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Structural Insight into the Optimization of Ethyl 5-Hydroxybenzo[g]indol-3-carboxylates and their Bioisosteric Analogues as 5-LO/m-PGES-1 Dual Inhibitors Able to Suppress Inflammation

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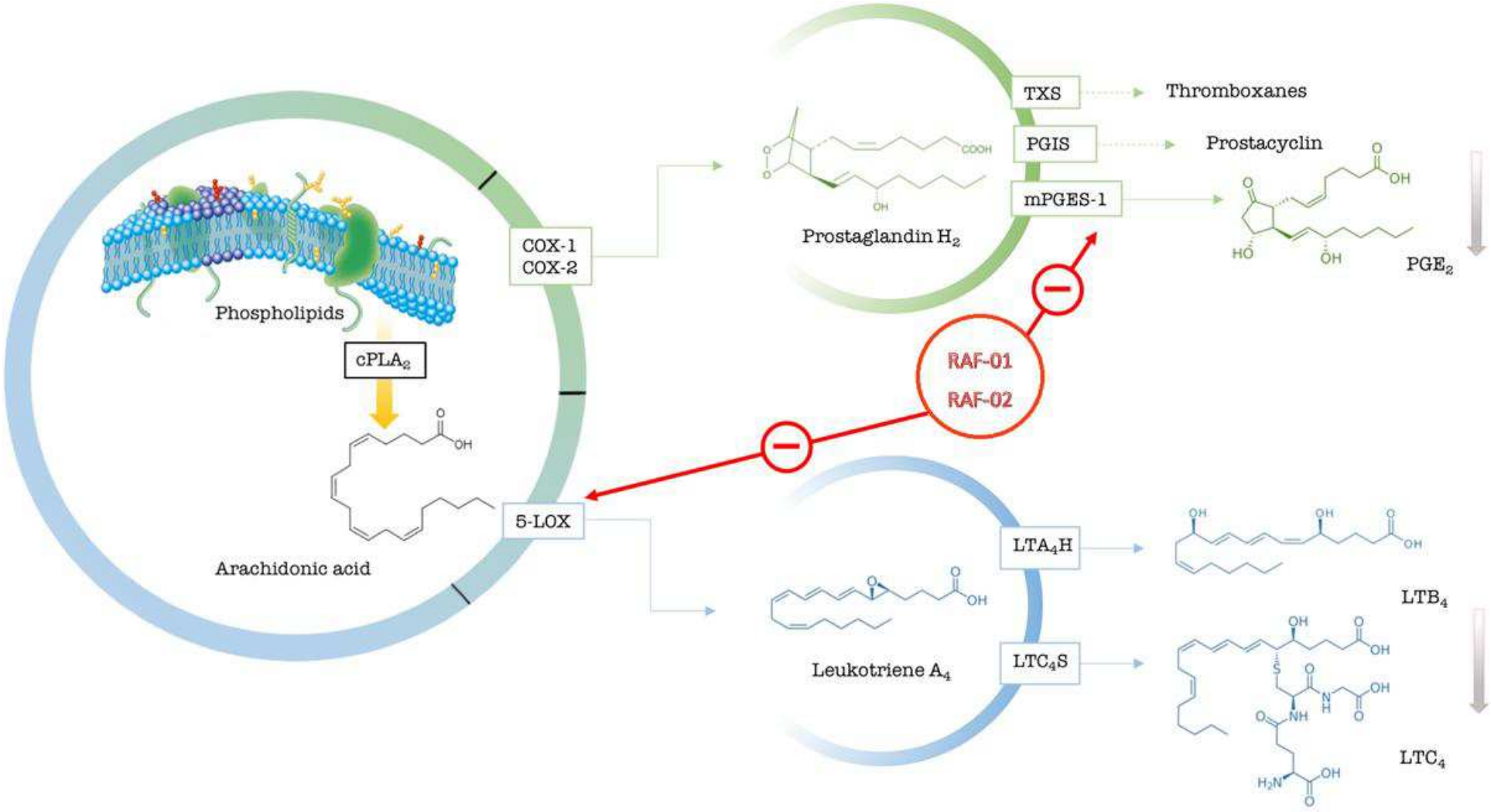
Abstract: The release of pro-inflammatory mediators, such as prostaglandines (PGs) and leukotrienes (LTs), arising from the arachidonic acid (AA) cascade, play a crucial role in initiating, maintaining, and regulating inflammatory processes.

New dual inhibitors of 5-lipoxygenase (5-LO) and microsomal prostaglandin E_2 synthase-1 (mPGES-1), that block, at the same time, the formation of PGE_2 and LTs, are currently emerged as a highly interesting drug candidates for better pharmacotherapie of inflammation-related disorders.

Following our previous studies, we here performed a detailed structure-based design of benzo[g]indol-3-carboxylate derivatives, disclosing several new key factors that affect both enzyme activity. Ethyl 2-(3,4-dichlorobenzyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (**4*b*, RAF-01)** and ethyl 2-(3,4-dichlorophenyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (**7*h*, RAF-02)** emerged as the most active compounds of the series. Additionally, together with selected structure based analogues, both derivatives displayed significant *in vivo* anti-inflammatory properties.

In conclusion, modeling and experimental studies lead to the discovery of new candidate compounds prone to further developments as multi-target inhibitors of the inflammatory pathway.


Phospholipids
cPLA2
Arachidonic acid
COX-1
COX-2
5-LOX
Prostaglandin H2
TXS
PGIS
mPGES-1
Thromboxanes
Prostacyclin
PGE2
RAF-01
RAF-02
Leukotriene A4
LTA4H
LTC4S
LTB4
LTC4

Introduction

5-Lipoxygenase (5-LO) is defined as a non-*heme* iron-containing dioxygenase involved in the biosynthesis of leukotrienes (LTs) from arachidonic acid (AA) [1,2]. Upon cell stimulation, the cytosolic PLA_2 ($cPLA_2$) releases AA that is converted by 5-LO with the help of the 5-LO-activating protein (FLAP) into an unstable intermediate LTA_4 that is further transformed, in enzymatic reactions, to LTB_4 or so-called cysteinyl (cys)-LTs.

LTB_4 acts as a potent pro-inflammatory agent by inducing chemotaxis and activation of leukocytes, whereas the cys-LTs essentially act on smooth muscles causing vaso and bronchoconstriction [3,4]. In view of the significant pathophysiological role of LTs, pharmacological intervention strategies have been developed to either block the action of LTs or to inhibit their biosynthesis, defining 5-LO and/or FLAP as valid drug targets for pharmacotherapy of inflammatory disorders [5,6].

Furthermore, recent studies have described age-dependent increase in 5-LO expression and oxidative stress in rat retina [7-10], it is described that light and trauma activate 5-LO, suggesting its involvement also in the pathogenesis of retinal diseases due to light damage [11,12].

Until now, in order to modulate cellular 5-LO product synthesis, three concrete pharmacological strategies can be basically pursued: inhibition of $cPLA_2$, inhibition of FLAP activity and direct inhibition of 5-LO [13].

Even if 5-LO is a reasonable target for the discover of antinflammatory drugs, taking in mind the multitude of pro-inflammatory and pro-resolving mediators produced from AA, the block of a single branch by a selective drug may cause redirection/shunting and even amplification of alternative pathways. The overcome of the “one-drug-hits-one-target” approach paved the way to the discover of drugs with polypharmacological modes of action able to modulate also the cyclooxygenase (COX) mediated formation of prostanoids [14,15]. Unfortunately, over the last years, several clinical evidences indicated that selective COX-2 inhibitors may be involved in serious cardiovascular accidents [16-19].

Deduced from previous studies, a novel pharmacological strategy was suggested by targeting mPGES-1 [20-22] having, this isoform, a considerable implications in inflammation and a pivotal role in the production of PGE_2 that mediates acute pain and fever during an inflammatory response [23-25]. Therefore, the rational development of chemical structures that contain fragments able to inhibit 5-LO and mPGES-1 is now day a highly interesting field of research with promise for better pharmacotherapies also over dual 5-LO/COX-2 inhibition in terms of higher anti-inflammatory efficacy and lower incidences of side effects.[26]

Our groups have been interested for a long time in the synthesis and biological evaluation of anti-cancer and anti-inflammatory agents [27-39]; in our drug discovery projects we have considered lead structures chemical compounds, either from medicinal plants [40, 41] or synthesized drugs, [42-45] which display pharmacological or biological activity likely to be therapeutically useful, but that may still have suboptimal structure modification to fit better to the respective target(s).

For instance, novel series of ethyl 5-hydroxyindole-3-carboxylate has led to the discovery and development of two potent inhibitors of human 5-LO (**Fig. 1**). Among all tested compounds, ethyl 5-hydroxy-2-(mesitylthiomethyl)-1-methyl-1H-indole-3-carboxylate (Compound **1**) was the most potent derivative which blocks 5-LO activity in cell-free assays with an IC_{50} of 0.7 μM, and suppressed 5-LO product synthesis in polymorphonuclear leukocytes with an IC_{50} of 0.23 μM, being equally potent to the well-recognized reference 5-LO inhibitor Zileuton, used as antiasthmatic drug in the clinics [46]. Furthermore, we have confirmed that the annelation of a [*g*]benzene ring to the indole ring in the series led to more potent inhibitors, exemplified by compounds **6f a**nd **6l** with IC_{50} of 0.17 and 0.20 μM in cell-free and 0.19 and 0.37 μM in cell-based 5-LO activity assays, respectively [47]. We also demonstrated that both derivatives directly inhibit mPGES-1 (1.33 μM and 1.93 μM) representing a novel class of dual 5-LO/mPGES-1 inhibitors. The anti-inflammatory property of the selected compounds **6g** and **6l** was also confirmed *in vivo* evaluating their ability to reduce carrageenan-induced paw edema in mice [47-49].

In our ongoing efforts to further elucidate the structural requirements of this class of compounds we focused our investigations on the effect caused by:

(i) Introduction of one or more halogens and methyl substituents on the phenyl ring;

(ii) Presence of oxygen or nitrogen in the heterocycle scaffold;

(iii) Change of the distance between the substituted-phenyl and the heterocycle rings.

In this study, we report the MW (MicroWaves) catalyzed synthesis of this third generation of benzo[g]indole derivatives together with their benzofuran and naphthofuran bioisosters, and their biological evaluation, leading to potentially novel, powerful compounds as multi-target inhibitors of the inflammatory pathway.

