Bioorganic & Medicinal Chemistry 21 (2013) 532–539

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Synthesis and biological evaluation of some 5-arylidene-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-ones as dual anti-inflammatory/antimicrobial agents

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ARTICLE INFO

*Article history:*
Received 14 September 2012
Accepted 31 October 2012
Available online 17 November 2012

*Keywords:*
Thiazoles
Thiazolidinones
Antimicrobial
Antifungal
Antiinflammatory
COX/LOX inhibitors

ABSTRACT

As a part of our ongoing studies in developing new derivatives as dual antimicrobial/anti-inflammatory agents we describe the synthesis of novel 5-arylidene-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-ones. All newly synthesized compounds were tested for their anti-inflammatory activity using carrageenan mouse paw edema bioassay. Their COX-1/LOX inhibitory activities were also determined. Moreover, all compounds were evaluated for their antimicrobial and antifungal activities against a panel of Gram positive, Gram negative bacteria and moulds. All tested compounds exhibited better antimicrobial activity than commercial drugs, bifonazole, ketoconazole, ampicillin and streptomycin.



## 1. Introduction

Inflammation represents a fundamental host response to a wide range of stimuli such as trauma, tissue injury, microbial activity, burns, surgery, sepsis, toxic entities, ischemia–reperfusion, post-ischemic or autoimmune injury.

While the role of microorganisms in inflammation is well recognized, therapeutic strategies with effects on both the microorganism and associated inflammation have received considerably less attention.[1] Therapeutic options investigated for inflammation control include neutralization of tumor-necrosis factor (TNF), blockers of leukotriene receptors, inhibitors of cytokines or leukotriene synthesis and other principal components of the inflammatory response for systemic conditions.

During the past 50 years significant efforts in the diagnosis and treatment of microbial diseases have been accomplished.[2] These efforts led to impressive gains in the treatment of microbial diseases introducing a range of therapeutic strategies in clinical practice. In spite of a large number of antibiotics and chemotherapeutics available for medical use at the same time the emergence of old and new antibiotics resistance created in the last decades a substantial medical need for new classes of antibacterial agents. A potential approach to overcome the resistance problem is to design innovative agents with different mode of action so that no cross-resistance with the present therapeuticals can occur.

Identification of novel compounds which treat both infectious and inflammatory states more effectively, and which lack side effects associated with current therapies, remains a major challenge in biomedical research.[3] The use of several drugs to treat inflammatory conditions associated with infection is a problem, especially in case of patients with impaired liver or kidney function or to avoid drug–drug interaction. In addition, from the pharmaco-economic cost-effective standpoint, and seeking for a better patient compliance, a dual anti-inflammatory/antimicrobial agent with minimum adverse effects and high safety margin is highly desirable. This promoted us to try to find out agents that have a dual effect as anti-inflammatory/antimicrobial agents.

Thiazole derivatives are known to posses anti-inflammatory, analgesic and antipyretic activities.[4–9] Thiabendazole and 2-(*p*-chlorophenyl) thiazole-4-acetic acid are widely used as anti-inflammatory drugs.[10] Meloxicam, for example, is a new NSAID with a thiazolyl group in its structure.[11,12] Furthermore, Niridazole[13] and some other thiazole derivatives[14–20] have been found to exhibit antimicrobial antifungal/antihelminthic activities.

As a part of our ongoing studies in developing new active antimicrobials,[17,21–23] we report herein the synthesis of a new 4-thiazolidinone derivatives incorporating two known bioactive heterocyclic nuclei such as thiazolyl and thiazolidinonyl[9,21–25] in an attempt to present also anti-inflammatory activities. The

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http://dx.doi.org/10.1016/j.bmc.2012.10.046

structural variations were selected by introducing, at the 5 position of thiazolidinone moiety, different arylidene substituents that we recently exploited as bioactive, on heterocyclic scaffolds, useful to encompass certain physico-chemical properties as hydrophobic and steric.[21,22,24]

Twenty one new 5-arylidene-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-ones (Scheme 1, IV-1-21) were synthesized and evaluated for their in vitro antimicrobial properties against Gram positive, Gram negative bacteria and fungi, as well as for their ability to act as anti-inflammatory agents.

## 2. Results and discussion

### 2.1. Chemistry

The compounds described in this paper were synthesized by the multi-step reaction protocol reported earlier by us.[21,22]

The synthesis of new compounds (Scheme 1) was carried out by the reaction of the appropriate amine with chloroacetylchloride in dry benzene under reflux for 3 h. Heterocyclization was performed in the presence of ammonium thiocyanate refluxing in ethanol and efficiently led to 2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-ones (**3**).[21,22] After completion of the reaction, (usually 1 h), the 2-(thiazol-2-ylimino)thiazolidin-4-ones were afforded in excellent yields and purity. The final compounds were obtained by refluxing the previous intermediates with commercially available aldehydes and anhydrous sodium acetate in glacial acetic acid. Reactions were monitored by thin layer chromatography.

All new compounds were characterized by elemental and spectrophotometric analysis (IR and $^1$H NMR). The results are consistent with the proposed structures and are in agreement with previous findings.[21]

In our previous papers[21,22] have been already proposed a reaction mechanism for the formation of the compounds under study.

### 2.2. Antimicrobial activity

The designed compounds were evaluated for their in vitro antimicrobial activity against seven bacterial and six fungal species using microdilution test. The minimal inhibitory concentrations that inhibited the growth of the tested microorganisms (MIC) and minimal bactericidal/fungicidal concentration (MBC/MFC) were detected.

The results of antimicrobial testing against a panel of selected Gram positive and Gram negative bacteria are reported in Table 1, along with those of reference drugs Ampicillin and Streptomycin.[2] The structural modifications to previously synthesized thiazolidinones[21] involve the nature of the substituent X as well as R.

