Pyridazines. XIII. Synthesis of 6-Aryl-5-Oxygenated Substituted-3(2*H*)-Pyridazinones and Evaluation as Platelet Aggregation Inhibitors

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Several 6-aryl-5-oxygenated substituted pyridazinones have been synthesized and evaluated *in vitro* for inhibition of platelet aggregation induced by adenosine 5′-diphosphate (ADP), thrombin and collagen. All the tested compounds (except 8 and 9) inhibited platelet aggregation in a dose-dependent manner. The IC_{50} of the most active substance, compound 2b, was around 60 μM against ADP and collagen as inducers. The inhibition of platelet aggregation caused by test compounds was dependent on the level of oxidation of the function at the 5-position, with the order of IC_{50} values being R-OH (2a, b, 5) < R-CHO (6, 7) ≪ R-COOH (8, 9). None of the tested compounds increased the intracellular levels of cAMP, indicating a lack of inhibitory activity on cAMP phosphodiesterase (PDE III) in intact cells. These results suggest that the group present at the 5 position of 6-aryl-5-substituted pyridazinones determines the platelet aggregation-inhibitory activity, and that a mechanism other than PDE inhibition is responsible for this effect.

Key words pyridazinone; platelet; phosphodiesterase; platelet aggregation; platelet aggregation inhibitor

6-Aryl-3(2*H*)-pyridazinones, as well as their 4,5-dihydro derivatives, show various pharmacological activities, such as reduction of blood pressure,[1-3] inhibition of platelet aggregation and antithrombotic properties,[3-5] positive inotropic activity,[6-8] and others. Pyridazinones such as imazodan, CI-930,[6] pimobendan,[9] bemoradan[10] and zardaverine,[11] which are active as cardiotonic agents, are structurally related to amrinone (5-amino-3,4′-bipyridine-6(1*H*)-one) and milrinone (1,6-dihydro-2-methyl-6-oxo-3,4′-bypiridine-5-carbonitrile), the prototypes of a series of non-glycoside, non-catecholamine cardiotonics with mixed inotropic/vasodilator activity which have been developed as promising agents for treatment of congestive heart failure.[12,13] Milrinone and related compounds also show antiplatelet activity; the inhibition of cyclic adenosine monophosphate (cAMP) phosphodiesterase (PDE III) in cardiac muscle and platelets is assumed to be the primary mechanism of these activities. The mechanism involved, however, has not yet been completely elucidated, and some differences in biological effects between milrinone and amrinone have recently been reported.[14]

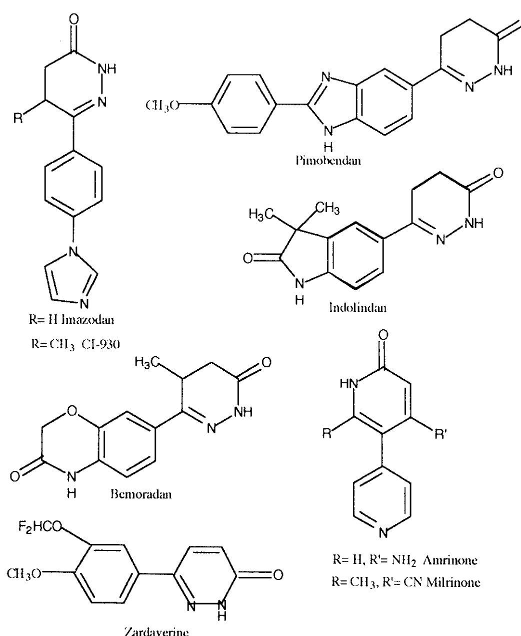
Moos *et al.*[15] have elaborated a pharmacophore five-point model for selective PDE III inhibitors where the following characteristics have a special relevance: a strong dipole (carbonyl group) in the azine system neighboring an NH group, a small lipophilic group at the 5-position, a hydrogen bonding region at the end of the molecule and a flat topography. Later, the model was refined and it was suggested that oxygenated substituents at the 5-position of the dihydropiridazinone could be more favorable than a small (methyl) lipophilic moiety.