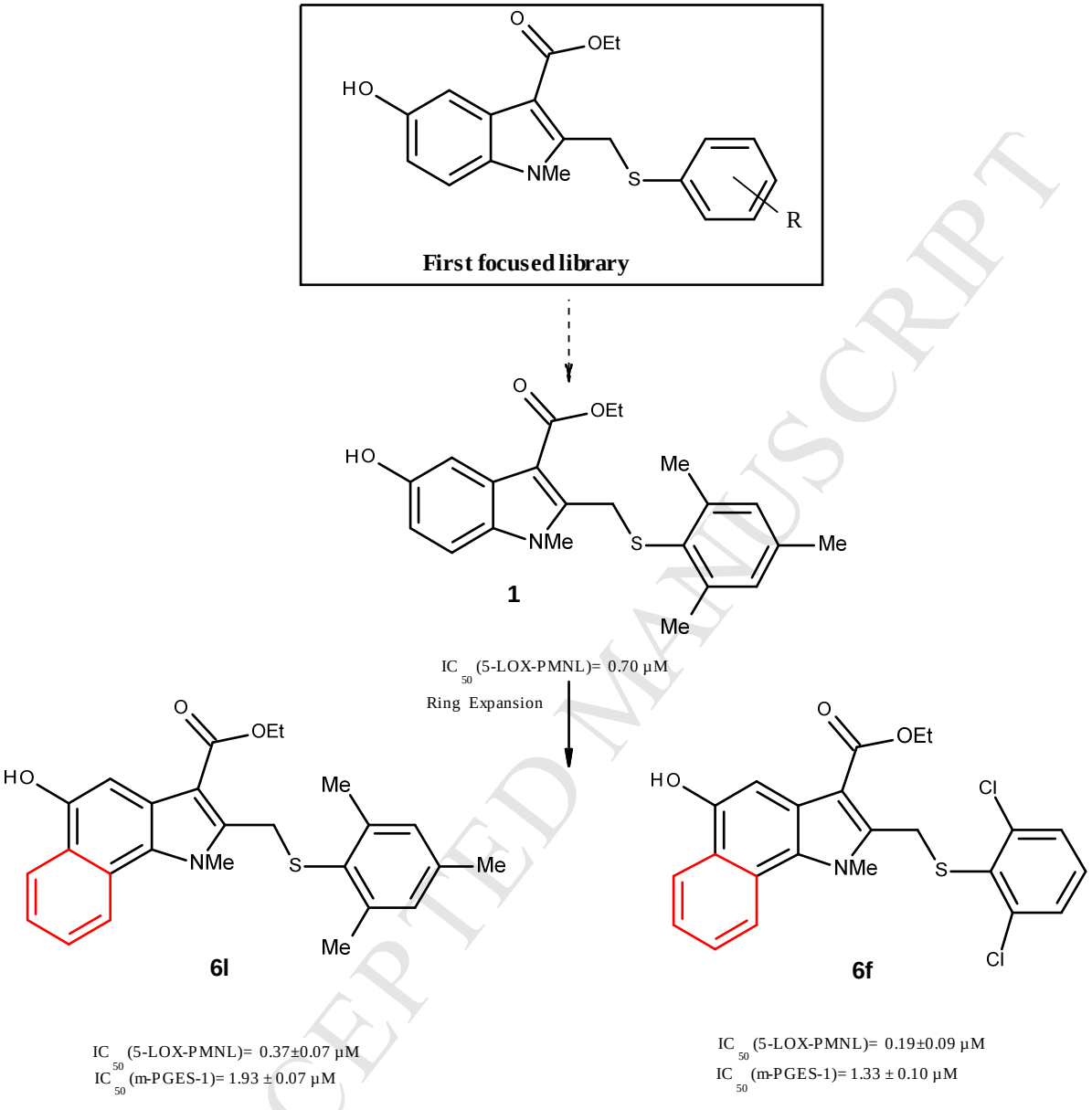


Fig.1

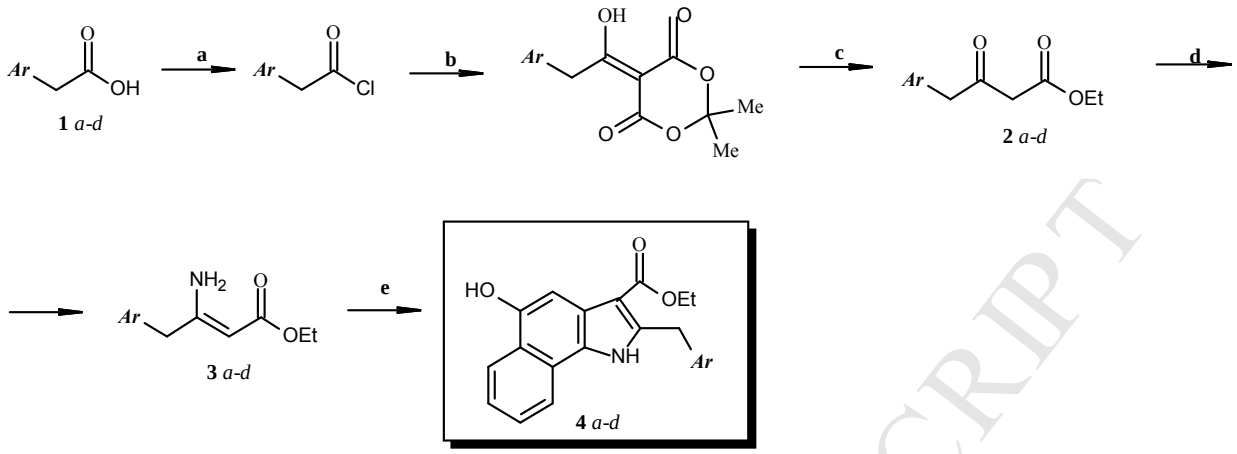
2. Results and discussion

2.1. Chemistry

Preparation of 2-substituted 5-hydroxybenzo[g]indole-3-carboxylic acid ethyl esters and their naphthofuran bioisosters is a good example of diversity-oriented synthesis (DOS) of exploratory libraries of "drug-like" compounds. "Synthetic trees" lying behind the target compounds are summarized in Schemes 1 and 2. First of them shows the synthesis of the corresponding 2-benzyl-5-hydroxybenzo[g]indole carboxylic acids ethyl esters, starting from the commercially available substituted arylacetic acids (**1 a-d**). The latter have been converted into the corresponding arylacetylchlorides, which were purified by distillation *in vacuo*, yielding the pure products in 86-99% yield. These were used in the synthesis of 4-aryl-3-oxobutyric acid ethyl esters according to the Moody's variation of Oikawa and Yonemitsu synthesis. Namely, 2,2-dimethyl-1,3-dioxan-4,6-dione (Meldrum's acid) solution in a mixture of anhydrous DCM and Hunig's base (*N*,*N*-diisopropyl-*N*-ethylamine) was treated with one of the above mentioned arylacetylchlorides, followed by subsequent work-up procedure and ethanolysis of the reaction product, yielding the desired technical grade 3-oxoesters (**2 a-d**). Purification of the intermedia has been achieved *via* flash-chromatography and/or fractional distillation under diminished pressure. It is necessary to mention, that synthesis of compounds **2a** and **2c** has been previously described in literature and carried out by a different method. Nevertheless, we preferred a Meldrum's acid based strategy, as it may widen the scope of the title compounds simply by varying the nature of the alcohol, used for solvolysis of an acylated 2,2-dimethyl-1,3-dioxane-4,6-dione. The thus obtained pure 3-oxoesters undergone successive transformation into the corresponding 3-amino-3-arylcrotonates upon treatment with NH_4OAc in PhMe in the presence of molecular sieves under microwave irradiation. The resulting 3-aminocrotonates have been purified by column chromatography and involved in Nenitzescu indole synthesis with 1,4-naphthoquinone in anhydrous DCM, catalyzed by ZnI_2 according to the method of Velezheva and co-authors.

A variety of ethyl 3-aryl-3-oxopropionates (**5a-n**), prepared according to the known procedures, served as starting material in the synthesis of the congeners of the above mentioned 2-benzylbenzo[g]indoles, containing an aryl-substituent at the C-2 position of the benzo[g]indole nucleus. As well, their benzo and naphthofuran bioisosters have been prepared. The total synthesis has much in common with the protocol, described above, but with several important peculiarities. As in the case of 4-aryl-3-oxobutyric acid ethyl esters, the corresponding ethyl 3-aryl-3-oxopropanoates undergone transformation into the desired 3-amino-3-arylacrylic acid ethyl esters upon treatment with NH_4Oac in PhMe under microwave irradiation and in the presence of molecular sieves. Their conversion into the target benzo[g]indoles was conducted according to the method of Velezheva and co-authors, as well. On the other side, the Nenitzescu synthesis of the target benzofurans and naphthofurans from ethyl 3-aryl-3-oxopropionates in the presence of ZnI_2 in DCM, required microwave irradiation. After the extractive work-up procedure the title compounds were purified *via* flash column chromatography. This modification of the literature procedure, allowed a significantly decreased reaction time, accompanied by higher yields of the target compounds (from about 60% to ~90%). In a number of entries, the same synthetic approach appeared to benefit with the preparation of benzo[g]indoles, too.

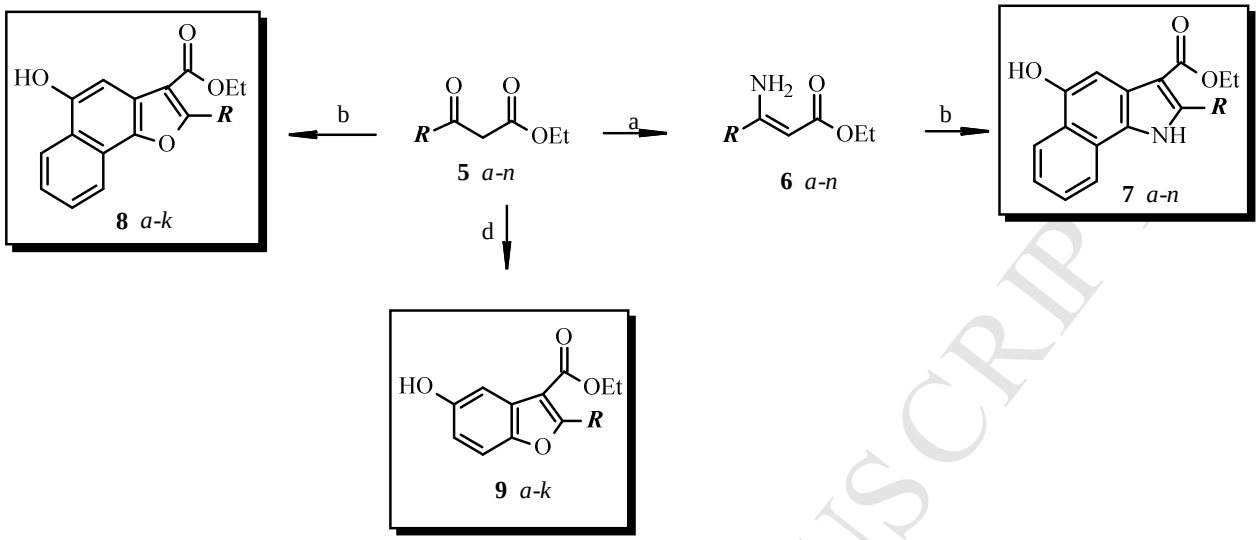
Scheme 1



Ar = 2,6-$Cl_2C_6H_3$ (***a***), 3,4-$Cl_2C_6H_3$ (***b***), 2,6-$F_2C_6H_3$ (***c***), 2,4,6-$Me_3C_6H_2$ (***d***)

Reagents and conditions**: a)** PCl_5, PhMe; **b)** Meldrum's acid, *i*-Pr_2NEt, DCM, 0-5°C, overnight; **c)** EtOH, reflux overnight; **d)** NH_4OAc , AcOH, dry PhMe, *molecularsieves*, 140 °C, 20 min, MW ; **e)** 1,4-Naphtoquinone , ZnI_2, DCM , reflux 30 min. and then 0 °C overnight.

Scheme 2



R = Me (***a***), 2-FC_6H_4 (***b***), 2-BrC_6H_4 (***c***), 2-ClC_6H_4 (***d***), 3-BrC_6H_4 (***e***), 3-ClC_6H_4 (***f***), 4-ClC_6H_4 (***g***), 3,4-$Cl_2C_6H_3$ (***h***), 4-Br-2-FC_6H_3 (***i***), 5-Br-2-FC_6H_3 (***j***), 2-$MeOC_6H_4$ (***k***), 2-MeC_6H_4 (***l***), 3-MeC_6H_4 (***m***), 3,5-$Me_2C_6H_3$ (***n***)

Reagent and conditions: **a)** NH_4OAc, PhMe, AcOH, *molecularsieves*, MW, 140 °C, 20min; **b)** 1,4-Naphtoquinone, ZnI_2, DCM, reflux 30 min then 0-5°C overnight; **c)** 1,4-Naphtoquinone, ZnI_2, DCM, MW, 110 °C, 30min, **c)** 1,4-Naphtoquinone , ZnI_2, DCM, MW, 110°C, 30 min; **d)** Benzoquinone, ZnI_2, DCM, MW, 110°C, 30 min

2.2 Evaluation of 5-LO and mPGES-1 activity and structure-activity relationships

Analysis of test compounds as 5-LO inhibitors was routinely carried out in two different test systems, a cell-free assay using isolated human recombinant 5-LO and a cell-based assay using human neutrophils. The cell-free assay allows identifying compounds that directly interfere with 5-LO catalytic activity, whereas the cell-based test system considers cellular regulatory aspects of 5-LO product synthesis, and as such offers several points of attack of a given compound (e.g., inhibition of FLAP, interference with 5-LO-activating lipid hydroperoxides, protein kinases or Ca^{2+} mobilization, and 5-LO translocation/membrane association). The reference 5-LO inhibitor N-[1-(1-benzothien-2-yl)ethyl]-N-hydroxyurea (**Zileuton**) was used as reference drug. For analysis of mPGES-1 inhibition, a well-established cell-free assay using microsomes of IL-1β-stimulated A549 cells as enzyme source and 20 µM PGH_2 as substrate was used [20].

In direct comparison to **16** and **19**, the corresponding analogues with a methylene bridge (**4d** and **4a**) were less potent against 5-LO in intact cells and cell-free assays, respectively. The strong impact of the thiomethylene moiety on the potency was also evident in the case of mPGES-1 evaluation. In contrast, variation of the halogen substituent chlorine *vs* fluorine (**4c**), leads to compound which is more potent both in cell-free and in cell-based 5-LO activity assays (IC_{50} 0.17 µM each *vs* 0.78 µM and 1.15 µM). Moreover, repositioning of the chlorine in *meta*- and *para*-positions of the C-2 phenyl ring, (**4b)** caused a remarkable increase in the potency versus **16**, yielding compounds with IC_{50} values in the range of 45 nM for intact cells and 54 nM for cell-free assays (**Table 1**). Of note, both **4c** and **4b** were most potent direct mPGES-1 inhibitors with IC_{50} values of 4.2 µM. Regarding the second series in which the phenyl moiety is directly attached to the benzoindoles core, the insertion of an activating group (i.e., methoxy) in *ortho*-position of the phenyl ring (**7k**) does not significantly alter the efficiency against 5-LO in intact cells and in the cell-free assay *vs* our previous lead **7d**. Compounds carrying deactivating groups, such as halogens -F, -Cl or -Br in *ortho*- and *para*-positions, are about equipotent to **7k** in the cell-free assay, with

IC_{50} values ranging from 0.13 to 0.16, respectively (except for **7f**) in the cell-based 5-LO test system, but markedly loose potency for mPGES-1 inhibition (**Table 2**).

In the case of polyhalogenated derivatives, the position of the substituents in an aromatic ring of the C-2 side chain is fundamental for the activity, especially in the cell-free assay for mPGES-1. In fact, compounds **7h** (3,4-Cl) and **7j** (5-Br, 2-F) showed the best activity, with IC_{50} values of 0.48 and 2.13 μM, respectively. In the case of polymethylated molecules **7l-n**, all derivatives were comparably active against 5-LO under cell-based conditions with IC_{50} of 0.42, 0.12 and 0.30 μM, respectively; otherwise only compound **7n** was active against mPGES-1 in the low micromolar range with an IC_{50} of 2.6 μM.

Finally, we replaced the indole by benzofurane. The respective derivatives moderately impaired potency against 5-LO in cell-free assays but substantial decrease 5-LO inhibitory activity in PMNL, implying that nitrogen is actually an important requirement in the aromatic system that may govern the 5-LO inhibitory potency (**Table 3**). Also, the annelation of benzene to the benzofuran, yielding corresponding dibenzofuran derivatives were less active as compared to the respective benzoindoles.

2.3 Docking studies and molecular dynamic (MD) simulations with mPGES-1 and 5-LO

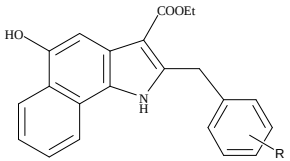
For rationalizing the inhibitory activities of **4b** and **7h** by means of molecular docking and MD simulations (10 ns) to 5-LO, we used the recently reported apo form of the human 5-LO crystal structure (PDB code: 3O8Y) [50]. We combined docking studies with four copies of MD simulations to investigate the binding modes of **4b** and **7h** taking into account ligand-induced conformational changes of 5-LO. The rationalization of the 5-LO binding mode was obtained considering the fundamental amino acids in the active site of the enzyme as described previously [51-53]. As a result of docking/MD studies for both molecules, we observe that **4b** and **7h** perfectly fits into the active site (**Figure 4A** and **4B**, respectively), and the ester carbonyl at 3-position and hydroxyl at 5-positon of benzoindole nucleus makes H-bonding interactions to Asn425 and Gln363, respectively, positioning the benzoindole nitrogen for H-bonding to Tyr181 in the upper part of the main binding cleft, which is a gatekeeper amino acid along with Phe177 at the active site entrance of 5-LO [54]. Additionally, annealed benzene ring lies in close proximity to His367 and His372, which engages through π-π interactions.

