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Synthesis and pharmacological evaluation of 1-methyl-5-[substituted-4(3*H*)-oxo-1,2,3-benzotriazin-3-yl]-1*H*-pyrazole-4-acetic acid derivatives

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Abstract

Several new 1-methyl-5-[substituted-4-oxo-1,2,3-benzotriazin-3-yl]-1*H*-pyrazole-4-acetic acids and their ethyl ester derivatives were prepared. The compounds were tested for analgesic and antiinflammatory activities, acute toxicity, ulcerogenic effect, and as in vitro inhibitors of 3α-hydroxysteroid dehydrogenase (3α-HSD), since it is claimed that the inhibition of such an enzyme predicts in vivo antiinflammatory activity. Some compounds were more active than phenylbutazone in the phenylbenzoquinone and acetic acid peritonitis tests, and equiactive to the same drug in the carrageenin paw edema test. All the compounds inhibited the 3α-HSD, but no correlation was observed with the paw edema inhibition values. The compounds proved to possess marginal or no ulcerogenic effect, as well as low systemic toxicity.

Keywords: 5-(1,2,3-Benzotriazinyl)-1*H*-pyrazole-4-acetic acid derivatives; Anti-inflammatory activity; Analgesic activity; 3α-Hydroxysteroid dehydrogenase inhibition

1. Introduction

Heteroarylalkanoic acids are a well-established class of nonsteroidal antiinflammatory agents, e.g. indomethacin and tolmetin, therapeutically useful in the treatment of acute as well as chronic inflammatory conditions [1].

The 4(3*H*)-1,2,3-benzotriazinones bearing an *N*(3)-heterocycle have drawn our attention for a long time due to their interesting antiinflammatory and analgesic activities as well as a lack of ulcerogenic effects [2].

On continuing our research program aimed to obtain new antiinflammatory agents as potent as the commonly used antiinflammatory drugs and, at the same time, characterized by reduced side effects, such as ulcerogenicity, we prepared and tested a series of 1-methyl-5-[6,7,8-substituted-4(3*H*)-oxo-1,2,3-benzotriazin-3-yl]-1*H*-pyrazole-4-acetic acid derivatives whose structure bears both the benzotriazinyl and the pyrazole acetic acid moieties.