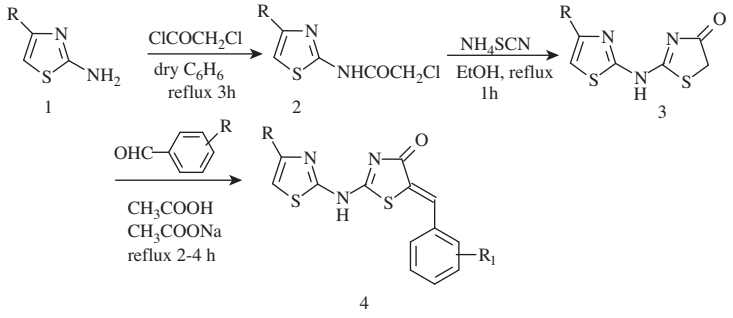
All the compounds tested showed strong antibacterial activity against all the species tested. MIC of compounds was ranged from 0.68–40.6 $\times 10^{-2}$ μmol/ml and MBC at 1.44–46.4 $\times 10^{-2}$ μmol/ml. The antibacterial potential could be presented as follows: **4h** > **4j** > **4s** > **4i** > **4k** > **4e** > **4c** > **4f** > **4g** > **4l** > **4m** > **4r** > **4q** > **4t** > **4u** > **4p** > **4o** > **4b** > **4d**. The best antibacterial effect was observed for compound **4h** with inhibitory activity at 0.73–2.56 $\times 10^{-2}$ μmol/ml and bactericidal effect at 1.44–2.92 $\times 10^{-2}$ μmol/ml, followed by compound **4j** with MIC 0.76–3.03 $\times 10^{-2}$ μmol/ml and MBC 1.56–6.06 $\times 10^{-2}$ μmol/ml, while **4o** exhibited the lowest inhibitory effect (0.73–23.3 $\times 10^{-2}$ μmol/ml) and bactericidal activity (5.8–34.8 $\times 10^{-2}$ μmol/ml).

The obtained results support that the tested compounds exhibited higher activity against all the screened bacteria, compared to Streptomycin (MIC 4.3–25.8 $\times 10^{-2}$ μmol/ml, and MBC 8.6–51.6 $\times 10^{-2}$ μmol/ml) and much better than Ampicillin (MIC 24.8–74.4 $\times 10^{-2}$ μmol/ml, and MBC 37.2–124.0 $\times 10^{-2}$ μmol/ml).

Gram (+), *Bacillus cereus* was the most sensitive bacterial species on compounds tested, while *Staphylococcus aureus* was the most resistant one.

Taking under consideration the relationships between the structure of the heterocyclic scaffold and the detected antibacterial properties, it seems clear, that the introduction of the bulky bromo, dimethylamino and chloro groups enhances the antibacterial potential. It should also be mentioned that the presence of a methoxy group is correlated with significant antibacterial activity (compound **3**).

Compounds **4h**, **4j**, **4s**, **4i**, **4k**, **4e** and **4c** which are either dimethylamino, or mono-chloro/bromo, mono-methoxy or




| N/N | R | $R_1$ | N/N | R | $R_1$ |
|---|---|---|---|---|---|
| 4a | H | 3-OH | 4k | H | 2,6-di-Cl |
| 4b | H | 3,5-di-$OCH_3$, 4-OH | 4l | H | 2,4-di-Cl |
| 4c | H | 2-$OCH_3$ | 4m | H | 2,3-di-Cl |
| 4d | H | 2,5-di-$OCH_3$ | 4n | 4-$CH_3$ | 2-Cl |
| 4e | H | 4-$CH_3$ | 4o | 4-$CH_3$ | 4-Cl |
| 4f | H | 3-F | 4p | 5-$CH_3$ | 4-$NO_2$ |
| 4g | H | 4-F | 4q | 4-Ph | H |
| 4h | H | 3-Br | 4r | 4-Ph | 2-Cl |
| 4i | H | 4-Br | 4s | 4-Ph | 3-Cl |
| 4j | H | $N(CH_3)_2$ | 4t | 4-Ph | 4-Cl |
| | | | 4u | H | 2-OH, 5-Br |

**Scheme 1.** Reagents and conditions: (a) $ClCOCH_2Cl$, dry $C_6H_6$, reflux,3 h; (b) $NH_4SCN$, EtOH, reflux, 1 h; (c) $RC_6H_4CHO$, $CH_3COOH$,$CH_3COONa$, reflux, 2–4 h.

**Table 1**
Antibacterial activity of unsubstituted/4-substituted 2-thiazolylimino-5-arylidene-4-thiazolidinones (MIC and MBC, μmol/ml × $10^{-2}$)

| Compounds | | *S. a.* | *B. c.* | *M. f* | *L. m.* | *Ps. aer.* | *S. typhi.* | *E. coli.* |
|---|---|---|---|---|---|---|---|---|
| **4b** | MIC | 11 | 5.5 | 11 | 11 | 11 | 11 | 27.5 |
| | MBC | 27.5 | 11 | 22 | 22 | 38.5 | 27.5 | 33 |
| **4c** | MIC | 3.15 | 3.15 | 3.15 | 3.15 | 3.15 | 3.15 | 3.15 |
| | MBC | 6.30 | 3.15 | 3.15 | 6.30 | 6.30 | 6.30 | 6.30 |
| **4d** | MIC | 23.2 | 23.2 | 23.2 | 5.8 | 29 | 11.5 | 40.6 |
| | MBC | 34.8 | 34.8 | 29 | 23 | 40.6 | 23 | 46.4 |
| **4e** | MIC | 2.47 | 1.65 | 6.60 | 6.60 | 1.65 | 3.30 | 3.30 |
| | MBC | 3.3 | 3.30 | 9.89 | 8.24 | 2.47 | 6.60 | 6.60 |
| **4f** | MIC | 3.27 | 2.46 | 6.54 | 8.19 | 2.46 | 6.54 | 3.27 |
| | MBC | 6.54 | 3.27 | 8.19 | 9.82 | 3.27 | 8.19 | 6.54 |
| **4g** | MIC | 3.27 | 2.46 | 6.54 | 8.19 | 3.27 | 3.27 | 3.27 |
| | MBC | 6.54 | 3.27 | 8.19 | 9.82 | 6.54 | 6.54 | 6.54 |
| **4h** | MIC | 0.73 | 2.56 | 1.83 | 1.44 | 1.46 | 1.46 | 1.46 |
| | MBC | 1.44 | 2.92 | 2.56 | 1.83 | 2.56 | 2.56 | 2.56 |
| **4i** | MIC | 1.37 | 2.05 | 2.73 | 2.73 | 2.73 | 2.73 | 2.73 |
| | MBC | 2.73 | 5.46 | 5.46 | 5.46 | 5.46 | 5.46 | 5.46 |
| **4j** | MIC | 3.03 | 0.76 | 0.76 | 3.03 | 3.03 | 1.52 | 0.76 |
| | MBC | 6.06 | 1.52 | 1.52 | 6.06 | 6.06 | 3.03 | 1.52 |
| **4k** | MIC | 2.81 | 2.11 | 5.62 | 5.62 | 2.11 | 2.81 | 2.81 |
| | MBC | 5.62 | 2.81 | 8.42 | 8.42 | 2.81 | 5.62 | 5.62 |
| **4l** | MIC | 5.62 | 2.81 | 8.42 | 7.02 | 2.81 | 2.81 | 2.11 |
| | MBC | 7.02 | 5.62 | 9.82 | 8.42 | 7.02 | 5.62 | 5.62 |
| **4m** | MIC | 7.02 | 5.62 | 5.62 | 7.02 | 2.81 | 2.81 | 5.62 |
| | MBC | 8.42 | 7.02 | 8.42 | 8.42 | 5.62 | 5.62 | 7.02 |
| **4n** | MIC | 5.96 | 5.96 | 2.98 | 11.92 | 5.96 | 11.92 | 11.92 |
| | MBC | 11.92 | 11.92 | 5.96 | 11.92 | 11.92 | 14.9 | 11.92 |
| **4o** | MIC | 12.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 12.00 |
| | MBC | 24.00 | 3.00 | 6.00 | 6.00 | 12.00 | 6.00 | 24.00 |
| **4p** | MIC | 11.60 | 2.90 | 2.90 | 2.90 | 2.90 | 2.90 | 11.60 |
| | MBC | 23.20 | 2.90 | 2.90 | 2.90 | 11.60 | 2.90 | 23.20 |
| **4q** | MIC | 5.40 | 0.68 | 2.70 | 2.70 | 2.70 | 2.70 | 5.40 |
| | MBC | 10.80 | 2.70 | 2.70 | 2.70 | 10.80 | 5.40 | 10.80 |
| **4r** | MIC | 5.00 | 2.50 | 2.50 | 2.50 | 5.00 | 2.50 | 5.00 |
| | MBC | 10.00 | 2.50 | 2.50 | 2.50 | 10.00 | 2.50 | 10.00 |
| **4s** | MIC | 2.52 | 2.52 | 2.52 | 2.52 | 2.52 | 2.52 | 2.52 |
| | MBC | 5.04 | 2.52 | 5.04 | 5.04 | 5.04 | 5.04 | 5.04 |
| **4t** | MIC | 5.00 | 2.50 | 2.50 | 5.00 | 5.00 | 5.00 | 5.00 |
| | MBC | 10.00 | 5.00 | 2.50 | 10.00 | 10.00 | 10.00 | 5.00 |
| **4u** | MIC | 3.4 | 0.9 | 3.4 | 1.7 | 1.7 | 1.7 | 6.8 |
| | MBC | 6.8 | 3.4 | 6.8 | 3.4 | 3.4 | 3.4 | 13.7 |
| Ampicilin | MIC | 24.80 | 24.80 | 24.80 | 37.20 | 74.40 | 24.80 | 37.20 |
| | MBC | 37.20 | 37.20 | 37.20 | 74.40 | 124.00 | 49.20 | 49.20 |
| Streptomycin | MIC | 17.20 | 4.30 | 8.60 | 25.80 | 17.20 | 17.20 | 17.20 |
| | MBC | 34.40 | 8.60 | 17.20 | 51.60 | 34.40 | 34.40 | 34.40 |