Luz *et al*. recently published co-crystal structures of mPGES-1 in complex with four diverse inhibitors provide important insights into inhibitor binding sites [55-56]. Based on the reported interaction network of these known inhibitors and to provide further insights into the interaction of **4b** and **7h** with mPGES-1, we performed docking studies in combination with four copies of MD simulations (10 ns) using the mPGES-1 crystal structure (PDB code: 4YL3) [56]. Since the available mPGES-1 x-ray structure does not include membrane coordinates, we analyzed the binding modes of mPGES-1/**4b** and mPGES-1/**7h** complex by means of MD simulations including membrane insertion (taken from OPM Database [57]) in the simulation systems (Figure **4C** and **4D**, respectively). By inclusion of the membrane bilayer, it was possible to observe its effect on the binding in which the adopted techniques have been widely used for the analysis of other membrane proteins [58].

Taking into account the considerations reported for these co-crystal structures, the binding specificities of **4b** and **7h** to mPGES-1 were conferred by specific polar interactions at the cytoplasmic part of the binding groove. For example, the C5-hydroxyl functionality of **4b** and **7h** forms direct or water-mediated H-bonds with the Arg52 and His53 at the cytoplasmic entrance orienting the rest of the molecule into the hydrophobic binding cavity for additional π-π contacts and hydrophobic interactions, i.e., the 3,4-dichlorophenyl group engages π-π contacts with Tyr130 while the ethyl carboxylate group at 3-position of the benzoindole lies in close proximity of Leu39 and Phe44 for hydrophobic interactions. Another stable H-bonding interaction is also observed between the side-chain of Gln36 and the NH of benzoindole nucleus (Figure **4C** and **4D**).

As can be seen from the Figure **4A**, **4B**, **4C** and **4D**, both molecules, **4b** and **7h**, show similar interaction network for both enzymes, although the inhibitory potency of the compound **4b** on mPGES-1 in comparison to **7h** was about 9-fold less potent (IC_{50}=4.2 vs. 0.48 μM). Based on these docking/MD simulations, it is still hard to make a conclusive explanation of this difference in activity, this may be related to the entropic cost upon binding, which may arise by the presence of a methylene bridge in **4b** causing instability inside the active site, as can be seen by the increased standard deviation trend of mPGES-1/**4b** complex's RMSD values given at Figure S1 (see supporting information).

Table 1 Inhibition of 5-LOX and inhibition of m-PGES-1 activity of compounds **4a-d**, **11** and Zileuton in a cell-based assay (intact PMNL) and in a cell-free assay. Data are given as mean ± S.E.M., n = 3–4.



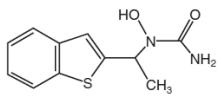
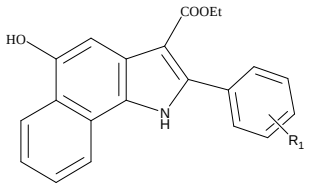
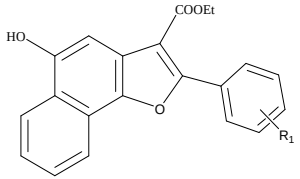
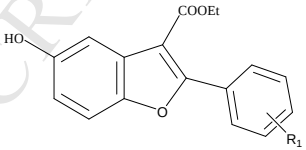
		Inhibition of 5LO activity (IC_{50} values [µM])		Inhibition of m-PGES1 activity(IC_{50} values [µM])
Cpd	**R**	**Intact**	**Cell-free**	**Cell-free**
4a	**2,6-Cl**	1.158±0.051	0.782±0.08	Remaining activity: 78.12 ± 13.7%
4b	**3,4-Cl**	0.045±0.018	0.054±0.025	4.2 ± 1.4
4c	**2,6-F**	0.094±0.026	0.169±0.021	4.2 ± 2.2
4d	**2,4,6-CH_3**	0.130±0.142	0.298±0.029	58.8 ± 4.9
11	**2-Cl**	0.301±0.029	0.317±0.052	0.96 ± 0.09
Zileuton		0.7 [46]	0.6[46]	-

Table 2 Inhibition of 5-LOX and inhibition of m-PGES-1 activity of compounds **7a-n** in a cell-based assay (intact PMNL) and in a cell-free assay. Data are given as mean ± S.E.M., n = 3–4.



Cpd	R	Inhibition of 5LOX activity (IC_{50} values [µM])		Inhibition of m-PGES1 activity (IC_{50} values [µM])
		Intact	**Cell-free**	**Cell-free**
7a	**CH_3**	0.42±0.01	0.48±0.02	n.d.
7b	**2-F**	0.15±0.01	0.10±0.04	50.58 ± 4,9%
7c	**2-Br**	0.20 ± 0.04	0.16 ± 0.02	2.54 ± 0.41
7d	**2-Cl**	0.24 ± 0.25	0.13 ± 0.07	No values
7e	**3-Br**	0.21 ± 0.06	0.13 ± 0.02	1.19 ± 0.1
7f	**3- Cl**	1.07±0.26	2.12 ± 0.65	6.01± 0.1
7g	**4-Cl**	0.32±0.11	0.07 ± 0.02	3.4*
7h	**3,4-Cl**	0.13 ± 0.03	0.13 ± 0.01	0.48 ± 0.13
7i	**4-Br,2-F**	0.86 ± 0.11	0.27 ± 0.05	3.43 ± 2.41
7j	**5-Br, 2-F**	0.23± 0.06	0.11 ± 0.07	2.13 ± 1.09
7k	**2-OCH_3**	0.21± 0.12	0.19 ± 0.05	8.6 ± 0.21
7l	**2-CH_3**	0.42 ± 0.1	0.11 ± 0.05	12.7 ± 0.24
7m	**3-CH_3**	0.12 ± 0.02	0.14 ± 0.02	2.56 ± 0.31
7n	**3,5-CH_3**	0.3 ± 0.03	0.12 ± 0.03	7.06 ± 0.53

Table 3 Inhibition of 5-LOX activity of compounds **8b-k** vs **9b-k** in a cell-based assay (intact PMNL) and in a cell-free assay. Data are given as mean ± S.E.M., n = 3–4.

		Inhibition of 5LO activity (IC_{50} values [µM])			Inhibition of 5LO activity (IC_{50} values [µM])	
						
Cpd	**R**	**Intact**	**Cell-free**	**Cpd**	**Intact**	**Cell-free**
8b	**2-F**	0.67 ± 0.17	0.37 ± 0.12	**9b**	116.9 ± 17.4	97.5 ± 7.6
8c	**2-Br**	0.79 ± 0.46	0.16 ± 0.04	**9c**	1.4 ± 0.11	6.39 ± 0.16
8d	**2-Cl**	1.86 ± 0.14	0.37 ± 0.09	**9d**	0.78 ± 0.14	59.5 ± 5.5
8e	**3-Br**	1.64 ± 0.01	0.86 ± 0.04	**9e**	3.3 ± 0.04	2.76 ± 0.06
8f	**3- Cl**	3.92 ± 0.11	0.46 ± 0.06	**9f**	1.79 ± 0.15	3.55 ± 1.35
8g	**4-Cl**	3.74 ± 0.75	0.13 ± 0.01	**9g**	2.49 ± 0.45	9.52 ± 0.6
8h	**3,4-Cl**	1.24 ± 0.66	1.70 ± 0.09	**9h**	4.76 ± 0.22	54.3 ± 6.0
8i	**4-Br, 2-F**	2.32 ± 0.23	1.06 ± 0.24	**9i**	1.86 ± 024	4.49 ± 0.8
8j	**5-Br, 2-F**	4.49 ± 1.49	2.03 ± 0.07	**9j**	2.19 ± 0.05	3.79 ± 0.08
8k	**2-OCH_3**	1.36 ± 0.31	0.22 ± 0.04	**9k**	4.71 ± 0.79	8.36 ± 1.53

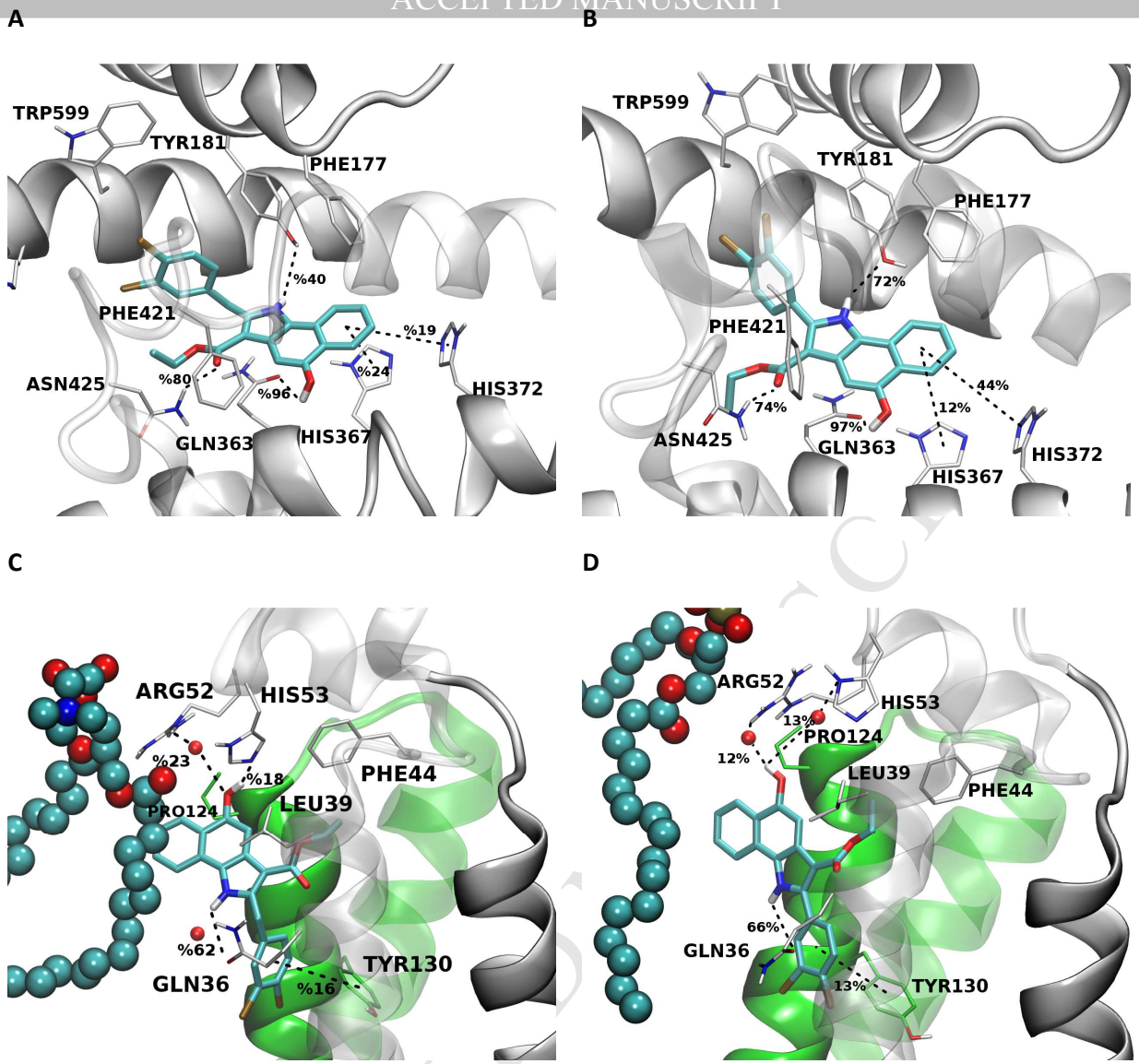


Figure 4. (A, B) Binding mode analysis of **4b** and **7h**, depicted with interacting 5-LO (PDB code 3O8Y) residues. (C, D) Binding mode analysis of **4b** and **7h** during interaction with the mPGES-1 (PDB code 4YL3) considering membrane residues. Main interactions are represented schematically with their occupancies calculated in the time window 0-10 ns.

2.3 Anti-inflammatory effectiveness of derivatives 4 b-c, 7c, 7e, 7 h-j, 7m in carrageenan-induced paw oedema

In order to verify the anti-inflammatory efficacy, we evaluated the most interesting compounds of the current series against carrageenan-induced paw oedema, which represents a well-established model of acute inflammation. [48] Intraplantar injection of carrageenan led to an increase in hind paw volume, expressed as oedema, monitored for a period of 6 h. The increase in paw volume (vehicle, i.p. treatment) reached its maximum at 4 h post-carrageenan application (**Fig. 6-7**). The vast majority of compounds (4 mg/kg, i.p.) significantly reduced paw oedema, in agreement with the results from *in vitro* studies.

In details, among the benzo[g]indol-3-carboxylates tested with C-2 benzylic branch (see **Table 1**), **4b** and **4c** were the most effective, in the *in vivo* experiment both compounds also significant reduced oedema formation by 73.0 and 81%, respectively, as compared with vehicle-treated animals, and they maintained their potency after 6 h of treatment (**Fig. 6**).

Regarding the second series, that is, the polyalogenated derivatives **7h**, **7i** and **7j (**4 mg/kg, i.p., each) the peak of the response to carrageenan at 4 h was reduced by 47.3, 69.35, and 73.95%, respectively (**Fig. 7 A**), while Zileuton (20 mg/kg, i.p.) used as reference, as previously reported by us, caused only 38.2% inhibition (data not shown).

Finally, the *ortho*-brominated derivatives **7c** and **7e** demonstrated the lowest anti-inflammatory effects with 47.0 and 18.1% inhibition, respectively. Of interest, compound **7m** was most effective, with a reduction of the response to carrageenan at 4 h by 75% (**Fig. 7 B**).

