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Nitric oxide releasing derivatives of tolfenamic acid with anti-inflammatory activity and safe gastrointestinal profile

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Abstract—Tolfenamic acid esters with nitrooxyalcohols are synthesized. They are anti-inflammatory agents reducing carrageenan rat paw edema, with low gastrointestinal and general toxicity. In vitro, they are nitric oxide donors, inhibitors of lipoxygenase and cyclooxygenases. A two to three carbon chain between carboxylic and nitric ester groups seems optimal for activity.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are still one of the first choices in the treatment of inflammatory conditions and among the most commonly prescribed drugs.[1,2] However, they are not devoid of adverse effects, gastrointestinal (GI) ulceration and renal damage being the most common, and even life-threatening.[3–10]

Several strategies have been followed to reduce these adverse effects, including enteric coating, parenteral administration, formulation of prodrugs that require hepatic metabolism for the cyclooxygenase (COX) activity to be unmasked and coadministration of either suppressors of acid secretion or exogenous prostaglandins (PGs), without the desired results.[11]

A more recent and promising approach is that of COX-2 inhibitors. It is found that prostaglandins, important for the gastrointestinal and renal function, are produced via COX-1, which is the constitutive isoform of COX, and prostaglandins responsible for inflammation are produced via COX-2, the inducible isoform of the enzyme.[12,13]

Therefore, an effort has been made for the development of COX-2 selective inhibitors, in order to suppress only the production of prostaglandins that mediate inflammation. These drugs are currently on the market and are considered GI safe and potent anti-inflammatory agents, but recent studies prove that they still have some important disadvantages.[14,15]

COX-2 is not only implicated in inflammatory process but also has a physiological role in neurons and in gastric mucosa where it is constitutively expressed, and it is considered to have gastroprtotective properties.[16–18] Furthermore, these drugs may lead to increased cardiovascular events because PGI_2, which is a potent antiaggregator of platelets, is mainly produced via COX-2 in vascular endothelial cells, while thromboxane A_2 (TXA_2), a potent aggregator of platelets and a vasoconstrictor, is mainly produced via COX-1 in platelets.[19–24]

Another approach to reduce the toxicity of NSAIDs is the linking of a nitric monoxide (NO) releasing moiety to these compounds, and thus, a new category of anti-inflammatory agents is emerging, the NO-NSAIDs.[25–28]

Nitric oxide is a small diatomic radical that plays an important physiological role in nervous, cardiovascular, and immune systems.[29] Its generation is controlled by the three isoforms of NO synthase (NOS), neuronal and endothelial NOS (nNOS and eNOS), which are constitutive and produce nanomolar amounts of NO important for normal cell function and tissue protection, and inducible NOS (iNOS) which generates high amounts of NO responsible for the destruction of invading pathogens as part of an overall inflammatory response.[30] The endogenous tissue NO, generated constitutively by

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GI eNOS and nNOS, appears to play a key role in the chronic maintenance of gastrointestinal tissue integrity and in adaptive cytoprotection to injurious stimuli, perhaps acting synergistically with other cytoprotective prostaglandins.[31–34] NO promotes several gastric defense mechanisms by increasing mucus and bicarbonate secretion in the GI tract, increasing mucosal blood flow, and inhibiting the pro-inflammatory activities of neutrophils and platelets.[30,35,36] Additionally, NO may reduce inflammation connected to oxidative stress by scavenging reactive oxygen species, which can adversely increase mucosal permeability and kill cells.[30] Furthermore, it is found that NO and NO-derived reactive nitrogen species interact with peroxidases[37–40] and lipoxygenases,[41–45] altering the generation of prostaglandins and leukotrienes, which are signaling molecules involved in inflammation.[46]

Several NO-NSAIDs, such as NO-aspirin, NO-diclofenac, NO-flurbiprofen, and NO-ketoprofen, have already been synthesized and clinical trials for some of them revealed protective activity against acute myocardial infraction[47] and osteoporosis (Paget's disease).[48] These compounds appear to have a safe GI profile and can be effective in a variety of diseases including cardiovascular, rheumatological, and lung diseases, Alzheimer's disease, and cancer. There is also evidence to suggest that these compounds release NO in a metabolic, and not spontaneous, way and therefore, they do not interfere with blood pressure regulation.[49]

Bearing in mind the above studies, we designed new derivatives of tolfenamic acid (TA) with a NO-donating moiety (Chart 1). In this investigation, we report the synthesis of TA derivatives esterified with various nitrooxyalcohols and the estimation of their anti-inflammatory, NO-donating, and antioxidant activities. Two derivatives of TA esterified with nitrophenylalcohols are also studied. Finally, we examine the GI toxicity of a representative compound compared to the parent NSAID.

2. Results and discussion

2.1. Chemistry

The formation of compounds **8**–**12** (Scheme 2) is a two-step procedure. Compounds **13** and **14** are prepared from commercially available 4- and 3-nitro-phenylethyl alcohols, respectively. Scheme 1 demonstrates two different methods used for preparing the nitrooxyalcohols. The reaction of silver nitrate with the appropriate alkyl chloride, yielding the nitric ester and silver chloride,[50] or of nitric acid with the corresponding alkyl alcohol,[51] has been applied. The second method is found to be the most favorable due to better yields and lower cost.

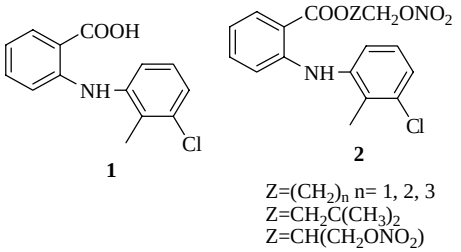
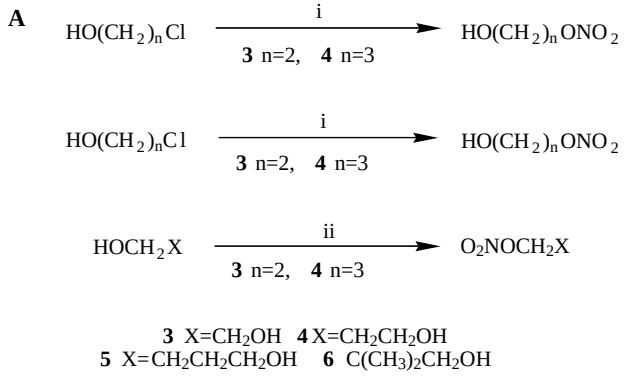


Chart 1. Structure of tolfenamic acid (**1**) and esters with NO-releasing moieties (**2**).

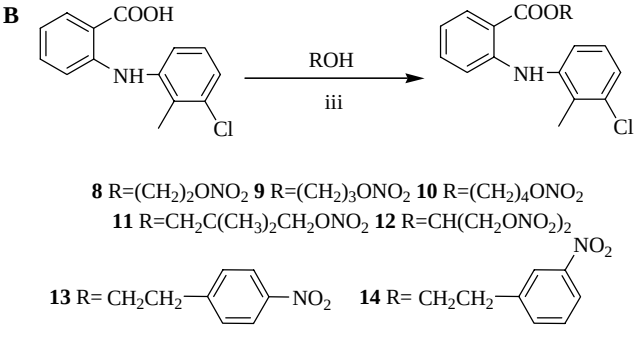
Scheme 2 demonstrates the final step in the synthesis of compounds **8**–**14**, which is an esterification of TA with the corresponding alcohol in the presence of 2,2-dicyclohexylcarbodiimide and catalytic amounts of 4-dimethylaminopyridine.[52]

2.2. In vivo experiments

2.2.1. Carrageenan induced inflammation. The effect of compounds **8**–**14** and TA on carrageenan induced edema is given in Table 1. They all show a significant anti-inflammatory action administered at a dose of 100 mg/kg [corresponding to 0.23–0.28 mmol/kg], the parent molecule being the less potent one. Compounds **9** and **11** are the most potent anti-inflammatory agents and in both a three carbon aliphatic chain connects the carboxylic and nitric ester groups. Compound **12** follows, which has two nitric ester moieties, each linked by a two carbon chain to the carboxylic group. Compounds **8** and **10**, which have a two or four carbon chain, respectively, demonstrate lower anti-inflammatory activity, with **8** being more potent. Finally,



Scheme 1. Synthesis of the intermediate nitrooxy-alcohols (i) Silver nitrate, acetonitrile, room temperature, 12 h. (ii) Nitric acid, glacial acetic acid, acetic anhydride, and ethyl acetate, room temperature, 12 h.



Scheme 2. Synthesis of the final TA esters. (iii) 2,2-dicyclohexylcarbodiimide, 4-dimethylaminopyridine, and dichloromethane, room temperature, 12 h.

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

Compound	Dose (mmol/kg)	% Weight increase[a]	% Edema inhibition[a]
Control	—	43.22	—
8	0.29	26.63	38.39
9	0.27	23.27	46.16
10	0.26	29.26	32.30
11	0.26	23.75	45.05
12	0.24	24.88	42.43
13	0.24	26.41	38.89
14	0.24	28.66	33.69
TA	0.25	30.71	28.97

[a] The effect on edema is expressed as percent of weight increase of hind paw (and as percent of inhibition of edema) in comparison to controls. Each value represents the mean obtained from 6 to 8 animals in two independent experiments. In all cases, significant difference from control: $P < 0.001$ (Student's t test).

compounds **13** and **14**, which are not NO-donors, still possess anti-inflammatory activity, which could be related to previous results indicating that amidation or esterification of acidic NSAIDs retains or enhances the anti-inflammatory activity of the parent drugs.[53]

These results indicate that the anti-inflammatory action of these compounds can be related to the number of carbon atoms of the aliphatic chain that separates the carboxylic from the nitric ester moiety. It seems that a chain of three carbon atoms is optimal (**9**) and methyl branching does not affect this activity (**11**). However, the ability of compounds to liberate NO enhances the anti-inflammatory action further since compound **12** with two nitrooxyethoxy groups is a more potent anti-inflammatory agent than compound **8** with only one such group.

2.2.2. Ulcerogenicity evaluation. TA and compound **9**, the most potent anti-inflammatory NO-donor, were administered sc in equimolar doses [0.76 mmol/kg body weight once daily for 4 days] to two groups of 6 rats. The ulcerogenic effect of these compounds was estimated as mortality (dead/total %), incidence of perforating ulcers (number of animals that developed perforating intestinal ulcers), and body weight change. TA showed a 66.7% of mortality (one of the animals died on the third day of treatment), a 100% incidence of perforating ulcers and a mean percent body weight decrease of 10.48% ± 1.636. On the other hand, compound **9** caused no mortality and perforating ulcers, and a mean percent body weight increase of 2.44% ± 0.167. These results indicate that the derivatives under investigation have a safer GI profile compared to that of the parent molecule at the same dose.

2.2.3. Toxicity. Acute toxicity of TA and compound **9** was estimated after the ip administration of equimolar doses [0.76 mmol/kg body weight] to groups of 8 animals. In the group of TA, 7 animals died within 24 h and one survived with perforating ulcers. In the group of compound **9**, all animals survived and looked normal both macroscopically and by autopsy. These results indicate that the synthesized compounds demonstrate a lower general toxicity compared to TA, which may be connected to the lower GI toxicity of these compounds.

2.3. In vitro experiments

2.3.1. Nitric oxide release. The ability of compounds **8**–**12** to release NO at different concentrations [from 31.25 to 500 μM] is shown in Table 2. *S*-nitroso-*N*-acetyl-penicillamine (SNAP), a known NO donor, is also presented in the same table as a reference compound. SNAP was dissolved in water or a 7/3 (v/v) mixture of DMSO/water (see experimental part). The liberated amount of NO was the same in both cases, indicating no interference of DMSO with the experiment. Compounds **13** and **14** are not included, because they are not NO donors. Compound **12**, which has two nitric ester moieties, is the most potent NO donor. The NO-releasing ability of compounds **8**–**11** is reduced as the aliphatic carbon chain, connecting the carboxylic with the nitric ester group, is increased. A nonlinear increase in the amount of released NO is observed with increasing compound concentration.

2.3.2. Inhibition of cyclooxygenase activity (isoforms 1 and 2). Cyclooxygenase enzymes contain both cyclooxygenase and peroxidase activities. COX catalyzes the first step in the biosynthesis of prostaglandins (PGs), thromboxane, and prostacyclins by the conversion of arachidonic acid to PGH_2.

It is well-known that there are two distinct isoforms of COX, COX-1, which is constitutively expressed in a variety of cell types and is involved in normal cellular homeostasis, and COX-2, which is the inducible isoform responsible for the biosynthesis of PGs under acute inflammatory conditions.

The inhibitory effect of compounds **8-14** [concentration of 100 μM] and TA [concentration of 1 μM for COX-1 and 10 μM for COX-2] in both COX isoforms is shown in Table 3. For the most active compound (**9**) and TA, more concentrations were tested for the determination of their IC_{50} value toward COX-1. The concentration of the substrate, arachidonic acid, was 1 and 0.1 μM in the case of COX-1 and COX-2, respectively, while for TA it was 1 μM for both COX isoforms. At higher concentrations of arachidonic acid [10 μM for COX-1 and 1 μM for COX-2], there was no significant inhibition.

Table 2. In vitro nitric oxide release

	Percent NO release				
Compound:	**8**	**9**	**10**	**11**	**12**
500 μM	15.7	3.7	3.1	3.5	21.4
250 μM	21.1	6.8	3.4	4.5	27.2
125 μM	23.0	9.4	5.0	5.0	38.2
62.5 μM	33.9	12.2	6.8	5.8	59.7
31.25 μM	36.5	—	—	—	66.2

All determinations are performed at least in triplicate and standard deviation is always within ±10% of the mean value. NO release from the reference NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) [100 μM in DMSO/water 7/3 (v/v) and in water] was found to be 56.3–56.7%.

Table 3. In vitro evaluation of anti-inflammatory activity

Compound	Percent cyclooxygenase inhibition (compound concentration 100 μM)		Lipoxygenase inhibition IC_{50} (μM)
	COX-1[a]	COX-2[b]	
8	44	61	59
9	67	85	67
10	51	28	150
11	2	1	92
12	61	56	35
13	1	1	200
14	0	0	262
TA[c]	69	64	170
NDGA	—	—	1.3

[a] Arachidonic acid 1 μM.
[b] Arachidonic acid 0.1 μM.
[c] **TA** 1 μM for COX-1 and 10 μM for COX-2, arachidonic acid 1 μM for both COX isoforms.

Most compounds showed inhibition of both COX isoforms, with the exception of **13** and **14**, which are not NO donors, and compound **11**, probably due to a steric hindrance affecting its interaction with the enzyme active site. TA is the most potent of all compounds (IC_{50} 0.6 μM for COX-1) and this was, in part, expected because of its free carboxylic moiety. Compound **9** is the most potent inhibitor of all TA derivatives for both COX isoforms, with an IC_{50} value of 57 μM for COX-1 inhibition under the described experimental conditions. Compound **12** followed, which has two NO donating moieties. The COX-2 inhibition was tested at a lower concentration of arachidonic acid [0.1 μM] than that used for COX-1 inhibition [1 μM] because at 1 μM all compounds showed no significant action on COX-2. Thus, these derivatives are mainly COX-1 inhibitors. However, the performed structural modifications were not able to offer high selectivity toward any of the COX isoforms.

2.3.3. Inhibition of lipoxygenase activity. Lipoxygenases oxidize certain fatty acids at specific positions to hydroperoxides that are the precursors of leukotrienes, which contain a conjugated triene structure. It is known that soybean lipoxygenase, which converts linoleic to 13-hydroperoxylinoleic acid, is inhibited by NSAIDs in a qualitatively similar way to that of the rat mast cell lipoxygenase and may be used in a reliable screen for such activity.[54] The IC_{50} values of compounds **8–14**, TA, and nordihydroguaiaretic acid (NDGA), an antioxidant compound acting as a nonspecific inhibitor of lipoxygenase, after 7 min of incubation are given in Table 3. The time course of lipoxygenase activity, as affected by various concentrations of three most potent compounds (**8**, **9**, and **12**) is given in Figure 1. It can be seen that the offered inhibition is time- and concentration-dependent. All compounds are more potent inhibitors than TA, with the exception of **13** and **14** which are not NO donors. The most potent inhibitor is compound **12**, which has two NO donating moieties. The other four compounds show an inhibitory activity that is reduced, as the aliphatic carbon chain connecting the carboxylic with the nitric ester moiety increases. The same effect of chain length has been observed on the liberation of NO from these compounds, indicating a relationship between the amount of NO released from these substances and their effect on lipoxygenase inhibition.

2.3.4. Antioxidant activity. Although oxygen-derived free radicals are implicated in inflammation and some anti-inflammatory agents, such as TA, have been found to possess antioxidant activity,[55] the synthesized TA esters exerted no significant inhibition on lipid peroxida-

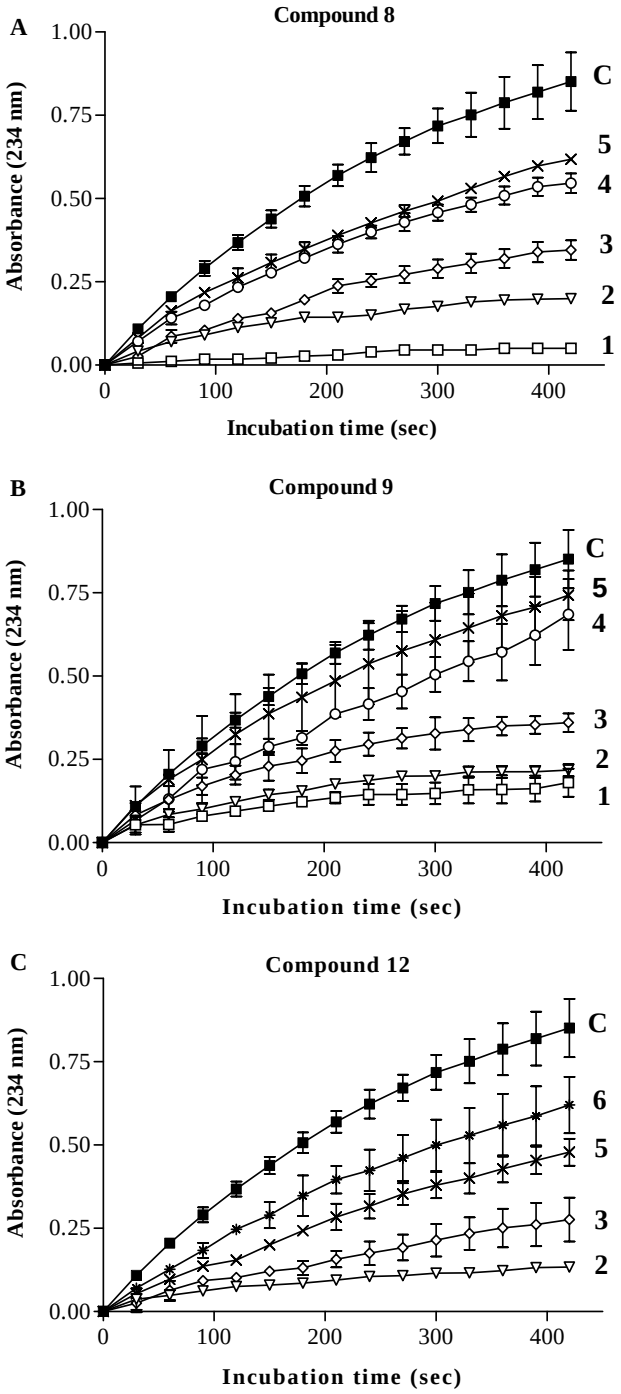


Figure 1. (A–C) Effect of various concentrations of compounds **8**, **9**, and **12** on lipoxygenase activity. C: Control, 1: 150 μM, 2: 100 μM, 3: 75 μM, 4: 50 μM, 5: 25 μM, and 6: 10 μM.

tion of rat hepatic microsomal membranes at 1 mM, and no interaction with the N-centered 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable free radical at equimolar concentrations. The above may indicate that other mechanisms are more important than the antioxidant ability in their mode of action.

From the in vitro and in vivo experiments, it could be concluded that a chain of two to three carbon atoms connecting the two ester oxygen atoms is important for all activities, which seem to be interrelated. Compound **12**, with two nitric ester groups connected via two chains of two carbons with the carboxylic oxygen, is the most active in all experiments. In general, four carbon chain or methyl branching in a three carbon chain does not favor activity in all the examined parameters. The in vivo and in vitro results for compounds **8** and **9** do not agree completely, however, differences in the rate of the metabolic liberation of NO in the organism and the possibility of other factors influencing their anti-inflammatory action can be a conceivable explanation for the observed deviations.

3. Conclusion

All the synthesized compounds are overall more active than tolfenamic acid and they seem to be less toxic, thus possessing a wider 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 appropriate purity. Tolfenamic acid was kindly donated from ELPEN Pharmaceutical Co. (Greece).

For the in vivo experiments, Fischer-344 rats [150–250 g] were used.

