Synthesis, Platelet Aggregation Inhibitory Activity, and in Vivo Antithrombotic Activity of New 1,4-Dihydropyridines[1]

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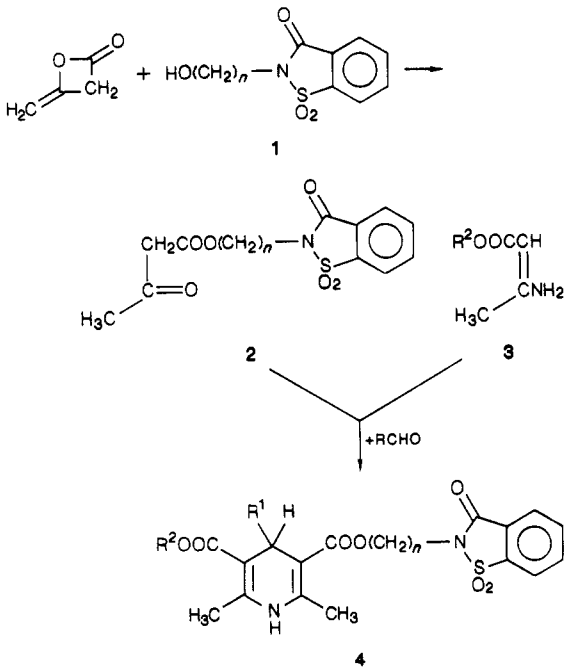
A series of 1,4-dihydropyridines (DHP) bound to 1,2-benzisothiazol-3-ones were synthesized and evaluated for their ability to inhibit platelet aggregation induced by collagen in human platelet-rich plasma (PRP) and to protect mice against experimental thrombosis. The results showed that the compounds were in vitro inhibitors of collagen-induced platelet aggregation. Most of them were also effective in reducing mortality in the mouse antithrombotic assay. 2-(1,1,3-Trioxo-2,3-dihydro-1,2-benzisothiazol-2-yl)ethyl 2,6-dimethyl-5-(ethoxycarbonyl)-4-methyl-1,4-dihydropyridinecarboxylate (**4A**) is the most promising compound. This compound did not show any cardiovascular effects either in the anesthetized cat or in the anesthetized rat at iv doses up to 750 or 500 μg/kg, respectively. Likewise, antiplatelet and cardiovascular effects of compound **4A** were simultaneously studied in anesthetized rats and compared with those of nitrendipine.

Discoveries that link platelet activation not only to thrombosis but also to the initial lesion in atherogenesis[2] have prompted a series of clinical studies that have provided better insight into the role of platelets in the clinical course of ischemic heart disease. They have stimulated an interest in the concept that drugs suppressing platelet activation might prevent or reduce clinical manifestations of thrombotic disease. However, most of those drugs have been first used for other indications, for example, aspirin,[3] clofibrate,[4] or dipyridamole,[5] and clinical experience with these sustances has not been conclusive and has not lead to a comprehensive understanding or a fully accepted treatment.[6] One of the reasons could be that none of these drugs strongly affects platelet function without affecting other systems. For these reasons, there is a considerable interest in the pharmaceutical industry in the development of antiplatelet agents as potential therapeutics for the prevention and treatment of the various forms of thrombosis and other diseases, the pathogenesis of which may be mediated by platelets. An attractive class of compounds is that of 1,4-dihydropyridines (DHP). In the past few years, several groups have reported antiplatelet actions of DHP currently used as calcium channel blockers.[7-9] Nevertheless, taking into account the platelet lack of voltage-operated calcium channels (VOC),[10] the antithrombotic activity of these drugs may be due to the summation of different effects on platelets and arteries rather than to calcium channel blocker activity.[11-13] Unfortunately, DHP have usually been used at concentrations 2–3 orders of magnitude higher than those needed to achieve pharmacological effects on the cardiovascular system, and therefore antiplatelet activity may be seriously disadvantaged by cardiovascular side effects.

In this paper, we report the synthesis and studies on activity of a new series of 1,4-DHP with enhanced antithrombotic activity and that is basically free of cardiovascular effects.

