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N,*N*-Dialkylaminosubstituted chromones and isoxazoles as potential anti-inflammatory agents

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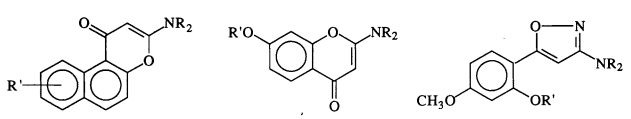
Abstract

The ability of some *N*,*N*-dialkylaminosubstituted chromones and isoxazoles to inhibit the protein kinase C (PKC) dependent signal transduction pathway was tested. As a cellular model, human neutrophils stimulated with either phorbol myristate acetate (PMA) or formylmethionine–leucine–phenylalanine (f-MLF) were used. The efficiency of the compounds was established by their capacity to reduce the O_2^- production by activated human neutrophils. Compounds carrying a 3-bis(2-methoxyethyl)amino group, a substituent found active in previously tested tricyclic compounds, do not show significant anti-PKC activity in this study. On the other hand, substitution with a 1-piperidinyl group leads all tested compounds to a high biological activity against stimulated neutrophils.

Keywords: Anti-PKC activity; Superoxide anion; Neutrophils; 1-Benzopyran-4-ones; 5-Phenylisoxazoles; Anti-inflammatory agents

1. Introduction

Previous work has shown that tricyclic compounds, such as 3-(dialkylamino)naphtho[2,1-*b*]pyran-1-ones (**A**), and bicyclic compounds, such as 2-(dialkylamino)-chromones (**B**), are interesting heterocycles endowed with various pharmacological activities. In fact, it was pointed out that by variation of these substituents, anti-depressive [1,2], anti-allergic [3,4] and anti-platelet [5–8] activities could be observed. In addition, compounds derived from the above-mentioned benzo- and naphthopyrans retain interesting pharmacological properties. In this connection, some Mannich bases were prepared from parent compounds in order to modify their original biological activity on the CNS [9]. Some heteropolycycles, obtained from compounds **B** by nucleophilic displacement of the dialkylamine, also possessed significant anti-allergic properties [10]. Furthermore, by opening the chromone ring with hydroxylamine hydrochloride, it was possible to prepare several 3-(dialkylamino)phenylisoxazoles (**C**) which exhibited specific anti-rhinovirus activity [11,12].



A B C

Due to the fact that many of the postulated biological activities of these compounds are confined to the membrane level and probably affect signal transduction pathways, we have analysed in detail these properties using human neutrophils stimulated with either phorbol myristate acetate (PMA) or formylmethionine–leucine–phenylalanine (f-MLF), as a cell model.

It is well known that these external stimuli induce a cell response through a protein kinase C (PKC)-dependent enzyme cascade. PKC is the collective name of a number of isozymes [13], most of which are localised in the cytosol fraction of the resting cells in inactive enzyme forms. Activation is accomplished by translocation of the kinase from the soluble fraction to the inner surface of plasma membranes. The signal triggering the change in PKC distribution is an increase in the intra-

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cellular concentration of free Ca^{2+}, normally induced by the second messenger IP_3 produced by G-protein activated phospholipase C. In the membrane-associated form, the calcium/PKC complex interacts with phospholipids and diacylglycerol, forming the active enzyme form and inducing a phosphorylation cascade, as cellular response. Activation of PKC has been reported as a limiting step in many biochemical processes, such as proliferation, differentiation, secretion, etc. [14].

In neutrophils, active PKC phosphorilates a membrane associated NADPH oxidase component, inducing reorganisation of the enzyme which acquires the active conformation. Detection of O_2^- produced by NADPH oxidase in a neutrophil suspension is in turn an indication of PKC activation. Consequently, the rate of the reaction catalysed by NADPH oxidase can be used to evaluate the efficiency of the intracellular phosphorylating capacity of PKC.

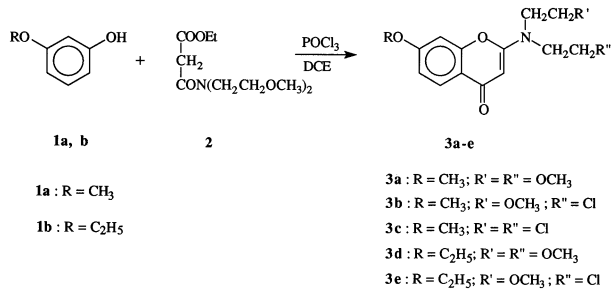
Under specific conditions, O_2^- radicals are believed to participate in the generation of tissue damage, characteristic of inflammatory states [15]. To prevent these unfavourable events and to reduce the negative effects of O_2^- radicals in target tissues, the inhibition of the limiting step in its production must be obtained.

In this regard, compounds in which the anti-inflammatory activity is due to the inhibition of PKC activation are extensively searched. Until now, the lead compounds in this type of action have been staurosporine and its derivatives [16]. Recently, we have obtained evidence that some naphtho[2,1-*b*]pyran derivatives displayed PKC inhibitory activity. Particularly, maximal activity was obtained when the dialkylamine substituent was bis(2-methoxyethyl)amine, though diethylamine and piperidine also had a relevant action [17].

