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New analogues of butylated hydroxytoluene as anti-inflammatory and antioxidant agents

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Abstract—Amine or amide derivatives bearing the 2,6-di-*tert*-butyl phenol moiety are synthesised. Almost all are antioxidants, reduce acute inflammation and inhibit COX-1 and lipoxygenase activity. The most potent anti-inflammatory, COX-1 inhibitor and antioxidant agent, with low toxicity, is 2,6-di-*tert*-butyl-4-thiomorpholin-4-ylmethyl-phenol.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used agents for the treatment of inflammation and highly prescribed medications.[1] However, they possess major and even life-threatening side effects, such as damage to the gastric mucosa, prolongation of bleeding time and renal failure.[2,3] It has been reported that there are about 17,000 deaths in the United States each year as a result of NSAID-related gastrointestinal complications.[2]

A number of approaches have been pursued to minimize these adverse effects, such as entering coating, parenteral administration, combination of conventional NSAIDs with a proton pump inhibitor or prodrug formulations, with moderate results.[4]

Cyclooxygenase (COX)-2 selective inhibitors have been evolved as agents that would treat inflammatory diseases via inhibition of the COX isoform responsible for the inflammatory response, without affecting the COX-1 mediated production of cytoprotective prostaglandins in the gastrointestinal tract.[5–7] Yet, emerging data suggest that selective COX-2 inhibitors could still cause serious side effects such as gastrointestinal toxicity,[8] hypersensitivity, elevated blood pressure and increased risk of cardiovascular events.[9]

A strategy recently followed involves combined COX and 5-lipoxygenase (5-LOX) inhibitors, interfering with the biosynthesis of both prostaglandins and leukotrienes. These agents are expected to possess potent anti-inflammatory activity with reduced prothrombotic effect and a good gastrointestinal safety profile.[10]

It is well known that reactive oxygen species are implicated in inflammation.[11] Superoxide anion radical has proinflammatory actions, such as endothelial cell damage and increased microvascular permeability,[12] formation of chemotactic factors,[13,14] recruitment of neutrophils at sites of inflammation,[15] lipid peroxidation and DNA damage.[16] Furthermore, since COX catalysis involves radical intermediates, a radical scavenger would be considered to interfere with the COX reaction. Linkage of the antioxidant di-*tert*-butyl phenol substructure with thiazolone, oxazolone[17] or thiadiazole moieties[18] gave non-ulcerogenic, orally active COX-2 inhibitors.

In addition, derivatives bearing the 3,5-di-*tert*-butyl-4-hydroxybenzilidene moiety have been found to be dual inhibitors of COX-2 and 5-LOX and to suppress interleukin-1 production.[19] Considering the above studies, we designed some new derivatives of 2,6-di-*tert*-butyl phenol (compounds **8**, **10**–**14**) (Chart 1) which are amines or amides of thiomorpholine, morpholine and proline residues. We also included in our series

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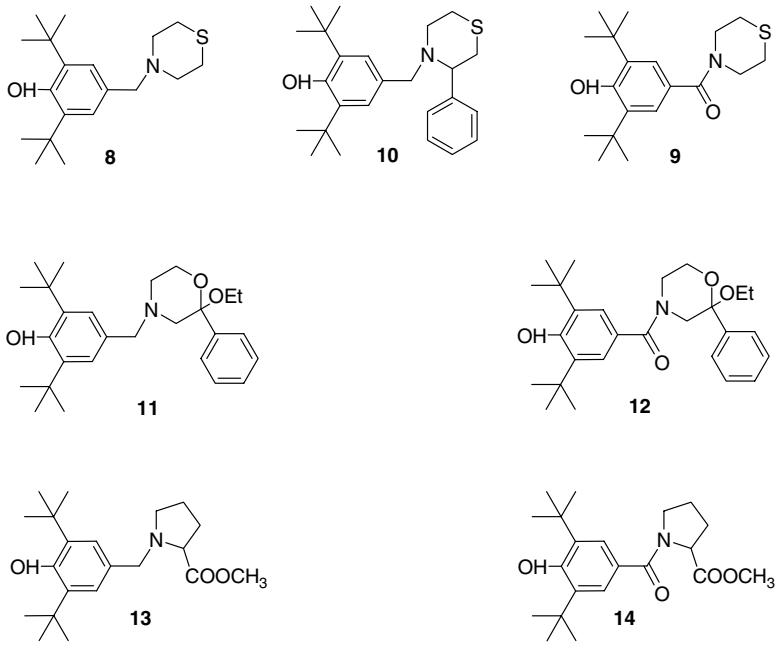


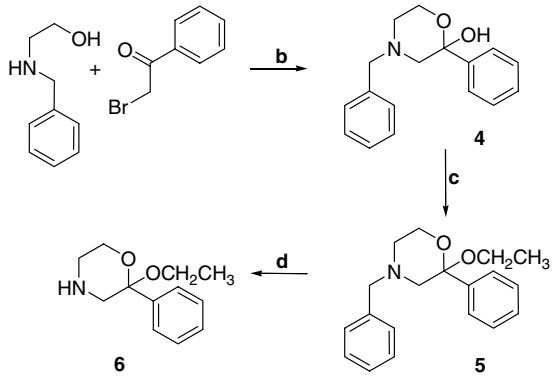
Chart 1. Structure of amine and amide derivatives (**8**–**14**) of butylated hydroxytoluene (BHT).

compound **9**, that has been prepared earlier.[20] We have previously found that some substituted morpholines possess anti-inflammatory[21] and antioxidant properties.[22,23] Thus, in this study, an effort was made to combine and increase these activities with the antioxidant potential that the phenolic moiety would convey, since butylated hydroxytoluene (BHT) is a well-known antioxidant with low toxicity. These compounds were tested in vitro and in vivo for the evaluation of their antioxidant and anti-inflammatory activities. Finally, the toxicity of **8**, one of the most active compounds, was examined, in relation to the parent molecule, BHT.