Scheme I



Chemistry

The 1,4-DHP derivatives were prepared by using a modification of Hantzch's synthesis reported by Fax et al.[14] as illustrated in Scheme I. Thus, compounds **1**, obtained by previously described procedures,[15,16] were made to react with diketene to yield the β-keto ester (compounds **2**). Finally, the reaction of compound **2** with 3-aminocrotonate (compound **3**) and the appropriate aldehyde gave the cy-

(1) Data on antiaggregatory and antithrombotic activity of compound **4A** were presented to the XIth International Congress on Thrombosis and Haemostasis held in Brussels, July 1987. Ortega, M. P.; Sunkel, C.; Priego, J. G. *Thromb. Haemostasis* **1987**, *58*(1), Abstr. 631, 632.

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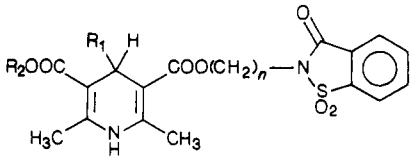
Table I. Chemical Data for 1,4-Dihydropyridines



compd	R_1	R_2	n	time, h	yield,[a] %	mp, °C	cryst[b] solvent	formula[c]
4A	CH_3	CH_3CH_2	2	10	81	144–6	A	$C_{21}H_{24}N_2O_7S$
4B	CH_3	CH_3	2	10	58	146–9	A	$C_{20}H_{22}N_2O_7S$
4C	CH_3	2-OHPhCONH$(CH_2)_2$	2	10	46	86–90	B	$C_{28}H_{29}N_3O_9S$
4D	CH_3	CH_3	1	8	58	89–92	C	$C_{19}H_{20}N_2O_7S \cdot C_2H_6O$
4E	CH_3	CH_3CH_2	1	8	40	162–4	A	$C_{20}H_{22}N_2O_7S$
4F	$CH_3(CH_2)_3$	CH_3	2	10	68	119–21	A	$C_{23}H_{28}N_2O_7S$

[a] Refers to the recrystallized product. [b] Key: A, ethyl acetate; B, CH_3OH; C, EtOH. [c] Analytical data were within ±0.4% of the theoretical values.

Table II. Antiplatelet and Antithrombotic Activity of Representative 1,4-Dihydropiridines and Standard Compounds

compound	IC_{50},[a] M	ED_{50},[b] μM/kg	ED_{50}, mg/kg
4A	10 ± 2	2.23	1.00
4B	100 ± 11	138.10	59.93
4C	500 ± 42	51.40	29.96
4D	230 ± 20	>220	>100
4E	180 ± 11	134.41	58.33
4F	200 ± 17	78.36	37.30
aspirin	70 ± 7	166.66[c]	30.00[c]
ticlopidine	>500	151.64	40.00
nifedipine	100 ± 16	6.93	2.40
dipyridamole	>500	142.69	72.00

[a] Concentration required to inhibit aggregation induced by 2 μg/mL of collagen by 50%. Figures are mean ± SD (n = 10). [b] Dose required to protect 50% tested mice. Data are calculated from dose–response curves. At least five experiments were performed for every compound. [c] This value is ED_{30}. No increase but decrease of protective effect was shown by aspirin when administered at higher doses.

clisized 1,4-DHP (compound 4). Although some variations of the reaction conditions were examined, best results were obtained in ethanolic medium.

Some aminocrotonates of type 3 were commercially available; otherwise they were synthesized according to methods reported in the literature.[17] All aldehydes RCHO were from commercial sources. The yields of the synthesis of 1,4-dihydropyridines 4 were in the range of 40–81%.

Pharmacology

The 1,4-DHP were tested for inhibition of in vitro collagen-induced platelet aggregation with human PRP. Results are shown in Table II. The evaluation of these data shows that all of the 1,4-DHP described in this paper are active in inhibiting collagen-induced platelet aggregation. However, this initial screening was not quite enough to substantiate any antithrombotic activity of the new compounds because it soon became clear that in vitro potency was not directly related to in vivo activity.

In vivo activity was assumed to give a better reflection of therapeutic potential. Therefore, the antithrombotic activity of compounds as compared with standard drugs was evaluated in a model of in vivo thrombosis in mice.[18]

Compounds failing at 0.22 mM/kg to protect mice from collagen/epinephrine-induced thrombosis were not usually further investigated.