Following our interest on synthetic substances useful as anti-inflammatory agents, we have now analysed the PKC inhibitory activity of some 2-(dialkylamino)-chromones and 3-(dialkylamino)isoxazoles, pointing our attention to a new series of chromones and isoxazoles bearing the bis(2-methoxyethyl)amine or other amines derived from it; in fact, we are interested in testing whether the relevance of this amino group in lowering the neutrophil activation is peculiar of tricyclic systems or may be extended to mono- or bicyclic systems.

2. Chemistry

As depicted in the Scheme 1, the reaction between 3-methoxyphenol (**1a**) and the malonamic acid ethyl ester **2** in the presence of phosphorus oxychloride in 1,2-dichloroethane (DCE) gives rise to the formation of the mixture of benzopyrans **3a–c**. The reaction conditions have already been extensively studied and lead in



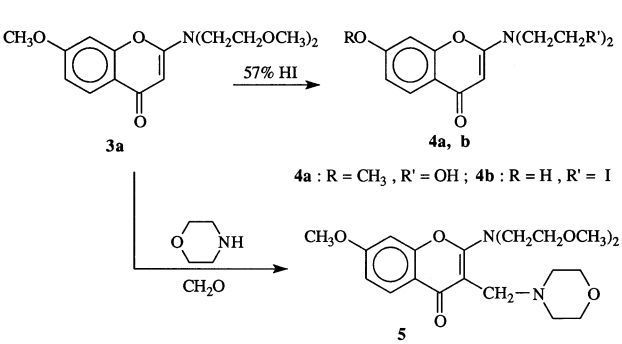
Scheme 1.

general to a single product [1–3,6]. In our case, the expected benzopyran-4-one is accompanied with by-products generated from the secondary reaction of phosphorus oxychloride on the methoxy groups, as shown by silica gel TLC (ethanol/ethyl acetate 1:1) of the crude product in which there are three spots. Then, the recovery of 2-[bis(2-methoxyethyl)amino]-7-methoxychromone (**3a**) is subjected to column chromatography to separate the monochloro derivative **3b** and the dichloro derivative **3c**. To minimise the formation of **3b** and **3c**, the amount of phosphorus oxychloride is lowered with respect to the amount used in the Refs. [1–3,6].

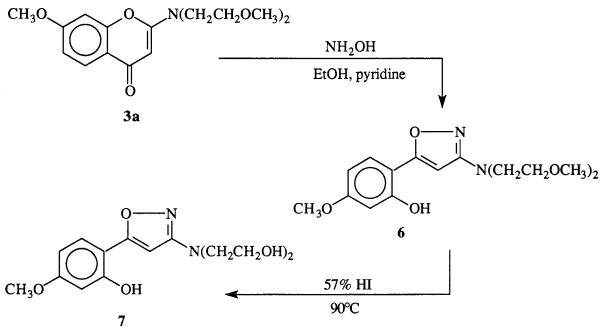
Using 3-ethoxyphenol (**1b**) as the starting product, the reaction pattern was similar: in fact, the TLC of the final mixture presented three spots in the same sequence as the previous reaction but the higher spot, attributable to the dichloro derivative, was in this case present only in trace amounts. Consequently, it was possible to isolate, by column chromatography, only the products which were quantitatively more abundant, namely the dimethoxy derivative **3d** and the monochloro derivative **3e**.

Compound **3a** was selected to obtain some modified chromones and to transform chromones into isoxazole derivatives.

When compound **3a** was submitted to the action of 57% hydriodic acid, it was possible to obtain different products depending on the reaction temperature (see Scheme 2). In fact, when mild conditions are used (90°C) it is possible to recover a compound (**4a**) only



Scheme 2.



Scheme 3.

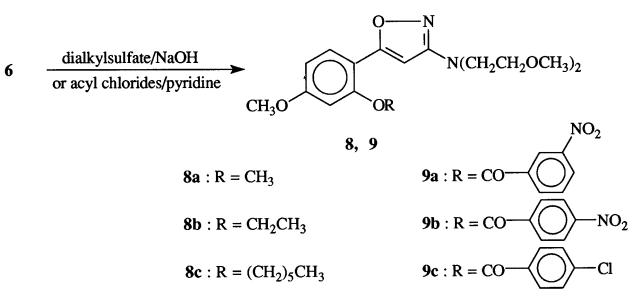
demethylated in the aliphatic chain; on the other hand, when the reaction is refluxed, the demethylation also occurs in the aromatic moiety whereas, as previously found in a similar case [18], the aliphatic hydroxy groups are substituted by iodine to yield **4b**. Then, by treating **3a** with morpholine in the presence of 40% formaldehyde, it is possible to recover the corresponding Mannich base **5** in good yield. As is known for these dialkylaminopyrans, the substitution involves position-3 [9,19]. Other substitutions occur only if the benzene ring bears a hydroxy group [19].

When the chromone **3a** was treated with hydroxylamine hydrochloride in ethanol and pyridine, nucleophilic attack of the hydroxylamine leads to the opening of the chromone ring and subsequently to the formation of an isoxazole ring [11,12].