As a continuation of our studies on the chemistry and pharmacology of 6-aryl-5-substituted-3(2*H*)-pyridazinones and 6-aryl-5-substituted-3(2*H*)-pyridazines,[16-22] we decided to prepare 6-aryl-3(2*H*)-pyridazinones with oxygenated substituents at the 5-position and to study the effect of the resulting compounds on platelet aggregation induced by agonists such as adenosine 5′-diphosphate (ADP), collagen and thrombin.

Experimental

Chemistry Melting points were determined with a Kofler's microscope hot stage and are uncorrected. Infrared spectra were recorded with a Perkin–Elmer 1600 FTIR spectrophotometer; main bands in cm^{-1}. Proton magnetic resonance (^{1}H-NMR) spectra were obtained with a Brucker WM-250 (250 MHz) or AMX-300 (300 MHz). Chemical shifts are reported in ppm relative to tetramethylsilane as an internal standard. Elemental analyses were performed on a Perkin–Elmer 240B apparatus at the Microanalyses Service of our University; all values were within 0.4% of theoretical. The progress of the reactions was monitored by thin layer chromatography with 2.5 mm Merck silica gel GF 254 strips, and the purified compounds each showed a single spot; unless otherwise stated iodine vapor and/or UV light were used for detection. Chromatographic separations were performed on a silica gel column by flash chromatography (Kieselgel 40, 0.040—0.063 mm).

6-Phenyl-5-hydroxymethyl-4,5-dihydro-3(2*H*)-pyridazinone Acetate (3): This compound was prepared by acetylation with acetic anhydride in dry pyridine as previously described,[19] mp 126—128 °C.



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6-Phenyl-5-hydroxymethyl-3(2*H*)-pyridazinone Acetate (**4**): A mixture of **3** (2.82 g, 11.5 mmol) and 10 g (115 mmol) of manganese dioxide in chloroform (50 ml) was refluxed for 3 h. After cooling, the oxidant was removed by filtration and washed with chloroform. The combined solution was concentrated to give a solid residue, which was purified by column chromatography on silica (ethyl acetate–hexane 8 : 2) and then recrystallized from ethanol to afford 6-phenyl-5-hydroxymethyl-3(2*H*)-pyridazinone acetate as white needles (2.35 g, 85% yield), mp 130—132 °C. IR (KBr) cm^{-1}: 1720 (CO ester), 1660 (CO pyridazinone), 1580 (C=C aromatics). ^{1}H-NMR ($CDCl_3$) δ: 7.49—7.39 (5H, m, Ph), 7.06 (1H, d, J=1.2 Hz, CH–CO), 4.91 (2H, d, J=1.2 Hz, CH_2–OAc), 2.12 (3H, s, CH_3). *Anal.* Calcd for $C_{13}H_{12}N_2O_3$: C, 63.92; H, 4.95; N, 11.47. Found: C, 63.38; H, 5.01; N, 11.37.

6-Phenyl-5-hydroxymethyl-3(2*H*)-pyridazinone (**5**): A solution of **4** (5 g, 20.5 mmol) in 25 ml of ethanol was treated with 10 ml of 2 N HCl and the mixture was heated to reflux for 3 h. After cooling, the reaction mixture was poured onto ice (60 g) and the precipitate was collected by filtration and recrystallized from ethanol to give 6-phenyl-5-hydroxymethyl-3(2*H*)-pyridazinone as white needles (3.93 g, 95% yield), mp 198—201 °C. IR (KBr) cm^{-1}: 3200—2900 (NH), 1650 (CO), 1590 (C=C aromatics). ^{1}H-NMR ($CDCl_3$–TFA) δ: 10.80 (1H, s, NH) 7.69 (1H, d, J=1.3 Hz, CH–CO), 7.56—7.53 (3H, m, *p*-, *m*-Ph), 7.40—7.36 (2H, m, *o*-Ph), 4.71 (2H, d, J=1.3 Hz, CH_2–OH). *Anal.* Calcd for $C_{11}H_9N_2O_2$: C, 63.33; H, 4.98; N, 13.86. Found: C, 63.68; H, 5.04; N, 13.98.

