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## New anti-inflammatory *N*-pyridinyl(alkyl)phthalimides acting as tumour necrosis factor- $\alpha$ production inhibitors

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**Abstract** – This paper describes the synthesis of *N*-pyridinyl(alkyl)phthalimides related to *N*-phenyl-4,5,6,7-tetrafluorophthalimides known to be inhibitors of tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) production. Pharmacomodulation at the phthalimide nitrogen led to the selection of two pharmacophoric fragments (2,4-lutidinyl and  $\beta$ -picolyl), allowing significant inhibition of TNF $\alpha$  production (compounds **12** and **17**). Variation of the substituents linked to the homocycle of their phthalimide scaffold indicated that high (TNF $\alpha$  production) inhibitory potency could be achieved, notably by 5-fluoro, 4- or 5-nitro, 5-amino and especially tetrafluoro substitution. The most active compound, *N*-(pyridin-3-ylmethyl)-4,5,6,7-tetrafluorophthalimide (**32**) (84% inhibition at 10  $\mu$ M), also produced an anti-oedematous effect in the PMA-induced mouse-ear swelling test. Although less active than dexamethasone, it exerted a marked reduction in ear thickness after oral administration (63% vs. 85% for dexamethasone at 0.2 mM kg<sup>-1</sup>) and remained efficient after topical application (46% vs. 96% for the dexamethasone). It also induced potent inhibition in the rat carrageenan foot oedema test with an ID<sub>50</sub> (0.14  $\mu$ M kg<sup>-1</sup>) comparable with that of *N*-(2,6-diisopropylphenyl)phthalimide (**4**) (0.15  $\mu$ M kg<sup>-1</sup>). © 2001 Éditions scientifiques et médicales Elsevier SAS

*N*-pyridinylphthalimides / non-acidic NSAIDs / TNF $\alpha$  inhibitors / PMA oedema / carrageenan-induced oedema

### 1. Introduction

Tumour necrosis factor- $\alpha$  (TNF $\alpha$ ), a protein secreted by monocytes/macrophages in response to many inflammatory stimuli, was initially detected because of its ability to induce hemorrhagic necrosis in transplanted solid tumours [1]. TNF $\alpha$  is now generally regarded as a cytokine that displays pleiotropic biological effects, such as anti-tumour activity, and that can also stimulate the immune system and generate pro-inflammatory activity. As a pro-inflammatory agent, TNF $\alpha$  elicits the production of other cytokines (interleukins IL-1, IL-6 and IL-8) and upregulates surface adhesion ligands on neutrophils and monocytes [2]. Excessive production of TNF $\alpha$  has also been

implicated in inflammatory processes and the pathogenesis of various human disorders such as cachexia (cancer, infection) [2], septic shock [3], multiple sclerosis [3], rheumatoid arthritis [4], brain injury and inflammation following stroke [5], psoriatic skin [6] or inflammatory bowel disease (Crohn's disease, ulcerative colitis) [7]. Drugs inhibiting the production or maturation of TNF $\alpha$  may have excellent therapeutic potential, constituting an interesting alternative to classical cyclooxygenase inhibitors in these pathologies.

In this context, thalidomide (**1**, *figure 1*), which was used as a hypnotic/sedative agent but withdrawn from the market because of its teratogenicity, could represent a lead compound. In fact, the effect of this drug on TNF $\alpha$  production is cell-type specific, i.e. thalidomide enhances TNF $\alpha$  production in phorbol myristate acetate-stimulated HL-60 cells [8], but inhibits

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TNF $\alpha$  production in lipopolysaccharide-stimulated human monocytes [9]. On the basis of these findings, a few groups have investigated the possible structural modifications of thalidomide in order to create more potent inhibitors of TNF $\alpha$  production. An increase in potency of about 100-fold was obtained by the replacement of the glutarimide moiety of thalidomide with the methyl 3-(3,4-dimethoxyphenyl)propionate synthon or the 2,6-diisopropylphenyl moiety, in conjunction with fluorine or amino substitution in the homocycle of thalidomide or other *N*-aryl-phthalimides (**2–4**) [10, 11]. Fluorine substitution might also prevent the formation of an arene oxide metabolite of thalidomide, thought to be responsible for the teratogenic effect of this drug [10].

We had described earlier the synthesis of structurally related compounds, *N*-(4,6-dimethylpyridin-2-yl) heteroarylcarboxamides, and evaluated their inhibitory activity on the production of TNF $\alpha$  by zymosan or lipopolysaccharide-activated macrophages [12]. Furan-2-carboxamide (**5**) was found to inhibit TNF $\alpha$  production at an IC<sub>50</sub> (ca. 70  $\mu$ M) lower than that of thalidomide (ca. 200  $\mu$ M). Cytokine-suppressing anti-inflammatory drugs (CSAIDs), typified by bicyclic imidazole (**6**) [13], are another class of compounds with high TNF $\alpha$  inhibitory activity. Like the most potent compounds in this series, imidazole is substituted by 4-pyridinyl and 4-fluorophenyl moieties.

These findings led us to synthesize the closely related *N*-azaaryl(alkyl)phthalimides incorporating amino(alkyl)pyridines and especially 6-amino-2,4-lutidine [14], which are known to enhance anti-inflammatory activity appreciably in this series [15].

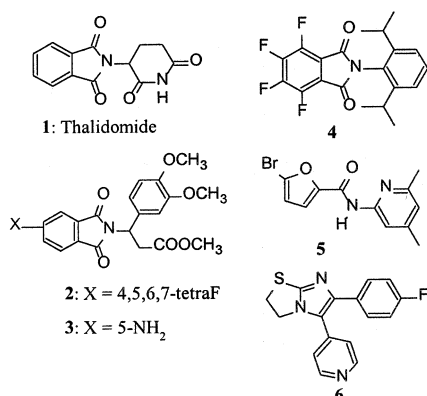


Figure 1. Inhibitors of TNF $\alpha$  production.

This paper describes the synthesis of a new series of *N*-pyridinyl(alkyl)phthalimides (**7–32**) and the evaluation of their *in vitro* activity in the inhibition of TNF $\alpha$  (tables I and II); a first approach to the structure–activity relationships will be presented. Nine phthalimides exhibiting an inhibition percentage of more than 30% at 10  $\mu$ M were evaluated as inflammation inhibitors after oral administration (table III) or topical application (for the most efficient compound, table IV). The five most potent compounds were studied for their systemic anti-inflammatory activity in the carrageenan-induced rat-paw oedema test (table V).