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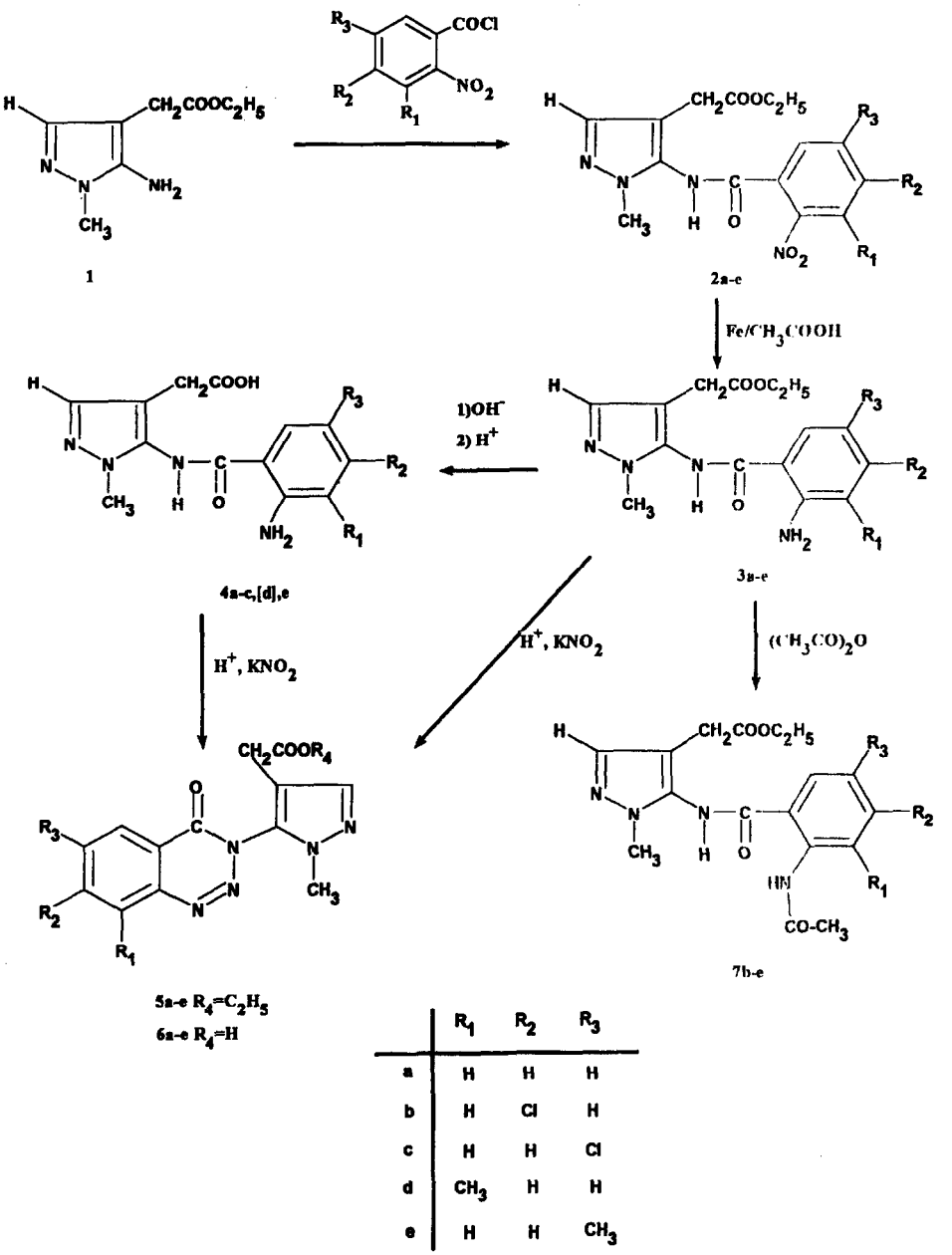
2. Chemistry

The synthesis of the benzotriazinones **5a–e** and **6a–e** was carried out following Scheme 1.

Ethyl 1-methyl-5-amino-1*H*-pyrazole-4-acetate **1** was reacted with 2-nitroaroyl chlorides in dry chloroform to yield ethyl 1-methyl-5-(2-nitrobenzamido)-1*H*-pyrazole-4-acetates **2a**[3],**b–e** which were reduced with iron filings in 5% aqueous acetic acid solution to afford the corresponding 2-amino derivatives **3a**[3],**b–e**. The latter compounds, used as crude products, by reaction with potassium nitrite in acetic acid afforded ethyl 5-benzotriazinylpyrazole-4-acetates **5a–e**. Compounds **3** were also hydrolyzed with 4% aqueous sodium hydroxide to yield the corresponding acids **4a–e** which, in turn, were transformed into 5-benzotriazinylpyrazole-4-acetic acid derivatives **6a–e** by the above procedure.

The structures of the new compounds, with the exception of **3b–e**, were confirmed by analytical and spectroscopic data. Compounds **3b–e**, obtained as impure oils, were identified as acetyl derivatives **7b–e**. The IR spectra of benzotriazinones **5** and **6** exhibited bands in the 1680–1745 cm^{-1} region, due



	R_1	R_2	R_3
a	H	H	H
b	H	Cl	H
c	H	H	Cl
d	CH_3	H	H
e	H	H	CH_3

Scheme 1. Synthesis of the benzotriazinones **5a–e** and **6a–e**.

to endocyclic and ester/acid carbonyl groups. Moreover, acid derivatives **6** showed a broad band attributable to the hydroxyl. ^{1}H NMR spectra of the ester and acid derivatives **5a–e** and **6a–e**, respectively, exhibited a set of signals in agreement with the proposed structures.

3. Pharmacology

All the new compounds **5a–e** and **6a–e** were screened in order to evaluate their analgesic and antiinflammmatory activities, behavioral effects, acute toxicity and ulcerogenic potential. Indomethacin and phenylbutazone (PBZ) were used as reference standards.

4. Results and discussion

Pharmacological data are reported in Tables 1 and 2.