6-Phenyl-5-formyl-3(2*H*)-pyridazinone (**6**): A solution of **5** (2.97 g, 14.8 mmol) in 15 ml of tetrahydrofuran was shaken with 12.8 g (148 mmol) of activated manganese dioxide (Fluka) for 50 h at room temperature. The suspension was filtered through Celite and the manganese dioxide was washed with tetrahydrofuran. The combined filtrate and washing were concentrated to give a solid residue, which was purified by column chromatography (ethyl acetate–hexane 8 : 2) and recrystallized from isopropanol to afford 6-phenyl-5-formyl-3(2*H*)-pyridazinone as yellow needles (2.36 g, 80% yield), mp 230—231 °C. IR (KBr) cm^{-1}: 3421 (NH), 1658 (CO pyridazinone), 1600 (CHO). ^{1}H-NMR ($CDCl_3$–TFA) δ: 11.34 (1H, NH deuterium oxide exchangeable), 8.40 (1H, s, CHO), 7.78 (1H, s, CH–CO), 7.56 (3H, m, *m*-, *p*-Ph), 7.50—7.46 (2H, dd, *o*-Ph). *Anal.* Calcd for $C_{11}H_8N_2O_2$: C, 65.98; H, 4.05; N, 14.0. Found: C, 65.68; H, 4.24; N, 13.98.

6-Phenyl-5-formyl-4,5-dihydro-3(2*H*)-pyridazinone (**7**): Freshly prepared pyridinium chlorochromate (PCC) (1.05 g, 4.9 mmol) was added in portions, with vigorous stirring, to a suspension of finely powdered **2a** (0.5 g, 2.4 mmol) in 50 ml of dry dichloromethane. The progress of the reaction was followed by thin-layer chromatography using a solution of cerium ammonium nitrate and ammonium molybdate in sulfuric acid for detection. After 7 h, 0.2 g of PCC was further added and the reaction mixture was stirred for an additional period of 12 h. The suspension was filtered through a pad of Florisil and the collected solid was washed several times with acetone. The filtrate and acetone washings were combined and evaporated *in vacuo* to give a solid residue, which was purified by column chromatography on silica (ethyl acetate–hexane 9 : 1). Recrystallization of isopropanol yielded 6-phenyl-5-formyl-4,5-dihydro-3(2*H*)-pyridazinone as a yellow crystalline solid (0.25 g, 50% yield), mp 215 °C. IR (KBr) cm^{-1}: 3421 (NH), 1658 (CO pyridazinone), 1600 (CHO). ^{1}H-NMR ($CDCl_3$–TFA) δ: 11.95 (1H, NH deuterium oxide exchangeable), 9.91 (1H, s, CHO), 7.42 (5H, m, Ph), 3.73 (1H, t, J=9.2, CH–CHO), 3.53 (2H, m, CH_2–CO). *Anal.* Calcd for $C_{11}H_{10}N_2O_2$: C, 65.32; H, 4.95; N,13.86. Found: C, 65.68; H, 5.04; N, 13.98.

6-Phenyl-5-carboxy-3(2*H*)-pyridazinone (**8**): A solution of the aldehyde **6** (6 g, 30 mmol) and silver nitrate (25.5 g, 150 mmol) in ethanol (20 ml) and water (25 ml) was stirred rapidly under an argon atmosphere. A 10% aqueous sodium hydroxide solution was added until the pH of the reaction mixture reached 12. The resulting suspension was stirred at room temperature for approximately 16 h and then filtered. The filtrate was concentrated under reduced pressure, then extracted with diethyl ether. The organic solution was acidified with hydrochloric acid to pH 3—4 to afford a solid, which was purified by column chromatography (dichloromethane–methanol 9 : 1) to give 3.18 g (50%) of 8, mp 243—245 °C. IR (KBr) cm^{-1}: 3600 (NH), 1681 (CO pyridazinone), 1679 (COOH). ^{1}H-NMR (DMSO-d_6) δ: 13.5 (1H, br s, NH, deuterium oxide exchangeable), 7.46 (5H, m, Ph), 7.15 (1H, s, CH–CO). *Anal.* Calcd for $C_{11}H_8N_2O_3$: C, 61.11; H, 3.70; N, 12.9. Found: C, 61.48; H, 4.31; N, 12.02.

