Synthesis and Study of the Anti-inflammatory Properties of Some Pyrazolo[1,5-*a*]pyrimidine Derivatives

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Abstract □ A series of pyrazolo[1,5-*a*]pyrimidin-7-ones (**1c–17c**) were synthesized to evaluate in vivo and in vitro effects induced by structural modifications at the 2 position of 4,7-dihydro-4-ethyl-2-phenylpyrazolo[1,5-*a*]pyrimidin-7-one (FPP028). This substance, which has been previously studied, is a weak inhibitor of prostaglandin biosynthesis and a nonacid analgesic and anti-inflammatory agent devoid of ulcerogenic properties. To gain more insight into the mechanism of action of this class of compounds, several in vivo tests were carried out, such as carrageenan-induced rat paw edema and pleurisy. In vitro tests include some studies of leukocyte functions, such as superoxide production and myeloperoxidase release. In vitro effects on arachidonic acid-, adenosine 5′-diphosphate-, and platelet-activating factor-induced platelet aggregation were also studied. Different anti-inflammatory activities were observed, depending on the nature of substituents at the 2 position; these differences are probably linked to the capacity of these compounds to inhibit leukotrienes and/or prostaglandin biosynthesis with different selectivity. 4,7-Dihydro-4-ethyl-2(2-thienyl)pyrazolo[1,5-*a*]pyrimidin-7-one (**7c**) proved to be the most interesting compound of the novel synthesized series, showing powerful pharmacological activity in vivo as well as in vitro, together with very weak acute toxicity.

Pyrazolo[1,5-*a*]pyrimidine is a condensed biheterocyclic system that has been widely studied from both chemical and pharmacological points of view. In fact, there are many papers about the synthesis and the chemical reactivity as well as the biological properties of this system.[1–3] A series of papers that reports studies of the inhibitory activity on phosphodiesterases[4,5] exhibited by several pyrazolo[1,5-*a*]pyrimidine derivatives is of particular interest. Another investigation[6] has pointed out the anxiolytic activity of some pyrazolo[1,5-*a*]pyrimidines that do not enhance depressant activity of ethanol or barbiturates on the central nervous system. We have reported[7] the synthesis of a number of 4,7-dihydro-4-alkyl-2-phenylpyrazolo[1,5-*a*]pyrimidin-7-ones, which were found to be endowed with interesting anti-inflammatory activity. Of these compounds, 4-ethyl-4,7-dihydro-2-phenylpyrazolo[1,5-*a*]pyrimidin-7-one (FPP028)[8] was the most active in a series of anti-inflammatory tests. Unlike the majority of known nonsteroidal anti-inflammatory drugs (NSAIDs), FPP028 showed nonulcerogenic properties and demonstrated a gastroprotective effect in rats. It is well known that the inhibition of cycloxygenase (CO) prevents the formation of prostaglandins, whereas inhibition of the enzyme 5-lipooxygenase (5-LO) blocks the synthesis of leukotrienes and constitutes a potential new treatment for diseases such as rheumatoid arthritis, asthma, and inflammatory bowel disease, in which high levels of leukotrienes are believed to play a role.[9–12] The lack of ulcerogenic activity of FPP028 is consistent with the finding that it does not inhibit prostaglandin biosynthesis in vitro or in vivo.[13] These observations are in good agreement[14] with reports that FPP028 did not interfere with arachidonic acid (AA)-induced platelet aggregation, further suggesting that this compound is a very weak inhibitor of CO. Previous papers of ours have pointed out that the anti-inflammatory activity of pyrazolo[1,5-*a*]pyrimidines is retained when the 3, 5, and 6 positions are free of substituents and the alkyl chain on the nitrogen in the 4 position contained no more than two carbon atoms. On the other hand, replacement of a phenyl ring of FPP028 with a methyl group or a hydrogen completely abolished the anti-inflammatory activity.[7] To evaluate the role played by the phenyl moiety, several structural modifications were carried out at the 2 position. We therefore synthesized a series of compounds bearing a substituted benzene or heterocyclic ring or a branched alkyl chain.

To clarify the mechanism of action of this class of compounds, the anti-inflammatory effect was studied on carrageenan-induced rat paw edema. In addition, as some preliminary results showed inhibitory properties of FPP028 on leukocyte chemotaxis,[15] we decided to investigate the effects of some more active compounds on rat carrageenan-induced pleurisy, an inflammatory model characterized by fluid and leukocyte accumulation.[16] Polymorphonuclear leukocytes (PMNs) respond to inflammatory stimuli by generation of superoxide anion radicals and secretion of their granular enzymes, such as β-glycuronidase or myeloperoxidase, and by prostaglandin and leukotriene biosynthesis.[17] Oxygen-free radicals, leukotriene B_4 (LTB_4), and proteolytic enzymes secreted by these PMNs are believed to be involved in inflammatory diseases. Because a number of anti-inflammatory drugs have been shown to modulate PMN[18] and platelet responses,[19–21] we also studied the effects of these new compounds on superoxide production and myeloperoxidase release of isolated neutrophils together with their platelet antiaggregant properties.

Results and Discussion

The synthesis of the pyrazolo[1,5-*a*]pyrimidine system was performed by condensation of suitable 3-substituted 5-aminopyrazoles with ethyl formylacetate sodium salt[7] (Scheme I). 3-Substituted 5-aminopyrazoles were, in turn, prepared by reacting β-ketonitriles[22–27] with hydrazine hydrate. 3-(4-Nitrophenyl)-5-aminopyrazole (**15a**) was, however, prepared according to a different, more recent method.[28] Whereas the known 3-(2-pyridyl)-5-aminopyrazole (**9a**) was obtained according to a previously described method,[29] particular difficulties were encountered during the synthesis of compounds bearing a 3- or 4-pyridyl substituent in position 2. First, condensation between ethyl nicotinate or isonicotinate with acetonitrile gave a very hygroscopic sodium salt from which it was impossible to isolate the free nicotinoyl and isonicotinoyl acetonitriles. Thus, the following reaction with hydrazine hydrate was performed with the intermediate sodium salts. In contrast to the literature,[30,31] it was impossible in our

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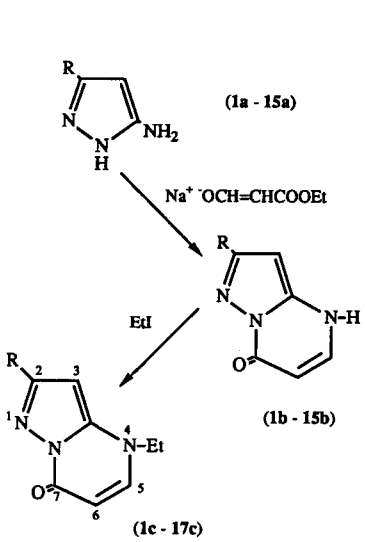

R = 2-bromophenyl	1
3-bromophenyl	2
4-bromophenyl	3
2-methoxyphenyl	4
3-methoxyphenyl	5
4-methoxyphenyl	6
2-thienyl	7
3-thienyl	8
2-pyridyl	9
3-pyridyl	10
4-pyridyl	11
cyclohexyl	12
ter. butyl	13
isopropyl	14
4-nitrophenyl	15
4-aminophenyl hydrocloride	16
3-ethylpyridinium iodide	17

Scheme I

hands to isolate pure **10a** and **11a** (Table I). These compounds were thus characterized through their oxalates and acetyl derivatives. The 2-substituted 4,7-diihydropyrazolo[1,5-*a*]pyrimidin-7-ones were obtained according to the literature[7] (Table II).

N-Alkylation was carried out according to a modification of a known method.[32] Following the above general procedure, ethylation of 3- and 4-pyridyl derivatives was somewhat troublesome. Moreover, the reaction of **10b** also gave alkylation on the pyridine nucleus to afford an ethylpyridinium quaternary salt **17c**. Substitution of potassium carbonate with sodium hydride afforded the selective alkylation in position 4. The resulting derivatives **10c** and **11c** were very difficult to isolate because they appeared to be very sensitive to air, heat, and light (Table III).

A number of functionalized 4,7-dihydro-4-alkylpyrazolo[1,5-*a*]pyrimidin-7-ones have been described as anti-inflammatory agents.[7] Previous work from our laboratory has focused on FPP028, a compound endowed with interesting anti-inflammatory activity without any inhibitory properties on prostaglandin biosynthesis.[8] To better evaluate the role of substituents at the 2 position of the phenylpyrazole nucleus, we synthesized two series of molecules in which the phenyl ring of FPP028 was functionalized with different subsituents (**3c, 6c, 13c, 16c**) or replaced by some heterocyclic (**7c, 8c, 9c, 10c, 11c**) or alkyl moieties (**12c, 13c, 14c**).

The anti-inflammatory activity of these compounds is shown in comparison with that of the parent compound FPP028 and indomethacin in Table IV. Substituents at the 4 position of the phenyl ring (**3c, 6c, 16c**) completely abolished the anti-inflammatory activity. A significant reduction of the pharmacological effect was also observed when the 2-phenyl ring was replaced by alkyl moieties with different lipophilic or steric properties (**13c, 14c**). On the other hand, the introduction of a six-member saturated ring (**12c**) instead of an aromatic one did not substantially modify the pharmacological properties of FPP028. Finally, substitution of the phenyl ring at the 2 position with either a thien-2-yl and a thien-3-yl group provided a significant increase in the anti-inflammatory activity only for the thien-2-yl isomer (**7c**). Similar results were also observed with the regioisomer of a pyridine (**9c, 10c, 11c**). The 2-pyridyl isomer (**9c**) provided the most notable anti-inflammatory activity.

Table V summarizes the activity of a number of pyrazolo[1,5-*a*]pyrimidines on in vitro human platelet aggregation and superoxide anion production with human PMNs. In agreement with previous results[14] obtained with FPP028 in AA-stimulated aggregation, many compounds were inactive. Similar results were obtained when 3 μM adenosine 5′-diphosphate (ADP) was used as a proaggregatory agent. As pointed out by many authors,[20] thromboxane A_2 (TXA_2) is believed to play a significant role in AA-induced platelet aggregation. CO is also known to be involved in aggregation induced by high doses of ADP (up to 2 μM) by stimulating intraplatelet TXA_2 biosynthesis, the release reaction, and the so-called "second-wave" of platelet aggregation.[21] Unlike indomethacin, FPP028 and related compounds were inactive in AA- or ADP-induced aggregation; this result strongly supports the view that these compounds are endowed with a different mechanism of action from CO inhibition. Nevertheless, it is worth mentioning that the introduction of a basic function in the 4 position of the phenyl ring (**16c**) or the substitution of this aromatic group with a pyridine in 2 position (**9c**) generates new compounds with antiaggregatory properties, the latter closely resembling those of indomethacin. Furthermore we observed that FPP028 and related compounds exert some inhibitory activity on platelet-activiting factor (PAF)-induced platelet aggregation. In this test, to rule out the platelet release reaction and therefore TXA_2 involvement, we considered the effect of the tested compounds on reversible aggregometric curves only according to Kloprogge et al.[33] This anti-PAF activity was not

Table I—Physical Properties and Yields for 3-Substituted 5-Aminopyrazoles (1a–15a)

Compound	Reaction Time, h	mp, °C (Recrystallization Solvent)	Derivative; mp, °C (solvent)	Yield, %	Molecular Formula[a]
1a	12	Oil	Diacetyl; 128–129 (H_2O)	61	$C_{13}H_{12}BrN_3O_2$
2a	12	149–150 (EtOH/H_2O)		55	$C_9H_8BrN_3$
3a	12	173–174 (EtOH/H_2O)[b]		41	$C_9H_8BrN_3$
4a	12	Oil	Diacetyl; 171–172 (EtOH)	81	$C_{14}H_{15}N_3O_3$
5a	8	88–89 (CCl_4)		72	$C_{10}H_{11}N_3O$
6a	8	140–141 [lit. 141][b] (EtOH/H_2O)		39	$C_{10}H_{11}N_3O$
7a	12	51–52 (EtOH)	Diacetyl; 148–149 (EtOH)	22	$C_{11}H_{11}N_3O_2S$
8a	12	126–127 (H_2O)		78	$C_7H_7N_3S$
9a	12	122–124 [lit. 124][c]		77	$C_8H_8N_4$
10a	3	Oil [lit. 76–77][d]	Acetyl; 295 (methoxyethanol)[f]	67	$C_{10}H_{10}N_4O$
11a	8	193–194 (H_2O) [lit. 193][e]	Oxalate	26	$C_8H_8N_4$
12a	12	Oil	Diacetyl; 205–206 (EtOAc)	51	$C_{13}H_{18}N_3O_2$
13a	8	69–70 (Cyclohexane)	Diacetyl; 111–113 (C_6H_{12})	66	$C_{11}H_{17}N_3O_2$
14a	8	Oil	Diacetyl; 185–186 (EtOH/H_2O)	65	$C_{10}H_{15}N_3O_2$
15a	8	249–253 [lit. 248–253][b] (MeOH)		63	$C_9H_8N_4O_2$

[a] Combustion analyses of C, H, and N were within ±0.4% of the calculated values; spectral data are in accordance with the proposed structures. [b] Reference 28. [c] Reference 29. [d] Reference 30. [e] Reference 31 [f] Oxalate.

Table II—Physical Properties and Yields for the 2-Substituted 4,7-Dihydropyrazolo[1,5-*a*]pyrimidin-7-ones (1b–15b)

Compound	Reaction Time, h	mp, °C (Recrystallization Solvent)	Yield, %	Molecular Formula[a]
1b	2	309–311 dec. (Methoxyethanol)	45	$C_{12}H_8BrN_3O$
2b	2	312–313 (DMF)	35	$C_{12}H_8BrN_3O$
3b	4	>320 (DMF)	48	$C_{12}H_8BrN_3O$
4b	3	244–245 (EtOH/H_2O)	42	$C_{13}H_{11}N_3O_2$
5b	2	312–314 (DMF/H_2O)	41	$C_{13}H_{11}N_3O_2$
6b	4	>300 (DMF)	26	$C_{13}H_{11}N_3O_2$
7b	4	>300 (DMF)	91	$C_{10}H_7N_3OS$
8b	3	337–338 (DMF)	61	$C_{10}H_7N_3OS$
9b	4	345 (Methoxyethanol)	50	$C_{11}H_8N_4O$
10b	6	>305 (EtOH/H_2O)	46	$C_{11}H_8N_4O$
11b	6	>330 dec. (MeOH)	33	$C_{11}H_8N_4O$
12b	8	325–327 dec. (EtOH/H_2O)	15	$C_{12}H_{15}N_3O$
13b	6	338–339 (AcOH)	24	$C_{10}H_{13}N_3O$
14b	6	292–293 (AcOH)	24	$C_9H_{11}N_3O$
15b	2	340 (AcOH)	26	$C_{12}H_8N_4O_3$

[a] Combustion analyses of C, H, and N were within ±0.4% of the calculated values; spectral data are in accordance with the proposed structures.

Table III—Physical Properties and Yields for the 2-Substituted 4,7-Dihydro-4-ethylpyrazolo[1,5-*a*]pyrimidin-7-ones (1c–17c)

Compound	Reaction Time, h	mp, °C (Recrystallization Solvent)	Yield, %	Molecular Formula[a]
1c	12	191–192 (EtOH)	39	$C_{14}H_{12}BrN_3O$
2c	9	199–200 (70% EtOH)	71	$C_{14}H_{12}BrN_3O$
3c	12	283–285 (EtOH)	63	$C_{14}H_{12}BrN_3O$
4c	12	184–185 (EtOH)	59	$C_{15}H_{15}N_3O_2$
5c	24	189–190 (EtOH)	63	$C_{15}H_{15}N_3O_2$
6c	12	204–205 (EtOH)	54	$C_{15}H_{15}N_3O_2$
7c	12	168–169 (H_2O)	39	$C_{12}H_{11}N_3OS$
8c	12	151–152 (EtOAc)	37	$C_{12}H_{11}N_3OS$
9c	6	165–167 (iPrOH)	25	$C_{13}H_{12}N_4O$
10c	12	167–169	6	$C_{13}H_{12}N_4O$
11c	12	—[b]	7	$C_{13}H_{12}N_4O$
12c	12	146–147 (H_2O)	90	$C_{14}H_{19}N_3O$
13c	6	188–189 (EtOH/H_2O)	83	$C_{12}H_{17}N_3O$
14c	6	124–125 (Cyclohexane)	51	$C_{11}H_{15}N_3O$
15c	12	288–289 (AcOH)	53	$C_{14}H_{12}N_4O_3$
16c[c]	12	296–298 (MeOH)	35	$C_{14}H_{14}N_4O$·HCl
17c[d]	2	250–253 (EtOH/iPrOH)	8	$C_{15}H_{17}IN_4O$

[a] Combustion analyses of C, H, and N were within ±0.4% of the calculated values; spectral data are in accordance with the proposed structures. [b] mp not determined, heating instable. [c] Obtained by reduction for 15 h. [d] Obtained as byproduct in the alkylation of **10b**.

further investigated in this study. However, we might point out that this effect, which is more evident with the highest concentrations of our compounds, was also shown by indomethacin, a compound which is substantially devoid of anti-PAF activity[33] at anti-inflammatory concentrations. Hence, the activity against PAF-induced aggregation (Table V) could be explained by a possible "nonspecific effect" of these compounds. In Table V there is also a summary of the results obtained by evaluating fMet-Leu-Phe (FMLP)-evoked superoxide anion production from human PMNs. In preliminary experiments, the drugs were evaluated for their interference of cytochrome C reduction by a xanthine oxidase superoxide generating system; results agree with the hypothesis that tested compounds do not act as chemical scavangers.

Compounds **3c**, **7c**, **12c**, and **13c** dose dependently inhibited $O_2^{\cdot-}$-production, with maximal effects comparable to those obtained with known NSAIDs (e.g., indomethacin and nimesulide). As a consequence of their interesting anti-inflammatory activity, also linked to a substantial lack of antiaggregatory properties, **12c** and **7c** were selected for additional pharmacological evaluation on carrageenan-induced pleurisy in rats. Table VI summarizes the oral activity of these two compounds. Similar to the results obtained in carrageenan-induced rat paw edema, **7c** shows the better anti-inflammatory effect by reducing both the exudate volume and cell accumulation, whereas **12c** is only a weak anti-inflammatory agent in this test (Figure 1).

Currently available data suggest that FPP028 acts as an anti-inflammatory agent without inhibiting prostaglandin biosynthesis in inflammatory exudates.[7] Our results in platelet aggregation experiments confirm these suggestions. In an attempt to formulate an alternative hypothesis for the mechanism of action, we assessed the inhibitory properties of our compounds on human PMN superoxide production. Results (Table V) indicate that FPP028 is about threefold less potent than indomethacin, with approximately the same potency order comparing ED_{50} (50% effective dose) values in carrageenan-induced rat paw edema. With reference to the activity of FPP028, all chemical modifications performed at the pyrazolo[1,5-*a*]pyrimidine ring gave new compounds that retain some inhibitory properties on superoxide production. In particular, the introduction of a Br atom in the 4 position of the phenyl ring or substitution with a thien-2-yl generated two compounds, **3c** and **7c**, that were more potent than the parent one. Similar effects were also observed for **7c** and **12c** on neutrophils taken from rat pleural exudates. Every compound in this test inhibited both superoxide production (Figure 1) and myeloperoxidase (MPO) release (data not shown) to a comparable extent: a maximal inhibition of ~30% was recorded at 30 μM for both compounds. By evaluating

Table IV—Anti-Inflammatory Activity[a]

Compound	Dose po, mg/kg	Inhibition of Carrageenan-Induced Rat Paw Edema, %	ED_{50} ± SEM, mg/kg
3c	50.0	0	—
6c	50.0	0	—
7c	5.0	25.0 ± 15.0	10.5 ±0.8[b]
	10.0	43.4 ± 14.1	
	15.0	57.0 ± 21.3	
	25.0	92.1 ± 15.6	
8c	50.0	35.0 ± 9.0	
9c	50.0	84.8 ± 11.6	
10c	50.0	22.1 ± 1.9	
11c	50.0	36.0 ± 11.2	
12c	10.0	20.1 ± 9.2	29.07±1.22
	25.0	36.6 ± 12.2	
	50.0	72.0 ± 15.2	
13c	10.0	3.3 ± 1.2	—[c]
	50.0	33.0 ± 10.7	
14c	5.0	15.0 ± 10.1	—
	10.0	20.0 ± 10.5	
	25.0	30.0 ± 11.6	
	50.0	32.4 ± 14.6	
16h	50.0	8.0 ± 3.1	—
INDO[d]	1.0	21.2 ± 6.7	3.09±1.22
	3.0	41.7 ± 4.0	
	5.0	68.4 ± 4.3	
FPP028	5.0	29.0 ± 6.20	15.4 ±1.07
	10.0	42.7 ± 4.9	
	25.0	61.5 ± 3.8	
	50.0	68.8 ± 4.2	
	100.0	83.4 ± 4.4	

[a] All values are expressed as mean ± standard deviation of 15 experiments. [b] Significantly different ($p < 0.001$) in comparison with the ED_{50} for FPP028. [c] —, Not determined. [d] Indomethacin.

MPO release from human PMNs, we observed that **12c** dose dependently inhibited this parameter with a maximum (~50%) at 30 μM, whereas **7c** was less active (~25% inhibition at 30 μM). In this test, both indomethacin and nimesulide acted dose dependently, with maximal inhibition (~50%) being achieved at 30 μM.

In preliminary experiments, the activity of **7c** and **12c** was also studied on LTB_4 and thromboxane B_2 (TXB_2) production induced by FMLP in rat PMNs. Compounds **7c** and **12c** were tested at 10 μM; this was the highest concentration tested and allowed us to rule out the solvent effect [<1% dimethyl sulfoxide (DMSO)]. On the other hand, it is known that at this concentration classic NSAIDs are able to inhibit CO in vitro.[34] As shown in Table VII, **7c** significantly reduced LTB_4 release without affecting TXB_2 production in pleural PMNs, whereas **12c** was inactive.

Comparing the results shown in Tables IV and V, an apparent lack of correlation can be seen between in vitro and in vivo experiments. For example, compounds (**3c**, **6c**, **13c**, and **16c**) that showed good activity against superoxide production, were inactive (**3c**, **6c**, and **16c**) or only weak inhibitors (**13c**) of carrageenan-induced edema in rats. This apparent disagreement between in vitro and in vivo tests could probably be explained by metabolic inactivation and/or different bioavailability processes.

Conclusions

In conclusion, this work demonstrates that chemical modifications introduced on the 2-phenyl position of FPP028 can modify its pharmacological properties. Some new compounds are able to modulate cellular activity in inflammation to different extents by inhibiting both granulocyte superoxide production and MPO release. This activity is probably linked to the inhibition of AA metabolism via the CO (**9c**, **16c**) and 5-LO (**7c**) pathways. In our opinion, selectivity within the series could be modulated by specific 2 position substitution, most notably with the analogue containing the 2-(thien-2-yl) substituent. The key compound arising from our preliminary studies is **7c**, which apparently combined 5-LO selectivity in vitro and anti-inflammatory properties in vivo. In addition, in acute toxicity experiment, this compound was endowed with a 50% lethal dose of >500 mg/kg, thus showing greater safety than the parent compound FPP028 that has a 50% lethal dose of 270 mg/kg.

Experimental Section

Chemistry—Melting points were determined on a Gallenkamp capillary melting point apparatus and are uncorrected. Elemental analyses were performed for C, H, and N with a Perkin-Elmer 260 C elemental analyzer, and results were within ±0.4% of the theoretical values. The IR spectra were taken in nujol mull with a Perkin-Elmer 681 spectrophotometer. The UV spectra were obtained with a Perkin-Elmer 552-S spectrophotometer. The ^{1}HNMR spectra were recorded with a Varian EM 360 instrument; chemical shifts are reported in δ (ppm) downfield from tetramethylsilane. Mass spectra were run on a 5970 A Hewlett-Packard mass spectrometer, coupled with a gas chromatograph equipped with a 30-m OV 101 capillary column. Column chromatography was carried out on silica gel Kieselgel 60 type (70–230 mesh ASTM, E. Merck A.G., Darmstadt, Germany).

All analytical samples were homogeneous as determined by thin-layer chromatography which was carried out on Merck silica gel F_{254} analytical plates (eluant $CHCl_3$:CH_3OH, 10:1).

Synthesis of 3-Substituted 5-Aminopyrazoles (1a–15a)—Compounds **1a–15a** were obtained according to reported methods.[28–31]

Synthesis of 2-Substituted 4,7-Dihydropyrazolo[1,5-*a*]pyrimidin-7-ones (1b–15b)—*General Procedure*—To the magnetically stirred solutions of **1a–10a** and **12a–15a** (0.02 mol) dissolved in ethanol (200 mL), ethyl formylacetate sodium salt (0.05 mol) was added, and the mixture was refluxed for several hours. The jelly-like product that was obtained was acidified to pH 5–6, and the solid was filtered and washed with water. In the event that any precipitate was obtained, the solvent was distilled under reduced pressure.

*Preparation of **11b***—The ethanol solution of **11a** oxalate (4.2 g) and ethyl formylacetate sodium salt (7 g; molar ratio, 1:3.5) was evaporated to a small volume under reduced pressure. The residual solid was filtered off, and the filtrate was acidified with acetic acid to pH 5–6. A crystalline precipitate separated out by addition of diethyl ether.

Alkylation of 3-Substituted 4,7-Dihydropyrazolo[1,5-*a*]pyrimidin-7-ones (1b–15b)—*General Procedure*—A mixture of 3-substituted 4,7-dihydropyrazolo[1,5-*a*]pyrimidin-7-ones (0.01 mol) dissolved or suspended in anhydrous *N,N*-dimethylformamide (25 mL), anhydrous potassum carbonate (0.01 mol), and ethyl iodide (0.02 mol) was heated to 40–50 °C with magnetic stirring for several hours in a 100-mL conical flask. Exclusion of external moisture was achieved by insertion of a $CaCl_2$ guard tube at the top of the condenser. The course of the reaction was monitored by thin-layer chromatography ($CHCl_3$:CH_3OH as eluant). On cooling, the product, which sometimes crystallized or was precipitated by the addition of water, was washed with water and dried.

In the event that the 4-ethylderivative, which is soluble in *N,N*-dimethylformamide, did not precipitate upon addition of water, the solvent was distilled off under reduced pressure, and the residual solid was treated with chloroform and filtered. The chloroform solution was dried with anhydrous sodium sulfate and evaporated under reduced pressure to give a solid substance or an oil that crystallized on treatment with diethyl ether or with acetone, respectively (**17c**).

*Preparation of **10c** and **11c***—Solutions of **10b–11b** (2.12 g, 0.01 mol) in anhydrous *N,N*-dimethylformamide (20 mL) were each reacted with a 50% dispersion of sodium hydride in mineral oil (0.48 g, 0.01 mol). Ethyl iodide (2.04 mL, 0.025 mol) was added in one portion, and the mixture was magnetically stirred for 12 h at 40–50 °C, with care being taken to insert a $CaCl_2$ guard tube at the top of the condenser. The solvent was distilled off at reduced pressure, and the residue was washed on the filter with $CHCl_3$ to dissolve tarry byproducts. The semisolid reaction product was dissolved in 2-propanol and filtered.

Table V—Effect of Tested Compounds on Platelet Aggregation and PMNs Superoxide Anion Production[a]

Compound	Concentration, μM	ID_{50} ± SEM, μM	% Inhibition of Human Platelet Aggregation Induced by:			Generation of Human PMNs $O_2^{\cdot-}$[b]
			AA[c]	ADP[d]	PAF[e]	
3c	1	—[f]	—	—	14.6 ± 0.5	8.3 ± 8.5
	3		—	—	—	7.4 ± 6.6
	10		1.2 ± 2.5	1.7 ± 3.5	36.3 ± 5.8	27.7 ± 11.1
	50		9.7 ± 2.5	14.0 ± 3.6	94.0 ± 10.3	47.5 ± 16.6
6c	1	—	—	—	7.0 ± 2.6	—
	10		6.1 ± 2.3	0	25.6 ± 4.5	0
	50		15.9 ± 4.2	11.0 ± 3.0	46.6 ± 13.5	—
	100		—	—	—	30.6 ± 2.5
7c	1	—	—	—	—	0
	3		—	—	—	0
	10		13.3 ± 1.5	10.7 ± 8.5	27.3 ± 3.7	5.5 ± 2.7
	30		—	—	—	14.4 ± 8.3
	50		35.3 ± 3.5	19.3 ± 3.0	50.7 ± 16.4	46.4 ± 7.2
8c	1	—	—	—	9.0 ± 2.6	—
	10		5.0 ± 2.6	9.2 ± 3.7	23.2 ± 10.1	0
	50		10.7 ± 1.5	11.0 ± 4.5	41.2 ± 9.3	—
	100		—	—	—	22.3 ± 9.0
9c	1	4.9 ± 1.4	28.6 ± 1.3	3.3 ± 1.8	—	—
	10		53.0 ± 9.8	14.3 ± 7.0	—	8.0 ± 2.0
	50		90.0 ± 1.1	20.0 ± 6.2	—	—
	100		—	—	—	31.6 ± 6.8
10c	10	—	11.7 ± 6.3	8.6 ± 2.2	5.6 ± 2.4	6.1 ± 2.6
	100		32.6 ± 16.0	20.8 ± 6.6	14.2 ± 3.0	6.6 ± 3.2
11c	10	—	22.6 ± 5.0	9.3 ± 1.5	3.9 ± 1.7	16.5 ± 8.3
	100		50.7 ± 14.8	23.2 ± 7.7	11.4 ± 6.0	19.0 ± 11.0
12c	1	—	—	—	18.6 ± 3.0	4.7 ± 1.2
	3		—	—	—	13.8 ± 3.6
	10		10 ± 8.1	17.5 ± 8.3	36.3 ± 11.5	21.9 ± 4.2
	30		—	—	—	28.2 ± 5.1
	50		17.3 ± 1.5	26.9 ± 1.1	66.3 ± 14.1	30.0 ± 19.0
	100		—	—	—	35.0 ± 12.0
13c	1	—	—	—	3.3 ± 2.5	6.2 ± 2.2
	3		—	—	—	12.1 ± 7.0
	10		0	0	20.6 ± 2.5	23.6 ± 6.5
	30		—	—	—	31.3 ± 16.1
	100		—	—	—	44.3 ± 23.1
14c	10	—	0	0	2.5 ± 1.0	0
	100		13.2 ± 6.0	6.5 ± 2.0	16.0 ± 4.4	3.3 ± 3.0
16c	0.1	0.8 ± 2.5	10.0 ± 2.0	0	0	—
	1		75.0 ± 5.0	0	0	—
	10		83.6 ± 3.2	14.6 ± 6.1	—	0
	50		88.0 ± 2.0	21.0 ± 3.5	—	0
	100		—	—	—	30.6 ± 2.5
1	0.5	—	—	—	2.5 ± 1.0	—
	1		—	—	16.6 ± 1.5	0
	3		—	—	—	—
	10		0	8.0 ± 5.5	28.3 ± 1.1	12.2 ± 4.0
	30		—	—	—	12.5 ± 6.2
	50		15.0 ± 10.6	18.3 ± 7.4	—	—
	100		100 ± 20.6	31.0 ± 12.6	—	39.0 ± 9.5
INDO[g]	0.5	1.9 ± 1.4	—	—	10.3 ± 1.5	—
	1		20.0 ± 6.2	0	30.6 ± 2.5	5.2 ± 3.0
	3		80.0 ± 13.0	10.0 ± 2.4	—	9.7 ± 3.7
	10		95 ± 22.9	28.0 ± 9.7	42.6 ± 3.0	20.0 ± 11.0
	30		—	—	—	31.2 ± 8.2
	50		—	—	—	—
NIME[h]	1	—	—	—	—	10.8 ± 6.2
	3		—	—	—	14.3 ± 7.8
	10		—	—	—	25.9 ± 8.6
	30		—	—	—	35.9 ± 9.5

[a] Values are expressed as mean ± standard deviation of 5–10 experiments. [b] Induced by FMLP (0.1 μM). [c] 1 μM. [d] 3 μM. [e] 1 μM. [f] —, Not determined. [g] Indomethacin. [h] Nimesulide.

The solvent was removed under reduced pressure, and the residue was treated with diethyl ether, giving a crystalline substance (**11c**) or a semisolid suspension that solidified on addition of a small amount of acetonitrile (**10c**).

Preparation of 16c—Compound **16c** was obtained by catalytic hydrogenation of **15c**. Thus, a suspension of **15c** (0.5 g, 2 mmol) in ethanol (150 mL) was hydrogenated in a Parr apparatus at room temperature and 3 atm for 12 h in the presence of 0.5 g of 5% Pd on C. The obtained solution was filtered, a few milliliters of concentrated hydrochloric acid were added, and the solvent was evaporated to dryness under reduced pressure. The light-brown residue was diluted with a little methanol and recrystallized from the same solvent.

Table VI—Carrageenan-Induced Rat Pleurisy[a]

Compound	Dose (po), mg/kg	Volume, mL	% Inhibition	Total Number of Cells ($\times 10^3$)	% Inhibition
7c	25	0.25 ± 0.06[b]	70.26	966 ± 1.13[c]	91.6
12c	50	0.63 ± 0.03[b]	22.30	8104 ± 7.16	11.8
Control	—	0.85 ± 0.03	0	11 265	0

[a] Values are means ± standard deviations of 10 experiments. [b] Significantly different than control ($p < 0.01$) as determined by the *t* test. [c] Significantly different than control ($p < 0.001$) as determined by the *t* test.

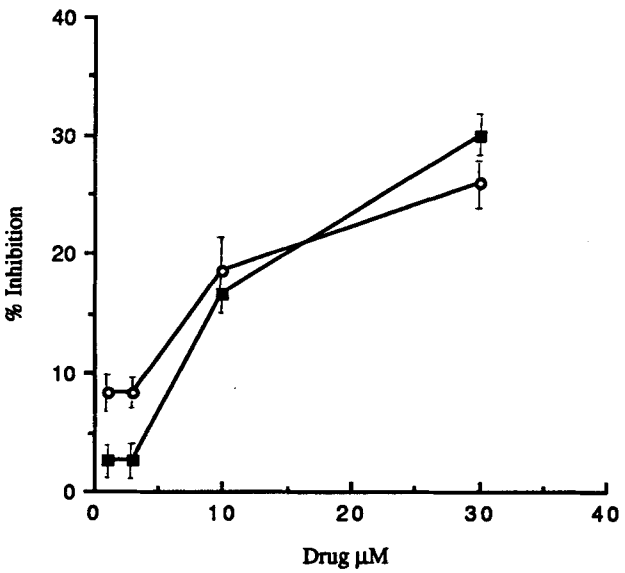

Figure 1—Inhibition of rat pleural PMNs superoxide production by **7c** (■) and **12c** (○). Values are means ± SEM of three experiments.

Table VII—Rat PMNs Pleurisy[a]

Compound	LTB_4	% I	TXB_2	% Inhibition
Control	3.73±0.4	—[b]	7.38±0.7	—
7c	1.55±0.4[c]	58.5	7.11±1.0	3.6
12c	3.66±1.1	1.8	5.17±1.1[d]	29.9

[a] 10^6 cells were incubated with CytB for 10 min and then stimulated by FMLP (10^{-7} M) in the presence or absence of 10^{-5} M drug; values are means ± SEM of three experiments. [b] —, Not applicable. [c] Significantly different than control ($p < 0.05$) as determined by the *t* test. [d] Significantly different than control ($p < 0.01$) as determined by the *t* test.

Pharmacology—*Preparation of Neutrophils*—Heparinized venous blood of healthy adult volunteers was used immediately for neutrophil isolation by standard techniques of dextran sedimentation (dextran T500, Pharmacia), Ficoll-Paque (Pharmacia, density = 1.077 g/ml) gradient centrifugation, and hypotonic lysis of erythrocytes. The purity of the final cell suspension averaged 98%; neutrophil viability (as assessed by trypan blue exclusion) was usually >95% and was not affected by any of the drug treatments.

Incubation Conditions—Neutrophils were suspended in a buffered salt solution (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) supplemented with glucose (1 mg/mL) and human serum albumin (1 mg/mL; Behringwerke). Incubations were performed at 37 °C in duplicate or triplicate. In all experiments, cells were treated for 5 min with cytochalasin B (5 μg/mL; Aldrich) before exposure to the drugs to allow lysosomal enzyme release and $O_2^{\cdot-}$ production without phagocytosis.[35] After cytochalasin B treatment, neutrophils were incubated with the drugs and then stimulated for further 5 min with FMLP (10^{-7} M). Drug solutions were prepared daily in DMSO.