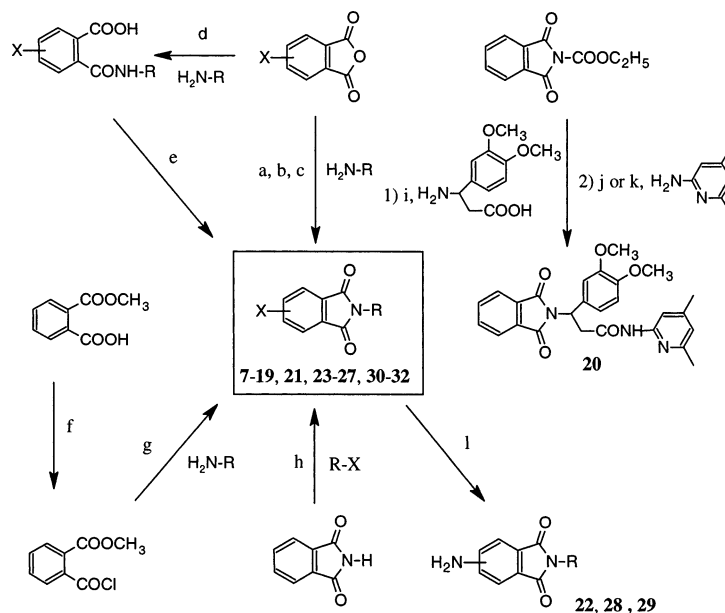
## 2. Chemistry

The preparation of target *N*-pyridinyl(alkyl)phthalimides is shown in figure 2. Classical condensation of amino(alkyl)pyridines with phthalic anhydrides in refluxing acetic acid (method a) afforded these imides in satisfactory yields that could sometimes be increased by adding acetic anhydride (method b) or by heating the mixture at 160–200 °C in the absence of solvent (method c). Alternative methods consisted of cyclizing the intermediate phthalamic acids (methods d and e) or the corresponding phthalamic ester (methods f and g). Attempted arylation in the presence of K<sub>2</sub>CO<sub>3</sub>–DMF (method h) gave poor results (18%) that could not be improved by operating under microwave. The amidic function in compound **20** was created after the activation of the corresponding acid by 1,1-carbonyldiimidazole (method j) or phenyl dichlorophosphate (method k). Physicochemical data for target compounds **7–32** are summarised in tables I and II.

## 3. Results and discussion

### 3.1. *In vitro* inhibition of TNF $\alpha$ production

As the azaaryl ring (present in compounds **5** and **6**) appears to be important for optimum potency against LPS-induced TNF $\alpha$  production by murine macrophages [16], we evaluated the influence of: (i) the variation of the position of the nitrogen atom; (ii) the number and position of methyl or bromine substituents; and (iii) the existence and length variation of an alkyl chain binding the *N*-azaaryl and phthalimide rings (table I). In  $\alpha$ -

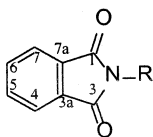


**Figure 2.** Synthetic procedure to compounds 7–32. Reagents and conditions: (a) AcOH, reflux; (b) AcOH, Ac<sub>2</sub>O, reflux; (c) 160–200 °C; (d) THF; (e) Dean Stark, toluene, reflux; (f) SOCl<sub>2</sub>, reflux; (g) TEA, THF, reflux; (h) (1) K<sub>2</sub>CO<sub>3</sub>, DMF, (2) R–X, 180 °C; (i) Na<sub>2</sub>CO<sub>3</sub>, H<sub>2</sub>O; (j) 1,1'-carbonyldiimidazole, THF; (k) phenyl dichlorophosphate, TEA, 1,2-dichloroethane; (l) H<sub>2</sub>, Pd/C, EtOH.

pyridinyl derivatives, unsubstituted *N*-pyridinylphthalimide (**7**) lacked significant inhibition (less than 10%) of TNF $\alpha$  production. The presence of a methyl substituent in position 3, 4, 5 or 6 (**8**–**11**) induced no increase in activity. The most potent compound in this series was obtained by the introduction at nitrogen of a 4,6-dimethylpyridin-2-yl moiety corresponding to our lead pharmacophore (**12**: 37% inhibition at 10  $\mu$ M) described earlier [15]. Introduction of one (**13**) or two (**14**) bromine atoms at positions 3 and 5 of **12** resulted in a total loss of activity. Displacement of the nitrogen atom to the  $\beta$  position (**16**) failed to enhance TNF $\alpha$  inhibition, whereas activity was clearly increased by the introduction of a methylene group between pyridine and phthalimide, leading to **17** (34% inhibition at 10  $\mu$ M). This phenomenon was not observed when the same spacer was introduced into the  $\alpha$  sub-series (**15** was inactive at the same concentration). Further elongation of the alkyl chain in this sub-series allowed a moderate increase in activity (20% inhibition with **19**). Although amide **20** incorporated the 3-(3,4-dimethoxyphenyl)-propionyl moiety present in leads **2** and **3**, it exerted very poor inhibition (<10%).

Once it was determined that 2,4-lutidinyl and  $\beta$ -picolyl moieties were the most potent of the different

pyridyl(alkyl) substituents introduced at the phthalimido nitrogen, we investigated the effect of substituents introduced at the phthalimide moiety of compounds **12** and **15** (table II). In the two sub-series (2,4-lutidinyl and  $\beta$ -picolyl series), higher activity was observed with 5-fluoro derivatives (**24**: 40% inhibition, and **31**: 41% inhibition) than with their 4-fluoro analogues (**23** and **30**: <10%). However, the introduction of the electron-withdrawing nitro group into the  $\beta$ -picolyl series exerted a positive effect when fixed at position 4 (**26**: 72% inhibition) or 5 (**27**: 73% inhibition), yielding activity equivalent to that of the reference compound (**4**: 73% inhibition). Contrary to the results obtained by Muller et al. [11], the introduction of an electron-donating group, such as an amino group, at position 4 in the 2,4-lutidinyl series (**22**) definitely decreased the TNF $\alpha$  inhibitory activity (less than 10%). Nevertheless, the corresponding 5-substituted analogue in the  $\beta$ -picolyl series (**29**) was more potent, showing moderately lower activity than the 5-nitro analogue (**27**: 62% vs. 73%). As reported earlier [10], TNF $\alpha$  inhibitory activity was increased more markedly by tetrafluorination of the phthalimide moiety (**25**: 50% inhibition, and **32**: 84% inhibition). Phthalimide **32** was the most potent compound of the series studied, showing slightly higher