4.1. Behavioral effects and acute toxicity in mice

The tested compounds did not show any significant gross behavioral effects at doses of up to 1000 mg/kg p.o. and 800 mg/kg i.p. in mice. At higher doses the most typical signs of acute intoxication were motor uncoordination, bradypnea and hypotonia. At these levels, death generally occurred 12–48 h after the administration of the drug in 40–60% of the animals, whereas the surviving mice appeared to be normal throughout the 7-day observation period.

Table 1
In vivo pharmacological data

Comp.	Acute toxicity		Analgesic activity		Antiinflammatory activity		Ulcerogenic score
	Approximate LD_{50} (mg/kg)		Phenylquinone writhing test [a]		Carrageenin paw rat oedema [a]	Acetic acid peritonitis [a]	400 mg/kg
	p.o.	i.p.	% Protection		% Inhibition	% Inhibition	
			1 mg/kg	10 mg/kg	100 mg/kg	10 mg/kg	
5a	>1000	>800	15	35*	59*	40*	0
5b	>1000	>800	33	59*	55*	33*	0
5c	>1000	~700	31	45*	59*	38*	25
5d	>1000	~700	25	46*	50*	40*	16
5e	>1000	>700	28	46*	68*	47*	25
6a	>1000	~800	18	35*	31*	18*	25
6b	>1000	>800	30	48*	58*	43*	0
6c	>1000	~800	30	45*	62*	45*	0
6d	>1000	>700	10	34*	30*	15*	16
6e	>1000	>800	32*	51*	66*	45*	0
PBZ	~700	~300	7	28*	58*	7*	250 [c]
Indomethacin	~25	15	55*		57 [b]*	60*	300 [c]

Oral administration for all tests.
[a] Values are percent of controls.
[b] Indomethacin: 10 mg/kg.
[c] PBZ 2×100 mg/kg; indomethacin 2×10 mg/kg.
*$p < 0.05$, Student's t-test versus controls.

Table 2
Antiinflammatory effects of compounds on croton oil-induced mouse ear edema

Drugs	Dose (mg/ear)	EW increase (mg ± S.E.)	Reduction (%)
Control		30.4 ± 1.0	
5a	0.5	20.6 ± 0.9*	32
	1.0	15.4 ± 0.9*	49
5b	0.5	15.2 ± 0.9*	50
	1.0	10.7 ± 1.1*	65
5c	0.5	15.2 ± 0.9*	50
	1.0	8.1 ± 1.0*	73
5d	0.5	15.3 ± 0.9*	49
	1.0	8.2 ± 1.0*	73
5e	0.5	15.0 ± 0.9*	50
	1.0	8.7 ± 1.0*	71
6a	0.5	14.4 ± 0.9*	53
	1.0	10.7 ± 1.0*	65
6b	0.5	16.5 ± 0.9*	46
	1.0	12.1 ± 1.0*	60
6c	0.5	15.2 ± 0.9*	50
	1.0	9.2 ± 0.7*	70
6d	0.5	12.3 ± 1.0*	59
	1.0	8.4 ± 1.0*	72
6e	0.5	16.6 ± 0.9*	45
	1.0	12.2 ± 1.0*	60
PBZ	0.5	14.5 ± 0.9*	52
	1.0	9.3 ± 0.7*	69
Indomethacin	0.05	16.4 ± 0.9*	46
	0.10	8.0 ± 1.0*	74

Drugs were given in situ together with the irritant. The ear weight (EW) increase was determined 6 h after application of croton oil. Results are the mean ± S.E. from six treated animals and twelve controls.
*$p < 0.05$, Dunnett's test vs. controls.

4.2. Analgesic activity

In a phenylbenzoquinone-induced writhing test in mice, compounds **5b–e** and **6b,c,e** were more active than the unsubstituted compounds **5a** and **6a**. However, among **5b–e** and **6b,c,e**, activity was slightly influenced by substitution pattern. Several compounds were more active than phenylbutazone.

4.3. Antiinflammatory activity

In the acetic acid peritonitis test, compounds **5** were much more active than PBZ, and less active than indomethacin. The acid derivatives **6b,c,e** were nearly equiactive to the corresponding esters **5**, while compounds **6a,d** were slightly active.