*S. a.—Staphylococcus aureus*, *B. c.—Bacillus cereus*, *M. f.—Micrococcus flavus*, *L. m.—Listeria monocytogenes*, *Ps. aer.—Pseudomonas aeruginosa*, *S. typhi.—Salmonella typhimurium*, *E. coli.—Escherichia coli*.

2,6-dichloro substituted derivatives, are presenting the most noteworthy properties.

The results revealed very strong antifungal activity better than commercial fungicides bifonazole (15- to 28-fold) and ketoconazole (15- to 250-fold) which were tested as positive standards (Table 2). Minimal inhibitory concentration (MIC) of these compounds is observed at the range of 0.73–23.2 × $10^{-2}$ μmol/ml and minimal fungicidal concentration MFC 1.52–38.5 × $10^{-2}$ μmol/ml. The antifungal potential could be presented as follows: **4j = 4o > 4r > 4n > 4i = 4t > 4h > 4q = 4s > 4p > 4c > 4l > 4m > 4f > 4e > 4u > 4k > 4g > 4b > 4d.** The best activity was obtained by compound **4j** with MIC at the range of 0.76–1.52 × $10^{-2}$ μmol/ml and MFC at the range of 1.52 × $10^{-2}$ μmol/ml, while **4d** showed the lowest antifungal potential with MIC 0.73–23.3 × $10^{-2}$ μmol/ml and MFC at 5.8–34.8 × $10^{-2}$ μmol/ml. The most resistant fungal species was *Aspergillus flavus,* while *Trichoderma viride* and *Penicillium ochrochloron* were the most sensitive species.

Again the observed results support the significant positive effect of bulky substituents to the antifungal potential.

It is worth to be mentioned that the position of chloro substituent plays important role in antifungal activity. Thus, in case of 5-(chlorobenzylidene)-2-(4-phenyl-1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-ones the most active appears to be 2-Cl, followed by 4-Cl and 3-Cl derivatives. The last one is less potent than the 5-(benzylidene)-2-(4-phenyl-1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one It was also observed that the nature of R-substituent affects the antifungal activity as well. Thus, replacement of 4-phenyl- (compound **4t**) with 4-methyl (**4o**) increased activity 1.6-fold.

It was found that the range of antibacterial activity of compounds tested was wider than that of antifungal activity.

## 2.3. Anti-inflammatory activity

### 2.3.1. In vivo inhibition of the carrageenin-induced edema

In acute toxicity experiments, the compounds examined in vivo did not present toxic effects in ip doses up to 0.5 mmol/kg body weight of the mouse. The in vivo anti-inflammatory effect of the tested compounds was assessed by using the functional model of carrageenin-induced mouse paw edema and is presented as the percentage of inhibition of induction of edema at the right hind paw (Table 3). Edema was measured by weight increase of the right hind paw in comparison to the uninjected left paw.

Carrageenin-induced edema is a nonspecific inflammation that is highly sensitive to nonsteroidal anti-inflammatory drugs (NSAIDs), and so carrageenin has been accepted as a suitable agent for