4.2. Synthesis

Melting points (m.p.) were obtained with 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 are obtained on a Bruker AW 80 MHz and 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. General procedure for the preparation of nitrooxyalcohols.

4.2.1.1. Method A. Silver nitrate [35 mmol] was added to a solution of the corresponding 2-chloroethanol or 3-chloropropanol [23 mmol] in acetonitrile [40 mL]. The reaction mixture was stirred at room temperature overnight and was light protected. The reaction mixture was filtered and the volatiles were removed under reduced pressure. The residue was dissolved in diethyl ether and washed with saturated NaCl solution. The organic layer was dried over calcium chloride, filtered, and concentrated. The obtained oil was purified by flash chromatography, eluting with petroleum ether-ethyl acetate.

4.2.1.2. Method B. Concentrated nitric acid [22 mmol], glacial acetic acid [5 mL], and acetic anhydride [5 mL] were consecutively added to an ice-cold solution of the appropriate diol [20 mmol] in 50 mL ethyl acetate under nitrogen. For the synthesis of **7**, double the amount of nitric acid, acetic acid, and acetic anhydride was used. The reaction mixture was stirred at room temperature overnight, and the solution was washed with a 10% aqueous solution of sodium bicarbonate. The organic layer was dried over potassium carbonate, filtered, and concentrated under reduced pressure. The oily residue was purified by flash chromatography, eluting with petroleum ether-ethyl acetate.

The IR spectra of all compounds show peaks at 3400, 2900, and 1630 cm^{-1}.

4.2.1.3. 2-Nitrooxy-ethanol (3).[56] Pale yellow oil, yield 16% (method A) or 38% (method B). ^{1}H NMR ($CDCl_3$): δ 2.0 (s, 1H, O**H**), δ 3.90–4.00 (t, J = 5 Hz, 2H, C**H**$_2$OH), δ 4.50–4.60 (t, J = 5 Hz, 2H, C**H**$_2$ONO$_2$).

4.2.1.4. 3-Nitrooxy-propan-1-ol (4).[57] Pale yellow oil, yield 40% (method A) or 55% (method B). ^{1}H NMR ($CDCl_3$): δ 1.90–1.96 (m, 2H, C**H**$_2$) δ 1.98 (s, 1H, O**H**), δ 3.71–3.74 (t, J = 1.50, 2H, C**H**$_2$OH) δ 4.54–4.57 (t, J = 1.58, 2H, C**H**$_2$ONO$_2$).

4.2.1.5. 4-Nitrooxy-butan-1-ol (5).[58] Pale yellow oil, yield 25% (method B). ^{1}H NMR ($CDCl_3$): δ 1.50 (s, 1H, O**H**), δ 1.60–1.65 (m, 2H, C**H**$_2$CH$_2$OH), δ 1.75–1.82 (m, 2H, C**H**$_2$CH$_2$ONO$_2$), δ 3.63–3.66 (t, J = 1.56, 2H, C**H**$_2$OH) δ 4.43–4.46 (t, J = 1.65, 2H, C**H**$_2$ONO$_2$).

4.2.1.6. 2,2-Dimethyl-3-nitrooxy-propan-1-ol (6). Pale yellow oil, yield 39% (method B). ^{1}H NMR ($CDCl_3$): δ 0.95 (s, 6H, C**H**$_3$), δ 2.40 (s, 1H, O**H**), δ 3.39 (s, 2H, C**H**$_2$OH), δ 4.28 (s, 2H, C**H**$_2$ONO$_2$).

4.2.1.7. 1,3-Bis-nitrooxy-propan-2-ol (7).[59] Pale yellow oil, yield 40% (method B). ^{1}H NMR ($CDCl_3$): δ 2.10 (s, 1H, O**H**), δ 4.25–4.40 (m, 1H, C**H**OH), δ 4.52–4.60 (d, J = 8 Hz, 4H, C**H**$_2$ONO$_2$).

4.2.2. General procedure for the preparation of the esters of 2-(3-chloro-2-methylphenylamino)-benzoic acid (tolfenamic acid) with the appropriate (nitrooxy) alcohols. Dicyclohexylcarbodiimide [2 mmol] and 4-dimethylaminopyridine [0.19 mmol] were added to a solution of tolfenamic acid [1 mmol] and the appropriate nitroxyalcohol [4 mmol] in dichloromethane. The reaction mixture was stirred at room temperature overnight, filtered to remove the formed dicyclohexylurea, and washed with a 10%

aqueous solution of potassium carbonate to remove excess tolfenamic acid. The organic layer was dried over sodium sulfate, filtered, and concentrated under reduced pressure. The resultant semisolid residue was purified by flash chromatography, eluting with petroleum ether-ethyl acetate.