The evaluation of data in Table II shows that compound **4A** was the most active of the tested 1,4-DHP, and therefore, its cardiovascular effects were assessed and compared with those of nifedipine or nitrendipine. Table III shows the effects of the compound **4A** versus nifedipine on vascular smooth muscle preparations of rabbit aorta. Compound **4A** had little inhibitory effect on contraction evoked by high K^+ concentrations (35 mM) when compared with nifedipine (IC_{50} = 10^{-6} and 3.4 × 10^{-9} M, respectively). Additionally inotropic and chronotropic activities were evaluated on left and right guinea pig atria, respectively. Compound **4A** showed no activity in this test even at 10^{-6} M (Tables IV and V).

A series of in vivo experiments were also carried out in anesthetized cats. Compound **4A** was administered by an ip or iv route (18 or 0.15 mg/kg per min, respectively). No modifications attributable to compound **4A** were observed in either electrocardiograms, mean arterial blood pressure,

Table III. Effect of Compound **4A** and Nifedipine on 35 mM K^+-Induced Contraction on Rabbit Aorta

	% inhibition[a]	
concn, mol/L	**4A**	nifedipine
10^{-9}	0	18.28 ± 2.42
3 × 10^{-9}	0	41.73 ± 3.54
10^{-8}	0	73.66 ± 7.10
3 × 10^{-8}	0	88.35 ± 8.31
10^{-7}	12.60 ± 5.22	100 ± 0
3 × 10^{-7}	23.89 ± 10.34	nt[b]
10^{-6}	48.13 ± 13.76	nt
3 × 10^{-6}	71.3 ± 18.42	nt

[a] Figures are mean ± SEM (n = 6). [b] Not tested.

Table IV. Effect of Compound **4A** versus Nitrendipine at Cumulative Doses on Guinea Pig Atria[a]

	4A		nitrendipine	
dose, M	beats/min	% control	beats/min	% control
0	183 ± 59	100	190 ± 56	100
10^{-9}	193 ± 65	105.6	190 ± 66	100
10^{-8}	176 ± 58	96.3	185 ± 55	97.5
10^{-7}	181 ± 42	98.6	179 ± 60	94
10^{-6}	181 ± 68	98.5	153 ± 72	81

[a] Data are mean ± standard deviation of four experiments.

Table V. Effects of compound **4A** (10^{-6} M) on Chronotropic Activity of Guinea Pig Atria[a]

time, min	beats/min	% control (zero time)	time, min	beats/min	% control (zero time)
0	137 ± 42	100.0	75	134 ± 48	97.8
15	138 ± 50	100.7	90	133 ± 49	97.1
30	138 ± 44	100.7	120	131 ± 47	95.6
45	138 ± 54	100.4	150	129 ± 44	94.2
60	136 ± 51	99.3	180	130 ± 44	94.9

[a] Data are mean ± standard deviation of four experiments.

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Table VI. Effects of a Continuous Intravenous Infusion of Compound **4A** (0.15 mg/kg per min) in Anesthetized Cats

time, min	dose, mg/kg	cardiac frequency, bpm[a]	respiratory frequency, rpm[b]	arterial pressure, mmHg		
				systolic	diastolic	mean
0	0.00	190	18	165	115	124
1	0.15	190	18	165	115	124
2	0.30	190	18	165	115	123
5	0.75	195	19	162	112	124
10	1.50	210	18	160	110	118
15	2.25	217	19	162	110	120
20	3.00	216	18	160	110	118

[a] Beats per minute. [b] Respiration per minute.