**Table 2**
Antifungal activity of unsubstituted/4-substituted 2-thiazolylimino-5-arylidene-4-thiazolidinones (MIC and MFC, μlmol/ml × $10^{-2}$)

| Compounds | | *P. f.* | *P. o.* | *T. v.* | *A. fum.* | *A. n.* | *A. fl.* |
|---|---|---|---|---|---|---|---|
| **4b** | MIC | 5.5 | 1.38 | 11 | 22 | 22 | 11 |
| | MFC | 11 | 2.75 | 22 | 33 | 38.5 | 22 |
| **4c** | MIC | 1.58 | 1.58 | 1.58 | 1.58 | 1.58 | 2.36 |
| | MFC | 3.15 | 3.15 | 3.15 | 3.15 | 3.15 | 3.15 |
| **4d** | MIC | 0.73 | 5.8 | 5.8 | 23.3 | 23.2 | 29 |
| | MFC | 5.8 | 11.6 | 34.8 | 34.8 | 29 | 34.8 |
| **4e** | MIC | 4.94 | 3.30 | 3.30 | 4.94 | 4.94 | 4.94 |
| | MFC | 6.60 | 6.60 | 4.94 | 6.60 | 6.60 | 6.60 |
| **4f** | MIC | 3.27 | 3.27 | 3.27 | 3.27 | 3.27 | 3.27 |
| | MFC | 6.54 | 6.54 | 6.54 | 4.91 | 6.54 | 6.54 |
| **4g** | MIC | 3.27 | 3.27 | 3.27 | 4.91 | 3.27 | 6.54 |
| | MFC | 6.54 | 6.54 | 6.54 | 6.54 | 6.54 | 8.19 |
| **4h** | MIC | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 | 2.20 |
| | MFC | 2.20 | 2.20 | 2.20 | 2.56 | 2.56 | 2.56 |
| **4i** | MIC | 2.05 | 1.37 | 1.37 | 1.37 | 1.37 | 1.37 |
| | MFC | 2.73 | 2.73 | 2.73 | 2.73 | 2.73 | 2.73 |
| **4j** | MIC | 0.76 | 0.76 | 0.76 | 1.52 | 1.52 | 1.52 |
| | MFC | 1.52 | 1.52 | 1.52 | 1.52 | 1.52 | 1.52 |
| **4k** | MIC | 2.81 | 2.81 | 2.11 | 5.62 | 5.62 | 5.62 |
| | MFC | 7.02 | 5.62 | 2.81 | 7.02 | 7.02 | 7.02 |
| **4l** | MIC | 2.81 | 4.21 | 2.81 | 4.21 | 4.21 | 4.21 |
| | MFC | 5.62 | 5.62 | 4.21 | 5.62 | 5.62 | 5.62 |
| **4m** | MIC | 2.81 | 4.21 | 2.81 | 5.62 | 4.21 | 4.21 |
| | MFC | 5.62 | 5.62 | 5.62 | 7.02 | 5.62 | 5.62 |
| **4n** | MIC | 1.79 | 1.49 | 1.79 | 1.79 | 1.49 | 2.09 |
| | MFC | 2.09 | 2.09 | 2.09 | 2.09 | 2.09 | 2.09 |
| **4o** | MIC | 0.76 | 0.76 | 0.76 | 1.52 | 1.52 | 1.52 |
| | MFC | 1.52 | 1.52 | 1.52 | 1.52 | 1.52 | 1.52 |
| **4p** | MIC | 1.58 | 1.58 | 1.58 | 1.58 | 1.58 | 1.58 |
| | MFC | 3.15 | 3.15 | 3.15 | 3.15 | 3.15 | 3.15 |
| **4q** | MIC | 1.90 | 1.90 | 1.26 | 1.90 | 1.90 | 1.90 |
| | MFC | 2.52 | 2.52 | 2.52 | 2.52 | 2.52 | 2.52 |
| **4r** | MIC | 1.76 | 1.17 | 1.17 | 1.76 | 1.76 | 1.76 |
| | MFC | 2.34 | 2.34 | 2.34 | 2.34 | 2.34 | 2.34 |
| **4s** | MIC | 1.90 | 1.90 | 1.26 | 1.90 | 1.90 | 1.90 |
| | MFC | 2.52 | 2.52 | 2.52 | 2.52 | 2.52 | 2.52 |
| **4t** | MIC | 2.05 | 1.37 | 1.37 | 1.37 | 1.37 | 1.37 |
| | MFC | 2.73 | 2.73 | 2.73 | 2.73 | 2.73 | 2.73 |
| **4u** | MIC | 1.7 | 1.7 | 0.85 | 1.7 | 3.4 | 3.4 |
| | MFC | 3.4 | 3.4 | 1.7 | 3.4 | 6.8 | 6.8 |
| Bif | MIC | 64.00 | 48.00 | 64.00 | 48.00 | 48.00 | 48.00 |
| | MFC | 80.00 | 64.00 | 80.00 | 64.00 | 64.00 | 64.00 |
| Ket | MIC | 38.00 | 380.00 | 475.00 | 38.00 | 38.00 | 285.00 |
| | MFC | 95.00 | 380.00 | 570.00 | 95.00 | 95.00 | 380.00 |

*P. f.—Penicillium funiculosum*, *P. o.—Penicillium ochrochloron*, *T. v.—Trichoderma viride*, *A. fum.—Aspergillus fumigatus*, *A. n.—Aspergillus niger*, *A. fl.—Aspergillus flatus*.

the study of new compounds with anti-inflammatory activity. As shown in Table 3, the majority of the investigated compounds induced protection against carrageenin-induced paw edema. The protection ranged up to 67.3%, while the reference drug, indomethacin exhibited 47% protection at an equivalent dose. Compound **4j** is the most potent followed by compounds **4q**, **4k** and **4p**. Among the 3-Cl and 4-Cl substituted derivatives, compounds **4s** and **4t** and the unsubstituted **4q**, most potent seems to be compound **4q**. It seems that low $\pi$ values of $R_1$ are correlated with higher anti-inflammatory activity; for example, for compounds **4j** and **4i**, 4-$N(CH_3)_2$ >4-Br ($\pi$-$N(CH_3)_2$ = 0.18; $\pi$-Br 0.86. Between the two 4-$CH_3$ substituted analogues (**4n** and **4o**) no differences are observed. Both compounds seem to be equipotent.

Based on very preliminary results, it seems that molar hydrophilicity (low lipophilicity values, *C*log*P*) partly affects the anti-inflammatory activity of the sub-group in which R = H. On the contrary, for the sub-group where R is phenyl, higher lipophilicity is correlated with potent anti-inflammatory activity in vivo.

### 2.3.2. In vitro soybean lipoxygenase LOX inhibition

Compounds were further evaluated for inhibition of soybean lipoxygenase LOX by the UV absorbance based enzyme assay. It has been shown that inhibition of plant LOX activity by NSAIDs is qualitatively similar to the inhibition of the rat mast cell LOX and can be used as a simple qualitative screen for such activity.[26]

Perusal of $IC_{50}$ values shows that compound **4d** is the most active within the set, followed by compounds **4t**, **4c**, **4o**, **4n** = **4p** (Table 3). The comparison of 4-Cl derivatives (**4o**) and (**4t**) revealed that compound **4t** demonstrates highly significant inhibition compared to the corresponding 4-$CH_3$ substituted derivative (**4o**).

