Original article

One-pot microwave assisted synthesis under green chemistry conditions, antioxidant screening, and cytotoxicity assessments of benzimidazole Schiff bases and pyrimido[1,2-a]benzimidazol-3(4H)-ones

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ABSTRACT

The synthesis of a number of benzimidazole Schiff bases 3 and 3-oxo-pyrimido[1,2-a]benzimidazoles 4 in excellent yields by a one-step sequence from the reaction of 2-aminobenzimidazole under green chemistry conditions is described. Structural assignments of the new compounds as well as complete assignment of ^1H and ^{13}C NMR signals have been unambiguously achieved based on the analysis of their ^1H and ^{13}C NMR (1D and 2D), IR, MS and elemental analysis data. To the synthesized Schiff bases the E-configuration was assigned on the basis of comparison of experimental and calculated (DFT) ^{13}C NMR chemical shifts. Compounds 3 and 4 were evaluated as inhibitors of lipoxygenase (LOX) and of lipid peroxidation (LPO). All the tested derivatives showed inhibition of lipid peroxidation, whereas most of them were found to have higher activation than the reference compound trolox; The Schiff bases 3e, 3h, and 3i, and the pyrimidobenzimidazoles 4a, 4e and 4f were found to be the most potent. The most potent LOX inhibitor within the subset of Schiff bases was found compound 3i, followed by 3f, whereas compounds 4a and 4g were found the most potent of the 3-oxo-pyrimido[1,2-a]benzimidazole group. Moreover, some cytotoxicity assessments were undertaken, whereupon it was found that Schiff base 3i and pyrimidobenzimidazoles 4e and 4f did not exhibit cytotoxicity at similar concentrations resembling thus the inhibitory activity of lipid peroxidation. The most cytotoxic Schiff base and pyrimidobenzimidazole were found to be 3d and 4c, respectively.

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1. Introduction

2-Aminobenzimidazole has attracted considerable attention since its derivatives such as astemizole, mebendazole, enviroxime, carbendazime, and benomyl have been widely used [1,2]. Concerning benzimidazoles it is well known that they exhibit antioxidant activities causing inhibition of NADPH-dependent lipid peroxidation (LPO) in the rat liver microsomes [3,4]. It seems that they have variable tissue dependent in vitro effects on LPO due to their distinct effects on CYP activities, but not on NADPH-cytochrome P450 reductase activity in rats [4]. They also have strong inhibitory effects on superoxide anion formation and act as effective scavengers of 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable free radical [5]. On the other hand, it has been reported that Schiff bases are known to display good level of anti-inflammatory activity as well as inhibition of lipoxygenase (LOX) [6–8]. Finally, dihydropyrimidine derivatives represent a family of heterocycles with significant therapeutic and medicinal properties [9–11]. Several marine alkaloids having the dihydropyrimidine core-unit were found to show interesting biological activities such as antiviral, antibacterial, antioxidant and anti-inflammatory [12,13].

Today, there is an increased interest in the combination of two pharmacophores on the same scaffold leading to hybrid molecules or conjugates. These hybrid drugs combine two drugs in a single molecule with the goal of creating a chemical entity more medically effective than its individual components [14–19]. As a result, pyrimido[1,2-a]benzimidazoles, which combine two biologically active heterocyclic cores, have been found to be of pharmacological interest

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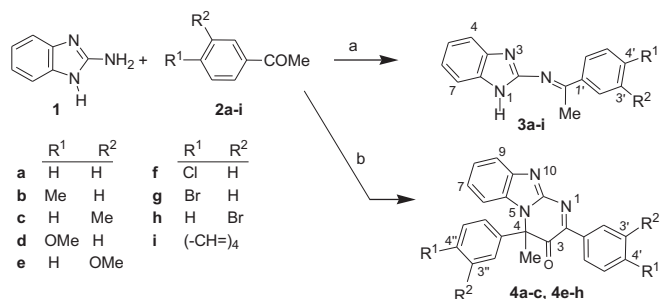
Scheme 1. Reagents and conditions: (a) MW irradiation, 160 °C, 4–10 min; (b) MW irradiation, 250 °C, 5–10 min including catalytic amount of acetic acid in both cases (molar ratio of benzimidazole:ketone:acetic acid = 1:2.1:0.2).

Table 1
Reaction conditions and products.

Ketone	R^1	R^2	Time (min)	Temp (°C)	Prod	Yield (%)
2a	H	H	5	160	**3a**	92
2b	Me	H	4	160	**3b**	91
2c	H	Me	4	160	**3c**	90
2d	OMe	H	10	160	**3d**	89
2e	H	OMe	4	160	**3e**	90
2f	Cl	H	5	160	**3f**	93
2g	Br	H	4	160	**3g**	85
2h	H	Br	4	160	**3h**	89
2i	1-Naphthyl		10	250	**3i**	92
2a	H	H	5	250	**4a**	81
2b	Me	H	7	250	**4b**	72
2c	H	Me	10	250	**4c**	86
2e	H	OMe	10	250	**4e**	70
2f	Cl	H	5	250	**4f**	92
2g	Br	H	5	200	**4g**	93
2h	H	Br	5	200	**4h**	88

for their diverse biological activities [20–22]. Therefore, recently considerable attention has been devoted towards their synthesis by a variety of synthetic procedures [23–26], the two predominating being either cyclocondensation reactions of 2-aminobenzimidazole with a variety of α,β-unsaturated carbonyl compounds [27–29] or multicomponent condensation reactions [30–33] involving 2-aminobenzimidazole. The synthesis of 3-oxo-pyrimido[1,2-a]benzimidazoles by the reaction of propiolic esters and α,β-unsaturated esters with 2-aminobenzimidazole has also been investigated since they were found to show immunotropic properties [34,35]. To the contrary, concerning the 3-oxo-pyrimido[1,2-a]benzimidazole derivatives to our knowledge only one reference appeared in the literature for the 4-methyl-2,4-diphenyl-derivative prepared by a two step reaction sequence [36]. Finally, although available data confirm that benzimidazole Schiff bases exhibit antineoplastic activity [37], the corresponding acetophenone-benzimidazole Schiff bases are not even reported in the literature.

Against this literature background, concerning the biological activity of benzimidazole and pyrimidobenzimidazole derivatives, in the present work we wish to describe a one-pot synthetic methodology for the preparation of a series of novel benzimidazole Schiff bases **3** and 3-oxo-pyrimido[1,2-a]benzimidazoles **4** in excellent yields occurring upon microwave irradiation in a simple and straight-forward manner (Scheme 1) along with a preliminary study of compounds **3** and **4** as inhibitors of lipoxygenase (LOX) and of lipid peroxidation (LPO). In addition, their potential of medical applications as inhibitors in certain cell metabolic pathways as lipid peroxidation or in the catalysis of dioxygenation of polyunsaturated fatty acids in lipids, is articulated and supported by cell toxicity assessments on human lung fibroblasts.