Superoxide Production—PMNs (1×10^6/1 mL) were preincubated in the absence or presence of increasing concentrations of each drug for 5 min at 37 °C and then stimulated with FMLP (10^{-7} M; Serva). The $O_2^{\cdot}$-production was continuously monitored spectrophotometrically at 37 °C by measuring superoxide dismutase (Boehringer-Mannheim) and inhibitable cytochrome C (Sigma) reduction, and was expressed as nmoles of cytochrome C reduced/10^6 cells/min [with ϵ_{550} (reduced-oxidized) = 21.1 mM^{-1}, where ϵ_{550} is 0.190].

The drugs were checked for interference in the assay by measuring their effects on cytochrome C reduction with a xanthine oxidase superoxide generating system. Assays were carried out in the same buffer as above, with 100 μM cytochrome C, 150 μM hypoxanthine, ~0.01 units of xanthine oxidase per milliliter, and appropriate concentrations of each drug.

Evaluation of MPO Net Release—MPO release was evaluated, both in pellet and supernatants, according to the method of Henson et al.[36] When a drug inhibited FMLP-evoked lysosomal enzyme release, the percent inhibition of enzyme release was calculated according to the following formula[37]: % inhibition = 1 − [(% enzyme release with drug)/(% enzyme release without drug)] × 100. Experiments were performed in duplicate or triplicate, and basal values (e.g., values obtained in the absence of any treatment) were subtracted from all determinations. All results are expressed as mean ± standard error of the mean (SEM), and significance was evaluated by the *t* test.

Induction and Collection of Pleural Exudates—Male Wistar rats (150–200 g, purchased from Morini, S. Polo breeding farm, commercially fed) were orally (po) administered indomethacin (1 mg/kg), tested compounds (5 mL of suspension in 1% acqueous Carbowax), or vehicle. One hour later, carrageenan (0.1 mL of 1% suspension in saline) was injected into the right pleural cavity after lightly anesthetizing the rat with ether. Four hours later, rats were sacrificed. The chest was cut open by lateral incision, the pleural exudate was harvested on ice, and the exudate volume was measured. Exudates were heparinized (10 IU/mL of exudate) and centrifuged at 500 × *g* for 5 min at 4 °C. After erythrocyte lysis, the neutrophils (16–20 × 10^6 cells/mL) were suspended in a buffered salt solution (NaCl, 138 mM; KCl, 2.7 mM; $CaCl_2$, 1 mM; $MgCl_2$, 0.5 mM; glucose, 1 mg/mL; pH 7.4) and stimulated in the presence of cytochalasin B (5 μg/mL; Aldrich) for 10 min at 37 °C with FMLP (0.1 μM; Serva) with or without drug.

Determinations of LTB_4 and TXB_2 Production by Rats PMNs—The amounts of TXB_2 and LTB_4 in the cell supernatant were determined by specific enzyme immunoassays. Suitable dilutions (1:10–1:50 for exudates) were made in enzyme immunoassay buffer without prior extraction or purification according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI). The threshold sensitivities of these assays were 20 pg/mL for TXB_2 and 6 pg/mL for LTB_4. Supernatants were collected, immediately frozen, and freeze-dried before being evaluated for $TXB_2/10^6$ and $LTB_4/10^6$.

Carrageenan-Induced Rat Paw Edema—Normal rats (180–200 g) were fasted for 18 h. According to Winter et al.,[38] the animals were dosed po with the test compounds (5 mL of suspension) 1 h before the injection of 0.05 mL of 1% carrageenan suspension into the right and left hind paws. The paw volume was measured by plethismography (Ugo Basile plethismography). Fifteen rats per dose were tested for each compound. The activity of the drugs was compared with that of indomethacin. All compounds were administered po in 1% aqueous suspension of Carbowax.

Acute Oral Toxicity of 7c—Compound **7c** was administered po to rats at doses of 50, 100, 200, and 500 mg/kg in a 1% Carbowax suspension. Five rats per dose were tested. The animals were observed for 1 week.

In Vitro Studies on Human Platelet Aggregation—Blood was obtained from healthy human volunteers who had not taken drugs for at least 2 weeks. Blood samples (9 mL) were collected in siliconated tubes containing 1 mL of 3.8% sodium citrate. The blood was centrifuged at 1000 rpm for 20 min to obtain platelet-rich plasma (PRP). Further centrifugation at 2500 rpm for 10 min afforded

platelet-poor plasma (PPP). The platelet count was adjusted to 3.10^8 cells/mL for aggregation studies. Measurement of platelet aggregation was performed according to the turbidimetric method of Born and Cross[39] with a PA 3210 aggregometer (Daiichi, Kyoto, Japan). All tested compounds were dissolved in DMSO and diluted until a maximum final concentration of 0.1% in residual solvent was obtained. This final concentration had no measurable effect on platelet aggregation in preliminary experiments. To induce platelet aggregation, a solution (0.050 mL) containing the aggregating agent (ADP, AA, or PAF-acether) was added to the cuvette containing 0.450 mL of PRP. The percentage of aggregation was calculated from the amplitude of the trace in comparison with PRP from control donors (100% aggregation) and PPP (0% aggregation).

Tha ADP (Daiichi Kyoto, Japan) was used at a final concentration of 3.0 μM, AA (A-6523 sodium salt, Sigma Chemical) at a final concentration of 1.0 mM, and PAF-acether (P-5029, Sigma Chemical) at a final concentration of 1.0 μM.

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