### 2.3.3. COX-1/COX-2 inhibition

Cycloxygenase inhibitory action was measured using ovine recombinant COX-1 and and human recombinant COX-2 isoenzymes included in 'COX inhibitor screening assay' kit purchased by Cayman. It was found that all tested compounds exhibited inhibitory activity ranging from 50% to 86%. The best inhibition was observed for compound **4m** (100%, $IC_{50}$ 10 μM) followed by compound **4a** (98%, $IC_{50}$ 16 μM). It seems that the presence of a chloro substituent (**4k**–**4o**, **4s**, **4t**) in general is favourable for COX inhibitory activity. It should be mentioned that two chloro atoms in the molecule leads to more active COX-1 inhibitors (97–100%) in comparison to mono chloro substitution (65–97%). The replacement of 3-Cl and 4-Cl with a fluoro substituent decreased activity two-folds, while in case of bromo derivatives inhibition seems to be dependent on position of the bromine atom in the molecule. Thus, 4-Br derivative exhibited moderate activity (67%) while 3-Br was almost inactive (2%) (Table 3). Good inhibitory activity was exhibited by 5-(4-(chlorobenzylidene)-2-(4-methyl-1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (**4e**).

On the other hand it is obvious that the presence of free OH group in $C_3$ of benzene ring is also beneficial for COX-1 inhibition. The insertion of methoxy group in 3,5 position led to dramatic decrease of inhibitory activity. The role of lipophilicity is not well defined.

All compounds showed no or low (0–47%) COX-2 inhibition when they were added to the assay mixture in a concentration of 200 μM. The results shown in Table 3 were obtained at an arachidonic acid substrate concentration of 0.1 μM. Increase of arachidonic acid concentration led to loss of activity, showing that the compounds are competitive inhibitors of the enzyme.

5-(2-Methoxybenzylidene)-2-(4-phenyl-1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-ones (**4c**) exhibited the best COX-2 inhibition (47%). It is only 1.4-fold less active than naproxene. Introduction of the second methoxy group led to compound **4d** which is completely inactive as COX-2 inhibitor. Inhibition was also exhibited by compound **4t** (34%) with 4-Cl substitution. The results show that lipophilicity does not play role in COX-2 inhibition.

## 3. Conclusion

This study has demonstrated that 5-arylidene-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-ones bearing a variety of substituents in the aromatic part combine anti-inflammatory, antibacterial and antifungal activities. All tested compounds exhibited good ability to inhibit bacteria and fungi, higher than commercial antimicrobial agents used as reference drugs. It is worth to notice that these compounds are more potent/selective against fungi.

Some of our derivatives proved to be potent in vitro inhibitors of soybean lipoxygenase and COX-1 enzyme. Compound **4d** is the most potent LOX inhibitor, whereas compound **4j** presents the highest anti-inflammatory activity and 50% COX-1 inhibition. Compound **4m** ($IC_{50}$ 10 μM) is the most potent COX-1 inhibitor**.** The influence of lipophilicity is not well documented.

However, further investigation is needed in order to gain insight into the mechanism of action of the examined compounds. Some of the synthesized compounds might constitute initial leads for the design of new more potent multi-target therapeutic agents.

**Table 3**
Anti-inflammatory and COX/LOX inhibitory activity of synthesized compounds

| Compound | CPE (%)[a] | COX-1 (%) | COX-1 ($IC_{50}$ μM) | COX-2 (%) | LOX (%) | LOX ($IC_{50}$ μM) | *C*log *P* |
|---|---|---|---|---|---|---|---|
| **4a** | 33.7 | 98.0 | 16.0 | 18.0 | 35.0 | >150 | |
| **4b** | 39.0 | 23.0 | — | 0 | 11.0 | >150 | 0.393 |
| **4c** | 50.0 ± 2.2 | 86.0 | — | 47.0 | | 63.0 | 1.530 |
| **4d** | 26.0 ± 1.1 | 85.0 | — | 0 | | 52.0 | 2.490 |
| **4e** | 47.0 | 99.0 | 51.0 | Nt | 67.0 | 92.0 | 1.927 |
| **4f** | 56.57 | 42.0 | — | 16.0 | 72.0 | 90.0 | 1.571 |
| **4g** | 65.6 | 44.0 | — | 11.0 | 35.0 | >150 | 1.571 |
| **4h** | 42.7 | 2.0 | — | 7.0 | 34.0 | >150 | 2.291 |
| **4i** | 42.2 ± 1.3 | 67.0 | — | 0 | | 66.0 | 2.291 |
| **4j** | 67.3 ± 4.5 | 50.0 | — | 0 | | 95.0 | 2.260 |
| **4k** | 58.5 | 100.0 | 78.0 | 22.0 | 43.0 | 137 | 2.854 |
| **4l** | 9.9 | 98.0 | 32.0 | Nt | 54.0 | 122.0 | 2.854 |
| **4m** | 45.0 | 100.0 | 10.0 | Nt | 41.0 | 143.0 | 2.734 |
| **4n** | 43.0 ± 3.4 | 97.0 | — | 0 | | 79.0 | 2.820 |
| **4o** | 42.0 ± 3.2 | 74.0 | — | 0 | | 76.0 | 2.820 |
| **4p** | 55.4 ± 3.1 | 68.0 | — | Nt | | 79.0 | |
| **4q** | 59.3 ± 2.1 | 67.0 | — | 0 | | 66.0 | 3.700 |
| **4s** | 51.0 ± 1.8 | 65.0 | — | 0 | | 88.0 | 4.420 |
| **4t** | 27.3± 1.0 | 78.0 | — | 34.0 | | 54.0 | 4.420 |
| **4u** | 54.4 | 73.0 | 65 | Nt | Nt | | |
| Indom/cine | 47.0 ± 1.1 | Nt | — | Nt | | Nt | |
| Naproxene | Nt | 46.0 | 49.0 | 65.0 | | Nt | |
| NDGA | Nt | Nt | — | Nt | | 32 | |

[a] Inhibitory activity on carrageenan-induced rat paw oedema. The results are expressed as mean ($n$ = 6–10) percentage reduction of the difference in weight between the carrageenan injected and uninjected paws following a 0.01 mmol/kg intraperitoneal injection of the test compound.

## 4. Experimental

### 4.1. Chemistry—general aspects

Melting points °C were determined with a MELTEMP II capillary apparatus (LAB Devices, Holliston, MA, USA) without correction. Elemental analyses were performed on Perkin–Elmer 2400 CHN elemental analyzer for all compounds synthesized and were within ±0.4% of theoretical values. IR spectra were recorded, in Nujol, on Perkin Elmer Spectrum BX and DR-8001 Shimadzu. Wave numbers in the IR spectra are given in $cm^{-1}$. $^1H$ NMR spectra of the newly synthesized compounds, in DMSO-$d_6$ solutions, were recorded on a Bruker AC 300 instrument (Bruker, Karlsruhe, Germany) at 298 K. Chemical shifts are reported as $\delta$ (ppm) relative to TMS as internal standard. Coupling constants $J$ are expressed in Hertz (Center of Instrumental Analysis of the University of Thessaloniki).

The reactions were monitored by TLC on $F_{254}$ silica-gel precoated sheets (Merck, Darmstadt, Germany) and the purified compounds each showed a single spot.

Solvents, unless otherwise specified were of analytical reagent grade or of the highest quality commercially available. Synthetic starting materials, reagents and solvents were purchased from Aldrich Chemie (Steinheim Germany).

#### 4.1.1. General procedure for synthesis of 5-arylidene-2-(4-substituted/unsubstituted-1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-ones (4a–4u)

A well-stirred solution of 0.8 g of 2-(thiazol-2-ylimino)thiazolidin-4-one (4 mmol) in 35 mL of acetic acid was buffered with sodium acetate (8 mmol) and added with the appropriate arylaldehyde (6 mmol). The solution was refluxed for 4 h and then poured into ice-cold water. The precipitate was filtered and washed with water and the resulting crude product was purified by recrystallisation from dioxane.

**4.1.1.1. 5-(3-Hydroxybenzylidene)-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4a).** Yield: 12%, mp 279–280 °C. IR (Nujol): 3084 (NH), 1702 (C=O), 1594 (C=N) $cm^{-1}$. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 6.88–6.91 (m, 1H, ArH), 7.04–7.10 (m, 2H, ArH), 7.33–7.38 (m, 1H, ArH), 7.49–7.62 (m, 2H, thiazole), 7.73 (s, 1H, =CH). Anal. Calcd for $C_{13}H_9N_3O_2S_2$ (303.01): C, 51.47; H, 2.99; N, 13.85. Found: C, 51.50; H, 3.01; N, 13.83.

**4.1.1.2. 5-(4-Hydroxy-3,5-dimethoxybenzylidene)-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4b).** Yield: 11%, mp 201–202 °C. IR (Nujol): 3089 (NH), 1702 (C=O), 1591 (C=N) $cm^{-1}$. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 3.84 (s, 6H, $OCH_3$), 6.97(s, 2H, ArH 2′ and 6′), 7.38–7.45 (m, 1H, thiazole 5′), 7.58–7.65 (m, 2H, thiazole 4′, =CH). Anal. Calcd for $C_{15}H_{13}N_3O_4S_2$ (363.03): C, 49.57; H, 3.61; N, 11.56. Found: C, 49.55; H, 3.59; N, 11.54.

**4.1.1.3. 5-(2-Methoxybenzylidene)-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4c).** Yield: 76%, mp 234–235 °C. IR (Nujol): 3110 (NH), 1690 (C=O), 1597 (C=N). $cm^{-1}$. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 3.90 (s, 3H, $OCH_3$), 7.15–7.18 (m, 2H, ArH), 7.46–7.53 (m, 3H, ArH, thiazole), 7.69–7.70 (m, 1H, ArH), 7.94 (s, 1H, =CH). Anal. Calcd for $C_{14}H_{11}N_3O_2S_2$ (317.39): C, 52.98; H, 3.49; N, 13.24. Found: C, 52.99; H, 3.52; N, 13.26.

**4.1.1.4. 5-(2,5-Dimethoxybenzylidene)-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4d).** Yield: 84%, mp 195–197 °C. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 3.78 (s, 3H, 5-OMe), 3.85 (s, 3H, 2-OMe), 7.04–7.1 (m, 3H, ArH), 7.47 (d, $J$ = 3.3 Hz, 1H, thiazole 5′), 7.7 (d, $J$ = 3.6 Hz, 1H, thiazole 4′), 7.87 (s, 1H, =CH). Anal. Calcd for $C_{15}H_{13}N_3O_3S_2$ (347.42): C, 51.86; H, 3.77; N, 12.10. Found: C, 51.82; H, 3.84; N, 12. 15.

**4.1.1.5. 5-(4-Methylbenzylidene)-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4e).** Yield: 13%, mp 211–213 °C. IR (Nujol): 3195 (NH), 1717 (C=O), 1584 (C=N) $cm^{-1}$. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 2.37 (s, 3H, Ar-$CH_3$), 7.30–7.39 (m, 2H, ArH 3′ and 5′), 7.52–7.55 (m, 3H, ArH 2′ and 6′, thiazole 5′), 7.68 (s, 1H thiazole 4′), 7.72 (s, 1H, =CH). Anal. Calcd for $C_{14}H_{11}N_3OS_2$ (301.03): C, 55.79; H, 3.68; N, 13.94. Found: C, 55.82; H, 3.71; N, 13. 97.

**4.1.1.6. 5-(3-Fluorobenzylidene)-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4f).** Yield: 34%, mp 217–219 °C. IR (Nujol): 3091 (NH), 1720 (C=O), 1596 (C=N) $cm^{-1}$. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 7.30–7.32 (m, 1H, ArH), 7.47–7.59 (m, 2H thiazole), 7.61–7.66 (m, 3H, ArH), 7.71 (s, 1H, =CH). Anal. Calcd for $C_{13}H_8FN_3OS_2$ (305.01): C, 51.13; H, 2.64; N, 13.76. Found: C, 51.16; H, 2.67; N, 13.79.

**4.1.1.7. 5-(4-Fluorobenzylidene)-2-(1,3-thiazol-2-ylimino)-1,3thiazolidin-4-one (4g).** Yield: 27%, mp 225–227 °C. IR (Nujol): 3091 (NH), 1720 (C=O), 1596 (C=N) $cm^{-1}$. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 7.39–7.58 (m, 4H, ArH), 7.69–7.73 (m, 3H, thiazole, =CH). Anal. Calcd for $C_{13}H_8FN_3OS_2$ (305.01): C, 51.13; H, 2.64; N, 13.76. Found: C, 51.15; H, 2.66; N, 13.78.

**4.1.1.8. 5-(3-Bromobenzylidene)-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4h).** Yield: 38%, mp 209–210 °C. IR (Nujol): 3098 (NH), 1709 (C=O), 1596 (C=N) $cm^{-1}$. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 7.51–7.57 (m, 2H, thiazole), 7.74 (d, $J$ = 7.5 Hz, 2H, ArH), 7.71 (s, 1H, ArH 2′), 7.62–7.67 (m, 1H, ArH), 7.87 (s, 1H, =CH). Anal. Calcd $C_{13}H_8BrN_3OS_2$ (364.93): C, 42.63; H, 2.20; N, 11.47. Found: C, 42.61; H, 2.18; N, 11.45.