The ester derivatives **5** were as active as PBZ at the same dose, and as indomethacin at 10 mg/kg, in the rat paw edema test. Among the acids, **6a,d** were scarcely active whereas activity of the remaining compounds was comparable to the corresponding esters.

Compounds **5** and **6** were also evaluated in the croton oil-induced mouse ear edema test. Except compound **5a**, they were substantially equiactive to PBZ, at the same doses, and to indomethacin at one tenth of the doses.

4.4. Biochemical assay of the antiinflammatory activity

Finally, antiinflammatory activity of esters **5** and acids **6** was also evaluated employing the rapid spectrophotometric assay developed by Penning et al. [4–6]. These authors claim

Table 3
Inhibition of 5β-dihydrocortisone reduction

Compounds (500 μM)	% Inhibition [a]
5a	10.4
6a	20.3
5b	7.4
6b	32.3
5c	7.9
6c	18.6
5e	13.8
6e	28.5
ASA	19.0

[a] Arithmetic mean of three determinations.

that the assay is based on the observation that 3α-hydroxysteroid dehydrogenase (3α-HSD) of rat liver cytosol can be inhibited in vitro by the major classes of nonsteroidal anti-inflammatory drugs (NSAIDs), proportionally to their in vivo potency.

The inhibition of 3α-HSD by compounds **5a–e** and **6a–e** at 500 μM is reported in Table 3. Compounds **5d** and **6d** were not tested owing to their high absorbance at 340 nm (see Section 6.2.7).

The highest enzyme inhibition was shown by derivatives **6**, probably because their carboxyl group interacts with the enzyme binding site. Compounds **6b,e** were slightly more active than acetylsalicylic acid (ASA).

In any case, no correlation was observed among in vitro enzyme inhibition and in vivo data.

4.5. Ulcerogenic activity

Compounds showed low or no harmful effects on the stomach at the tested dose of 400 mg/kg p.o., when administered twice at a 2 h interval in fasted rats. On the contrary, PBZ and indomethacin, at lower doses, produced serious gastric ulcers in all animals.

5. Conclusions

Pharmacological assays showed that some of the screened compounds are endowed with good analgesic and antiinflammatory activities, low systemic toxicity, and very poor or no ulcerogenic effect.

6. Experimental

6.1. Chemistry

All melting points were taken on a Büchi-530 capillary apparatus and are uncorrected. IR spectra were recorded on a Jasco IR-810 spectrometer as nujol or hexachlorobutadiene mulls. ^{1}H NMR spectra were obtained in DMSO-d_6 or $CDCl_3$ solutions on a Brüker AC-E 250 MHz spectrometer using tetramethylsilane as internal reference. Microanalyses were performed in the laboratories of the Institut de Chimie Pharmaceutique, Université de Genève, Geneva, Switzerland and were within ±0.4% of theoretical values.

*6.1.1. Ethyl 1-methyl-5-(substituted-2-nitrobenzamido)-1H-pyrazole-4-acetates **2b–e***

Equimolar amounts of compound **1** (33 mmol) and the appropriate substituted-2-nitrobenzoylchloride (obtained from the corresponding acid and thionyl chloride) in dry chloroform (150 ml) were refluxed for 5 h. After the first hour of reflux, triethylamine (4.5 ml) was added in four aliquots (2.2, 1.1, 2×0.6 ml, respectively), at intervals of 1 h between each addition. The solution was evaporated under reduced pressure and the residue was washed with 2×100 ml of water and then crystallized to give compound **2**; yields 50–90% (see Table 4).

^{1}H NMR ($CDCl_3$) of **2b** taken as representative compound: δ (ppm) 1.26 (3H, t, CH_3, J=7.1 Hz), 3.63–3.65 (5H, superimposed singlets, CH_2 and CH_3), 4.13 (2H, q, CH_2, J=7.1 Hz), 7.27–8.07 (4H, a set of signals, C_6H_3 and pyrazole H-3), 9.19 (1H, s, NH).