4.2.2.1. 2-Nitrooxyethyl 2-(3-chloro-2-methylphenylamino)benzoate (8). Pale yellow solid, yield 44%, m.p. 58–59 °C. IR: 3300, 1680, 1630, 1590 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.35 (s, 3H, C**H**$_3$) δ 4.50–4.60 (t, J = 5 Hz, 2H, C**H**$_2$ONO$_2$) δ 4.70–4.80 (t, J = 5 Hz, 2H, COOC**H**$_2$) δ 6.6–8.00 (m, 7Harom) δ 9.17 (s, 1H, N**H**). Analysis for $C_{16}H_{15}ClN_2O_5$ calculated: C 54.79%, H 4.31%, N 7.99%. Found: C 54.58%, H 4.26%, N 7.70%.

4.2.2.2. 3-Nitrooxypropyl 2-(3-chloro-2-methylphenylamino)benzoate (9). Pale yellow solid, yield 51%, m.p. 53–54 °C. IR: 3300, 1680, 1630, 1590 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.05–2.55 (m, 5H, C**H**$_3$, COOCH$_2$C**H**$_2$-CH$_2$ONO$_2$) δ 4.40–4.50 (t, J = 5 Hz, 2H, C**H**$_2$ONO$_2$) δ 4.60–4.70 (t, J = 5 Hz, 2H, COOC**H**$_2$) δ 6.60–8.00 (m, 7Harom) δ 9.27 (s, 1H, N**H**). Analysis for $C_{17}H_{17}ClN_2O_5$ calculated: C 55.97%, H 4.70%, N 7.68%. Found: C 56.09%, H 4.29%, N 7.33%.

4.2.2.3. 4-Nitrooxybutyl 2-(3-chloro-2-methylphenylamino)benzoate (10). Yellow oil, yield 82%. IR: 3300, 1680, 1630, 1590 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.83–2.00 (m, 4H, CH$_2$C**H**$_2$C**H**$_2$CH$_2$) δ 2.35 (s, 3H, C**H**$_3$) δ 4.30–4.40 (t, J = 5 Hz, 2H, C**H**$_2$ONO$_2$),δ 4.50–4.60 (t, J = 5 Hz, 2H, COOC**H**$_2$), δ 6.60–8.00 (m, 7Harom) δ 9.30 (s, 1H, N**H**). Analysis for $C_{18}H_{19}ClN_2O_5$ calculated: C 57.07%, H 5.05%, N 7.40%. Found: C 57.06%, H 4.83%, N 7.38%.

4.2.2.4. 2,2-Dimethyl-3-nitrooxypropyl 2-(3-chloro-2-methylphenylamino)benzoate (11). Pale yellow solid, yield 70%, m.p. 64–65 °C. IR: 3300, 1680, 1630, 1590 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.12 (s, 6H, C(C**H**$_3$)$_2$) δ 2.35 (s, 3H, C**H**$_3$) δ 4.15 (s, 2H, C**H**$_2$ONO$_2$) δ 4.45 (s, 2H, COOC**H**$_2$) δ 6.60–8.00 (m, 7Harom) δ 9.30 (s, 1H, N**H**). Analysis for $C_{19}H_{21}ClN_2O_5$ calculated: C 58.09%, H 5.39%, N 7.13%. Found: C 58.45%, H 5.48%, N 7.15%.

4.2.2.5. 2-Nitrooxy-1-nitrooxymethylethyl 2-(3-chloro-2-methylphenylamino)benzoate (12). Yellow solid, yield 79%, m.p. 60–61 °C. IR: 3300, 1680, 1630, 1590 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.40 (s, 3H, C**H**$_3$) δ 4.90–5.00 (d, J = 10 Hz, 4H, C**H**$_2$ONO$_2$) δ 5.70–6.00 (m, 1H, COOC**H**) δ 6.80–8.10 (m, 7Harom) δ 9.30 (s, 1H, N**H**). Analysis for $C_{17}H_{16}ClN_3O_8$ calculated: C 47.96%, H 3.79%, N 9.87%. Found: C 48.10%, H 3.85%, N 9.86%.

