hexylamine

To obtain compound **9**, compound **8** (167.7 mg) was dissolved in 15 mL of methanol. The mixture was stirred at room temperature and 1 mL of a 1:1 mixture of HCl and methanol was added dropwise. The reaction took place for 24 h. Methanol was removed under reduced pressure and a solution of $NaHCO_3$ 10% was added dropwise, under stirring in an ice bath, until the pH of the mixture reached a value between 8 and 9 (60 mL of $NaHCO_3$ 10%). The mixture was extracted with ethyl acetate (3 x 70 mL) and washed with water (3 x 70 mL), dried over anhydrous Na_2SO_4, filtered and concentrated, giving 21.0 mg of compound **9** in 17% yield. Due to such a low yield, the final reaction to obtain compound **10** was not attempted.

ASSESSMENT OF COX-INHIBITING ACTIVITY OF THE SYNTHESIZED HEXYLAMIDES, RESULTS AND DISCUSSION

COX-inhibiting activity was assessed for amides **4**, **5** and **6** by an *in vitro* whole blood assay, sampled from healthy human volunteers after informed consent. Different assays were performed for COX-1 and COX-2. In each case, conversion of arachidonic acid into PGH_2 was activated and PGE_2 (derived from PGH_2) was quantified.

Table 3: Results of COX-inhibiting activity assays.

Compound	COX-1 inhibition (%)	COX-2 inhibition (%)	log P *
4	no activity (100 µM)	no activity (100 µM)	2.91
5	12.5 µM - 56±9 % 100 µM - 85±6 %	12.5 µM - 10±5 % 100 µM - 79±7 %	2.63
6	no activity (100 µM)	no activity (100 µM)	3.37

* Estimate of log P obtained by ChemDraw® (PerkinElmer, Inc.)

The most immediate finding is that compounds **4** and **6** are inactive in the inhibition of both COX isoforms, while compound **5** is the only active hexylamide among those assessed. Its maximum inhibiton rate, within the studied concentration range, is higher for COX-1 than for COX-2, which means that this compound exhibits a relative degree of selectivity towards COX-1.
All three hexylamides fulfill the criteria of the Lipinski Rule, being assumed that all of them were able to cross biological membranes, enter blood cells and reach both isoforms of COX, which are located intracellularly, bound to membranes. Therefore, the notorious differences among their COX-inhibiting activities are owed to their intrinsic ability to inhibit COX, which is determined by their structure.
The only structural differences among the compounds reside in their aromatic ring substituents, which suggests that these were responsible for their COX-inhibiting activity or lack of it. Since compounds **4** and **6** are inactive, it can be assumed that functional groups 3-methoxyl, 4-methoxyl and 3,4-methylenedioxy do not provide COX-inhibiting activity to a cinnamic acid hexylamide. However, a comparison between the structures of compounds **4** and **5** reveals that the only difference between them is that a 3-methyl group is replaced by a 3-hydroxyl. Since compound **5**, unlike compound **4**, is active in the inhibition of COX, it appears that a 3-hydroxyl group is highly important to provide or increase the COX-inhibiting activity of a cinnamic acid hexylamide, probably due to the possibility of establishing hydrogen bonds with a COX active site. This conclusion agrees with the findings of other studies about different activities of cinnamic acid hexylamides.

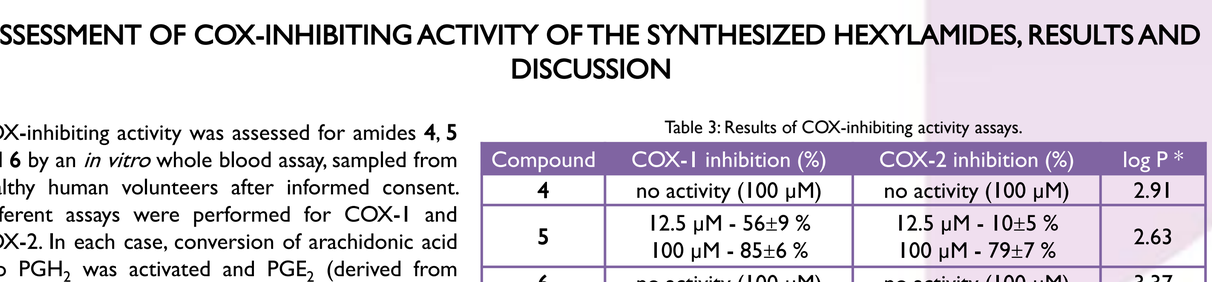

Figure 8: Structure-activity relationships of cinnamic acid hexylamides concerning their COX-inhibiting activity.

The main goal of the work described was to obtain cinnamic acid hexylamides with selectivity towards COX-2 (due to its role in inflammation), which was not accomplished. However, colaborators of this research project synthesized hexylamides of other cinnamic acids, some of which exhibited interesting activities in that aspect. In addition, no hexylamide compounds with two cinnamic acid units were assessed for their COX-inhibiting activities, due to the very low yield of the second reaction of the proposed synthesis methodology. If a new attempt to synthesize that kind of compounds is to be made, it is likely that changes to the used method should be considered.

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[a] UCIBIO/REQUIMTE, Laboratory of Applied Chemistry, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto
[b] Pharmaceutical Chemistry Laboratory, Faculty of Pharmacy, University of Coimbra
[c] CIEPQPF Centre for Chemical Processes Engineering and Forest Products, University of Coimbra

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