*6.1.2. Ethyl 1-methyl-5-(substituted-2-aminobenzamido)-1H-pyrazole-4-acetates **3b–e***

A suspension of iron filings (80 g) in 5% aqueous acetic acid (100 ml) was heated over a steam-bath, with stirring, until no more hydrogen was evolved. Compound **2** (0.13 mol) was slowly added, and the mixture was stirred over the steam-bath for 1 h. The suspension was neutralized with aqueous sodium hydrogen carbonate and filtered. After filtration the residue was extracted with hot chloroform (3×400 ml). The combined extracts were evaporated under reduced pressure to produce the crude amine **3** as oil; yields 75–80%.

*6.1.3. 1-Methyl-5-(substituted-2-aminobenzamido)-1H-pyrazole-4-acetic acids **4a–e***

0.02 mol of crude compound **3a**[3],**b–e** in ethanol (40 ml) and aqueous sodium hydroxide (8% wt./vol., 40 ml) were stirred for 24 h at room temperature. The suspension thus obtained was concentrated under vacuum to half its original volume and water was then added (40 ml). The solution obtained was treated with aqueous hydrochloric acid solution (37% wt./wt.). At this point the procedure depended on the compound. For compounds **4b,c** the pH was adjusted to 2 whereas for compound **4e** it was set to 4.5. The crude acid which separated was filtered off. As regards the acids **4a** and **4d**, the pH was adjusted to 3.5 and the solutions were extracted with ethyl acetate and chloroform, respectively. The organic extracts were dried (Na_2SO_4) and evaporated under reduced pressure to afford crude intermediates **4a,d**. Only **4a–c,e** could be crystallized (ethanol) (see Table 4); yields 35–65%.

^{1}H NMR (DMSO-d_6) of **4a** taken as representative compound: δ (ppm) 3.31 (2H, s, CH_2), 3.76 (3H, s, CH_3), 6.42–

Table 4
Physical data of compounds **2b–e**, **4a–e**, **5a–e**, **6a–e** and **7b–e**

Comp.	M.p. (°C)	Crystallization solvent	Formula	IR [a] (cm^{-1})		
				NH, NH_2	OH	CO
2b	181–183	ethanol	$C_{15}H_{15}N_4O_5Cl$	3260–3220		1730, 1660–1650
2c	105–107	ethanol	$C_{15}H_{15}N_4O_5Cl$	3460–3080		1725, 1660
2d	96–100	ethyl acetate	$C_{16}H_{18}N_4O_5$	3520–3100		1725, 1665
2e	114–116	ethyl acetate	$C_{16}H_{18}N_4O_5$	3220–3140		1740, 1650
4a	180–182	ethanol	$C_{13}H_{14}N_4O_3$	3460–3300	3200–2500	1700, 1660–1650
4b	237–238	ethanol	$C_{13}H_{13}N_4O_3Cl$	3470–3320	3200–2500	1715, 1660–1650
4c	232–234	ethanol	$C_{13}H_{13}N_4O_3Cl$	3460–3340	3200–2500	1700, 1645
4e	241–244	ethanol	$C_{14}H_{16}N_4O_3$	3440–3320	3100–2500	1695, 1645
5a	120–121	ethanol	$C_{15}H_{15}N_5O_3$			1740, 1695
5b	125–127	ethanol	$C_{15}H_{14}N_5O_3Cl$			1740, 1695
5c	120–121	ethanol	$C_{15}H_{14}N_5O_3Cl$			1740, 1700
5d	142–144	ethanol	$C_{16}H_{17}N_5O_3$			1730, 1685
5e	103–105	ethanol	$C_{16}H_{17}N_5O_3$			1745, 1700
6a	237–239	ethanol	$C_{13}H_{11}N_5O_3$		3100–2500	1745, 1700
6b	206–209	ethanol	$C_{13}H_{10}N_5O_3Cl$		3100–2500	1720–1680
6c	224–226	ethanol	$C_{13}H_{10}N_5O_3Cl$		3100–2500	1730–1680
6d	199–201	ethanol	$C_{14}H_{13}N_5O_3$		3100–2500	1710–1680
6e	237–239	ethanol	$C_{14}H_{13}N_5O_3$		3100–2500	1730–1690
7b	163–166	ethanol	$C_{17}H_{19}N_4O_4Cl$	3220		1730, 1700, 1690
7c	152–155	ethanol	$C_{17}H_{19}N_4O_4Cl$	3200–3100		1740, 1665–1645
7d	190–193	ethyl acetate	$C_{18}H_{22}N_4O_4$	3240–3040		1735, 1670, 1650
7e	154–156	ethanol	$C_{18}H_{22}N_4O_4$	3360–3100		1730, 1690, 1650

[a] As mulls: in nujol for compounds **2**, **5**, and **7**; in hexachlorobutadiene for compounds **4** and **6**.