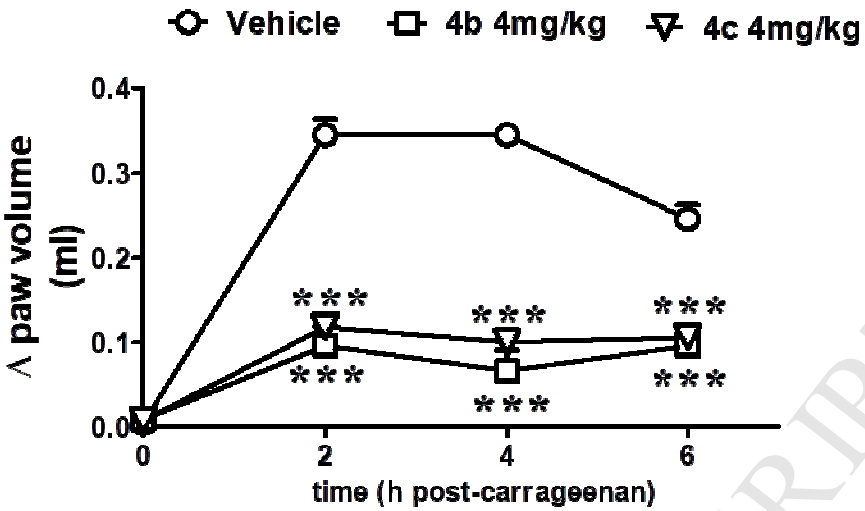


Fig.6 Effect of compounds 4b and 4c (4 mg/kg, i.p.) on carrageenan-induced paw oedema 0–6 h after carrageenan injection. Data are expressed as mean ± SEM (n = 6 for each group). *** indicate significant ($P < 0.001$) differences vs carrageenan+ vehicle-treated animals.

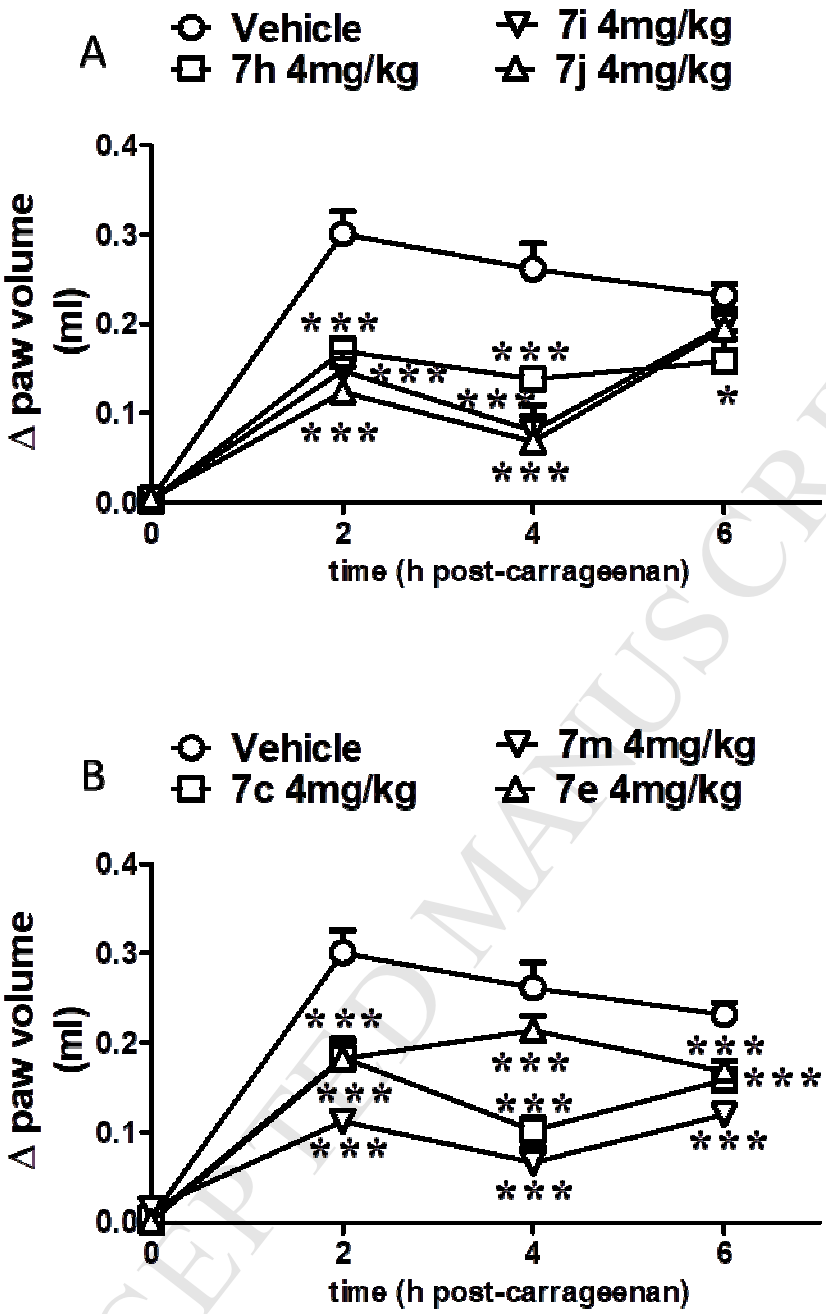


Fig.7 Effect of compounds 7i, 7h and 7j (A) or 7m, 7c and 7e (B) (4 mg/kg, i.p.) on carrageenan-induced paw oedema 0–6 h after carrageenan injection. Data are expressed as mean ± SEM (n= 6 for each group). * or *** indicate significant ($P < 0.05$ or $P < 0.001$, respectively) differences vs carrageenan + vehicle-treated animals.

Conclusion

A series of new benzoindole, benzofuran and naphthofuran derivatives were prepared and evaluated for their ability to inhibit both 5-LO and *m*-PGES-1 activity. Considering the biological data, we were able to perform a detailed structure based design of a revisited version of benzo[*g*]indol-3-carboxylic acid derivatives. Moreover, we disclosed new key factors that may govern the 5-LO/m-PGES-1 inhibitory potency of the target structures.

Our studies allowed us to complete SAR profile and to optimize chemical structures of the title compounds of the series. In particular, according to the above mentioned results, it is possible to conclude that:

- the thiomethylene linker between the phenyl ring and C-2 position of benzo[*g*]indole heterocyclic system may be omitted, allowing the direct connection of 3,4-chlorophenyl-substituent to heterocycle, leading to **7h** with an increased inhibitory activity *vs* previous hits **(16** and **19**) in the nanomolar range for both enzymes;
- the presence of nitrogen of the indole or benzo[*g*]indole-3-carboxylic acid derivatives remains an indispensable requirement for the activity, in fact, its substitution for benzofuran or naphthofuran bioisoster reduces the inhibitory activity of the target compounds (**7h** vs **8h**)
- the substitution of the phenyl ring radical influences the inhibitory activity. The simultaneous presence of chlorine in *meta*- or *para*-positions of the C-2 benzylic aromatic ring (**4b**, and **7h**), as well as the introduction of methyl groups (**7m**) (in *meta*- position on the phenyl ring), causes a marked increase of 5-LOX and mPGES-1 inhibitory activity in *cell-free* assay.

The antiflammatory properties of selected compounds were confirmed by *in vivo* evaluation of their ability to reduce carrageenan-induced paw oedema. In particular, methylated compound **7m** and its dichloro-substituted congener **4b** exhibit antinflammatory activity in lower doses than our previous hit compounds.

4.Experimental section

4.1 Chemistry

All reagents were analytical grade and purchased from Sigma-Aldrich (Milano-Italy). All microwave irradiation experiments were carried out in a Biotage® Initiator+ Microwave synthesizer (Biotage, Sweden AB, Uppsala, Sweden). The reactions were carried out in 10 mL glass tubes, sealed with aluminium/Teflon crimp tops, which can be exposed to 300 °C and 30 bar internal pressure. After the irradiation period, the reaction vessel was cooled rapidly(60-120s) to ambient temperature by gas jet cooling. Flash chromatography was performed on Carlo Erba silica gel 60 (230-400 mesh; CarloErba, Milan, Italy). TLC was carried out using plates coated with silica gel 60 F254 nm purchased from Merck (Darmstadt, Germany). Melting points were determined in open capillary tubes on Stuart®SMP30 Melting Point Apparatus and are uncorrected. Reaction yields refer to chromatographically and spectroscopically pure products. ^{1}H and ^{13}C NMR spectra were registered on a Bruker AC 300. Chemical shifts are reported in ppm relative to tetramethylsilane The abbreviations used are follows: s, singlet; d, doublet; dd double doublet; bs, broad signal. MS spectrometry analysis ESI-MS was carried out on a Finnigan LCQ Deca ion trap instrument. Microanalyses were carried out on a Carlo Erba 1106 elemental analyzer.

The following specific procedures illustrate the general methods for the preparation of key intermediates and title compounds ethyl 4-aryl-3-oxobutanoates

4.2 General procedure for the synthesis of Ethyl 4-aryl-3-oxobutanoates (2*a-d*)

Step-1

A mixture of 10 g (48.7 mmol) of 2-substituted phenyl acetic acid, 40 ml of anhydrous PhMe was treated with 11.2 g (53.8 mmol) PCl_5 at ambient temperature under protection of moisture. After the effervescence ceased, the resulting clear yellow solution was heated at reflux for 2 hours, only in the case of 2-(2,4,6-trimethylphenyl)acetyl chloride the reaction was conducted at room

temperature overnight. Due to their hydrolytic lability, all intermedia were not analyzed, but used directly in the next step.

Step-2

A stirred solution of 7.7 g (53.5 mmol) 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid) in 150 ml of anhydrous CH_2Cl_2 was chilled in an ice chest and treated with 25 ml (~18.6 g, 143.5 mmol) *i*-Pr_2NEt (Hunig's base). The resulting mixture was cooled to – 8 ^oC in an ice-salt bath with stirring, followed by dropwise addition of dichloromethane solution of 2-substituted phenyl acetyl chloride (50.9 mmol). During the addition of the acylating agent, the inner temperature was kept below 0 ^oC. After the addition of chloroanhydride was complete, the whole reaction mixture was stirred for 2 hours more at the above mentioned temperature and then left overnight. The next day it was poured into a mixture of 150 ml of 1M aqueous H_2SO_4 and crushed ice. The two layers formed were vigorously shaken, and the lower organic layer was separated. The aqueous layer was discarded, while the organic layer was washed successively with ice-cold dilute H_2SO_4 and water, and dried over anhydrous Na_2SO_4. After filtration, the solution was stripped down from the solvent at ordinary, and then – slightly reduced pressure and the residue was diluted with 150 ml of absolute EtOH. This was refluxed under the protection of atmospheric moisture until the evolution of CO_2 has ceased and treated with 1 g of activated carbon. The resulting mixture was reflux for one more hour, filtered and evaporated under the diminished pressure, leaving the title product of technical grade as a straw-colored oil. The latter was redissolved in 200 ml of *t*-BuOMe, washed successively with saturated $NaHCO_3$ solution, water and dried over anhydrous Na_2SO_4. The latter was removed by filtration through a TLC-grade silica-gel pad and evaporated. The thus obtained 3-oxoester was subjected to vacuum distillation collecting a fraction in a wide range, which was redistilled with a short Vigreux column, to remove the undesired by products.

An analytically pure substance was prepared by gravity elution silica gel column chromatography of this products, using Hexan-EtOAc (9:1, v/v) mixture as a mobile phase.

4.2.1 Ethyl 4-(2,6-dichlorophenyl)-3-oxobutanoate (**2*a***)

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.25 (t, 3H, *J*=7.40Hz); 3.47 (s, 2H); 3.79 (s, 2H); 4.16 (q, 2H, *J*=7.42Hz); 7.00 (d, 1H, *J*=8.23Hz); 7.27 (s, 1H); 7.35 (d, 1H, *J*=7.78Hz). Yield: 75%.

4.2.2 Ethyl 4-(3,4-dichlorophenyl)-3-oxobutanoate (**2*b***)

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.30 (t, 3H, *J*=7.13Hz); 3.26 (s, 2H); 3.58 (s, 2H); 4.23 (q, 2H, *J*=7.29Hz); 7.19 (t, 1H, *J*=8.85Hz); 7.33 (d, 2H, *J*=7.70Hz). Yield: 69%.

4.2.3 Ethyl 4-(2,6-difluorophenyl)-3-oxobutanoate (**2*c***)

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.27 (t, 3H, *J*=7.37Hz); 3.54 (s, 2H); 3.92 (s, 2H); 4.19 (q, 2H, *J*=7.51Hz); 6.88 (t, 2H, *J*=8.24Hz); 7.27 (m, 1H). Yield: 88%.

*4.2.4 Ethyl 3-oxo-4-(2,4,6-trimethylphenyl)butanoate (**2*d***)*

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.92 (t, 3H, *J*=6.90Hz); 2.28 (s, 6H); 2.31 (s, 3H); 3.47 (s, 2H); 3.51 (s, 2H); 4.21 (q, 2H, *J*=7.06Hz); 6.90 (s, 2H). Yield: 57%.

4.3 General procedure for the synthesis of Ethyl 3-amino-4-arylcrotonates (3*a-d*)

A 10 ml glass vial, equipped with a magnetic stirring bar and an aluminium-Teflon crimp top, was charged with ethyl 4-(2,6-difluorophenyl)-3-oxobutyrate, NH_4OAc, PhMe, a catalytic amount of glacial AcOH and appropriate molecular sieves. The reaction mixture was stirred at 140 ^oC under 60 W microwave irradiation for 20 minutes, cooled to 50 ^oC and partitioned between *t*-BuOMe and saturated $NaHCO_3$ aqueous solution. The resulting mixture was vigorously stirred, filtered on a Schott funnel to break the resulting emulsion and the upper organic layer was separated. An aqueous layer was extracted with *t*-BuOMe and the combined organic phases were washed with water (until the neutral pH), dried over anhydrous Na_2SO_4 and filtered. The resulting filtrate was stripped down from the solvent under reduced pressure, yielding a crude product, which was purified by flash chromatography on silica gel column, using Exan-EtOAc (4:1, V/V) mixture as a mobile phase. The yield of pure ethyl 3-amino-4-(2,6-difluorophenyl) crotonate was 88%.

4.3.1 Ethyl 3-amino-4-(2,6-dichlorophenyl)but-2-enoate (**3*a***)

Compound **3*a*** was synthesized from Ethyl 4-(2,6-dichlorophenyl)-3-oxobutanoate **2*a*** (1.0equiv.), Ammonium acetate (3.0 equiv.) and acetic acid (5 gtt.). Flash chromatography with hexan/EtOAc (80:20) afforded analytically pure product. Yield: 88%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) =1.26 (t, 3H, *J*=7.19Hz); 3.87 (s, 2H); 4.11 (q, 2H, *J*=7.19Hz); 4.48 (s, 1H); 7.22 (t, 1H, *J*=8.71Hz); 7.39 (d, 2H, *J*=7.79Hz).