**Table I.** Physical properties and TNF $\alpha$  inhibitory activity of *N*-pyridinyl-(alkyl)phthalimides **7–19**.

Compound	R	Formula $M_r$	Method Yield (%)	Melting point (°C) Ethanol	TNF $\alpha$ % inhibition <sup>a</sup> at 10 $\mu$ M
7		C <sub>13</sub> H <sub>8</sub> N <sub>2</sub> O <sub>2</sub> 224.22	A: 65 B: 83 D, E: 90	232	< 10
8		C <sub>14</sub> H <sub>10</sub> N <sub>2</sub> O <sub>2</sub> 238.25	A : 77	175	< 10
9		C <sub>14</sub> H <sub>10</sub> N <sub>2</sub> O <sub>2</sub> 238.25	A: 74 D, E: 70 F, G: 42	176	< 10
10		C <sub>14</sub> H <sub>10</sub> N <sub>2</sub> O <sub>2</sub> 238.25	A: 70	173	< 10
11		C <sub>14</sub> H <sub>10</sub> N <sub>2</sub> O <sub>2</sub> 238.25	A: 65	194	< 10
12		C <sub>15</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub> 252.27	A: 65 D, E: 80 H: 18	192	37 $\pm$ 10
13		C <sub>15</sub> H <sub>11</sub> BrN <sub>2</sub> O <sub>2</sub> 331.18	A: 44	122	< 10
14		C <sub>15</sub> H <sub>10</sub> Br <sub>2</sub> N <sub>2</sub> O <sub>2</sub> 410.08	A: 40	207	< 10
15		C <sub>14</sub> H <sub>10</sub> N <sub>2</sub> O <sub>2</sub> 238.25	C: 70 F, G: 81	124	< 10
16		C <sub>13</sub> H <sub>8</sub> N <sub>2</sub> O <sub>2</sub> 224.22	A: 62	177	< 10
17		C <sub>14</sub> H <sub>10</sub> N <sub>2</sub> O <sub>2</sub> 238.25	B: 89 C: 82	156	34 $\pm$ 5
18		C <sub>14</sub> H <sub>10</sub> N <sub>2</sub> O <sub>2</sub> 238.25	B: 91 C: 80	166	< 10
19		C <sub>15</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub> 252.27	C: 84	98	20 $\pm$ 6

<sup>a</sup>Mean of three individual experiments performed in triplicate ( $\pm$  SEM).

**Table II.** Physical properties and TNF $\alpha$  inhibitory activity of *N*-pyridinyl (alkyl)phthalimides **20** and **22–32**.

Compound	X	R	Formula $M_r$	Method Yield (%)	Melting point (°C) solvent	TNF $\alpha$ % inhibition <sup>a</sup> at 10 $\mu$ M
<b>20</b>	H		C <sub>26</sub> H <sub>25</sub> N <sub>3</sub> O <sub>3</sub> 459.50	J: 61 K: 73	157 <sup>c</sup>	< 10
<b>22</b>	4-NH <sub>2</sub>		C <sub>15</sub> H <sub>13</sub> N <sub>2</sub> O <sub>2</sub> 267.29	L: 72	200 <sup>b</sup>	< 10
<b>23</b>	4-F	"	C <sub>15</sub> H <sub>11</sub> FN <sub>2</sub> O <sub>2</sub> 270.26	A: 64	134 <sup>b</sup>	< 10
<b>24</b>	5-F	"	C <sub>15</sub> H <sub>11</sub> FN <sub>2</sub> O <sub>2</sub> 270.26	A: 43	135 <sup>b</sup>	40 $\pm$ 7
<b>25</b>	4, 5, 6, 7 -tetraF	"	C <sub>15</sub> H <sub>8</sub> F <sub>4</sub> N <sub>2</sub> O <sub>2</sub> 324.24	B: 67	117 <sup>b</sup>	50 $\pm$ 8
<b>26</b>	4-NO <sub>2</sub>		C <sub>14</sub> H <sub>9</sub> N <sub>3</sub> O <sub>4</sub> 283.24	A: 85	102 <sup>b</sup>	72 $\pm$ 8
<b>27</b>	5-NO <sub>2</sub>	"	C <sub>14</sub> H <sub>9</sub> N <sub>3</sub> O <sub>4</sub> 283.24	A: 92	133 <sup>b</sup>	73 $\pm$ 2
<b>28</b>	4-NH <sub>2</sub>	"	C <sub>14</sub> H <sub>11</sub> N <sub>3</sub> O <sub>2</sub> 253.26	L: 77	186 <sup>c</sup>	NS <sup>d</sup>
<b>29</b>	5-NH <sub>2</sub>	"	C <sub>14</sub> H <sub>11</sub> N <sub>3</sub> O <sub>2</sub> 253.26	L: 70	168 <sup>c</sup>	62 $\pm$ 5
<b>30</b>	4-F	"	C <sub>14</sub> H <sub>9</sub> FN <sub>2</sub> O <sub>2</sub> 256.25	A: 80	124 <sup>b</sup>	< 10
<b>31</b>	5-F	"	C <sub>14</sub> H <sub>9</sub> FN <sub>2</sub> O <sub>2</sub> 256.25	A: 61	157 <sup>b</sup>	41 $\pm$ 3
<b>32</b>	4, 5, 6, 7 -tetraF	"	C <sub>14</sub> H <sub>6</sub> F <sub>4</sub> N <sub>2</sub> O <sub>2</sub> 310.21	A: 56 C: 72	128 <sup>b</sup>	84 $\pm$ 12
<b>4<sup>e</sup></b>						73 $\pm$ 4

<sup>a</sup> Mean of three individual experiments performed in triplicate ( $\pm$  SEM).<sup>b</sup> Ethanol.<sup>c</sup> Diisopropyl ether.<sup>d</sup> NS: no solubility.<sup>e</sup> See reference (10).

inhibition of  $\text{TNF}\alpha$  production than lead compound **4** [11] (84 and 73% inhibition, respectively).