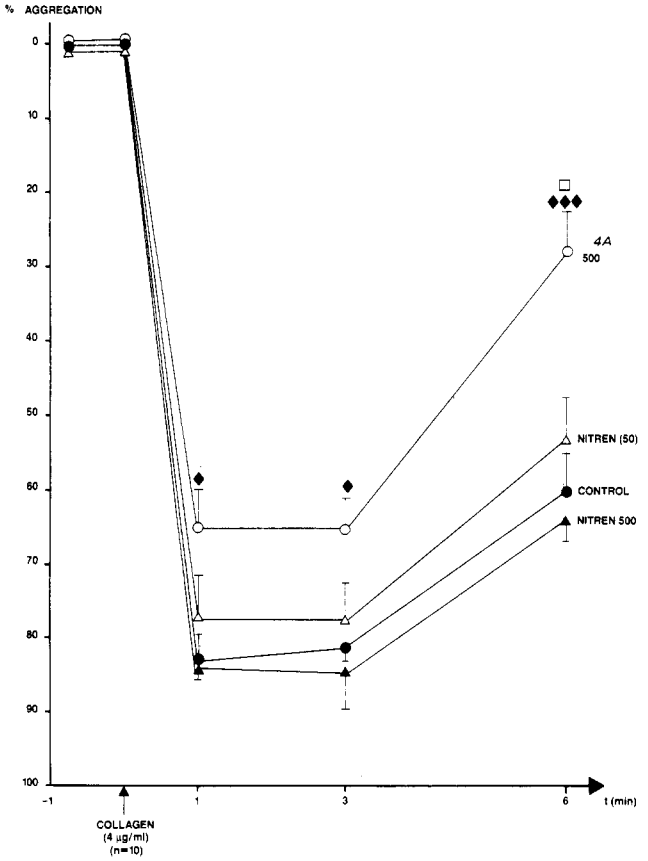

Figure 1. Kinetics of platelet aggregation induced by collagen (3 μg/mL) on rat whole blood as measured by change in platelet counts. Statistical significance between aggregation of control and treated platelets: (◆) p (t) < 0.05; (◆ ◆ ◆) p (t) < 0.001. Compound **4A** versus nitrendipine, 500 μg/kg: (□) p (t) < 0.01.

Table VII. Effects of an Intraperitoneal Dose (200 mg/kg) of Compound **4A** on the Anesthetized Cat

time, min	cardiac frequency, bpm[a]	respiratory frequency,[b] rpm	arterial pressure, mmHg		
			systolic	diastolic	mean
0	260	14	130	85	102
15	254	14	130	90	100
30	245	13	135	90	96
45	245	14	125	85	95
60	256	13	130	85	95
75	255	12	137	90	102
90	256	12	138	85	101
105	259	12	140	87	93
120	261	13	145	85	105
135	271	15	140	80	94
150	265	15	140	80	93
165	278	15	142	80	91
180	276	15	145	80	96

[a] Beats per minute. [b] Respirations per minute.

or respiratory frequency. A slight decrease in blood pressure values and a 14% increase in heart rate observed at doses over 750 μg/kg were also present in control cats receiving only vehicle (PEG-400, 200 μL/kg per min). Results are shown in Tables VI and VII.

Finally, in studies performed in anesthetized rats, platelets from animals treated with compound **4A** aggregated weaker and recovered faster upon stimulation with collagen than platelets from control or nitrendipine-treated rats (Figure 1).

Figure 2 demonstrates the hypotensive response to a single 50 or 500 μg/kg bolus infusion of nitrendipine as compared with the lack of action of compound **4A** (500 μg/kg). Additionally, intravenous injection of nitrendipine (50 or 500 μg/kg) led to an increase (20% and 36%, respectively) in heart rate while no changes were associated with injection of compound **4A** (500 μg/kg) or vehicle