4.3.2 Ethyl 3-amino-4-(3,4-dichlorophenyl)but-2-enoate (**3*b***)

Compound **3*b*** was synthesized from Ethyl 4-(3,5-dichlorophenyl)-3-oxobutanoate **2*b*** (1.0equiv.), Ammonium acetate (3.5 equiv.) and acetic acid (4 gtt.). Flash chromatography with hexan/EtOAc (80:20) afforded analytically pure product. Yield: 87%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) =1.15 (t, 3H, *J*=7.11Hz); 3.40 (s, 2H); 4.05 (q, 2H, *J*=7.26Hz); 4.58 (s, 1H); 7.03 (d, 1H, *J*=8.27Hz); 7.24 (s, 1H); 7.31 (d, 1H, *J*=8.34Hz).

4.3.3 Ethyl 3-amino-4-(2,6-difluorophenyl)but-2-enoate (**3*c***)

Compound **3*c*** was synthesized from Ethyl 4-(2,6-difluorophenyl)-3-oxobutanoate **2*c*** (1.0equiv.), Ammonium acetate (3.3 equiv.) and acetic acid (4 gtt.). Flash chromatography with hexan/EtOAc (80:20) afforded analytically pure product. Yield: 82%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) =1.24 (t, 3H, *J*=7.36Hz); 3.51 (s, 2H); 4.09 (q, 2H, *J*=7.21Hz); 4.60 (s, 1H); 6.91 (t, 2H, *J*=7.46Hz); 7.33(m, 1H).

4.3.4 Ethyl 3-amino-4-mesitylbut-2-enoate (**3*d***)

Compound **3*d*** was synthesized from Ethyl 4-mesityl-3-oxobutanoate **2*d*** (1.0equiv.), Ammonium acetate (3.0 equiv.) and acetic acid (5 gtt.). Flash chromatography with hexan/EtOAc (80:20) afforded analytically pure product. Yield: 89%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) =1.37(t, 3H, *J*=6.68Hz); 2.31 (s, 6H); 2.37 (s, 3H); 3.62(s, 2H); 4.11 (q, 2H, *J*=7.07Hz); 4.47 (s, 1H); 6.89 (s, 1H); 6.91 (s, 1H).

4.4 General procedure for the synthesis of 5-Hydroxy-1*H*-benzo[*g*]indole-3-carboxylate (4*a-d*)

To a solution of 1,4-naphthoquinone (1.0 equiv.) in 5mL CH_2Cl_2, ZnI_2 (0.1equiv.) was added and the resulting mixture was heated to boiling temperature. A solution of β-enaminoesters **3*a-d***, respectively (1.0 equiv.), in 2 mL of CH_2Cl_2 was added drop by drop under stirring for 5-10 min. After refluxing for additional 30 min, the mixture was cooled to 0-5 °C over night. The precipitated crystals were filtered off and washed with CH_2Cl_2.

4.4.1 Ethyl 2-(2,6-dichlorobenzyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (**4*a***)

Yield: 79%. ^{1}H NMR (Acetone-*d6*) δ (ppm) =1.46 (t, 3H, J =7.23Hz); 3.53 (s, 2H); 4.42 (q, 2H, J =7.59Hz); 5.02 (s, 1H, OH); 7.37 (t, 1H, J =6.21Hz); 7.39 (d, 2H, *J*=7.79Hz); 7.42-7.49 (m, 2H); 7.77 (s, 1H); 8.01 (dd, 2H, J = 1.5, 8.20Hz); 11.03 (s, 1H, NH). 13C NMR (Acetone-*d6*) δ (ppm) = 14.8; 17.3; 60.5; 101.7; 104.8; 113.1; 115.9; 126.6; 127.9; 128.1; 128.6; 130.3; 136.7; 139.4; 140.5; 144.3; 149.2; 168.0 MS-ESI (m/z): 413.06 [M^-]. Anal. Calcd. For $C_{22}H_{17}Cl_2NO_3$ C, 63.78; H, 4.14; Cl, 17.12; N, 3.38; O, 11.59 found C, 63.88; H, 4.15; Cl, 17.18; N, 3.65; O, 11.74

4.4.2 Ethyl 2-(3,4-dichlorobenzyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (**4*b***)

Yield: 65%. ^{1}H NMR (Acetone-*d6*) δ (ppm) =1.44(t, 3H, J =7.19); 4.22 (s, 2H); 4.35 (q, 2H, J =7.22); 5.15 (s, 1H, OH); 7.25 (d, 1H J =7.24); 7.31(d, 1H, J =7.24); 7.40 (s, 1H); 7.50-7.58 (m, 2H); 7.75 (s, 1H); 7.90 (dd, 2H, J =1.39; 8.15 Hz); 11.39 (s, 1H, NH). 13C NMR (Acetone-*d6*) δ (ppm) = 18.2; 35.5; 61.8; 99.4; 105.2; 112.4; 114.8; 127.9; 128.3; 128.7; 130.1; 130.3; 131.0; 132.2; 132.5; 135.4; 137.4; 138.1; 144.1; 147.0; 168.0 MS-ESI (m/z): 413.06[M^-]. Anal. Calcd. For $C_{22}H_{17}Cl_2NO_3$ C, 63.78; H, 4.14; Cl, 17.12; N, 3.38; O, 11.59 found C, 64.01; H, 4.24; Cl, 17.12; N, 3.55; O, 11.68

4.4.3 Ethyl 2-(2,6-difluorobenzyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (**4*c***)

Yield: 68%. ^{1}H NMR (Acetone-*d6*) δ (ppm) = 1.39 (t, 3H, J = 7.28); 3.71 (s, 2H); 4.44 (q, 2H, J = 7.15); 7.01 (t, 2H, J =7.89); 7.34-7.49 (m, 3H); 7.62-7.69 (m, 2H), 7.74 (s, 1H), 7.90 (dd, 2H, J =2.22; 9.03Hz), 11.30 (s, 1H, NH). 13C NMR (Acetone-*d6*) δ (ppm) = 17.0; 19.5; 72.1; 100.9; 111.4; 112.3; 112.6; 113.1; 113.8; 114.1; 128.4; 129.6; 129.8; 130.7; 130.9; 131.0; 138.2; 144.8;

149.7; 165.4; 165.9; 170.1 MS-ESI (m/z): 381.12[M$^-$]. Anal. Calcd. For $C_{22}H_{17}F_2NO_3$ C, 69.29; H, 4.49; F, 9.96; N, 3.67; O, 12.59 found C, 69.32; H, 4.68; F, 10.15; N, 3.68; O, 12.59

4.4.4 Ethyl 2-(2,4,6 trimethylbenzyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (**4*d***)

Yield: 77%. ^{1}H NMR (Acetone-*d6*) δ (ppm) =1.48 (t, 3H, J=7.11); 2.45 (s, 6H); 2.49 (s, 3H); 4.47 (s, 2H); 4.41 (q, 2H, J =7.12); 5.50 (s, 1H, OH); 6.90 (s, 2H); 7.36-7.40 (m, 2H); 7.77 (s, 1H); 8.07-8.14 (m, 2H); 10.57(s, 1H, NH). ^{13}C NMR (Acetone-*d6*) δ (ppm) = 20.1; 24.2; 25.4; 26.7; 27.0; 70.0; 99.4; 103.0; 111.3; 115.4; 125.6; 125.9; 127.8; 127.9, 128.9; 130.8; 136.8; 137.0; 138.4; 144.0; 149.3; 166.1 MS-ESI (m/z): 387.18[M$^-$]. Anal. Calcd. For $C_{25}H_{25}NO_3$ C, 77.49; H, 6.50; N, 3.61; O, 12.39 found C, 77.51; H, 6.45; N, 3.81; O, 12.40.

4.5 General procedure for the microwave irradiated synthesis of β-enaminoesters derivatives (6*a*-*n*)

β-keto esters **5** ***a*-*n*** (1.0 equiv.), Ammonium acetate (3.0 equiv.) and Acetic acid (gtt.) were dissolved in dry Toluol (6mL) in a 10mL reaction glass vial containing a tiny stirring magnet and *molecular sieves* . The vial was sealed tightly with an aluminium-Teflon® crimp top and the mixture was irradiated for 20 min at a pre-selected temperature of 140°C, with an irradiation power of 60W. After the reaction, the vial was cooled to 50°C by gas jet cooling. The crude mixture was portioned between ethyl acetate and saturated solution of Sodium bicarbonate (15mL of each) and the aqueous layer was extracted with ethyl acetate (3 x 15mL). The combined organic layer were dried on Sodium sulfate anhydrous, filtered and the solvent was removed under reduce pressure. Then, final crude compounds were purified by flash chromatography over silica gel.

4.5.1 Ethyl 3-aminobut-2-enoate (**6*a***)

Compound **6*a*** was synthesized from Ethyl 3-(2-fluorophenyl)-3-oxopropanoate **5*a***(1.0equiv.), Ammonium acetate (4.0equiv.) and acetic acid (7 gtt.). Flash chromatography with hexan/EtOAc (80:20 to 70:30) afforded analytically pure product as 89 %.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) =1.24 (t, 3H, *J*=7.36Hz), 3.51 (s, 3H), 4.09 (q, 2H, *J*=7.28Hz), 4.60 (s, 1H).

4.5.2 Ethyl 3-amino-3-(2-fluorophenyl)acrylate (**6*b***)

Compound **6*b*** was synthesized from Ethyl 3-(2-fluorophenyl)-3-oxopropanoate **5*b*** (1.0equiv.), Ammonium acetate (3.0equiv.) and acetic acid (5 gtt.). Flash chromatography with hexan/EtOAc (80:20 to 70:30) afforded analytically pure product as 72%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.36 (t, 3H, J = 7.1Hz); 4.38 (q, 2H, J = 7.1Hz); 4.98 (s, 1H); 7.12 (dd, 1H, J = 5.4, 11.5Hz); 7.20 (t, 1H, J = 5.4Hz); 7.42-7.51 (m, 1H); 7.50 (t, 1H, J = 2.1Hz).

4.5.3Ethyl 3-amino-3-(2-bromophenyl)acrylate (**6*c***)

Compound **6*c*** was synthesized from Ethyl 3-(2-bromophenyl)-3-oxopropanoate **5*c*** (1.0equiv.), Ammonium acetate (3.5equiv.) and acetic acid (6 gtt.). Flash chromatography with hexan/EtOAc (80:20 to 70:30) afforded analytically pure product as 63%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.42 (t, 3H, J = 7.3Hz); 1.80 (s, NH_2); 4.23 (q, 2H, J = 7.3Hz); 4.74 (s, 1H); 7.29(t, 1H, J = 3.5Hz); 7.39-7.45 (m, 2H); 7.65 (d, 1H, J = 4.8Hz).

4.5.4Ethyl 3-amino-3-(2-chlorophenyl)acrylate (**6*d***)

Compound **6*d*** was synthesized from Ethyl 3-(2-chlorophenyl)-3-oxopropanoate **5*d*** (1.0equiv.), Ammonium acetate (3.5equiv.) and acetic acid (5 gtt.). Flash chromatography with hexan/EtOAc (80:20 to 70:30) afforded analytically pure product as 53%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.38 (t, 3H, J = 7.1Hz); 4.42 (q, 2H, J = 7.1Hz); 4.84 (s, 1H); 7.38-7.48 (m, 2H); 7.49-7.50 (m, 2H).

4.5.5Ethyl 3-amino-3-(3-bromophenyl)acrylate (**6*e***)

Compound **6*e*** was synthesized from Ethyl 3-(3-bromophenyl)-3-oxopropanoate **5*e*** (1.0equiv.), Ammonium acetate (3.0equiv.) and acetic acid (6 gtt.). Flash chromatography with hexan/EtOAc (90:10 to 60:40) afforded analytically pure product as 58%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.37 (t, 3H, J = 6.8Hz); 4.25 (q, 2H, J = 6.8Hz); 5.00 (s, 1H); 7.45 (t, 1H, J = 2.5Hz); 7.54 (d, 1H, J = 5.3Hz); 7.62 (d, 1H, J = 5.3Hz); 7.74 (s, 1H).

4.5.6 Ethyl 3-amino-3-(3-chlorophenyl)acrylate (**6*f***)

Compound **6*f*** was synthesized from Ethyl 3-(3-chlorophenyl)-3-oxopropanoate **5*f*** (1.0equiv.), Ammonium acetate (3.5equiv.) and acetic acid (4 gtt.). Flash chromatography with hexan/EtOAc (90:10 to 8:20) afforded analytically pure product as 59%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.28 (t, 3H, J = 7.1Hz); 3.89 (q, 2H, J = 7.1Hz); 4.87 (s, 1H); 7.48-7.54 (m, 3H); 7.65 (s, 1H).

4.5.7 Ethyl 3-amino-3-(4-chlorophenyl)acrylate (**6*g***)

Compound **6*g*** was synthesized from Ethyl 3-(4chlorophenyl)-3-oxopropanoate **5*g*** (1.0equiv.), Ammonium acetate (3.0equiv.) and acetic acid (6 gtt.). Flash chromatography with hexan/EtOAc (90:10 to 70:30) afforded analytically pure product as 68%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.47 (t, 3H, J = 6.5Hz); 2.00 (s, NH_2); 4.44 (q, 2H, J = 6.5Hz); 5.12 (s, 1H), 7.35 (d, 2H, J = 5.4Hz); 7.42 (d, 2H, J = 5.8Hz).

4.5.8 Ethyl 3-amino-3-(3,4-dichlorophenyl)acrylate (**6*h***)

Compound **6*h*** was synthesized from Ethyl 3-(3,4-dichlorophenyl)-3-oxopropanoate **5*h*** (1.0equiv.), Ammonium acetate (3.5equiv.) and acetic acid (7 gtt.). Flash chromatography with hexan/EtOAc (90:10 to 70:30) afforded analytically pure product as 71%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.30 (t, 3H, J = 6.8Hz), 4.26 (q, 2H, J = 6.8Hz); 5.00 (s, 1H); 7.39(d, 1H, J = 3.5Hz); 7.51 (d, 1H, J = 3.7Hz); 7.71(s, 1H).