In turn, the isoxazole **6** was easily demethylated by means of 57% hydriodic acid in mild conditions to give **7** (see Scheme 3).

The isoxazole **6** was treated with some alkylating and acylating reagents. The use of dimethyl and diethylsulfate in alkaline medium yielded **8a** and **8b**, respectively. To obtain a longer aliphatic chain (**8c**), 1-bromohexane was used in the presence of acetone and anhydrous potassium carbonate. The esters **9a**–**c** were prepared by treating **6** with suitable acyl chlorides in anhydrous pyridine (see Scheme 4). All esterification reactions were performed at 100°C for a few minutes. Yields ranged between 90 and 95%.

All compounds described herein are white crystals whose structures are in agreement with elemental analyses and spectral data (see Section 3).



Scheme 4.

3. Experimental

Melting points were determined using a Fisher–Johns apparatus and are uncorrected. Microanalyses were carried out on a Carlo Erba 1106 elemental analyser. The results of elemental analysis were within $\pm 0.3\%$ for C and ± 0.1 for H and N of the theoretical values. ^{1}H NMR spectra were performed on a Hitachi Perkin–Elmer R 600 (60 MHz) spectrometer using TMS as internal standard ($\delta = 0$). IR spectra were recorded on a Perkin–Elmer 398 spectrophotometer.

*3.1. 2-bis(2-Methoxyethyl)amino-7-methoxy-4H-benzopyran-4-one (**3a**), 2-(2-chloroethyl-2-methoxyethyl)-amino-7-methoxy-4H-benzopyran-4-one (**3b**) and 2-bis-(2-chloroethyl)amino-7-methoxy-4H-benzopyran-4-one (**3c**)*

In an ice cooled flask, protected from moisture with a calcium chloride drying tube, 7.0 ml (76.5 mmol) of phosphorus oxychloride were added dropwise under stirring to 13.6 g (55.0 mmol) of 3-bis(2-methoxyethyl)amino-3-oxo-propanoic acid ethyl ester **1** [18]. After the addition, the mixture was removed from the ice bath and maintained at room temperature for 0.5 h. To the resulting yellow mixture, a solution of 6.2 g (50.0 mmol) of 6-methoxyphenol in 40 ml of DCE was added slowly under stirring. The reaction mixture was then heated for 5 h at reflux. After cooling, a solution of 68 g of sodium acetate trihydrate in 200 ml of water was added and the mixture was then heated for 1.5 h at 70°C. After cooling, the organic phase was discarded and the aqueous one was extracted several times with chloroform. The pooled organic extracts were washed with water, dried and evaporated under reduced pressure to give a dark red oil. The oil was stirred at room temperature for 2 h with 200 ml of 2 N NaOH and 50 ml of light petroleum ether. The resulting solid was filtered and washed with water. The solid was chromatographed on a silica gel column with ethyl acetate as eluent. In the first 200 ml of eluent the dichloro derivative **3c** was recovered; then in the second amount of eluent (200 ml) the derivative **3b** was recovered; finally, changing the solvent to ethyl acetate/ethanol (1:1) the dimethoxy derivative **3a** was recovered. The three compounds were crystallised from ethyl acetate obtaining **3a** (m.p. 95–96°C, 34% yield), **3b** (m.p. 110–111°C, 22% yield) and **3c** (m.p. 138–139°C, 14% yield).

3a: ^{1}H NMR ($CDCl_3$), δ: 3.28 (s, 6H, CH_2OCH_3), 3.55 (near s, 8H, CH_2), 3.77 (s, 3H, 7-OCH_3), 5.29 (s, 1H, H-3), 6.58–6.95 (m, 2H, H-6, 8), 7.96 (d, 1H, H-5). IR (KBr) ν (cm^{-1}): 1610, 1590, 1550. *Anal.* $C_{16}H_{21}NO_5$: C, H, N.

3b: ^{1}H NMR ($CDCl_3$), δ: 3.30 (s, 3H, CH_2OCH_3), 3.55 (s, 3H, CH_2OCH_3), 3.55 (near s, 4H, NCH_2CH_2O), 3.70 (near s, 4H, NCH_2CH_2Cl), 3.79 (s, 3H, 7-OCH_3), 5.28 (s, 1H, H-3), 6.60–6.98 (m, 2H, H-6, 8), 7.98 (d, 1H, H-5). IR (KBr) ν (cm^{-1}): 1612, 1592, 1555. *Anal.* $C_{15}H_{18}ClNO_4$: C, H, N.

3c: ^{1}H NMR ($CDCl_3$), δ: 3.70–4.10 (m, 11H, CH_2, CH_3), 5.38 (s, 1H, H-3), 6.68–7.07 (m, 2H, H-6, 8), 8.06 (d, 1H, H-5). IR (KBr) ν (cm^{-1}): 1612, 1594, 1555. *Anal.* $C_{14}H_{15}Cl_2NO_3$: C, H, N.