2. Chemistry

All reactions were carried out by environmentally friendly conditions ('green chemistry') by application of microwave irradiation without solvent. The syntheses were carried out simply by mixing the 2-aminobenzimidazole (1 mmol) with the acetophenone **2** (2.1 mmol) in the presence of 0.2 mmol of acetic acid and irradiating in the microwave oven, whereupon the benzimidazole derivatives **3** or **4** were obtained in very good yields and were purified by column chromatography. The desired reaction conditions (temperature and time) were set as reported in Table 1. Analytically, initial studies were conducted with acetophenone **2a** whereupon at 160 °C only the Schiff base **3a** was isolated (92% yield). At higher temperatures (180–230 °C), mixtures of the two products **3a** and **4a**, their ratio depending upon the reaction temperature, were formed, whereas finally at 250 °C only the pyrimidobenzimidazole **4a** was isolated (81% yield). After the reaction conditions were optimized, the generality of the reaction was investigated, and the results (Table 1) show that the reaction has a broad applicability. In all studied reactions at 160 °C the Schiff bases **3a**–**h** were formed with the exception of the 1-naphthyl-derivative **3i** for the formation of which a higher temperature (250 °C) was required. Generally, at 250 °C pyrimidobenzimidazoles **4a**–**c** and **4e**–**f** were the isolated reaction products, with exception of the bromo derivatives **4g** and **4h** for which the optimal reaction
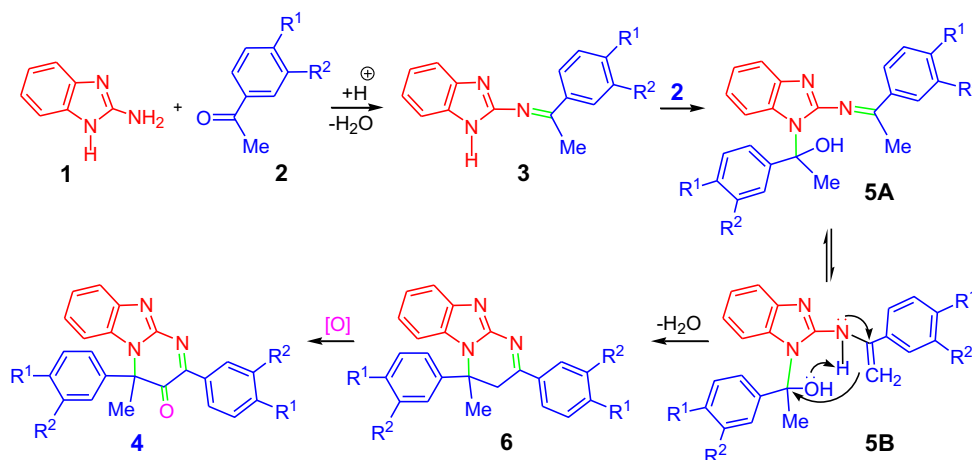
Scheme 2. Plausible mechanism for the formation of compounds **3** and **4**.
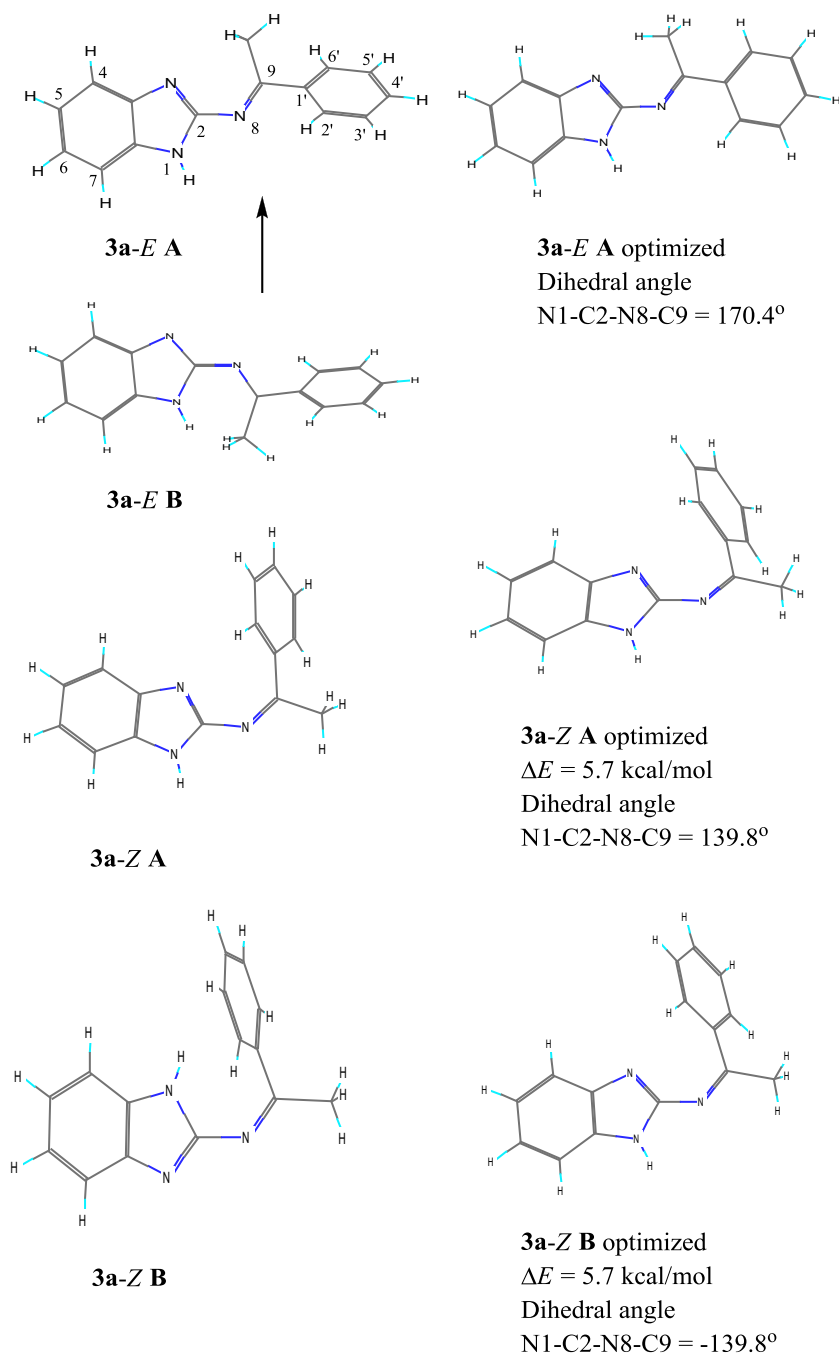
Fig. 1. Chemical structure and selected numbering for the four conformers investigated in the gas phase by DFT method (B3LYP/6-311G(df,p)). ΔE is the energy difference relative to **3a**-*E* **A**.

temperature was found 200 °C, and the lack of formation of the 4-methoxy-substituted compound **4d** and the naphthlyl derivative **4i**.

Concerning the reaction mechanism, for the formation of the pyrimidobenzimidazoles **4**, the benzimidazole amino-group attacks the acetophenone carbonyl group, whereas the NH moiety attacks a second acetophenone molecule to form the imine intermediate **5A**, which undergoes further tautomerization to the enamine **5B**. Cyclization under water abstraction and formation of the 6-membered ring gives rise to the colourless compound **6** (Scheme 2). Quite unexpectedly the 3-methylene group of the pyrimidine ring is air oxidized to give finally the yellow or orange 3-oxo-pyrimido[1,2-*a*]benzimidazole **4**. An analogous methylene group air oxidation has been previously observed in some benzodiazepine derivatives [38]. Concerning the lack of formation of the

4-methoxy derivative **4d** the reduced nucleophilicity of the NH moiety (compared to that of NH_2), renders attack to the comparatively poor electrophilic 4-methoxy-acetophenone carbonyl carbon prohibited, therefore only the Schiff base **3d** is always formed. To the contrary, the enhanced electrophilicity of the 4-bromo-acetophenone carbonyl carbon allows the reaction to proceed successfully at lower temperature (200 °C). Finally, the behavior of the 1-acetylnaphthalene (**2i**) could be attributed to stereochemical reasons.

Concerning the *Z*- or *E*-configuration of Schiff bases, there is generally an ambiguity on account of the many different experimental and theoretical studies [39–41], with exception cases where a particular configuration is favoured [40] because of formation of an intramolecular hydrogen bond between

substituents and heteroatoms. It has also been reported that imines having E- or Z-configuration in the solid state, afford in solution at ambient temperature equilibrium mixtures, even in non polar solvents (cyclohexane) [42]. Concerning the geometry of the isolated Schiff bases **3a**–**i** a theoretical approach was undertaken, since NOE measurements were not applicable.

In Fig. 1 for both isomers **3a**-E and **3a**-Z, where the imino group is coplanar with the benzimidazole ring, two conformers **A** and **B** are presented, with the NH group anti or syn to the imino double bond, respectively.

Initially, all structures were optimized at the AM1 level [43, 44], whereupon conformer **3a**-E **B** was converted to conformer **3a**-E **A**, probably due to stereochemical interactions of 9-CH$_3$ with 1-H. For the two conformers of **3a**-Z, after optimization, a small difference in heat of formation ($\Delta\Delta H_f = 1.85$ kcal/mol) is calculated to favour the **3a**-Z **A**. Finally, **3a**-E **A** is calculated to be more stable from **3a**-Z **A** by about 1.4 kcal/mol. Since from the above results no definite conclusion could be drawn, quantum chemical calculations were performed using DFT at the B3LYP/6-311G(df,p) level of theory in order to find out which structure is preferred, using as input the geometries produced from the AM1 treatment. After optimization each geometry was checked by frequency calculation to assure that it does not represent a transition state (no imaginary frequencies). The optimized geometry for **3a**-Z **B** is calculated to be similar to that of **3a**-Z **A**, with the substituents on the imino-carbon in symmetric position to the N1–C2–N8 plane (Fig. 1). The final geometry for **3a**-E **A** is calculated to be favoured over **3a**-Z **A** (or **B**) by 5.7 kcal/mol. Since this energy difference is small to lead to an unambiguous conclusion the experimental ^{13}C NMR chemical shifts were compared with the calculated ones (DFT, GIAO) [45–47]. The comparison is limited only to the nuclei around the imino group (Table 2).

Comparing the calculated NMR chemical shifts with the experimental ones a much better approximation is observed for the methyl carbon of **3a**-E **A**, where the methyl group is closer to the C2=N3 and most probably shielded from the magnetic anisotropic effect of this double bond. Furthermore, from the better approximation for C9 it can be concluded that in solution (CDCl$_3$) the prepared Schiff bases most probably exist in the E-configuration and consequently the same configuration is adopted in the solid state.

The assigned molecular structures of all new compounds **3** and **4** are based on rigorous spectroscopic analysis including IR, NMR (^{1}H, ^{13}C, COSY, NOESY, HETCOR or HMQC and COLOC or HMBC), MS and elemental analysis data.