6-Phenyl-5-carboxy-4,5-dihydro-3(2*H*)-pyridazinone (**9**): This compound was prepared using the same procedure as described for the carboxylic acid **8**, in 65% yield, mp 238—240 °C. IR (KBr) cm^{-1}: 3600 (NH), 1660 (CO pyridazinone), 1689 (COOH). ^{1}H-NMR (DMSO-d_6) δ: 13.02 (1H, br s, NH, deuterium oxide exchangeable), 7.53—7.46 (5H, m, Ph), 3.82 (1H, m, CH), 3.06 (2H, m, CH_2–CO).

Pharmacology Preparation of Platelet-Rich Plasma: Blood was collected by cardiac puncture from sodium pentobarbital-anaesthetized male Sprague–Dawley rats (60 mg/kg, i.p.) and anticoagulated with 3.13% citrate (1 to 9 vol of blood). Platelet-rich plasma (PRP) was prepared by centrifugation (400 × ***g***, 20 min) of the citrated blood. Platelets were suspended to a cell density of 3×10^8/ml by diluting the PRP with autologous platelet-poor plasma (2700 × ***g***, 10 min).

Preparation of Washed Platelets: Blood was collected on acid citrate dextrose (ACD) (85 mM trisodium citrate; 65 mM citric acid and 111 mM glucose). Platelets were pelleted from PRP (1000 × ***g***, 18 min), washed in a Ca^{2+}-free modified Tyrode–HEPES solution (107 mM NaCl, 2.68 mM KCl, 1 mM $MgCl_2$, 3.8 mM $NaPO_4H_2$, 20 mM HEPES, 5.5 mM glucose, 0.35% (w/w) bovine serum albumin and 1 : 6 ACD; pH 7.4) and finally resuspended at an appropriate density in the same buffer minus albumin and ACD, and with NaCl increased to 132.5 mM to maintain osmolality (300 ± 10 mOsm/kg). One hour prior to the experiments $CaCl_2$ (2 mM) was added. For ADP-induced aggregation studies, fibrinogen (0.25 mg/ml) was present in the platelet suspension.

Measurement of Platelet Aggregation: PRP and washed platelet aggregation was measured as a percentage of the maximum change in light transmission in a dual channel aggregometer (Chrono–Log; Havertown, PA, U.S.A.) according to the turbidimetric method of Born.[23] All experiments were performed at 37 °C under constant stirring. Platelet suspensions were prewarmed before the addition of a test compound or vehicle (control) and then incubated for 5 min before addition of the stimulus.

Measurement of cAMP in Platelets: Each incubation tube contained 500 μl of washed platelet suspension (350—400 × $10^3/\mu$l) and 50 μl of drug or vehicle (control). At the end of incubation (37 °C and 1000 rpm), 500 μl of trichloroacetic acid (10% w/v) was quickly added. The protein precipitate after centrifugation (6500 × ***g***, 2 min) was discarded. The supernatant was collected and trichloroacetic acid was extracted by three diethyl ether extractions. The aqueous phases of the extracts were lyophilized and kept at −30 °C until the assay could be performed. cAMP was measured by enzyme immunoassay using a cAMP EIA kit from Amersham as described by the manufacturer. Results were expressed as pmol/10^8 cells.

Chemicals: Adenosine 5′-diphosphate (ADP), collagen, thrombin from bovine plasma, bovine fibrinogen, (±)-sulfinpyrazone and 3-isobutyl-1-methylxanthine (MIX) were purchased from Sigma; metrizamide was from Nycomed. Compounds under study were dissolved in dimethyl sulfoxide (DMSO) (Sigma).