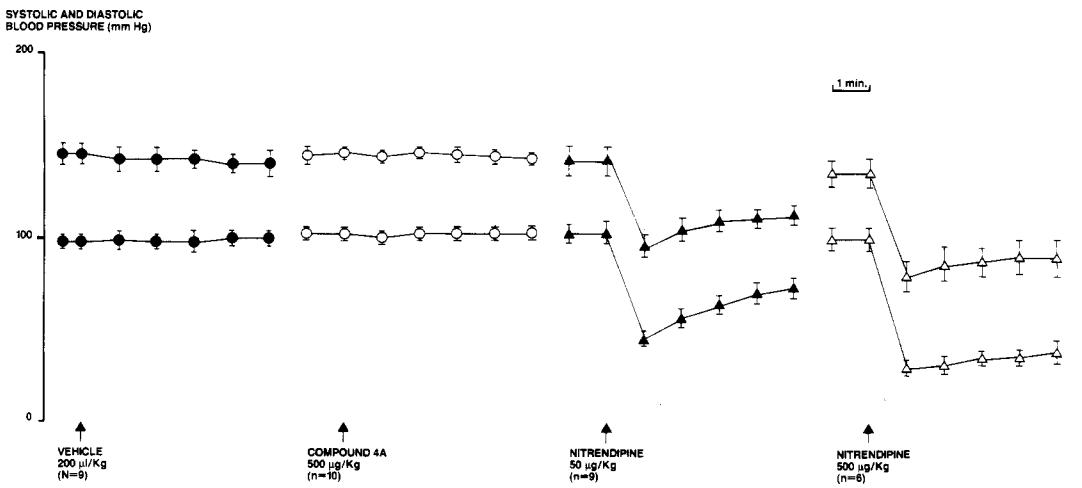

Figure 2. Effects of intravenous administration of compound **4A** and nitrendipine on systolic and diastolic blood pressure in anesthetized rats.

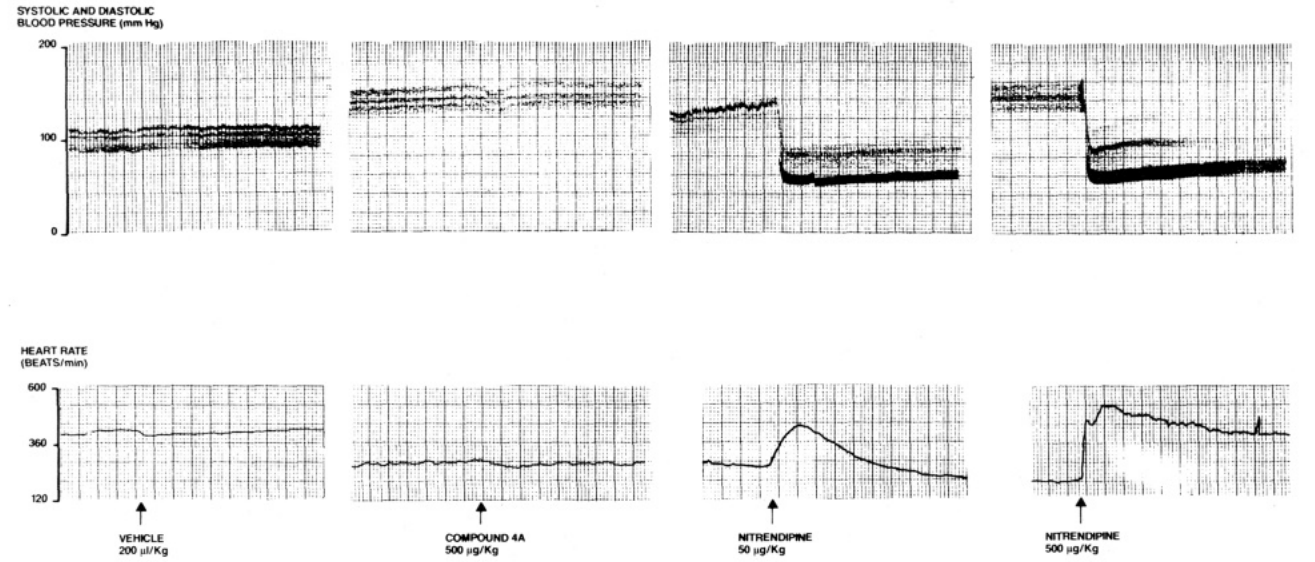

Figure 3. Effects of compound **4A** versus nitrendipine on pulse blood pressure and heart rate of anesthetized rats. (Data of a representative experiment.)

(PEG-400–distilled water, 1:1, 200 µL/kg).

A representative experiment is shown in Figure 3.

Discussion

Although many drugs have inhibitory effects on platelet function, none of them have generally been accepted as protective against any of the various forms of thrombosis.