7.67 (7H, a set of signals, C_6H_4, pyrazole H-3 and NH_2), 9.88 (1H, s, exchangeable, NH); 12.16 (1H, br, exchangeable, COOH).

*6.1.4. Ethyl 1-methyl-5-[substituted-4(3H)-oxo-benzotriazin-3-yl]-1H-pyrazole-4-acetates **5a-e***

A potassium nitrite solution (20 mmol in 5 ml of water) was added dropwise to a magnetically stirred cold (ice bath, 0–5°C) solution of amide **3** (10 mmol) in 15 ml of 2 M HCl. Stirring was continued for 1 h at 0–5°C. The reaction mixture was filtered, the residue washed with water and then purified by flash-chromatography; eluent ethyl acetate/chloroform (1:1) for **5b,c,d** and ethyl acetate for **5a,e**. Compounds **5** were crystallized from ethanol; yields 25–50% (see Table 4).

^{1}H NMR ($CDCl_3$) of **5a** taken as representative compound: δ (ppm) 1.12 (3H, t, CH_3, $J=7.3$ Hz), 3.48 (2H, s, CH_2), 3.98 (3H, s, CH_3), 4.04 (2H, q, CH_2, $J=7.3$ Hz), 7.61–8.44 (5H, a set of signals, C_6H_4, and pyrazole H-3).

*6.1.5. 1-Methyl-5-[substituted-4(3H)-oxo-benzotriazin-3-yl]-1H-pyrazole-4-acetic acids **6a–e***

Compounds **6a–e** were obtained as described for compounds **5a–e**, starting from a solution of compound **4** (10 mmol) in 2 M HCl (70 ml for **4b,c** and 35 ml for **4a,d,e**). The crude products were crystallized from ethanol; yields 60–70% (see Table 4).

^{1}H NMR (DMSO-d_6) of **6a** taken as representative compound: δ (ppm) 3.36 (2H, s, CH_2), 3.92 (3H, s, CH_3), 7.84–8.34 (5H, a set of signals, C_6H_4, and pyrazole H-3), 12.25 (1H, br, exchangeable, COOH).

*6.1.6. Ethyl 1-methyl-5-(substituted-2-acetoamidobenz-amido)-1H-pyrazole-4-acetates **7b,c,d,e***

The crude amine **3** (1.5 g) and acetic anhydride (6 ml) were stirred at room temperature for 24 h. After this time ethanol (20 ml) was added and the mixture was stirred for 1 h and then evaporated under reduced pressure. The solid residue was treated with a saturated aqueous solution of sodium hydrogen carbonate until the pH was adjusted to 7. The suspension was filtered off and the solid was washed with water, air dried and crystallized; yields 65–80% (see Table 4).

^{1}H NMR ($CDCl_3$) of **7b** taken as representative compound: δ (ppm) 1.26 (3H, t, CH_3, $J=7.1$ Hz), 2.18 (3H, s, CH_3), 3.51 (2H, s, CH_2), 3.86 (3H, s, CH_3), 4.16 (2H, q, CH_2, $J=7.1$ Hz), 7.08–8.78 (4H, a set of signals, C_6H_3 and pyrazole H-3), 9.04 (1H, s, exchangeable, NH), 11.30 (1H, s, exchangeable, NH).

6.2. Pharmacology

6.2.1. Materials and methods

Swiss male mice (20–23 g) and Sprague–Dawley rats (130–160 g) were used. The animals were starved for about 12 h before administration and maintained at a temperature of 22 ± 2°C. Tested compounds, indomethacin and phenylbutazone (PBZ) were suspended in 0.5% aqueous methyl

cellulose solution and administered orally or intraperitoneally. Control animals received the same amounts of the vehicle.