Concerning the structure of the isolated 3-oxo-pyrimido[1,2-a] benzimidazoles **4** the assignment of **4b** is described. In the IR spectrum the presence of a carbonyl (1710 cm^{-1}) was observed. In the ^{1}H NMR spectrum, in addition to the two p-substituted phenyl rings, the presence of the four benzimidazole aromatic protons resonating as a double doublet of doublets at $\delta = 7.90$ ($J = 8.2$, 1.0, 0.5 Hz, 9-H) [48], a double doublet of doublets at $\delta = 7.32$ ($J = 8.2$, 7.2, 0.95 Hz, 8-H), a double doublet of doublets at $\delta = 7.20$ ($J = 8.2$, 7.2, 1.0 Hz, 7-H) and as a double doublet of doublets at $\delta = 7.06$ ($J = 8.2$, 0.95, 0.5 Hz, 6-H), with their carbons resonating at 121.8, 123.4, 124.9, and 112.2 ppm, respectively, was identified. Moreover, the 4-position methyl group appears as a singlet at $\delta = 2.33$ (C at
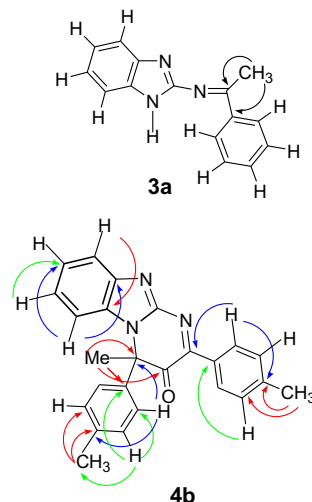

Fig. 2. COLOC correlations between protons and carbons (via $^2J_{CH}$ and $^3J_{CH}$) in compound **4b**. Only critical COLOC correlations in **3a** are depicted.

23.8 ppm). Long-range C–H correlations (COLOC) spectra were optimized for $J = 8$ Hz, thus aromatic protons show COLOC correlations mainly via $^3J_{CH}$, whereas protons on saturated carbons show COLOC via $^2J_{CH}$ and $^3J_{CH}$ couplings. Consequently, the 4-methyl group protons gave COLOC correlations with the quaternary carbons at 188.7 (CO), 133.6 (C-1″), and 69.9 (C-4) ppm. With the C-4 carbon (69.9 ppm) correlated also the two o-protons ($\delta = 6.93$) of the one p-substituted phenyl ring, whereas the o-protons ($\delta = 8.01$) of the second p-substituted phenyl ring correlated with the quaternary carbon at 158.5 ppm (C-2). In Fig. 2 all the observed COLOC correlations in compound **4b** and some critical correlations in compound **3a** are depicted.

3. Pharmacology

3.1. Antioxidant activity

In the present investigation compounds **3** and **4** are tested in vitro, with regard to their antioxidant ability as well as their ability to inhibit soybean LOX. All results are shown in Table 3 and are discussed in terms of structural characteristics.

There is a growing body of evidence on the possible role of free radicals in the inflammatory process [49], in the etiology of atherosclerosis, nephritis and carcinogenesis [50]. It is also known that lipid peroxidation is a free radical chain reaction [51], which causes the degeneration of cell membranes. Most products of lipid peroxidation are known to have mutagenic and/or carcinogenic properties. Antioxidants are defined as substances that even at low concentration significantly delay or prevent oxidation of easy oxidizable substrates. There is an increased interest of using antioxidants for medical purposes in the recent years. Thus, drugs possessing antioxidant and free radical scavenging properties are considered for preventing and/or treatment of diseases, which are directly related to the lack of the antioxidant capacity of the organisms. Many non-steroidal anti-inflammatory drugs have been reported to act either as inhibitors of free radical production or as radical scavengers [52].

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. For these reasons several assays should be used in order to assess in vitro antioxidant activity [53]. Thus, we have used two different types of assays to measure the antioxidant

Table 2
Comparison of experimental and calculated ^{13}C NMR chemical shifts for critical carbon atoms for the E- and Z-isomers of imine **3a**.

Carbon	Expl	**3a**-E **A**	**3a**-Z **A**
2	155.0	158.7	156.6
9	174.3	177.0	183.2
1′	139.5	146.4	143.2
9-Me	19.8	20.5	35.3

Table 3
Interaction % with DPPH; *In vitro* % inhibition of lipid peroxidation (AAPH); *In vitro* % inhibition of soybean lipoxygenase (LOX % inh).

		R¹	R²		R¹	R²
a		H	H	**f**	Cl	H
b		Me	H	**g**	Br	H
c		H	Me	**h**	H	Br
d		OMe	H	**i**	(-CH=)4	
e		H	OMe			
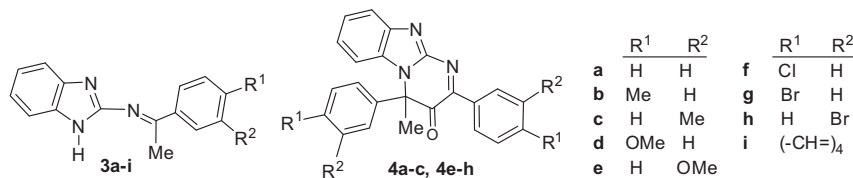
Compds	C log P	DPPH % 0.05 mM		AAPH % inhibition		LOX % inhibition	
		20 min	60 min	0.01 mM	0.1 mM	0.01 mM	0.1 mM
3a	3.51	0.3	5	18	67	No[a]	No
3b	4.01	No	8	47	84	No	No
3c	4.01	No	No	63	77	No	8
3d	3.88	4	3	12	68	22	65
3e	3.59	No	No	69	100	No	42
3f	4.60	3	4	nt	26	62	85
3g	4.37	No	4	22	50	No	18
3h	4.37	No	6	65	97	No	No
3i	5.06	5	7	26	97	18	98
4a	5.03	2	1	11	100	49	100
4b	6.03	No	No	8	80	No	40
4c	6.03	4	5	35	84	No	19
4e	4.87	3	2	3	100	No	No
4f	6.45	2	2	33	97	No	No
4g	6.75	3	2	9	78	51	61
4h	6.56	No	3	51	88	No	12
NDGA	3.92	81	83			40	84
BHT	5.43	31	60				
Trolox	3.09				63		

[a] No activity detectable under the reported experimental conditions. Each *in vitro* experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the mean.

activity of compounds **3** and **4**: a) Interaction with 1,1-diphenyl-2-picrylhydrazyl stable free radical (DPPH) and b) interaction with the water soluble azo compound 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and the results are compared to well known antioxidant agents e.g. nordihydroguairetic acid (NDGA), butylated hydroxyl toluene (BHT) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox).

Both protocols require a spectrophotometric measurement and a certain reaction time in order to obtain reproducible results [54]. DPPH has been used in a radical scavenging measuring method. In its oxidized form, the DPPH radical has an absorbance maximum centred at about 517 nm [55]. The DPPH method is described as a simple, rapid and convenient method independent of sample polarity for screening many samples for radical scavenging activity [56]. Azo compounds generating free radicals through spontaneous thermal decomposition are useful for free radical production studies *in vitro* [57]. The water soluble azo compound AAPH has been extensively used as a clean and controllable source of thermally produced alkylperoxyl free radicals. We have used AAPH as a free radical initiator to follow oxidative changes of linoleic acid to conjugated diene hydroperoxide.

The interaction of the examined compounds with the stable free radical DPPH indicates their radical scavenging ability and electron donating activity in an iron free system. All compounds presented very low, if any, interaction to DPPH at 0.05 mM. The low interaction values of the derivatives, compared to BHT, should be mainly attributed to the absence of easily oxidized functionalities like the ones present in BHT. The absence of hydrogen atoms in phenolic hydroxyl groups, which could be donated to stabilize the DPPH radical is obvious within the given results.

The majority of the studied compounds **3** and **4** effectively inhibit AAPH induced lipid peroxidation (LPO), showing higher activity than the reference compound trolox. This activity is concentration dependent. Comparing the anti-lipid peroxidation activities of the tested Schiff bases, compound **3e** is the most potent followed by **3h** and **3i**, whereas the unsubstituted Schiff base **3a** presents moderate activity. Although high inhibition values are observed for Schiff bases (**3e**, **3h**, **3i**) with high lipophilicities (C log P 3.59–5.06), there are some exceptions, such as compound **3f** presenting high C log P 4.60 and low anti-LPO activity. Perusal of the % inhibition values at 0.1 mM (Table 3) did not show significant differences among the pyrimido [1,2-a]benzimidazoles **4** (78–100%), **4a** and **4e** (100%) being the most potent followed by **4f** (97%). Compound **4e** with the lower C log P value (4.87) within the **4** series is very potent. The standard inhibitor trolox obviously exerts its inhibitory effect on lipid peroxidation mainly through the ability of its 6-hydroxy-5,7,8-trimethylchromane moiety to break the radical chain. Although no phenol moieties are present within these compounds, they could break the radical chain through the initial abstraction of a hydrogen. The thus created new radicals are efficiently stabilized through resonance.

The role of molar lipophilicity, as theoretically calculated C log P [58] in the series of pyrimido[1,2-a]benzimidazoles **4**, is contradictory since the three most active analogs **4a**, **4e** and **4f** present big differences in terms of calculated values of lipophilicity, e.g. C log P values 5.03, 4.87 and 6.45. It is worth to mention that between the two isomers **3b** and **3c** as well as **4b** and **4c**, not big differences are observed. On the contrary, significant differences are noticed between isomers **3g** and **3h**.