Perhaps the most readily accepted common denominator is the significance of Ca^{2+} in platelet function.[19] Therefore, calcium channel blocking agents have recently received considerable attention as possible therapeutic agents in patients suffering risk of any form of thrombosis.[20,21] Nevertheless, the antiplatelet effects of 1,4-DHP with calcium channel blocking activity are currently a matter of controversy since, in most studies reported, concentrations of drug necessary to achieve antiplatelet activity were 2 or 3 orders of magnitude higher than the known therapeutic plasma levels of these drugs in humans; therefore undesirable cardiovascular side effects may appear at such high concentrations.

The compounds described in this paper showed enhanced antiplatelet activity in vitro and were basically devoid of cardiovascular effects, probably due to the lack of a bulky substituent in position 4 of the dihydropyridine ring that seems to be essential for the characteristic mode of action of 1,4-DHP with calcium channel blocking activity.[22] On the other hand, the chemical bond of 1,4-DHP and benzisothiazolone groups seems also significant, since neither molecule shows antiplatelet activity by itself.[23] Moreover, the presence of the ethylenic bridge in compound **4A** could be an important feature, taking into account the weaker effect of compound **4E** (Table II).

Experimental Section

Chemistry. All melting points were determined in a Büchi 510 open capillary melting point apparatus and are uncorrected. The structures of all compounds were supported by IR and NMR spectra. IR spectra were obtained in a Beckman Acculab-4 recording spectrometer and NMR spectra were recorded on a Varian T-60 spectrometer. All elemental analysis were within ±0.4%.

2-(1,1,3-Trioxo-2,3-dihydro-1,2-benzisothiazol-2-yl)ethyl Acetylacetate. A mixture of 30 g (0.13 mol) of 2-(2-hydroxyethyl)-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide and 0.2 mL (0.0014 mol) of triethylamine was stirred at 80 °C, and 10.1 mL (0.13 mol) of diketene was added dropwise in order to keep the temperature between 85 and 90 °C. The pasty mixture was stirred at 90 °C for a further 3 h, then 500 mL of CH_2Cl_2 was added, and the mixture was washed with 2 × 500 mL of water, filtered on activated charcoal/diatomaceous earth, and dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure, yielding a yellow oil, which solidified slowly to white crystals. The product was recrystallized from ethanol to give 35.6 g (88%) of compound **2A**.

(1,1,3-Trioxo-2,3-dihydro-1,2-benzisothiazol-2-yl)methyl Acetylacetate. A 128.69-g (0.60 mol) portion of 2-(hydroxymethyl)-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide and 9.66 g (0.03 mol) of mercuric acetate were dissolved in 138 mL (2.41 mmol) of acetic acid. Diketene (60.9, 0.72 mol) was added dropwise. The mixture was stirred at room temperature for a further 8 h and then left overnight. The solid precipitate was collected, washed with water, and dried, yielding 158.7 g (89%) of compound **2B**.

2-(2-Salicylamido)ethyl 3-Aminocrotonate (3). To a stirred mixture of 69.82 g (0.39 mol) of *N*-(2-hydroxyethyl)salicylamide and 0.4 mL (0.0028 mol) of triethylamine heated at 80 °C was added dropwise 29.5 mL (0.39 mol) of diketene to keep the temperature between 85 and 90 °C. After the mixture was stirred at 90 °C for a further 3 h, 15 mL of boiling methanol was added and the mixture was then cooled at −10 °C. The precipitate was collected and recrystallized from methyl *tert*-butyl ether, yielding 2-(2-salicylamido)ethyl acetoacetate (44.0 g, 76%) as white needles, mp 58–60 °C.

A 55.43-g (0.21 mol) portion of 2-(2-salicylamido)ethyl acetoacetate was dissolved in 250 mL of dry methanol and NH_3 was passed into the solution for 3 h. Excess NH_3 and the solvent were removed under reduced pressure; the residue was a liquid that quickly became solid. It was recrystallized from ethanol, yielding white crystalline flakes of compound **3**; yield 77.5 g (80%); mp 124–126 °C.