Statistics: Comparison of means was done with Student's *t* test.

Results and Discussion

Chemistry 6-Phenyl-5-hydroxymethyl-3(2*H*)-pyridazinone (**2a**)[19] and 6-(2-thienyl)-5-hydroxymethyl-3(2*H*)-pyridazinone (**2b**)[19] were prepared by hydrazinolysis of β-benzoyl-γ-butyrolactone (**1a**) and β-thenoyl-γ-butyrolactone, respectively according to previously described procedures (Chart 1).[19,24,25] Dehydrogenation of **2a** to the aromatic derivative **4** was carried out with manganese dioxide after protection of the alcoholic hydroxyl group, in excellent yield (85%). This procedure gave better results than one previously described by us.[19] Direct dehydrogenation of **2a** to **5** was done by using selenium oxide as an oxidant in a rather troublesome procedure, as we have also previously reported.[19] Several oxidants were examined for preparing the aldehydes **6** and **7**. The best results for synthesis of **6** were obtained with manganese dioxide (quantitative yield) and for **7** with pyridinium chlorochromate (35—40%). Manganese dioxide oxidation of **2a** gave 6-phenyl-5-methyl-3(2*H*)-pyridazinone. Finally, silver oxide oxidation of the saturated aldehyde **6** and the unsaturated aldehyde **7** gave the cor-

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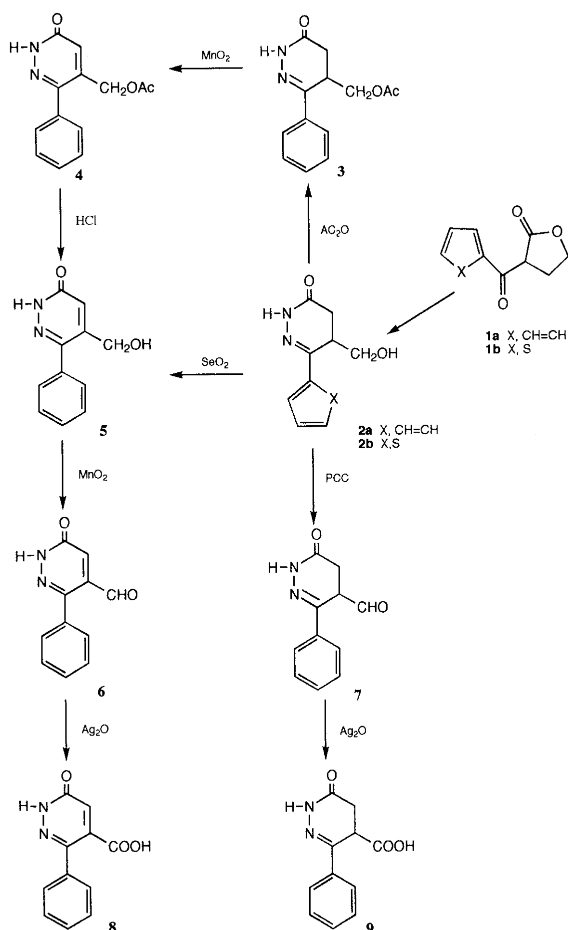

Chart 1

Table 1. Effect of Test Compounds on Aggregation Induced by 2 μM ADP in Rat PRP

Compound	Concentration (mM)	% inhibition of platelet aggregation
2a	3	72.6 ± 3.2
	2	64.3 ± 3.5
	1	49.8 ± 2.7
	0.3	$19.5 \pm 3.9^{a)}$
	0.1	4.7 ± 0.5 (ns)
2b	3	81.3 ± 5.1
	2	71.1 ± 7.4
	1	56.2 ± 2.0
	0.3	25.2 ± 2.5
	0.1	$10.1 \pm 2.4^{a)}$
5	3	80.4 ± 2.8
	2	73.7 ± 2.6
	1	50.5 ± 3.9
	0.3	20.1 ± 2.6
	0.1	5.8 ± 1.9 (ns)
6	2	69.1 ± 5.3
	1	45.1 ± 3.9
	0.3	22.6 ± 1.7
7	3	56.6 ± 6.4
	1.5	35.0 ± 6.1
	1	23.9 ± 6.1
	0.5	11.0 ± 1.4
8	2	2.2 ± 1.6 (ns)
9	2	-11.3 ± 6.0 (ns)
	1	10.1 ± 3.2 (ns)
	0.1	1.5 ± 0.7 (ns)
Sulfinpyrazone	3.5	28.3 ± 5.6
	2.5	15.0 ± 2.4
	1.5	0.6 ± 0.4 (ns)