*3.2. 2-bis(2-Methoxyethyl)amino-7-ethoxy-4H-benzopyran-4-one (**3d**) and 2-(2-chloroethyl-2-methoxyethyl)-amino-7-ethoxy-4H-benzopyran-4-one (**3e**)*

Following the procedure described for **3a–c**, but using 6.9 g (50.0 mmol) of 3-ethoxyphenol, a solid was obtained which was chromatographed on a silica gel column with ethyl acetate as eluent. In the first 300 ml of eluent the monochloro derivative **3e** was recovered; then, on changing the solvent to ethyl acetate/ethanol (1:1), the dimethoxy derivative **3d** was recovered. The two compounds were crystallised from ethyl acetate obtaining **3d** (m.p. 125–126°C, 23% yield) and **3e** (m.p. 122–123°C, 18% yield).

3d: ^{1}H NMR ($CDCl_3$), δ: 1.30 (t, 3H, CH_2CH_3), 3.26 (s, 6H, CH_2OCH_3), 3.53 (near s, 8H, CH_2), 3.97 (q, 2H, OCH_2), 5.26 (s, 1H, H-3), 6.56–6.91 (m, 2H, H-6, 8), 7.95 (d, 1H, H-5). IR (KBr) ν (cm^{-1}): 1610, 1590, 1545. *Anal.* $C_{17}H_{23}NO_5$: C, H, N.

3e: ^{1}H NMR ($CDCl_3$), δ: 1.45 (t, 3H, CH_2CH_3), 3.38 (s, 3H, OCH_3), 3.65 (near s, 4H, NCH_2CH_2O), 3.80 (near s, 4H, NCH_2CH_2Cl), 4.15 (q, 2H, OCH_2CH_3), 5.42 (s, 1H, H-3), 6.68–7.10 (m, 2H, H-6, 8), 8.09 (d, 1H, H-5). IR (KBr) ν (cm^{-1}): 1610, 1590, 1550. *Anal.* $C_{16}H_{20}ClNO_4$: C, H, N.

*3.3. 3-bis(2-Hydroxyethyl)amino-7-methoxy-4H-benzopyran-4-one (**4a**) and 3-bis(2-iodoethyl)amino-7-hydroxy-4H-benzopyran-4-one (**4b**)*

A solution containing 1 g of 2-bis(2-methoxyethyl)-amino-7-methoxy-4*H*-benzopyran-4-one (**3a**) in 15 ml of 57% HI was heated at 95°C for 0.5 h. After cooling, the final mixture was treated with a saturated solution of $NaHCO_3$ until neutrality and the obtained precipitate was filtered, washed with water and recrystallised from ethanol; the dihydroxy derivative **4a** was obtained (m.p. 178–179°C, 25% yield). The neutral aqueous solution was acidified with hydrochloric acid (1:1) obtaining a second precipitate, which was crystallised from ethanol, obtaining the diiodo derivative **4b** (m.p. 257–258°C, 18% yield).

4a: ^{1}H NMR (DMSO-d_6), δ: 3.81 (m, 8H, CH_2), 3.97 (s, 3H, OCH_3), 5.68 (broad s, 2H, OH), 6.22 (s, 1H, H-3), 7.06–7.55 (m, 2H, H-6,8), 7.97 (d, 1H, H-5). IR (KBr) ν (cm^{-1}): 3400 (broad), 1610, 1580, 1545. *Anal.* $C_{14}H_{17}NO_5$: C, H, N.

4b: ^{1}H NMR (DMSO-d_6), δ: 3.35–4.10 (m, 8H, CH_2), 5.49 (s, 1H, H-3), 6.60–6.98 (m, 2H, H-6, 8), 7.75 (d, 1H, H-5), 11.30 (s, 1H, OH). IR (KBr) ν (cm^{-1}): 1619, 1600, 1565. *Anal.* $C_{13}H_{13}I_2NO_3$: C, H, N.

*3.4. 2-bis(2-Methoxyethyl)amino-7-methoxy-4-morpholinomethyl-4H-benzopyran-4-one (**5**)*

To 0.82 g (2.68 mmol) of **3a** dissolved in 15 ml of ethanol, 0.80 g (9.2 mmol) of morpholine, 1.28 ml (17.0 mmol) of 40% formaldehyde and 0.26 ml (4.6 mmol) of acetic acid were added. The resulting mixture was refluxed for 24 h. After cooling, the solvent was evaporated under reduced pressure and the obtained pale yellow oil was chromatographed through a silica gel column using ethyl acetate/cyclohexane (2:1) as eluent. After discarding the first 100 ml of eluate, a second fraction of 70 ml was collected. The removal of the solvent left a white solid (0.63 g, 55.7% yield) which, after recrystallisation from ethyl acetate, yielded **5** (m.p. 87–88°C).

^{1}H NMR ($CDCl_3$), δ: 2.37–2.70 (m, 4H, β-morpholine CH_2), 3.39 (s, 6H, CH_2OCH_3), 3.42–3.80 (m, 12H, α-morpholine $CH_2 + NCH_2CH_2OCH_3$), 3.91 (s, 3H, 7-OCH_3), 4.02 (s, 2H, CH_2 bridge), 6.73–7.12 (m, 2H, H-6, 8), 8.13 (d, 1H, H-5). IR (KBr) ν (cm^{-1}): 1615, 1590, 1545. *Anal.* $C_{21}H_{30}N_2O_6$: C, H, N.