### 3.2. *In vivo pharmacological studies*

Psoriatic skin shares many of the pathologic features of phorbol ester-treated mouse skin, including elevated levels of arachidonic acid metabolism products, inflammatory cells and cell proliferation. The nine most potent  $\text{TNF}\alpha$  inhibitors (% inhibition: >30% at 10  $\mu\text{M}$ ) and

**Table III.** Inhibition of phorbol myristate acetate-induced oedema in mice (orally administered drugs).

Compound	Mouse-ear oedema (% inhibition)	
	0.2 mM kg <sup>-1</sup>	0.4 mM kg <sup>-1</sup>
<b>12</b>	55 ± 4	69 ± 8
<b>17</b>	58 ± 6	65 ± 4
<b>24</b>	52 ± 3	56 ± 5
<b>25</b>	55 ± 5	70 ± 3
<b>26</b>	54 ± 8	83 ± 1
<b>27</b>	41 ± 5	66 ± 5
<b>28</b>	47 ± 4	80 ± 2
<b>29</b>	76 ± 2	94 ± 3
<b>31</b>	53 ± 5	59 ± 4
<b>32</b>	63 ± 3	95 ± 2
<b>4</b>	61 ± 7	79 ± 4
Ibuprofen	56 ± 4	86 ± 6
Dexamethasone	85 ± 2	100

**Table IV.** Inhibition of phorbol myristate acetate-induced oedema in mice (topically applied drugs).

Compound	Mouse-ear oedema (% inhibition)	
	2 × 100 $\mu\text{g}$	2 × 500 $\mu\text{g}$
<b>32</b>	46 ± 5	79 ± 1
Dexamethasone	96 ± 2	100

**Table V.** Inhibition of carrageenan-induced oedema in rats (orally administered drugs).

Compound	Rat-paw oedema ID <sub>50</sub> (mM kg <sup>-1</sup> )
<b>25</b>	0.18 ± 0.06
<b>26</b>	0.21 ± 0.06
<b>28</b>	0.47 ± 0.04
<b>29</b>	0.20 ± 0.05
<b>32</b>	0.14 ± 0.04
<b>4</b>	0.15 ± 0.05
Ibuprofen	0.20 ± 0.01
Dexamethasone	0.023 ± 0.003

5-aminophthalimide **28** (which could not be tested in the *in vitro* assay because of its very low solubility in the solvent system used) were evaluated in the acute phorbol myristyl acetate (PMA)-induced mouse-ear oedema model of topical inflammation [17]. The activity of these inhibitors was compared with that of phthalimide **4** and reference drugs (dexamethasone and ibuprofen). Although ibuprofen induced a marked increase of  $\text{TNF}\alpha$  production, it was selected, as a classical arylalkanoic NSAID for comparison with the  $\text{TNF}\alpha$  inhibitors studied (*table III*). Although the correlation between inhibition percentages after oral administration at 0.2 and 0.4 mM kg<sup>-1</sup> was not quite satisfactory, all the compounds tested exhibited significant ear oedema reduction at a lower concentration. Determination of the ID<sub>50</sub> of the two most potent compounds (**29** and **32**) and comparison with the reference compound **4** and ibuprofen brought to the fore that they were essentially equiactive: 0.12 ± 0.03, 0.14 ± 0.02, 0.13 ± 0.01 and 0.18 ± 0.05 mM kg<sup>-1</sup>). *N*- $\beta$ -Picolyltetrafluorophthalimide (**32**) also proved to be highly efficient in mouse-ear thickness reduction after topical application of 2 × 500  $\mu\text{g}$  (79% inhibition). Although less potent than dexamethasone, it remained significantly active at 2 × 100  $\mu\text{g}$  (46% vs. 96%) (*table IV*).

The five most active phthalimides in the mouse-ear swelling test (**25**, **26**, **28**, **29** and **32**) were selected, together with the reference drugs (**4**, ibuprofen and dexamethasone), for the determination of their inhibitory effect on systemic inflammation. Evaluation of their ID<sub>50</sub> in the carrageenan-induced rat-paw oedema model [18] after oral administration (*table V*) confirmed that compounds **25**, **26** and **29** possess an activity level comparable with that of ibuprofen (ID<sub>50</sub> = 0.20 mM kg<sup>-1</sup>). Phthalimide **32** was equipotent to reference compound **4** (0.14 vs. 0.15 mM kg<sup>-1</sup>).

Finally, the acute toxicity of the new lead compound **32** was evaluated in male rats, showing an LD<sub>50</sub> of 1.05 ± 0.27 mM kg<sup>-1</sup> after oral administration. This indicated that its therapeutic coefficient, compared to that of ibuprofen [19], remained moderate (8 vs. 25).

## 4. Conclusion

Overexpression of inflammatory cytokines, particularly  $\text{TNF}\alpha$  and IL-1, appears to play a key role in the progression of inflammatory disease. In accordance with its inhibitory activity *in vitro* on  $\text{TNF}\alpha$  production, **32** potently inhibited MPA-induced

mouse-ear swelling and carrageenan rat-paw oedema. This tetrafluorophthalimide is now being evaluated in our laboratory in a mouse model of multiple PMA-induced chronic inflammation [20] and a rat model of trinitrobenzenesulfonic acid (TNBS)-induced colitis [21], which are considered to be relevant for human psoriasis and Crohn's disease, respectively. The objective of the ongoing pharmacomodulation studies in this series is to identify new low-molecular-weight TNF $\alpha$  inhibitors with enhanced efficacy and better tolerability. As the incorporation of amino acid residues into 2-amino-4,6-dimethylpyridine has produced potent topical inflammation inhibitors [22], studies are now in progress to incorporate such moieties into our compounds.

## 5. Experimental protocols

### 5.1. Chemistry

Melting points (m.p. (dec.)) determined on a Tottoli–Büchi apparatus were uncorrected. The structures of the products described were confirmed by IR,  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR and microanalytical data. IR spectra were run with KBr pellets on a Beckman IR 4230 IR spectrophotometer.  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra were recorded on a Bruker AC 250 spectrometer (250 and 62 MHz), using  $\text{CDCl}_3$  or  $\text{Me}_2\text{SO}-d_6$  as the solvent. Chemical shifts ( $\delta$ ) are reported in parts per million (ppm) downfield from tetramethylsilane ( $\text{Me}_4\text{Si}$ , 0.00 ppm) as the internal standard, and coupling constants in hertz. Microanalyses were performed on a Perkin–Elmer CHN 240 apparatus. Analyses indicated by the symbols of the elements were within  $\pm 0.4\%$  of the theoretical values. Analytical thin-layer chromatography was performed on pre-coated silica-gel aluminium plates (0.2 mm, GF254, E Merck). Spots were located by UV illumination. Evaporations were performed in vacuo (rotary evaporator). Sodium sulphate or phosphorus pentoxide was used as the drying agent. Most crude products were passed through short silica gel columns (silica gel 60, 70–230 mesh, E Merck), using an appropriate mixture of dichloromethane and ethanol.

Commercially available solvents and chemicals were used for syntheses, with the exception of the following polyfunctional amines that were prepared using methods reported in the literature.