Results are expressed as percent inhibition (mean+S.E.M. of at least four experiments). $p < 0.01$ unless otherwise indicated. *a*) $p < 0.05$. ns, not significant.

responding carboxylic acids in yields of 40—50%.

Pharmacology The activity of the synthesized compounds as inhibitors of platelet aggregation was initially tested in PRP using 2 μM ADP as the inducer. In this system, it was evident that compounds **2b** and **2a** were the most active substances, while **8** and **9** were inactive (Table 1).

Compounds **2a** and **2b** both exhibited a dose-dependent inhibition of PRP aggregation caused by 2 and 20 μM ADP (Tables 1 and 2). These concentrations of ADP induced reversible and irreversible aggregation, respectively. The greatest inhibitory effect of both compounds was seen against the reversible aggregation induced by 2 μM ADP, with a IC_{50} of 751 μM for **2b** and 1.1 mM for **2a**. The effect of both compounds on the aggregation induced by 20 μM ADP was smaller. However in this case, irreversible aggregation induced by 20 μM ADP was converted to reversible aggregation in the presence of the compounds.

For compounds **2b** and **2a**, aggregation was also studied using washed platelets resuspended in Tyrode–HEPES buffer, to determine if the effect of these compounds on platelet function is due to their direct action on the platelets or whether plasma components also participate. In addition, studies of platelet activation induced by thrombin have to be performed using washed platelets, as fibrinogen present in PRP, when in contact with thrombin, can lead to clotting, masking platelet responses.[26] Under these conditions, compounds **2b** and **2a** each inhibited platelet aggregation induced by ADP, collagen and thrombin in a dose-dependent manner. The intensity of the effect varied according to the stimulus used. Compound **2b** showed a greater inhibitory effect than **2a** on 20 μM ADP-induced aggregation ($IC_{50} = 58.3\,\mu$M and $IC_{50} = 640\,\mu$M respectively). In contrast, compound **2a** was more potent in inhibiting the aggregation induced by thrombin, with $IC_{50} = 52\,\mu$M compared to $IC_{50} = 140\,\mu$M for **2b**. Both compounds showed similar potency on the aggregation stimulated by collagen, with IC_{50} of 60 and 70 μM for **2b** and **2a**, respectively (Table 2). In all cases, the anti-aggregatory activity showed by compounds **2b** and **2a** was higher than that obtained with the standard drug sulfinpyrazone. Moreover, the inhibitory activity of **2a** and **2b** was higher on washed platelets resuspended in buffered medium than in PRP; this may reflect binding to plasma proteins.

Both compounds inhibited platelet aggregation induced by all agonists used. These agonists not only have well-defined patterns of activation, such as adhesion-dependent (collagen), aggregation-dependent (ADP) and direct (thrombin) but also have different activation mechanisms.[27] The results suggest that **2a** and **2b** may interfere in a step common to all agonist activation mechanisms. Structurally related compounds have an inhibitory effect on cAMP phosphodiesterase activity.[6,7,28] In platelets, a decrease in cAMP phosphodiesterase activity implies an increase in the intracellular concentration of cAMP, causing a general inhibitory effect on platelet responses.[29]

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Table 2. Effect of **2b** and **2a** on Aggregation Induced by ADP 20 μM in PRP and by ADP, Collagen and Thrombin in Washed Rat Platelets