2. Results and discussion

2.1. Chemistry

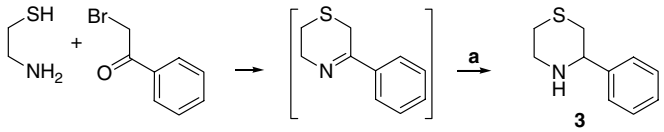
Preparation of the intermediate amines **3** and **6** is demonstrated in Schemes 1 and 2. 3-Phenylthiomorpholine (**3**) was prepared by the hydrogenation of the intermediate imine, obtained from the reaction of 2-aminoethanethiol with 2-bromo-1-phenylethanone in alkaline environment. 2-Ethoxy-2-phenyl morpholine (**6**) was obtained after hydrogenolysis of the 4-benzyl analogue. The final reaction for the synthesis of compounds **8**–**14** is demonstrated in Scheme 3. For the amides **9**, **12** and **14**, direct amidation of the carboxylic group of 3,5-di-*tert*-butyl-4-hydroxybenzoic acid in the presence of 2,2-dicyclohexylcarbodiimide could not be accomplished.



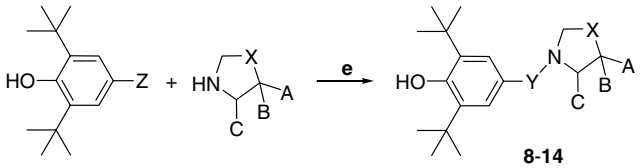
Scheme 2. Synthesis of the substituted morpholine. Reagents and conditions: (b) Ethyl ether/acetone, room temperature, 12 h; (c) Ethanol, hydrobromic acid/ethyl ether, reflux, 3 h; (d) Ethanol, Pd/C under hydrogen, room temperature, 3 h.

2.2. In vivo experiments

2.2.1. Carrageenan-induced inflammation. The effect of compounds **8**–**14** and BHT on carrageenan induced edema is presented in Table 1. They showed a significant anti-inflammatory action administered at a dose of 0.28 mmol/kg, with the exception of



Scheme 1. Synthesis of the substituted thiomorpholine. Reagents and conditions: (a) Potassium hydroxide, methanol, room temperature under nitrogen, 2 h, sodium borohydride, 1 h.



$Z=CH_2Cl$[f]
$Z=COCl$[g]

8: **Y**: CH_2, **X**: CH_2S, **A**, **B**, **C**: H
9: **Y**: C=O, **X**: CH_2S, **A**, **B**, **C**: H
10: **Y**: CH_2, **X**: CH_2S, **A**, **B**: H, **C**: Ph
11: **Y**: CH_2, **X**: CH_2O, **A**: Ph, **B**: OCH_2CH_3, **C**: H
12: **Y**: C=O, **X**: CH_2O, **A**: Ph, **B**: OCH_2CH_3, **C**: H
13: **Y**: CH_2, **X**: CH_2, **A**, **B**: H, **C**: $COOCH_3$
14: **Y**: C=O, **X**: CH_2, **A**, **B**: H, **C**: $COOCH_3$

Scheme 3. Synthesis of the final compounds. Reagents and conditions: (e) Ethyl ether, triethylamine, room temperature, 12 h; (f) Prepared from 3,5-di-*tert* butyl-4-hydroxybenzyl alcohol, hydrochloric acid, in petroleum ether, room temperature, 12 h; (g) Prepared from 3,5-di-*tert*-butyl-4-hydroxybenzoic acid sodium salt, thionyl chloride, in petroleum ether, room temperature, 2–3 h.

Table 1. Effect of compounds on hind paw edema induced by carrageenan in rats

Compound	Percent paw weight increase[a]	Percent edema inhibition[a]
Control	43.22	—
8	23.56	45.49*
9	21.12	51.13*
10	29.58	31.56*
11	40.60	6.06
12	24.87	42.46*
13	26.98	37.58*
14	27.96	35.31*
BHT	43.10	0.00

[a] The effect on edema is expressed as percent weight increase of hind paw and as percent of inhibition of edema in comparison to controls. All compounds were administered ip at a dose of 0.28 mmol/kg of body weight. Each value represents the mean obtained from 6 to 8 animals in two independent experiments.
* $P < 0.001$ (Student's *t*-test).

compound **11**, the amine derivative of substituted morpholine, and the parent molecule (BHT) which were not active. Under the same conditions, indomethacin produced 54% edema inhibition.[24] Compounds **8** and **9**, which are derivatives of thiomorpholine, were the most potent anti-inflammatory agents of this series, with **9** being the most active. Compound **12**, which is the amide derivative of substituted morpholine, followed closely the activity of compounds **8** and **9**, while the amine (compound **11**) had no action. The addition of a phenyl moiety to the thiomorpholine ring of compound **8** decreased its activity (compound **10**). Compounds **13** and **14**, the derivatives of substituted proline, demonstrated an anti-inflammatory activity similar to that of compound **10**.

2.2.2. Toxicity. Acute toxicity of BHT and compound **8** was estimated after the ip administration of equimolar doses (1 mmol/kg body weight) to rats. In both groups, all animals survived and looked normal both macroscopically and by autopsy. These results indicate that the synthesised compounds seem to possess a wide safety margin.

2.3. In vitro experiments

2.3.1. Inhibition of cyclooxygenase activity (isoforms 1 and 2). Cyclooxygenase, one of the two activities of prostaglandin endoperoxide synthase, is the key enzyme in the conversion of arachidonic acid (AA) to prostaglandins (PGs) and other eicosanoids, important inflammatory mediators, also playing a major role in gastric mucosa protection, platelet aggregation and kidney function. It has been established that there are two distinct isoforms of COX. COX-1 is the constitutive isoenzyme found in most tissues under physiological conditions which maintains cellular homeostasis. COX-2 is highly inducible in response to proinflammatory stimuli, resulting in exaggerated PG release.