6.2.2. Behavioral effects and acute toxicity in mice [7]

Irwin's multidimensional screening–evaluative procedure was used on groups of five animals. The compounds were administered at three doses orally (500, 700, 1000 mg/kg) or intraperitoneally (250, 500, 800 mg/kg). The animals were kept under observation for 6 h and the symptomatology was checked again 24 h later. The approximate LD_{50} was obtained from mortality 7 days later.

6.2.3. Analgesic activity

6.2.3.1. Phenylbenzoquinone writhing test [8]

Analgesia was assessed by means of phenylbenzoquinone (PBQ)-induced writhing on groups of five male mice. Each mouse was given i.p. 0.25 ml of 0.02% PBQ in 5% ethanol and the number of writhes was counted for 5 min, beginning 5 min after the injection. The tested compounds, indomethacin and PBZ were administered orally (1–10 mg/kg) 60 min before PBQ. The analgesic effect was expressed as the percentage of protection in comparison with the controls.

6.2.4. Antiinflammatory activity

6.2.4.1. Carrageenin-induced edema [9]

Groups of four rats were used. The tested compounds and PBZ were given orally at 100 mg/kg, while indomethacin was given at 10 mg/kg. 60 min later, 0.1 ml of 1% carrageenin solution was injected into the subplantar tissue of the right hind paw. The volume was measured by a mercury plethysmometer prior to the injection of carrageenin and 3 h later. The increase in volume of the paw 3 h after the injection of carrageenin was adopted as a measure of edema. Swelling in treated animals was calculated as the percentage of inhibition in comparison with the controls.

6.2.4.2. Acetic acid peritonitis [10]

Groups of four rats were tested. Peritonitis was produced by an i.p. injection of acetic acid (10 ml/kg of 0.5% solution). 30 min later, rats were killed by ether and peritoneal exudate was collected and measured. The tested compounds, PBZ and indomethacin were given orally at the dose of 10 mg/kg, 60 min before the injection of the acetic acid. The antiexudative response was expressed and the percentage of the exudate volume reduction compared with controls.

6.2.4.3. Mouse ear inflammation induced by croton oil [11]

The inflammation was induced in anesthetized mice (ketamine HCl 150 mg/kg i.p.) by applying an acetone solution of croton oil (CO, 35 μg/15 μl) to the inner surface of the right ear. The compounds and PBZ were dissolved in 15 μl of the irritant solution at doses of 0.5 mg/ear and 1 mg/ear, and indomethacin at doses of 0.05 mg/ear and 0.1 mg/ear. The left ear (control) remained untreated, after preliminary experiments had shown that acetone by itself did not affect the weight of the ear. The animals were killed 6 h after CO challenge. Both ears were quickly excised at the hair line and weighed. The difference in weight between the inflamed and non-inflamed ear was taken as the measure of the inflammatory response.

6.2.5. Ulcerogenic activity [12]

Groups of four rats were used. The compounds were given orally (400 mg/kg) to animals fasted for 24 h and after 2 h the treatment was repeated again; indomethacin was administered twice at 10 mg/kg and PBZ was administered twice at 100 mg/kg. Six hours after the first dose each rat was sacrificed by ether inhalation, the stomach removed, opened along the greater curvature and examined with a dissecting microscope for the presence of gastric ulcers. The severity of mucosal damage (ulcerogenic index) was graded by means of scores from 0 (no lesion) to 4 (exceptionally severe lesions). In order to take into account the percentage of rats having ulcers, an index of ulceration was calculated on the basis of the following formula:

$$\frac{\text{mean degree of ulcers} \times \text{no. of animals with ulcers}}{\text{no. of animals}} \times 100$$

6.2.6. Statistical analysis

The results are expressed as mean ± S.E.; one-way analysis with Dunnett's comparison with controls and the unpaired Student *t*-test were used to determine statistical significance.

6.2.7. Biological tests

The activity of 3α-HSD was determined by using 5β-dihydrocortisone as substrate and β-nicotinamide adenine dinucleotide phosphate (β-NADPH) as coenzyme.