Lipoxygenases (LOXs) play a role in membrane lipid peroxidation by forming hydroperoxides in the lipid bilayer [59,60]. Leukotrienes play an important role as mediators of a variety of inflammatory and allergic reactions and are derived from the biotransformation of arachidonic acid catalyzed by LOXs. Inhibitors of LOX have attracted attention initially as potential agents for the treatment of inflammatory and allergic diseases but their therapeutic potential has now been expanded to certain types of cancer and cardiovascular diseases

[61,62]. Thus, we decided to further evaluate the synthesized molecules for their ability to inhibit soybean LOX by the UV absorbance based enzyme assay [63]. Most of the LOX inhibitors are antioxidants or free radical scavengers [64]. Other studies suggest a relationship between LOX inhibition and the ability of the inhibitors to reduce Fe^{3+} at the active site to the catalytically inactive Fe^{2+}. Several LOX inhibitors are excellent ligands for Fe^{3+} and this inhibition is related to their ability to reduce the iron species in the active site to the catalytically inactive ferrous form [64]. It seems that the inhibition increases with the concentration's increase. Perusal of the % inhibition values (Table 3) shows that among the Schiff bases the most potent inhibitor is the naphthyl substituted compound **3i** followed by **3f** and **3d**, whereas **4a** and **4g** are the most active among the pyrimido[1,2-*a*] benzimidazoles. Notably, the parent compound **3a** seemed essentially inactive under our experimental conditions, while the parent **4a** showed the higher % LOX inhibition. It is worth to mention that between the two isomers **4g** and **4h** most potent is the *p*-analogue. Although lipophilicity is referred to as an important physicochemical property for LOX inhibitors [65], herein the most potent compounds **3i** and **4a** with 98% and 100% inhibition at 0.1 mM present *C* log *P* values 5.06 and 5.03 and do not follow this concept. Overall lipophilicity, as *C* log *P* values does not influence biological activity.

Compared to the standard inhibitor NDGA and taking into consideration the mode of action of LOX, the most active analogs of the present set of compounds do not contain a catechol moiety, which efficiently scavenges free radicals and might also chelate Fe^{3+} and reduce it to the catalytically inactive Fe^{2+}. Our experimental findings suggest that the tested compounds may bind to the active site of the enzyme or simultaneously act as antioxidants.

The present study indicates that LOX or LPO inhibitory activity is not always accompanied by DPPH radical scavenging activity. Thus, compounds **3f**, **3i**, **4a** and **4g** are potent LOX inhibitors possessing very low DPPH radical scavenging activity. This is in accordance with the finding of Curini et al. [66] and Hadjipavlou-Litina et al. [67], who have studied antioxidant and LOX inhibitory activity and showed that the most efficient LOX inhibitors are not the most active DPPH radical scavenger. However, a better correlation exists between LOX and LPO inhibitory activity.

3.2. Cytotoxicity in human lung fibroblasts

The cytotoxicity of Schiff bases **3d**, **3g** and **3i**, as well as of pyrimidobenzimidazoles **4c**, **4e** and **4f** was assessed by means of a colorimetric microculture assay (MTT assay) in human lung fibroblast passage 19. The investigated compounds were dissolved in DMSO (its end concentration in the culture medium did not
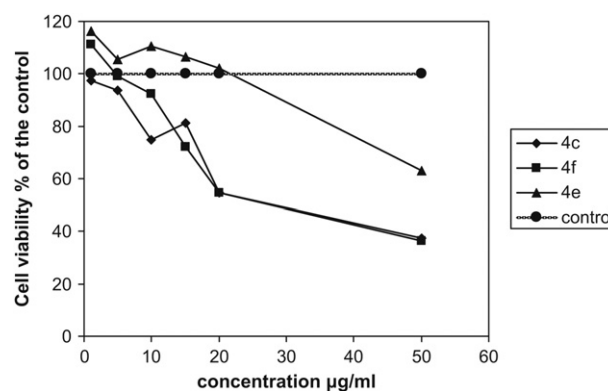

Fig. 4. Cytotoxic activity of pyrimidobenzimidazoles **4c**, **4e** and **4f** assessed by MTT assay on human lung fibroblasts over an incubation period of 24 h. Values shown are the standard deviation of at least three measurements.

exceed 1%) and after 24 h of cell adherence/culture, under the described conditions in the experimental part of this work, the compounds were added at different concentrations from 1 μg/mL to 50 μg/mL to each vial. The results obtained after 24 h by performing the MTT assay are indicated below in Figs. 3 and 4 for Schiff bases **3d**, **3g**, **3i** and pyrimidobenzimidazoles **4c**, **4e**, **4f**, respectively. In particular, Schiff bases **3i** and **3g** exhibited a non toxic uptake by the fibroblasts at 0.003–0.017 mM and 0.003–0.015 mM, respectively. In higher concentrations 0.17 mM and 0.16 mM cell viability was decreased, but however remained higher than 50% (62.5% and 73.6%, respectively). These assessments are in accordance with those obtained for LOX or AAPH inhibition and corroborate a possible medical use of these compounds. On the contrary, cell viability studies of **3d** indicated that at 0.003–0.037 mM cell viability was over 50% (96–56%, respectively), while for higher concentrations (>0.037 mM–0.18 mM) the viability was ranked between 33% and 29%, respectively. It has to be mentioned that this compound exhibited a promising activity profile for concentrations up to 0.1 mM for LOX or AAPH inhibition.

Concerning the pyrimidobenzimidazoles, compounds **4f** and **4e** (Fig. 4) exhibited a non toxic uptake by fibroblasts up to 0.02 mM and 0.04 mM, respectively. In higher concentrations cell viability was reduced to 37% and 63%, respectively. Additionally, **4e** seems to be a good candidate for medical use, taken into consideration its AAPH inhibitory activity at 0.1 mM being 100%. The compound **4c** showed a similar cell viability profile as **4f** at lower however concentrations.

4. Conclusion

In conclusion, we have developed a direct method for the synthesis of a series of benzimidazole Schiff bases **3** and 3-oxo-pyrimido[1,2-*a*]benzimidazoles **4** in excellent yields by a one-pot synthetic procedure from the reaction of 2-aminobenzimidazole with acetophenones by applying environmentally friendly conditions ('green chemistry') namely, microwave irradiation without solvent. In addition, a highly unusual air oxidation of a methylene group to carbonyl was observed. Full assignment of all ^1H and ^{13}C NMR chemical shifts has been unambiguously achieved. To the Schiff bases **3** the *E*-configuration was assigned comparing the experimental and calculated (by DFT) ^{13}C NMR chemical shifts in the proximity of the imino bond. In addition, compounds **3** and **4** were evaluated as inhibitors of lipoxygenase (LOX) and of lipid peroxidation (LPO). All the tested derivatives showed inhibition of lipid peroxidation, whereas most of them were found to have higher activation than the reference compound trolox. The Schiff
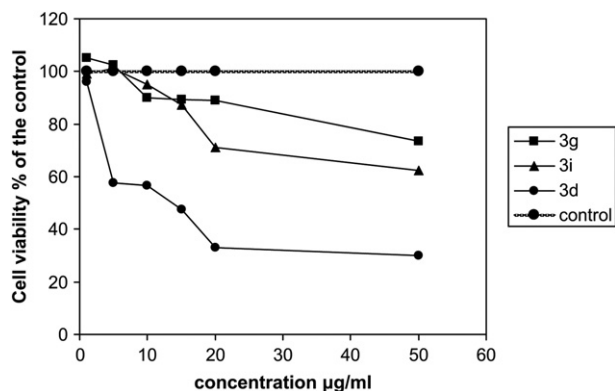

Fig. 3. Cytotoxic activity of Schiff bases **3d**, **3g** and **3i**, assessed by MTT assay on human lung fibroblasts over an incubation period of 24 h. Values shown are the standard deviation of at least three measurements.

bases **3e**, **3h**, and **3i** as well as the pyrimidobenzimidazoles **4a**, **4e** and **4f** were found to be the most potent. The most potent LOX inhibitor within the subset of Schiff bases was found compound **3i**, followed by **3f**, whereas compounds **4a** and **4g** are the most potent of the 3-oxo-pyrimido[1,2-*a*]benzimidazole group. Compounds **3i** and **4a** can be used as lead molecules for the design of agents with excellent LOX and LPO inhibitory activities. The role of lipophilicity is contradictory.

Moreover, based on cell viability investigations it seems that **3i** and **3g** could be possible candidates for medical use concerning their inhibitory activity for LOX or AAPH. On the contrary, **3d** seems to be toxic for the cells and therefore its inhibitory activity cannot be recommended for medical applications, at least at the moment. On the other hand, the uptake of pyrimidobenzimidazole **4e** from fibroblasts points out a promising compound for medical applications. The pyrimidobenzimidazoles **4c** and **4f** could be used as inhibitors at lower concentrations than **4e**.