*3.5. 3-bis(2-Methoxyethyl)amino-5-(2-hydroxy-4-methoxyphenyl)isoxazole (**6**)*

A total of 1 g of hydroxylamine hydrochloride and 1.5 ml of pyridine were added to a solution of 1.2 g (3.90 mmol) of **3a** in 40 ml of ethanol. The mixture was refluxed for 24 h. The final solution was evaporated under reduced pressure to yield a solid. The latter was dissolved in a little amount of 2 N NaOH, filtering off any impurity or unreacted starting product. The alkaline solution was then acidified with 6 N HCl obtaining a white precipitate, which was filtered and washed with water. Compound **6** was obtained after recrystallisation from ethanol (m.p. 151–152°C, 75.2% yield).

^{1}H NMR ($CDCl_3$), δ: 3.36 (s, 6H, CH_2OCH_3), 3.61 (m, 8H, CH_2), 3.80 (s, 3H, 4′-OCH_3), 6.30 (s, 1H, H-4), 6.40–6.72 (m, 2H, H-3′, 5′), 7.65 (d, 1H, H-6′), 8.00 (broad s, 1H, OH). IR (KBr) ν (cm^{-1}): 2920 (broad), 1620, 1590, 1546. *Anal.* $C_{16}H_{22}N_2O_5$: C, H, N.

*3.6. 3-bis(2-Hydroxyethyl)amino-5-(2-hydroxy-4-methoxyphenyl)isoxazole (**7**)*

A solution of 1 g (3.10 mmol) of 3-bis(2-methoxyethyl)amino-5-(2-hydroxy-4-methoxyphenyl)-isoxazole

(**6**) in 15 ml of 57% HI was heated for 0.5 h at 90°C. After cooling, the final mixture was treated with a saturated solution of $NaHCO_3$ until neutrality and the obtained precipitate was filtered, washed with water and recrystallised from ethanol/ethyl acetate (m.p. 167–168°C, 65.0% yield).

^{1}H NMR ($CDCl_3$), δ: 3.25–3.70 (m, 8H, CH_2), 3.81 (s, 3H, OCH_6), 4.07 (broad s, 2H, CH_2OH), 6.30–6.75 (m, 3H, H-4, H-3′–5′), 7.68 (d, 1H, H-6′), 10.52 (broad s, 1H, OH). IR (KBr) ν (cm^{-1}): 3300 (broad), 1615, 1595, 1535. *Anal.* $C_{14}H_{18}N_2O_5$: C, H, N.

3.7. 3-bis(2-Methoxyethyl)amino-5-(2,4-dimethoxyphenyl)isoxazole ***(8a)***

To 1 g (3.10 mmol) of **6** dissolved in 20 ml of water containing 0.17 g (3.1 mmol) of KOH, 0.39 g (3.1 mmol) of dimethyl sulfate was added dropwise under stirring. The solution was stirred for 2 h at 40°C. The resulting oil was extracted three times with chloroform. The pooled organic extracts were washed with water, dried and evaporated under reduced pressure. The resulting solid was recrystallised from ethyl acetate/cyclohexane (1:1) (m.p. 73–75°C, 84.8% yield).

^{1}H NMR ($CDCl_3$), δ: 3.37 (s, 6H, CH_2OCH_3), 3.60 (m, 8H, CH_2), 3.85 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 6.32 (s, 1H, H-4), 6.43–6.76 (m, 2H, H-3′, 5′), 7.84 (d, 1H, H-6′). IR (KBr) ν (cm^{-1}): 1620, 1580, 1545. *Anal.* $C_{17}H_{24}N_2O_5$: C, H, N.

3.8. 3-bis(2-Methoxyethyl)amino-5-(2-ethoxy-4-methoxyphenyl)isoxazole ***(8b)***

To 1 g (3.10 mmol) of **6** dissolved in 20 ml of water containing 0.17 g (3.1 mmol) of KOH, 0.48 g (3.1 mmol) of diethyl sulfate was added dropwise under stirring. The solution was stirred for 2 h at 75°C. The resulting oil was extracted three times with chloroform. The pooled organic extracts were washed with water, dried and evaporated under reduced pressure. The resulting solid was recrystallised from ethyl acetate/cyclohexane (1:1) (m.p. 54–55°C, 78.6% yield).

^{1}H NMR ($CDCl_3$), δ: 1.43 (t, 3H, CH_2CH_3), 3.30 (s, 6H, CH_2OCH_3), 3.51 (m, 8H, CH_2), 3.77 (s, 3H, 4′-OCH_3), 4.10 (q, 2H, CH_2CH_3), 6.31 (s, 1H, H-4), 6.40–6.68 (m, 2H, H-3′, 5′), 7.80 (d, 1H, H-6′). IR (KBr) ν (cm^{-1}): 1620, 1578, 1546. *Anal.* $C_{18}H_{26}N_2O_5$: C, H, N.