#### 5.1.1. Synthesis of intermediary amines

##### 5.1.1.1. 6-Amino-3-bromo-2,4-lutidine

Monobromination of 6-amino-2,4-lutidine, according to Fox et al. [23] led to the 3-bromo derivative. M.p. (dec.): 143 °C; lit. value [23]: 145–146 °C;  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ ):  $\delta$  2.28 (s, 3H, 4- $\text{CH}_3$ ), 2.50 (s, 3H, 6- $\text{CH}_3$ ), 4.32 (s, 2H,  $\text{NH}_2$ ), 6.25 (s, 1H, Pyr H $^3$ ).

##### 5.1.1.2. 6-Amino-3,5-dibromo-2,4-lutidine

Dibromination of 6-amino-2,4-lutidine was carried out using the same protocol [23], with the addition of 2 equiv. of bromine (instead of 1). M.p. (dec.): 135 °C; lit. value [24]: 136–137 °C;  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ ):  $\delta$  2.49 (s, 3H, 4- $\text{CH}_3$ ), 2.53 (s, 3H, 6- $\text{CH}_3$ ), 4.92 (s, 2H,  $\text{NH}_2$ ).

##### 5.1.1.3. 3-Amino-3-(3,4-dimethoxyphenyl)propionic acid

This  $\beta$ -amino- $\beta$ -aryl acid was prepared by the treatment of 3,4-dimethoxybenzaldehyde with ammonium acetate and malonic acid in refluxing ethanol [25]. M.p. (dec.): 203 °C.  $^1\text{H}$ -NMR ( $\text{D}_2\text{O}$ ):  $\delta$  2.76–2.84 (m, 2H,  $\text{CH}_2$ ), 3.79 (s, 3H,  $\text{CH}_3\text{O}$ ), 3.81 (s, 3H,  $\text{CH}_3\text{O}$ ), 4.55 (m, 1H, CH), 6.90–7.02 (m, 3H, Ar-H).

#### 5.1.2. Synthesis of phthalimides

The following experimental protocols illustrate the general procedures used to synthesise all the compounds.

##### 5.1.2.1. Method A: 2-(4,6-dimethylpyridin-2-yl)-4-nitro-1H-isoindole-1,3-dione (**21**)

A solution of 2-amino-4,6-dimethylpyridine (1.83 g, 15 mmol) and 3-nitrophthalic anhydride (2.88 g, 15 mmol) in glacial acetic acid (15 mL) was heated under reflux for 24 h. After the evaporation of the reaction mixture to dryness under reduced pressure, the residue was neutralised by a solution of sodium hydrogenocarbonate (4%) until effervescence ceased. The precipitate obtained was washed with water, dried ( $\text{P}_2\text{O}_5$ ) and recrystallised from diisopropyl ether to produce **21** (3.1 g, 70%). M.p. (dec.): 184 °C. IR (KBr,  $\text{cm}^{-1}$ ):  $\nu_s(\text{C=O imide})$  1780,  $\nu_{as}(\text{C=O imide})$  1742,  $\nu_{as}(\text{NO}_2)$  1532,  $\nu_s(\text{NO}_2)$  1372.  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ ):  $\delta$  2.40 (s, 3H, 4- $\text{CH}_3$ ), 2.56 (s, 3H, 6- $\text{CH}_3$ ), 7.03 (s, 1H, Pyr H $^5$ ), 7.10 (s, 1H, Pyr H $^3$ ), 7.98 (dd, 1H, H $^6$ ,  $J_{\text{H}^6\text{H}^5} = 8.1$  Hz,  $J_{\text{H}^6\text{H}^7} = 8.0$  Hz), 8.16 (dd, 1H, H $^7$ ,  $J_{\text{H}^7\text{H}^6} = 8.0$  Hz,  $J_{\text{H}^7\text{H}^5} = 1.1$  Hz), 8.22 (dd, 1H, H $^5$ ,  $J_{\text{H}^5\text{H}^6} = 8.1$  Hz,  $J_{\text{H}^5\text{H}^7} = 1.1$  Hz). Anal. ( $\text{C}_{15}\text{H}_{11}\text{N}_3\text{O}_4$ ): C, H, N.



### 5.1.2.2. Method B:

#### 2-(pyridin-4-ylmethyl)-1H-isoindole-1,3-dione (**18**)

A mixture of 4-aminomethylpyridine (2.16 g, 20 mmol) and phthalic anhydride (2.96 g, 20 mmol) in glacial acetic acid (20 mL) was refluxed for 3 h. The solvent was removed under reduced pressure until the volume reached ca. 6 mL. After addition of 7 mL of acetic anhydride, the solution was refluxed again for 15 h. The solvent was removed under reduced pressure. The residue was neutralised by a solution of sodium hydrogenocarbonate (4%) until effervescence ceased. The precipitate obtained was washed with water, dried ( $P_2O_5$ ) and recrystallised from ethanol 95% to give **18** (4.3 g, 91%). M.p. (dec.): 166 °C. IR (KBr,  $cm^{-1}$ ):  $\nu_s(C=O \text{ imide})$  1740,  $\nu_{as}(C=O \text{ imide})$  1720.  $^1H$ -NMR ( $CDCl_3$ ):  $\delta$  5.02 (s, 1H, N-CH<sub>2</sub>), 7.51 (d, 2H, Pyr H<sup>3</sup>, Pyr H<sup>5</sup>,  $J_{H^3H^2} = J_{H^5H^6} = 5$  Hz), 8.01 (m, 4H, Ar-H), 8.55 (d, 2H, Pyr H<sup>2</sup>, Pyr H<sup>6</sup>,  $J_{H^2H^3} = J_{H^6H^5} = 5$  Hz).  $^{13}C$ -NMR ( $DMSO-d_6$ ):  $\delta$  40.24 (N-CH<sub>2</sub>), 122.38 (C<sup>4</sup>, C<sup>7</sup>), 123.66 (Pyr C<sup>3</sup>, C<sup>5</sup>), 131.95 (C<sup>3a</sup>, C<sup>7a</sup>), 134.95 (C<sup>5</sup>, C<sup>6</sup>), 145.75 (Pyr C<sup>4</sup>), 150.15 (Pyr C<sup>2</sup>, C<sup>6</sup>), 168.00 (C<sup>1</sup> and C<sup>3</sup>). Anal. (C<sub>14</sub>H<sub>10</sub>N<sub>2</sub>O<sub>2</sub>): C, H, N.