5. Experimental

5.1. General

DPPH, trolox, BHT and NDGA were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Soybean LOX and linoleic acid sodium salt were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DMEM was purchased from Invitrogen, Carlsbad, CA. FBS was purchased from Intergen, NY, and penicillin and streptomycin were purchased from GIBCO. For the *in vitro* tests a Lambda 20 (Perkin–Elmer) UV–Vis double beam spectrophotometer was used. Melting points were measured on a Kofler hot-stage and are uncorrected. Column chromatography was carried out using Merck silica gel. TLC was performed using precoated silica gel glass plates 0.25 mm containing fluorescent indicator UV_{254} purchased from Macherey–Nagel using a 3:1 mixture of petroleum ether–ethyl acetate. Petroleum ether refers to the fraction boiling between 60 and 80 °C. NMR spectra were recorded at room temperature on a Bruker AM 300 or AVANCE 300 spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C, respectively, using $CDCl_3$ as solvent. Chemical shifts are expressed in δ values (ppm) relative to TMS as internal standard for ^{1}H and relative to TMS (0.00 ppm) or to $CDCl_3$ (77.05 ppm) for ^{13}C NMR spectra. Coupling constants $^n J$ are reported in Hz. Second order ^{1}H NMR spectra were analyzed by simulation [48]. IR spectra were recorded on a Perkin–Elmer 1600 series FTIR spectrometer and are reported in wave numbers (cm^{-1}). Low-resolution LC–MS (ESI, 1.65 eV) mass spectra were recorded on LCMS-2010 EV system (Shimadzu). Elemental analyses performed with a Perkin–Elmer 2400-II CHN analyzer.

5.2. Chemistry

5.2.1. General procedure for the microwave synthesis of Schiff bases 3 and of pyrimido[1,2-a]benzimidazoles 4

The syntheses were carried out simply by mixing thoroughly the 2-aminobenzimidazole **1** (1 mmol) with the acetophenone derivative **2** (2.1 mmol) in the presence of catalytic amount of acetic acid (one drop, ~0.2 mmol) and irradiating in a Biotage Initiator 2.0 microwave oven at a power and time indicated in Table 1, whereupon the benzimidazole derivatives were obtained in very good yields. At the temperature of 160 °C only the Schiff bases **3** were isolated, with exception of the 1-naphthyl derivative **3i**, which was formed at 250 °C. Increasing the temperature to 250 °C led to the synthesis of only derivatives **4**, with exception of the bromo-substituted ones, **4g** and **4h**, which were formed at 200 °C. The crude products were purified by column chromatography on silica

gel using petroleum ether–ethyl acetate (10:1) of slowly increased polarity to afford in elution order compounds **3** or **4**.

5.2.2. N-[(1E)-1-phenylethylidene]-1H-benzimidazol-2-amine 3a

Yield: 0.216 g (92%); white crystals; mp 185–187 °C (ethanol). ^{1}H NMR ($CDCl_3$): $\delta = 2.85$ (s, 3H, N=C–CH_3), 7.15–7.25 (m, 2H, 5,6-H), 7.32 (d, $J = 6.4$ Hz, 1H, 7-H), 7.42–7.52 (m, 2H, 3′,5′-H), 7.51 (t, $J = 7.5$ Hz, 1H, 4′-H), 7.73 (d, $J = 6.5$ Hz, 1H, 4-H), 8.08 (d, $J = 7.5$ Hz, 2H, 2′,6′-H), 9.52 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): $\delta = 19.8$ (N=C–CH_3), 110.1 (C-7), 119.5 (C-4), 122.2 (C-5),* 122.5 (C-6),* 127.9 (C-2′,6′), 128.6 (C-3′,5′), 131.6 (C-4′), 133.1 (C-7a), 139.5 (C-1′), 143.4 (C-3a), 155.0 (C-2), 174.3 (2-N=C). LC–MS (ESI, 1.65 eV): m/z (%) = 268 ($M^+ + H + MeOH$, 8), 258 ($M^+ + Na$, 100), 236 ($M^+ + H$, 100). Anal. Calcd for $C_{15}H_{13}N_3$ (235.28): C, 76.57; H, 5.57; N, 17.86%. Found: C, 76.65; H, 5.70; N, 17.68%. (*The assignments may be interchanged*).

5.2.3. N-[(1E)-1-(4-methylphenyl)ethylidene]-1H-benzimidazol-2-amine 3b

Yield: 0.227 g (91%); yellow crystals; mp 175–176 °C (ethanol). ^{1}H NMR ($CDCl_3$): $\delta = 2.41$ (s, 3H, 4′-CH_3), 2.79 (s, 3H, N=C–CH_3), 7.15–7.23 (m, 2H, 5,6-H), 7.24 (d, $J = 7.3$ Hz, 2H, 3′,5′-H), 7.50–7.55 (m, 2H, 4,7-H), 7.92 (d, $J = 7.3$ Hz, 2H, 2′,6′-H), 9.40 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): $\delta = 19.7$ (N=C–CH_3), 21.5 (4′-CH_3), 114.6 (br, C-4,7), 122.3 (C-5,6), 127.9 (C-3′,5′), 129.3 (C-2′,6′), 136.6 (C-1′), 138.0 (br, C-3a,7a), 142.3 (C-4′), 155.0 (C-2), 174.4 (2-N=C). LC–MS (ESI, 1.65 eV): m/z (%) = 272 ($M^+ + Na$, 35), 250 ($M^+ + H$, 100). Anal. Calcd for $C_{16}H_{15}N_3$ (249.31): C, 77.08; H, 6.06; N, 16.85%. Found: C, 77.15; H, 5.96; N, 16.90%.

5.2.4. N-[(1E)-1-(3-methylphenyl)ethylidene]-1H-benzimidazol-2-amine 3c

Yield: 0.224 g (90%); yellow crystals; mp 155–157 °C (ethanol). ^{1}H NMR ($CDCl_3$): $\delta = 2.40$ (s, 3H, 3′-CH_3), 2.79 (s, 3H, N=C–CH_3), 7.17–7.23 (m, 2H, 5,6-H), 7.29–7.37 (m, 2H, 4′,5′-H), 7.48–7.56 (m, 2H, 4,7-H), 7.77–7.83 (m, 1H, 6′-H,). 7.84 (br s, 1H, 2′-H), 9.33 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): $\delta = 19.7$ (N=C–CH_3), 21.4 (3′-CH_3), 114.6 (br, C-4,7), 122.2 (C-5,6), 125.1 (C-6′), 128.3 (C-2′), 128.4 (C-5′), 132.4 (C-4′), 138.0 (br, C-3a,7a), 138.2 (C-3′), 139.1 (C-1′), 154.9 (C-2), 174.7 (2-N=C). LC–MS (ESI, 1.65 eV): m/z (%) = 250 ($M^+ + H$, 100). Anal. Calcd for $C_{16}H_{15}N_3$ (249.31): C, 77.08; H, 6.06; N, 16.85%. Found: C, 77.19; H, 6.11; N, 16.71%.

5.2.5. N-[(1E)-1-(4-methoxyphenyl)ethylidene]-1H-benzimidazol-2-amine 3d

Yield: 0.236 g (89%); yellow crystals; mp 210–212 °C (ethanol). ^{1}H NMR ($CDCl_3$): $\delta = 2.74$ (s, 3H, N=C–CH_3), 3.86 (s, 3H, O–CH_3), 6.93 (dt, $J = 8.8$, 2.0 Hz, 2H, 3′,5′-H), 7.17–7.22 (m, 2H, 5,6-H), 7.48–7.53 (m, 2H, 4,7-H), 7.99 (dt, $J = 8.8$, 1.9 Hz, 2H, 2′,6′-H), 12.67 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): $\delta = 19.5$ (N=C–CH_3), 55.5 (O–CH_3), 113.8 (C-3′,5′), 114.9 (C-7), 122.0 (C-4,5,6), 129.7 (C-2′,6′), 131.5 (C-1′), 132.1 (C-7a), 144.2 (C-3a), 155.3 (C-2), 162.6 (C-4′), 173.4 (2-N=C). LC–MS (ESI, 1.65 eV): m/z (%) = 320 ($M^+ + Na + MeOH$, 40), 288 ($M^+ + Na$, 35), 266 ($M^+ + H$, 100). Anal. Calcd for $C_{16}H_{15}N_3O$ (265.31): C, 72.43; H, 5.70; N, 15.84%. Found: C, 72.55; H, 5.62; N, 15.77%.

5.2.6. N-[(1E)-1-(3-methoxyphenyl)ethylidene]-1H-benzimidazol-2-amine 3e

Yield: 0.239 g (90%); brownish solid; mp 181–183 °C (ethanol). ^{1}H NMR ($CDCl_3$): $\delta = 2.78$ (s, 3H, N=C–CH_3), 3.83 (s, 3H, O–CH_3), 7.05 (dd, $J = 6.4$, 2.3 Hz, 1H, 4′-H), 7.18–7.25 (m, 2H, 5,6-H), 7.34 (t, $J = 8.1$ Hz, 1H, 5′-H), 7.52–7.62 (m, 5H, 4,7,2′,6′-H,NH). ^{13}C NMR ($CDCl_3$): $\delta = 20.0$ (N=C–CH_3), 55.4 (O–CH_3), 112.5 (C-2′), 114.6 (br, C-4,7), 117.8 (C-4′), 120.5 (C-6′), 122.3 (C-5,6), 129.6 (C-5′), 133.4 (br, C-7a), 138.0 (br, C-3a), 140.4 (C-1′), 154.8 (C-2), 159.7 (C-3′), 174.3

(2-N═C). LC−MS (ESI, 1.65 eV): m/z (%) = 298 (M$^+$ + H + MeOH, 8), 266 (M$^+$ + H, 100). Anal Calcd for $C_{16}H_{15}N_3O$ (265.31): C, 72.43; H, 5.70; N, 15.84%. Found: C, 72.59; H, 5.78; N, 15.70%.