3.9. 3-bis(2-Methoxyethyl)amino-5-(2-n-hexyloxy-4-methoxyphenyl)isoxazole ***(8c)***

To 1.2 g (3.72 mmol) of **6** dissolved in 30 ml of acetone, 0.43 g (7.27 mmol) of 1-bromohexane and 0.27 g of anhydrous potassium carbonate were added with stirring. The mixture was refluxed for 24 h. At the end the solvent was distilled under reduced pressure and the pale yellow oil was stirred with 40 ml of 2 N sodium hydroxide. The solid (**8c**) which separated out was filtered and recrystallised from cyclohexane (m.p. 57–58°C, 86.2% yield).

^{1}H NMR ($CDCl_3$), δ: 0.95 (t, 3H, CH_2CH_3), 1.15–2.12 (m, 8H, $(CH_2)_4CH_3$), 3.37 (s, 6H, CH_2OCH_3), 3.60 (m, 8H, NCH_2CH_2O), 3.85 (s, 3H, 4′-OCH_3), 4.10 (t, 2H, OCH_2), 6.40 (s, 1H, H-4), 6.49–6.78 (m, 2H, H-3′, 5′), 7.90 (d, 1H, H-6′). IR (KBr) ν (cm^{-1}): 1622, 1572, 1535. *Anal.* $C_{22}H_{34}N_2O_5$: C, H, N.

3.10. 3-bis(2-Methoxyethyl)amino-5-(2-m-nitrobenzoyloxy-4-methoxy)isoxazole ***(9a)***

To a solution of 0.60 g (1.86 mmol) of **6** in 4 ml of anhydrous pyridine, 0.4 g (2.28 mmol) of 3-nitrobenzoyl chloride were added and the mixture was heated at 100°C for 7 min under stirring. The final solution was then poured into 15 ml of water and the mixture was stirred for 30 min, obtaining an oil which was extracted many times with chloroform. The pooled organic extracts were washed with water, dried and evaporated under reduced pressure. The resulting solid was crystallised from ethyl acetate/cyclohexane (1:3) obtaining **9a** (m.p. 86–87°C, 88.3% yield).

^{1}H NMR ($CDCl_3$), δ: 3.24 (s, 6H, CH_2OCH_3), 3.42 (m, 8H, CH_2), 3.89 (s, 3H, 4′-OCH_3), 6.07 (s, 1H, H-4), 6.85–9.22 (m, 7H, H arom.). IR (KBr) ν (cm^{-1}): 1745, 1620, 1604. *Anal.* $C_{23}H_{25}N_3O_8$: C, H, N.

3.11. 3-bis(2-Methoxyethyl)amino-5-(2-p-nitrobenzoyloxy-4-methoxy)isoxazole ***(9b)***

Following the procedure described for **9a** but using 0.4 g of *p*-nitrobenzoyl chloride, a solid was obtained which was crystallised from ethyl acetate/cyclohexane (1:3) obtaining **9b** (m.p. 74–75°C, 86.0% yield).

^{1}H NMR ($CDCl_3$), δ: 3.25 (s, 6H, CH_2OCH_3), 3.44 (m, 8H, CH_2), 3.89 (s, 3H, 4′-OCH_3), 6.08 (s, 1H, H-4), 6.85–9.24 (m, 7H, H arom.). IR (KBr) ν (cm^{-1}): 1750, 1623, 1604. *Anal.* $C_{23}H_{25}N_3O_8$: C, H, N.

3.12. 3-bis(2-Methoxyethyl)amino-5-(2-p-chlorobenzoyloxy-4-methoxy)isoxazole ***(9c)***

Following the procedure described for **9a** but using 0.4 g of *p*-chlorobenzoyl chloride, a solid was obtained which was crystallised from ethyl acetate cyclohexane (1:3) obtaining **9c** (m.p. 96–97°C, 86.7% yield).

^{1}H NMR ($CDCl_3$), δ: 3.24 (s, 6H, CH_2OCH_3), 3.40 (m, 8H, CH_2), 3.86 (s, 3H, 4′-OCH_3), 6.02 (s, 1H, H-4), 6.82–8.45 (m, 7H, H arom.). IR (KBr) ν (cm^{-1}): 1746, 1621, 1602. *Anal.* $C_{23}H_{25}ClN_2O_6$: C, H, N.

4. Biology

4.1. Isolation of neutrophils

Freshly collected heparinised human blood (100 ml) from healthy donors was treated with 1.6% dextran (final concentration) and left at 25–28°C to sedimentate for 1 h. The supernatants (40 ml) were collected and layered onto 10 ml of 6% Ficoll 400 solution containing 0.17% (v/v) Urovison and centrifuged at $800 \times g$ for 20 min. The pellets containing mostly neutrophils and contaminating red cells were resuspended in 10 ml of hypotonic 0.2% NaCl. After 30 s, 10 ml of hypertonic 1.6% NaCl were added to normalise the osmotic pressure. This treatment lyses all contaminating red cells. The white cells were recovered and washed three times with 0.01 M sodium phosphate (pH 7.4), 5 mM KCl, 0.12 M NaCl, 24 mM $NaHCO_3$ and 5 mM glucose. Prior to use, the cells were maintained in an ice bath in the same medium at a concentration of $15–20 \times 10^6$ cells/ml. The cell population obtained consisted of more than 96% neutrophils, as evaluated by microscopic examination. The remaining percentages consisted of eosinophils and monocytes.