4.5.9 Ethyl 3-amino-3-(4-bromo-2-fluorophenyl)acrylate (**6*i***)

Compound **6*i*** was synthesized from Ethyl 3-(4-bromo-2-fluorophenyl)-3-oxopropanoate **5*i*** (1.0equiv.), Ammonium acetate (3.0equiv.) and acetic acid (5 gtt.). It was necessary two mw-irradiation cycles to obtain the compound. Flash chromatography with hexan/EtOAc (80:20) afforded analytically pure product as 47%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.45 (t, 3H, J = 7.5Hz); 4.33(q, 2H, J = 7.5Hz); 4.87 (s, 1H); 7.46(s, 1H); 7.54-7.60 (m, 2H).

4.5.10 Ethyl 3-amino-3-(5-bromo-2-fluorophenyl)acrylate (**6*j***)

Compound **6*j*** was synthesized from Ethyl 3-(5-bromo-2-fluorophenyl)-3-oxopropanoate **5*j*** (1.0equiv.), Ammonium acetate (3.0 equiv.) and acetic acid (5 gtt.). It was necessary two mw-irradiation cycles to obtain the compound. Flash chromatography with hexan/EtOAc (80:20) afforded analytically pure product as 54%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.42 (t, 3H, J = 6.9Hz); 4.54 (q, 2H, J = 6.9Hz); 4.71 (s, 1H); 7.31 (t, 1H, J=2.1Hz); 7.47-7.51 (m, 2H).

4.5.11Ethyl 3-amino-3-(2-methoxyphenyl)acrylate (**6*k***)

Compound **6*k*** was synthesized from Ethyl 3-(5-bromo-2-fluorophenyl)-3-oxopropanoate **5*k*** (1.0equiv.), Ammonium acetate (3.0equiv.) and acetic acid (6 gtt.). Flash chromatography with hexan/EtOAc (90:10) afforded analytically pure product as 67%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.28 (t, 3H, J = 7.1Hz); 1.69 (s, NH_2); 3.95 (s, 3H); 4.29 (q, 2H, J = 7.1Hz); 4.82 (s, 1H); 6.98-7.09-(m, 2H); 7.44-7.50 (m, 2H).

4.5.12Ethyl 3-amino-3-o-tolylacrylate (**6*l***)

Compound **6*l*** was synthesized from Ethyl 3-oxo-3-o-tolylpropanoate **6*l*** (1.0equiv.), Ammonium acetate (3.8equiv.) and acetic acid (7 gtt.). Flash chromatography with hexan/EtOAc (90:10 to 80:20) afforded analytically pure product as 49%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.31 (t, 3H, J =6.4Hz); 2.45 (s, 3H); 4.35 (q, 2H, J = 6.4Hz); 4.68 (s, 1H); 7.10-7.25(m, 3H); 7.39 (d, 1H, J = 3.2Hz).

4.5.13Ethyl 3-amino-3-m-tolylacrylate (**6*m***)

Compound **6*m*** was synthesized from Ethyl 3-oxo-3-m-tolylpropanoate **5*m*** (1.0equiv.), Ammonium acetate (3.0equiv.) and acetic acid (5gtt.). Flash chromatography with hexan/EtOAc (80:20) afforded analytically pure product as 64%.

^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.44 (t, 3H, J =6.8Hz); 2.51 (s, 3H); 4.40 (q, 2H, J = 6.9Hz); 5.71 (s, 1H); 7.38(d, 1H, J = 2.8Hz); 7.41 (t, 1H, J = 3.2Hz); 7.62(d, 1H, J = 3.3Hz); 7.71 (s, 1H).

4.5.14 Ethyl 3-amino-3-(3,5-dimethylphenyl)acrylate (**6*n***)

Compound **6*n*** was synthesized from Ethyl 3-(3,5-dimethylphenyl)-3-oxopropanoate **5*n*** (1.0equiv.), Ammonium acetate (3.5equiv.) and acetic acid (7gtt.). It was necessary two mw-irradiation cycles to obtain the compound. Flash chromatography with hexan/EtOAc (80:20) afforded analytically pure product as 44%. ^{1}H NMR ($CDCl_3$, 300MHz) δ (ppm) = 1.31 (t, 3H, J =7.2Hz); 1.64 (s, NH_2); 2.44 (s, 6H); 4.22 (q, 2H, J = 7.2Hz); 4.98 (s, 1H); 7.17 (s, 2H); 7.37 (s, 1H).

***4.6 General procedure for synthesis of 5-Hydroxy-1H-benzo[g]indole-3-carboxylate* (7*a-n*)**

To a solution of 1,4-naphthoquinone (1.0equiv.) in 5 mL of CH_2Cl_2, ZnI_2 (0.1equiv.) was added and the resulting mixture was heated to boiling temperature. A solution of *β*-enaminoesters 6*a-n*, respectively (1.0 equiv.), in 2 mL of CH_2Cl_2 was added drop by drop under stirring for 5-10 min. After refluxing for additional 30 min, the mixture was cooled to 0-5 °C over night. The crude mixture was portioned between methylenchloride and saturated solution of Sodium chloride (10mL of each) and the aqueous layer was extracted with CH_2Cl_2 (3 x 10mL). The combined organic layer were dried on Sodium sulfate anhydrous, filtered and the solvent was removed under reduce pressure. Then, the pure product was obtained by crystallization of the crude product in a mixture of Hexane-Chloroform 90:10.

4.6.1 Ethyl 5-hydroxy-2-methyl-1H-benzo[g]indole-3-carboxylate (7*a*)

Yield: 58%. ^{1}H NMR (Acetone-d_6) δ (ppm) =1.42 (t, 3H, *J* =7.2Hz); 2.70 (s, 3H); 4.39 (q, 2H, *J* =7.3Hz), 4.90 (s, 1H, OH); 7.34-7.48 (m, 2H); 7.50 (s, 1H); 7.94 (dd, 2H, *J* =1.9, 8.8Hz); 11.00 (s, 1H, NH). ^{13}C NMR (Acetone-d_6) δ (ppm) = 11.9; 20.0; 64.3.; 100.7; 11.4; 113.9; 114.5; 127.3; 128.4; 129.6; 130.8; 131.8; 143.2; 148.1; 167.0 MS-ESI (m/z): 269,11[M^-]. Anal. Calcd. For $C_{16}H_{15}NO_3$ C, 71.36; H, 5.61; N, 5.20; O, 17.82 found C, 71.68; H, 5.72; N, 5.20; O, 17.88.

4.6.2 Ethyl 2-(2-fluorophenyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (7*b*)

Yield: 79%. ^{1}H NMR (Acetone-d_6) δ (ppm) = 1.34 (t, 3H, J=7.1Hz); 4.31 (q, 2H, J = 7.1 Hz); 7.19 (dd, 1H, J = 8.4, 10.5Hz); 7.31 (t, 2H, J = 8.1Hz); 7.22 (d, 1H, J = 8.1Hz); 7.64-7.71 (m, 2H); 7.80 (s, 1H); 8.2(dd, 2H J = 6.9, 12.2Hz); 10.39 (s, 1H, NH). ^{13}C NMR (Acetone-d_6) δ (ppm) = 14.6;

61.4; 100.1; 102.8; 111.4; 112.8; 117.1; 123.5; 124.2; 125.2; 126.7; 126.8; 129.4; 129.8; 131.2; 143.0; 147.5; 158.8; 168.2. MS-ESI (m/z): 349,11 [M^-] Anal. Calcd. for $C_{21}H_{16}FNO_3$ C, 72.20; H, 4.62; F, 5.44; N, 4.01; O, 13.74 found C, 73.20; H, 4.68; F, 5.64; N, 4.42; O, 14.02.

4.6.3 Ethyl 2-(2-bromophenyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (7*c*)

Yield: 68%. ^{1}H NMR (Acetone-d_6) δ (ppm) = 1.48 (t, 3H, J=7.5Hz); 4.22 (q, 2H, J = 7.5Hz); 5.09 (s,1H,OH) 7.21 (dd, 1H, J = 8.4, 10.5Hz); 7.39 (t, 2H, J = 8.1Hz); 7.44 (d, 1H, J = 8.1Hz); 7.66-7.70 (m, 2H); 7.8 (s, 1H); 8.4(dd, 2H J = 6.9, 12.2Hz); 11.02 (s, 1H, NH). ^{13}C NMR (Acetone-d_6) δ (ppm) = 15.1; 60.8; 99.7; 101.8; 110.4; 115.3; 120.4; 122.7; 126.0; 126.4; 128.7; 128.9; 130.1; 132.5; 133.9; 140.1; 142.9; 148.8; 167.2. MS-ESI (m/z): 409.03[M^-] Anal. Calcd. for $C_{21}H_{16}BrNO_3$ C, 61.48; H, 3.93; Br, 19.48; N, 3.41; O, 11.70 found C, 62.58; H, 3.91; Br, 19.68; N, 4.21; O, 12.30.

4.6.4 Ethyl 2-(2-chlorophenyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (7*d*)

Yield: 81%. ^{1}H NMR (Acetone-d_6) δ (ppm) = 1.25 (t, 3H, J= 7.2Hz); 4.13 (q, 2H, J = 7.2Hz); 4.50 (s,1H,OH); 7.40-7.51 (m, 2H); 7.55 (t, 2H, J = 8.2Hz); 7.44 (d, 2H, J = 6.23Hz); 7.70 (s, 1H); 8.4(dd, 2H J = 7.2, 10.2Hz); 10.31 (s, 1H, NH). ^{13}C NMR (Acetone-d_6) δ (ppm) = 14.8; 60.9; 98.8; 102.2; 111.4; 112.0; 124.2; 127.0; 127.4; 128.6; 129.4; 129.8; 130.5; 130.9; 133.3; 142.8; 148.7; 166.5 MS-ESI (m/z): 365,08 [M^-] Anal. Calcd. for $C_{21}H_{16}ClNO_3$ C, 68.95; H, 4.41; Cl, 9.69; N, 3.83; O, 13.12 found C, 68.31; H, 4.44; Cl, 9.72; N, 3.92; O, 13.16.

4.6.5 Ethyl 2-(3-bromophenyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (7*e*)

Yield: 79%. ^{1}H NMR (Acetone-d_6) δ (ppm) = 1.34 (t, 3H, J= 7.5Hz); 4.20 (q, 2H, J = 7.5Hz); 5.00 (s,1H,OH); 7.30 (t, 1H, J = 6.4Hz); 7.40 (t, 2H, J = 7.2Hz); 7.54-7.61(m, 2H); 7.68 (s,1H); 7.80 (dd, 2H J = 7.2, 9.2Hz); 11.41 (s, 1H, NH). ^{13}C NMR (Acetone-d_6) δ (ppm) = 13.9; 61.2; 99.4; 102.8; 115.2; 117.4; 128.6; 128.8; 129.7; 129.9; 130.3; 131.9; 132.4; 133.8; 134.1; 134.9; 142.8; 148.1; 167.2 MS-ESI (m/z): 409,03[M^-] Anal. Calcd. for $C_{21}H_{16}BrNO_3$ C, 61.48; H, 3.93; Br, 19.48; N, 3.41; O, 11.70 found C, 61.58; H, 3.89; Br, 19.67; N, 3.45; O, 11.80.

4.6.6 Ethyl 2-(3-chlorophenyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (7*f*)

Yield: 81%. ^{1}H NMR and ^{13}C NMR data are in agreement with those reported in literature [48]

4.6.7 Ethyl 2-(4-chlorophenyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (7*g*)

Yield: 64%. ^{1}H NMR and ^{13}C NMR data are in agreement with those reported in literature [48]

4.6.8 Ethyl 2-(3,4-dichlorophenyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (7*h*)

Yield: 64%. ^{1}H NMR (Acetone-d_6) δ (ppm) = 1.33 (t, 3H, J= 7.5Hz); 4.29 (q, 2H, J = 7.5Hz); 7.51(t, 1H, J= 6.8Hz); 7.60(t, 1H, J= 7.2Hz); 7.62 (d, 1H, J=5.4Hz); 7.68 (d, 1H, J=6.3Hz); 7.72 (s, 1H); 8.0 (s, 1H); 8.30-8.41(m, 2H); 9.22 (s, 1H, NH). ^{13}C NMR (Acetone-d_6) δ (ppm) = 14.8; 61.4; 99.3; 103.2; 110.4; 115.4; 124.8; 127.4; 126.8;128.4; 129.5; 130.7; 133.9; 135.5; 135.9; 143.3; 148.4; 166.0 MS-ESI (m/z): 399,04 [M^-] Anal. Calcd. for $C_{21}H_{15}Cl_2NO_3$ C, 63.02; H, 3.78; Cl, 17.72; N, 3.50; O, 11.99 found C, 62.92; H, 3.84; Cl, 17.82; N, 3.51; O, 11.91.

4.6.9Ethyl 2-(4-bromo-2-fluorophenyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (7*i*)

Yield: 72%. ^{1}H NMR (Acetone-d_6) δ (ppm) = 1.34 (t, 3H, J=7.1Hz); 4.31 (q, 2H, J = 7.1 Hz); 4.82 (s,1H,OH); 7.42 (s, 1H); 7.48-7.61 (m, 4H); 7.70 (s, 1H); 7.79 (dd, 1H, J = 2.2, 10.5Hz); 7.82 (dd, 1H, J = 2.4, 9.9Hz) 9.40 (s, 1H, NH). ^{13}C NMR (Acetone-d_6) δ (ppm) = 14.3; 60.7;100.1; 102.4; 110.0; 112.7; 122.4; 123.4; 124.7; 125.2; 126.3; 127.9; 128.8; 133.3; 134.9; 143.1; 148.9; 161.1; 167.4 MS-ESI (m/z): 427,02 [M^-] Anal. Calcd. for $C_{21}H_{15}BrFNO_3$ C, 58.90; H, 3.53; Br, 18.66; F, 4.44; N, 3.27; O, 11.21 found C, 58.88; H, 3.56; Br, 18.74; F, 4.84; N, 3.38; O, 11.51.