5.2.7. N-[(1E)-1-(4-chlorophenyl)ethylidene]-1H-benzimidazol-2-amine 3f

Yield: 0.251 g (93%); yellow crystals; mp 218−220 °C (ethanol). ^{1}H NMR (CDCl$_3$): δ = 2.80 (s, 3H, N═C−CH$_3$), 7.15−7.25 (br m, 2H, 5,6-H), 7.40 (d, J = 8.2 Hz, 2H, 3′,5′-H), 7.54 (br m, 2H, 4,7-H), 7.95 (d, J = 8.2 Hz, 2H, 2′,6′-H), 9.05 (br s, 1H, NH). ^{13}C NMR (CDCl$_3$): δ = 19.6 (N═C−CH$_3$), 112.5 (br, C-4,7), 122.5 (C-5,6), 128.8 (C-2′,6′),* 128.9 (C-3′,5′),* 137.8 (C-1′), 138.0 (C-4′), 142.1 (br, C-3a,7a), 154.6 (C-2), 172.7 (2-N═C). LC−MS (ESI, 1.65 eV): m/z (%) = 292/294 (M$^+$ + Na, 10), 270/272 (M$^+$ + H, 100). Anal Calcd for $C_{15}H_{12}ClN_3$ (269.73): C, 66.79; H, 4.48; N, 15.58%. Found: C, 66.58; H, 4.37; N, 15.39%. (**The assignments may be interchanged*).

5.2.8. N-[(1E)-1-(4-bromophenyl)ethylidene]-1H-benzimidazol-2-amine 3g

Yield: 0.267 g (85%); green crystals; mp 220−221 °C (ethanol). ^{1}H NMR (CDCl$_3$): δ = 2.79 (s, 3H, N═C−CH$_3$), 7.19−7.28 (m, 2H, 5,6-H), 7.51−7.62 (m, 4H, 4,7,3′,5′-H), 7.87 (d, J = 7.7 Hz, 2H, 2′,6′-H), 9.15 (br s, 1H, NH). ^{13}C NMR (CDCl$_3$): δ = 19.7 (N═C−CH$_3$), 114.8 (br, C-4,7), 122.5 (C-5,6), 126.6 (C-4′), 129.3 (C-2′,6′), 131.8 (C-3′,5′), 137.9 (br, C-3a,7a), 138.0 (C-1′), 154.4 (C-2), 173.1 (2-N═C). LC−MS (ESI, 1.65 eV): m/z (%) = 336/338 (M$^+$ + Na, 15), 314/316 (M$^+$ + H, 100). Anal Calcd for $C_{15}H_{12}BrN_3$ (314.18): C, 57.34; H, 3.85; N, 13.37%. Found: C, 57.45; H, 3.77; N, 13.41%.

5.2.9. N-[(1E)-1-(3-bromophenyl)ethylidene]-1H-benzimidazol-2-amine 3h

Yield: 0.280 g (89%); yellow crystals; mp 145−146 °C (ethanol). ^{1}H NMR (CDCl$_3$): δ = 2.73 (s, 3H, N═C−CH$_3$), 7.15−7.23 (m, 2H, 5,6-H), 7.25 (t, J = 7.9 Hz, 1H, 5′-H), 7.48−7.57 (m, 2H, 4,7-H), 7.59 (d, J = 7.9 Hz, 1H, 4′-H), 7.83 (dd, J = 7.9, 1.6 Hz, 1H, 6′-H), 8.13 (d, J = 1.6 Hz, 1H, 2′-H), 9.05 (br s, 1H, NH). ^{13}C NMR (CDCl$_3$): δ = 19.7 (N═C−CH$_3$), 114.7 (br, C-4,7), 122.5 (C-5,6), 122.8 (C-3′), 126.4 (C-6′), 130.0 (C-5′), 130.7 (C-2′), 134.4 (C-4′), 138.0 (br, C-3a,7a), 140.9 (C-1′), 154.5 (C-2), 172.4 (2-N═C). LC−MS (ESI, 1.65 eV): m/z (%) = 336/338 (M$^+$ + Na, 10), 314/316 (M$^+$ + H, 100). Anal Calcd for $C_{15}H_{12}BrN_3$ (314.18): C, 57.34; H, 3.85; N, 13.37%. Found: C, 57.47; H, 3.70; N, 13.48%.

5.2.10. N-[(1E)-1-(1-naphthyl)ethylidene]-1H-benzimidazol-2-amine 3i

Yield: 0.262 g (92%); yellow crystals; mp 134−136 °C (ethanol). ^{1}H NMR (CDCl$_3$): δ = 3.02 (s, 3H, 4-Me), 6.78 (d, J = 7.8 Hz, 1H, 7-H), 7.10 (dd, J = 8.0, 7.3 Hz, 1H, 5-H), 7.19 (dd, J = 7.8, 7.3 Hz, 1H, 6-H), 7.48−7.58 (m, 3H, 3′,6′,7′-H), 7.69 (dd, J = 7.1, 0.9 Hz, 1H, 5′-H), 7.75 (d, J = 8.0 Hz, 1H, 4-H), 7.91 (dd, J = 6.2, 3.5 Hz, 1H, 4′-H), 7.95 (dd, J = 8.0, 0.9 Hz, 1H, 8′-H), 8.36 (dd, J = 6.2, 3.5 Hz, 1H, 2′-H), 10.28 (br s, 1H, NH). ^{13}C NMR (CDCl$_3$): δ = 25.2 (4-Me), 110.3 (C-7), 119.6 (C-4), 122.1 (C-6),* 122.4 (C-5),* 125.0 (C-3′), 125.5 (C-2′,8′), 126.3 (C-6′), 127.0 (C-7′), 128.7 (C-5′), 130.2 (C-8a′), 130.3 (C-4′), 134.2 (C-4a′), 139.1 (C-1′), 143.3 (C-3a), 147.5 (C-10a), 154.2 (C-2), 178.7 (2-N═C). LC−MS (ESI, 1.65 eV): m/z (%) = 308 (M$^+$ + Na, 20), 286 (M$^+$ + H, 100). Anal. Calcd for $C_{19}H_{15}N_3$ (285.34): C, 79.98; H, 5.30; N, 14.73%. Found: C, 80.15; H, 5.43; N, 14.77%. (**The assignments may be interchanged*).

5.2.11. 4-Methyl-2,4-diphenylpyrimido[1,2-a]benzimidazole-3(4H)-one 4a

Yield: 0.285 g (81%); orange crystals; mp 115−117 °C (ethanol). IR (KBr): 1703 (C═O) cm^{-1}. ^{1}H NMR (CDCl$_3$): δ = 2.36 (s, 3H, 4-Me), 7.05 (dd, J = 8.2, 1.0 Hz, 1H, 6-H), 7.05−7.10 (m, 2H, 2″,6″-H), 7.20 (ddd, J = 8.2, 7.2, 1.1 Hz, 1H, 7-H), 7.28−7.37 (m, 4H, 8,3″,4″,5″-H),

7.42 (tt, J = 7.2, 1.5 Hz, 2H, 3′,5′-H), 7.50 (tt, J = 7.2, 1.5 Hz, 1H, 4′-H), 7.94 (dd, J = 8.2, 1.0 Hz, 1H, 9-H), 8.08 (dt, J = 7.2, 1.5 Hz, 2H, 2′,6′-H). ^{13}C NMR (CDCl$_3$): δ = 23.9 (4-Me), 70.1 (C-4), 112.1 (C-6), 122.0 (C-9), 123.5 (C-8), 125.1 (C-7), 126.0 (C-2″,6″), 128.4 (C-3″,5″), 129.1 (C-4″), 129.5 (C-3′,5′),* 129.9 (C-2′,6′),* 132.3 (C-4′), 132.7 (C-1′), 134.0 (C-5a), 136.5 (C-1″), 144.2 (C-9a), 147.9 (C-10a), 158.5 (C-2), 188.4 (C-3). LC−MS (ESI, 1.65 eV): m/z (%) = 370 (M$^+$ + H + H$_2$O, 30). 352 (M$^+$ + H, 5), 270 (100) Anal. Calcd for $C_{23}H_{17}N_3O$ (351.40): C, 78.61; H, 4.88; N, 11.96%. Found: C, 78.75; H, 4.73; N, 11.87%. (**The assignments may be interchanged*).