**4.1.1.9. 5-(4-Bromobenzylidene)-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4i).** Yield: 80, mp 268–269 °C. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 7.51 (d, $J$ = 3 Hz, 1H, thiazole 5′), 7.58 (d, $J$ = 9 Hz, 2H, ArH 3′ and 5′), 7.68–7.72 (m, 1H, thiazole 4′), 7.73 (s, 1H, =CH), 7.78 (d, $J$ = 9 Hz, 2H, ArH 2′ and 6′). Anal. Calcd for $C_{13}H_8BrN_3OS_2$ (364.93): C, 42.63; H, 2.20; N, 11.47. Found: C, 42.67; H, 2.23; N, 11.50.

**4.1.1.10. 5-(4-(Dimethylamino)benzylidene)-2-(1,3-thiazol-2-ylimino-1,3-thiazolidin-4-one (4j).** Yield: 69%, mp 176–177 °C. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 3.03 (s, 6H, $N(CH_3)_2$), 6.86 (d, $J$ = 7.2 Hz, 2H, ArH 3′ and 5′), 7.44–7.70 (m, 5H, ArH 2′ and 6′, thiazole, =CH). Anal. Calcd for $C_{15}H_{14}N_4OS_2$ (330.43): C, 54.52; H, 4.27; N, 16.96. Found: C, 54.62; H, 4.29; N, 16.87.

**4.1.1.11. 5-(2,6-Dichlorobenzylidene)-2-(1,3-thiazol-2-ylimino)1,3-thiazolidin-4-one (4k).** Yield: 74%, mp 255–257 °C. IR (Nujol): 3079 (NH), 1693 (C=O), 1590 (C=N) $cm^{-1}$. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 7.47–7.54 (m, 2H, thiazole), 7.62–7.64 (m, 4H, ArH and =CH). Anal. Calcd for $C_{13}H_7Cl_2N_3OS_2$ (354.94): C, 43.83; H, 1.98; N, 11.80. Found: C, 43.80; H, 1.95; N, 11.95.

**4.1.1.12. 5-(2,4-Dichlorobenzylidene)-2-(1,3-thiazol-2-ylimino-1,3-thiazolidin-4-one (4l).** Yield: 75%, mp 259–261 °C. IR (Nujol): 3081 (NH), 1696 (C=O), 1593 (C=N) $cm^{-1}$. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 7.48–7.49 (m, 1H, thiazole 5′), 7.50–7.68 (m, 3H, thiazole 4′, ArH), 7.70–7.79 (m, 2H, ArH, =CH). Anal. Calcd for $C_{13}H_7Cl_2N_3OS_2$ (354.94): C, 43.83; H, 1.98; N, 11.80. Found: C, 43.82; H, 1.97; N, 11.79.

**4.1.1.13. 5-(2,3-Dichlorobenzylidene)-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4m).** Yield: 83%, mp 264–265 °C. IR (Nujol): 3084 (NH), 1671 (C=O), 1595 (C=N) $cm^{-1}$. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 7.49 (d, $J$ = 3 Hz, 1H, thiazole 5′), 7.59–7.69 (m, 3H, thiazole 4′, ArH), 7.75 (d, $J$ = 6 Hz, 1H, ArH), 7.83 (s, 1H, =CH). Anal. Calcd for $C_{13}H_7Cl_2N_3OS_2$ (354.94): C, 43.83; H, 1.98; N, 11.80. Found: C, 43.82; H, 1.97; N, 11.77.

**4.1.1.14. 5-(2-Chlorobenzylidene)-2-(4-methyl-1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4n).** Yield: 65%, mp 241–243 °C. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 2.26 (s, 3H, thiazole 4-$CH_3$), 7.01 (d, 1H, ArH), 7.48–7.69 (m, 4H, ArH, =CH), 7.84 (s, 1H, thiazole). Anal. Calcd for $C_{14}H_{10}ClN_3OS_2$ (335.83): C, 50.07; H, 3.00; N, 10.56. Found: C, 50.05; H, 2.98; N, 10.54.

**4.1.1.15. 5-(4-Chlorobenzylidene)-2-(4-methyl-1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4o).** Yield: 77%, mp 270–271 °C. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 2.37 (s, 3H, $CH_3$), 7.64–7.70 (m, 6H, ArH, thiazole, =CH). Anal. Calcd for $C_{14}H_{10}ClN_3OS_2$ (335,83): C, 50.07; H, 3.00; N, 12.51. Found: C, 50.10; H, 3.03; N, 12.54.

**4.1.1.16. 5-(4-Nitrobenzylidene)-2-2-(5-methy-1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4p).** Yield: 52%, mp 301–303 °C. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 2.37 (s, 3H, $CH_3$), 7.64–7.69 (m, 6H, ArH, thiazole, =CH). Anal. Calcd for $C_{14}H_{10}N_4O_3S_2$ (346.38): C, 48.54; H, 2.91; N, 16.17. Found: C, 48.50; H, 3.03; N, 16.24.

**4.1.1.17. 5-Benzylidene-2-(4-phenyl-1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4q).** Yield: 84%, mp 285–287 °C. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 7.39–7.75 (m, 9H, ArH, thiazole), 7.89 (s, 1H, =CH), 8.01 (d, $J$ = 7.5 Hz, 2H, ArH). Anal. Calcd for $C_{19}H_{13}N_3OS_2$ (363.46): C, 62.79; H, 3.61; N, 11.56. Found: C, 62.84; H, 3.71; N, 11.60

**4.1.1.18. 5-(2-Chlorobenzylidene)-2-(4-phenyl-1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4r).** Yield: 64%, mp 256–258 °C. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 7.34–7.69 (m, 6H, ArH), 7.80 (d, 1H, ArH), 7.87 (s, 1H, =CH), 7.93–7.97 (m, 3H, thiazole, ArH). Anal. Calcd for $C_{19}H_{12}ClN_3OS_2$ (397.90): C, 57.35; H, 3.04; N, 10.56. Found: C, 57.42; H, 3.12; N, 10.49.

**4.1.1.19. 5-(3-Chlorobenzylidene)-2-(4-phenyl-1,3-lthiazol-2-ylimino)-1,3-thiazolidin-4-one (4s).** Yield: 76%, mp 304–305 °C. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 7.36–7.72 (m, 7H, ArH), 7.78 (s, 1H, =CH), 7.94 (s, 1H, thiazole), 8.01 (d, 2H, ArH). Anal. Calcd for $C_{19}H_{12}ClN_3OS_2$ (3971): C, 57.35; H, 3.04; N, 10.56. Found: C, 57.38; H, 3.09; N, 10.59.