4.2.2.6. 2-(4-Nitrophenyl)ethyl 2-(3-chloro-2-methylphenylamino)benzoate (13). White solid, yield 73%, m.p. 104–105 °C. IR: 3300, 1680, 1590 cm^{-1}. ^1H NMR ($CDCl_3$): 2.30 (s, 3H, C**H**$_3$) δ 3.15–3.25 (t, J = 5 Hz, 2H, C**H**$_2$Ar) δ 4.50–4.60 (t, J = 5 Hz, 2H, COOC**H**$_2$) δ 6.60–8.25 (m, 11Harom) δ 9.25 (s, 1H, N**H**). Analysis for $C_{22}H_{19}ClN_2O_4$ calculated: C 64.37%, H 4.67%, N 6.83%. Found: C 64.13%, H 4.52%, N 6.76%.

4.2.2.7. 2-(3-Nitrophenyl)ethyl 2-(3-chloro-2-methylphenylamino)benzoate (14). Pale yellow solid, yield 80%, m.p. 91–92 °C. IR: 3300, 1680, 1590 cm^{-1}. ^1H NMR ($CDCl_3$): 2.30 (s, 3H, C**H**$_3$) δ 3.15–3.25 (t, J = 5 Hz, 2H, C**H**$_2$Ar) δ 4.50–4.60 (t, J = 5 Hz, 2H, COOC**H**$_2$) δ 6.60–8.20 (m, 11Harom) δ 9.25 (s, 1H, N**H**). Analysis for $C_{22}H_{19}ClN_2O_4$ calculated: C 64.31%, H 4.66%, N 6.82%. Found: C 64.29%, H 4.39%, N 6.83%.

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 [0.23–0.28 mmol/kg of body weight] 5 min prior to the carrageenan injection. Rats were euthanized 3.5 h later, and the hind paws were excised and weighed separately. The produced edema was estimated as paw weight increase. The parent anti-inflammatory drug, at the same dose, was used as reference in addition to the absolute control.[60]

4.4. Ulcerogenicity evaluation

Equimolar doses [0.76 mmol/kg of body weight] of TA and compound **9** were administered sc once daily for 4 days to female animals. The incidence of perforating GI ulcers, body weight change, and mortality were recorded 24 h after the last treatment.[61]

4.5. Toxicity

Equimolar doses [0.76 mmol/kg] of TA and compound **9** were administered ip to 5–8 female animals and their toxicity was evaluated by mortality, GI ulcers, and body weight change after 24 h.

4.6. In vitro nitric oxide release

Due to their very poor water solubility, compounds **8**–**14** were dissolved in 7/3 (v/v) mixture of DMSO/water at various concentrations and incubated overnight at room temperature in the presence of cadmium. Aliquots were taken from each sample and added to an equal volume of *N*-naphthylaminoethylamine [0.2%] and sulfanilamide [2%] solution in 3 N hydrochloric acid (Griess reagent). Appropriate sodium nitrite solutions in the same DMSO/water mixture were used for the construction of the standard curve and values were identical to those obtained with aqueous solutions. Nitric oxide release was estimated spectrophotometrically [540 nm].[62]

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

The COX-1 and COX-2 activity of the compounds **8**–**14** 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 quanti-

fied 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 100 μM, unless otherwise mentioned.

4.8. In vitro evaluation of lipoxygenase activity

The reaction mixture contained (final concentration) the test compounds, dissolved in propyleneglycol in concentrations of 10–300 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 at 234 nm. The performance of the assay was checked using nordihydroguaiaretic acid as a reference.[54] under exactly the same experimental conditions.

4.9. In vitro lipid peroxidation

The incubation mixture contained heat-inactivated rat hepatic microsomal fraction (corresponding to 2.5 mg of hepatic protein per milliliter 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 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-thiobarbituric acid reactive material. All compounds and solvents were tested and found not to interfere with the assay.[63]

4.10. In vitro interaction with the stable radical DPPH

Compounds, dissolved in absolute ethanol, were added to an equal volume of an ethanolic solution of DPPH [final concentration 0.2 mM] at room temperature [22 ± 2 °C]. Absorbance [517 nm] was recorded at different time intervals for 4 h.[64]

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