Compound	Concentration (mM)	Agonist: ADP 20 μM PRP	ADP 20 μM WP	Thrombin 0.2 u/ml	Collagen 20 μg/ml
2a	3	41.5±2.9			
	2	36.9±2.5			
	1	29.2±1.9	67.3±3.7	97.7±2.3	100± 0.0
	0.3	17.0±0.8[a]	28.9±1.9	82.0±4.9	95.2± 4.8
	0.1	9.6±2.2 (ns)	17.8±1.9	62.1±6.9	56.5±12.3
	0.03		7.1±2.9 (ns)	19.2±4.5	26.9± 3.3
	0.01		3.6±0.3 (ns)	20.8±4.1	5.5± 2.3 (ns)
2b	3	41.4±2.0			
	2	36.6±2.1			
	1	28.2±1.5	80.1±2.2	94.7±1.5	100± 0.0
	0.3	17.1±1.7[a]	77.0±4.0	62.4±8.1	96.5± 1.5
	0.1	12.8±0.6 (ns)	62.7±0.2	39.9±8.7	73.5± 6.6
	0.03		35.0±3.4	21.6±5.2[a]	25.8± 4.8
	0.01		21.0±2.2	−3.7±0.3 (ns)	12.4± 3.9 (ns)
Sulfinpyrazone	3		80.2±1.5		77.5±12.4
	2.5			80.8±0.6	
	2		46.4±1.3		
	1		29.1±0.8	57.3±0.4	47.1±11.5
	0.5		12.7±1.1[a]		
	0.3				13.6± 6.5 (ns)
	0.25			32.0±2.0	
	0.1			11.8±1.3	

Results are expressed as percent inhibition and shown as mean±S.E.M. from at least four experiments. WP: washed platelets. $p<0.01$ unless otherwise indicated. *a*) $p<0.05$. ns, not significant.

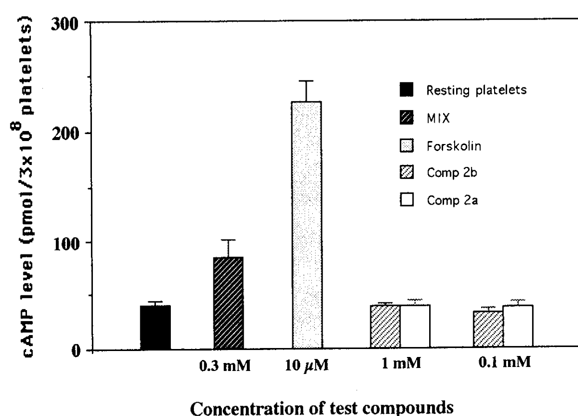

Fig. 1. Effect of Test Compounds **2b**, **2a**, MIX and Forskolin on Intracellular cAMP Level in Rat Platelets

Therefore the effect of **2b** and **2a** on platelet cAMP levels was studied. Figure 1 shows that none of the tested compounds modified platelet cAMP concentrations after 5 min of incubation; the same results were obtained in platelets incubated for 5 min with the compounds and then stimulated by thrombin (data not shown). In contrast, MIX and forskoline, two specific pharmacological tools which induce intracellular cAMP accumulation *via* different mechanisms, increase the cAMP levels from 40.6 pmol/3.10^8 platelets under basal conditions to 84.6 and 226.46 pmol/3.10^8 platelets, respectively after incubation for 5 min.

In conclusion, 1) the platelet aggregation-inhibitory properties of the compounds under study reveal structure–activity relationships critically dependent on the level of oxidation at the 5-position. The dihydropyridazinones **2a** and **2b** are the most active compounds, followed by the aromatic derivative **5** and the aldehydes **6** and **7**. No significant inhibitory effects are exhibited by the carboxylic acids **8** and **9**; 2) the inhibitory effects caused by compounds **2b** and **2a** on platelet aggregation are not due to cAMP phosphodiesterase inhibition, in contrast to those of other structurally related compounds; both compounds may affect one or more steps in platelet signal transduction common to all the agonists used. We found that compounds **2a** and **2b** decreased intracellular calcium level after platelet activation.[30] Further work on this subject is in progress.

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