General Procedure for the Preparation of (1,1,3-Trioxo-2,3-dihydro-1,2-benzisothiazol-2-yl)alkyl 5-(Alkoxycarbonyl)-4-alkyl-2,6-dimethyl-1,4-dihydropyridine-3-carboxylate (4). The reaction of β-keto esters from **2** with 3-aminocrotonates from **3** in the presence of the appropriate aldehyde RCHO afforded the corresponding 1,4-DHP derivatives as illustrated in Scheme I. Reactions were carried out in the darkness to protect the dihydropyridine ring against oxidation.

A mixture of equimolar amounts of compounds **2** and **3** and aldehyde in absolute ethanol (100 mL/mol of reagent) was stirred

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and boiled under reflux for periods of time given in Table I. Afterward, the reaction mixture was treated in one of three ways: (1) cooled and filtered, (2) concentrated under reduced pressure, and (3) concentrated to dryness. The solid product was recrystallized from the appropriate solvent given in Table I.

Biological Methods. In Vitro Determination of Inhibition of Platelet Aggregation. Citrated human blood was centrifuged to prepare platelet-rich plasma (PRP) and the count adjusted to 250 000/μL with autologous platelet-poor plasma (PPP) as previously described.[24] Collagen-induced platelet aggregation was measured photometrically[25] in a Lumiaggregometer (Chrono-Log Co., Haventon, PA) at 37 °C and at a stirring rate of 1100 rpm. Fresh solutions of collagen from bovine tendon (DIC, Kyoto, Daiichi, Japan) were prepared daily in pH 7.35 Tris–HCl buffer (20 μg/mL, final concentration). Aliquots of PRP (450 μL) were preincubated with 2 μL of solutions of compounds in dimethyl sulfoxide (DMSO) for 15 min before addition of collagen suspension (50 μL). DMSO was included in the control samples of PRP. Responses to collagen were quantified by measuring the maximum increase in light transmission 5 min after the addition of collagen preparation. Compounds and standard drugs were studied at several concentrations. The IC_{50} (concentration producing 50% inhibition) of each compound was determined from linear regression analysis from log concentration–percent inhibition curves.

Mouse Antithrombotic Assay. Effects of 1,4-DHP and standard drugs were evaluated by a modification of the technique described by DiMinno and Silver.[18] Male COBS/CD-1 Charles River mice weighing 30 ± 2 g were used. Doses of aggregating agents were 15 μg of collagen plus 1.8 μg of epinephrine per mouse. One hundred microliters of collagen–epinephrine solution in saline was injected in mice in one of the four tail veins. One single doses of either vehicle (0.5% (carboxymethyl)cellulose) or drug was given to mice orally. Six groups (control and five levels of dosage) of 10 mice per group were studied in every experiment. Six mice, one of each group, were challenged 1 h after dosing and then every 20 min for the next 3 h. Animals were observed for more than 15 min, and characteristics and length of attacks were recorded by a trained observer. The effect to be overcome was death or paralysis of the hind limbs within 15 min. From the data obtained, the percentage of mice protected in every 4-h interval could be calculated and a comparison made between values obtained with compounds or vehicle alone. Curves log dose–percent protected mice were represented and ED_{50} (dose required to protect 50% tested mice) was graphically calculated for each compound.

Vascular Effects. The experimental protocol is similar to another previously described.[26,27] Male New Zealand rabbits weighing 2–2.5 kg were sacrificed, and the thoracic aorta was removed and placed in Krebs–bicarbonate buffer. Excess fat and tissue were removed, and the aorta was cut in helicoidal strips.[28] Strips were mounted in 15-mL organ muscle baths under a 2.5-g preload, which was applied and maintained during a 2 h equilibration period. Bath temperature was kept at 37 °C and Krebs–bicarbonate was bubbled with 95% O_2 + 5% CO_2 (final pH 7.4). The aorta was washed every 20 min to avoid interference of metabolites.[29]

The strips were stimulated by replacing the normal Krebs solution with a high K^+ (35 mmol of KCl) Krebs in which NaCl was reduced by an equimolar amount. After attainment of a steady plateau tension, strips were exposed to increasing concentrations of compound **4A** or nifedipine.[30]

Relaxant responses were recorded and normalized with respect to initial recorded tensions. IC_{50} values were determined from concentration–response curves by the method of Finney.[31]

Isolated Guinea Pig Atria. Left and right atria were dissected from hearts of adult guinea pigs weighing 500–600 g and mounted in an organ bath (30 mL) containing Krebs–bicarbonate, gassed with 95% O_2 plus 5% CO_2 (final pH 7.4). The bath was maintained at 37 °C. Inotropic responses were recorded from electrically stimulated left atrial preparations (1 Hz, 1 ms, 20% threshold voltage). Right atria were used in studies on the chronotropic activity of compound **4A**. Auricles were allowed to equilibrate for 1 h before starting experiments, and then compound **4A** was added to the bath in cumulative doses according to the method described by van Rosum.[30] Stock solutions of compounds (10^{-2} M) were prepared in DMSO and then diluted in Krebs–bicarbonate (final concentration of DMSO in organ bath ≤ 0.1%).

Studies in Anesthetized Cats. Male cats weighing 2–2.5 kg were superficially anesthetized with ether, and the right femoral vein was cannulated. D-Glucochloralose solution (80 mg/kg) was then injected via the femoral vein. The left femoral artery was cannulated and connected to a blood pressure transducer (Hewlett-Packard 1280 C) attached to a Hewlett-Packard polygraph. Tracheotomy was performed and the tracheal cannula was connected to a pneumotachograph.

Electrocardiograms were registered with a bioelectric amplifier (Hewlett-Packard). Blood pressure and respiratory rate were allowed to stabilize for 1 h and then compound **4A** was injected as a continuous infusion (0.15 mg/kg per min) through the femoral vein.

In another series of experiments, 20 mg/kg of compound **4A** was administered by the intraperitoneal route. Recordings of mean arterial blood pressure, electrocardiogram, and respiratory rate were performed within 6 h after administration of compounds.

Studies in Anesthetized Rats. Male Sprague–Dawley rats weighing 200 ± 20 g were anesthetized ip with pentobarbital (50 mg/kg). A polyethylene catheter was inserted into a femoral vein for intravenous applications. For blood pressure measurements, the carotid artery was connected, via a polyethylene catheter, to a pressure transducer (Statham P 23 Db). Five minutes after drug infusion, blood samples were drawn from the carotid artery and used immediately in platelet aggregation studies. Platelet aggregation in whole blood was carried out with 400-μL samples of carotid blood collected into plastic tubes containing heparin (10 INHU/mL blood) and maintained at 37 °C under constant stirring with a magnetic bar. The number of platelets was determined 1 min before and 1, 3, and 6 min after addition of collagen (4 μg/mL). Platelet counts were performed in 20-μL blood samples collected at each indicated time interval and diluted in 2 mL of Isoton (Coultronics) containing 1% formaldehyde. After centrifugation at 200*g* for 3 min, the number of free platelets in the supernatant was determined with a Coulter Counter ZM.

Statistical Methods. Paired Student's *t* test was used to show any significant difference in in vitro experiments. The χ^2 test with the Yate's correction was used in series of in vivo experiments.

Acknowledgment. This work was supported by a grant from the Fondo Nacional para el Desarrollo de la Investigación Científica (CAICYT No. 4/82). We wish to acknowledge the excellent technical assistance of M. P. Blasco, M. C. de la Cal, G. Gabriel, M. L. Ortega, M. Pérez, A. Ponga, M. T. Pontijas, T. de la Puente, and M. J. Simón. We also are grateful to C. Montero for typing the manuscript and to A. de la Noriega for her careful review of this paper.

Registry No. **1** (*n* = 2), 6343-81-3; **1** (*n* = 1), 13947-20-1; **2** (*n* = 2), 113658-92-7; **2** (*n* = 1), 113658-93-8; **3**, 92565-10-1; **3** (R^2 = Et), 7318-00-5; **3** (R^2 = Me), 14205-39-1; **4A**, 113658-85-8; **4B**, 113658-86-9; **4C**, 113658-87-0; **4D**, 113658-88-1; **4E**, 113658-89-2; **4F**, 113658-90-5; MeCHO, 75-07-0; BuCHO, 110-62-3; diketene, 674-82-8; *N*-(2-hydroxyethyl)salicylamide, 24207-38-3; 2-(2-salicylamido)ethyl acetoacetate, 115288-98-7.

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