4.6.10Ethyl 2-(5-bromo-2-fluorophenyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate (7*j*)

Yield: 90%. ^{1}H NMR (Acetone-d_6) δ (ppm) = 1.33 (t, 3H, J=7.5Hz); 4.47 (q, 2H, J = 7.5 Hz); 4.98 (s,1H,OH); 7.02 (t, 1H, 8.5Hz); 7.32-7.37 (m, 3H); 7.65 (s, 1H); 7.73 (s, 1H); 8.00 (dd, 1H, J = 1.8, 9.5Hz); 8.10 (dd, 1H, J = 1.9, 8.4Hz) 10.40 (s, 1H, NH). ^{13}C NMR (Acetone-d_6) δ (ppm) = 14.8; 61.2; 99.4; 103.3; 110.7; 115.0; 119.1; 119.9; 124.2; 126.6; 126.9; 127.9; 134.6; 135.5; 144.8; 149.4; 157.7; 166.8 MS-ESI (m/z): 427,02 [M^-] Anal. Calcd. for $C_{21}H_{15}BrFNO_3$ C, 58.90; H, 3.53; Br, 18.66; F, 4.44; N, 3.27; O, 11.21 found C, 58.98; H, 3.66; Br, 18.64; F, 4.54; N, 3.27; O, 11.31.

4.6.11 Ethyl 5-hydroxy-2-(2-methoxyphenyl)-1H-benzo[g]indole-3-carboxylate (7*k*)

Yield: 86%. ^{1}H NMR (Acetone-d_6) δ (ppm) = 1.30 (t, 3H, J=7.8Hz); 3.38 (s,3H); 4.51 (q, 2H, J = 7.5 Hz); 5.01 (s,1H,OH); 6.83-6.88 (m, 2H); 7.12 (t, 1H, 8.5Hz); 7.65 -7.73 (m, 3H); 7.78 (s, 1H); 7.82 (dd, 1H, J = 2.8, 8.8Hz); 7.90 (dd, 1H, J = 2.5, 9.1Hz); 10.01 (s, 1H, NH). ^{13}C NMR (Acetone-d_6) δ (ppm) = 15.1; 57.3; 60.8; 100.1; 102.7; 114.9; 115.7; 120.0; 122.6; 124.4; 126.0; 127.3; 128.1; 129.5; 130.2; 144.8; 147.6; 158.1; 168.3 MS-ESI (m/z): 361,13[M$^-$] Anal. Calcd. for $C_{22}H_{19}NO_4$ C, 73.12; H, 5.30; N, 3.88; O, 17.71 found C, 73.22; H, 5.39; N, 3.95; O, 18.01.

4.6.12 Ethyl 5-hydroxy-2-o-tolyl-1H-benzo[g]indole-3-carboxylate (7*l*)

Yield: 70%. ^{1}H NMR (Acetone-d_6) δ (ppm) = 1.41 (t, 3H, J=7.4Hz); 2.25 (s,3H); 4.16 (q, 2H, J = 7.4 Hz); 5.01 (s,1H,OH); 7.10-7.12 (m, 3H); 7.42-7.49 (m, 3H); 7.61 (s, 1H); 8.31 (dd, 1H, J = 1.9, 7.7Hz); 8.39(dd, 1H, J = 1.5, 7.9Hz) 9.91 (s, 1H, NH). ^{13}C NMR (Acetone-d_6) δ (ppm) = 14.3; 17.9; 61.8; 98.8; 103.2; 111.1; 114.4; 123.7; 127.0; 127.4; 128.5; 129.4; 130.8; 131.9; 142.4; 149.1; 155.4; 168.0 MS-ESI (m/z): 345,14 [M$^-$] Anal. Calcd. for $C_{22}H_{19}NO_3$ C, 76.50; H, 5.54; N, 4.06; O, 13.90 found C, 76.55; H, 5.60; N, 4.12; O, 13.89.

4.6.13 Ethyl 5-hydroxy-2-m-tolyl-1H-benzo[g]indole-3-carboxylate (7*m*)

Yield: 94%. ^{1}H NMR (Acetone-d_6) δ (ppm) = 1.30 (t, 3H, J=6.9Hz); 2.44 (s,3H); 4.23 (q, 2H, J = 6.9 Hz); 4.89 (s,1H,OH); 7.34 (d, 1H, J = 7.9Hz); 7.40 (s,1H); 7.43-7.55(m, 4H); 7.61 (s, 1H); 8.31 (dd, 1H, J = 2.1, 7.9Hz); 8.39(dd, 1H, J = 1.6, 7.9Hz) 11.01 (s, 1H, NH). ^{13}C NMR (Acetone-d_6) δ (ppm) = 15.8; 24.4; 61.8; 102.2; 104.8; 112.0; 116.1; 125.1; 126.5; 128.1; 128.7; 130.3; 131.1; 131.7; 133.2; 139.5; 144.9; 148.3; 171.1 MS-ESI (m/z): 345,14 [M$^-$] Anal. Calcd. for $C_{22}H_{19}NO_3$ C, 76.50; H, 5.54; N, 4.06; O, 13.90 found C, 76.50; H, 5.95; N, 4.08; O, 13.94.

4.6.14 Ethyl 5-hydroxy-2-(3,5-dimethylphenyl)-1H-benzo[g]indole-3-carboxylate (7*n*)

Yield: 81%. ^{1}H NMR (Acetone-d_6) δ (ppm) = 1.31 (t, 3H, J=7.3Hz); 2.40 (s,6H); 4.27 (q, 2H, J = 7.3 Hz); 5.54 (s,1H,OH); 7.10 (s, 1H); 7.30 (s, 2H); 7.41(t, 2H, J=8.4 Hz); 7.70 (s, 1H); 7.91 (dd, 1H, J = 1.2, 7.7Hz); 8.04(dd, 1H, J = 1.8, 7.4Hz) 9.91 (s, 1H, NH). ^{13}C NMR (Acetone-d_6) δ (ppm) = 14.4; 25.5; 25.9; 64.4; 99.8; 101.3; 109.9; 113.8; 123.2; 127.9; 128.2; 129.5; 129.9; 131.5; 132.0;

133.9; 139.6; 139.9; 144.0; 148.1; 166.0 MS-ESI (m/z): 359,15 [M$^-$] Anal. Calcd. for $C_{23}H_{21}NO_3$ C, 76.86; H, 5.89; N, 3.90; O, 13.35 found C, 77.50; H, 5.95; N, 3.88; O, 14.04.

***4.7 General procedure for the microwave irradiated synthesis of Ethyl 5-hydroxynaphtho[1,2-b]furan-3-carboxylate* (8*a-n*)**

1,4-naphthoquinone (1.5equiv.) ZnI_2 (0.7equiv.) and β-keto esters **5** ***a-n*** (1.0 equiv.) were dissolved in dry CH_2Cl_2(6mL) in a 10mL reaction glass vial containing a tiny stirring magnet The vial was sealed tightly with an aluminium-Teflon® crimp top and the mixture was irradiated for 30 min at a pre-selected temperature of 110°C, with an irradiation power of 60W. After the reaction, the vial was cooled to 50°C by gas jet cooling. The crude mixture was portioned between CH_2Cl_2 and saturated solution of sodium chloride (6mL) and the aqueous layer was extracted with methylen chloride (3 x 6mL). The combined organic layer were dried on Sodium sulfate anhydrous, filtered and the solvent was removed under reduce pressure. Then, final crude compounds were purified by flash chromatography over silica gel.

4.7.1 Ethyl 5-hydroxy-2-methylnaphtho[1,2-b]furan-3-carboxylate (**8*a***)

Flash chromatography with hexan/EtOAc (90:10) afforded analytically pure product as 54%.

^{1}H NMR (DMSO-d_6) δ (ppm) = 1.14 (t, 3H, J=7.1Hz); 2.21(s, 3H); 4.41 (q, 2H, J = 7.1 Hz); 5.42(s, 1H, OH); 6.88 (s, 1H); 7.34-7.38 (m, 2H); 7.49 (dd, 2H, J =6.4, 10.2Hz); ^{13}C NMR (DMSO-d_6) δ (ppm) = 8.4; 14.7; 62.4; 102.4; 111.4; 125.2; 128.7; 128.9; 130.9; 131.3; 145.7; 149.4; 149.9; 159.5 MS-ESI (m/z): 270,09 [M$^-$] Anal. Calcd. for $C_{16}H_{14}O_4$ C, 71.10; H, 5.22; O, 23.68 found C, 71.48; H, 5.88; O, 24.0.

4.7.2 Ethyl 2-(2-fluorophenyl)-5-hydroxynaphtho[1,2-b]furan-3-carboxylate (**8*b***)

Flash chromatography with hexan/EtOAc (90:10) afforded analytically pure product as 55%.

^{1}H NMR (DMSO-d_6) δ (ppm) = 1.39 (t, 3H, J=7.4Hz); 4.54 (q, 2H, J = 7.4 Hz); 5.30(s, 1H, OH); 6.75 (s, 1H); 7.31-7.44 (m, 2H); 7.51 (t, 1H, J =6.5Hz); 7.66-7.70 (m, 2H); 7.72 (t, 1H, J =6.9Hz); 7.89 (dd, 2H, J =5.5, 8.4Hz); ^{13}C NMR (DMSO-d_6) δ (ppm) = 15.2; 63.3; 102.4; 109.6; 117.6; 121.1; 124.9; 125.3; 126.8; 127.9; 128.8; 130.0; 130.5; 132.4; 142.9; 150.4; 159.9; 160.0; 169.2

MS-ESI (m/z): 350,1[M^-] Anal. Calcd. for $C_{21}H_{15}FO_4$ C, 71.99; H, 4.32; F, 5.42; O, 18.27 found C, 72.02; H, 4.33; F, 5.68; O, 18.84.

4.7.3 Ethyl 2-(2-bromophenyl)-5-hydroxynaphtho[1,2-b]furan-3-carboxylate (**8*c***)

Column chromatography [elution with hexan/EtOAc (80:20)] afforded analytically pure product as 39%.

^{1}H NMR (DMSO-d_6) δ (ppm) = 1.24 (t, 3H, J=6.5Hz); 4.54 (q, 2H, J = 6.6 Hz); 5.61(s, 1H, OH); 7.11 (s, 1H); 7.32 (t, 1H, J=2.4Hz); 7.55-7.69 (m, 4H); 7.74 (dd, 1H, J =1.5, 7.4Hz); 7.78 (dd, 2H, J =4.5, 8.8Hz); ^{13}C NMR (DMSO-d_6) δ (ppm) = 14.9; 61.7; 102.2; 108.0; 122.5; 123.1; 126.8; 128.5; 130.5; 130.7; 131.4;131.8; 132.2; 133.3; 133.9; 140.8; 158.7; 169.7 MS-ESI (m/z): 410,02 [M^-] Anal. Calcd. for $C_{21}H_{15}BrO_4$ C, 61.33; H, 3.68; Br, 19.43; O, 15.56 found C, 61.45; H, 4.08; Br, 19.44; O, 15.75.

4.7.4 Ethyl 2-(2-chlorophenyl)-5-hydroxynaphtho[1,2-b]furan-3-carboxylate (**8*d***)

Flash chromatography with hexan/EtOAc (80:20)] afforded analytically pure product as 78%.

^{1}H NMR (DMSO-d_6) δ (ppm) = 1.14 (t, 3H, J=6.5Hz); 4.19 (q, 2H, J = 6.6 Hz); 5.88(s, 1H, OH); 7.48(s, 1H); 7.52-7.60 (m, 2H); 7.65-7.71 (m, 2H); 7.74 (dd, 1H, J =1.2, 4.5Hz); 8.01 (dd, 2H, J =3.2, 8.2Hz); ^{13}C NMR (DMSO-d_6) δ (ppm) = 15.5; 66.8 101.7; 107.8; 120.5; 124.6; 127.8; 128.8; 129.5; 130.1; 130.6;131.2; 131.2; 134.3; 136.9; 144.8; 148.7; 159.7; 170.1 MS-ESI (m/z): 366,07 [M^-] Anl. Calcd. for $C_{21}H_{15}ClO_4$ C, 68.76; H, 4.12; Cl, 9.67; O, 17.45 found C, 69.02; H, 4.12; Cl, 9.87; O, 17.55.

4.7.5 Ethyl 2-(3-bromophenyl)-5-hydroxynaphtho[1,2-b]furan-3-carboxylate (**8*e***)

Flash chromatography with hexan/EtOAc (70:30) afforded analytically pure product as 44%.

^{1}H NMR (DMSO-d_6) δ (ppm) = 1.47 (t, 3H, J=7.2Hz); 4.45 (q, 2H, J = 7.2Hz); 5.64 (s, 1H, OH); 7.61(s, 1H); 7.52-7.60 (m, 2H); 7.65-7.71 (m, 2H); 7.74 (s, 1H); 8.01 (dd, 2H, J =3.2, 8.2Hz); ^{13}C NMR (DMSO-d_6) δ (ppm) = 16.3; 62.0, 102.5; 110.4; 122.7; 125.4; 126.0; 127.3; 127.9; 128.5; 129.9; 132.7; 133.0; 135.2; 136.9; 145.5; 150.2; 160.0; 168.8 MS-ESI (m/z): 410,02 [M^-] Anal.

Calcd. for $C_{21}H_{15}BrO_4$ C, 61.33; H, 3.68; Br, 19.43; O, 15.56 found C, 61.86; H, 3.70; Br, 19.54; O, 15.62

4.7.6 Ethyl 2-(3-chlorophenyl)-5-hydroxynaphtho[1,2-b]furan-3-carboxylate (**8*f***)

Flash chromatography with hexan/EtOAc (90:10) afforded analytically pure product as 90%.

^{1}H NMR (DMSO-d_6) δ (ppm) = 1.38 (t, 3H, J=6.9Hz); 4.37 (q, 2H, J = 7.1Hz); 4.83 (s, 1H, OH); 7.59(s, 1H); 7.64 (dd, 1H, J =1.4, 3.2Hz); 7.70-7.85 (m, 4H); 7.90 (s, 1H); 8.32(dd, 2H, J =3.5, 7.2Hz); ^{13}C NMR (DMSO-d_6) δ (ppm) = 15.7; 60.8, 105.4; 109.9; 120.8; 125.6; 127.8; 128.5; 129.4; 130.3; 130.8; 131.1; 132.4; 133.8; 137.1; 144.2; 149.9; 160.7; 169.8 MS-ESI (m/z): 366,07 [M$^-$] Anal. Calcd. for $C_{21}H_{15}ClO_4$ C, 68.76; H, 4.12; Cl, 9.67; O, 17.45 found C, 68.78; H, 4.89; Cl, 9.84; O, 17.45.

4.7.7 Ethyl 2-(4-chlorophenyl)-5-hydroxynaphtho[1,2-b]furan-3-carboxylate (**8*g***)

Flash chromatography with hexan/EtOAc (80:20) afforded analytically pure product as 61%.

^{1}H NMR (DMSO-d_6) δ (ppm) = 1.44 (t, 3H, J=7.8Hz); 4.62 (q, 2H, J = 7.8Hz); 5.11 (s, 1H, OH); 7.50(s, 1H); 7.61-7.72 (m, 4H); 8.01(d, 2H, J =7.1Hz); 8.11 (dd, 1H, J =4.4, 6.2Hz) ^{13}C NMR (DMSO-d_6) δ (ppm) = 17.0; 61.1, 102.3; 110.1; 120.7; 124.1; 128.8; 129.4; 129.8; 130.2; 130.8; 131.2; 131.4; 133.8; 136.7; 144.5; 149.7; 160.0; 170.1 MS-ESI (m/z): 366,07 [M$^-$] Anal. Calcd. for $C_{21}H_{15}ClO_4$ C, 68.76; H, 4.12; Cl, 9.67; O, 17.45found C, 69.01; H, 4.11; Cl, 9.67; O, 17.55.

4.7.8 Ethyl 2-(3,4-dichlorophenyl)-5-hydroxynaphtho[1,2-b]furan-3-carboxylate (**8*h***)

Flash chromatography with hexan/EtOAc (95:05) afforded analytically pure product as 47%.

^{1}H NMR (DMSO-d_6) δ (ppm) = 1.39 (t, 3H, J=6.7Hz); 4.40 (q, 2H, J = 6.8Hz); 5.04 (s, 1H, OH); 7.51(s, 1H); 7.66-7.91(m, 4H); 7.98 (s, 1H); 8.23 (dd, 1H, J =2.9, 7.2Hz) ^{13}C NMR (DMSO-d_6) δ (ppm) = 15.4; 72.9, 104.3; 109.5; 120.8; 125.1; 126.3;127.8; 128.8; 129.1; 129.4; 130.2; 131.4; 135.7; 135.8; 136.0; 145.9; 149.3; 158.0; 169.4 MS-ESI (m/z): 400,03 [M$^-$] Anal. Calcd. for $C_{21}H_{14}Cl_2O_4$ C, 62.86; H, 3.52; Cl, 17.67; O, 15.95 found C, 62.89; H, 4.02; Cl, 17.88; O, 15.96.

4.7.9 Ethyl 2-(4-bromo-2-fluorophenyl)-5-hydroxynaphtho[1,2-b]furan-3-carboxylate (**8*i***)

Flash chromatography with hexan/EtOAc (80:20) afforded analytically pure product as 39%.

^{1}H NMR (DMSO-d_6) δ (ppm) = 1.32 (t, 3H, J=7.9Hz); 4.49(q, 2H, J = 7.7Hz); 7.65(s, 1H); 7.69 (s, 1H); 7.84 (d, 1H, J=5.4Hz); 7.88-7.93(m, 3H); 8.41 (dd, 1H, J =3.5, 9.4Hz) ^{13}C NMR (DMSO-d_6) δ (ppm) = 13.2; 65.0, 101.8; 108.1; 120.4; 122.4; 123.6; 125.8; 126.0; 128.5; 129.4; 130.7; 131.3; 135.7; 136.6; 144.7; 150.9; 159.3; 168.0; 169.4 MS-ESI (m/z): 428,01 [M$^-$] Anal. Calcd. for $C_{21}H_{14}BrFO_4$ C, 58.76; H, 3.29; Br, 18.62; F, 4.43; O, 14.91 found C, 58.82; H, 3.30; Br, 18.65; F, 4.49; O, 14.95

4.7.10 Ethyl 2-(5-bromo-2-fluorophenyl)-5-hydroxynaphtho[1,2-b]furan-3-carboxylate (**8*j***)

Flash chromatography with hexan/EtOAc (70:30) afforded analytically pure product as 51%.

^{1}H NMR (DMSO-d_6) δ (ppm) = 1.34 (t, 3H, J=7.4Hz); 4.37 (q, 2H, J = 7.4Hz); 5.21 (s, 1H, OH); 7.48 (s, 1H); 7.58 (t, 1H, J=7.1Hz); 7.65-7.72 (m, 3H); 7.79(s, 1H); 7.94 (dd, 1H, J =1.8, 9.2Hz) ^{13}C NMR (DMSO-d_6) δ (ppm) = 19.6; 65.4, 100.7; 108.5; 116.0; 117.1; 120.7; 123.5; 125.4; 126.1; 126.4; 127.2; 130.4; 134.8; 144.9; 150.0; 157.9; 159.3; 168.1 MS-ESI (m/z): 428,01 [M$^-$] Anal. Calcd. for $C_{21}H_{14}BrFO_4$ C, 58.76; H, 3.29; Br, 18.62; F, 4.43; O, 14.91 found C, 58.78; H, 3.29; Br, 18.80; F, 4.42 O, 14.93

4.7.11 Ethyl 5-hydroxy-2-(2-methoxyphenyl)naphtho[1,2-b]furan-3-carboxylate (**8*k***)

Flash chromatography with hexan/EtOAc (90:10 to 80:20) afforded analytically pure product as 55%. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.26 (t, 3H, J=7.6Hz); 3.85 (s, 3H); 4.35 (q, 2H, J = 7.7Hz); 6.00(s, 1H, OH); 6.90 (s, 1H); 7.05 (d, 1H, J=5.5Hz); 7.10 (t, 1H, J=5.8Hz); 7.55-7.70 (m, 3H); 8.12 (dd, 2H, J =2.1, 9.8Hz); ^{13}C NMR (DMSO-d_6) δ (ppm) = 14.4; 55.5; 60.8; 103.6; 108.9; 115.5; 115.9; 121.7; 125.8; 126.3; 128.1; 129.0;131.4; 144.2; 148.9; 157.7; 161.0; 172.7; MS-ESI (m/z): 362,12 [M$^-$] Anal. Calcd. for $C_{22}H_{18}O_5$ C, 72.92; H, 5.01; O, 22.08 found C, 72.94; H, 5.15; O, 22.14

4.7.12 Ethyl 5-hydroxy-2-o-tolylnaphtho[1,2-b]furan-3-carboxylate (**8*l***)

Flash chromatography with hexan/EtOAc (90:10) afforded analytically pure product as 47%. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.39 (t, 3H, J=6.9Hz); 2.28(s, 3H); 4.48 (q, 2H, J = 7.2Hz); 5.02 (s, 1H, OH); 7.02 (s, 1H); 7.21- 7.28 (m, 3H); 7.40-7.45 (m, 3H); 7.72 (dd, 2H, J =3.5, 10.1Hz); ^{13}C

NMR (DMSO-d_6) δ (ppm) = 14.4; 19.0; 61.2;108.5; 122.6; 125.4; 127.9;128.2; 128.7; 128.9; 129.9; 130.7; 131.1; 131.5; 133.8; 136.9; 146.0; 149.0; 159.3; 169.3 MS-ESI (m/z): 346,12 [M^-] Anal. Calcd. for $C_{22}H_{18}O_4$ C, 76.29; H, 5.24; O, 18.48 found C, 76.33; H, 5.78; O, 18.58.

4.7.13 Ethyl 5-hydroxy-2-m-tolylnaphtho[1,2-b]furan-3-carboxylate (**8*m***)

Flash chromatography with hexan/EtOAc (90:10) afforded analytically pure product as 66%. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.29 (t, 3H, J=7.2Hz); 2.40 (s, 3H); 4.55 (q, 2H, J = 7.2Hz); 5.49 (s, 1H, OH); 7.00 (s, 1H); 7.12(d, 1H, J=5.5Hz); 7.39 (t, 1H, J=6.1Hz); 7.45- 7.57(m, 4H); 7.81 (dd, 2H, J =1.9, 8.8Hz); ^{13}C NMR (DMSO-d_6) δ (ppm) = 15.5; 29.3; 67.4; 102.7; 108.5; 121.2; 125.4; 125.6; 127.5; 127.9; 130.1; 130.3; 130.9; 131.9; 132.2; 139.4; 144.4; 149.7; 155.5; 172.1 MS-ESI (m/z): 346,12 [M^-] Anal. Calcd. for $C_{22}H_{18}O_4$ C, 76.29; H, 5.24; O, 18.48 found C, 76.30; H, 5.25; O, 18.49.

4.7.14 Ethyl 5-hydroxy-2-(3,5-dimethylphenyl)naphtho[1,2-b]furan-3-carboxylate (**8*n***)

Flash chromatography with hexan/EtOAc (80:20) afforded analytically pure product as 51%. ^{1}H NMR (DMSO-d_6) δ (ppm) = 1.38 (t, 3H, J= 6.8Hz); 2.21 (s, 3H); 2.28 (s, 3H); 4.49 (q, 2H, J = 7.1Hz); 6.88 (s,1H); 7.03 (s, 1H); 7.28(s, 2H); 7.36 - 7.40(m, 2H); 7.68 (dd, 2H, J =2.5, 9.1Hz); ^{13}C NMR (DMSO-d_6) δ (ppm) = 14.4; 25.8; 26.2; 121.8; 125.9; 126.7; 128.4; 128.6; 129.3; 129.5; 130.2; 130.7; 140.2; 141.0; 149.5; 160.4; 170.4 MS-ESI (m/z): 360,14 [M^-] Anal. Calcd. for $C_{23}H_{20}O_4$ C, 76.65; H, 5.59; O, 17.76 found C, 76.68; H, 6.01; O, 17.87.

4.8 Biological evaluation and assay systems

4.8.1. Materials

Zileuton was purchased from Sequoia Research Products (Oxford, UK), zymosan from Sigma (Milan, Italy) and LTC_4 enzyme immunoassay (EIA) from Cayman Chemical, (Inalco, Milan, Italy).

4.8.2. Cells and isolation

Neutrophils were freshly isolated from leukocyte concentrates obtained from the Institute of Transfusion Medicine, University Hospital Jena as described [47]. Briefly, human peripheral blood was collected in heparinized tubes (16 I.E. heparin/mL blood) by venipuncture from fasted (12 h) adult healthy volunteers, with consent, and leukocyte concentrates were prepared by centrifugation (4000*g*×mg, 20 min, 20 °C). The subjects had no apparent inflammatory conditions and had not taken anti-inflammatory drugs for at least ten days prior to blood collection. Neutrophils were immediately isolated by dextran sedimentation and centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria) and hypotonic lysis of erythrocytes was performed as described [47]. Neutrophils were finally resuspended in PBS pH 7.4 containing 1 mg/Ml glucose and 1 mM $CaCl_2$ (PGC buffer) (purity > 96-97%).

4.8.3. Animals

Male ICR mice 8 weeks old (Envigo), were housed 5 per cage under controlled illumination (12:12 h light:dark cycle; light on 06.00 h) and standard environmental conditions (room temperature 22 ± 1 C, humidity 60 ± 10%) for at least 1 week before experimental use. Mouse chow and tap water were available ad libitum. The experimental procedures were approved by the Animal Ethics Committee of the University of Campania, Naples. Animal care was in compliance with the IASP and European Community (E.C. L358/1 18/12/86) guidelines on the use and protection of animals in experimental research. All efforts were made to minimize animal suffering and to reduce the number of animals used.

4.8.4. Mouse paw oedema

Oedema was induced by carrageenan injection (20 mL/paw, 2% w/v in saline) into the plantar surface of the right hind paw. The paw volume was measured using a hydroplethysmometer (Ugo Basile, Varese, Italy) before treatment and at 2-4-6 h following carrageenan. Mice (n =6 per group) were treated with 4b and 4c or 7i, 7h, 7j or 7m, 7c and 7e (4 mg/kg, i.p.), or vehicle (DMSO), 30 min before the carrageenan injection.

4.8.5. 5-LO activity assays

For analysis of 5-LO products in intact cells, neutrophils (5×10^6) were resuspended in 1 mL PGC buffer, preincubated for 15 min at 37 °C with test compounds or vehicle (0.1% DMSO) and Ca^{2+}-ionophore A23187 (2.5 µM) plus 20 mM arachidonic acid was added. After 10 min at 37 °C the reaction was stopped on ice by addition of 1 mL of methanol. 30 ml 1 N HCl and 500 µl PBS, and 200 ng prostaglandin (PG)B1 were added and the samples were subjected to solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). 5-LO products (LTB_4 and its trans-isomers, and 5-H(P)ETE) were analyzed by RP-HPLC and quantities calculated on the basis of the internal standard PGB_1. Cys-LTs C_4, D_4 and E_4 were not detected (amounts were below detection limit), and oxidation products of LTB_4 were not determined.

For analysis of 5-LO activity in cell-free assays, E. coli BL21 cells were transformed with pT3-5-LO plasmid (provided by Dr. Olof Radmark, Karolinska Institute, Stockholm, Sweden), human recombinant 5-LO protein was expressed and purified on an ATP-agarose column as described previously [28]. Aliquots of semi-purified 5-LO (0.5 µg) were diluted with ice-cold PBS containing 1 mM EDTA, and 1 mM ATP was added (final volume was 1 mL). Samples were preincubated with the test compounds or vehicle (0.1% DMSO) as indicated. After 15 min at 4 °C, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$ plus 20 µM arachidonic acid were added to initiate 5-LO product formation. After 10 min at 37 °C, the reaction was stopped by addition of 1 mL ice-cold methanol, and the formed metabolites were analyzed by RP-HPLC as described [48]. 5-LO products include the all-trans isomers of LTB4 and 5-H(P)ETE.

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Highlights

Molecular modification and synthetic optimitation process aiming to improve 5-LO and m-PGES-1 inhibition.

Further developments of multi-target inhibitors of the inflammatory pathway

Docking studies and molecular dynamic (MD) simulations with mPGES-1 and 5-LO of selective active compounds

in vivo anti-inflammatory evaluation