4.2. Activation of neutrophils and assay of a superoxide anion (O_2^-)

A total of 10^6 cells were diluted in 1 ml of 10 mM HEPES, pH 7.4, containing 0.15 M NaCl, 5.0 mM glucose, 1.0 mM Ca^{2+} and 0.625 mg/ml of Cytochrome C (Fe^{3+}) and incubated at 37°C for 2 min. PMA (100 mg) or f-MLF (0.1 μM final concentration) were then added. The absorbance at 550 nm was continuously monitored for 10 min. The amount of O_2^- produced was calculated by the difference in absorbance at zero time and at the end of the reaction.

4.3. Samples

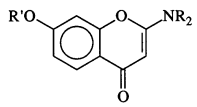
The compounds were diluted in DMSO at standard concentration of 20 mM. When tested on neutrophils, 1 μl of standard solution was added to 1 ml of cell suspension. As a control, 1 μl of DMSO was added to a blank sample.

5. Results and discussion

5.1. Effects of substituted chromones on neutrophil activation

To understand the role of dialkylamino substituents in mono- (isoxazoles) and bicyclic (chromones) compounds better, these molecules were tested for their ability to affect the O_2^- production by human neutrophils stimulated with either PMA or f-MLF.

Table 1
Effect of the chromones **B1–12** on O_2^- production by human neutrophils stimulated with f-MLF or PMA [a]



B1-12

Comp.	NR_2	R′	f-MLF	PMA
B1	NMe_2	H	2	20
B2	NMe_2	CH_3	3	26
B3	NHEt	H	43	5
B4	NEt_2	H	34	4
B5	NEt_2	CH_3	40	48
B6	NEt_2	$CH_2CH_2CH_3$	66	3
B7	NEtAc	CH_3	2	35
B8	NEtAr	CH_3	100	44
B9	1-pyrrolidinyl	H	29	39
B10	1-pyrrolidinyl	CH_3	37	18
B11	1-piperidinyl	H	100	25
B12	1-piperidinyl	CH_3	100	38

[a] Neutrophils (10^6 cells/ml) were stimulated with 100 ng/ml PMA or with 0.1 μmol f-MLF, as described in Section 4, in the presence of the incubated compounds at the conc. of 20 μmol. Data refer to the % inhibition of O_2^- production with respect to controls. The assays were carried out in triplicate. Values are the arithmetical mean of three determinations.

As shown in Table 1, in the absence of substituents, the chromones express very low inhibitory activity on O_2^- production. However, the substitution of methyl residue (**B5**) with propyl residue (**B6**) promotes a significant increase in inhibition of O_2^- production by neutrophils stimulated with f-MLF. In this context, the activity is very high when an aromatic substituent is present on the amino group (**B7**) but unfortunately there is only one such substitution in our series. Furthermore, chromones having a pyrrolidino residue at the NR_2 position (**B9**, **B10**) show good inhibitory efficiency on neutrophils stimulated with both PMA and f-MLF.

The introduction of a piperidino group (**B11**, **B12**) largely increases the inhibitory effect of f-MLF stimulated neutrophils, without affecting PMA-stimulated neutrophils.

Taken together, these findings indicate that an increase in hydrophobicity of the substituents is correlated with the ability of the compounds to inhibit neutrophil activation. In fact, replacement of dimethylamino (**B1**, **B2**) with diethylamino groups (**B4–6**) promotes the appearance of biological activity of the compounds, which becomes maximal when the substitution is carried out with the *N*-arylamino and piperidino groups.

5.2. Effects of substituted isoxazoles on neutrophil activation

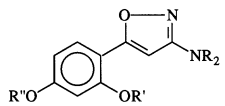
As depicted in Table 2, compound **C2** shows inhibitory activity against both PMA and f-MLF activated neutrophils. However, the presence of a methyl group on the R″ position (**C3**, **C4**) significantly reduces the inhibitory efficiency of the resulting compound on PMA stimulated neutrophils. When R″ is a methyl residue, the activity against PMA is generally low, while the activity against f-MLF is maintained at a good level, particularly when R″ is a large substituent, e.g. in benzoyl derivatives (**C6**, **C9–13**, **C15**, **C18**). When a heterocycle is present in the R″ position (**C16**, **C17**), activity is maintained. Low efficiency against f-MLF stimulated neutrophils has been observed with compounds having charged substituting groups at position R′ (**C11**, **C14**). The presence of a large hydrocarbon tail, such as *n*-octyl (**C19**) and oleyl (**C20**) chains, reduces the biological activity of the compounds. As already shown for the chromones (see Table 1), compounds having a piperidino residue at R″ position, even in the absence of substituents at R′ and in the presence of short alkyl groups at R″ (**C22**, **C23**), show the highest inhibitory activity against PMA and f-MLF activated neutrophils.

5.3. Effects of bis(2-substituted-ethyl)amino chromones and isoxazoles on neutrophil activation

We have previously shown that tricyclic compounds having a bis(2-methoxyethyl)amino group as the substituent were very efficient in preventing neutrophil activation by PMA and f-MLF [17]. In order to establish whether the introduction of this substitution in mono and bicyclic compounds could promote the appearance of biological activity against human neutrophils, we synthesised some new derivatives.