5.2.12. 4-Methyl-2,4-bis(4-methylphenyl)pyrimido[1,2-a]benzimidazole-3(4H)-one 4b

Yield: 0.273 g (72%); yellow crystals; mp 237−239 °C (ethanol). IR (KBr): 1710 (C═O) cm^{-1}. ^{1}H NMR (CDCl$_3$): δ = 2.28 (s, 3H, 4″-Me), 2.33 (s, 3H, 4-Me), 2.39 (s, 3H, 4′-Me), 6.93 (d, J = 8.4 Hz, 2H, 2″,6″-H), 7.06 (ddd, J = 8.2, 0.95, 0.5 Hz, 1H, 6-H), 7.11 (d, J = 8.4 Hz, 2H, 3″,5″-H), 7.20 (ddd, J = 8.2, 7.2, 1.0 Hz, 1H, 7-H), 7.22 (d, J = 8.4 Hz, 2H, 3′,5′-H), 7.32 (ddd, J = 8.2, 7.2, 0.95 Hz, 1H, 8-H), 7.90 (ddd, J = 8.2, 1.0, 0.5 Hz, 1H, 9-H), 8.01 (d, J = 8.4 Hz, 2H, 2′,6′-H). ^{13}C NMR (CDCl$_3$): δ = 21.0 (4″-Me), 21.7 (4′-Me), 23.8 (4-Me), 69.9 (C-4), 112.1 (C-6), 121.8 (C-9), 123.4 (C-8), 124.9 (C-7), 126.0 (C-2″,6″), 129.3 (C-3′,5′), 129.9 (C-2′,6′), 130.1 (C-1′), 130.3 (C-3″,5″), 133.6 (C-1″), 134.1 (C-5a), 139.1 (C-4″), 143.3 (C-4′), 144.2 (C-9a), 148.2 (C-10a), 158.5 (C-2), 188.7 (C-3). LC−MS (ESI, 1.65 eV): m/z (%) = 434 (M$^+$ + Na + MeOH, 100), 402 (M$^+$ + Na, 65), 380 (M$^+$ + H, 70). Anal. Calcd for $C_{25}H_{21}N_3O$ (379.45): C, 79.13; H, 5.58; N, 11.07%. Found: C, 79.05; H, 5.53; N, 11.22%.

5.2.13. 4-Methyl-2,4-bis(3-methylphenyl)pyrimido[1,2-a]benzimidazole-3(4H)-one 4c

Yield: 0.326 g (86%); yellow crystals; mp 160−162 °C (ethanol). IR (KBr): 1706 (C═O) cm^{-1}. ^{1}H NMR (CDCl$_3$): δ = 2.27 (s, 3H, 3″-Me), 2.35 (s, 3H, 4-Me), 2.39 (s, 3H, 3′-Me), 6.84 (dd, J = 7.7, 1.2 Hz, 1H, 6″-H), 6.89 (d, J = 1.2 Hz, 1H, 2″-H), 7.05 (dd, J = 8.2, 1.0 Hz, 1H, 6-H), 7.13 (dd, J = 7.6, 1.2 Hz, 1H, 4″-H), 7.20 (ddd, J = 8.2, 7.2, 1.0 Hz, 1H, 7-H), 7.21 (t, J = 7.6 Hz, 1H, 5″-H), 7.25−7.36 (m, 3H, 8,4′,5′-H), 7.83−7.88 (m, 1H, 6′-H), 7.93 (dd, J = 8.4, 1.0 Hz, 1H, 9-H), 7.96 (d, J = 1.2 Hz, 1H, 2′-H). ^{13}C NMR (CDCl$_3$): δ = 21.4 (3″-Me), 21.6 (3′-Me), 23.9 (4-Me), 70.0 (C-4), 112.1 (C-6), 121.9 (C-9), 123.1 (C-6″), 123.4 (C-8), 125.0 (C-7), 126.6 (C-6′), 127.3 (C-2″), 128.3 (C-2′), 129.4 (C-4″), 129.9 (C-5′), 130.3 (C-5″), 132.7 (C-1′), 133.2 (C-4′), 134.1 (C-5a), 136.6 (C-1″), 138.2 (C-3′), 139.5 (C-3″), 144.2 (C-9a), 148.0 (C-10a), 158.7 (C-2), 188.6 (C-3). LC−MS (ESI, 1.65 eV): m/z (%) = 412 (M$^+$ + H + MeOH, 50), 402 (M$^+$ + Na, 10), 380 (M$^+$ + H, 100). Anal Calcd for $C_{25}H_{21}N_3O$ (379.45): C, 79.13; H, 5.58; N, 11.07%. Found: C, 79.21; H, 5.50; N, 10.97%.

5.2.14. 4-Methyl-2,4-bis(3-methoxyphenyl)pyrimido[1,2-a]benzimidazole-3(4H)-one 4e

Yield: 0.288 g (70%); orange crystals; mp 58−60 °C (ethanol). IR (KBr): 1707 (C═O) cm^{-1}. ^{1}H NMR (CDCl$_3$): δ = 2.36 (s, 3H, 4-Me), 3.71 (s, 3H, 3″-OMe), 3.87 (s, 3H, 3′-OMe), 6.61 (dd, J = 7.6, 1.3 Hz, 1H, 6″-H), 6.62 (dd, J = 2.1, 1.3 Hz, 1H, 2″-H), 6.86 (dd, J = 7.6, 2.1 Hz,, 1H, 4″-H), 7.08 (dd, J = 7.6, 2.1 Hz, 1H, 4′-H), 7.11 (dd, J = 8.1, 1.0 Hz, 1H, 6-H), 7.22 (t, J = 7.6 Hz, 1H, 7-H), 7.24 (ddd, J = 8.1, 7.0, 1.0 Hz, 1H, 5″-H), 7.33 (t, J = 7.6 Hz, 1H, 5′-H), 7.34 (ddd, J = 8.1, 7.0, 1.0 Hz, 1H, 8-H), 7.64 (m, 1H, 6′-H), 7.74 (dd, J = 2.1, 1.3 Hz, 1H, 2′-H), 7.94 (d, J = 8.1 Hz, 1H, 9-H). ^{13}C NMR (CDCl$_3$): δ = 23.8 (4-Me), 55.3 (3″-Me), 55.5 (3′-Me), 70.0 (C-4), 112.1 (C-6),* 112.3 (C-2″),* 113.4 (C-4″), 114.0 (C-2′), 118.2 (C-4′),* 119.6 (C-6″),* 122.0 (C-9), 123.0 (C-6′),* 123.5 (C-8),* 125.2 (C-7), 129.4 (C-5″), 130.6 (C-5′), 134.0 (C-1′),* 134.1 (C-5a),* 138.1 (C-1″), 144.2 (C-9a), 147.9 (C-10a), 158.3 (C-2), 159.7 (C-3″),* 160.4 (C-3′),* 188.2 (C-3). LC−MS (ESI, 1.65 eV): m/z (%) = 430 (M$^+$ + H + H$_2$O, 100), 412 (M$^+$ + H, 20). Anal Calcd for

$C_{25}H_{21}N_3O_3$ (411.45): C, 72.98; H, 5.14; N, 10.21%. Found: C, 73.11; H, 5.22; N, 10.07%. (*The assignments may be interchanged).

5.2.15. 4-Methyl-2,4-bis(4-chlorophenyl)pyrimido[1,2-a] benzimidazole-3(4H)-one 4f

Yield: 0.387 g (92%); orange crystals; mp 215–217 °C (ethanol). IR (KBr): 1698 (C=O) cm^{-1}. ^{1}H NMR (CDCl$_3$): δ = 2.34 (s, 3H, 4-Me), 6.99 (dd, J = 8.1, 1.1 Hz, 1H, 6-H), 7.01 (d, J = 8.9 Hz, 2H, 2″,6″-H), 7.24 (ddd, J = 8.1, 7.0, 1.1 Hz, 1H, 7-H), 7.32 (d, J = 8.9 Hz, 2H, 3″,5″-H), 7.35 (ddd, J = 8.1, 7.0, 1.1 Hz, 1H, 8-H), 7.42 (d, J = 8.9 Hz, 2H, 3′,5′-H), 7.95 (dd, J = 8.1, 1.1 Hz, 1H, 9-H), 8.09 (d, J = 8.9 Hz, 2H, 2′,6′-H). ^{13}C NMR (CDCl$_3$): δ = 24.3 (4-Me), 69.7 (C-4), 112.0 (C-6), 122.3 (C-9), 123.8 (C-8), 125.6 (C-7), 127.6 (C-2″,6″), 128.9 (C-3″,5″), 129.8 (C-3′,5′), 131.1 (C-1′), 131.3 (C-2′,6′), 133.9 (C-5a), 135.2 (C-1″), 135.6 (C-4″), 139.1 (C-4′), 144.4 (C-9a), 147.5 (C-10a), 156.8 (C-2), 188.1 (C-3). LC–MS (ESI, 1.65 eV): m/z (%) = 474/476/478 (M$^+$ + Na + MeOH, 40), 452/454/456 (M$^+$ + H + MeOH, 50), 442/444/446 (M$^+$ + Na, 35), 420/422/424 (M$^+$ + H, 100). Anal. Calcd for $C_{23}H_{15}Cl_2N_3O$ (420.29): C, 65.73; H, 3.60; N, 10.00%. Found: C, 65.65; H, 3.53; N, 10.17%.