The effect of compounds **8**–**14** (concentration of 10 μM) and indomethacin (IND) (concentration of 1 μM for COX-1 and 10 μM for COX-2) on both COX isoforms has been examined. The concentration of the substrate, arachidonic acid, was 0.1 μM for both COX isoforms and 1 μM for IND. At higher concentrations of arachidonic acid there was no significant inhibition. For compound **8**, more concentrations were tested for the determination of its IC_{50} value towards COX-1.

Compounds **8**–**14** and BHT had negligible effect on COX-2. However, as shown in Table 2, most

Table 2. In vitro evaluation of anti-inflammatory activity

Compound[a]	Percent cyclooxygenase inhibition COX-1[b]	Lipoxygenase inhibition IC_{50} (μM)
8	74	70
9	50	300
10	32	112
11	5	30
12	8	100
13	24	230
14	25	250
BHT	—	192
NDGA	—	1.3
IND[c]	59	—

[a] Compound concentration 10 μM.
[b] Arachidonic acid 0.1 μM.
[c] IND 1 μM, arachidonic acid 1 μM.

compounds showed significant inhibition of the COX-1 isoform with the exception of **11** and **12**, the substituted morpholine derivatives. Compound **8**, the thiomorpholine amine derivative, was the most potent inhibitor, with an IC_{50} value of 6.5 μM for COX-1 inhibition. Compound **9**, the thiomorpholine amide analogue, closely followed **8**. Phenyl substitution on the thiomorpholine ring (**10**) reduced activity, in agreement with their in vivo results. The same effect of ring substitution on COX-1 activity may also explain the lack of inhibitory action of the morpholine derivatives **11** and **12**. Proline derivatives showed some activity against COX-1 isoform, although diminished, compared to the thiomorpholine derivatives. When arachidonic acid was used at a concentration of 100 μM, which is slightly higher than the saturating substrate concentration, no inhibition was observed, under the same experimental conditions. These results indicate that the examined compounds act as competitive inhibitors of COX-1, since inhibition can be overcome by increasing substrate concentration.

Indomethacin, used as a reference, was a potent inhibitor of both COX isoforms (59% and 33% for COX-1 and COX-2, respectively) at a lower concentration (1 μM for COX-1 and 10 μM for COX-2) using 1 μM concentration of AA for both COX isoforms.

2.3.2. Inhibition of lipoxygenase activity. Lipoxygenases (LOX) catalyse the regio- and stereo-specific dioxygenation of polyunsaturated fatty acids containing 1(*Z*), 4(*Z*) pentadiene system, to form hydroperoxides, which are leukotriene precursors. The dioxygenase activity of LOX displays a rather broad substrate specificity. The IC_{50} values of compounds **8**–**14**, BHT and nordihydroguaiaretic acid (NDGA) towards soybean LOX after 7 min of incubation are given in Table 2. The time course of lipoxygenase activity, as affected by various concentrations of four most potent compounds (**8**, **10**, **11**, and **12**), is depicted in Figure 1. Most compounds were more potent inhibitors than BHT, with the exception of **9**, **13** and **14**, which were the amide derivative of thiomorpholine and the derivatives of substituted proline, respectively. The most potent inhibitor was compound **11**, the amine derivative of substituted morpholine, followed by compound **8**, the amine derivative of thiomorpholine, and compounds **10** and **12** which had similar activity. These results indicate that the amine derivatives are more potent inhibitors than the respective amide analogues. In general, the substituted morpholine derivatives appeared to be the most potent, followed by the thiomorpholine derivatives, while the addition of a phenyl moiety to the thiomorpholine ring slightly reduced activity.

2.3.3. Antioxidant activity. Reactive oxygen species may be important contributors to tissue injury in inflammatory diseases. Thus, antioxidant properties may contribute to the anti-inflammatory action of compounds.

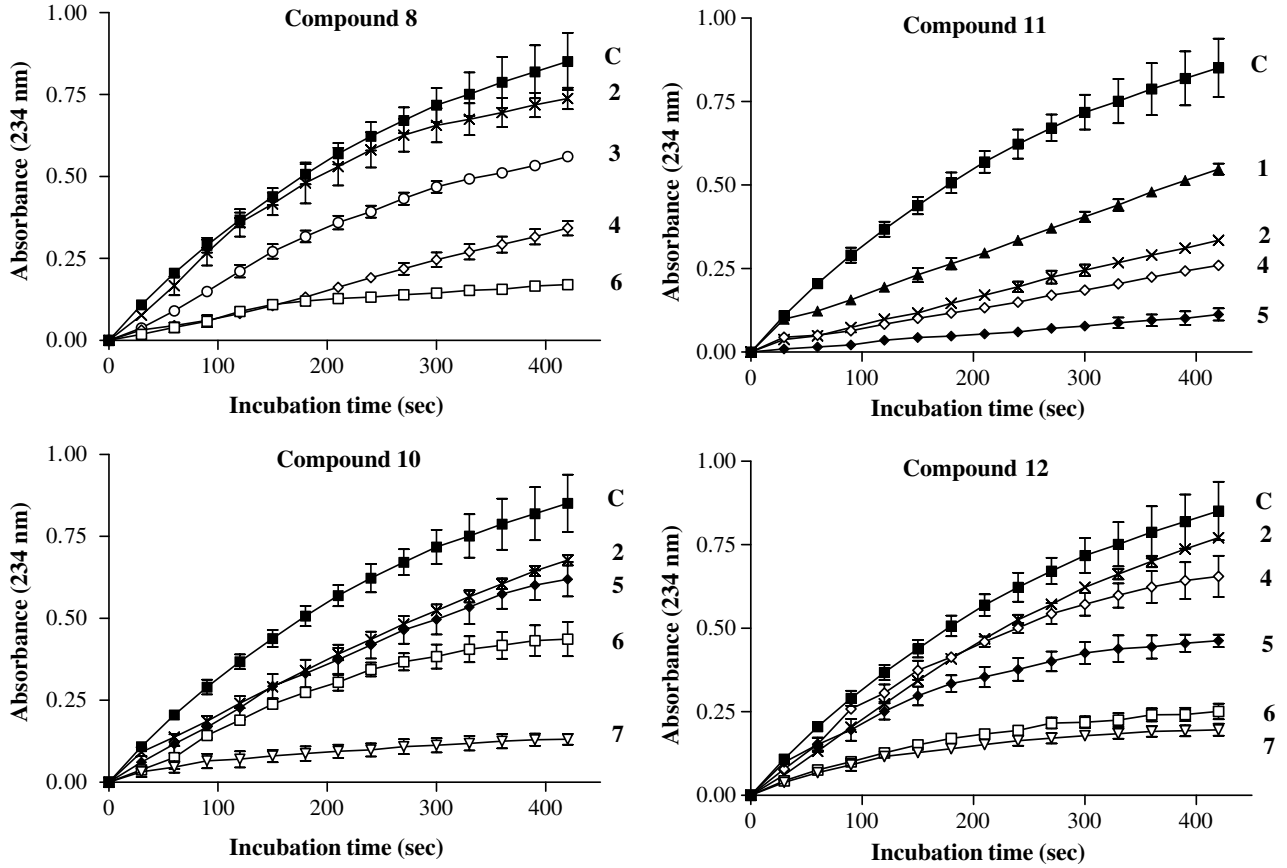


Figure 1. Effect of various concentrations of compounds **8**, **10**, **11** and **12** on lipoxygenase activity. C, control; 1, 10 μM; 2, 50 μM; 3, 60 μM; 4, 75 μM; 5, 100 μM; 6, 125 μM; 7, 150 μM.

BHT, a known strong antioxidant, was used as a parent molecule and some of the final compounds **8**–**14** showed antioxidant activity similar to this molecule. The antioxidant activity of these compounds was estimated by their inhibition of rat hepatic microsomal membrane lipid peroxidation and by their interaction with the N-centred 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable free radical. The IC_{50} values of the final compounds in the above tests are shown in Table 3.

2.3.3.1. In vitro inhibition of lipid peroxidation. Compound **8**, the amine derivative of thiomorpholine, was the most potent inhibitor, with an action comparable to that of BHT. The amide derivative of thiomorpholine (compound **9**) showed a low action which is lost at a concentration of 500 μM. Compounds **11** and **12**, which are the substituted morpholine derivatives, were very potent inhibitors of lipid peroxidation, with the amine derivative being slightly better. Compound **12** was the only amide derivative that had an antioxidant activity. The substitution of the thiomorpholine ring with a phenyl moiety slightly reduced activity (compound **10**). Finally, compound **13**, the proline derivative, lost its activity at a concentration of 300 μM, while the amide derivative of this group was not active (compound **14**). The inhibitory activity of compounds **8**, **10**, **11** and **12** was time dependent and it is demonstrated in Figure 2.

Table 3. In vitro evaluation of antioxidant activity

Compound	Inhibition of lipid peroxidation IC_{50} (μM)	Interaction with DPPH (200 μM) IC_{50} (μM)
8	7.5	75
9	>750	>200
10	22.5	>200
11	18.5	55
12	19.5	>200
13	95	>500
14	>1000	>200
BHT	5	45

2.3.3.2. In vitro interaction with the stable radical DPPH. Compounds **8** and **11**, the thiomorpholine and substituted morpholine derivatives, showed the greatest interaction with DPPH, **11** being the most potent, having activity similar to BHT, while compound **13**, which had a substituted proline moiety, showed a diminished activity. The interaction of these two compounds with DPPH at various concentrations, which is time dependent, is shown in Figure 3. Compound **10**, the derivative of the substituted thiomorpholine, showed a very low activity only at an equimolar dose (0.2 mM). Compounds **9**, **11**, and **14**, which are the amide derivatives, did not interact with DPPH.

The results on lipid peroxidation largely correlate with the effect on LOX. Thus, compound **11** is a LOX inhibitor and a potent antioxidant, while **9** demonstrated weak LOX inhibition and negligible antioxidant proper-

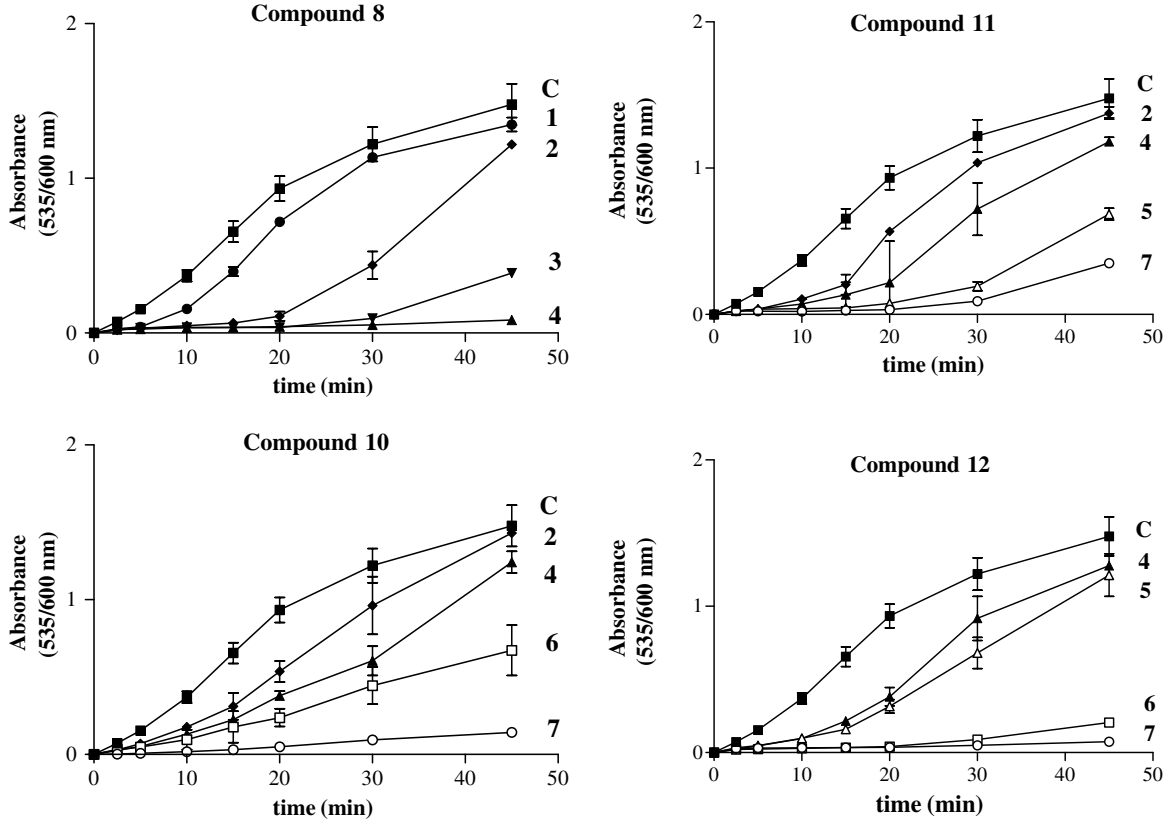


Figure 2. Time course of lipid peroxidation as affected by compounds **8**, **10**, **11** and **12**. C, control; 1, 5 μM; 2, 10 μM; 3, 12.5 μM; 4, 15 μM; 5, 20 μM; 6, 25 μM; 7, 30 μM.

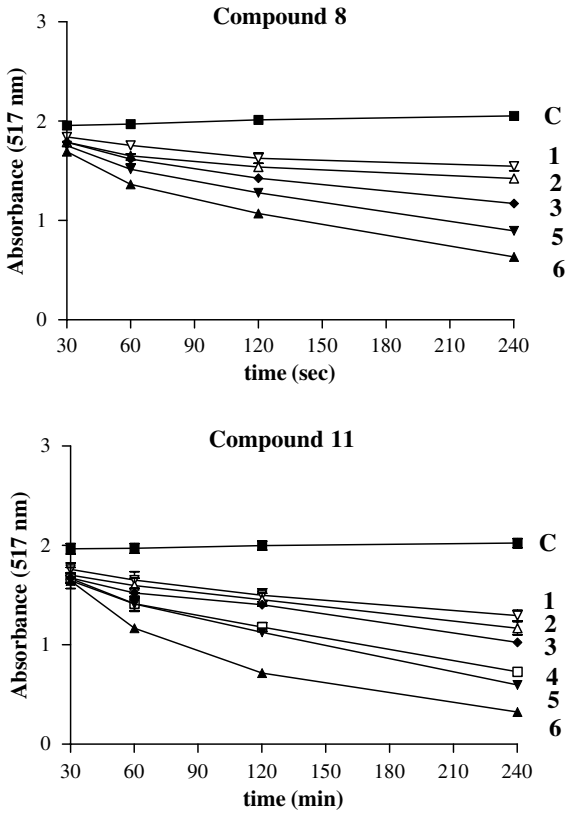


Figure 3. Time dependent interaction of DPPH (200 μM) with compounds **8** and **11**. C, control; 1, 25 μM; 2, 30 μM; 3, 50 μM; 4, 75 μM; 5, 100 μM; 6, 200 μM.

ties. In general, amines are more potent antioxidants than their amide counterparts, in agreement with their effect on LOX activity. Thus, these compounds may be able to offer protection against free radical attack by direct inhibition of lipid peroxidation as well as by inhibiting LOX, which is considered to play a significant contributory role in the generation of cellular oxidative stress.[25]

3. Conclusion

From the in vitro and in vivo experiments it could be concluded that BHT has only antioxidant activity and that substitution with a thiomorpholine, substituted thiomorpholine, morpholine or proline moiety offered anti-inflammatory properties. The most potent derivatives possess a thiomorpholine moiety and are followed by the substituted morpholine compounds, while the addition of a phenyl group to the thiomorpholine moiety reduced activity. Finally, the introduction of a five-membered ring without the presence of a sulfur or oxygen atom, like that of proline, significantly reduced activity. It seems that the in vivo anti-inflammatory activity correlates more closely with COX inhibition, while LOX inhibitory action and antioxidant activity appear to be interrelated.

All the synthesised compounds are more active anti-inflammatory agents than BHT and they seem to be non-toxic, thus possibly possessing a wide safety margin. Consequently, their design could offer more alternatives to the medicinal treatment of inflammatory conditions.

4. Experimental

4.1. Materials

All commercially available chemicals are of the appropriate purity. For the in vivo experiments, Fischer-344 rats (150–250 g) were used.

4.2. Synthesis

Melting points (mp) were obtained on a MEL-TEMP II (Laboratory Devices) apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer 597 infrared spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained with a Bruker 400 MHz spectrometer. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane (TMS), and signals are given as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Elemental analyses are performed with a Perkin-Elmer 2400 CHN analyzer.

4.2.1. Synthesis of the intermediates (1–7).

4.2.1.1. 2,6-Di-*tert*-butyl-4-chloromethyl-phenol (1)[26]. Concentrated hydrochloric acid (1 mL, 0.001 mol) was added to a slurry of 2,6-di-*tert*-butyl-4-hydroxymethyl-phenol (0.786 g, 0.003 mol) in petroleum ether (40 mL) and stirred at ambient temperature overnight under nitrogen. The resulting two layers were separated and the petroleum ether layer was washed with water, dried ($MgSO_4$) and evaporated to give 0.704 g of the chloride. Yellow oil, yield 83%. IR (neat): 3625, 2950, 1430, 1230, 700 cm^{-1}.^{1}H NMR ($CDCl_3$): δ 1.45 (s, 18 H, CH_3) δ 4.55 (s, 2H, CH_2Cl) δ 5.25 (s, 1H, O*H*) δ 7.15 (s, 2*H* arom).

4.2.1.2. 3,5-Di-*tert*-butyl-4-hydroxy-benzoyl chloride (2)[27]. 3,5-Di-*tert*-butyl-4-hydroxy-benzoic acid (5 g, 0.020 mol) was added to a solution of NaOH (0.8 g, 0.020 mol) in 95% aqueous methanol and the mixture was evaporated under reduced pressure, pulverised and dried to yield 5.4 g of the corresponding salt. The salt was treated with petroleum ether (50 mL), cooled to 0 °C and then 10 mL of distilled thionyl chloride was added to the mixture and the reaction was left at room temperature for 2 h. The volatiles were removed under reduced pressure below 50 °C and the residue was treated with 100 mL of petroleum ether. The mixture was filtered and the filtrate was concentrated under reduced pressure yielding 4.7 g of the acid chloride. White solid, yield 80%, mp 96 °C. IR (Nujol): 3545, 1725, 1590 cm^{-1}.

4.2.1.3. 3-Phenyl-thiomorpholine (3)[28]. 2-Amino-ethanethiol hydrochloride (2.3 g, 0.02 mol) was added to a solution of potassium hydroxide (2.2 g, 0.04 mol) in 50 mL of methanol cooled to 0–5 °C under nitrogen. To this mixture a solution of 2-bromo-1-phenyl-etha-

none (4 g, 0.02 mol) in 10 mL of methanol was added. The reaction mixture was stirred below 5 °C for 1 h and then acidified with 30% methanolic hydrochloric acid. After stirring for an additional 1 h at 0 °C, sodium borohydride (1.5 g, 0.04 mol) was slowly added and the mixture was stirred for 30 min. The hydrolysis was carried out with aqueous hydrochloric acid, the solvent was evaporated to dryness and the residue was treated with water. The aqueous layer was made alkaline with a saturated solution of sodium bicarbonate and extracted with chloroform. The extracts were dried and the solvent was removed at reduced pressure to give the final product. White solid, yield 52%, mp 62–64 °C. IR (Nujol): 2705, 2641, 2598, 2449 cm^{-1}.

4.2.1.4. 4-Benzyl-2-phenyl-morpholin-2-ol hydrobromide (4)[29]. 2-Bromo-1-phenyl-ethanone (5.2 g, 0.026 mol) was dissolved in a mixture of ethyl ether and acetone 10:1 and added to a solution of 2-benzylamino-ethanol (8.2 g, 0.054 mol) in diethyl ether. The reaction mixture was stirred overnight at room temperature. It was then filtered, the filtrate washed with a saturated aqueous NaCl solution. The organic layer was dried over potassium carbonate, concentrated and finally the amine was acidified with a 10% solution of HBr in diethyl ether to give the corresponding salt. The salt was recrystallised from ethyl ether/acetone. White solid, yield 88%, mp 150–151 °C. IR (Nujol): 3160, 2760, 2725, 2650, 2500, 2425, 1600 cm^{-1}.

4.2.1.5. 4-Benzyl-2-ethoxy-2-phenyl-morpholine hydrobromide (5)[29]. A solution of 4-benzyl-2-phenyl-morpholin-2-ol hydrobromide (3 g, 0.0085 mol) in ethanol (150 mL) was acidified with a 10% solution of HBr in diethyl ether and the mixture was refluxed for 3 h. Then, the solvent was removed under reduced pressure and the residue was recrystallised from diethyl ether/ethanol to give the final product. White solid, yield 93%, mp 163–164 °C. IR (Nujol): 2725, 2675, 2650, 2625, 2580, 1600 cm^{-1}.

4.2.1.6. 2-Ethoxy-2-phenyl-morpholine hydrobromide (6)[29]. 4-Benzyl-2-ethoxy-2-phenyl-morpholine hydrobromide (2.7 g, 0.007 mol) was dissolved in absolute ethanol (150 mL), palladised charcoal (0.2 g, 10%) was added and hydrogen was introduced with shaking. The hydrogenation was stopped when the theoretical volume of hydrogen was consumed. The solution was filtered, the filtrate concentrated and alkalified with potassium carbonate to obtain the free base, which was purified by flash chromatography, eluting with ethyl acetate containing 1% triethylamine. The purified base was then acidified with a 10% solution of HBr in ethyl ether to obtain the corresponding salt. White solid, yield 64%, mp 125–126 °C. IR (Nujol): 2760, 2725, 2650, 2500, 2425, 1600 cm^{-1}.

4.2.1.7. Pyrrolidine-2-carboxylic acid methyl ester (7)[30]. Pyrrolidine-2-carboxylic acid (11.5 g, 0.1 mol) was dissolved in 125 mL of anhydrous methanol and cooled to 0 °C. The solution was acidified with the addition of a 30% methanolic solution of HCl and stirred overnight. The solution was then concentrated under reduced pressure. The acidification and evaporation procedures were repeated and the oily residue was dried over P_2O_5 and NaOH overnight. The oil was crystallised and the crystals were washed thoroughly with diethyl ether. White solid, yield 29%, mp 151–152 °C. IR (Nujol): 3400, 2760, 1750 cm^{-1}.

4.2.2. General procedure for the preparation of the amines of 2,6-di-*tert*-butyl-4-(chloromethyl)phenol and the amides of 3,5 di-*tert*-butyl-4-hydroxybenzoyl chloride (8–14).[31] A solution of 2,6-di-*tert*-butyl-4-(chloromethyl)-phenol (**1**) or 3,5-di-*tert*-butyl-4-hydroxybenzoyl chloride (**2**) (1 mol) was slowly added to a solution of the appropriate amine (thiomorpholine, 3-phenyl-thiomorpholine, 2-ethoxy-2-phenyl-morpholine, or pyrrolidine-2-carboxylic acid methyl ester, 1 mol) and triethylamine (1.1 mol) in diethyl ether (30 mL). The reaction mixture was stirred overnight at room temperature and filtered to remove triethylamine hydrochloride. The filtrate was then washed with a saturated aqueous NaCl solution, dried over $CaCl_2$ and evaporated under reduced pressure. The final product, in the case of amines, was either isolated as hydrochloride, recrystallised from acetone/diethyl ether, or purified by flash chromatography, eluting with petroleum ether/ethyl acetate containing 1% triethylamine. Amides were purified by flash chromatography, eluting with petroleum ether/ethyl acetate.

4.2.2.1. 2,6-Di-*tert*-butyl-4-thiomorpholin-4-ylmethyl-phenol hydrochloride (8). White solid, purified by recrystallisation from acetone/diethyl ether, yield 66%, mp 235–236 °C. IR (Nujol): 3624, 2700, 2650, 2470, 2406 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.39 (s, 18H, CH_3) δ 2.54–2.83 (m, 4H, CH_2SCH_2) δ 3.63–3.70 (m, 4H, CH_2NCH_2) δ 4.01 (s, 2H, NCH_2Ar) δ 5.41 (s, 1H, O*H*) δ 7.27 (s, 2*H* arom) δ 12.67 (s, 1H, *H*Cl). Anal. Calcd for $C_{19}H_{32}ClNOS$: C, 63.75; H, 9.01; N, 3.91. Found: C, 63.46; H, 9.24, N, 3.87.

4.2.2.2. (3,5-Di-*tert*-butyl-4-hydroxy-phenyl)-thiomorpholin-4-yl-methanone (9)[20]. White solid, purified by flash chromatography, eluting with petroleum ether/ethyl acetate 6:1, yield 49%, mp 181–182 °C. IR (Nujol): 3538, 1636, 1606 cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.45 (s, 18H, CH_3) δ 2.58–2.70 (m, 4H, CH_2SCH_2) δ 3.75–3.90 (m, 4H, CH_2NCH_2) δ 6.70 (s, 1H, O*H*) δ 7.20 (s, 2*H* arom). Anal. Calcd for $C_{19}H_{29}NO_2S$: C, 68.02; H, 8.71; N, 4.18. Found: C, 68.14; H, 9.13; N, 4.27.

4.2.2.3. 2,6-Di-*tert*-butyl-4-(3-phenyl-thiomorpholin-4-ylmethyl)-phenol hydrochloride (10). White solid, purified by recrystallisation from acetone/diethyl ether, yield 83%, mp 194–195 °C IR (Nujol): 3624, 2705, 2662, 2534, 2448, 1611 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.36 (s, 18H, CH_3) δ 2.56–2.62 (t, $J = 3.0$ Hz, 2H, NCH_2CH_2S) δ 2.93–2.96 (d, 1H, NCH(Ar)CH_2S) δ 3.73–3.87 (m, 3H, NCH_2CH_2S, NCH(Ar)CH_2S) δ 4.02–4.08 (t, $J = 3.0$ Hz, 3H, $NCH(Ar)CH_2S$, NCH_2Ar) δ 5.38 (s, 1H, O*H*) δ 6.95–7.48 (m, 7*H* arom) δ 12.86 (s, 1H, *H*Cl). Anal. Calcd for

$C_{25}H_{35}NOS$ (base): C, 75.52; H, 8.87; N, 3.52. Found: C, 75.05; H, 8.80; N, 3.17.

4.2.2.4. 2,6-Di-*tert*-butyl-4-(2-ethoxy-2-phenyl-morpholin-4-ylmethyl)-phenol hydrochloride (11). White solid, purified by flash chromatography, eluting with petroleum ether, or by recrystallisation from acetone/diethyl ether, yield 82%, mp 124–125 °C. IR (Nujol): 3645, 2726, 2598, 2350, 1606 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.31–1.50 (m, 21H, OCH_2CH_3, CH_3) δ 2.53–3.53 (m, 4H, OCH_2CH_2N, $NCH_2C(Ar)$-OCH_2CH_3) δ 3.71–4.85 (m, 6H, OCH_2CH_3, NCH_2Ar, OCH_2CH_2N) δ 5.46 (s, 1H, O*H*) δ 7.27–7.61 (m, 7*H* arom) δ 12.66 (s, 1H, *H*Cl). Anal. Calcd for $C_{27}H_{40}ClNO_3$: C, 70.18; H, 8.73; N, 3.03. Found: C, 69.81; H, 8.44; N, 3.22.

4.2.2.5. (3,5-Di-*tert*-butyl-4-hydroxy-phenyl)-(2-ethoxy-2-phenyl-morpholin-4-yl)-methanone (12). White solid, purified by flash chromatography, eluting with petroleum ether/ethyl acetate 6:1, yield 43%, mp 143–144 °C. IR (Nujol): 3624, 1645, 1607 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.05–1.18 (t, $J = 6.5$ Hz, 3H, OCH_2CH_3) δ 1.34 (s, 18H, CH_3) δ 2.43–2.98 (m, 4H, OCH_2CH_3, OCH_2CH_2N) δ 3.83–4.25 (m, 4H, OCH_2CH_2N, $NCH_2C(Ar)OCH_2CH_3$) δ 5.68 (s, 1H, O*H*) δ 7.26–7.31 (m, 7*H* arom). Anal. Calcd for $C_{27}H_{37}NO_4$: C, 73.77; H, 8.48; N, 3.19. Found: C, 73.35; H, 8.12; N, 2.96.

4.2.2.6. 1-(3,5-Di-*tert*-butyl-4-hydroxy-benzyl)-pyrrolidine-2-carboxylic acid methyl ester hydrochloride (13). White solid, purified by flash chromatography, eluting with petroleum ether/ethyl acetate 7:1 containing 1% triethylamine, or by recrystallisation from acetone/diethyl ether, yield 69%, mp 150–151 °C. IR (Nujol): 3646, 2727, 2683, 2598, 1748, 1611 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.38 (s, 18H, CH_3) δ 1.95–2.70 (m, 4H, CH_2CH_2) δ 3.35–3.40 (t, $J = 2.5$ Hz, 1H, $NCHCOOCH_3$) δ 3.72 (s, 5H, NCH_2Ar $COOCH_3$) δ 4.20–4.40 (m, 2H, NCH_2) δ 5.36 (s, 1H, O*H*) δ 7.34 (s, 2*H* arom) δ 13.00 (s, 1H, *H*Cl). Anal. Calcd for $C_{21}H_{34}ClNO_3 \cdot 0.3\ H_2O$: C, 64.78; H, 8.96; N, 3.60. Found: C, 64.71; H, 8.91; N, 3.59.

4.2.2.7. 1-(3,5-Di-*tert*-butyl-4-hydroxy-benzoyl)-pyrrolidine-2-carboxylic acid methyl ester (14). White solid, purified by flash chromatography, eluting with petroleum ether/ethyl acetate 10:1, or by recrystallisation from acetone/petroleum ether, yield 61%, mp 135–136 °C. IR (Nujol): 3581, 1765, 1620, 1602 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.37 (s, 18H, CH_3) δ 1.82–2.26 (m, 4H, CH_2CH_2) δ 3.56–3.67 (m, 2H, NCH_2) δ 3.70 (s, 3H, $COOCH_3$) δ 4.55–4.59 (t, $J = 2.0$ Hz, 1H, $NCHCOOCH_3$) δ 5.40 (s, 1H, O*H*) δ 7.39 (s, 2*H* arom). Anal. Calcd for $C_{21}H_{31}NO_4$: C, 69.78; H, 8.64; N, 3.87. Found: C, 69.31; H, 8.44; N, 3.62.

4.3. Carrageenan paw edema

An aqueous solution of carrageenan was prepared (1% w/v) and 0.1 mL of this was injected into the right hind paw of male rats, the left paw serving as control. The tested compounds (dissolved or suspended in water with a few drops of Tween 80) were given ip (0.28 mmol/kg of body weight) 5 min prior to the carrageenan injection. After 3.5 h, the hind paws were excised and weighed separately. The produced edema was estimated as paw weight increase.[21]

4.4. Toxicity

A dose of 1 mmol/kg of compound **8** and BHT was administered ip to female animals and their toxicity was evaluated by mortality, GI ulcers and body weight change after 24 h.

4.5. In vitro evaluation of cyclooxygenase activity (isoforms 1 and 2)

The effect of compounds **8**–**14** on COX-1 and COX-2 activity was 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, USA). The assay directly measures PGF_{2a} produced by $SnCl_2$ reduction of COX-derived PGH_2. The prostanoid product is quantified via enzyme immunoassay using a broadly specific antibody that binds to all the major prostaglandin compounds.

The inhibitory activity of the compounds was measured at various concentrations of arachidonic acid (0.1–1 μM). The compounds were added to the reaction mixture at a final concentration of 10 μM, unless otherwise mentioned. COX, 10^{-9} IU/ml for both isoforms.

4.6. In vitro evaluation of lipoxygenase activity

The reaction mixture contained (final concentration) the test compounds, dissolved in propyleneglycol at concentrations of 10–450 μM, or the solvent (control), soybean lipoxygenase, dissolved in 0.9% NaCl solution (250 u/mL) and sodium linoleate (100 μM), in Tris–HCl buffer, pH 9.0. The reaction was monitored for 7 min at 28 °C, by recording the absorbance of a conjugated diene structure at 234 nm, due to the formation of 13-hydroperoxy-linoleic acid. The performance of the assay was checked using nordihydroguaiaretic acid as a reference.[32]

4.7. In vitro lipid peroxidation

The incubation mixture contained heat-inactivated rat hepatic microsomal fraction (corresponding to 2.5 mg of hepatic protein per millilitre or 4 mM fatty acid residues), ascorbic acid (0.2 mM) in Tris–HCl/KCl buffer (50 mM/150 mM, pH 7.4) and the studied compounds in dimethylsulfoxide at concentrations of 5 μM–1.0 mM. The peroxidation was started with the addition of a freshly prepared $FeSO_4$ solution (10 μM), and aliquots were taken from the incubation mixture (37 °C) at various time intervals for 45 min. Lipid peroxidation was assessed by spectrophotometric (535 against 600 nm) determination of the 2-thio-

barbituric acid reactive material. All compounds and solvents were tested and found not to interfere with the assay.[33]

4.8. In vitro interaction with the stable radical DPPH

Compounds, dissolved in absolute ethanol, at concentrations of 25–200 μM, were added to an equal volume of an ethanolic solution of DPPH (final concentration 200 μM) at room temperature (22 ± 2 °C). Absorbance (517 nm) was recorded at different time intervals for 4 h.[34]

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