As depicted in Table 3, in contrast with those previously tested, it seems possible with these compounds to discriminate the activation by PMA from that of f-MLF. Since the two stimuli utilise different signal transduction pathways [20], including the step involved in the membrane crossing, it can be postulated that changes in substituent groups might affect the biological specificity of the compounds. In fact, in the chromone series, while the more lypophilic compounds **3a–d** are inactive, compounds **4a** and **4b** carrying one

Table 2
Effect of the isoxazoles **C1–23** on O_2^- production by human neutrophils stimulated with f-MLF or PMA [a]

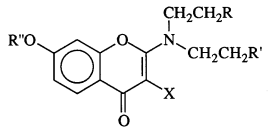


C1-23

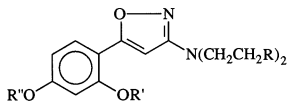
Comp.	NR_2	R′	R″	f-MLF	PMA
C1	NMe_2	$COCH_3$	$COCH_3$	ND	75
C2	NEt_2	H	H	100	80
C3	NEt_2	$CH_2CH{=}CH_2$	CH_3	70	38
C4	NEt_2	$CH_2C{\equiv}CH$	CH_3	89	21
C5	NEt_2	benzyl	CH_3	ND	42
C6	NEt_2	benzoyl	CH_3	90	31
C7	NEt_2	phenoxyacetyl	CH_3	45	10
C8	NEt_2	*p*-chlorobenzoyl	CH_3	ND	51
C9	NEt_2	*m*-chlorobenzoyl	CH_3	86	49
C10	NEt_2	*p*-nitrobenzoyl	CH_3	100	66
C11	NEt_2	*p*-aminobenzoyl	CH_3	16	2
C12	NEt_2	*p*-acetamidobenzoyl	CH_3	88	13
C13	NEt_2	*m*-nitrobenzoyl	CH_3	95	80
C14	NEt_2	*m*-aminobenzoyl	CH_3	47	35
C15	NEt_2	*m*-acetamidobenzoyl	CH_3	100	16
C16	NEt_2	2-furoyl	CH_3	100	77
C17	NEt_2	2-thiophenecarbonyl	CH_3	95	66
C18	NEt_2	3,4,5-trimethoxybenzoyl	CH_3	84	30
C19	NEt_2	*n*-octyl	CH_3	ND	62
C20	NEt_2	oleoyl	CH_3	ND	70
C21	1-pyrrolidinyl	H	CH_3	71	100
C22	1-piperidinyl	H	CH_3	82	95
C23	1-piperidinyl	H	C_2H_5	100	100

[a] See Table 1; ND, not determined.

Table 3
Effect of the chromones **3–5** and isoxazoles **6–9** on O_2^- production by human neutrophils stimulated with f-MLF or PMA [a]



3-5



6-9

Comp.	R	R′	R″	X	f-MLF	PMA
3a	OCH_3	OCH_3	CH_3	H	22	4
3b	OCH_3	Cl	CH_3	H	6	7
3c	Cl	Cl	CH_3	H	71	3
3d	OCH_3	OCH_3	C_2H_5	H	18	28
4a	OH	OH	CH_3	H	100	3
4b	I	I	H	H	100	2
5	OCH_3	OCH_3	CH_3	morpholinomethyl	25	12
6	OCH_3	H	CH_3		45	90
7	OH	H	CH_3		30	91
8a	OCH_3	CH_3	CH_3		12	75
8b	OCH_3	CH_2CH_3	CH_3		9	53
8c	OCH_3	$(CH_2)_5CH_3$	CH_3		18	100
9a	OCH_3	*m*-nitrobenzoyl	CH_3		45	92
9b	OCH_3	*p*-nitrobenzoyl	CH_3		14	32
9c	OCH_3	*p*-chlorobenzoyl	CH_3		23	35

[a] See Table 1.

and two hydroxy groups, respectively, are very effective against f-MLF stimulated neutrophils. On the other hand, in the isoxazoles series, compounds **6**–**8** are more active in PMA stimulated neutrophils. The maximum of inhibitory efficiency is achieved when R′ is a *n*-hexyl substituent (**8c**). As in previous **C** isoxazoles, the introduction of benzoyl groups (**9a–c**) does not produce a positive effect against the f-MLF stimulus; however, the resulting compounds show some inhibitory activities against the PMA stimulus (especially for **9a**).

6. Conclusions

A comparison of the results obtained with the compounds tested in this paper (compare Tables 1–3) shows that the introduction of a piperidino group in all compounds always leads to derivatives with high biological activity against stimulated neutrophils. On the contrary, substitution with the bis(2-methoxyethyl)amino group produces derivatives almost completely lacking this biological activity.

Taken together, these data indicate that the piperidino group improves the inhibitory efficiency of the chromones and isoxazoles tested in this paper, as well as the naphtho[2,1-*b*]pyrans previously reported [17].

In conclusion, it can be suggested that piperidino substituted derivatives, both in the chromone and isoxazole series, might be the base for the development of drugs controlling inflammatory diseases.

Acknowledgements

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