5.2.16. 4-Methyl-2,4-bis(4-bromophenyl)pyrimido[1,2-a] benzimidazole-3(4H)-one 4g

Yield: 0.474 g (93%); orange crystals; mp 210–212 °C (ethanol). IR (KBr): 1705 (C=O) cm^{-1}. ^{1}H NMR (CDCl$_3$): δ = 2.34 (s, 3H, 4-Me), 6.94 (d, J = 8.4 Hz, 2H, 2″,6″-H), 6.99 (dd, J = 8.1, 1.1 Hz, 1H, 6-H), 7.24 (dd, J = 8.1, 7.1, 1.1 Hz, 1H, 7-H), 7.35 (ddd, J = 8.1, 7.1, 1.1 Hz, 1H, 8-H), 7.48 (d, J = 8.3 Hz, 2H, 3″,5″-H), 7.58 (d, J = 8.5 Hz, 2H, 3′,5′-H), 7.95 (dd, J = 8.1, 1.1 Hz, 1H, 9-H), 8.01 (d, J = 8.5 Hz, 2H, 2′,6′-H),. ^{13}C NMR (CDCl$_3$): δ = 24.2 (4-Me), 69.8 (C-4), 112.0 (C-6), 122.3 (C-9), 123.8 (C-4″), 123.9 (C-8), 125.7 (C-7), 127.9 (C-2″,6″), 128.0 (C-4′), 131.4 (C-3″,5″), 131.5 (C-1′), 131.9 (C-2′,6′), 132.8 (C-3′,5′), 133.9 (C-5a), 135.7 (C-1″), 144.4 (C-9a), 147.5 (C-10a), 156.9 (C-2), 187.9 (C-3). LC–MS (ESI, 1.65 eV): m/z (%) = 562/564/566 (M$^+$ + Na + MeOH, 30), 540/542/544 (M$^+$ + H + MeOH, 50), 530/532/534 (M$^+$ + Na, 20), 508/510/512 (M$^+$ + H, 100). Anal. Calcd for $C_{23}H_{15}Br_2N_3O$ (509.19): C, 54.25; H, 2.97; N, 8.25%. Found: C, 54.36; H, 3.03; N, 8.37%.

5.2.17. 4-Methyl-2,4-bis(3-bromophenyl)pyrimido[1,2-a] benzimidazole-3(4H)-one 4h

Yield: 0.448 g (88%); orange crystals; mp 62–64 °C (ethanol). IR (KBr): 1705 (C=O) cm^{-1}. ^{1}H NMR (CDCl$_3$): δ = 2.32 (s, 3H, 4-Me), 6.91 (ddd, J = 7.9, 1.7, 1.0 Hz, 1H, 6″-H), 6.97 (dd, J = 8.1 Hz, 1H, 6-H), 7.18 (t, J = 7.9 Hz, 1H, 5″-H), 7.23 (dd, J = 8.1, 7.2 Hz, 1H, 7-H), 7.30 (t, J = 7.9 Hz, 1H, 5′-H), 7.33 (dd, J = 8.1, 7.2 Hz, 1H, 8-H), 7.37 (t, J = 1.7 Hz, 1H, 2″-H), 7.48 (ddd, J = 7.9, 1.7, 1.0 Hz, 1H, 4″-H), 7.64 (ddd, J = 7.9, 1.7, 1.0 Hz, 1H, 4′-H), 7.94 (dd, J = 8.1 Hz, 1H, 9-H), 8.07 (ddd, J = 7.9, 1.7, 1.1 Hz, 1H, 6′-H), 8.33 (t, J = 1.7 Hz, 1H, 2′-H). ^{13}C NMR (CDCl$_3$): δ = 24.4 (4-Me), 69.7 (C-4), 112.0 (C-6), 122.5 (C-9), 122.7 (C-3″), 123.93 (C-3′), 123.96 (C-8), 124.8 (C-6″), 125.8 (C-7), 128.6 (C-6′), 129.4 (C-2″),* 130.0 (C-5″),* 131.0 (C-5′), 132.6 (C-4″),* 132.7 (C-2′),* 133.9 (C-5a), 134.6 (C-1′), 135.2 (C-4′), 139.0 (C-1″), 144.5 (C-9a), 147.3 (C-10a), 156.2 (C-2), 187.8 (C-3). LC–MS (ESI, 1.65 eV, pos. polarity): m/z (%) = 562/564/566 (M$^+$ + Na + MeOH, 1), 540/542/544 (M$^+$ + H + MeOH, 3), 508/510/512 (M$^+$ + H, 5), 153 (100). LC–MS (ESI, 1.65 eV, neg. polarity): m/z (%) = 538/540/542 (M$^+$ − H + MeOH, 100), 524/526/528 (30), 508/510/512 (M$^+$ + H, 30), 314/316 (60), 205 (98). Anal. Calcd for $C_{23}H_{15}Br_2N_3O$ (509.19): C, 54.25; H, 2.97; N, 8.25%. Found: C, 54.12; H, 3.12; N, 8.15%. (*The assignments may be interchanged).

5.3. Physicochemical studies

5.3.1. Determination of lipophilicity as C log P values

Since lipophilicity is a significant physicochemical property determining distribution, bioavailability, metabolic activity and elimination, we tried to calculate theoretically the lipophilicity

values of compounds 3 and 4 as C log P values in n-octanol/buffer by C log P Programme of Biobyte Corp. [43].

5.4. Antioxidant activity

Each in vitro experiment was performed at least in triplicate, the results were averaged and the standard deviation of absorbance was less than 10% of the mean and the results are presented in Table 2.

5.4.1. Determination of the reducing activity of the stable radical DPPH [49]

To an ethanolic solution of DPPH (0.05 mM) in absolute ethanol an equal volume of the compounds (final concentration 0.05 mM) dissolved in DMSO was added. The mixture was shaken vigorously and allowed to stand for 20 min 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 were averaged (Table 2).

5.4.2. Inhibition of linoleic acid lipid peroxidation [42]

The water soluble azo compound AAPH is used as a free radical initiator for in vitro studies of free radical production. Production of conjugated diene hydroperoxide by oxidation of linoleic acid sodium salt in an aqueous solution is monitored at 234 nm. This assay can be used to follow oxidative changes and to understand the contribution of each tested compound.

10 µL 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 aliquots (10 µL) 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.

5.4.3. Soybean LOX inhibition study in vitro [49]

The tested compounds dissolved in DMSO were incubated at RT with sodium linoleate (0.1 mL) and 0.2 mL of enzyme solution ($1/9 \times 10^{-4}$ w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor.

5.4.4. Cytotoxicity in human lung fibroblast cell line
5.4.4.1. Cell culture.
Human lung fibroblasts p19 were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics penicillin and streptomycin. Cell line was maintained in continuous culture at 37 °C, in 5% CO$_2$, in a fully humidified incubator, using standard aseptic techniques and cell growth was monitored by determining the cell number/mL with the use of a Coulter counter model ZBI.

5.4.4.2. MTT assays.
Cell proliferation was assessed by monitoring the conversion of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan. The reduction of MTT is catalyzed by mitochondrial dehydrogenase enzyme and is therefore a measure for cell viability [68]. The MTT assay was used as a cytotoxicity assay for lung fibroblastic cells grown in the presence of the studied compounds. Briefly, cells (3×10^4 cells mL^{-1}) were seeded in 48-well plates and after 24 h were treated with varying concentrations of the studied compounds (1–50 µg/mL) for 24 h. Briefly, 400 mL of freshly prepared MTT solution (5 mg/mL in PBS) were added to each well − after removal of culture medium and washing with PBS. Following incubation at 37 °C for 4–6 h in a fully humidified atmosphere at 5% CO$_2$, MTT was taken up by active cells

and reduced in the mitochondria to insoluble formazan granules. Subsequently, the medium was discarded and the precipitated formazan was dissolved in isopropanol (400 μL/well). The Organon Teknika @ Enterprise Ireland plate was used to measure the absorbance of the plates at 570 nm. Values shown are the standard deviation of at least three measurements.

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Abbreviations

AAPH: 2,2′-azobis(2-amidinopropane) dihydrochloride
BHT: butylated hydroxy toluene
C log P: logarithm of partition coeficient $\log(C_{octanol}/C_{water})$
COLOC: correlation via long-range coupling $^2J_{CH}$ or $^3J_{CH}$
COSY: 2D H–H correlation spectroscopy via $^nJ_{HH}$
CYP: cytochrome P450
DMEM: Dulbecco's/Vogt modified eagle minimal essential medium
DPPH: 2,2′-diphenyl-1-picrylhydrazyl
FBS: fetal bovine serum
GIAO: gauge independent atomic orbital
HETCOR: 2D heteronuclear correlation via $^1J_{CH}$
HMQC: heteronuclear multiple quantum coherence, 2-D inverse H,C correlation
HMBC: heteronuclear multiple bond coherence, 2-D inverse H,C correlation
LOX: lipoxygenase peroxydation
LPO: lipid peroxydation
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW: microwave irradiation
NADPH: nicotinamide adenine dinucleotide phosphate
NDGA: nordihydroguairetic acid
Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid