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Exploring the 2′-Hydroxy-Chalcone Framework for the Development of Dual Antioxidant and Soybean Lipoxygenase Inhibitory Agents

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Abstract: 2′-Hydroxy-chalcones are naturally occurring compounds with a wide array of bioactivity. In an effort to delineate the structural features that favor antioxidant and lipooxygenase (LOX) inhibitory activity, the design, synthesis, and bioactivity profile of a series of 2′-hydroxy-chalcones bearing diverse substituents on rings A and B, are presented. Among all the synthesized derivatives, chalcone 4b, bearing two hydroxyl substituents on ring B, was found to possess the best combined activity (82.4% DPPH radical scavenging ability, 82.3% inhibition of lipid peroxidation, and satisfactory LOX inhibition value (IC50 = 70 µM). Chalcone 3c, possessing a methoxymethylene substituent on ring A, and three methoxy groups on ring B, exhibited the most promising LOX inhibitory activity (IC50 = 45 µM). A combination of in silico techniques were utilized in an effort to explore the crucial binding characteristics of the most active compound 3c and its analogue 3b, to LOX. A common H-bond interaction pattern, orienting the hydroxyl and carbonyl groups of the aromatic ring A towards Asp768 and Asn128, respectively, was observed. Regarding the analogue 3c, the bulky (-OMOM) group does not seem to participate in a direct binding, but it induces an orientation capable to form H-bonds between the methoxy groups of the aromatic ring B with Trp130 and Gly247.

Keywords: chalcones; aurones; butein; sulfuretin; antioxidant activity; lipoxygenase inhibition; molecular docking

1. Introduction
Chalcones (1,3-diaryl-2-propan-1-ones) (Figure 1) constitute a large and important group of bioactive compounds belonging to the flavonoid family. They possess an important role in medicinal chemistry since they are privileged scaffolds used as lead compounds for the discovery of new drugs. Thus, the isolation from natural sources (plants, flowers, fruits, vegetables etc.), as well as the organic synthesis of chalcones have been intensified during the last decades due to their wide spectrum of bioactivity. The presence of a α,β-unsaturated carbonyl system in chalcone derivatives enable them to exhibit a wide range of biological activities such as antioxidant, anti-inflammatory, [1], anticancer [2], antibacterial [3], antimalarial [4], tyrosinase inhibitory activity [5], anti-HIV [6], anti-leishmanial [7], while they are also potential neuroprotective agents [8,9]. The chalcone derivatives which
possess hydroxyl and methoxy substitution on aromatic rings A and B have demonstrated potential anti-leishmanial and anti-trypanosomal activities by acting on a number of molecular targets. Moreover, free hydroxyl groups are essential to increase their activity (such as antioxidant and antimicrobial) while methoxy groups decrease it. Regarding antitumor activity, it is possibly positively affected in the presence of methoxy substitutions on the aromatic ring [10].

Another flavonoid subclass, aurones ((Z)-2-benzylidenebenzofuran-3-(2H)-ones) (Figure 1) are oxidative cyclization products of chalcones that rarely appear in nature and are less studied than chalcones. So far aurones have been exploited via isolation from flowering plants [11,12], invasive weeds [13], mosses [14], and marine brown algae [15], via organic synthesis [16,17] and biosynthesis from chalcone precursors by the key enzyme aureusidin synthase [18]. Sulfuretin [19] and aureusidin [20] consist representative naturally occurring aurones, bearing different hydroxylation patterns, while some methoxy-aurones also occur in nature [21,22]. Aurones constitute promising bioactive compounds exhibiting among others, antioxidant [17], antifungal [23], anticancer [24], antimicrobial [25], anti-leishmanial [16], antimalarial [26], anti-inflammatory activity [27], and consist potential effective agents for the treatment of Alzheimer’s disease [28]. Consequently, during the last decades, there is an increase in publications concerning their isolation, synthesis, and bioactivity evaluation.

In this work, among the synthesized compounds the synthesis of two naturally occurring chalcones and aurones, such as butein (4a) and sulfuretin (7a), is also presented. Butein (2′,4′,3,4-tetrahydroxy-chalcone) occurs in many unrelated genera including Butea Dahlia, Coreopsis, and Searsia and has been isolated in good yields [29]. Chemically, butein has been synthesized through various organic synthetic methods including microwave irradiation in the presence of PdCl2(PPh3)2, ammonium formate in THF [30], aldol condensation catalyzed by the novel acid catalyst thionyl chloride (SOCl2)/ethyl alcohol (EtOH) [31], Claisen–Schmidt condensation between protected 2,4-di-hydroxyacetophenone and 3,4-dihydroxybenzaldehyde in the presence of basic catalyst, and subsequent removal of the protecting groups [30,32].

Among naturally occurring aurones, sulfuretin (6,3′,4′-tri-hydroxyaurone) is known to have anti-inflammatory effects and has been isolated among others from the heartwood of Rhus verniciflua, Bidens, Cosmos, and Dahlia flowers [33,34]. Furthermore, the total synthesis of sulfuretin has been exploited by scientists starting by the chemical synthesis of a 3-coumaranone, obtained by gold(I)-catalyzed oxidative cyclization of an o-ethynylanisole and its condensation with 3,4-dimethoxybenzaldehyde in basic conditions, followed by demethylation with BBr3 [35]. To our knowledge, the synthesis of sulfuretin has not been previously achieved using the oxidative cyclization of the OMOM protected butein precursor.

Natural and synthetic chalcones have shown to possess strong radical scavenging potential, while some of them, such as 2,3,4,6-tetrahydroxychalcone and butein (2′,4′,3,4-tetrahydroxy-chalcone), exhibit more potent radical-scavenging activity than reference antioxidants such as vitamin C or α-tocopherol [36,37]. It is considered that hydroxyl
groups consist key moieties which can enhance the antioxidant activity of chalcones [23,38]. Aurones have been less studied than chalcones concerning their antioxidant and anti-inflammatory activity whereas their ability to inhibit soybean lipooxygenase (LOX) and histone deacetylase (HDAC) have been recently reported [27,39–41].

The combined anti-inflammatory and antioxidant activity of novel drugs could be an asset for the treatment of several diseases and several natural and synthetic antioxidants have been shown to possess potent anti-inflammatory properties [42,43]. In this framework, and as a continuation of our studies towards the synthesis and biological evaluation of novel bioactive compounds [16,17,44,45], we present herein the synthesis and structure identification of synthetic and naturally occurring 2′-hydroxy-chalcones and selected aurones. Among the 2′-hydroxy-chalcones series (3a–3l) and aurones (6a–6b and 7a–7b), the chalcones 3c, 3h, 4b, 5 and the aurone 6a are presented, to our knowledge, for the first time in the literature. The novel chalcones were prepared via Claisen–Schmidt condensation in alkaline media (Method A: 20% w/v KOH, EtOH, Method B: 60% w/v NaH, DMF). Chalcones 3f–3g and 3i–3l, were prepared through the Claisen–Schmidt condensation reaction between appropriately substituted 2′-hydroxy-acetophenones and benzaldehydes carried out using aqueous KOH in ethanol, as previously reported in the literature [41,46–49]. Aurones 6b and 7b were synthesized via oxidative cyclization of the corresponding chalcones using mercury (II) acetate in pyridine followed by deprotection of the methoxylated aurone using BBr3 in CH2Cl2, respectively. The synthesis of aurones 6b and 7b has been previously presented [50,51] via Knoevenagel condensation of benzofuranone with the appropriate benzaldehyde using polyphosphoric acid (PPA).

The synthesized compounds were evaluated in vitro for their antioxidant activity as well as their ability to inhibit soybean LOX. Structure/activity relationship and in silico studies were undertaken, attempting to discover the structural characteristics of compounds 3b and 3c that contribute to their LOX inhibitory activity.

2. Results and Discussion

2.1. Chemistry

First, 2-Hydroxy-4-(methoxymethoxy)-acetophenone 1a and 3,4-bis(methoxymethoxy)-benzaldehyde 2a were synthesized from the corresponding hydroxy-compounds using MOM-Cl and K2CO3 in acetone. Further, 2′-hydroxy-acetophenones 1b–1e and benzaldehydes 2b–2h were commercially available.

Chalcones 3a–3l were obtained in 22–85% yields via Claisen–Schmidt condensation of 2-hydroxy-acetophenones 1a–1e with appropriately substituted benzaldehydes 2a–2h in basic conditions (Scheme 1).

![Scheme 1](https://example.com/scheme1.png)

**Scheme 1.** Synthetic procedure of 2′-hydroxychalcones 3a–3l.
The hydroxylated chalcones 4a and 4b were prepared by deprotection of the corresponding chalcones 3a and 3h, respectively, after removal of the methoxymethyl-protecting group, using 10% aqueous HCl in methanol. Chalcone 5 was synthesized by Fischer esterification of the corresponding chalcone 3k in methanol, using H₂SO₄ as a catalyst (Scheme 2).

Furthermore, aurones 6a and 6b were obtained in moderate yields (60 and 74% respectively) via the oxidative cyclization of the corresponding chalcones 3a and 3b, using mercury(II) acetate in pyridine as has been described in detail in one of our previous publications [17]. Aurone 7a was prepared by deprotection of the corresponding aurone 6a, by removing the methoxymethyl-protecting group using 10% aqueous HCl in MeOH, whereas aurone 7b was prepared by deprotection of the methoxylated aurone 6b using BBr₃ in CH₂Cl₂ as the deprotecting agent (Scheme 3).
The structural characterization of the synthesized compounds was confirmed by $^1$H- and $^{13}$C-NMR, MS/ESI (+/−), and HRMS spectroscopy (for those compounds that have not been reported in the literature).

The $^1$H-NMR spectra of $2'$-hydroxy-chalcones are characterized by a signal at a low field ($\approx 13$ ppm) attributed to the proton of the $2'$-OH group which is deshielded as a result of taking part in a strong intramolecular hydrogen bond with the neighboring carbonyl group, and the pair of AB doublets with characteristic $J$ values of 15.0–17.0 Hz, confirming the E-geometry of the double bond. In the $^1$H-NMR spectra of chalcones possessing methoxy group on position 2 (chalcones 3b, 3c, 3e), a significant downfield shift of the AB doublets is observed for the vinyl protons which appear at 8.01–8.21 ppm (for Hb) and 7.54–7.92 ppm (for Ha) as compared to the same signals in the spectra of the chalcones (3a–3d, 3f–3i, 3k, 3l, 4a, 4b) without substitution at position 2 (7.68–8.05 ppm for Hb and 7.45–7.87 ppm for Ha, respectively). This can be attributed to the ‘ortho’ effect of the methoxy group. In the $^1$H-NMR spectra of the synthesized aurones, the most characteristic peak is the singlet owed to the exocyclic vinylic proton at $\delta$ 6.78–7.50 ppm for the substituted on ring A. The presence of a substituent on position $2'$ in aurones 6b and 7b causes a downfield shift of the vinylic proton signal at 7.78 ppm and 7.74 ppm, respectively. This difference in chemical shifts is also attributed to the ‘ortho’ effect [17].

2.2. In Silico Determination of Lipophilicity as ClogP

Lipophilicity is an important physicochemical property related to the biological activity and ADME properties. We theoretically calculated the lipophilicity values of all compounds as ClogP using the CLOGP Program of Biobyte Corp. [52].

2.3. Bioactivity Assessment

In this work, we evaluated in vitro the bioactivity of a series of chalcones and aurones that were expected to offer protection against inflammation and free radical attack.

Free radicals, which are generated during the biochemical function of all aerobic organisms, are very reactive species that can attack and damage biological targets such as lipids and DNA. Normally, the endogenous antioxidant mechanisms of the organisms (redox enzymes and small molecules such as vitamins) scavenge the free radicals, however these mechanisms are not effective enough when excessive free radical production occurs. In this case, exogenous antioxidants have to be used in order to prevent damage caused by oxidative stress. Antioxidants are defined as substances that even at low concentration, significantly delay or prevent oxidation of easily oxidizable substrates [53,54].

There is an increased interest of using antioxidants for medicinal purposes in the recent years, as it is widely accepted that free radicals play a crucial role in many pathological conditions such as inflammation, cancer, and neurodegenerative disorders [55–58]. Moreover, several nonsteroidal anti-inflammatory agents have been reported to act either as inhibitors of free radical production or as radical scavengers [59]. Consequently, compounds possessing antioxidant properties could be expected to offer protection in inflammation and to lead to potentially effective drugs.

In order to estimate the antioxidant activity of a compound, different experimental approaches are used. In this way, factors such as solubility or steric hindrance, which may be of overriding importance in one environment but not in another, can be varied and the antioxidant ability of a compound in a variety of milieus may be evaluated. Most of them require a spectrophotometric measurement and a certain reaction time in order to obtain reproducible results [60].

In this context, the in vitro antioxidant capacity of the synthesized chalcones and aurones (Table 1) was monitored in terms of: (a) the interaction with the stable free radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH); (b) the anti-lipid peroxidation activity; (c) the hydroxyl radical scavenging ability; and (d) the 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation (ABTS•+) reduction-decolorization ability.
Table 1. Substituents of all the synthesized chalcone and aurone derivatives.

| Compound | R₁ | R₂ | R₃  | R₄  | R₅  | R₆  | R₇  |
|----------|----|----|-----|-----|-----|-----|-----|
| 3a       | H  | OMOM | H   | H   | OMOM | OMOM | H   |
| 3b       | H  | H   | H   | OCH₃ | H   | OCH₃ | OCH₃ |
| 3c       | H  | OMOM | H   | OCH₃ | H   | OCH₃ | OCH₃ |
| 3d       | H  | H   | Cl  | H   | OCH₃ | OCH₃ | H   |
| 3e       | H  | H   | Cl  | OCH₃ | OCH₃ | H   | H   |
| 3f       | H  | H   | Br  | H   | OCH₃ | OCH₃ | H   |
| 3g       | Br | H   | Br  | H   | OCH₃ | OCH₃ | H   |
| 3h       | H  | H   | Br  | H   | OMOM | OMOM | H   |
| 3i       | Br | H   | Br  | H   | OMOM | OMOM | H   |
| 3j       | H  | H   | H   | H   | H   | COOH | H   |
| 3k       | H  | H   | Cl  | H   | H   | COOH | H   |
| 3l       | H  | H   | H   | H   | H   | H   | H   |
| 4a       | H  | OH  | H   | H   | OH  | OH  | H   |
| 4b       | H  | H   | Br  | H   | OH  | OH  | H   |
| 5        | H  | H   | H   | H   | H   | COOCH₃ | H |

The first assay refers to the interaction of the compounds with the stable free radical DPPH and it is a fast, simple, cost-effective, and widely used method, where the DPPH radical reacts directly with the antioxidant and is decolorized. This interaction indicates their radical scavenging ability in an iron-free system. The scavenging effect of the synthesized analogues on the DPPH radical was evaluated according to the methods of Hadjipavlou et al. [61].

The second technique is based on the ability of the compounds to inhibit lipid peroxidation of linoleic acid induced by 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH)
radical (Table 2). AAPH-induced linoleic acid oxidation has been developed as a quick and reliable method for measuring the antioxidant activity. The thermal free radical producer (AAPH) generates free radicals in the solution which cause the oxidation of linoleic acid, and the method is a measure of how effective antioxidants protect against lipid peroxidation in vitro. Oxidation of linoleic acid by AAPH is followed by UV spectrophotometry in a highly diluted sample [61].

Table 2. In vitro antioxidant evaluation of the synthesized compounds, through the DPPH scavenging ability and the inhibition of linoleic acid assays, as well as antioxidant activity of selected analogues via their ability to scavenge hydroxyl radicals and the ABTS radical cation decolorization assay.

| Compound | Interaction with the Free Radical DPPH (%) | Inhibition of Lipid Peroxidation of Linoleic Acid Induced by AAPH Radical (%) 100 µM | HO (%) 100 µM | ABTS+ % 100 µM |
|----------|------------------------------------------|----------------------------------------------------------------------------------|---------------|----------------|
| 3a       | 18.9                                     | 11.4                                                                             | 40            | 93             |
| 3b       | no                                       | 67.3                                                                             | no            | 4              |
| 3c       | no                                       | 39.4                                                                             | *             | *              |
| 3d       | no                                       | no                                                                                | *             | *              |
| 3e       | no                                       | 20.0                                                                             | *             | *              |
| 3f       | no                                       | no                                                                                | *             | *              |
| 3g       | no                                       | 31.0                                                                             | *             | *              |
| 3h       | 5.3                                      | 65.6                                                                             | *             | *              |
| 3i       | no                                       | 93.0                                                                             | *             | *              |
| 3j       | no                                       | no                                                                                | *             | *              |
| 3k       | no                                       | no                                                                                | *             | *              |
| 3l       | no                                       | 87.6                                                                             | *             | *              |
| 4a       | 95.7                                     | 24.4                                                                             | 68            | 86             |
| 4b       | 82.4                                     | 82.3                                                                             | 100           | no             |
| 5        | no                                       | 100                                                                               | *             | *              |
| 6a       | no                                       | 18.5                                                                             | 43            | 86             |
| 6b       | no                                       | 29.4                                                                             | no            | 64             |
| 7a       | 89.0                                     | 53.2                                                                             | no            | 71             |
| 7b       | 100                                      | 13.4                                                                             | 77            | no             |
| NDGA     | 87.0                                     |                                    |               |                |
| Trolox   | 93.0                                     | 92.0                                                                             | 82            | 93             |

no: no action under the experimental conditions; *: not tested.

Among the reactive oxygen species (ROS), the hydroxyl (•OH) free radical is possibly the most toxic, reacting with several biological important molecules such as DNA, lipids, or carbohydrates. Polyunsaturated fatty acids are particularly vulnerable by free radicals. Iron released from damaged cells is more likely to be readily available to catalyze the generation of •OH radicals. Thus, we tried to test the ability of our compounds to scavenge hydroxyl radicals determining the competition of selected chalcones and aurones with DMSO for hydroxyl radicals. Hydroxyl radicals were generated using the Fe3+/ascorbic acid system and expressed as a percentage inhibition of formaldehyde production in the presence of each compound at 100 µM.

The ABTS radical cation decolorization assay was used as another antioxidant activity estimation protocol (Table 2). Oxidation of ABTS with potassium persulfate generates directly the radical with no involvement of an intermediate radical, which is then reduced upon adding electron-donating antioxidants.

Furthermore, as an indication of their anti-inflammatory activity, all the molecules were tested as inhibitors of soybean lipoxygenase (LOX). The enzyme lipoxygenase cat-
analyzes the first two steps in the metabolism of arachidonic acid, which is cleaved from membrane phospholipids to leukotrienes (LTB4). LTB4 generation is considered to be important in the pathogenesis of neutrophil-mediated inflammatory diseases. For this study, the UV absorbance-based soybean LOX assay was used, which is a plant enzyme with satisfactory homology with the human 5-LOX [61–66].

2.3.1. Antioxidant Activity

The results of the DPPH scavenging ability of all the synthesized compounds (Table 2) revealed that, for the active analogues, the result was not time dependent (from 20 to 60 min). In the DPPH assay, the basic chemical reaction involved is the reduction of the DPPH radical by a single electron transfer (ET) from the antioxidant. Particularly effective antioxidants via this mechanism are the phenoxide anions derived from phenolic compounds, like catechol and derivatives, such as Nordihydroguaiaretic acid (NDGA). Chalcone analogues 3a and 3f–3i, which possess -OCH3 or -OMOM substituents at ring B, seem either to be totally inactive (3f, 3g, 3i) in scavenging the DPPH radical, or to possess weak activity (3a, 3h); whereas their hydroxylated analogues 4a and 4b exhibit significant interaction with the radical showing DPPH radical scavenging ability (95.7% and 82.4%, respectively).

The evaluation of the antioxidant activity of aurone derivatives via the DPPH method shows that aurones 6a and 6b exhibit no interaction with the radical, whereas their hydroxylated analogues, sulfuretin (7a) and aurone 7b, are potent DPPH scavengers with 89% and 100% inhibition, respectively. NDGA was used as a reference compound (Table 2).

All the synthesized chalcones possess a phenolic hydroxyl group at position 2′ of the aromatic ring A. However, this OH cannot effectively interact with the DPPH radical as it is involved in a strong hydrogen bond with the adjacent carbonyl oxygen. The antioxidant activity of 2′-hydroxy-chalcones arises mainly from the other substituents on rings A and B, as was also observed in our previous work regarding quinolinone-chalcone hybrids [67].

Furthermore, it seems that the presence of bromo-substituent in chalcone derivatives, in combination with the presence of hydroxyl groups, enhances the antioxidant activity of the compounds. The chalcones 4a and 4b exhibit high antioxidant activity regarding the DPPH assay (95.7% and 82.0%, respectively), while the 4b derivative, which possesses one bromo-substituent at position 5′ of the aromatic ring A, shows significantly higher lipid peroxidation inhibitory activity (82.3%), than the 4a analogue, which is almost inactive (24.4%). The insertion of the Br substituent renders the chalcone 4b more lipophilic (ClogP 3.14) than chalcone 4a (ClogP 1.65) and this can possibly explain the enhanced lipid peroxidation inhibitory activity of 4b. Aurones 6a and 6b did not show any DPPH radical scavenging ability, exactly as their chalcone precursors 3a and 3b.

Sulfuretin (7a) showed potent DPPH scavenging activity (89.0/91.2%) while the 2,4,5-trihydroxy aurone 7b was one of the most active compounds in this assay, showing 100% inhibition.

Lipophilicity as ClogP values does not seem to play any significant role in the DPPH scavenging ability of the compounds (Table 5).

As far as the lipid peroxidation inhibitory ability of the tested chalcones is concerned, it seems that the presence of bromo-substituents enhances anti-lipid peroxidation. Chalcone 3h, which bears two -OMOM substituents at positions 3 and 4 of ring B and 3,4-dihydroxychalcone derivative 4b, which both possess a bromo-substituent at position 5′, exhibit satisfactory inhibitory activity of 65.6% and 82.3%, respectively. The introduction of one more bromo-substituent at position 3′, leads to compound 3i, which is strong inhibitor of lipid peroxidation (93%). However, chalcones 3f and 3g, which also possess bromo-substituents, are inactive against lipid peroxidation. This is maybe attributed to the existence of two methoxy groups at the aromatic ring B, which seem to lead to inactive inhibitors, as it also happens in the case of chalcones 3d and 3e. Furthermore, according to Table 2, it seems that the presence of a chloro-substituent at the aromatic ring A of chalcone moiety leads to inactive molecules. Among all the evaluated chalcones, compound 5 with a
methoxy-carbonyl substituent at aromatic ring B showed the best inhibitory activity (100%). The studied aurones exhibited low activity overall, with the exception of aurone 7b which showed a moderate lipid peroxidation inhibition of 53.2%. With some exceptions anti-lipid peroxidation goes in parallel to lipophilicity values.

The % values for DPPH scavenging activity and AAPH anti-lipid peroxidation activity in relation to time, for the most potent compounds are given in Tables 3 and 4. Both activities seem to be constantly high during the 10 min of observation and measurement. These results verify the strong antioxidant capacity of the tested compounds, which is exhibited immediately after their addition to the assay medium (t = 0 min) and rapidly reaches a plateau at t = 9 min.

Table 3. In vitro % values for DPPH scavenging activity of the most potent compounds in relation to time.

| Compound | Interaction with the Free Radical DPPH (%) |
|----------|------------------------------------------|
|          | 0 min | 3 min | 6 min | 9 min |
| 4a       | 95.4  | 95.4  | 94.9  | 95.4  |
| 4b       | 100   | 100   | 100   | 100   |
| 7a       | 92.3  | 94.4  | 94.9  | 94.9  |
| 7b       | 98.2  | 99.3  | 99.5  | 99.8  |

Table 4. In vitro % values for AAPH anti-lipid peroxidation activity of the most potent compounds in relation to time.

| Compound | Inhibition of Lipid Peroxidation of Linoleic Acid Induced by AAPH Radical (%) |
|----------|-----------------------------------------------------------------------------|
|          | 0 min | 3 min | 6 min | 9 min |
| 3i       | 95.6  | 98.6  | 96.9  | 99.2  |
| 3l       | 87.6  | 93.2  | 95.7  | 94.3  |
| 4b       | 81.8  | 82.6  | 82.5  | 81.5  |
| 5        | 95.4  | 96.9  | 92.5  | 98.9  |

Representative compounds were selected for the competition with DMSO for hydroxyl radicals assay (Table 2). The best activity was shown by chalcone 4b whereas aurone 7b, with a 2′,4′,5′-trihydroxy-substitution pattern on ring B, follows. The presence of a hydroxyl substituent at ring A of the aurone scaffold seems to affect this type of activity: the natural product sulfuretin (7a), with a 5-OH at ring A and a catecholic moiety at ring B was inactive in this assay. Chalcone 4a, the precursor of sulfuretin (7a) shows 68% competition with DMSO for hydroxyl radicals. The highly oxygenated aurone 6a presents moderate activity, equal to its precursor, chalcone 3a (43 and 40%, respectively). Trolox was used as a reference compound (Table 2).

In the ABTS radical cation (ABTS**) decolorization assay (Table 2), the tested chalcones showed better activity than the corresponding aurones (compare 3a with 6a and 4a with 7a), with the exception of the 2′,4′,5′-trimethoxy chalcone 3b and chalcone 4b, which were both inactive, whereas aurone 6b showed 64% activity. Trolox was used as a reference compound (Table 2).

2.3.2. Soybean LOX Inhibitory Activity

In the present series of compounds, chalcones were generally found to possess significantly higher LOX inhibitory activity than aurones (Table 5). This is in accordance with our previous study [17].
Table 5. In vitro determination of soybean inhibition; theoretically calculated lipophilicity as ClogP values.

| Compound | Inhibition of Soybean Lipoxygenase 100 µM (%) or IC₅₀ µM | ClogP $^*$ |
|----------|------------------------------------------------------|-----------|
| 3a       | (40.2% at 100 µM) #                                   | 2.30      |
| 3b       | IC₅₀ = 75 µM                                        | 3.48      |
| 3c       | IC₅₀ = 45 µM                                        | 3.08      |
| 3d       | (32.0% at 100 µM) #                                 | 4.49      |
| 3e       | IC₅₀ = 100 µM                                       | 4.69      |
| 3f       | (28.0% at 100 µM) #                                 | 4.64      |
| 3g       | IC₅₀ = 68.5 µM                                      | 5.33      |
| 3h       | IC₅₀ = 55.0 µM                                      | 3.86      |
| 3i       | IC₅₀ = 67.5 µM                                      | 4.54      |
| 3j       | IC₅₀ = 100 µM                                       | 3.77      |
| 3k       | IC₅₀ = 67.5 µM                                      | 4.00      |
| 3l       | IC₅₀ = 100 µM                                       | 3.42      |
| 4a       | (39.3% at 100 µM) #                                 | 1.65      |
| 4b       | IC₅₀ = 70.0 µM                                      | 3.14      |
| 5        | IC₅₀ = 100 µM                                       | 3.39      |
| 6a       | (24.4% at 100 µM) #                                 | 1.72      |
| 6b       | (13.0% at 100 µM) #                                 | 2.96      |
| 7a       | (24.5% at 100 µM) #                                 | 1.97      |
| 7b       | no                                                   | 1.31      |
| NDGA     | IC₅₀ = 0.45 µM                                      | 3.92      |

No: no activity under the reported experimental conditions; #: These compounds were less potent therefore no IC₅₀ values were determined for them; $^*$: theoretically calculated ClogP values using the C-QSAR Program, Biobyte [36].

Among all the tested chalcones, the most potent lipoxygenase inhibitor is chalcone 3c (IC₅₀ = 45 µM) which possesses one -OMOM group at position 4′ of ring A and three methoxy groups at positions 2, 4, and 5 at ring B. The -OMOM groups seems to play an important role in the LOX inhibitory activity of this series of 2′-hydroxy-chalcones: Chalcone 3b, which possesses the same methoxy substitution pattern as chalcone 3c but lacks the -OMOM group at ring A, shows lower activity (IC₅₀ = 75 µM) than 3c. Chalcone 3a, with a 4′-OMOM and 3,4-di-OMOM substitution is a poor LOX inhibitor (40.2%). The presence of two -OMOM substituents at positions 3 and 4 of ring B favors LOX inhibitory activity in the case of chalcones 3h and 3i, which also bear bromine substituents at ring A. In fact, chalcone 3h with a 5′-Br substitution pattern is the second most active LOX inhibitor of the series (IC₅₀ = 55 µM) whereas 3i, with an additional Br atom at position 3′, shows IC₅₀ = 67.5 µM. NDGA was used as a reference compound (Table 5).

The -OMOM group seems also to be a structural feature that affects activity depending on the substituents at ring A: Chalcone 3i, with 3′, 5′-dibromo pattern at ring A and 3,4-di-OMOM at ring B, is almost equipotent with its 3,4-dimethoxy analogue 3g (IC₅₀ = 67.5 and 68.5 µM, respectively). However, chalcone 3h, one of the most active of the series with one bromine substituent at position 5′, is transformed to an inactive compound when the -OMOM groups are replaced by -OCH₃ (chalcone 3f, 38% inhibition). On the other hand, removal of the -MOM protecting group results to the active 3,4-dihydroxy chalcone analogue 4b (IC₅₀ = 70 µM).
Chalcone 3d, the chlorine-containing analogue of 3f, is also inactive but a change of the position of the methoxy groups at ring B results to a significant improvement in activity: chalcone 3e with a 2,3-dimethoxy substitution pattern shows \( \text{IC}_{50} = 100 \ \mu\text{M} \).

The transformation of chalcone 3b to the analogous aurone 6b diminishes the activity, showing only 13.0% LOX inhibition. The same decrease in inhibition is observed in the transformation of 4a to 7a and of 3a to 6a.

As a general remark, lower inhibition results are observed by the compounds that possess lower lipophilicity values.

2.4. In Silico Studies

2.4.1. Molecular Docking Studies

The lack of a co-crystallized ligand in the crystal structure of soybean lipoxygenase isoform 1 (LOX-1) (PDB ID: 3PZW) imposes the identification of potential allosteric binding sites apart from the iron-binding site and the substrate-binding cavity. To this extent, three putative binding sites were identified using SiteMap’s approach [68–70]. In particular, Site 1 is located and mapped at the interface of the amino terminal \( \beta \)-barrel (PLAT domain) and of the \( \alpha \)-helical domain (houses the catalytic iron) while Sites 2 and 3 belong to the \( \alpha \)-helical domain (Figure S1, Supplementary Materials) [71]. Evaluation of Site Score, indicate Site 1 (site score 1.113) as a putative allosteric site for further docking studies. Interestingly, Site 1 has been previously identified and utilized for docking studies on LOX-1, as presented in recent publications [67,72].

Molecular docking was utilized to study the binding mode of the most promising synthesized chalcone 3c and its analogue 3b, which does not possess an -OMOM substituent, in an effort to provide a structure-activity explanation on soybean LOX-1 inhibitory activity. Additionally, for comparison reasons, the binding mode of the standard LOX inhibitor NDGA (\( \text{IC}_{50} = 0.45 \ \mu\text{M} \)) was determined. In general, the results suggested that the studied compounds interact with the soybean LOX-1 through allosteric interactions.

Especially, the analysis of the docked poses of the examined compounds 3b (\( \text{IC}_{50} = 75 \ \mu\text{M} \)) and 3c (\( \text{IC}_{50} = 45 \ \mu\text{M} \)) illustrated a common H-bond interaction pattern as depicted in Figure 2, orienting the hydroxyl (-OH) and carbonyl (-CO) groups of their aromatic ring A towards Asp768 and Asn128, respectively. The binding motif of NDGA includes the formation of H-bonds between the hydroxyl groups (-OH) of the aromatic ring A with Asp768 and Asn128, highlighting their crucial role in the binding and potentially in the inhibitory activity. Additionally, the hydroxyl groups (-OH) of the aromatic ring B of NDGA create a strong hydrogen bond network, including Cys127, Arg182, Arg141, and Ile142, which is not presented in the examined 3b and 3c. However, in the case of 3c, although the bulky (-OMOM) group seems not to participate in a direct binding, it induces an orientation capable to form H-bonds between the methoxy (-OCH\(_3\)) groups in positions 4 and 5 of the aromatic ring B with Trp130 and Gly247. In the case of 3b only, the methoxy (-OCH\(_3\)) group in position 3 forms a H-bond with Phe144, probably inducing the reduction of the inhibitory activity (Figure 2).

2.4.2. Molecular Dynamics Simulations Studies

In a step further, in an effort to evaluate (a) the stability of the tested complexes, (b) the binding mode, and (c) the influence of -OMOM substitution in position 4’ of compound 3c, short length (10 ns) molecular dynamics simulations were implemented in the docking poses of compounds 3b and 3c. LOX-1 interactions as derived from the MD simulations with the testing compounds are depicted in Figure 3. The RMSD results indicated that the structural model backbone of LOX-1, as well as compounds 3b and 3c, remain stable during the simulation (Figure S2, Supplementary Materials).
The binding motif of NDGA includes the —OCH3 groups in position 3 and —OCH(OH) group in position 4 of the aromatic ring B, as well as compounds 3b and 3c are depicted. Hydrogen bonds are illustrated with pink (2D) and yellow (3D) color lines. Figure made with MAESTRO (Schrödinger Release 2020-4: Maestro, Schrödinger, LLC, New York, NY, USA, 2020).

**Figure 2.** 2D and 3D representative binding poses of (A) nordihydroguaiaretic acid (NDGA), (B) Compound 3b, and (C) Compound 3c are depicted. Hydrogen bonds are illustrated with pink (2D) and yellow (3D) color lines. Figure made with MAESTRO (Schrödinger Release 2020-4: Maestro, Schrödinger, LLC, New York, NY, USA, 2020).

Both compounds exhibit the interactions presented in docking studies (Figure S3, Supplementary Materials). Specifically, the MD results indicate that both 3b and 3c preserve the direct H-bonds of their hydroxyl and carbonyl groups with the side chains of Asp768.
and Asn128 although not in high percentage (Figure 3). In addition, their methoxy (-OCH$_3$) groups in position 2 interact with the backbone (in the case of 3b) and the side chain (in the case of 3c) of Asn128, respectively. Moreover, a weaker water-bridged H-bond is formed between the hydroxyl group of compound 3b and the backbone of Cys127, while the same interaction is shown in the case of compound 3c with the side chain of Glu244. Regarding compound 3c, its carbonyl and methoxy (-OCH$_3$) group in position 2 interact through H-bonds with the side chains of Asn769 and Arg533, respectively. In contrast, the described interactions are not present for compound 3b, offering a putative explanation of the higher inhibitory activity of compound 3c.

Regarding the interactions of methoxy (-OCH$_3$) groups at positions 4 and 5 of 3b and 3c, a different profile is observed in agreement with the docking results. Especially, for compound 3b, the methoxy (-OCH$_3$) groups share H-bonds with Phe144 backbone while in the case of compound 3c, H-bonds are formed with Gly246 and Trp130.

With regard to the -OMOM chain of compounds 3c, the MD simulations indicates the formation of a hydrogen bond with Tyr525.

Overall, the binding of 3b and 3c is reinforced by additional hydrophobic interactions. In detail, compound 3b interacts with Val126, Val520, and Trp772 while compound 3c develops interactions with Leu246 and Phe108 (Figure S3, Supplementary Materials).

3. Materials and Methods

NMR spectroscopy: Synthesized compounds were structurally elucidated using Varian Gemini 300 MHz (Palo Alto, CA, USA) at the School of Chemical Engineering, NTUA and Varian 600 MHz (Palo Alto, CA, USA) at the Institute of Chemical Biology, NHRF, NMR spectrometers using DMSO-d$_6$, CDCl$_3$ 99.9 atom % D and CD$_3$OD-d$_4$ as solvents. ¹H-NMR spectra were recorded at 298 K under the following parameters. Spectral width (SW) = −2–14 ppm, number of scans (ns) = 32. Coupling constants (J) are expressed in hertz (Hz). Chemical shifts (δ) of NMR are reported in parts per million (ppm) units relative to the solvent.

Melting points were determined on a Gallenkamp MFB-595 melting point apparatus (London, UK) and are uncorrected. ESI-MS spectra were recorded on a Varian 500 MS IT Mass Spectrometer (Palo Alto, CA, USA). For the in vitro tests, a Lambda 20 (Perkin–Elmer, Norwalk, CT, USA) UV–Vis double beam spectrophotometer was used.

3.1. Synthesis and General Procedures

2-hydroxy-4-(methoxymethoxy)-acetophenone (1a) [17]: To a mixture of 2,4-dihydroxy-acetophenone (531.1 mg, 3.5 mmol) and anhydrous K$_2$CO$_3$ (1.9 g, 10.0 mmol) in dry acetone (39 mL) was added dropwise chloromethyl methylether (CH$_3$OCH$_2$Cl) (0.4 mL, 5.3 mmol). The mixture was refluxed for 24 h, cooled to room temperature, filtered, washed with acetone, and the solvent was evaporated. The resulting orange oil was purified using flash column chromatography (petroleum ether/ethyl acetate 9:1) to give (1a) as a low melting yellow solid. Yield: 548 mg (80%); ¹H-NMR (300 MHz, CDCl$_3$): δ (ppm) 12.58 (s, 1H, -OH), 7.65 (d, $J$ = 8.8 Hz, 1H, H-6), 6.60 (d, $J$ = 2.4 Hz, 1H, H-3), 6.56 (dd, $J$ = 8.8, 2.4 Hz, 1H, H-5), 5.22 (s, 2H, 2 × -OCH$_2$-), 3.50 (s, 3H, 3 × -OCH$_3$), 2.59 (s, 3H, 3 × -CH$_3$).

Note: Chloromethyl methylether is a highly toxic compound and should not be ingested or inhaled. All contact with this material should be avoided. It is a flammable material, corrosive to eyes and skin on contact. It is a possible carcinogen. It is lachrymator; Heat and sources of ignition should be avoided.

3,4-bis(methoxymethoxy)-benzaldehyde (2a) [17]: Prepared following the procedure described for the synthesis of acetophenone 1a, compound 2a was prepared from 3,4-dihydroxy-benzaldehyde (500 mg, 3.6 mmol). After evaporation of the solvent, the oily resulting product was purified using flash column chromatography (petroleum ether/ethyl acetate 8:2 (2 × 100 mL) and 7:3 (2 × 100 mL) with dry packing to give benzaldehyde 2a as a low melting white solid. Yield: 503.6 mg (62%). mp 61.2–62 °C; ¹H-NMR (300 MHz, CDCl$_3$): δ (ppm) 9.82 (s, 1H, HC = O), 7.64 (d, $J$ = 1.9 Hz, 1H, H-3), 7.47 (dd, $J$ = 8.3, 1.9 Hz, 1H,
3.1.1. General Procedure for the Synthesis of Chalcones 3a–3l

(1) Method A

To a stirred solution of the appropriate 2-hydroxy-acetophenone (1 eq) and a substituted benzaldehyde (1 eq) in ethanol and added KOH (20% w/v aqueous solution). The mixture is refluxed for 4 h or stirred in room temperature for 24 h. The completion of the reaction is monitored by TLC. At the end of the reaction, the mixture is cooled to 0 °C (ice-water bath) and acidified with HCl (10% v/v aqueous solution). An orange-yellow oil is formed and the mixture is extracted with diethyl ether; the extracts are dried with Na$_2$SO$_4$ and the solvent is evaporated under reduced pressure to give the chalcone as an orange-yellow solid.

Note: NaH dispersed in oil was used for the reaction. Special attention must be paid when using NaH, as it can ignite in air, especially upon contact with water to release hydrogen, which is also flammable.

2'-hydroxy-4',3,4'-methoxymethoxy-chalcone (3a): Prepared according to the general procedure (method B) starting from 2-hydroxy-4-(methoxymethoxy)-acetophenone (1a) (418 mg, 2.1 mmol) and 3,4-bis(methoxymethoxy)benzaldehyde (2a) (504 mg, 2.1 mmol), dissolved in 4.5 mL dry DMF and NaH (60% w/w, 255.43 mg). After the work-up procedure, the product was obtained upon recrystallization from hexane/ethyl acetate as an orange-yellow powder. The orange-yellow solid was recrystallized from hexane/ethyl acetate. Yield: 342 mg (40%). m.p. 114.95, 108.116, 103.93, 95.54, 95.10, 94.01, 56.37, 56.35, 56.33.

2'-hydroxy-2,4,5-trimethoxy-chalcone (3b): Prepared according to the general procedure (method A) starting from 2-hydroxy-acetophenone (1b) (500 mg, 3.7 mmol) and 2,4,5-trimethoxy-benzaldehyde (2b) (721 mg, 3.1 mmol), dissolved in 6 mL ethanol and 3.1 mL 20% aqueous solution KOH. The mixture was refluxed for 4 h. After the work-up procedure, the mixture was obtained upon recrystallization from hexane/ethyl acetate as an orange-yellow solid. Yield: 145 mg (39%). m.p. 86.2–86.8 °C; 1H-NMR (600 MHz, CDCl$_3$): δ (ppm) 13.04 (s, 1H, -OH), 8.22 (d, J = 8.5 Hz, 1H, H-3'), 6.54 (s, 1H, H-3), 3.96 (s, 3H, -OCH$_3$), 3.94 (s, 3H, -OCH$_3$).

2'-hydroxy-4'-methoxymethoxy-2,4,5-trimethoxy-chalcone (3c): Prepared according to the general procedure (method B) starting from 2-hydroxy-4-(methoxymethoxy)-acetophenone (1a) (60 mg, 0.3 mmol) and 2,4,5-trimethoxy-benzaldehyde (2b) (60 mg, 0.3 mmol), dissolved in 0.8 mL dry DMF and NaH (60% w/w, 36.67 mg). After the work-up procedure, the residue was purified using flash column chromatography (petroleum ether/ethyl acetate 7:5:2.5) to give 3c as an orange solid. Yield: 145 mg (39%). mp 86.2–86.8 °C; 1H-NMR (600 MHz, CDCl$_3$):
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5′-chloro-3,4-dimethoxy-2′-hydroxy-chalcone (3d): Prepared according to the general procedure (method A) starting from 5-chloro-2-hydroxyacetophenone (1e) (500 mg, 2.9 mmol) and 3,4-dimethoxy-benzaldehyde (2e) (486.9 mg, 2.9 mmol), dissolved in 7.2 mL ethanol and 2.5 mL of 20% w/v aqueous solution of KOH. The mixture was stirred at room temperature for 24 h. After the work-up procedure, the product was obtained upon recrystallization from methanol/dichloromethane as a yellow powder. Yield: 630 mg (72%); m.p. 170–172 °C (ref. [73] m.p. 167–168 °C). 1H-NMR (600 MHz, DMSO-d6): δ (ppm) 12.56 (s, 1H, -OH), 8.25 (d, J = 2.6 Hz, 1H, H-6′), 7.87 (d, J = 15.0 Hz, 1H, Hb), 7.81 (d, J = 15.6 Hz, 1H, Ha), 7.57 (m, 2H, Ar), 7.47 (dd, J = 7.8, 1.2 Hz, 1H, Ar), 7.04 (m, 2H, Ar), 3.86 (s, 3H, -OCH3), 3.83 (s, 3H, -OCH3); 13C-NMR (150 MHz, DMSO-d6): δ (ppm) 192.46, 160.08, 151.73, 148.94, 146.17, 135.45, 129.38, 127.09, 124.43, 122.70, 119.71, 119.11, 111.61, 111.15, 55.83, 55.50.

5′-chloro-2,3-dimethoxy-2′-hydroxy-chalcone (3e) [31]: Prepared according to the general procedure (method A) starting from 5-chloro-2-hydroxyacetophenone (1e) (341 mg, 2.0 mmol) and 2,3-dimethoxy-benzaldehyde (2d) (330 mg, 2.0 mmol), dissolved in 5 mL ethanol and 1.6 mL of 20% w/v aqueous solution of KOH. The mixture was stirred at room temperature for 24 h. After the work-up procedure, the product was obtained upon recrystallization from methanol/dichloromethane as a yellow powder. Yield: 390 mg (50%); m.p. 133–134 °C; 1H-NMR (600 MHz, DMSO-d6): δ (ppm) 12.18 (s, 1H, -OH), 8.18 (d, J = 2.4 Hz, 1H, H-6′), 8.06 (d, J = 15.6 Hz, 1H, Hb), 7.75 (d, J = 15.6 Hz, 1H, Ha), 7.66 (dd, J = 7.2, 1.8 Hz, 1H, H-6), 7.57 (dd, J = 9.0, 2.4 Hz, 1H, H-4′), 7.17 (m, 2H, H-4, H-5), 7.04 (d, J = 9.0 Hz, 1H, H-3′), 3.85 (s, 3H, -OCH3), 3.80 (s, 3H, -OCH3); 13C-NMR (75 MHz, DMSO-d6): δ (ppm) 192.30, 162.50, 155.80, 148.30, 138.60, 135.10, 129.70, 127.80, 125.50, 124.10, 122.90, 115.60, 112.40, 112.16, 60.80, 55.58.

5′-bromo-3,4-dimethoxy-2′-hydroxy-chalcone (3f) [41]: Prepared according to the general procedure (method A) starting from 5-bromo-2-hydroxyacetophenone (1d) (500 mg, 2.0 mmol) and 3,4-dimethoxy-benzaldehyde (2c) (330 mg, 2.0 mmol), dissolved in 5 mL ethanol and 1.6 mL of 20% w/v aqueous solution of KOH. The mixture was stirred at room temperature for 24 h. After the work-up procedure, the product was obtained upon recrystallization from methanol/dichloromethane as a yellow powder. Yield: 300 mg (50%); m.p. 178–179 °C; 1H-NMR (600 MHz, DMSO-d6): δ (ppm) 12.56 (s, 1H, -OH), 8.33 (s, 1H, H-6′), 7.86 (d, J = 15.6 Hz, 1H, Hb), 7.80 (d, J = 15.0 Hz, 1H, Ha), 7.68 (dd, J = 9.0, 2.4 Hz, 1H, H-4′), 7.55 (d, J = 1.8 Hz, 1H, H-2), 7.47 (dd, J = 8.4, 1.8 Hz, 1H, H-6), 7.04 (d, J = 8.4 Hz, 1H, H-5), 6.97 (d, J = 8.4 Hz, 1H, H-3′), 3.86 (s, 3H, -OCH3), 3.83 (s, 3H, -OCH3); 13C-NMR (75 MHz, DMSO-d6): δ (ppm) 192.90, 160.50, 152.10, 149.60, 146.30, 138.40, 132.30, 127.10, 124.50, 123.00, 119.30, 112.02, 111.58, 55.80, 55.50.

3′,5′-dibromo-3,4-dimethoxy-2′-hydroxy-chalcone (3g) [41]: Prepared according to the general procedure (method A) starting from 3,5-dibromo-2-hydroxyacetophenone (1e) (500 mg, 1.7 mmol) and 3,4-dimethoxy-benzaldehyde (2e) (282.5 mg, 1.7 mmol), dissolved in 5 mL ethanol and 1.4 mL of 20% w/v aqueous solution of KOH. The mixture was stirred at room temperature for 24 h. After the work-up procedure, the product was obtained upon recrystallization from methanol/dichloromethane as a yellow powder. Yield: 470 mg (63%); m.p. 165–166 °C; 1H-NMR (600 MHz, DMSO-d6): δ (ppm) 13.75 (s, 1H, -OH), 8.55 (d, J = 2.4 Hz, 1H, H-6′), 8.11 (d, J = 2.4 Hz, 1H, H-4′), 7.95 (d, J = 15.6 Hz, 1H, Hb), 7.91 (d, J = 15.6 Hz, 1H, Ha), 7.60 (d, J = 1.8 Hz, 1H, H-2), 7.53 (dd, J = 8.4, 1.8 Hz, Hz, 1H, H-6), 7.06 (d, J = 8.4 Hz, 1H, H-5), 3.87 (s, 3H, -OCH3), 3.84 (s, 3H, -OCH3); 13C-NMR (75 MHz,
DMSO-d$_6$): $\delta$ (ppm) 195.30, 161.60, 155.20, 152.00, 150.80, 143.50, 135.10, 130.20, 128.20, 121.00, 119.20, 115.50, 114.70, 114.50, 113.00, 58.80, 58.60.

$5$'-bromo-$3,4$-dimethoxymethoxy-$2$'-hydroxy-chalcone (3h): Prepared according to the general procedure (method A) starting from $5$-bromo-$2$-hydroxy-acetophenone (1d) (430 mg, 2.0 mmol) and $3,4$-dimethoxymethoxy-benzaldehyde (2a) (500 mg, 2.0 mmol), dissolved in 5 mL ethanol and 1.6 mL of 20% w/v aqueous solution of KOH. The mixture was stirred at room temperature for 24 h. After the work-up procedure, the product was purified using flash column chromatography (petroleum ether/ethyl acetate 8:2) to give 3h as a yellow solid. Yield: 38 mg (22%). mp 105–106 °C; $^1$H-NMR (600 MHz, DMSO-d$_6$): $\delta$ (ppm) 13.73 (s, 1H, -OH), 8.28 (d, $J = 2.4$ Hz, 1H, H-6'), 7.83 (d, $J = 15.0$ Hz, 1H, Ha), 7.75 (d, $J = 15.6$ Hz, 1H, Ha), 7.68 (dd, $J = 9.0$, 2.4 Hz, 1H, H-4'), 7.66 (d, $J = 1.8$ Hz, 1H, H-2), 7.55 (dd, $J = 8.4$, 1.2 Hz, 1H, H-6), 7.18 (d, $J = 8.4$ Hz, 1H, H-3'), 5.98 (d, $J = 8.4$ Hz, 1H, H-5), 5.29 (s, 4H, 2 × -OCH$_3$-), 3.44 (s, 3H, -OCH$_3$). $^{13}$C-NMR (150 MHz, DMSO-d$_6$): $\delta$ (ppm) 192.51, 166.35, 145.65, 139.38, 135.09, 124.86, 124.96, 123.24, 120.48, 120.06, 117.53, 116.28, 110.28, 94.92, 94.42, 55.94, 55.91; HR-MS m/z (neg): 421.0288 C$_{19}$H$_{19}$BrO$_6$ (calcld. 422.0365).

$3$,5$'$-bromo-$3,4$-dimethoxymethoxy-$2$'-hydroxy-chalcone (3i): Prepared according to the general procedure (method A) starting from $3,5$-dibromo-$2$-hydroxy-acetophenone (1e) (588 mg, 2.0 mmol) and $3,4$-dimethoxymethoxy-benzaldehyde (2a) (500 mg, 2.0 mmol), dissolved in 5 mL ethanol and KOH 1.6 mL of 20% w/v aqueous solution of KOH. The mixture was stirred at room temperature for 24 h. After the work-up procedure, the product was obtained as a yellow powder. Yield: 38 g (22%). $^{1}$H-NMR (600 MHz, DMSO-d$_6$): $\delta$ (ppm) 13.63 (s, 1H, -OH), 7.98 (d, $J = 2.4$ Hz, 1H, H-6), 7.92 (d, $J = 15.0$ Hz, 1H, Ha), 7.87 (d, $J = 2.4$ Hz, 1H, H-4'), 7.48 (d, $J = 1.8$ Hz, 1H, H-2), 7.39 (d, $J = 15.0$ Hz, 1H, Ha), 7.33 (dd, $J = 8.4$, 1.2 Hz, 1H, H-6), 7.22 (d, $J = 8.4$ Hz, 1H, H-5), 5.31 (br, 4H, 2 × -OCH$_2$-O). 3.56 (s, 3H, -OCH$_3$). 3.53 (s, 3H, -OCH$_3$).

$4$-carboxy-$2$'-hydroxy-chalcone (3j) [41]: Prepared according to the general procedure (method A) starting from $2$-hydroxy-acetophenone (1b) (1 g, 7.3 mmol) and $4$-carboxy-benzaldehyde (2g) (1.102 g, 7.3 mmol), dissolved in 18 mL ethanol and 6 mL of 20% w/v aqueous solution of KOH. The mixture was stirred at room temperature for 24 h. After the work-up procedure, the product was obtained upon recrystallization from methanol/dichloromethane as a yellow powder. Yield: 813 mg (63%). $^{1}$H-NMR (600 MHz, CDCl$_3$): $\delta$ (ppm) 13.63 (s, 1H, -OH), 7.98 (d, $J = 2.4$ Hz, 1H, H-6), 7.92 (d, $J = 15.0$ Hz, 1H, Ha), 7.87 (d, $J = 2.4$ Hz, 1H, H-4'), 7.48 (d, $J = 1.8$ Hz, 1H, H-2), 7.39 (d, $J = 15.0$ Hz, 1H, Ha), 7.33 (dd, $J = 8.4$, 1.2 Hz, 1H, H-6), 7.22 (d, $J = 8.4$ Hz, 1H, H-5), 5.31 (br, 4H, 2 × -OCH$_2$-O). 3.56 (s, 3H, -OCH$_3$), 3.53 (s, 3H, -OCH$_3$).

$4$-carboxy-$5$'-chloro-$2$'-hydroxy-chalcone (3k) [48]: Prepared according to the general procedure (method A) starting from $5$-chloro-$2$-hydroxy-acetophenone (1e) (500 mg, 2.9 mmol) and $4$-carboxy-benzaldehyde (2g) (440 mg, 2.9 mmol), dissolved in 7.2 mL ethanol and 2.5 mL of 20% w/v aqueous solution of KOH. The mixture was stirred at room temperature for 24 h. After the work-up procedure, the product was obtained upon recrystallization from methanol/dichloromethane as a yellow powder. Yield: 710 mg (85%). $^{1}$H-NMR (600 MHz, DMSO-d$_6$): $\delta$ (ppm) 13.15 (s, 1H, -COOH), 12.15 (s, 1H, -OH), 8.23 (d, $J = 3.0$ Hz, 1H, H-2, H-3); 6.80 (d, $J = 15.6$ Hz, 1H, H-6), 8.01 (m, 4H, H-2, H-3, H-5, H-6), 7.84 (d, $J = 15.6$ Hz, 1H, Ha), 7.56 (t, $J = 7.8$ Hz, 1H, H-5'), 6.99 (m, 2H, H-3', H-4'), $^{13}$C-NMR (150 MHz, DMSO-d$_6$): $\delta$ (ppm) 192.51, 166.33, 161.30, 142.98, 138.51, 136.47, 132.41, 131.08, 129.86, 129.26, 124.44, 121.19, 119.5, 118.05.

$2$'-hydroxy-chalcone (3l): Prepared according to the general procedure (method A) starting from $2$-hydroxy-acetophenone (1b) (500 mg, 3.7 mmol) and benzaldehyde (2h) (392.6 mg, 3.7 mmol), dissolved in 6 mL ethanol and 3.1 mL of 20% w/v aqueous solution of KOH. The mixture was stirred at room temperature for 24 h. After the work-up procedure, the product...
was obtained upon recrystallization from methanol/dichloromethane as a yellow powder. Yield: 697 mg (84%); m.p. 85–86 °C (ref. [49] m.p. 82–85 °C); 1H-NMR (600 MHz, CDCl3): \(\delta\) (ppm) 12.83 (s, 1H, -OH), 7.93 (m, 2H, aromatic H), 7.67 (m, 4H, aromatic H and Hb), 7.51 (dd, \(J = 8.4, 7.2, 1.2\) Hz, 1H, aromatic H), 7.45 (m, 3H, aromatic H and Ha), 7.04 (d, \(J = 8.4\) Hz, 1H, aromatic H), 6.96 (t, \(J = 7.2\) Hz, 1H aromatic H).

3.1.2. General Procedure for the Synthesis of Chalcones 4a and 4b

Chalcones 4a and 4b were produced by removing the MOM protecting group. The corresponding methoxymethoxy-chalcone was dissolved in methanol and 10% aqueous HCl was added dropwise. The mixture was stirred at 65 °C for 15 min. The reaction was monitored by TLC. After completion of the reaction, the mixture was diluted with water and extracted with ethyl acetate. The organic extracts were dried with Na2SO4 and the solvent was evaporated under reduced pressure to give the chalcone as a solid product.

2′,3,4′,4′-tetrahydroxy-chalcone (Buten) (4a): Prepared according to the general procedure, chalcone (3a) (47.5 mg, 0.1 mmol) was dissolved in 2.2 mL methanol followed by dropwise addition HCl (10% aqueous solution, 1 mL). The mixture was refluxed for 15 min, until a clear red solution was obtained. After the work-up procedure, a red solid was acquired. Yield: 22.4 mg (70%). m.p. > 180 °C.

3.1.3. Synthetic Procedure of 4-Methoxycarbonyl-2′-Hydroxy-Chalcone (5)

Chalcone (3j) (500 mg, 1.9 mmol) was added to methanol (50 mL), followed by dropwise addition of H2SO4 (5 mL). The mixture was stirred at 70 °C for 24 h. The reaction was monitored by TLC. After completion of the reaction, the solvent was evaporated under reduced pressure and the residue was cooled at -19 °C. The precipitate formed was filtered and washed with methanol. Yield: 317 mg (60%); m.p. 128–131 °C; 1H-NMR (600 MHz, DMSO-d6): \(\delta\) (ppm) 12.31 (s, 1H, -OH), 8.24 (dd, \(J = 8.4, 1.2\) Hz, 1H, H-6′), 8.14 (d, \(J = 15.6\) Hz, 1H, Hb), 8.04 (m, 4H, H-2′,H-3′,H-5′,H-6), 7.86 (d, \(J = 15.6\) Hz, 1H, Ha), 7.58 (ddd, \(J = 8.4, 7.8, 1.8\) Hz, 1H, H-4′), 7.02 (m, 2H, H-3′, H-5′), 3.88 (s, 3H, -COOCH3); 13C-NMR (150 MHz, DMSO-d6): \(\delta\) (ppm) 193.3, 165.7, 161.7, 142.8, 139.0, 136.5, 131.0, 129.6, 129.2, 124.6, 121.0, 119.2, 117.7, 109.6, 52.3; HR-MS m/z (neg): 281.0892 (calcld. 282.0892).

3.1.4. General Procedure for the Synthesis of Aurones 6a and 6b

To a solution of mercuric acetate (1.25 eq) in pyridine was added the desirable chalcone (1 eq) at room temperature and the mixture was stirred at 110 °C for 1 h. The reaction was monitored by TLC. The cooled reaction mixture was poured into ice cold water and acidified with HCl (10% aqueous solution). The precipitated solid was extracted with
dichloromethane, the extracts were dried (Na$_2$SO$_4$) and the solvent was evaporated to give a solid which was further purified by recrystallization from methanol/dichloromethane.

Note: Pyridine is a harmful reactant and should be handled with care as it can be harmful in case of inhalation, ingestion, or when in contact with the skin. Pyridine is also known to reduce male fertility. Pyridine has a very noticeable and unpleasant smell, therefore, good ventilation is essential, when used. To neutralize pyridine addition of a weak acid, such as 10% aqueous HCl, at 0 °C is suggested.

Mercuric acetate is a harmful reagent. It is fatal if swallowed, in contact with skin or if inhaled. It may cause damage to organs through prolonged or repeated exposure. It is very toxic to aquatic life with long lasting effects. Protective gloves/protective clothing should be worn throughout the period of handling this reagent.

3′,4′,6-tri(methoxymethoxy)-aurone (6a): Prepared according to the general procedure starting from chalcone (3a) (140.4 mg, 0.3 mmol), dissolved in 3.5 mL pyridine. After the work-up procedure, the product was obtained upon recrystallization from methanol/dichloromethane as a yellow solid. Yield: 84 mg (60%). mp 140.2–141.4 °C; $^1$H-NMR (300 MHz, CDCl$_3$): δ (ppm) 7.71 (m, 2H, H-4, H-10), 7.55 (d, $J = 8.4$ Hz, 1H, H-5), 7.22 (d, $J = 8.7$ Hz, 1H, H-6'), 6.95 (s, 1H, H-7), 6.86 (d, $J = 8.4$ Hz, 1H, H-6'), 6.78 (s, 1H, H-2'), 5.31 (s, 6H, 3 × -OCH$_2$O-), 3.57 (s, 3H, -CH$_3$), 3.53 (br, 6H, 2 × -CH$_2$OCH$_3$); $^{13}$C-NMR (150 MHz, CDCl$_3$): δ (ppm) 183.16, 168.09, 164.82, 147.29, 147.22, 126.98, 126.84, 125.93, 119.99, 116.47, 116.04, 113.27, 112.24, 99.48, 95.90, 95.29, 94.59, 56.58, 56.54, 56.48; HR-MS m/z (pos): 403.1352 C$_{21}$H$_{22}$O$_{8}$ (calcd. 403.1352).

2′,4′,5′-trimethoxy-aurone (6b): Prepared according to the general procedure starting from chalcone (3b) (150 mg, 0.4 mmol) and mercury acetate (184.25 mg, 0.6 mmol) dissolved in 4.8 mL pyridine. After the work-up procedure, the product was obtained upon recrystallization from methanol/dichloromethane as a yellow solid. Yield: 112.7 mg (74%). mp 181.2–183.4 °C (ref. [50] m.p. 191–193 °C); $^1$H-NMR (300 MHz, DMSO-d$_6$): δ (ppm) 7.78 (s, 1H, H-10), 7.75 (m, 2H, H-4, H-5) 7.56 (d, $J = 8.4$ Hz, 1H, H-7), 7.28 (t, $J = 7.5$ Hz, 1H, H-6), 7.17 (s, 1H, H-6'), 6.77 (s, 1H, H-3'), 3.91 (br, 6H, 2 × -OCH$_3$), 3.82 (s, 3H, -OCH$_3$).

3.1.5. Synthetic Procedure of 3′,4′,6-Trihydroxy-Aurone (Sulforutilin) (7a)

Aurone (7a) was prepared via deprotection reaction of aurone (6a). Aurone (6a) (48.2 mg, 0.1 mmol) was added to 2.25 mL methanol followed by dropwise addition of HCl (10% aqueous solution, 1 mL). The mixture was refluxed for 15 min, until a clear orange solution was obtained. The reaction was monitored by TLC. After completion of the reaction, the mixture was diluted with water and extracted with ethyl acetate. The organic extracts were dried with Na$_2$SO$_4$ and the solvent was evaporated under reduced pressure to give the product as an orange solid. Yield: 29.6 mg (92%). mp > 250 °C; $^1$H-NMR (300 MHz, CD$_3$OD-d$_4$): δ (ppm) 11.04 (s, 1H, OH-3'), 9.60 (s, 1H, OH-4'), 9.23 (s, 1H, OH-6), 7.57 (d, $J = 8.1$ Hz, 1H, H-4'), 7.42 (d, $J = 1.8$ Hz, 1H, H-7), 7.22 (dd, $J = 8.4$, 1.8 Hz, 1H, H-5), 6.81 (d, $J = 8.4$ Hz, 1H, H-5'), 6.72 (d, $J = 1.5$ Hz, 1H, H-2'), 6.68 (dd, $J = 8.4$, 1.8 Hz, 1H, H-6'), 6.61 (s, 1H, H-10); $^{13}$C-NMR (150 MHz, CD$_3$OD-d$_4$): δ (ppm) 184.47, 169.77, 168.24, 149.37, 147.69, 146.70, 126.83, 126.40, 125.53, 118.94, 116.66, 114.90, 114.70, 114.03; MS (ESI) (positive): m/z = 271.14 (67%) [M + 1]$^+$, 562.75 (42%) [2 × M + Na]$^+$; MS (ESI) (negative): m/z = 269.23 (33%) [M − 1]$^+$, 538.86 (100%) [2 × (M − 1)]$^+$. 

3.1.6. Synthetic Procedure of 2′,4′,5′-Trihydroxy-Aurone (7b)

Aurone (7b) was prepared via deprotection reaction of aurone (6b). Aurone (6b) (50 mg, 0.2 mmol) was added to 2 mL dry dichloromethane followed by addition of BBr$_3$ (2.4 mL, 2.4 mmol). The mixture was stirred for 2 h at 0 °C, until a clear orange solution was obtained. The reaction was monitored by TLC. After completion of the reaction, the resulting mixture was poured into ice-cold water and left to stand for 25 min. The residue was extracted with ethyl acetate, the organic extracts were dried with Na$_2$SO$_4$, and the solvent was evaporated under reduced pressure to give the product as a red solid.
Yield: 26.8 mg (62%). mp > 250 °C; ¹H-NMR (300 MHz, CD₃OD-d₄): δ (ppm) 7.74 (s, 1H, H-10), 7.70 (m, 2H, H-4, H-5), 7.44 (s, 1H, H-6′), 7.37 (d, J = 8.4 Hz, 1H, H-7), 7.24 (t, J = 7.2 Hz, 1H, H-6), 6.38 (s, 1H, H-3′); MS (ESI): m/z = 271.2 (55%) [M + 1]^+, 255.3 (100%) [M − 1 × OH + 1]^+.

3.2. Biological In Vitro Assays

Each in vitro experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the mean. For the in vitro assays, a stock solution (1% DMSO in the appropriate buffer with the tested compound diluted under sonification) was prepared from which several dilutions were made with the appropriate buffer.

3.2.1. Determination of the Reducing Activity of the Stable Radical 2,2-Diphenyl-1-Picrylhydrazyl (DPPH)

To an ethanolic solution of DPPH (100 µM) in absolute ethanol, an equal volume of the compounds dissolved in DMSO was added (100 µM). The mixture was shaken vigorously and allowed to stand for 20 or 60 min; absorbance at 517 nm was determined spectrophotometrically, and the percentage of activity was calculated. All tests were undertaken on three replicates, and the results presented in Table 2 were averaged and compared with the appropriate standard nordihydroguaiaretic acid (NDGA) [61].

3.2.2. Inhibition of Linoleic Acid Lipid Peroxidation

Production of conjugated diene hydroperoxide by oxidation of sodium linoleate in an aqueous solution is monitored at 234 nm. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was used as a free radical initiator. Ten microliters of the 16 mM linoleic acid sodium salt solution was added to the UV cuvette containing 0.93 mL of 0.05 M phosphate buffer, pH 7.4 prethermostated at 37 °C. The oxidation reaction was initiated at 37 °C under air by the addition of 50 µL of 40 mM AAPH solution. Oxidation was carried out in the presence of the synthesized compounds (10 µL, from a stock solution of 10 mM in DMSO). In the assay without antioxidant, lipid oxidation was measured in the presence of the same level of DMSO. The rate of oxidation at 37 °C was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides and compared with the appropriate standard trolox [61].

3.2.3. Competition of the Tested Compounds with DMSO for Hydroxyl Radicals

The hydroxyl radicals generated by the Fe³⁺ / ascorbic acid system, were detected by the determination of formaldehyde produced from the oxidation of DMSO (Table 2). A negative control using the same % DMSO was used. Trolox was used as reference compound [75].

3.2.4. ABTS⁺—Decolorization Assay for Antioxidant Activity

In order to produce the ABTS radical cation (ABTS⁺), ABTS stock solution in water (7 mM) was mixed with potassium persulfate (2.45 mM) and left in the dark at room temperature for 12–16 h before use. Our published experimental technique was used (1). The results are recorded after 1 min of the mixing solutions at 734 nm. The results were compared to the appropriate standard inhibitor Trolox [75].

3.2.5. Soybean LOX Inhibition Study In Vitro

The tested compounds dissolved in DMSO were incubated at room temperature with sodium linoleate (0.1 mL) and 0.2 mL of enzyme solution (1/9 × 10⁻⁴ w/v in saline) in Tris buffer pH 9. The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor NDGA [61].
3.3. In Silico Studies

3.3.1. Molecular Docking Studies on Soybean Lipoxygenase Seed Isoform 1 (LOX-1)

The synthesized compounds \(3b\) and \(3c\) were primarily sketched in 2D format and prepared at the optimum \(pH = 7.0 \pm 0.5\), using LigPrep program of Schrödinger platform. [76] Subsequently, molecular docking simulations were carried out applying the Schrödinger induced fit docking (IFD) protocol [76,77]. The referred protocol, based on glide and prime modules, offers an accurate prediction of ligand favorable binding modes with concomitant structural changes in the receptor (simultaneous flexibility of the side chains, backbone, or both). The crystal structure of soybean LOX-1 (PDB ID: 3PZW) [63] complexed with ferrous iron (\(\text{Fe}^{2+}\)—inactive form) was subjected to protein preparation [78,79]. Particularly, all water molecules were removed, missing residues and hydrogens were added, and restrained energy minimization was followed using OPLS3 force field. Identification of potential allosteric binding sites apart from the iron-binding site and the substrate-binding cavity was performed using SiteMap calculations [68–70]. The maximum number of docking poses was set equal to 10, they were visually inspected and their binding modes were analyzed.

3.3.2. Molecular Dynamics Simulations

Molecular dynamics simulations (MD) of the tested ligand-enzyme complexes were performed using the Desmond software of the Schrödinger platform, release 2020-3 [80]. For the examined systems setup, TIP4P was defined as the solvent model and the examined complexes were inserted in an orthorhombic box with volume \(850,054 \text{ Å}^3\). Additionally, OPLS2003 force field was utilized and the systems were neutralized by adding 7 Na\(^+\) ions. Subsequently, modeled systems were relaxed and then subjected to 10 ns MD simulations in the NPT ensemble class. During the simulation, the temperature was maintained at 300 K using the Nose–Hoover chain thermostat method with a 1.0 ps relaxation time and the pressure was maintained at 1 bar using Martyna–Tobias–Klein barostat method. The root mean square deviation (RMSDs) was calculated for both the docked compounds and Ca atoms of the enzyme, and also interactions and contacts (H-bonds, hydrophobic, ionic, water bridges) of the examined compounds with the LOX-1 residues were monitored throughout the simulation.

4. Conclusions

In conclusion, this project reports the synthesis of sixteen 2′-hydroxy chalcone derivatives and four aurone analogues, from which, to our knowledge, compounds \(3c\), \(3b\), \(4b\), \(5\), and \(6a\) are presented in the literature for the first time. The synthesized chalcones and aurones were evaluated for their antioxidant activity using different in vitro techniques, while they were also tested as inhibitors of soybean LOX, as an indication of their anti-inflammatory effect. The bioassays’ results revealed that compound \(4b\) is the most promising antioxidant agent (82.4% for DPPH scavenging ability and 82.3% in lipid peroxidation inhibition and 100% scavenging of hydroxyl radicals), while analogue \(3c\) is the most potent LOX inhibitor (\(\text{IC}_{50} = 45 \mu\text{M}\)). Chalcones \(3c\) and \(3b\), which share the common structural feature of a 2,4,5-trimethoxy substitution at ring B, were further studied to explore their mechanism of action on the soybean LOX-1 enzyme using molecular docking studies and molecular dynamics simulations. The results indicate that these compounds share common binding characteristics and interact with LOX-1 enzyme through allosteric interactions.

Supplementary Materials: The following are available online: Figure S1: Predicted binding sites of Soybean Lipoxygenase seed isoform 1 (LOX-1), Figure S2: RMSD calculations for (A) all Ca enzyme atoms (blue) in LOX-1-Compound 3b complex and for all atoms of compound 3b (red), (B) all Ca enzyme atoms (blue) in LOX-1-Compound 3c complex and for all atoms of compound 3c (red), Figure S3: Categorization of (A) LOX-1-Compound 3b and (B) LOX-1-Compound 3c interactions, during the molecular dynamics simulation (10 ns). The \(^1\text{H}-\text{NMR}\) spectra of all the synthesized compounds and \(^{13}\text{C}-\text{NMR}\) spectra of the novel synthesized compounds.
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