**4.1.1.20. 5-(4-Chlorobenzylidene)-2-(4-phenyl-1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4t).** Yield: 77%, mp 247–8 °C. IR (Nujol): 3200 (NH), 1690 (C=O), 1650 (C=N) $cm^{-1}$. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 7.48–7.77 (m, 8H, ArH, thiazole), 7.93–8.02 (m, 3H, =CH, ArH). Anal. Calcd for $C_{19}H_{12}ClN_3OS_2$ (397.91): C, 57.35; H, 3.04; N, 10.56. Found: C, 57.40; H, 3.10; N, 10.60.

**4.1.1.21. 5-(2-Hydroxy-5-bromobenzylidene)-2-(1,3-thiazol-2-ylimino)-1,3-thiazolidin-4-one (4u).** Yield: 79%, mp 185–186 °C. IR (Nujol): 3200 (NH), 1690 (C=O), 1650 (C=N) $cm^{-1}$. $^1H$ NMR ($\delta$ ppm, DMSO-$d_6$, 300 MHz): 6.98 (d, $J$ = 8.7 Hz, 1H, ArH), 7.59 (d, $J$ = 3.6 Hz, 1H, thiazole 5′), 7.64 (d, $J$ = 2.7 Hz, 1H, ArH), 7.67 (d, $J$ = 2.4 Hz, 1H, thiazole 4′), 7.71 (d, $J$ = 2.7 Hz, 2H, =CH, ArH), 10.21 (s, 1H, OH). Anal. Calcd for $C_{13}H_8BrN_3O_2S_2$ (382.25): C, 40.85; H, 2.11; N, 10.99. Found: C, 40.87; H, 2.14;N, 11.015.

### 4.2. Antibacterial activity

The following Gram-negative bacteria were used: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), and the following Gram-positive bacteria: *Listeria monocytogenes* (NCTC 7973), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Staphylococcus aureus* (ATCC 6538). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research 'Siniša Stanković', Belgrade, Serbia.

The antibacterial assay was carried out by microdilution method[27–29] in order to determine the antibacterial activity of compounds tested against the human pathogenic bacteria.

The bacterial suspensions were adjusted with sterile saline to a concentration of $1.0 \times 10^5$ cfu/ml. The inocula were prepared daily and stored at +4 °C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum.

#### 4.2.1. Microdilution test

The minimum inhibitory and bactericidal concentrations (MICs and MBCs) were determined using 96-well microtitre plates. The bacterial suspension was adjusted with sterile saline to a concentration of $1.0 \times 10^5$ cfu/ml. Compounds to be investigated were dissolved in broth LB medium (100 μl) with bacterial inoculum ($1.0 \times 10^4$ cfu per well) to achieve the wanted concentrations (1 mg/ml). The microplates were incubated for 24 h at 48 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The compounds investigated were dissolved in 5% DMSO (1 mg/ml) and added in LB medium with inoculum. The MBCs were determined by serial sub-cultivation of 2 μl into microtitre plates containing 100 μl of broth per well and further incubation for 72 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by Microplate manager 4.0 (Bio-Rad Laboratories) and compared with a blank and the positive control. Streptomycin and ampicillin were used as a positive control (1 mg/ml). All experiments were performed in duplicate and repeated three times.

### 4.3. Antifungal activity

For the antifungal bioassays, eight fungi were used: *Aspergillus flavus* (ATCC 9643), *Aspergillus fumigatus* (plant isolate), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Trichoderma viride* (IAM 5061). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia.

The micromycetes were maintained on malt agar and the cultures stored at 4 °C and sub-cultured once a month[30] In order to investigate the antifungal activity of the extracts, a modified microdilution technique was used.[27–29] The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately $1.0 \times 10^5$ in a final volume of 100 μl per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid malt agar to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. The compounds investigated were dissolved in 5% DMSO (1 mg/ml) and added in broth Malt medium with inoculum. The microplates were incubated for 72 h at 28 °C, respectively. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs.

The fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2 μl into microtiter plates containing 100 μl of broth per well and further incubation 72 h at 28 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. DMSO was used as a negative control, commercial fungicides, bifonazole and ketoconazole, were used as positive controls (1–3000 μg/ml). All experiments were performed in duplicate and repeated three times.

### 4.4. Biological assays: in vitro experiments

In the in vitro assays each experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the average values.

#### 4.4.1. Soybean lipoxygenase inhibition study in vitro[31]

Lipoxygenase inhibition was evaluated as reported previously.[9] The tested compounds, dissolved in DMSO, were added to the reaction mixture at several concentrations and were preincubated for 4 min at 28 °C with soybean lipoxygenase at a concentration of $7 \times 10^{-7}$ w/v. $IC_{50}$ values were determined. Enzyme reaction was initiated by the addition of sodium linolate at a final concentration of 100 μM. The conversion of sodium linoleate to 13-hydroperoxylinoleic acid was measured at 234 nm. Nordihydroguaretic acid, an appropriate standard inhibitor, was used as positive control (inhibition) 94.4% at 100 μM.

#### 4.4.2. COX inhibitor screening assay[32]

The COX-1 and COX-2 activities of the compounds were measured using ovine COX-1 and human recombinant COX-2 enzymes included in the 'COX inhibitor screening assay' kit provided by Cayman (Cayman Chemical Co., Ann Arbor, MI). The assay directly measures PGF2a produced by $SnCl_2$ reduction of COX-derived PGH2. The prostanoids production was quantified via enzyme immunoassay using a broadly specific antibody that binds to all the major prostaglandin compounds. COX-1/COX-2 inhibitory activity was tested at an arachidonic acid concentration of 0.1 μM which is much lower from the saturating concentration. $IC_{50}$ values were calculated for the most active compounds. Naproxen was used as positive control.

#### 4.4.3. In vivo experiments: inhibition of the carrageenin-induced edema

Edema was induced in the right hind paw of mice (AKR) by the intradermal injection of 0.05 mL of 2% carrageenin in water. Both sexes were used, but pregnant females were excluded. Each group was composed of 6–10 animals. The animals, which have been bred in our laboratory, were housed under standard conditions and received a diet of commercial food pellets and water ad libitum prior to experimentation, but they were fasted during the experimental period. The tested compounds (0.01 mmol/kg body weight) were suspended in water with a few drops of Tween-80 and ground in a mortar before use and were given intraperitoneally simultaneously with the carrageenin injection. The mice were euthanized 3.5 h after the carrageenin injection. The difference between the weight of the injected and uninjected paws was calculated for each animal. It was compared with that in control animals (treated with water) and expressed as a percent inhibition of the edema CPE% values (Table 3). Each experiment was performed in duplicate, and the standard deviation was less than 10%.

## Acknowledgments

We thank Dr. A. Leo and Biobyte Corp., Claremont, California, for free access to the C-QSAR program.

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