Reduction of 5β-dihydrocortisone was measured at 25°C by monitoring the decrease in absorbance of β-NADPH at 340 nm. The reaction mixture contained 2.5 ml of distilled water, 0.3 ml of 1 M potassium phosphate buffer (pH 6.0), 60 μl of 9 mM β-NADPH, 30 μl of 5 mM 5β-dihydrocortisone and 90 μl of compound solution in the tests or DMSO (used to dissolve compounds) in control experiments.

Each assay was initiated by the addition of 30 μl of a crude preparation of rat liver cytosol [6] and the optical density was followed for 5 min. Enzyme activity was expressed as the decrease in optical density per minute (OD/min). The percentage of inhibition of 3α-HSD was calculated using the following equation:

$$\%\ \text{Inhibition} = \frac{(\text{OD/min control} - \text{OD/min sample})}{\text{OD/min control}} \times 100$$

Percent inhibition (mean values of three experiments) of compounds **5a–e** and **6a–e** and ASA, tested at 0.5 mM, is reported in Table 3. None of the compounds showed absorbance at 340 nm at these concentrations; moreover, the pres-

ence at the same time of coenzyme NADPH and substrate 5β-dihydrocortisone were required before the cytosol promoted a change in absorbance.

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References

[1] P.F. Juby, in: R.A. Scerrer and M.W. Whitehouse (Eds.), Antiinflammatory Agents, vol. I, Academic Press, New York, 1984, pp. 91–127.
[2] G. Daidone, S. Plescia, D. Raffa, M.L. Bajardi, M. Matera, A. Caruso, M.G. Leone, Researches on antiinflammatory agents. Studies on some new 3-(pyrazol-5-yl)-1,2,3-benzotriazin-4(3*H*)-ones and -quinazolin-4(3*H*)-ones, Farmaco 45 (1990) 391–398.
[3] G. Daidone, B. Maggio, D. Raffa, S. Plescia, M.L. Bajardi, A. Caruso, V.M.C. Cutuli, M. Amico-Roxas, Synthesis and pharmacological study of ethyl 1-methyl-5-[2-substituted-4-oxo-3(4*H*)-quinazolinyl]-1*H*-pyrazole-4-acetates, Eur. J. Med. Chem. 29 (1994) 707–711.
[4] T.M. Penning, I. Mukarjihi, S. Barrows, P. Talalay, Purification and properties of a 3α-hydroxysteroid dehydrogenase of rat liver cytosol and its inhibition by antiinflammatory drugs, Biochem. J. 222 (1984) 601–611.
[5] T.M. Penning, P. Talalay, Inhibition of a major NAD(P)-linked oxidoreductase from rat liver cytosol by steroidal and nonsteroidal antiinflammatory agents and by prostaglandins, Proc. Natl. Acad. Sci. USA 80 (1983) 4504–4508.
[6] T.M. Penning, Inhibition of 5β-dihydrocortisone reduction in rat liver cytosol: a rapid spectrophotometric screen for nonsteroidal antiinflammatory drug potency, J. Pharm. Sci. 74 (1985) 651–653.
[7] S. Irwin, Comprehensive observational assessment: 1a. A systematic, quantitative procedure for assessing the behavioural and physiologic state of the mouse, Psycopharmacologia (Berlin) 13 (1968) 222.
[8] B.A. Berkowitz, A.D. Fink, S.H. Ngai, Nitrous oxide analgesia: reversal by naloxone and development of tolerance, J. Pharmacol. Exp. Ther. 203 (1977) 539–547.
[9] C.A. Winter, E.A. Risley, G.W. Nuss, Carrageenin induced edema in hind paw of the rat as an assay for antiinflammatory drugs, Proc. Soc. Exp. Biol. Med. 111 (1962) 544–547.
[10] E. Arrigoni-Martelli, Problemi di valutazione preclinica degli effetti analgesici di farmaci antiinfiammatori non steroidei, Boll. Chim. Farm. 107 (1968) 29–42.
[11] G. Tonelli, L. Thibault, J. Ringler, A bio-assay for the concomitant assessment of the antiphlogistic and thymolytic activities of topically applied corticoids, Endocrinology 77 (1965) 625.
[12] R. Domenioz, The pharmacology of phenylbutazone analogues, Ann. N.Y. Acad. Sci. 86 (1960) 263.