### 5.1.2.3. Method C:

#### 2-(pyridin-4-ylmethyl)-1H-isoindole-1,3-dione (**18**)

A mixture of 4-aminomethylpyridine (1.08 g, 10 mmol) and phthalic anhydride (1.48 g, 10 mmol) was heated to 180 °C for 2 h. The oil was cooled and recrystallised from ethanol 95%; 1.9 g of pure product **18** was obtained: Yield: 80%; m.p. (dec.): 165 °C.

### 5.1.2.4. Methods D and E:

#### 2-(4-methylpyridin-2-yl)-1H-isoindole-1,3-dione (**9**)

2-Amino-4-methylpyridine (1.08 g, 10 mmol) and phthalic anhydride (1.48 g, 10 mmol) were dissolved in 20 mL of dry THF. The reaction mixture was stirred at room temperature (r.t.) for 4 h and cooled. The suspension was then filtered, and the precipitate was washed with THF and dried ( $P_2O_5$ ), yielding 2.01 g (82%) of *N*-(4-methylpyridin-2-yl) phthalamic acid. A suspension of 2.01 g (8.2 mmol) of *N*-(4-methylpyridin-2-yl) phthalamic acid in dry toluene (20 mL) was heated under reflux for 72 h, using a Dean Stark to separate the theoretical quantity of water (1.44 mL). The solvent was removed under reduced pressure. The residue was neutralised by a solution of sodium hydrogenocarbonate (4%) until effervescence ceased. The collected solid was washed with water and dried ( $P_2O_5$ ). By recrystallization from 95% ethanol, 1.36 g of pure white product was obtained. Yield: 70%; m.p. (dec.): 176 °C. IR (KBr,

$cm^{-1}$ ):  $\nu_s(C=O \text{ imide})$  1740,  $\nu_{as}(C=O \text{ imide})$  1730.  $^1H$ -NMR ( $CDCl_3$ ):  $\delta$  2.48 (s, 3H, 4-CH<sub>3</sub>), 7.43 (dd, 1H, Pyr H<sup>5</sup>,  $J_{H^5H^6} = 4.8$  Hz,  $J_{H^5H^3} = 1.6$  Hz), 7.45 (dd, 1H, Pyr H<sup>3</sup>,  $J_{H^3H^6} = 1.0$  Hz,  $J_{H^3H^5} = 1.6$  Hz), 8.08 (m, 4H, Ar-H), 8.58 (dd, 1H, Pyr H<sup>6</sup>,  $J_{H^6H^5} = 4.8$  Hz,  $J_{H^6H^3} = 1.0$  Hz).  $^{13}C$ -NMR ( $DMSO-d_6$ ):  $\delta$  20.75 (CH<sub>3</sub>), 123.91 (Pyr C<sup>3</sup>), 124.00 (C<sup>4</sup>, C<sup>7</sup>), 125.30 (Pyr C<sup>5</sup>), 131.71 (C<sup>3a</sup>, C<sup>7a</sup>), 135.34 (C<sup>5</sup>, C<sup>6</sup>), 146.28 (Pyr C<sup>4</sup>), 149.37 (Pyr C<sup>6</sup>), 150.07 (Pyr C<sup>2</sup>), 166.86 (C<sup>1</sup>, C<sup>3</sup>). Anal. (C<sub>14</sub>H<sub>10</sub>N<sub>2</sub>O<sub>2</sub>): C, H, N.

### 5.1.2.5. Methods F and G:

#### 2-(4-methylpyridin-2-yl)-1H-isoindole-1,3-dione (**9**)

Methyl hydrogenophthalate (1.8 g, 10 mmol) was dissolved in thionyl chloride (5 mL), and the solution was refluxed for 1 h. The solvent was removed, and the acid chloride obtained was dissolved in THF (5 mL) and added to a mixture of 2-amino-4-methylpyridine (1.08 g, 10 mmol) and triethylamine (1.45 mL, 10 mmol) in THF (20 mL). The solution was heated under reflux for 15 h. The precipitate of triethylamine hydrochloride was separated by filtration. The solvent was removed under reduced pressure. The residue was washed with water and dried ( $P_2O_5$ ). Recrystallization from 95% ethanol afforded **9** (1 g, 42%). M.p. (dec.): 176 °C.

### 5.1.2.6. Method H:

#### 2-(4,6-dimethylpyridin-2-yl)-1H-isoindole-1,3-dione (**12**)

A mixture of phthalimide (1.47 g, 10 mmol) and K<sub>2</sub>CO<sub>3</sub> (1.37 g, 10 mmol) in DMF (15 mL) was stirred at r.t. for 6 h. 2-Bromo-4,6-dimethylpyridine (1.86 g, 10 mmol) was then added and the solution was heated under reflux for 8 h. The solvent was removed under reduced pressure and the residue obtained was washed with water, dried ( $P_2O_5$ ) and purified by column chromatography using dichloromethane–ethanol 95:5 as the eluent. Recrystallization from 95% ethanol afforded **12** (0.45 g, 18%). M.p. (dec.): 191–193 °C. IR (KBr,  $cm^{-1}$ ):  $\nu_s(C=O \text{ imide})$  1775,  $\nu_{as}(C=O \text{ imide})$  1725.  $^1H$ -NMR ( $CDCl_3$ ):  $\delta$  2.39 (s, 3H, 4-CH<sub>3</sub>), 2.56 (s, 3H, 6-CH<sub>3</sub>), 7.02 (s, 1H, Pyr H<sup>5</sup>), 7.05 (s, 1H, Pyr H<sup>3</sup>), 7.78–7.93 (m, 4H, Ar-H).  $^{13}C$ -NMR ( $DMSO-d_6$ ):  $\delta$  20.65 (4-CH<sub>3</sub>), 23.85 (6-CH<sub>3</sub>), 121.20 (Pyr C<sup>3</sup>), 123.95 (C<sup>4</sup>, C<sup>7</sup>), 124.72 (Pyr C<sup>5</sup>), 131.73 (C<sup>3a</sup>, C<sup>7a</sup>), 135.31 (C<sup>5</sup>, C<sup>6</sup>), 145.55 (Pyr C<sup>4</sup>), 150.11 (Pyr C<sup>6</sup>), 158.25 (Pyr C<sup>2</sup>), 166.98 (C<sup>1</sup>, C<sup>3</sup>). Anal. (C<sub>15</sub>H<sub>12</sub>N<sub>2</sub>O<sub>3</sub>): C, H, N.

### 5.1.2.7. Methods I and J: *N*-(4,6-dimethylpyridin-2-yl)-3-phthalimido-3-(3,4-dimethoxyphenyl) propanamide (**20**)

A mixture of 3-amino-3-(3,4-dimethoxyphenyl) propi-

onic acid (1 g, 4.46 mmol),  $\text{Na}_2\text{CO}_3$  (0.46 g, 4.46 mmol) and *N*-carbethoxyphthalimide (0.96 g, 4.46 mmol) in water (10 mL) was stirred for 20 min at r.t. The solution was acidified by 6 M HCl and extracted with diethyl ether. The organic layer was dried ( $\text{Na}_2\text{SO}_4$ ) and evaporated. Crystallization from diisopropyl ether gave 1.1 g of 3-phthalimido-3-(3,4-dimethoxyphenyl) propionic acid. Yield: 69%; m.p. (dec.): 120–121 °C. 1,1-Carbonyldiimidazole (0.81 g, 5 mmol) was added portionwise to a solution of 3-phthalimido-3-(3,4-dimethoxyphenyl) propionic acid (1.77 g, 5 mmol). The mixture was stirred for 1 h. 2-Amino-4,6-dimethylpyridine (0.79 g, 6.5 mmol) was then added to the solution. Stirring was continued for 24 h at r.t. The solvent was removed under reduced pressure. After column chromatography with dichloromethane–ethanol 95:5 as the solvent system, crystallization from diisopropyl ether gave 1.4 g of pure product **20**. Yield: 61%; m.p. (dec.): 157 °C. IR (KBr,  $\text{cm}^{-1}$ ):  $\nu(\text{NH})$  3340,  $\nu_s(\text{C=O imide})$  1775,  $\nu_{\text{as}}(\text{C=O imide})$  1700,  $\nu(\text{C=O amide})$  1650,  $\delta(\text{NH})$  1540.  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ ):  $\delta$  2.24 (s, 3H, 4- $\text{CH}_3$ ), 2.35 (s, 3H, 6- $\text{CH}_3$ ), 3.28 (dd, 1H,  $\text{CH}_2$ ,  $^3J = 5.9$  Hz,  $^2J = 15.7$  Hz), 3.83–3.87 (m, 6H, 3- $\text{OCH}_3$ , 4- $\text{OCH}_3$ ), 3.91 (m, 1H,  $\text{CH}_2$ ), 5.93 (dd, 1H, CH,  $^3J = 5.9$  Hz,  $^3J = 5.8$  Hz), 6.68 (s, 1H, Ph  $\text{H}^2$ ), 6.80 (d, 1H, Ph  $\text{H}^5$ ,  $J = 7.5$  Hz), 7.13 (s, 1H, Pyr  $\text{H}^5$ ), 7.15 (d, 1H, Ph  $\text{H}^6$ ,  $J = 7.1$  Hz), 7.66–7.78 (m, 4H, Ar-H), 7.81 (s, 1H, Pyr  $\text{H}^3$ ), 8.06 (s, 1H, NH).  $^{13}\text{C-NMR}$  ( $\text{DMSO}-d_6$ ):  $\delta$  21.05 (4- $\text{CH}_3$ ), 23.67 (6- $\text{CH}_3$ ), 38.31 ( $\text{CH}_2$  CO), 50.62 (N-CH), 55.87 (3- $\text{OCH}_3$  and 4- $\text{OCH}_3$ ), 111.22 (Pyr  $\text{C}^3$ ), 111.68 (Ph  $\text{C}^2$ ), 112.07 (Ph  $\text{C}^5$ ), 119.78 (Ph  $\text{C}^6$ ), 119.89 (Pyr  $\text{C}^5$ ), 123.50 ( $\text{C}^4, \text{C}^7$ ), 131.56 (Ph  $\text{C}^1$ ), 131.87 ( $\text{C}^{3a}, \text{C}^{7a}$ ), 134.94 ( $\text{C}^5, \text{C}^6$ ), 148.71 (Ph  $\text{C}^3$ ), 148.94 (Ph  $\text{C}^4$ ), 149.34 (Pyr  $\text{C}^4$ ), 151.57 (Pyr  $\text{C}^6$ ), 156.34 (Pyr  $\text{C}^2$ ), 168.05 ( $\text{C}^1, \text{C}^3$ ), 169.44 (CONH), Anal. ( $\text{C}_{26}\text{H}_{25}\text{N}_3\text{O}_5$ ): C, H, N.

#### 5.1.2.8. Method K: *N*-(4,6-dimethylpyridin-2-yl)-3-phthalimido-3-(3,4-dimethoxyphenyl) propanamide (**20**)

A solution of 3-phthalimido-3-(3,4-dimethoxyphenyl) propionic acid (2.02 g, 5.7 mmol), 2-amino-4,6-dimethylpyridine (0.69 g, 5.7 mmol) and triethylamine (2.4 mL, 17.1 mmol) in dry dichloroethane (70 mL) was cooled in an ice bath. Phenyl dichlorophosphate (0.85 mL, 5.7 mmol) was added dropwise. The mixture was stirred at r.t. for 24 h. The solvent was removed under reduced pressure. The residue was purified by column chromatography using dichloromethane–ethanol 95:5 as the eluent. By recrystallization from diisopropyl ether, 1.9 g of the pure product was obtained. Yield: 73%; m.p. (dec.): 157 °C.

#### 5.1.2.9. Method L: 2-(4,6-dimethylpyridin-2-yl)-4-amino-1*H*-isoindole-1,3-dione (**22**)

Palladium on charcoal (5%, 0.24 g) was added to a solution of **21** (2.38 g, 8 mmol) in ethanol (160 mL). The mixture was stirred under an  $\text{H}_2$  atmosphere. When the consumption of hydrogen ceased, the catalyst was filtered and the solvent was evaporated under reduced pressure. The resulting solid was recrystallised from 95% ethanol to afford **22** (1.54 g, 72%). M.p. (dec.): 200 °C. IR (KBr,  $\text{cm}^{-1}$ ):  $\nu(\text{NH})$  3740,  $\nu_s(\text{C=O imide})$  1760,  $\nu_{\text{as}}(\text{C=O imide})$  1715,  $\delta(\text{NH})$  1645.  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ ):  $\delta$  2.39 (s, 3H, 4- $\text{CH}_3$ ), 2.58 (s, 3H, 6- $\text{CH}_3$ ), 5.34 (s, 2H,  $\text{NH}_2$ ), 6.88 (dd, 1H, Ar  $\text{H}^5$ ,  $J_{\text{H}^5\text{H}^6} = 8.2$  Hz,  $J_{\text{H}^5\text{H}^7} = 0.47$  Hz), 7.02 (s, 1H, Pyr  $\text{H}^5$ ), 7.05 (s, 1H, Pyr  $\text{H}^3$ ), 7.22 (dd, 1H, Ar  $\text{H}^7$ ,  $J_{\text{H}^7\text{H}^6} = 7.1$  Hz,  $J_{\text{H}^7\text{H}^5} = 0.47$  Hz), 7.46 (dd, 1H, Ar  $\text{H}^6$ ,  $J_{\text{H}^7\text{H}^6} = 7.1$  Hz,  $J_{\text{H}^5\text{H}^6} = 8.2$  Hz).  $^{13}\text{C-NMR}$  ( $\text{DMSO}-d_6$ ):  $\delta$  20.61 (4- $\text{CH}_3$ ), 23.86 (6- $\text{CH}_3$ ), 109.00 ( $\text{C}^{3a}$ ), 111.46 (Pyr  $\text{C}^3$ ), 121.17 (Pyr  $\text{C}^5$ ), 122.04 ( $\text{C}^5$ ), 124.39 ( $\text{C}^7$ ), 132.41 ( $\text{C}^{7a}$ ), 135.94 ( $\text{C}^6$ ), 145.77 ( $\text{C}^4$ ), 147.42 (Pyr  $\text{C}^4$ ), 149.92 (Pyr  $\text{C}^6$ ), 158.11 (Pyr  $\text{C}^2$ ), 167.12 ( $\text{C}^3$ ), 168.40 ( $\text{C}^1$ ). Anal. ( $\text{C}_{15}\text{H}_{13}\text{N}_3\text{O}_2$ ): C, H, N.

## 5.2. Pharmacology

### 5.2.1. *TNF $\alpha$* inhibitory activity

The effect of the different compounds on the production of the major pro-inflammatory cytokine *TNF $\alpha$*  by in vitro activated peritoneal macrophages (M $\Phi$ ) was quantified as described earlier in Ref. [16]. Adult male Swiss CF mice (18–25 g) were used. Briefly, thioglycolate-elicited mouse M $\Phi$  were isolated by peritoneal washing with PBS without Ca–Mg, after cervical dislocation. The cell suspension ( $1\text{--}2 \times 10^6$  cell per well) was incubated in a 24 well culture plate in RPMI 10% FCS for 2 h at 37 °C, 5%  $\text{CO}_2$ . After three washes with PBS, mice M $\Phi$  were pre-incubated with the different compounds solubilised in ethanol (maximal final concentration of ethanol: 0.5%) at 10  $\mu\text{M}$ . M $\Phi$  were then stimulated by bacterial lipopolysaccharide or LPS (0.1  $\text{mg mL}^{-1}$  — from *Salmonella typhimurium*, Sigma) for 4 h. Supernatants were collected and assessed for *TNF $\alpha$*  content using the Wehi164 clone 13 cytotoxic assay [16].

### 5.2.2. PMA-induced mouse-ear oedema (orally administered drugs)

Induction of mouse-ear oedema was based on the method of Carlson et al. [17] with some modifications. Groups of five male Swiss mice weighing 20–25 g were used. All animals were fasted 24 h before the experiments and maintained in suitable environmental condi-

tions throughout the experiments. PMA (phorbol-12-myristate-13-acetate) was dissolved in 80% aqueous ethanol at a concentration of  $250 \mu\text{g mL}^{-1}$ ;  $10 \mu\text{L}^{-1}$  was applied topically to the anterior and posterior surfaces of the right ear of each mouse. The left ear (control) received the vehicle ( $10 \mu\text{L}$  of 80% aqueous ethanol). The products studied were orally administered 1 h before the PMA application. Ear thickness was measured with a model micrometer gauge (Oditest Kroeplin) 3 h and 30 min after treatment. Ear oedema, calculated by subtracting the thickness of the left ear (vehicle) from the thickness of the right ear (PMA), was expressed as an increase in ear thickness. The percentage of inhibition of the inflammatory reaction was determined for each animal by the comparison of ear oedema in treated and non-treated animals.

#### 5.2.3. PMA-induced mouse-ear oedema (topically applied drugs)

Groups of five male Swiss mice weighing 20–25 g were used. Mice were briefly anaesthetised with ether for ear application. PMA (phorbol-12-myristate-13-acetate) was dissolved in 80% aqueous ethanol at a concentration of  $250 \mu\text{g mL}^{-1}$ ;  $10 \mu\text{L}$  was applied topically to the anterior and posterior surfaces of the right ear of each mouse. The left ear (control) received the vehicle ( $10 \mu\text{L}$  of 80% aqueous ethanol). A solution of  $100 \mu\text{g}$  or  $500 \mu\text{g}$  of drug in  $10 \mu\text{L}$  of ethanol was applied to the inner surface of the right ear of treated animals, and  $10 \mu\text{L}$  of vehicle (ethanol) to the contralateral ear as the control. These applications were made 30 min before PMA application, and then again 5 min later. Ear thickness was measured with a model micrometer gauge (Oditest Kroeplin) 3 h and 30 min after treatment. Ear oedema, calculated by subtracting the thickness of the left ear (vehicle) from the thickness of the right ear (PMA), was expressed as an increase in ear thickness. The percentage of inhibition of the inflammatory reaction was determined for each animal by the comparison of ear oedema in treated and non-treated animals.

#### 5.2.4. Carrageenan-induced rat-paw oedema

Anti-inflammatory activity against carrageenan-induced rat-paw oedema was assayed in adult male Wistar CF rats weighing 180–220 g, according to the method of Winter et al. [18] with slight modifications. The drugs were orally administered 1 h before the injection of 0.05 mL of a 1% suspension of carrageenan saline into the subcutaneous tissue of one hind paw. The other hind paw was injected in the same way with 0.05 mL of a

saline solution. Rats were fasted 24 h before the experiment, and water ( $1.5 \text{ mL}/100 \text{ g}$  body weight) was orally administered twice (20 and 4 h before injections). The volume of both the hind paws of control and treated animals were measured with a plethysmograph 3 h after injection. Rats were kept in the same experimental conditions. The inhibition percentage of the inflammatory reaction was determined for each animal by comparison with controls, and calculated by the formula  $I (\%) = 100 \times (1 - dt/dc)$ , where  $dt$  is the difference in paw volume in the drug-treated group and  $dc$  the difference in the control group.

#### 5.2.5. Acute toxicity ( $LD_{50}$ )

Tests were carried out on rats fasted for 24 h. Groups of eight animals were treated orally with the compounds studied at various dose levels. Animals were watched for mortality until 48 h, and for symptoms until day 8. The value of the lethal dose ( $LD_{50}$ ) was determined according to the method of Miller and Tainter [26].

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